School of Pharmacy

Pigment Epithelium-derived Factor and Insulin Crosstalk in Skeletal Muscle Biology

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Declaration

To the best of my knowledge and belief this thesis contains no material previously published by any other person except where due acknowledgment has been made. This thesis contains no material which has been accepted for the award of any other degree or diploma in any university. The research presented and reported in this thesis was conducted in compliance with the National Health and Medical Research Council Australian code for the care and use of animals for scientific purposes 8th edition (2013). The proposed research study received animal ethics approval from the Curtin University Animal Ethics Committee, Approval number #AEC_2016_35

Revathy Carnagarin
5th September 2017

Dedication

To my lovely husband and soul mate, Papumama

For his endless love, support and encouragement.

Acknowledgement

God has been so kind and I have been granted the greatest opportunities life has to offer and have been incredibly fortunate with the people who have chosen to make themselves part of my life. This thesis is a testament to all the people whom I have shared my life with.

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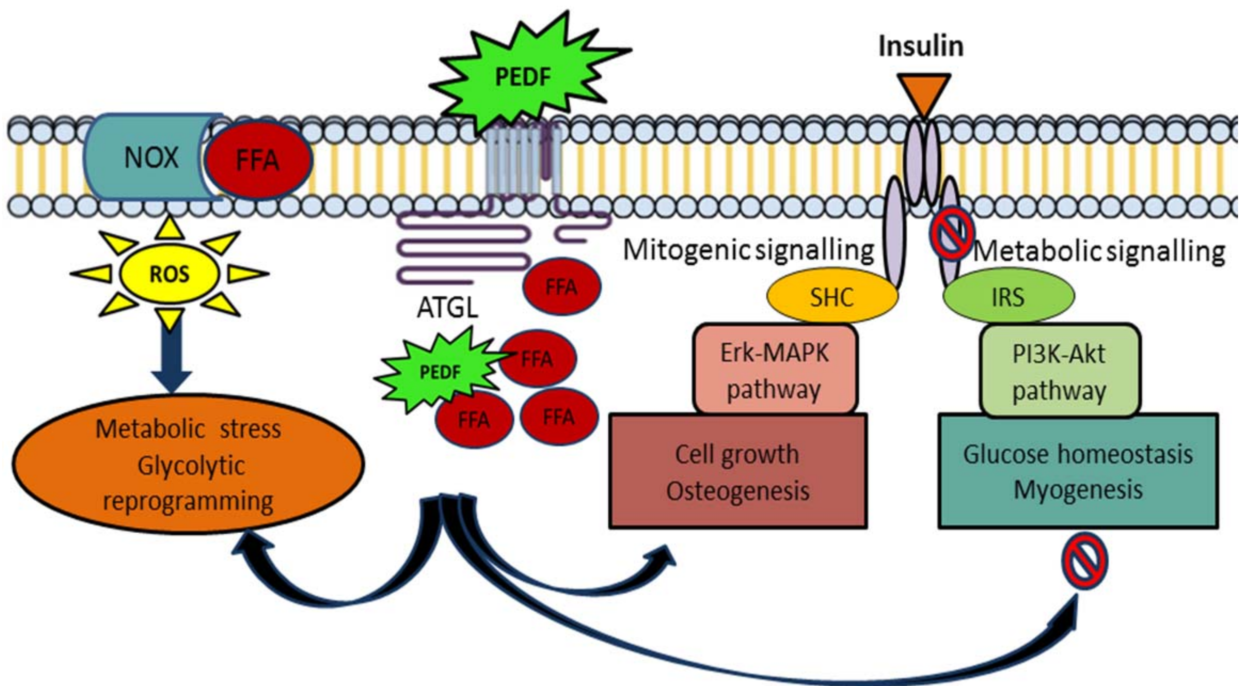
Graphical abstract

Pigment epithelium-derived factor (PEDF) and insulin crosstalk in skeletal muscle biology



Abstract

Pigment epithelium derived factor (PEDF) is an endogenous multifunctional glycoprotein with a spectrum of biological effects across diverse pathologies. Recent evidence points to the involvement of PEDF in metabolic dysfunction and reveals a definitive association between PEDF and metabolic health in animal models and humans. However, little evidence exists to support PEDF playing a direct role in mediating the metabolic impairment in peripheral tissue such as skeletal muscle. Since there has been a proven link between PEDF and insulin resistance, it was pertinent to conduct further research on this in various aspects of skeletal muscle biology. The specific aims of this study were to identify the effect of PEDF in insulin-dependent molecular pathways of glucose homeostasis in skeletal myocytes, to identify if PEDF induces metabolic stress in skeletal myocytes, and to characterise the end biological effect of PEDF and insulin crosstalk in skeletal myocytes.

To this end, I identified that PEDF antagonised metabolic insulin signal transduction and prevented activation of its phosphorylation, from the receptor to its metabolic effectors through Akt/PKB-dependent and -independent pathways in murine and human skeletal myocytes. PEDF-treated skeletal myocytes exhibited attenuated insulin-dependent autophosphorylation of insulin receptor (IR), tyrosine phosphorylation of insulin receptor substrate 1 (IRS1), and dual loop phosphorylation-activation of the atypical protein kinase, Akt/PKB. PEDF significantly blocked the glycogenic insulin axis via glycogen synthase kinase and turned off both the molecular switches of glucose uptake through glucose transporter 4 (GLUT4): IRS-Akt/PKB-AS160-mediated and IR-pCbl-dependent GLUT4 translocation. These findings seminally provided evidence of the direct effect of PEDF on multiple insulin-dependent molecular mechanisms of glucose homeostasis in skeletal muscle cells, thereby enabling it to contribute to peripheral cellular insulin resistance.

As part of the second objective, the effect of PEDF on skeletal myocellular metabolism was studied. PEDF enhanced phospholipase A2 activity, accompanied with increased nicotinamide adenine dinucleotide phosphate (NADPH) oxidase-dependent reactive oxygen species (ROS) production that triggered a metabolic reprogramming. PEDF-induced ROS triggered a metabolic shift towards a more glycolytic phenotype. Extracellular flux analysis and glucose consumption assays demonstrated that PEDF treatment induced glycolytic stress but did not change mitochondrial respiration. I conclude that skeletal muscle cells express a PEDF-

inducible oxidant-generating system that enhances glycolysis but is sensitive to antioxidants and NADPH oxidase inhibition.

The sequential attenuation of all the insulin-dependent molecular mechanisms indicate and support the notion that PEDF is antagonistic to insulin and I explored the effect on the other critical molecular insulin axis, namely mitogenesis. PEDF did not impact mitogenic signalling evident from the viability assays and constitutively activated the extracellular regulatory/mitogen activated protein kinase (Erk-MAPK). So, as a part of the last objective of the study, the net biological effect of PEDF and insulin crosstalk in skeletal myocytes was investigated both in vitro and in vivo. PEDF inhibited myogenic migration and downregulated myogenic differentiation markers such as myogenin and MyoD. PEDF induced Erk MAPK-dependent transdifferentiation of myocytes into the osteogenic lineage. Insulin antagonised PEDF-induced upregulation of osteogenic markers such as osteocalcin and alkaline phosphatase and enhanced mineral deposition in skeletal myocytes. I was able to demonstrate bone formation in murine gastrocnemius muscle pockets and the surrounding adipose tissue of the PEDF group and an ablated effect in PEDF + insulin group. The results of this study provide new insights into the molecular mechanisms underpinning PEDF's ability to modulate the differentiation commitment of musculoskeletal cells towards osteogenesis. Such activity has not been observed with any other potent bone-forming factors, not even bone morphogenetic protein 2 (BMP-2). This could pave the way for new strategies such as PEDF-incorporated muscle grafts and flaps, which could overcome the limitations of existing therapies to accelerate bone regeneration. However, these results also indicate that diffusion of PEDF into muscle tissues around bone injury sites may lead to heterotopic ossification, which would be a major limitation for PEDF usage but could be effectively counteracted by insulin, a positive regulator for bone regeneration. This study is a step towards developing PEDF as an effective clinical therapy along with insulin for bone regeneration whilst inhibiting muscle heterotopic ossification.

List of publications by the candidate included as a part of the thesis

- **Carnagarin R**, Dharmarajan AM, Dass CR (2015). Molecular aspects of glucose homeostasis in skeletal muscle – A focus on the molecular mechanisms of insulin resistance. Molecular and Cellular Endocrinology 417: 52-62

- **Carnagarin R**, Dharmarajan AM, Dass CR (2015). PEDF-induced alteration of metabolism leading to insulin resistance. Molecular and Cellular Endocrinology 401: 98-104

- **Carnagarin R**, Dharmarajan AM, Dass CR (2016). PEDF attenuates insulin-dependent molecular pathways of glucose homeostasis in skeletal myocytes. Molecular and Cellular Endocrinology 422: 115-124

- **Carnagarin R**, Carlessi R, Newsholme P, Dharmarajan AM, Dass CR (2016). Pigment epithelium-derived factor stimulates skeletal muscle glycolytic activity through NADPH oxidase-dependent reactive oxygen species production. International Journal of Biochemistry and Cell Biology 78: 229-236

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Relevant publications to the thesis but not forming a part of it

- Chen Y, Carlessi R, Walz N, Cruzat VF, Keane K, John AN, **Carnagarin R**, Dass CR, Newsholme P (2016). Pigment epithelium-derived factor (PEDF) regulates metabolism and insulin secretion from a clonal rat pancreatic beta cell line BRIN-BD11 and mouse islets. Molecular and Cellular Endocrinology 426: 50-60

- Wong CY, Martinez J, **Carnagarin R**, Dass CR (2016). In vitro evaluation of enteric coated insulin tablets containing absorption enhancer and enzyme inhibitor. Journal of Pharmacy and Pharmacology 69: 285-294

- Lin D, **Carnagarin R**, Dharmarajan AM, and Dass CR. Transdifferentiation of myoblasts into osteoblasts – possible use for bone therapy. Journal of Pharmacy and Pharmacology, DOI:10.1111/jphp.12790

- **Carnagarin R,** Sabapathy T, Mamotte C, Dharmarajan AM and Dass CR (2017). Pigment epithelium-derived factor alters cholesterol homeostasis to impair insulin binding - *in preparation.*

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Conference proceedings and awards

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Statement of contribution of others

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Revathy Carnagarin
5th of September 2017

Abbreviation

ACTN4	Actinin4
ALP	Alkaline phosphatase
AMD	Age related macular degeneration
ANOVA	Analysis of variance
aPKC	Atypical protein kinase C
ARS	Alizarin red
AS160	AKT substrate
ATGL	Adipose triglyceride lipase
BCA	Bicinchoninic acid
BMI	Body mass index
BMP-2	Bone morphogenetic protein-2
cAMP	Cyclic adenosine monophosphate
CAP	Cbl-associated protein
Cdc42	Cell division cycle 42
cDNA	Complementary DNA
CHO	Chinese hamster ovary
cMBCD	Cholesterol-loaded MBCD
CM-H2DCFDA	Chloromethyl 2′,7′-dichlorodihydrofluorescein diacetate
DNA	Deoxyribonucleic acid
ECAR	Extracellular acidification rate
ELISA	Enzyme linked immunosorbent assay
ERK1/2	Extracellular signal-regulated kinases 1/2
FCCP	Cyanide-4-trifluoromethoxy phenyhydrazone
FFAs	Free fatty acids
GAP	GTPase-activating protein

GEF	Guanine nucleotide exchange factor
GLUTs	Glucose transporters
GSK3β	Glycogen synthase kinase 3β
GTP	Guanosine triphosphate
HDL	High density lipoprotein
HO	Heterotrophic ossification
HSD	Hydroxysteroid dehydrogenase
HSMMs	Human skeletal muscle myoblasts
HUVECs	Human umbilical vein endothelial cells
IF	Immunofluorescent
IGF1	Insulin-like growth factor 1
IKK	IkappaB kinase complex
IKKβ	Inhibitor of nuclear factor kappa-B kinase subunit beta
IR	Insulin receptor
IRS	Insulin receptor substrate
IκB	I kappa B kinase
JNKs	c-Jun N-terminal kinases
MAPK	Mitogen activated protein kinase
MBCD	Methyl-β-cyclodextrin
MDCK	Madin-Darby canine kidney cell
MSC	Mesenchymal stem cell
MTORC2	Mammalian target of rapamycin 2
NADPH	Nicotinamide adenine dinucleotide phosphate
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NOXs	NADPH oxidases

OCN	Osteocalcin
OCR	Oxygen consumption rate
p38 MAPK	p38 mitogen-activated protein kinases
PBS	Phosphate buffered saline
PCOS	Polycystic ovarian syndrome
PCR	Polymerase chain reaction
PDK1	Phosphoinositide-dependent kinase 1
PEDF	Pigment epithelium-derived factor
PFA	Paraformaldehyde
PH	Plecktrin homology
PI3K	Phosphatidylinositol-3-kinase
PIP3	Phosphatidylinositol-3,4,5-trisphosphate
PKB	Protein kinase B
PKC	Protein kinase C
PNPLA2	Patatin-like phospholipase domain-containing 2
PPAR	Peroxisome proliferator-activated receptors
PTB	Phosphotyrosine-binding domain
PTH	Parathyroid hormone
PVDF	Polyvinylidene fluoride
qPCR	Quantitative polymerase chain reaction
RCL	Reactive central loop
RPE	Retinal pigment epithelial cells
RNA	Ribonucleic acid
SCL2A	Solute carrier family 2A
Serpin	Serine protease inhibitor

SH2	Src homology 2
TBS	Transfer buffer solution
TBST	TBS + 0.1% Tween-20
TCF7L2	Transcription factor 7-like 2
TGF-β	Transforming growth factor beta
TNF-α	Tumour necrosis factor alpha
VEGF	Vascular endothelial growth factor

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Chapter 1: Pigment epithelium-derived factor (PEDF)

History of PEDF

PEDF is a multifunctional protein, discovered in 1990 as a 50kDa secreted glycoprotein from the conditioned medium of foetal human retinal pigment epithelial cells [Tombran-Tink et al 1991]. PEDF was identified to be a potent neurotrophic and neurodifferentiating factor and was stipulated to exert a paracrine effect in the retina. In 1993, PEDF was reported to protect and maintain retinal homeostasis [Becerra et al 1993]. PEDF emerged as an ocular guardian, a potent inhibitor of angiogenesis that maintained avascularity of regions responsible for visual acuity in the eye [Bouck et al 2002]. Following this, researchers focused on the antiangiogenic property of PEDF and PEDF emerged to be the most potent antiangiogenic factor known to date, two times more potent than angiostatin and seven times more potent than endostatin [Dawson et al 1999]. In 2001, the crystal structure of PEDF was obtained with the identification of its probable functional domains [Simonovic et al 2001]. In 2002, the ability of PEDF to discriminate between remodelling and quiescent vasculature and to induce apoptosis selectively in Fas-positive endothelial cells was identified, underscoring the therapeutic utility of PEDF [Volpert et al 2002]. The antiangiogenic N-terminal of PEDF was identified in 2005 [Filleur et al 2005]. In 2005, it was discovered that decreased PEDF expression resulted in extracellular matrix overproduction and deregulation of transforming growth factor β (TGFβ) which caused diabetic nephropathy [Wang et al 2005]. In 2005, the osteogenic potential of PEDF and its localisation within the proliferative and hypertrophic zones of the epiphyseal growth plate and in the bony spicule was identified [Quan et al 2005]. In 2006, the positive association between circulating PEDF levels and metabolic diseases in humans was established [Yamagishi et al 2006]. In 2006, the Becerra laboratory discovered the first PEDF-signalling receptor – the patatin like phospholipase A2 / adipose triglyceride lipase (PNPLA2/ATGL) [Notari et al 2006]. In the same year, Farinas and colleagues discovered that PEDF maintains the stem cell niche in the brain of mice [Ramírez-Castillejo et al 2006]. In 2007 and 2009, the Choong [Ek et al 2007] and Jimenez [Orgaz et al 2009] laboratories discovered the antimetastatic effect of PEDF in osteosarcoma and melanoma respectively and attributed this mechanism to three-pronged phenomena; the effect of PEDF on angiogenesis, tumour cell invasion and tumour cell differentiation.

In 2008, Crawford and colleagues demonstrated that PEDF regulates lipid metabolism and

homeostasis via PNPLA2/ATGL in cultured human hepatocytes and PEDF null mice [Chung et al 2008]. The additional PEDF receptors were discovered as follows: the Bernard lab identified the laminin receptor in 2009 [Bernard et al 2009], F1-ATP synthase by Becerra lab in 2010 [Notari et al 2010] and PEDF signalling via LRP6, a Wnt co-receptor in 2011 [Park et al 2011]. In 2009, the Watt laboratory demonstrated that PEDF administration reduced insulin sensitivity in mice [Crowe et al 2009] and PEDF was identified by Famulla and colleagues in 2011 to induce inflammatory signalling in muscle and fat cells [Famulla et al 2011]. [Famulla et al 2011]. The determination of stem cell fate to osteogenesis by PEDF was reported by Li et al and the Dass laboratory who demonstrated its in vivo-bone forming potential in 2013 [Li et al 2013] and 2016 [Elahy et al 2016] respectively. In the same years, the Belinsky laboratory discovered that the PEDF null mutation caused mineralisation defects in bone [Belinsky et al 2016]. In 2014 and 2015, the potential of PEDF to induce ROS production via NADPH oxidase in human ovary [Kampfer et al 2013] and skeletal muscle [Carnagarin et al 2016b] was identified. PEDF emerged as the single potent biomarker to identify insulin resistance clinically in 2017 [Toloza et al 2017].

PEDF – structure and function

PEDF is an endogenously produced soluble monomeric glycoprotein widely expressed in foetal and adult tissues [Becerra 2006]. PEDF is a non-inhibitory serine protease that possesses a tertiary structure with three β sheets and 10 α helices [Simonovic et al 2001]. It has a typical protease-sensitive, reactive central loop (RCL: residues 373-380) near the C terminus which lacks the stressed to relaxed conformational transition like ovalbumin [Broadhead et al 2010]. PEDF has a molecular weight of 50kDa, and is a 418-amino acid polypeptide encoded by the SERPINF1 gene located on chromosome 17p13 [Tombran-Tink et al 1994]. The encoded PEDF polypeptide includes an amino-terminal secretion signal peptide, one N-glycosylation site (Asn^{285}) and a signature serpin sequence ($YHLNQPFIFVL^{398}$) [Steele et al 1993, Becerra 2006]. PEDF is a secretory protein and the truncation of the RCL bearing C-terminus results in the exposure of Asp414 to the negatively charged C-terminus and disrupts the hydrophobic interactions of Pro415 which is usually buried to interact with Phe231 and Leu223, impairing PEDF secretion. Gly 376 and Leu377 of RCL are also highly indispensable for efficient PEDF secretion [Shao et al 2003].

PEDF is biologically active at a concentration of 1-100nM and is found in the

interphotoreceptor matrix (IPM) and body fluids such as serum, vitreous and aqueous humour of the eye, cerebrospinal fluid and tears at physiologically relevant concentrations [Petersen et al 2003, Yasui et al 2003, Meyer et al 2002]. The activity of PEDF is determined by its interaction with its binding partners such as cell surface receptors and various complex proteins, glycosaminoglycans and proteoglycans **(Table 1)**. The PEDF crystal structure shows an asymmetric charge distribution with the lysine-populated basic residues (exposed to the surface located between β sheet and F helix) interacting with glycosaminoglycans and the three clustered amino acids binding heparin to induce a conformational change in PEDF structure that facilitates interaction with receptors [Alberdi et al 1999, Yasui et al 2003] whereas the acidic regions populated with aspartic and glutamic acid residues act as collagen binding sites [Meyer et al 2002]. PEDF is the only serpin that contains hyaluronan-binding sites [Becerra et al 2006] and the non-inhibitory serpin nature of PEDF is conferred by the presence of alanine and N-terminus residues at the active serpin site along with the three proline residues in the RCL which block PEDF access to target proteases [Kawaguchi et al 2010]. The study of PEDF structure-function relationship with recombinant DNA technology and chemical proteolysis has given a better understanding of PEDF activity. And a variety of PEDF-derived peptides have so far been demonstrated to exert diverse biological effects such as neurotrophic, anti-angiogenic, anti-tumorigenic and retinoprotective effects **(Table 2)**.

Decreased PEDF expression is associated with more aggressive cancers of the pancreas, breast, and prostate, is associated with poorer prognosis [Doll et al 2003, Uehara et al 2004, Zhou et al 2010], and it is implicated in G_o arrest in fibroblastic cell types [Pignolo et al 2003]. PEDF ablation studies revealed its significance in angiogenesis and stromal-epithelial homeostasis and metabolism. The angio-inhibitory role of PEDF is critical in pathological hyperangiogenic states such as augmented oxygen-induced retinopathy of PEDF null retinas [Huang et al 2008] and excessive neovascularisation associated with epithelial hyperplasia in the prostate and pancreas of PEDF-deficient mice [Doll et al 2003]. PEDF is an important metabolic factor as well with more studies demonstrating its role in obesity [Crowe et al 2009], type 2 diabetes mellitus [Nakamura et al 2009], metabolic syndrome [Chen et al 2010, Yamagishi et al 2006], polycystic ovarian syndrome [Yang et al 2011], hepatic disorders [Yamagishi et al 2010], essential hypertension [Nakamura et al 2010], and cardiovascular diseases such as atherosclerosis and acute coronary syndrome [Rychli et al 2009]. PEDF exerts its metabolic effects via ATGL interaction [Borg et al 2011]. PEDF and ATGL co-localise to the lipid droplets in HepG2 hepatocytes and ATGL null mice were used to elucidate the metabolic

effects of PEDF in obesity [Chung et al 2008].

PEDF, stem cells and their therapeutic implications.

PEDF regulation is necessary to maintain normal stem cell properties **(Table 3)** and their proliferative capacity in different organ systems [Doyon et al 2009]. Stem cells are characterised by self-renewal and totipotency – the ability to differentiate into specialised functional cell types [Alberdi et al 1999]. Stem cells are broadly categorised based on their origins: embryonic stem cells (ESCs) form the inner cell mass of the blastocyst and produce all three embryonic germ layers: ectoderm, endoderm, and mesoderm [Bilak et al 2002] whereas adult stem cells are ubiquitously distributed almost in all tissues to support tissue repair and regeneration during the normal course of injury and senescence [Smith 2001, Li and Xie 2005].

PEDF maintains ESC stemness, growth and pluripotency and has been implicated as a central mediator of the neurovascular stem cell niche [Pumiglia et al 2006] and in the proliferation/differentiation of tissue resident stem cells to multiple cell types [Notari et al 2006, Bernard et al 2009]. In addition to the stem cells, PEDF is also secreted by stromal cells such as the fibroblast feeder cells to support the growth of human ESCs [Ramírez-Castillejo et al 2006]. Human ESCs secrete PEDF in high levels to support the survival of retinal progenitors [Zhu et al 2011]. Periocular PEDF gene transfer and PEDF-expressing bone marrow MSCs reduce choroid neovascularisation (CNV) in experimental models [Saishin et al 2005, Hou et al 2010]. Subretinal injection of PEDF-transfected bmMSCs resulted in their differentiation into PEDF-secreting retinal pigment epithelial cells (RPE) replacing damaged cells [Arnhold et 2006] identifying the therapeutic potential of PEDF and stem cells in diseases such as age related macular degeneration (AMD) and retinopathies [Holekamp et al 2002, Lu et al 2009].

Mesenchymal stem cells (MSCs), are the multipotent progenitors found in various tissues such as placenta, tendons, skeletal muscle, fat, umbilical cord blood and amniotic fluid, capable of differentiating into mesodermal cells such as osteoblasts, adipocytes, chondrocytes, or myocytes and abundantly secrete PEDF [Otto et al 2011]. PEDF is an important differentiation factor for MSCs. MSC-derived PEDF attracts fibroblasts and avidly binds to the components of extracellular matrix to generate a localised niche for MSC differentiation. PEDF depletion completely ablates fibroblastic migration in the neural stem cell niche [Sarojini et al 2007]. PEDF-secreting MSCs derived from human umbilical cord not only differentiate into

endothelial cells in vitro but also participate in vascular repair in in vivo experimental vascular injury [Wang et al 2009]. In a mouse model of myocardial infarction, PEDF-secreting MSCs stimulated the migration and proliferation of cardiac fibroblasts to replace lost myocardial fibers following myocardial infarction [Liang et al 2011]. MSCs expressing high levels of PEDF demonstrated potent three-pronged tumour-suppressing effects [Zolochevska et al 2011] in cancers of human prostate and breast [Alberdi et al 1998], as well as hepatocellular carcinoma [Gao et al 2010], indicating that PEDF creates a formidable barrier to cancer development.

Activation of one pathway occurs at the expense of another, leading to concomitant blockade of competing pathways responsible for alternative lineage specification. PEDF treatment of MSCs results in Wnt activation, sclerostin (a Wnt antagonist) suppression and PPARγ suppression, favouring osteoblast over adipocyte lineage specification [Ross et al 2000, Li et al 2015]. PEDF mutation is the key defect in osteogenesis imperfecta [Belinsky et al 2016]. PEDF upregulates osteogenic genes and directs MSCs towards the osteoblast lineage [Li et al 2013, Elahy et al 2016]. Moreover, PEDF knockdown significantly decreases osteoblast differentiation and mineralisation, which is rescued by exogenous PEDF [Cai et al 2006] indicating the critical role of PEDF in bone formation. In a rodent model of soleus muscle necrosis, PEDF and PEDF peptide (Ser93-Leu112) promoted muscle regeneration in vivo demonstrating PEDF responsiveness in mesoderm-derived tissue sites such as muscle [Ho et al 2015]. Thus, PEDF regulates progenitor cell populations to direct musculoskeletal regeneration and bone formation.

The specific aims of this study:

1. To identify the effect of PEDF in insulin-dependent molecular pathways of glucose homeostasis in skeletal myocytes.
2. To identify if PEDF induces metabolic stress in skeletal myocytes.
3. To characterise the PEDF and insulin crosstalk in skeletal myocytes.

Summary of the thesis

PEDF is an important regulator of skeletal muscle metabolism, in particular the insulin–dependent metabolic processes. The attenuation of PEDF signalling is an effective approach to achieve better control over the insulin resistance of metabolic disorders and the PEDF-insulin antagonistic cross talk involved in the modulation of skeletal muscle biology could be an effective strategy to achieve controlled bone formation counteracting the PEDF-induced heterotrophic ossification.

Table 1: The interaction of PEDF with other proteins

Binding partners of PEDF	Binding residues of PEDF	References
Cell surface receptors: • PNPLA2/desnutrin/ATGL/iPLA2 • Laminin • F1ATPase/synthase • Low-density lipoprotein receptor-related protein 6 (LRP6)	• NH_2 terminus • 44-mer peptide (residues 58-101) Critical aa:Glu81, Ile83, Leu92, Ser95 • 25mer (aa46-70) • aa78-121	Alberdi et al 1999 Bernard et al 2009 Notari et al 2010 Filleur et al 2005
Extracellular matrix components: • Heparin binding • Hyaluronan binding • Collagen binding • Critical sites to collagen I binding	• Arg145, Lys146,Arg148 – three clustered • Lys^{189}, Lys^{191}, Arg^{194}, Lys^{197} • Glu^{41}, Glu^{42}, Glu^{43}, Asp^{44}, Asp^{64}, Asp^{256}, Asp^{258}, Glu^{290}, Glu^{291}, Glu^{296}, Asp^{300}, Glu^{304} • Asp255, Asp257, Asp299	Becerra et al 2006 Yasui et al 2003 Meyer et al 2002
Interaction with proteins of endoplasmic reticulum	Gly^{376}, Leu^{377}	Shao et al 2003
Neurotrophic protein kinase site (CK2)	Ser^{24}, Ser^{114}	Simonovic et al 2001
Antiangiogenic PKA phosphorylation site	Ser^{227}	Maik-Rachline et al 2006
c-jun-NH2 kinase	34-mer (aa24-57) Shorter proximal peptide TGA (aa16-26)	Filleur et al 2005
Nuclear molecules	Aa146-149	Tombran-Tink et al 2005

Table 2: The functional domains of PEDF

PEDF functional domains	Biological activity	References
PEDF truncation at the carboxy terminal: BH:Asp44-Pro418, BP:Asp44-Pro267 BA: Asp44-Thr121	Neuronal differentiation and survival	Becerra and Notario 2013
Synthetic peptides: at the NH_2 terminal:		
• PEDF 44-mer peptide (residues 58-101) Val78-Thr121	Neuroendocrine differentiation, proangiogenic and retinoprotective	Liu et al 2004
• a fragment within the 44-mer (ERT, residues 78-94) (Ser93-Leu112)	Neurotrophic and antiangiogenic	Aymerich et al 2001
• 34-mer (residues 24-57) Asp44-Asn77	Apoptosis and angioinhibitory	Alberdi et al 1999
• a shorter proximal peptide (TGA, residues 16-26) with the critical stretch L19VEEED24	Angioinhibitory	Filleur et al 2005
25-mer synthetic PEDF-derived peptides (termed StVOrth-1, -2 -3, and -4)		
• StVOrth-2 (residues 78–102)	Antitumorigenic and antimetastatic in osteosarcoma	Ek et al 2007
• StVOrth-3 (residues 90–114)	Increased cellular adhesion to type-1 collagen and suppressed VEGF expression Antitumorigenic and antimetastatic in osteosarcoma	
• StVOrth-4 (residues 387–411)	Inhibition of Matrigel invasion and suppressed VEGF expression	
• StVOrth-1 (residues 40–64)	Osteoblastic differentiation	

Table 3: PEDF and stem cells

PEDF - stem cell niche	Effect	Regulatory signalling	Therapeutic implications
Human embryonic stem cells	Self-renewal process	TGFβ/Activin/Nodal pathway [James et al 2005].	Diabetic retinopathy (DR) or age-related macular degeneration (AMD) [Ohno-Matsui et al 2001]
Neural stem cell niche	Self-renewal process Stemness	Non-canonical activation transcriptional activation of NF-κB [Ramírez-Castillejo et al 2006]	Neurodegenerative disorders
Endothelial stem cell niche	Self-renewal process Stemness	NSC-vascular crosstalk Notch signalling [Shen et al 2004]	Neurovascular disorders
Retinal progenitor stem cells Ciliary body Limbus of the cornea	Self-renewal in neurosphere cultures	Chx10 gene regulation [Marso et al 2010]	Retinopathies
Intestinal stem cells	Impairs proliferation	Nuclear receptor co-repressor (NCoR) dependent PEDF suppression	Gut Malignancies [Doyon et al 2009]
MSCs	Tumour suppressive [Zolochevska et al 2011]	Upregulation of the CXCR4/SDF1 (CXCL-12) axis	Cancers [Menon et al 2007]
Bone marrow-derived MSCs	Inhibited pathologic angiogenesis	Anti-angiogenic	Hepatocellular carcinoma [Gao et al 2010]
Adipose tissue-derived MSCs	Treatment of myocardial injury	PEDF knockdown improves MSCs therapeutic efficacy	Heart disease [Liang et al 2011]
	Tumour suppressive		Prostate and breast cancer [Alberdi et al 1998]
Human umbilical cord-derived MSCs	Differentiated into endothelial cells	Anti-inflammatory PEDF signalling	Vascular injury [Wang et al 2009]
Human MSCs	Osteogenesis	ERK/MAPK signalling	Bone healing and repair [Li et al 2013]

Graphical abstract

Chapter 2: Molecular aspects of glucose homeostasis in skeletal muscle – A focus on the molecular mechanisms of insulin resistance

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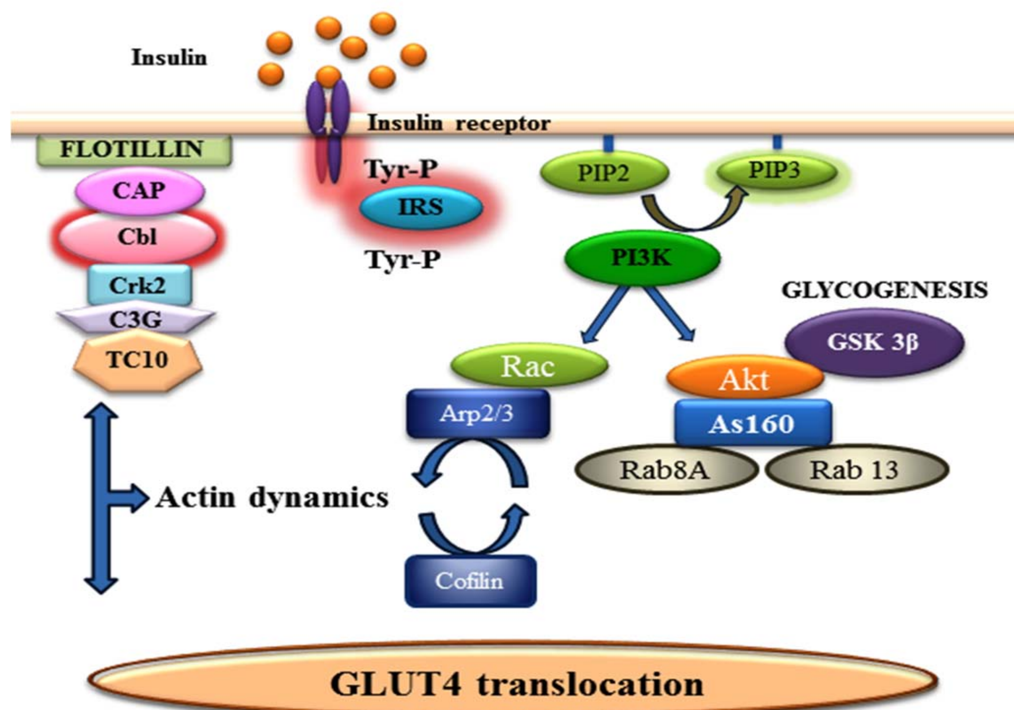

Insulin signalling is an intricate molecular network that redistributes the glucose available from the nutrient metabolism to generate energy and store the excess. Insulin-dependent glucose uptake is mediated through dynamic actin remodeling that moblises glucose transporter protein 4 (GLUT4) from intracellular storage site to the plasma membrane. The storage of excess glucose in the form of glycogen is mediated by insulin-dependent phosphorylation inactivation of glycogen synthase kinase 3β (GSK 3β), a rate limiting step of glycogenesis. Genetic and epigenetic modifications of the insulin signaling targets result in the complex phenomenon of insulin resistance.

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Review

Molecular aspects of glucose homeostasis in skeletal muscle – A focus on the molecular mechanisms of insulin resistance

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ABSTRACT

Among all the varied actions of insulin, regulation of glucose homeostasis is the most critical and intensively studied. With the availability of glucose from nutrient metabolism, insulin action in muscle results in increased glucose disposal *via* uptake from the circulation and storage of excess, thereby maintaining euglycemia. This major action of insulin is executed by redistribution of the glucose transporter protein, GLUT4 from intracellular storage sites to the plasma membrane and storage of glucose in the form of glycogen which also involves modulation of actin dynamics that govern trafficking of all the signal proteins of insulin signal transduction. The cellular mechanisms responsible for these trafficking events and the defects associated with insulin resistance are largely enigmatic, and this review provides a consolidated overview of the various molecular mechanisms involved in insulin-dependent glucose homeostasis in skeletal muscle, as insulin resistance at this major peripheral site impacts whole body glucose homeostasis.

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The skeletal muscle is the largest insulin-sensitive human organ that accounts for 85% of whole body glucose uptake and plays a crucial role in maintaining systemic glucose homeostasis. Skeletal muscle is the major peripheral site of insulin-stimulated glucose metabolism and plays a critical role in insulin sensitivity through interactive cross-talk with hepatic and adipose tissues. Peripheral insulin resistance is the central pathogenesis of major metabolic disorders, endocrine, cardiovascular, hepatic disorders, infections and cancer among others and insulin resistance in skeletal muscle impacts whole body glucose homeostasis (DeFronzo and Tripathy, 2009).

In skeletal muscles, the impaired glucose homeostasis is due to suboptimal insulin signalling that mediates various events of glucose metabolism and the underlying pathogenic mechanism can be at various levels of the signal transduction pathway including the ligand–receptor interaction. The insulin signalling network is a highly complex integrated network that controls various processes such as growth, development and nutrient metabolism. Insulin signal transduction at the cellular level involves three distinct biochemical steps: the hormone receptor interaction that incites the signal on receptor activation, transformation of this signal into the intracellular message which results in the final biological insulin responses such as chemical modification of various enzymes and transport systems. This review focuses on the critical molecular aspects of insulin signal transduction that co-ordinate the varied biochemical mechanisms to establish insulin mediated-glucose homeostasis in skeletal muscle.

1. An overview of the insulin-mediated molecular events involved in glucose homeostasis

The signal transduction process is initiated in the presence of insulin with the activation of the intrinsic tyrosine kinase of the insulin receptor (IR). Two major pathways of glucose metabolism are propagated in response to the activation of the insulin receptor kinase: phosphatidylinositol-3-kinase (PI3K) and the Cas-Br-murine ecotropic retroviral transforming sequence homologue Cbl pathway. The insulin-induced tyrosine phosphorylated insulin receptor substrate proteins (IRS) function as a signalling scaffold that binds to the Src homology 2 (SH2) domains of the downstream effectors. The tyrosine-phosphorylated IRS recruits PI3K which hydrolyses the membrane bound phosphatidylinositol bisphosphate (PIP2) to phosphatidylinositol-3,4,5-trisphosphate (PIP3) that recruits phosphoinositide-dependent kinase 1 (PDK1). PDK1 phosphorylates AKT at threonine 308, partially activating it and PDK2, now identified as mammalian target of rapamycin 2 (MTORC2) which in complex with Rictor and Sin completes AKT activation by phosphorylating it at its serine 473 residue (Saltiel and Kahn, 2001).

PI3K–AKT is a critical node which upon activation, phosphorylates and inactivates two downstream mediators: glycogen synthase kinase 3β (GSK3β) and AKT substrate (AS160). Cbl is activated upon IR-dependent tyrosine phosphorylation and associates with CAP (Cbl-associated protein) to activate GTP-binding TC10 and recruits CIP4/Gappex-5 complex to the plasma membrane regulating the protein trafficking of insulin signal transduction (Lodhi et al., 2007). A schematic overview of insulin-dependent molecular mechanisms involved in glucose homeostasis of skeletal muscle is given in Fig. 1.

2. The onset of the signal transduction process – factors at the level of insulin receptor that contribute to insulin resistance

2.1. Functional structure of the IR

The IR is a dynamic component of the cellular machinery which is regulated by various intracellular and extracellular signals including insulin itself. The IR is versatile in distribution and this integral protein is found in all cells of vertebrate species, but more concentrated on foetal tissues, skeletal myocytes, adipocytes and hepatocytes (Kahn, 1980). The IR is a heterotetrameric glycoprotein with α2β2 configuration. The α–β disulphide linkage is formed

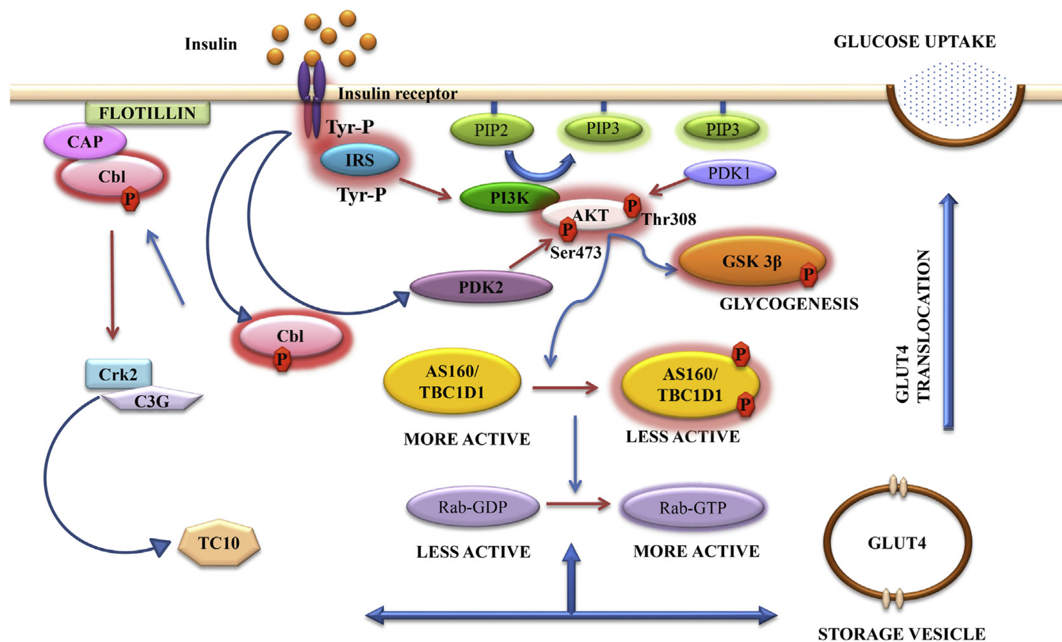

Fig. 1. Schematic overview of insulin-mediated molecular events involved in glucose homeostasis. Insulin binds and activates IR which tyrosine phosphorylates IRS to initiate the critical events of glucose metabolism. Insulin-dependent glucose metabolism can be dissected into two molecular axes: one governing glycogenesis which is Akt/PKB dependent phosphorylation-inactivation of GSK3β and the other governing GLUT4 translocation which is both PI3K–Akt-dependent AS160 phosphorylation and -independent involving Cbl phosphorylation.

between Cys-647 of the α subunit with Cys-872 of the β subunit and the inter-subunit disulphide bond is formed between Cys-524 and the corresponding residue of the adjacent α subunit that connects the α–β monomers.

The IR is coded by a gene located on the short arm of chromosome 19, and that contains 21 introns and 22 exons. The N-terminal exons 1–2, cysteine-rich domains containing exons 3–5 in combination with the carboxy terminal residues and the 12 amino acids sequence encoded by exon 11 determine the binding affinity of insulin (Kristensen et al., 1998). The α subunit is entirely extracellular and serves as the high affinity insulin binding site and also acts as a regulatory subunit of the intracellular catalytic subunit. In the absence of insulin, the subunit inflicts a structural constraint on the constitutively active kinase of the β subunit inhibiting its intrinsic tyrosine kinase activity. Deletion mutations of the α subunit or its proteolytic cleavage relieve its inhibitory effect on the intrinsic kinase activity of the β subunit.

The β-subunit of the IR is composed of a short extracellular domain, a transmembrane domain and an ATP-binding cytoplasmic domain with intrinsic tyrosine kinase activity. The activation of the intrinsic tyrosine kinase activity of the IR is a series of intramolecular sequential transphosphorylation reactions of adjacent tyrosine residues of the receptor and represents the essential first step of insulin-dependent glucose metabolism (Pessin and Saltiel, 2000).

The significant autophosphorylation sites of IR are located in its intracellular juxtamembrane region (Tyr-960) that resides in the NPXY-motif creates the recognition site for IRS proteins and is also essential for receptor internalisation. Mutations involving this region inhibit insulin stimulated tyrosine phosphorylation of downstream molecules such as IRS and Shc (White and Kahn, 1994). The next is the regulatory region (Tyr-1146, Tyr-1150 and Tyr-1151) of which Tyr-1150 phosphorylation plays an important role in receptor kinase activation and in a dephosphorylated state causes enzyme inactivation by occupying the catalytic site and preventing ATP binding (Hubbard et al., 1994). The phosphorylation of Tyr-1158, Tyr-1162, and Tyr-1163 is required for amplification of the kinase activity. The other phosphorylation sites include Tyr-1316 and Tyr-1322 at the carboxyl terminus (White et al., 1988).

2.2. IR defects in insulin resistance

The IR is a cognitive molecule which upon insulin binding generates the signal that kick starts the transduction process. Receptor defects in insulin resistance include receptor down-regulation and reduced affinity for insulin as observed in type 2 diabetics and impaired intrinsic tyrosine kinase activity of the IR which is acquired secondary to hyperglycaemia and other metabolic disturbances and also by inducers of insulin resistance such as TNFα (Hotamisligil et al., 1994) as seen in muscle biopsies of type 2 diabetics. Improved tyrosine kinase activity was observed upon weight loss (Freidenberg et al., 1988).

Insulin is the chief regulator of its receptor concentration and exerts the phenomenon of negative feedback regulation. Cells cultured in media containing insulin exhibited time and temperature dependent down regulation of insulin receptors and this was further confirmed by *in vivo* receptor studies in diseases involving high insulin levels (Bar et al., 1979). Basal hyperinsulinemia of metabolic disorders such as obesity, type 2 diabetes mellitus, acromegaly, islet tumors and chronic administration of exogenous insulin resulted in IR downregulation whereas elevated insulin binding was observed in hypoinsulinemic conditions such as anorexia nervosa and type 1 diabetes (Grunberger et al., 1983). Other factors such as diet, muscular exercise, thyroid hormones, glucocorticoids, androgens, oestrogens and cyclic nucleotides also regulate IR concentration and insulin binding (Kaplan, 1984).

IR is majorly governed by hormonal factors but in autoimmune conditions such as Graves disease, Myasthenia gravis and syndrome of extreme insulin resistance with acanthosis nigricans, circulating antibodies to IR causes insulin resistance (O'Rahilly et al., 1991; Cocozza et al., 1992). Mutations of the IR gene have been observed in leprechaunism and Rabson–Mendenhall syndrome (Taylor et al., 1990). IR gene polymorphisms such as Val–Met at residue 985, Lys–Glu at 1068 and Arg–Glu at 1164 were also seen in diabetics that could result in impaired ligand affinity and receptor activity (O'Rahilly et al., 1992). IR also exists in two isoforms as a consequence of alternate splicing of exon 11 determined by the presence or absence of 12 amino acid sequences in the carboxyl terminus of the α subunit as Ex11+ or Ex11− respectively. Ex11− binds insulin with two fold higher affinity and exhibited higher rates of receptor internalisation and recycling. Alternate splicing is determined by hormonal and metabolic factors and Ex11+ is positively correlated with body mass index, fasting glucose and fasting insulin levels. Alteration in the abundance of these isoforms also contributes to insulin resistance (Sesti et al., 2001). Insulin and insulin like growth factor1 (IGF1) form hybrid receptors that behave as IGF1 holoreceptors and are increased in skeletal muscle of type 2 diabetics, insulinomas and in the obese with high fasting insulin levels (Federici et al., 1998). Though subject to continuous modulation by various intracellular and extracellular events, IR is a major *in vivo* target site for cellular insulin response and IR phosphorylation and subsequent activation of its intrinsic kinase is the first step in the action of insulin on glucose metabolism.

3. IRS – the key adapter molecule of the intracellular transduction process governing insulin mediated glucose metabolism

The various intracellular substrates of IR involved in maintaining glucose homeostasis include the 180 kDa IRS that has been termed IRS1-6 and Cbl. Of the six different IRS isoforms, the widely distributed IRS1 is the predominant isoform mediating signal transduction governing the metabolic effect of insulin in skeletal muscle (Bouzakri et al., 2006) while IRS2 is linked with MAPK activation (Huang et al., 2005). The others like IRS3 are limited to adipocytes and brain while IRS5 and IRS6 have limited tissue expression and functional role in insulin signalling (Cai et al., 2003). The IRS proteins play the major role of translating the insulin signal to effector response through the PI3K pathway.

The IRS serve to be the major target of both autologous and heterologous feedback mechanisms modulating insulin signalling which involves serine/threonine phosphorylation, phosphatase-mediated dephosphorylation and ubiquitin-dependent, proteasome-mediated degradation (Haruta et al., 2000; Elcheby et al., 1999). Most of the human studies identified alterations majorly on IRS1 and few IRS2 dysfunctions in insulin resistance and moreover the protein isoforms differ in their activation kinetics (Ogihara et al., 1997), cellular compartmentalisation (Inoue et al., 1998) and structurally in case of IRS2 that binds to IR through unique kinase-regulatory-loop binding domain accounting for its special effects (Verhelle et al., 1996).

3.1. Functional structure of the IRS

The IRS proteins are characterised by the presence of plecktrin homology (PH) at the amino terminal end, phosphotyrosine-binding domain (PTB) and a variable length carboxyl tail. The PH domain is critical for sensitive IR–IRS interaction with the mediation of cytoskeletal elements and membrane phospholipids (Fallah et al., 2002). The PH domain contains the IRS homology domain 1

(Myers et al., 1995) and maintains crosstalk with G-proteins by interacting with Gβγ subunits (Luttrell et al., 1995; Pitcher et al., 1995).

The PTB domain accounts for high substrate affinity for IR. It binds to the NPXY motif of IR and provides the mechanism for receptor engagement at the juxtamembrane domain of IR regulated by Tyr-960 phosphorylation of IR. It houses the IRS homology domain 2 as well (Sun et al., 1995) which contains the several serine/threonine sites and bears similarity to the PTB domain of Shc (Gustafson et al., 1995). The carboxyl tail houses several tyrosine residues that gather into (YMXM or YXXM) tyr-phosphorylated consensus motifs that serve as docking sites for SH2 domains of the effectors such as p85 regulatory subunit of PI3K, phosphotyrosine phosphatases and Crk2 that binds to nucleotide exchange factors and activate G proteins (Zick, 2001), pertaining to glucose metabolism.

3.2. The involvement of IRS in the development of insulin resistance

Decreased levels of IRS expression was observed in hyperinsulinaemic states both in cell culture and mice models which is a consequence of IRS degradation as well as inhibition of protein synthesis at the transcriptional level (Hirashima et al., 2003). This reduced IRS levels coupled with reduced IR contributes to the insulin-resistant state of metabolic disorders in both rodents and humans such as in diabetes (Shimomura et al., 2000).

Serine phosphorylation of IRS is yet another important mechanism of insulin resistance. This sequential cascade mechanism is an array phenomenon which takes place in more than 50 potential phosphorylation sites identified so far, leading to a complex regulation of its activation state modulated by the IRS kinases (Copps and White, 2012). Serine phosphorylation of IRS in close proximity to its functional domains interrupts spatial matching required for protein–protein interaction which thereby prevents the signal-promoting IRS tyrosine phosphorylation blocking the normal conductance of metabolic insulin signalling. IRS serine phosphorylation is the principal negative feedback control mechanism adopted by the mediators of insulin signalling in the process of auto-regulatory inhibition in prolonged insulin stimulation as well as by the inducers of insulin resistance in the case of heterologous desensitisation mechanisms (Gual et al., 2005).

IRS serine phosphorylation at its PH domain interrupts binding to plasma membrane and IR, while serine phosphorylation of the PTB or the carboxyl domain induces IR–IRS1 dissociation with concomitant degradation and also interrupts IRS1 binding with its downstream effectors. IRS serine phosphorylation also mediates crosstalk between signalling systems (Halfon and Zick, 2009). The serine phosphorylation sites mediating skeletal muscle insulin resistance are discussed in Table 1.

4. PI3 kinase – AKT mediated signalling events of glucose homeostasis

4.1. Phosphatidylinositol-3,4,5-trisphosphate (PIP3) generation is a critical event

The selection and differentiation of insulin signal further towards metabolic events is achieved through interaction of SH2 domain-containing proteins to the tyrosine-phosphorylated IRS motifs. Phosphatidylinositol 3-kinase (PI3K) is the most important, immediate effector molecule that associates with the pTyr–IRS following its recruitment to the plasma membrane by the insulin–IR interaction.

PI3K is a class 1a 3-kinase, a heterodimer consisting of an 85-kDa regulatory subunit and a tightly-associated 110-kDa catalytic subunit. Class IA contains p110α, β and δ isoforms and p85α, p85β and p55γ isoforms encoded by three genes: PIK3R1, PIK3R2 and PIK3R3 genes respectively, with three splice variants of p85α: including p85α itself, p55α and p50α. However, p85α is the most abundant isoform and the predominant responsive element of the insulin pathway (Shepherd et al., 1998). The regulatory subunit isoforms share a common C-terminus, an inter-SH2 domain that has a p110-binding domain and a divergent N terminus varying in length and composition. The 339 amino acid long N terminus of p85α contain SH3 domain, breakpoint cluster-region homology domain and two proline-rich domains. This unique amino terminal of p85α binds to varied molecules regulating insulin sensitivity such as c-Cbl, Rac 1 GTPase and cell division cycle 42 (Cdc42) (Fang and Liu, 2001; Zheng et al., 1994) emphasising the importance of p85α-mediated regulation of insulin sensitivity besides its conventional regulatory role.

The functional heterodimeric PI3K possesses serine kinase activity that enables interaction with other signalling proteins and once activated catalyses the formation of phosphatidylinositol-

Table 1
Serine phosphorylation sites involved in negative regulation of insulin signalling.

Domain	Physiological significance	Serine phosphorylation sites	Inducers	References
pH	Critical for IR–IRS complex formation p85α association with IRS IRS1 binding to phosphatidyl inositol-4, 5-bisphosphate (PIP2)	Ser-24 (m)	Mouse pellet-like kinase, homologue of human IL-1 receptor-associated kinase	(Kim et al., 2005)
			Protein kinase Cs	(Kim et al., 2003; Nawaratne et al., 2006; Greene et al., 2006)
PTB	IR–IRS interaction Insulin-stimulated tyrosine phosphorylation. Insulin mediated-glucose uptake	Ser-307 (m)/Ser-312 (h)	Tumor necrosis factor α JNK IKKβ Free fatty acids	(Aguirre et al., 2002; Aguirre et al., 2000; Yuan et al., 2001; Kim et al., 2003)
COOH terminal domain	Docking and binding of downstream effectors to specific tyrosine residues at the carboxyl tail of IRS	Ser-522 (r) Ser-570 (m)	PKC ζ	(Giraud et al., 2007; Sommerfeld et al., 2004)
		Ser-612 (m) Ser-632 (m) Ser-662 (m) Ser-731 (m)	MAPK	(Mothe and Van Obberghen, 1996)
		Ser-636 (h)	ERK1/2	(Bouzakri et al., 2003)
		Ser-636 (h)/Ser-639 (m)	MTOR complex 1 S6 kinase 1	(Um et al., 2004)
		Ser-1101(m)	S6 kinase 1	(Tremblay et al., 2007)

3,4,5-trisphosphate (PIP3), a lipid second messenger that acts as a docking site for PH region of varied signalling molecules thereby altering their subcellular localisation or activity (Lietzke et al., 2000). The insulin-mediated PIP3 production influences multiple aspects of the insulin-dependent glucose metabolism. PIP3 is involved in the recruitment of cytoplasmic proteins to membrane localised signalosomes (Vanhaesebroeck et al., 2001) and as well as in the regulation of three main classes of downstream signalling molecules such as the AGC family of ser/thr kinases (Peterson and Schreiber, 1999), guanine nucleotide-exchange Rho GTPases (Mackay and Hall, 1998) and the tyrosine kinases of TEC family (Ziegler et al., 1993).

The negative regulation of the PI3K-mediated insulin signalling is almost exclusively by its p85 subunit and to an extent by various phosphatases (Fig. 2). The p85 subunit possesses two SH2 domains for interaction with tyrosine-phosphorylated IRS family and the upregulation of which has emerged as a second molecular mechanism of insulin resistance (Draznin, 2006). P110 is required for AKT activation and sustained response following insulin stimulation and inhibition of which induces severe insulin resistance with disruption of glucose homeostasis eventually resulting in diabetes (Braccini et al., 2012). Normally p85 subunits exist in stoichiometric excess with a balance maintained between the free p85 monomer pool and the p85–p100 heterodimers and in their competition for the tyrosine-phosphorylated IRS sites, with the latter being responsible for the PI3K activity.

An imbalance between the abundance of p85 and p110 altered PI3K activity in L6 cultured skeletal muscles. Dexamethasone treatment resulted in fourfold increase of p85α monomers, significantly decreasing PI3K activity as a result of competitive binding over the p85α–p110 heterodimer (Giorgino et al., 1997). Subsequently, animals were more sensitive to insulin with targeted disruption of p85α (Luo et al., 2005). Insulin resistance of pregnancy is also identified to be the consequence of human placental growth hormone-mediated increase in skeletal muscle p85α expression (Barbour et al., 2005). Increased expression of p85α, p85α to p110 ratio and reduced insulin sensitivity is observed in nutritionally-induced insulin resistance identifying increased p85α expression as an early molecular mechanism in the pathogenesis of insulin resistance (Cornier et al., 2006).

Other mechanisms of insulin resistance at this level include IRS–p85α sequestration and crosstalk between the p85α and stress kinase pathway. Monomeric p85 sequesters IRS1 into inert cellular foci incapable of PIP3 generation (Luo et al., 2005). It also activates JNK independent of its role as a PI3K holoenzyme component and may play an important role in whole body insulin sensitivity (Ueki et al., 2003). Inhibitory proteins are recruited by p85α which causes phospholipid phosphatase-mediated degradation of PIP3 (Elcheby et al., 1999). Summarily, a combination of IRS serine phosphorylation and enhanced p85α expression is adequate enough to induce clinical insulin resistance.

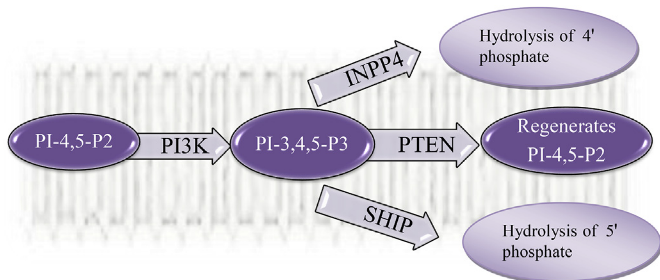

Fig. 2. Regulation of the active lipid second messenger (PI-3,4,5-P3). Insulin interaction with its receptor recruits cytosolic phosphatidylinositol-3-kinase (PI3K) to the plasma membrane which preferentially phosphorylates phosphatidylinositol-4,5-bisphosphate to yield the versatile second messenger, phosphatidylinositol-3,4,5 trisphosphate (PI-3,4,5-P3). PIP3 accumulates locally at the inner leaflet of the plasma membrane to attract proteins containing plecktrin homology (PH) domain. The phosphoinositide phosphatases involved in the negative modulation of PI3K signalling: phosphatase and tensin homolog (PTEN) that dephosphorylates PI-3,4,5-P3 to PI-4,5-P2, Src-homology 2 (SH2)-containing inositol 5′ phosphatase (SHIP) which dephosphorylates PIP3 to PI-3,4-P2 and inositol pyrophosphate 4-phosphatase type 2 (INPP4B) which hydrolyses 4′ phosphate of PI-3,4-P2.

5. AKT is a highly-regulated point of divergence for downstream signalling in metabolic homeostasis

The critical PH domain containing proximal transducers of the PI3K signalling include the best characterised serine/threonine kinase AKT/PKB and its juxtaposed upstream activator, PDK1 (Katso et al., 2001). PDK1 is a 63 KDa Ser/Thr kinase with carboxyl terminal PH domain that binds with high affinity to PIP3-containing regions of plasma membrane and phosphorylates the colocalised AKT at its threonine 308 residue partially activating it (Mora et al., 2004). The AKT activation is completed by its phosphorylation at serine 473 by phosphoinositide dependent kinase 2 (PDK 2) which is the mammalian target of rapamycin in complex with the rapamycin-insensitive companion of mTOR, Rictor, forming the mTORC 2 complex (Sarbassov et al., 2005).

Akt/PKB, a 57 KDa protein also known as the cellular homologue of the viral oncogene (v-Akt) is the member of cAMP-dependent protein kinase A/protein kinase G/protein kinase C (AGC) superfamily that plays a crucial role in PI3K-mediated metabolic actions of insulin by its varied substrate phosphorylation that includes transcription factors, kinases and other signalling proteins (Hajduch et al., 2001). Akt exists in three isoforms: α, β and γ (Akt 1, Akt2 and Akt3 respectively) with α being the major isoform expressed in skeletal muscle, β in adipocytes and γ is variably stimulated insignificant to skeletal muscle or adipocyte (Walkeret al., 1998). Each isoform possesses a PH domain containing an amino-terminal, a kinase domain and a regulatory domain at the carboxyl end. Insulin-dependent production of PIP3 causes the translocation of the cytosolic PKB to the plasma membrane inducing a conformational change resulting in site-specific phosphorylation: Thr-308 in the kinase domain and Ser-473 in the carboxy regulatory domain activating it (Coffer et al., 1998) and these activation sites are also involved in Akt regulation as shown in Fig. 3.

One of the most important biological responses of insulin in skeletal muscle is glucose disposal which involves either the oxidation of sugar or its storage in the form of glycogen. These critical events are Akt-mediated and involve phosphorylation-inactivation of two downstream targets. The primary physiological target of Akt/PKB is GSK3 which is implicated in the regulation of various cellular processes such as transcription factor activity and synthesis of protein and glycogen. Thus PI3K–PDK–Akt/PKB–GSK3 constitutes one important signalling arm of the insulin cascade regulating glycogenesis thereby glucose homeostasis. Akt/PKB also regulates glucose uptake by phosphorylation mediated inhibition of the Rab-GTPase-activating, 160 kDa Akt substrate proteins (AS160) which constitutes the other important signalling arm of glucose uptake (Thong et al., 2005).

Impaired insulin-stimulated Akt kinase activity has been implicated as an important component in the pathogenesis of insulin resistance despite unchanged total Akt protein levels in the skeletal muscle of diabetic mice (Shao et al., 2000) and this was same in the case of Goto–Kakizaki diabetic rats and in skeletal muscle from type 2 diabetic human subjects where Akt expression levels were similar to healthy controls (Krook et al., 1997). However insulin-stimulated Akt Ser-473 phosphorylation which closely

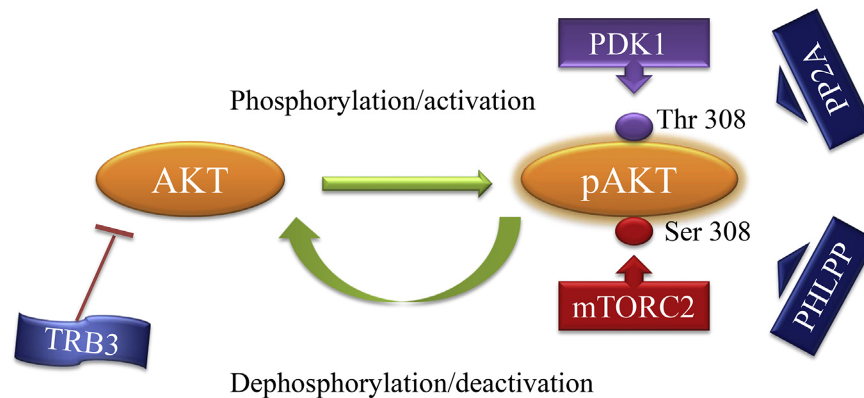

Fig. 3. The inhibitory regulation of Akt/PKB: several inhibitory molecules like protein phosphatase 2A (PP2A) selectively target Thr-308 (Kuo et al., 2008) and the PH domain leucine-rich repeat protein phosphatase (PHLPP) targets Ser-473 (Gao et al., 2005) directly dephosphorylating and deactivating Akt. A mammalian homolog of drosophila tribbles, tribble-3 (TRB3) is produced in the liver, binds to unphosphorylated Akt and prevents its phosphorylation and activation contributing to insulin resistance (Du et al., 2003).

follows Akt kinase activity is decreased by 32% in the skeletal muscle of diabetic mice (Shao et al., 2000). Such an effect was also observed in TNFα (Teruel et al., 2001), hyperglycaemia (Kurowski et al., 1999) or free fatty acid (Storz et al., 1999)-induced insulin resistance with significant impairment in downstream effects such as GLUT 4 recruitment to plasma membrane and these effects do not accompany the mitogenic branches of insulin signalling.

6. GSK 3 is a critical downstream effector, negatively regulated by insulin

Glycogen synthase kinase3 (GSK3) is a constitutively active cytosolic serine/threonine kinase, ubiquitous in distribution and acts as suppressor of insulin signalling. GSK3 is a key regulator of glycogen synthesis and exists in two isoforms- α and β that share 98% homology in their catalytic domain. GSK 3α has an extended N-terminal glycine rich domain and has a slightly higher molecular weight (Woodgett, 1990). Both isoforms have an inhibitory serine phosphorylation site at their N terminal and a facilitative tyrosine site, Y279 for α and Y216 for β at their catalytic loop. The insulin-mediated PI3K–AKT dependent phosphorylation-inactivation of GSK3 is achieved through phosphorylation of Ser-21 and Ser-9 of GSK3α and GSK3β respectively, while mutation of these sites to alanine results in constitutively-active GSK 3 (Cross et al., 1995). Insulin-dependent GSK 3 phosphorylation decreases its activity towards glycogen synthase thereby promoting dephosphorylation of glycogen synthase resulting in increased glycogenesis (Frame and Cohen, 2001). Other protein kinases such as protein kinase C (Goode et al., 1992), c-AMP dependent protein kinase A (PKA) (Fang et al., 2000) and S6 ribosomal protein kinase (Eldar-Finkelman et al., 1995) also inhibit GSK by phosphorylation of the same residues resulting in varied physiological consequences. However the PI3K/Akt is the predominant pathway of insulin-dependent GSK3 inhibition required to diminish its inhibitory effect towards its downstream substrate, glycogen synthase - the rate limiting enzyme for glycogen synthesis (Eldar-Finkelman and Kaidanovich, 2002).

GSK3 hyperactivity is demonstrated in high fat-fed and obesity-induced insulin resistance and is suggested as a potential factor contributing to the susceptibility of animals towards developing diabetes. This priming kinase is also an important physiological mediator in adipose differentiation and its heightened activity in diabetes causes adipose accumulation in insulin resistant conditions (Eldar-Finkelman et al., 1999). In humans, the expression and activity levels of GSK3 is significantly higher in the skeletal muscle of type 2 diabetics as compared to healthier controls (Nikoulina et al., 2000). The molecular mechanisms involved in GSK3-mediated insulin resistance involve phosphorylation of various proteins such as metabolic enzymes, signalling and transcription factors and by regulating pathways other than glycogen synthesis (Fig. 4). GSK3 modulates proximal insulin signalling by promoting serine phosphorylation of IRS1 (Qiao et al., 1999) and inhibits insulin-induced GLUT4 translocation to the plasma membrane through phosphorylation-inactivation of kinesin, a protein involved in GLUT4 membrane trafficking (Morfini et al., 2002). GSK3 also regulates transcription factors such as NFκB and participates in inflammation-induced insulin resistance (Yuan et al., 2001). Taken together, GSK3 regulates insulin resistance at multiple steps in multiple signalling pathways and targeting which could yield considerable clinical outcome.

7. Insulin-dependent GLUT4 trafficking involves discrete signalling pathways in skeletal muscle

The initial step of insulin mediated glucose uptake or storage involves the regulated cellular glucose entry mediated by the facilitative glucose transporter isoform, GLUT4. Insulin stimulated glucose transport into the skeletal muscle is the major cellular mechanism that regulates blood glucose concentration with nutrient ingestion which is oxidised to produce energy and the excess stored as glycogen. GLUT4 is an acutely regulated physiological parameter with unique characteristic of acute redistribution to plasma membrane with insulin stimulation and rapid intracellular disposition following the withdrawal of the stimuli (Bryant et al., 2002). The GLUT4 subsists in a complex interconnected tubulo-vesicular network, stationed majorly in the perinuclear region as highly insulin responsive GLUT4 storage vesicles (GSV) and remain linked to the endosomal-trans golgi network (TGN) system (Bryant et al., 2002). The GSV scaffold and compartmentalised GLUT4 is activated by insulin docking and fusing with the plasma membrane, getting exposed to the extracellular environment and serves as major targets for insulin mediated-GLUT4 mobilisation from within the cell to the plasma membrane in insulin-sensitive tissues (Olson et al., 1997).

GLUT4 is one of the 13 sugar transporters (GLUT1-12) encoded by SCL2A4 (solute carrier family 2A) and contains 12 membrane spanning domains (Joost and Thorens, 2001; Wood and Trayhurn, 2003). In skeletal muscle, GLUT 4 is the predominant isoform that enables facilitative diffusion of glucose in an ATP-independent manner (Hruz and Mueckler, 2001) along with GLUT1, GLUT5 and GLUT 12 which may also play a significant role in skeletal muscle (Stuart et al., 2000; Stuart et al., 2006). GLUT 4 belongs to

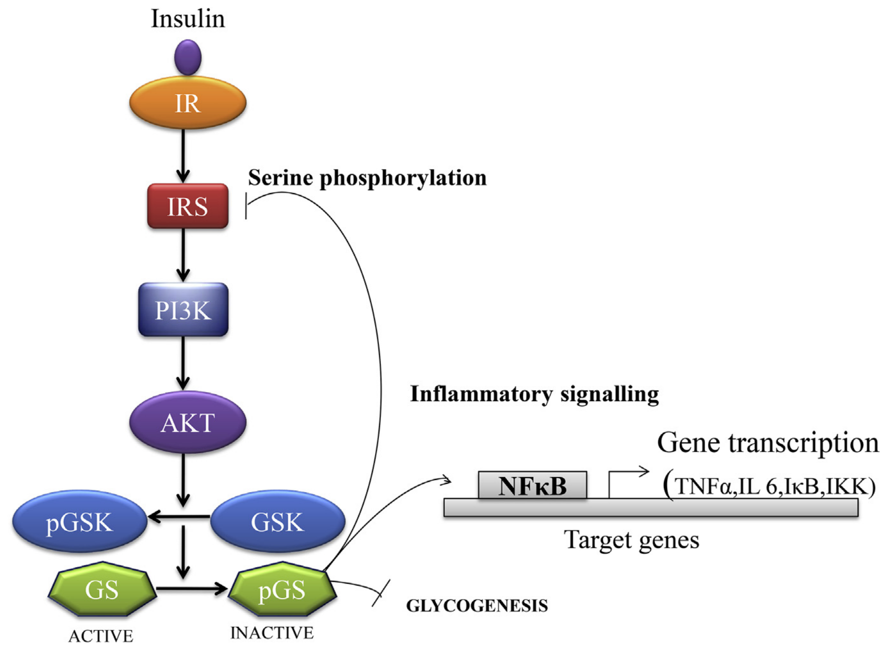

Fig. 4. Role of GSK3 in insulin resistance. With insulin stimulation, linear activation of IR/IRS1/Akt inactivates GSK3, resulting in dephosphorylation of glycogen synthase, in turn increasing glycogen synthesis. GSK3 induces serine phosphorylation of IRS and plays a positive role in NFκB activation, thereby inflammatory signalling. IR, insulin receptor, IRS, insulin receptor substrate, TNF, tumor necrosis factor, IL6, interleukin 6, IKK, IκBα kinase, IκB, inhibitor of NFκB.

class 1 glucose transporters based on its sequence homology and has both its amino and carboxyl terminals in the cytosol. The unique sequences that govern the kinetic aspect of the GLUT4 trafficking machinery includes the critical phenylalanine residue at the N-terminal (Melvin et al., 1999), a major cytoplasmic loop that harbours a phosphorylation site which together with phenylalanine motif plays an important role in GLUT4 endocytosis (Mohan et al., 2009). At the carboxyl end, the dileucine motif recruits clathrin adapters that initiates the packaging process of membrane proteins into clathrin-coated vesicles for further transport to the plasma membrane (Lalioti et al., 2001) and the acid targeting motif regulates tubule-vesicle trafficking of GLUT4 (Lim et al., 2001).

GLUT4 translocation is governed by discrete signalling pathways which act as molecular motors involving dynamic remodelling of the microtubular and actin cytoskeleton in response to insulin. The first involves the lipid kinase-PI3K signalling which bifurcates; one arm targets the serine/threonine kinase Akt/PKB-dependent phosphorylation-inhibition of AS160 and consequent activation of the RabGTPases which are Rab8A and Rab13 in skeletal muscle (Ishikura et al., 2007). The other arm involves a Rho family GTPase, Rac1-mediated cytoskeleton-regulating cascade involving dynamic cycle of cortical actin remodelling through the ARP2/3 complex and cofilin (Chiu et al., 2010). The second involves the proto-oncoprotein c-Cbl. The Cbl/CAP cascade is an independent yet significant pathway which along with PI3K–Akt, has evolved as the predominant pathways of insulin-dependent glucose uptake mediated by GLUT4 (Ribon et al., 1998).

7.1. Joint activation of Akt and Rac1 signalling is required for insulin-stimulated GLUT4 translocation in skeletal muscle

Insulin signalling downstream of PI3K segregates into two independent arms typified by the activation of Akt and Rac that jointly results in insulin-dependent GLUT4 translocation to the plasma membrane.

7.1.1. Rac1 mediates insulin dependent cytoskeletal remodelling

The actin cytoskeleton acts as a dynamic protein scaffold for the insulin signalling proteins and GLUT4 lies beneath the plasma membrane along the actin filaments (Randhawa et al., 2008). Any dysregulation at the level of cortical actin cytoskeletal remodelling impairs insulin signalling and is one of the central defects in peripheral insulin resistance (Ploug et al., 1998). Rac1is an obligatory actin skeleton-regulating Rho family GTPase which when activated by insulin–GTP loading and relocalisation, initiates its effector pathways: ARP2/3 (Chiu et al., 2010), P21 activated protein kinase (PAK) (JeBailey et al., 2004) and RalA (Nozaki et al., 2012) that enact a dynamic cycle of actin remodelling and rearrangement involved in insulin signalling network and insulin mediated-GLUT4 trafficking (Sylow et al., 2013). Rac1 exclusively regulates GLUT4 translocation in skeletal muscle cells (Marcusohn et al., 1995) and insulin-dependent Rac1 activation induces an iterative cycle of actin branching and depolymerisation mediated by the Arp2/3 complex. Arp2/3 complex increases actin polymerisation forming dynamic membrane ruffles and sets up a feedback reaction with the activation of phosphatases that dephosphorylate and thereby activate cofilin, the actin-depolymerising enzyme (Chiu et al., 2010). PAK is a serine/threonine kinase that exists in a feed forward-loop with Rac1. Insulin-activated Rac1 relieves PAK autoinhibition and induces its autophosphorylation on Thr-423/402 (PAK1/2) and redistribution from the cytosol to cortical actin (Dharmawardhane et al., 1997). Activated PAK modulates various kinases of actin dynamics that in turn phosphorylates and strips cofilin of its actin depolymerisation activity (Bokoch, 2003). Rac1 activation also causes subcellular redistribution and GTP loading of the small GTPase RalA which is involved in GLUT4 plasma-membrane targeting and exocytic transport machinery *via* Myo1C interaction in skeletal muscle (Chen et al., 2007). Ral A acts downstream of Rac1 and plays a pivotal role in insulin-dependent glucose uptake in muscle cells. A consolidated overview of Rac-signalling proteins involved in insulin resistance is given in Table 2.

Table 2
The proteins of insulin-dependent Rac signalling involved in insulin resistance.

Signalling proteins	Function	Supporting literature	Defects in insulin signalling
Rac	Cortical actin reorganisation	Overexpression of dominant negative Rac mutant (Khayat et al., 2000) SiRNA-mediated Rac knockdown (JeBailey et al., 2007)	Prevents actin remodelling and diminishes insulin-dependent GLUT4 translocation
Arp2/3 complex	Cortical actin polymerisation	SiRNA-mediated Arp2/3 knockdown (Chiu et al., 2010)	Failed to elicit actin remodelling at cell periphery and reduces insulin-mediated GLUT4 gain on the cell surface with unaltered basal GLUT4 levels.
Cofilin	Localises with insulin-induced remodelled actin to depolymerise	Cofilin knockdown (Chiu et al., 2010) or downregulation (Nishita et al., 2005; Sidani et al., 2007)	Promotes F-actin accumulation and decreases insulin-mediated GLUT4 translocation.
PAK		PAK 1 knockout mice (Wang et al., 2011)	Insulin resistance
Ral A	Resides in GLUT4 vesicles and regulates GLUT4 targeting to the plasma membrane and exocytosis in response to insulin	Ral a mutant or SiRNA-mediated knockdown (Nozaki et al., 2012)	Ablates insulin-dependent glucose uptake

7.2. Insulin-regulated AS160/TBC1D4acts as constitutive brake in signal transmission

As160 contains two phospho-tyrosine binding domain, RabGTPase activating domain (GAP) and carboxyl domain with several regulatory Ser/Thr residues of which Thr-642 and Ser-588 are key insulin dependent Akt-mediated phosphorylation sites required for GLUT4 translocation. Insulin-dependent Akt-mediated AS160 phosphorylation eliminates the GAP activity, effectively allowing activation and prevalence of Rab G proteins in skeletal muscle promoting GLUT4 exocytosis to the plasma-membrane thereby glucose transport (Kramer et al., 2006). Rab G proteins are quintessential molecular switches which on GTP loading engages mechanical effectors to regulate vesicle budding, mobilisation and fusion (Seabra and Wasmeier, 2004). The specific targets of the GAP activity downstream of AS160 in muscle cells include Rab8A and Rab13 which are suppressed in the basal state and upon insulin stimulation; Akt-dependent phosphorylation-inactivation of AS160 enables the GDP/GTP exchange factor (GEF)-mediated activation of the downstream Rabs to prevail, as required for GLUT4 translocation.

Activated Rab8A engages the myosin V molecules (Ishikura and Klip, 2008), which empowered by its intrinsic ATPase activity act as molecular motors to propel GLUT4 exocytosis aided by processing along actin filaments (Semenova et al., 2008). GTP-loaded Rab13 engages the adaptor protein Mical-L2 and delivers GLUT4 vesicles in proximity to cortical actin by interaction with actin binding filament, α-actinin4 (ACTN4) enabling cortical retention of GLUT4 and the vesicles tethers with actin through direct interaction with Myo1c (Foster et al., 2006). Recent studies in muscle cells have identified AS160 mutants such as AS160-4A, which cannot get phosphorylated by Akt, functions as constitutively active GAPs in a dominant fashion which prevents insulin-dependent GLUT4 translocation in adipocytes and muscle cells (Sano et al., 2003). Conversely, AS160 silencing by siRNA in muscle causes a gain in surface GLUT4 at the basal state and overexpression of Rab8A or Rab13 in muscle cells rescued insulin-dependent GLUT4 translocation from inhibition by constitutively active AS160-4A (Sun et al., 2010).

7.3. CAP–CBl may be a second signalling pathway required for insulin-stimulated glucose transport

Though the signalling pathways that interconnect the insulin receptor to GLUT4 trafficking involves PI3K as the critical node, Cbl pathway has evolved as yet another major signalling network that completes insulin-mediated cytoskeletal and GLUT4 events. The Cbl axis is wortmannin-independent and TC10-dependent, which functions in parallel with PI3K to complete the insulin-dependent Glut4 translocation cascade. This pathway involves several adapter molecules such as CAP, C3G and Crk2 with definitive roles that complement and complete the IR–Cbl–GLUT4 axis. The binding of insulin with its receptor initiates two simultaneous events of this pathway – recruitment of Cbl to the IR site *via* CAP interaction and TC10 localisation to the lipid raft subdomain of the plasma membrane.

Insulin mediated-tyrosine phosphorylation of Cbl (Ribon et al., 1998) dissociates the CAP–Cbl complex from the receptor site and generates a specific docking site for SH2 domain of CRK2. The carboxy terminal domain of CAP binds Cbl while the amino-terminal specifically interacts with flotillin, an integral membrane protein localised at the lipid rafts of the plasma membrane and forms the flotillin–CAP–Cbl ternary complex. The CAP binds with Cbl resulting in the recruitment of Cbl and co-recruitment of Crk2–C3G complex to the flotilin/caveolin-enriched compartments of the plasma membrane (Baumann et al., 2000). The C3G is the insulin-regulated guanine nucleotide exchange factor (GEF), specific for TC10 which switches the inactive GDP-bound state of TC10 to its activated GTP bound state. The TC10 activation is temporally and spatially correlated with C3G recruitment. Tyrosine phosphorylated Cbl associates with the CrkII adaptor protein bound to C3G GTPase exchange factor resulting in GTP/GDP exchange and activation of TC10GTPase in an insulin-dependent manner (Knudsen et al., 1994). CRK 2 docks Cbl at the amino-terminal end of its SH2 domain and the SH3 domain of Crk2 interacts with C3G at its proline-rich domain forming the constitutive Crk2–C3G complex (Gotoh et al., 1995) when translocated into lipid rafts, C3G comes into proximity with TC10, activating it (Chiang et al., 2001).

TC10 activation is GTP-dependent (Neudauer et al., 1151) and *via* CAP–Cbl–CrkII ultimately impinges on actin dynamics and GLUT4 translocation, as overexpression of CAP and TC10 mutants interfered with one or both of these outcomes (Chiang et al., 2001). TC10 activation was also linked to PI(3)P production through activation of class II PI3K downstream to TC10 (Maffucci et al., 2003). TC10 may affect GLUT4 translocation irrespective of its Cbl–CrkII-activated GTPase activity as well (Chunqiu Hou and Pessin, 2003). However, the participation of the CAP–Cbl–TC10 link in skeletal muscle requires further studies as controversies exist such as the finding that Cbl is not phosphorylated in muscle tissue in response to *in vivo* insulin challenge (Thirone et al., 2004). Similarly, L6 myoblasts that do not express CAP mount an insulin-dependent GLUT4 translocation, and overexpression of TC10 mutants in

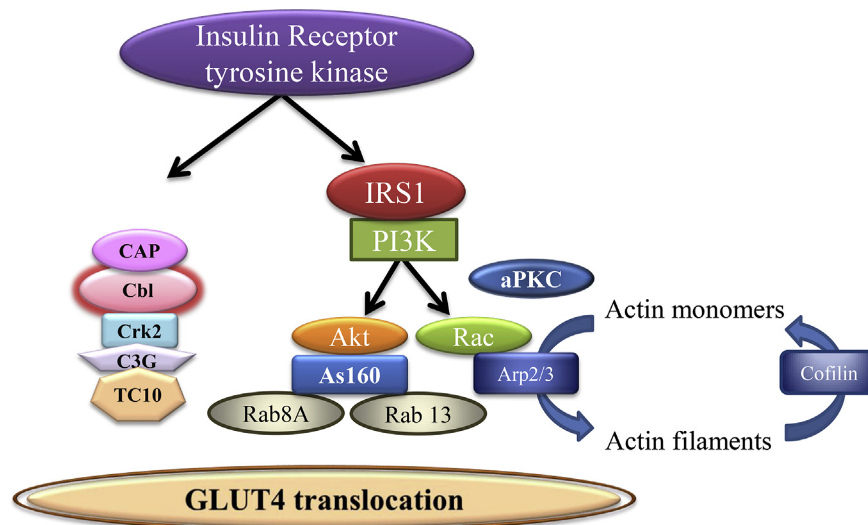

Fig. 5. Consolidated overview of insulin signalling pathways involved in GLUT4 translocation in muscle cells. The first involves the phosphatidylinositol 3-kinase (PI3K), and the second involves the proto-oncoprotein c-Cbl. The important PI3K targets in muscle cells that play a role in insulin-stimulated GLUT4 translocation are the serine/threonine kinase Akt/protein kinase B (PKB), Rac and the atypical protein kinase C (PKC) isoform, PKCζ. The mechanism of activation of PKCζ, although not clear, may involve its recruitment to intracellular membranes, and its presence has been shown in intracellular GLUT4-containing vesicles. Although Akt and PKCζ have both been implicated in insulin action, there are numerous downstream targets of PI3K — including proteins such as Rac that play a pivotal role in membrane transport through modulation of actin dynamics with the activation of it downstream effectors such as Arp2/3, PAK and Ral A. The second putative insulin-dependent signalling pathway that influences GLUT4 translocation operates independent of PI3K and involves the formation of a dimeric complex that comprises c-Cbl and the c-Cbl-associated protein CAP. Insulin triggers the movement of c-Cbl–CAP complex into cell-surface lipid rafts through association with flotillin, a raft protein. Tyrosine-phosphorylated c-Cbl then recruits a complex of CrkII and C3G into lipid rafts. C3G is a guanine-nucleotide exchange factor for TC10 that is constitutively localised in lipid rafts, and that catalyses GTP loading and subsequent activation.

these cells fails to prevent such translocation, unlike in adipocytes (JeBailey et al., 2004).

Other signalling pathways such as atypical protein kinase C-PKCζ has also been implicated in insulin-induced glucose transport in muscle cells through actin remodelling (Liu et al., 2006). PI3K also activates PKCζ which accumulate in insulin-treated cells and the expression of which induces GLUT4 translocation where as dominant-interfering PKCλ inhibits GLUT4 translocation (Kotani et al., 1998; Kitamura et al., 1998). Though PI3-kinase activation is critical, the protein kinase targets that mediate the effects of this pathway requires to be elucidated further. PI3K activation is essential for GLUT4 translocation in response to insulin, and its downstream effectors Akt and Rac control GLUT4 traffic within and from intracellular compartments and at the plasma membrane as well. This signalling axis was validated in all muscle cell systems studied so far. The contribution of the CAP–Cbl–TC10 link in insulin-dependent GLUT 4 mobilisation warrants more studies from a skeletal muscle perspective. A summary of the pathways involved in insulin-dependent GLUT 4 translocation is described in Fig. 5.

8. Conclusion

Insulin signalling is an intricate molecular network and exploring the molecules involved that could play a potential role in insulin resistance is a big challenge. Insulin resistance by itself is a complex phenomenon subject to both genetic and epigenetic regulation and it is necessary to consider that there may be no single or common defect that underlies peripheral insulin resistance. The cellular signalling systems and the molecular targets that are modified during insulin resistance are however worthy of exploration despite the fact that there is no evidence for a common mutation in any signalling pathway. A substantial body of evidence supports the involvement of the above discussed proteins in the pathogenesis of type 2 diabetes and other metabolic disorders. However, it is uncertain whether these defects represent primary lesions contributing to insulin resistance or results secondary to hyperinsulinaemia. The relative contribution of the several molecular insulin targets involved in glucose homeostasis can only be resolved by stringent techniques such as quantitative determination of the involved proteins right from receptor to the effectors and a wide population-based analysis to elicit their pathogenic role. Obviously, this approach can only be applied to known effector proteins and this makes exploring the insulin signal transduction proteins a worthwhile effort. Identifying newer molecules would pave the way for identifying novel targets and newer strategies in handling the complex phenomenon of insulin resistance.

Conflicts of interest

The authors declare no conflict of interest.

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References

Aguirre, V., et al., 2000. The c-Jun NH(2)-terminal kinase promotes insulin resistance during association with insulin receptor substrate-1 and phosphorylation of Ser(307). J. Biol. Chem. 275, 9047–9054.

Aguirre, V., et al., 2002. Phosphorylation of Ser307 in insulin receptor substrate-1 blocks interactions with the insulin receptor and inhibits insulin action. J. Biol. Chem. 277, 1531–1537.

Bar, R.S., et al., 1979. Regulation of insulin receptors in normal and abnormal physiology in humans. Adv. Intern. Med. 24, 23–52.

Barbour, L., et al., 2005. Increased P85alpha is a potent negative regulator of skeletal muscle insulin signaling and induces in vivo insulin resistance associated with growth hormone excess. J. Biol. Chem. 280, 37489–37494.

Baumann, C.A., et al., 2000. CAP defines a second signalling pathway required for insulin-stimulated glucose transport. Nature 407, 202–207.

Bokoch, G.M., 2003. Biology of the p21-activated kinases. Ann. Rev. Biochem. 72,

743–781.
Bouzakri, K., et al., 2003. Reduced activation of phosphatidylinositol-3 kinase and increased serine 636 phosphorylation of insulin receptor substrate-1 in primary culture of skeletal muscle cells from patients with type 2 diabetes. Diabetes 52, 1319–1325.
Bouzakri, K., et al., 2006. siRNA-based gene silencing reveals specialized roles of IRS-1/Akt2 and IRS-2/Akt1 in glucose and lipid metabolism in human skeletal muscle. Cell Metab. 4, 89–96.
Braccini, L., et al., 2012. PI3K keeps the balance between metabolism and cancer. Adv. Biol. Regul. 52, 389–405.
Bryant, N.J., et al., 2002. Regulated transport of the glucose transporter GLUT4. Nat. Rev. Mol. Cell Biol. 3, 267–277.
Cai, D., et al., 2003. Two new substrates in insulin signaling, IRS5/DOK4 and IRS6/DOK. J. Biol. Chem. 278, 25323–25330.
Chen, X.W., et al., 2007. Activation of RalA is required for insulin-stimulated Glut4 trafficking to the plasma membrane via the exocyst and the motor protein Myo1c. Dev. Cell. 13, 391–404.
Chiang, S.H., et al., 2001. Insulin-stimulated GLUT4 translocation requires the CAP-dependent activation of TC10. Nature 410, 944–948.
Chiu, T.T., et al., 2010. Arp2/3- and cofilin-coordinated actin dynamics is required for insulin-mediated GLUT4 translocation to the surface of muscle cells. Mol. Biol. Cell 21, 3529–3539.
Chunqiu Hou, J., Pessin, J.E., 2003. Lipid raft targeting of the TC10 amino terminal domain is responsible for disruption of adipocyte cortical actin. Mol. Biol. Cell 14, 3578–3591.
Cocozza, S., et al., 1992. NIDDM associated with mutation in tyrosine kinase domain of insulin receptor gene. Diabetes 41, 521–526.
Coffer, P.J., et al., 1998. Protein kinase B (c-Akt): a multifunctional mediator of phosphatidylinositol 3-kinase activation. Biochem. J. 335, 1–13.
Copps, K.D., White, M.F., 2012. Regulation of insulin sensitivity by serine/threonine phosphorylation of insulin receptor substrate proteins IRS1 and IRS2. Diabetologia 55, 2565–2582.
Cornier, M.A., et al., 2006. Nutritional up-regulation of p85α expression is an early molecular manifestation of insulin resistance. Diabetologia 49, 748–754.
Cross, D.A., et al., 1995. Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B. Nature 378, 785–789.
DeFronzo, R.A., Tripathy, D., 2009. Skeletal muscle insulin resistance is the primary defect in type 2 diabetes. Diabetes Care 32, S157–S163.
Dharmawardhane, S., et al., 1997. Localization of p21-Activated kinase 1 (PAK1) to pinocytic vesicles and cortical actin structures in stimulated cells. J. Cell Biol. 138, 1265–1278.
Draznin, B., 2006. Molecular mechanisms of insulin resistance: serine phosphorylation of insulin receptor substrate-1 and increased expression of p85α: the two sides of a Coin. Diabetes 55, 2392–2397.
Du, K., et al., 2003. TRB3: a tribbles homolog that inhibits Akt/PKB activation by insulin in liver. Science 300, 1574–1577.
Elcheby, M., et al., 1999. Increased insulin sensitivity and obesity resistance in mice lacking the protein tyrosine phosphatase-1B gene. Science 283, 1544–1548.
Eldar-Finkelman, H., et al., 1995. Inactivation of glycogen synthase kinase-3 by epidermal growth factor is mediated by mitogen-activated protein kinase/p90 ribosomal protein S6 kinase signaling pathway in NIH/3T3 cells. J. Biol. Chem. 270, 987–990.
Eldar-Finkelman, H., et al., 1999. Increased glycogen synthase kinase-3 activity in diabetes- and obesity-prone C57BL/6J mice. Diabetes 48, 1662–1666.
Eldar-Finkelman, H., Kaidanovich, O., 2002. The role of glycogen synthase kinase-3 in insulin resistance and Type 2 diabetes. Expert Opin. Ther. Targets 6, 555–561.
Fallah, F.J., et al., 2002. The pleckstrin homology (PH) domain-interacting protein couples the insulin receptor substrate 1 PH Domain to insulin signaling pathways leading to mitogenesis and GLUT4 translocation. Mol. Cell Biol. 22, 7325–7336.
Fang, X., et al., 2000. Phosphorylation and inactivation of glycogen synthase kinase 3 by protein kinase A. Proc. Natl. Acad. Sci U. S. A. 97, 11960–11965.
Fang, D., Liu, Y.C., 2001. Proteolysis-independent regulation of PI3K by Cbl-b-mediated ubiquitination in T cells. Nat. Immunol. 2, 870–875.
Federici, M., et al., 1998. Expression of insulin/IGF-I hybrid receptors is increased in skeletal muscle of patients with chronic primary hyperinsulinemia. Diabetes 47, 87–92.
Foster, L.J., et al., 2006. Insulin-dependent interactions of proteins withGLUT4 revealed through stable isotope labeling by amino acids in cell culture (SILAC). J. Proteome Res. 5, 64–75.
Frame, S., Cohen, P., 2001. GSK3 takes centre stage more than 20 years after its discovery. Biochem. J. 359, 1–16.
Freidenberg, G.R., et al., 1988. Reversibility of defective adipocyte insulin receptor kinase activity in non-insulin-dependent diabetes mellitus. Effect of weight loss. J. Clin. Invest. 82, 1398–1406.
Gao, T., et al., 2005. PHLPP: a phosphatase that directly dephosphorylates Akt, promotes apoptosis, and suppresses tumor growth. Mol. Cell 18, 13–24.
Giorgino, F., et al., 1997. Specific increase in p85α expression in response to dexamethazone is associated with inhibition of insulin-like growth factor-I stimulated phosphatidylinositol 3-kinase activity in cultured muscle cells. J. Biol. Chem. 272, 7455–7463.
Giraud, J., et al., 2007. Phosphorylation of Irs1 at SER-522 inhibits insulin signaling. Mol. Endocrinol. 21, 2294–2302.
Goode, N., et al., 1992. Differential regulation of glycogen synthase kinase-3 beta by protein kinase C isotypes. J. Biol. Chem. 267, 16878–16882.
Gotoh, T., et al., 1995. Identification of Rap1 as a target for the Crk SH3 domain-binding guanine nucleotide-releasing factor C3G. Mol. Cell Biol. 15, 6746–6753.
Greene, M.W., et al., 2006. PKCδ-mediated IRS-1 Ser24 phosphorylation negatively regulates IRS-1 function. Biochem. Res. Commun. 349, 976–986.
Grunberger, G., et al., 1983. Insulin receptors in normal and disease states. Clin. Endocrinol. Metab. 12, 191–219.
Gual, P., et al., 2005. Positive and negative regulation of insulin signaling through IRS-1 phosphorylation. Biochimie 87, 99–109.
Gustafson, T.A., et al., 1995. Phosphotyrosine-dependent interaction of SHC and insulin receptor substrate 1 with the NPEY motif of the insulin receptor via a novel non-SH2 domain. Mol. Cell Biol. 15, 2500–2508.
Hajduch, E., et al., 2001. Protein kinase B (PKB/Akt) – a key regulator of glucose transport? FEBS Lett. 492, 199–203.
Halfon, B.S., Zick, Y., 2009. Phosphorylation of IRS proteins, insulin action, and insulin resistance. Am. J. Physiol. Endocrinol. Metab. 296, E581–E591.
Haruta, T., et al., 2000. A rapamycin-sensitive pathway down-regulates insulin signaling via phosphorylation and proteasomal degradation of insulin receptor substrate-1. Mol. Endocrinol. 14, 783–794.
Hirashima, Y., et al., 2003. Insulin down-regulates insulin receptor substrate-2 expression through the phosphatidylinositol 3-kinase/Akt pathway. J. Endocrinol. 179, 253–266.
Hotamisligil, G.S., et al., 1994. Reduced tyrosine kinase activity of the insulin receptor in obesity-diabetes. Central role of tumor necrosis factor-alpha. J. Clin. Invest. 94, 1543–1549.
Hruz, P.W., Mueckler, M.M., 2001. Structural analysis of the GLUT1 facilitative glucose transporter (review). Mol. Membr. Biol. 18, 183–193.
Huang, C., et al., 2005. Differential contribution of insulin receptor substrates 1 versus 2 to insulin signaling and glucose uptake in L6 myotubes. J. Biol. Chem. 280, 19426–19435.
Hubbard, S.R., et al., 1994. Crystal structure of the tyrosine kinase domain of the human insulin receptor. Nature 372, 746–754.
Inoue, G., et al., 1998. Dynamics of insulin signaling in 3T3-L1 adipocytes. Differential compartmentalization and trafficking of insulin receptor substrate (IRS)-1 and IRS-2. J. Biol. Chem. 273, 11548–11555.
Ishikura, S., et al., 2007. Rabs 8A and 14 are targets of the insulin-regulated Rab-GAP AS160 regulating GLUT4 traffic in muscle cells. Biochem. Res. Commun. 353, 1074–1079.
Ishikura, S., Klip, A., 2008. Muscle cells engage Rab8A and myosin Vb in insulin-dependent GLUT4 translocation. Am. J. Physiol. Cell Physiol. 295, C1016–C1025.
JeBailey, L., et al., 2004. Skeletal muscle cells and adipocytes differ in their Reliance on TC10 and Rac for insulin-induced actin remodeling. Mol. Endocrinol. 18, 359–372.
JeBailey, L., et al., 2007. Ceramide- and oxidant-induced insulin resistance involve loss of insulin-dependent Rac-activation and actin remodeling in muscle cells. Diabetes 56, 394–403.
Joost, H.G., Thorens, B., 2001. The extended GLUT-family of sugar/polyol transport facilitators: nomenclature, sequence characteristics, and potential function of its novel members (review). Mol. Membr. Biol. 18, 247–256.
Kahn, C.R., 1980. Role of insulin receptors in insulin-resistant states. Metab. Clin. Exp. 29, 455–466.
Kaplan, S.A., 1984. The insulin receptor. J. Pediatr. 104, 327–336.
Katso, R., et al., 2001. Cellular function of phosphoinositide 3-kinases: implications for development, homeostasis, and cancer. Ann. Rev. Cell Dev. Biol. 17, 615–675.
Khayat, Z.A., et al., 2000. Insulin-induced actin filament remodelingcolocalizes actin with phosphatidylinositol 3-kinase and GLUT4 in L6 myotubes. J. Cell Sci. 113, 279–290.
Kim, Y.B., et al., 2003. Insulin-stimulated protein kinase C lambda/zeta activity is reduced in skeletal muscle of humans with obesity and type 2 diabetes: reversal with weight reduction. Diabetes 52, 1935–1942.
Kim, J., et al., 2005. Phosphorylation of Ser24 in the pleckstrin homology domain of insulin receptor substrate-1 by mouse pelle-like kinase/interleukin-1 receptor-associated kinase: cross-talk between inflammatory signaling and insulin signaling that may contribute to insulin resistance. J. Biol. Chem. 280, 23173–23183.
Kitamura, T., et al., 1998. Requirement for activation of the serine-threonine kinase Akt (protein kinase B) in insulin stimulation of protein synthesis but not of glucose transport. Mol. Cell Biol. 18, 3708–3717.
Knudsen, B.S., et al., 1994. Four proline-rich sequences of the guanine-nucleotide exchange factor C3G bind with unique specificity to the first Src homology 3 domain of Crk. J. Biol. Chem. 269, 32781–32787.
Kotani, K., et al., 1998. Requirement of atypical protein kinase clambda for insulin stimulation of glucose uptake but not for Akt activation in 3T3-L1 adipocytes. Mol. Cell Biol. 18, 6971–6982.
Kramer, H.F., et al., 2006. Distinct signals regulate AS160 phosphorylation in response to insulin, AICAR, and contraction in mouse skeletal muscle. Diabetes 55, 2067–2076.
Kristensen, C., et al., 1998. Expression and characterization of a 70-kDa fragment of the Insulin receptor that binds insulin: minimizing ligand binding domain of the insulin receptor. J. Biol. Chem. 273, 17780–17786.
Krook, A., et al., 1997. Improved glucose tolerance restores insulin-stimulated Akt kinase activity and glucose transport in skeletal muscle from diabetic Goto–Kakizaki rats. Diabetes 46, 2110–2114.
Kuo, Y.C., et al., 2008. Regulation of phosphorylation of Thr-308 of Akt, cell proliferation, and survival by the B55alpha regulatory subunit targeting of the protein phosphatase 2A holoenzyme to Akt. J. Biol. Chem. 283, 1882–1892.

Kurowski, T.G., et al., 1999. Hyperglycemia inhibits insulin activation of Akt/protein kinase B but not phosphatidylinositol 3-kinase in rat skeletal muscle. Diabetes 48, 658–663.
Lalioti, V., et al., 2001. Targeting motifs in GLUT4 (review). Mol. Membr. Biol. 18, 257–264.
Lietzke, S.E., et al., 2000. Structural basis of 3-phosphoinositide recognition by pleckstrin homology domains. Mol. Cell 6, 385–394.
Lim, S.N., et al., 2001. Identification of discrete classes of endosome-derived small vesicles as a major cellular pool for recycling membrane proteins. Mol. Biol. Cell 12, 981–995.
Liu, L.Z., et al., 2006. Protein kinase Czeta mediates insulin-induced glucose transport through actin remodeling in L6 muscle cells. Mol. Biol. Cell 17, 2322–2330.
Lodhi, I.J., et al., 2007. Gapex-5, a Rab31 guanine nucleotide exchange factor that regulates Glut4 trafficking in adipocytes. Cell Metab. 5, 59–72.
Luo, J., et al., 2005. The p85 regulatory subunit of phosphoinositide 3-kinase down-regulates IRS-1 signaling via the formation of a sequestration complex. J. Cell Biol. 170, 455–464.
Luttrell, L., et al., 1995. Effect of cellular expression of pleckstrin homology domains on Gi-coupled receptor signaling. J. Biol. Chem. 270, 12984–12989.
Mackay, D.J., Hall, A., 1998. Rho GTPases. J. Biol. Chem. 273, 20685–20688.
Maffucci, T., et al., 2003. Insulin induces phosphatidylinositol-3-phosphate formation through TC10 activation. EMBO J. 22, 4178–4189.
Marcusohn, J., et al., 1995. The GTP-binding protein Rac does not couple PI 3-kinase to insulin-stimulated glucose transport in adipocytes. Curr. Biol. 5, 1296–1302.
Melvin, D.R., et al., 1999. Analysis of amino and carboxy terminal GLUT-4 targeting motifs in 3T3-L1 adipocytes using an endosomal ablation technique. Biochemistry 38, 1456–1462.
Mohan, S.S., et al., 2009. Homology modeling of GLUT4, an insulin regulated facilitated glucose transporter and docking studies with ATP and its inhibitors. J. Biomol. Struct. Dyn. 26, 455–464.
Mora, A., et al., 2004. PDK1, the master regulator of AGC kinase signal transduction. Semin. Cell Dev. Biol. 15, 161–170.
Morfini, G., et al., 2002. Glycogen synthase kinase 3 phosphorylates kinesin light chains and negatively regulates kinesin-based motility. EMBO J. 21, 281–293.
Mothe, I., Van Obberghen, E., 1996. Phosphorylation of insulin receptor substrate-1 on multiple serine residues, 612, 632, 662, and 731, modulates insulin action. J. Biol. Chem. 271, 11222–11227.
Myers, M.G., et al., 1995. The pleckstrin homology domain in IRS-l sensitizes insulin signaling. J. Biol. Chem. 270, 11715–11718.
Nawaratne, R., et al., 2006. Regulation of insulin receptor substrate 1 pleckstrin homology domain by protein kinase C: role of serine 24 phosphorylation. Mol. Endocrinol. 20, 1838–1852.
Neudauer CL., et al.. Distinct cellular effects and interactions of the Rho-family GTPase TC10. Curr. Biol. 199; 8:1151–1160.
Nikoulina, S.E., et al., 2000. Potential role of glycogen synthase kinase-3 in skeletal muscle insulin resistance of type 2 diabetes. Diabetes 49, 263–271.
Nishita, M., et al., 2005. Spatial and temporal regulation of cofilin activity by LIM kinase and Slingshot is critical for directional cell migration. J. Cell Biol. 171, 349–359.
Nozaki, S., et al., 2012. Role of RalA downstream of Rac1 in insulin-dependent glucose uptake in muscle cells. Cell Signal 24, 2111–2117.
Ogihara, T., et al., 1997. 14-3-3 protein binds to insulin receptor substrate-1, one of the binding sites of which is in the phosphotyrosine binding domain. J. Biol. Chem. 272, 25267–25274.
Olson, A.L., et al., 1997. Syntaxin 4, VAMP2, and/or VAMP3/cellubrevin are functional target membrane and vesicle SNAP receptors for insulin-stimulated GLUT4 translocation in adipocytes. Mol. Cell Biol. 17, 2425–2435.
O'Rahilly, S., et al., 1991. Detection of mutations in insulin-receptor gene in NIDDM patients by analysis of single-stranded conformation polymorphisms. Diabetes 40, 777–782.
O'Rahilly, S., et al., 1992. Insulin receptor and insulin-responsive glucose transporter (GLUT 4) mutations and polymorphisms in a welsh type 2 (non-insulin-dependent) diabetic population. Diabetologia 35, 486–489.
Pessin, J.E., Saltiel, A.R., 2000. Signaling pathways in insulin action: molecular targets of insulin resistance. J. Clin. Invest. 106, 165–169.
Peterson, R.T., Schreiber, S.L., 1999. Kinase phosphorylation: keeping it all in the family. Curr. Biol. 9, R521–R524.
Pitcher, J.A., et al., 1995. Pleckstrin homology domain-mediated membrane association and activation of the beta-adrenergic receptor kinase requires coordinate interaction with G-beta/gamma subunits and lipid. J. Biol. Chem. 270, 11707–11710.
Ploug, T., et al., 1998. Analysis of GLUT4 distribution in whole skeletal muscle fibers: identification of distinct storage compartments that are recruited by insulin and muscle contractions. J. Cell Biol. 142, 1429–1446.
Qiao, L.Y., et al., 1999. Identification of enhanced serine kinase activity in insulin resistance. J. Biol. Chem. 274, 10625–10632.
Randhawa, V.K., et al., 2008. GLUT4 vesicle recruitment and fusion are differentially regulated by Rac, AS160, and Rab8A in muscle cells. J. Biol. Chem. 283, 27208–27219.
Ribon, V., et al., 1998. A novel, multifuntional c-Cbl binding protein in insulin receptor signaling in 3T3-L1 adipocytes. Mol. Cell Biol. 18, 872–879.
Saltiel, A.R., Kahn, C.R., 2001. Insulin signalling and the regulation of glucose and lipid metabolism. Nature 414, 799–806.
Sano, H., et al., 2003. Insulin-stimulated phosphorylation of a RabGTPase-activating protein regulates GLUT4 translocation. J. Biol. Chem. 278, 14599–14602.
Sarbassov, D.D., et al., 2005. Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex. Science 307, 1098–1101.
Seabra, M.C., Wasmeier, C., 2004. Controlling the location and activation of RabGTPases. Curr. Opin. Cell Biol. 16, 451–457.
Semenova, I., et al., 2008. Actin dynamics is essential for myosin-based transport of membrane organelles. Curr. Biol. 18, 1581–1586.
Sesti, G., et al., 2001. Molecular mechanism of insulin resistance in type 2 diabetes mellitus: role of the insulin receptor variant forms. Diabetes Metab. Res. Rev. 17, 363–373.
Shao, J., et al., 2000. Decreased Akt kinase activity and insulin resistance in C57BL/KsJ-Leprdb/db mice. J. Endocrinol. 167, 107–115.
Shepherd, P.R., et al., 1998. Phosphoinositide 3-kinase: the key switch mechanism in insulin signalling. Biochem. J. 333, 471–490.
Shimomura, I., et al., 2000. Decreased IRS-2 and increased SREBP-1c Lead to mixed insulin resistance and sensitivity in livers of lipodystrophic and ob/ob mice. Mol. Cell 6, 77–86.
Sidani, M., et al., 2007. Cofilin determines the migration behavior and turning frequency of metastatic cancer cells. J. Cell Biol. 179, 777–791.
Sommerfeld, M.R., et al., 2004. Vitro phosphorylation of insulin receptor substrate 1 by protein kinase C-ζ: functional analysis and identification of novel phosphorylation sites. Biochemistry 43, 5888–5901.
Storz, P., et al., 1999. Cross-talk mechanisms in the development of insulin resistance of skeletal muscle cells. Palmitate rather than tumor necrosis factor inhibits insulin-dependent protein kinase B (PKB)/Akt stimulation and glucose uptake. Eur. J. Biochem. 266, 17–25.
Stuart, C.A., et al., 2000. Comparison of GLUT1, GLUT3, and GLUT4 mRNA and the subcellular distribution of their proteins in normal human muscle. Metab. Clin. Exp. 49, 1604–1609.
Stuart, C.A., et al., 2006. Hexose transporter mRNAs for GLUT4, GLUT5, and GLUT12 predominate in human muscle. Am. J. Physiol. Endocrinol. Metab. 291, E1067–E1073.
Sun, X.J., et al., 1995. Role of IRS-2 in insulin and cytokine signalling. Nature 377, 173–177.
Sun, Y., et al., 2010. Rab8A and Rab13 are activated by insulin and regulate GLUT4 translocation in muscle cells. Proc. Natl. Acad. Sci. U. S. A. 107, 19909–19914.
Sylow, L., et al., 2013. Rac1 signaling is required for insulin-stimulated glucose uptake and is dysregulated in insulin-resistant murine and human skeletal muscle. Diabetes 62, 1865–1875.
Taylor, S.I., et al., 1990. Mutations in insulin-receptor gene in insulin-resistant patients. Diabetes Care 13, 257–279.
Teruel, T., et al., 2001. Ceramide mediates insulin resistance by tumor necrosis factor-α in brown adipocytes by maintaining Akt in an inactive dephosphorylated state. Diabetes 50, 2563–2571.
Thirone, A.C., et al., 2004. Regulation of Cbl-associated protein/Cbl pathway in muscle and adipose tissues of two animal models of insulin resistance. Endocrinology 145, 281–293.
Thong, F.S., et al., 2005. Turning signals on and off: GLUT4 traffic in the insulin-signaling highway. Physiol. Bethesda 20, 271–284.
Tremblay, F., et al., 2007. Identification of IRS-1 Ser-1101 as a target of S6K1 in nutrient- and obesity-induced insulin resistance. Proc. Natl. Acad. Sci. U. S. A. 104, 14056–14061.
Ueki, K., et al., 2003. Positive and negative roles of p85α and p85β regulatory subunits of phosphoinositide 3-kinase in insulin signaling. J. Biol. Chem. 278, 48453–48466.
Um, S.H., et al., 2004. Absence of S6K1 protects against age- and diet-induced obesity while enhancing insulin sensitivity. Nature 43, 200–205.
Vanhaesebroeck, B., et al., 2001. Synthesis and function of 3-phosphorylated inositol lipids. Ann. Rev. Biochem. 70, 535–602.
Verhelle, S.D., et al., 1996. Insulin receptor substrate-2 binds to the insulin receptor through its phosphotyrosine-binding domain and through a newly identified domain comprising amino acids 591786. J. Biol. Chem. 271, 5980–5983.
Walker, et al., 1998. Activation of protein kinase B β and γ isoforms by insulin in vivo and by 3-phosphoinositide-dependent protein kinase-1 in vitro : comparison with protein kinase B α. Biochem. J. 331, 299–308.
Wang, Z., et al., 2011. Inhibition or ablation of p21-activated kinase (PAK1) disrupts glucose homeostatic mechanisms in vivo. J. Biol. Chem. 286, 41359–41367.
White, M.F., et al., 1988. A cascade of tyrosine autophosphorylation in the beta-subunit activates the phosphotransferase of the insulin receptor. J. Biol. Chem. 263, 2969–2980.
White, M.F., Kahn, C.R., 1994. The insulin signaling system. J. Biol. Chem. 269, 1–4.
Wood, I.S., Trayhurn, P., 2003. Glucose transporters (GLUT and SGLT): expanded families of sugar transport proteins. Br. J. Nutr. 89, 3–9.
Woodgett, J.R., 1990. Molecular cloning and expression of glycogen synthase kinase-3/factor A. EMBO J. 9, 2431–2438.
Yuan, M., et al., 2001. Reversal of obesity- and diet-induced insulin resistance with salicylates or targeted disruption of Ikkβ. Science 293, 1673–1677.
Zheng, Y., et al., 1994. Activation of phosphoinositide 3-kinase activity by Cdc42Hs binding to p85. J. Biol. Chem. 269, 18727–18730.
Zick, Y., 2001. Insulin resistance: a phosphorylation-based uncoupling of insulin signaling. Trends Cell Biol. 11, 437–441.
Ziegler, S.F., et al., 1993. Molecular cloning and characterization of a novel receptor protein tyrosine kinase from human placenta. Oncogene 8, 663–670.

Graphical abstract

Chapter 3: PEDF-induced alteration of metabolism leading to insulin resistance

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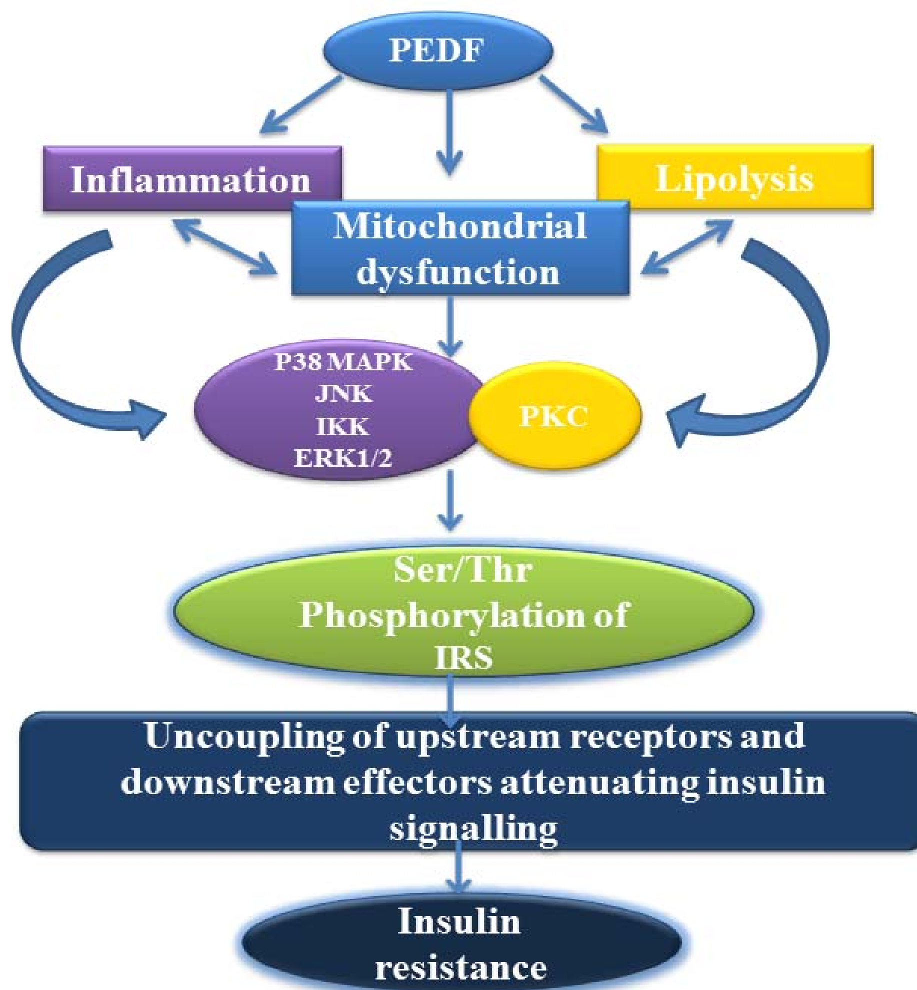

Summary of the literature search: PEDF- induced insulin resistance is triggered by a combination of various potential mechanisms such as metabolic inflammation, lipotoxicity and free radical injury and mitochondrial dysfunction in peripheral tissues - these mechanisms converge to attenuate insulin signaling and induce insulin resistance.

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Review

PEDF-induced alteration of metabolism leading to insulin resistance

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A B S T R A C T

Pigment epithelium-derived factor (PEDF) is an anti-angiogenic, immunomodulatory, and neurotrophic serine protease inhibitor protein. PEDF is evolving as a novel metabolic regulatory protein that plays a causal role in insulin resistance. Insulin resistance is the central pathogenesis of metabolic disorders such as obesity, type 2 diabetes mellitus, polycystic ovarian disease, and metabolic syndrome, and PEDF is associated with them. The current evidence suggests that PEDF administration to animals induces insulin resistance, whereas neutralisation improves insulin sensitivity. Inflammation, lipolytic free fatty acid mobilisation, and mitochondrial dysfunction are the proposed mechanism of PEDF-mediated insulin resistance. This review summarises the probable mechanisms adopted by PEDF to induce insulin resistance, and identifies PEDF as a potential therapeutic target in ameliorating insulin resistance.

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1. Introduction

Pigment epithelium-derived factor (PEDF) is a 50 kDa serine protease inhibitor (serpin) originally identified as a growth factor with neuronal differentiating activity (Tombran-Tink et al., 1991). The protein has since been found to possess a variety of useful properties in cancer therapy (Becerra and Notario, 2013), anti-angiogenesis (Dawson et al., 1999), stem cell support (Elahy et al., 2012), and as antioxidant in the eye (Yamagishi and Matsui, 2011).

2. PEDF biology

PEDF is a multifunctional, non-inhibitory serine protease discovered as a factor secreted by the human retinal pigment epithelial cells (Tombran-Tink et al., 1991). It emerged as one of the most potent anti-angiogenic molecule with great tumour specificity revolutionising cancer therapeutics (Becerra and Notario, 2013). PEDF was depicted as an ocular guardian owing to its ability to differentiate between remodelling and quiescent vasculature by causing apoptosis in the Fas-positive retinal endothelium sparing the pre-existing vasculature. PEDF exerts its angio-inhibitory effect by modulating the vascular endothelial growth factor (VEGF) and its receptors (VEGFR1 and VEGFR2) mediated signalling events directly by inhibiting VEGFR1 phosphorylation and by enhancing

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γ secretase-dependent cleavage of VEGFR1 and indirectly affects VEGFR2 by modulating VEGFR1 (Cai et al., 2006).

This endogenously secreted glycoprotein is a 418-amino acid polypeptide encoded by SERPINF1 gene on chromosome17p13 expressed by various human tissues. Most of the amino acids of PEDF form secondary structure except the first 35 residues (1–35) at the N-terminus and possess a tertiary structure with ten α-helices and three β-sheets. The reactive central loop (RCL) located near the C-terminus (RCL residues; 373–380) is protease sensitive and lacks conformational change when cleaved as is typical of an inhibitory serpin (Kawaguchi et al., 2010).

The first binding partner of PEDF is identified to be a triglyceride lipase (ATGL), a cell surface transmembrane protein with phospholipase activity which is activated upon PEDF binding to release bioactive lipids (Subramanian et al., 2013). The other additional PEDF receptors include laminin receptor (Bernard et al., 2009), membrane linked F1-ATP synthase (Notari et al., 2010) and PEDF also signals through LRP6, a co-receptor for Wnt (Park et al., 2011). The biological function of PEDF is governed by its interaction with these receptors and their downstream signalling pathways and is regulated both by its intrinsic properties such as post-translational modification and the extrinsic effectors altering the cellular microenvironment such as hormones, REDOX status, composition of extracellular matrix and lipids (Becerra and Notario, 2013; Dawson et al., 1999).

3. PEDF and its relevance to insulin resistance

PEDF is a metabolic regulatory protein that modulates whole body homeostasis and is associated with leading metabolic disorders such as obesity (Crowe et al., 2009), polycystic ovarian syndrome (PCOS) (Yang et al., 2011), type 2 diabetes mellitus (Nakamura et al., 2009), and metabolic syndrome (Chen et al., 2010; Yamagishi et al., 2006), where it is found to contribute to the central pathogenesis of insulin resistance (IR). Clinically, PEDF is positively associated with several metabolic risk factors such as body mass index (BMI), waist circumference, fasting triglycerides, glucose, and insulin, and is negatively correlated with circulating high density lipoprotein (HDL), and levels of this serpin decrease markedly after weight loss (Sabater et al., 2010). PEDF also plays a pathological role in hepatic insulin resistance and hepatic disorders (Yamagishi et al., 2010). PEDF is an independent determinant of insulin resistance in patients with essential hypertension (Nakamura et al., 2010). PEDF is also associated with cardiovascular pathologies such as acute coronary syndrome and was detected in atherosclerotic plaques of humans (Rychli et al., 2009). Recently, there have been an increasing number of studies focussing on the potential physiologic and pathologic associations of PEDF with adipocyte biology, lipid metabolism, and insulin sensitivity.

But the conclusion whether PEDF is the cause or effect of the impaired metabolism still remains debated. When there is enough evidence suggesting PEDF as a potential molecule mediating insulin resistance, there are emerging studies that contradict this fact. This article will review the evidence that supports or refutes the hypothesis that PEDF plays a causal role in the development of insulin resistance in metabolic disorders.

4. PEDF and metabolic diseases

PEDF is a pluripotent molecule with varied behaviour in different tissues (Crawford et al., 2013). This includes its potent anti-inflammatory action in the context of angio-inhibitory signalling (Zhang et al., 2006) which plays an inflammatory role in metabolism (Chen et al., 2010; Nakamura et al., 2009; Yamagishi et al., 2006; Yang et al., 2011). PEDF also modulates both the β-catenin mediated canonical and the JNK mediated noncanonical Wnt signalling pathways (Clevers et al., 2012; Gattu et al., 2014; Park et al., 2011). This suggest that PEDF mediated pathway of metabolic inflammation may congregate with Wnt signalling that is primarily a developmental pathway, with transcription factor 7-like 2 (TCF7L2) gene polymorphisms emerging as a strong risk factor for the development of type 2 diabetes mellitus (Grant et al., 2006) .

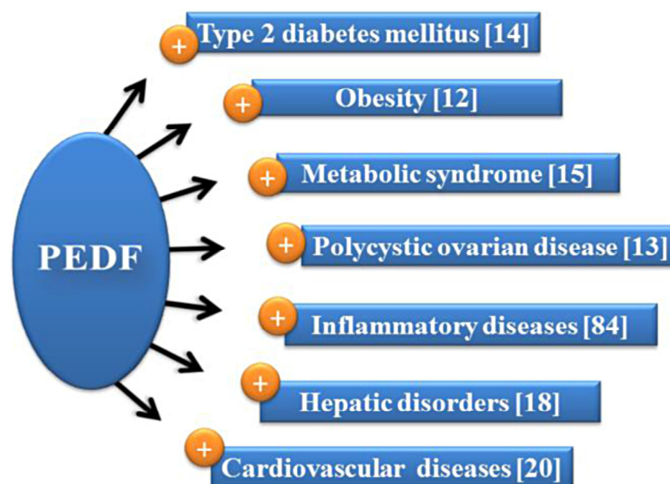

Fig. 1. Association of PEDF with metabolic disorders.

Circulating levels of PEDF are elevated in various metabolic disorders (Fig. 1) including prediabetics and type 2 diabetics (Jenkins et al., 2008). It has been shown that factors related to visceral obesity such as waist circumference, triglycerides, and tumour necrosis factor-alpha (TNF-α) are significantly and independently related to PEDF levels in type 2 diabetes patients' sera (Nakamura et al., 2009). PEDF is also positively correlated with fasting insulin, homeostasis model assessment of insulin resistance (HOMA-IR), BMI, fat mass, obesity, and cardiometabolic risk factors in the young and paediatric population (Sunderland et al., 2012). PEDF is elevated in obesity and reduced upon weight loss and insulin sensitisation (Crowe et al., 2009). These authors suggested that immature adipocytes in visceral adipose tissues may be one of the main sources of serum PEDF in diabetic patients.

In addition to hepatocytes (Matsumoto et al., 2004) and adipocytes (Famulla et al., 2011), skeletal myocytes are also potential sources of circulating PEDF especially during contraction or exercise (Norheim et al., 2011). Hepatic PEDF gene expression co-related with circulating PEDF levels in obese and insulin resistant type 2 diabetics was positively associated with hepatic injury and its markers such as alanine transaminase and aspartate transaminase suggesting liver as the major source of circulating PEDF linked with insulin resistance in humans (Moreno-Navarrete et al., 2013). PEDF is highly involved in adipogenic events and recent work with adipocytes in culture suggests that PEDF may be the signal through which excess adipose tissue promotes IR and metabolic dysfunction (Borg et al., 2011). Exogenous administration of recombinant PEDF to lean mice reduced insulin sensitivity during hyperinsulinemic–euglycemic clamp method which is the gold standard assessment of *in vivo* insulin sensitivity, whereas neutralisation of PEDF restored insulin sensitivity (Crowe et al., 2009). But on the other hand adipocyte generated PEDF enhanced lipolysis but did not contribute to the development of systemic metabolic derangements and improved metabolic derangements in obese type 2 diabetic rats (Lakeland et al., 2014; Matsui et al., 2014). The mechanism by which PEDF induces IR is not yet clear but acute administration of PEDF in rodents induces IR *in vivo*, and this onset of IR is accompanied by an increase in serum TNF and other cytokine levels. TNF infusions in humans lead to impairment of whole body insulin-mediated glucose

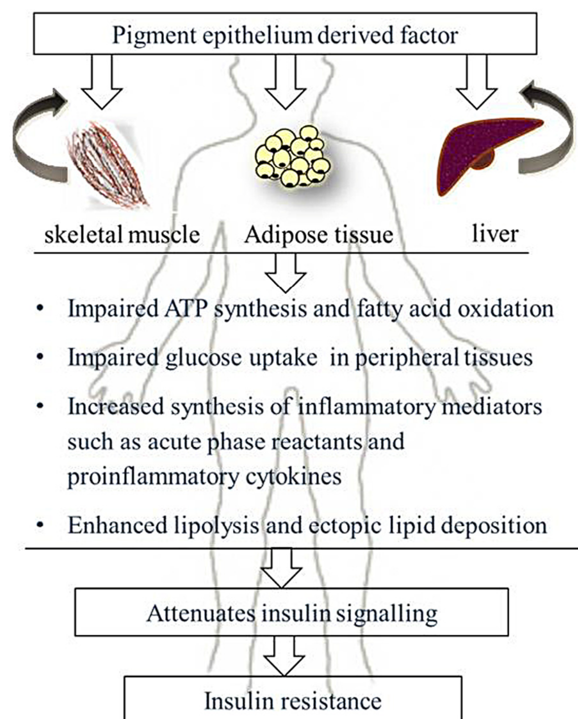

Fig. 2. An overview of PEDF-mediated mechanisms leading to the development of insulin resistance.

uptake (Lang et al., 1992), so it could be a downstream modulator for PEDF-mediated IR (Fig. 2).

In a recent clinical study, serum PEDF was reduced by 15% in newly diagnosed type 2 diabetic patients after insulin therapy. Insulin inhibits PEDF by downregulating 11β-hydroxysteroid dehydrogenase 1 [11β-HSD1] expression promotion by NF-κB (Zhou et al., 2013). Furthermore, PEDF levels could be decreased both in serum and in adipose tissue by insulin treatment. In adipocytes *in vitro*, PEDF is induced by TNF-α through activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) (Yabe et al., 2005).

Serum PEDF levels are also elevated in metabolic syndrome (Yamagishi et al., 2006) and in women with PCOS, and are associated with IR (Yang et al., 2011). However, a more recent study found that PEDF is not elevated in obese PCOS patients but had positive correlations with hypertension and dyslipidaemia (Joham et al., 2012). Serum PEDF levels are closely associated with high sensitivity C-reactive protein in women with PCOS, signifying a role of the serpin in the development of chronic inflammation in PCOS (Cheng et al., 2013). The correlation of PEDF with various health parameters is given in Table 1.

PEDF concentration was also inversely associated with insulin sensitivity and metabolic flexibility (the ability to switch between lipid and carbohydrate as a primary fuel source) in healthy humans (Richards et al., 2010).

The available evidence suggests the involvement of PEDF in metabolic dysfunction and reveals a definitive association between PEDF and metabolic health in animal models and humans (Box 1). Therefore, characterising the pathophysiological roles of PEDF is of high priority.

5. Mechanism by which PEDF mediates IR and its regulation during the process

5.1. PEDF has a marked effect on systemic lipid metabolism

PEDF is a lipolytic adipocytokine that mobilises free fatty acids and lipid ligands into the systemic circulation resulting in inflammation and ectopic lipid deposition. PEDF promotes lipolysis in an adipose triglyceride lipase [ATGL]-dependent manner and also reduces fatty acid oxidation in the muscle (Borg et al., 2011). The serpin relies on ATGL for the development of IR. ATGL serves as a putative PEDF receptor, and is a highly conserved triacylglycerol lipase that plays a key role in lipid (Zimmermann et al., 2004) and glucose (Haemmerle et al., 2006) homeostasis. Exogenously-added PEDF colocalises with ATGL in lipid droplets, and physically interacts with ATGL (Chung et al., 2008a), indicating that ATGL mediates the negative effects of PEDF on cell metabolic homeostasis.

The direct association of PEDF with elevated triglycerides in healthy individuals (Sabater et al., 2010) suggests that reduced *in vivo* insulin sensitivity might be a consequence of prolonged exposure to high PEDF levels. Prolonged PEDF infusion in mice resulted

Box 1 Summary of literature review associating PEDF and insulin resistance.

Elevated in metabolic disorders
Positively correlates with cardio-metabolic risk factors
Patented as therapeutic target for inflammatory disorders
Directly linked to IR and its inhibition restores insulin sensitivity
Lipolytic activity mediated by ATGL results in elevated free fatty acids
PEDF may also cause mitochondrial dysfunction

Table 1
Correlation of circulating PEDF concentration with various health parameters.

Positive correlation	Negative correlation	Associated pathology	Refs.
Lifestyle			
Weight	Weight	Obesity	(Cheng et al., 2013;
BMI	loss	PCOD	Yang et al., 2011)
Fat mass			
WHR			
BP			
Systolic BP		HT	(Nakamura et al., 2009)
Diastolic BP		Arterial	
baPWV		stiffness	
Diabetic parameters			
Fasting insulin		Diabetes	(Sabater et al., 2010;
Blood glucose		MS	Yamagishi et al., 2006)
HbA1c			
HOMA-IR			
IGT			
Lipid parameters			
TC	HDL	Atherogenic	(Sabater et al., 2010)
LDL		dyslipidaemia	
Fasting TGL			
Renal parameters			
BUN	Albumin	Renal	(Arimura et al., 2011;
Creatinine	eGFR	impairment	Motomiya et al., 2006)
UA			
Liver parameters			
ALT		Liver disorders	(Choi et al., 2012)
AST			
Other			
HsCrp		Inflammatory	(Cheng et al., 2013)
		marker	
		Diabetes, PCOD, HT	

BMI, body mass index; WHR, waist–hip ratio; PCOD, polycystic ovarian disease; BP, blood pressure; baPWV, brachial ankle pulse wave velocity; HT, hypertension; HbA1c, glycated haemoglobin; HOMA-IR, homeostatic model assessment of insulin resistance; IGT, impaired glucose tolerance; MS, metabolic syndrome; TC, total cholesterol; LDL, low density glycoprotein; TGL, triglycerides; HDL, high density lipoprotein; BUN, blood urea nitrogen; UA, uric acid; eGFR, estimated glomerular filtration rate; ALT, alanine amino transferase; AST, aspartate aminotransferase; HsCrp, high sensitive C-reactive protein.

in elevated free fatty acids (FFAs) coupled with lipid deposition in skeletal muscle and liver. This was accompanied by impaired insulin-mediated whole body glucose uptake and disposal, leaving the basal 2-deoxyglucose uptake and disposal undisturbed along with blunted hepatic glucose production in mice. Studies in mice also proved that PEDF had no effect on basal plasma glucose concentrations but decreased whole body insulin sensitivity (Crowe et al., 2009). Administration of PEDF neutralising antibody reverted all the effects mentioned earlier. Moreover, the presence of PEDF in adipocyte-conditioned media caused IR in myotubes, and these effects were reverted upon addition of PEDF-neutralising antibody.

PEDF gene expression and secretion increased during differentiation of human preadipocytes (Lakeland et al., 2014). PEDF exclusively targets the initial adipogenic events such as repression of adipogenic transcription factors PPARγ and C/EBP-alpha and ERK1/2 activation (Yamagishi et al., 2008). This finding contrasted an earlier study that showed that PEDF is synthesised by adipose tissue and is downregulated during differentiation to mature adipocytes (Kratchmarova et al., 2002). PEDF can block mesenchymal stem cell (MSC) differentiation to adipocytes in order to facilitate differentiation to osteoblasts (Gattu et al., 2013). In PEDF –/– mice, total body adiposity was increased by >50% compared with controls, illustrating its systemic role as a negative regulator of adipogenesis. Several adipose tissue-secreted factors that are elevated in obesity are implicated in the pathogenesis of metabolic dysfunction and IR, including TNF-α (Hotamisligil et al., 1993) . The fact that PEDF acts as an inhibitor of adipogenesis indicates a direct autocrine effect of PEDF on insulin action rather than evoking a secondary paracrine or endocrine effect, though this is yet to be proven.

5.2. PEDF impairs insulin signal transduction

In humans, elevated FFAs decrease insulin-mediated glucose transport in skeletal muscle and hepatic glycogenolysis (Boden, 2002). In skeletal muscle and hepatocytes, the elevated FFA (Kratchmarova et al., 2002) inhibits insulin transduction not only directly by decreasing insulin-induced phosphoinositide 3-kinase (PI3K) activation but also mechanistically due to ectopic lipid deposition in peripheral tissues. Intracellular lipid accumulation allosterically activates protein kinase C-β, δ, and θ (PKCs) (Griffin et al., 1999). The increase in PKCθ activity was found to occur concomitantly with insulin-stimulated insulin receptor substrate (IRS) tyrosine phosphorylation and reduced glucose transport (Yu et al., 2002). PKCs are known to activate the inhibitor of nuclear factor kappa-B kinase subunit beta (IKKβ) and c-Jun N-terminal kinases (JNKs) at the molecular level (Kim et al., 2004; Sun et al., 2000). Fatty acid-mediated decrease of IRS-1 associated PI3K activity has also been observed in biopsies from healthy human subjects when exposed to fatty acids (Bruce et al., 2006).

In a study focussing on the insulin-targeted peripheral tissues (Crowe et al., 2009), PEDF was associated with the activation of JNK, IKK and ERK1/2 in adipocytes, skeletal muscle cells, and cultured myotubes. JNK is an inhibitory Ser/Thr kinase that phosphorylates serine/threonine residues of insulin receptor substrate-1 (IRS-1), and its phosphorylation interferes with the interaction of insulin receptor with IRS-1, thus preventing tyrosine phosphorylation of IRS-1 (Aguirre et al., 2002). The I kappa B kinase (IκB) complexes with IκKβ that phosphorylates and degrades IκB, allowing NF-κB activation (Karin, 1999). IκKβ plays a central role in hepatic (Yuan et al., 2001) and systemic IR (Cai et al., 2005). IκKβ complexes with IRS-1, which has function in TNFα-mediated IR (Nakamori et al., 2006). The ERK1/2 are the other potential targets of PEDF (Wang et al., 2009) that are known to cause serine phosphorylation of IRS-1, and reduce its association with PI3K and inhibit tyrosine phosphorylation as well as attenuate insulin signalling (Bouzakri et al., 2011).

PEDF is also involved in the serine 307 phosphorylation of insulin receptor substrate with phosphorylation and activation of JNK, c-Jun and IKK by blocking advance glycation end products (AGE) mediated activation of Rac-1 (Yoshida et al., 2008). On the contrary PEDF reverses AGE mediated attenuation of tyrosine phosphorylation of IRS-1 and its association with PI3K-P85 alpha revealing a positive impact on the insulin-mediated glycogenesis (Zhang et al., 2006). PEDF also acts to preserve insulin sensitivity by blocking the phagocyte oxidase (PHox and P22PHOX)-mediated free radical events (Yoshida et al., 2009) and by reactivating PPAR receptors as well (Chung et al., 2008b; Gaetano et al., 2007). These studies suggest that PEDF ameliorates insulin resistance by its anti-oxidative properties, however these effects are endothelial. Moreover, PEDF blocks NF-κB and alleviates insulin resistance in hepatitis C infection (Kawaguchi et al., 2009). PEDF is also found to suppress IL-1beta mediated c-Jun N-terminal kinase activation in hepatocytes reducing hyperglycemia thereby improving hepatocyte insulin signalling (Gattu et al., 2014). Though several studies provide controversial results, these were extrapolated from mice to humans and the overwhelming evidence from clinical studies links PEDF with insulin resistance.

5.3. PEDF mediates mitochondrial dysfunction

The other distinct mechanism adopted by PEDF might be mitochondrial dysfunction. Reduced fatty acid oxidation was observed in the skeletal muscle of obese and type 2 diabetics (Sparks et al., 2005). Studies in cultured myotubes and isolated muscles revealed that PEDF reduces fatty acid oxidation, eventually generating a "lipotoxic" environment. FFAs are known inducers of mitochondrial dysfunction in 3T3-L1 adipocytes (Gao et al., 2010). PEDF decreases the expression of genes associated with fatty acid β-oxidation and mitochondrial biogenesis, which is unexpected as stimulation of ATGL would produce fatty acid ligands that activate peroxisome proliferator-activated receptors (PPARs) and increase the expression of genes involved in mitochondrial fat oxidation (Holloway et al., 2007). PPARs are master regulators of lipid metabolism (Ahmadian et al., 2013), and as a side-note, the anti-angiogenic property of PEDF involves an increase in expression and transcriptional activity of PPARγ for apoptosis of human umbilical vein endothelial cells (HUVECs) (Ho et al., 2007). In the long-term, PEDF acts by inducing transcriptional suppression of several genes involved with β-oxidation and oxidative phosphorylation. PEDF inhibits endothelial cell surface ATP synthesis activity (Notari et al., 2010). PEDF also induces mitochondrial dysfunction secondary to activation of PKCs due to intracellular accumulation of lipid metabolites (Shi et al., 2006). The dysfunctional mitochondria produce reactive oxygen species, which also contribute to inhibition of insulin signalling. The insulin-resistant peripheral tissues such as skeletal muscle, liver, and adipocytes had a decreased number of mitochondria, decreased mitochondrial gene expression, abnormal mitochondrial morphology, and abnormality in oxidative phosphorylation (Kim et al., 2008). Mitochondrial dysfunction is a proven pathologic mechanism of IR. PEDF-induced mitochondrial dysfunction impairs insulin signalling, and it also contributes both directly and indirectly to impairment of mitochondrial biogenesis.

5.4. PEDF is involved in metabolic inflammation

Metabolic disorders are now characterised as a state of chronic low grade inflammation. This state is sustained by various cytokines and chemokines, secreted during the course of these diseases, which contribute to the pathogenesis involved in the mortality and morbidity of the metabolic disease spectra and its related complications. PEDF has evolved as a protein capable of promoting metabolic inflammation. Be it a direct effect on the secreting cells or the

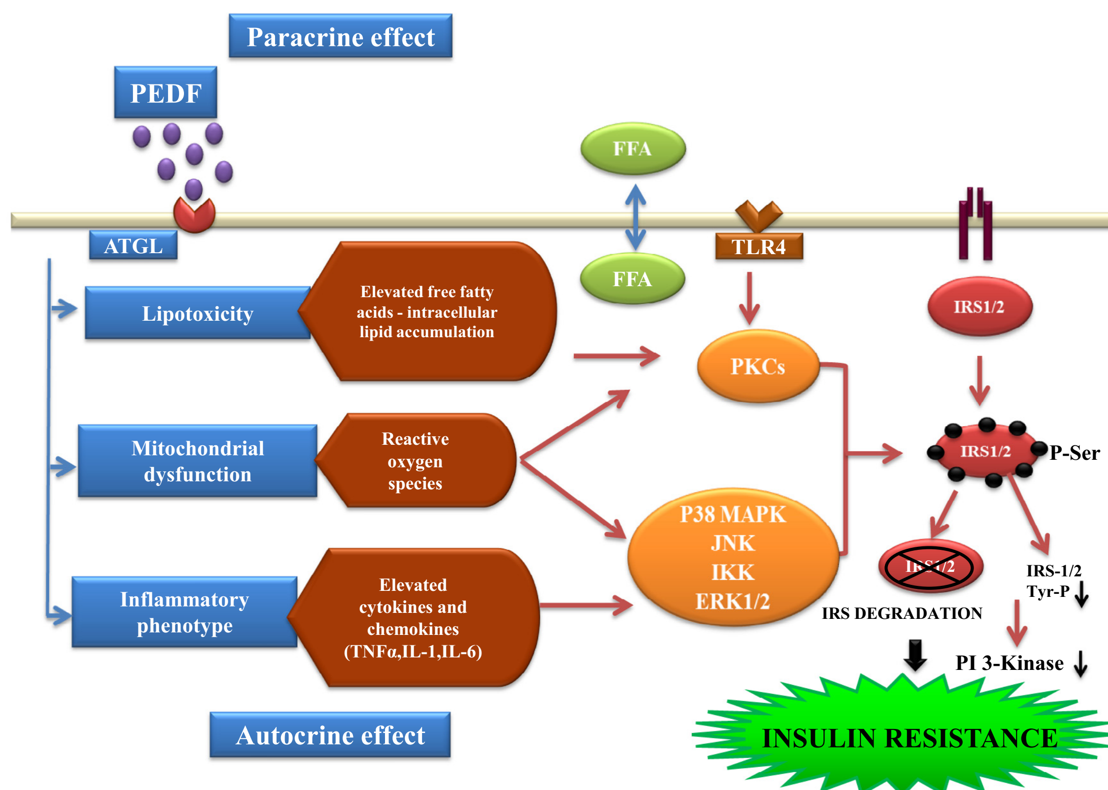

Fig. 3. PEDF acts to provoke mechanisms such as lipolytic mobilisation of free fatty acids, inflammation and mitochondrial dysfunction (Borg et al., 2011; Crowe et al., 2009; Halfon and Zick, 2009) which function synergistically to abrogate insulin action. These mechanisms take advantage of the negative feedback control of insulin signalling by activating the Ser/Thr kinases (PKCs, JNK, p38MAPK, IKK and ERK1/2) that, by mediating phosphorylation of IRS proteins, inhibit function of IRS proteins, terminate insulin action and induce insulin resistance. *Abbreviations*: FFA, free fatty acids; PKCs, protein kinases C; JNK, c-Jun N-terminal kinases; p38MAPK, p38 mitogen-activated protein kinases; IKK, IkappaB kinase complex; ERK1/2, extracellular signal-regulated kinases 1/2.

secondary effect of PEDF such as inflammation, lipolytic mobilisation of FFA and mitochondrial dysfunction converge onto the activation of cellular kinases (Fig. 3).

FFAs generated from PEDF mediated lipolysis could stimulate Toll-like receptor-mediated inflammatory signalling, which activates IKKβ and JNKs, that in turn stimulates the production of several cytokines including TNFα and interleukins (IL) especially, IL-1β and IL-6 (Kim et al., 2007).

PEDF has been identified to play a key role in initiating and maintaining the inflammatory cascade that contributes to the 'cytokine storm' in inflammatory disorders (Tracey, 2002). PEDF administration induces an inflammatory phenotype in mice, resulting in the recruitment and activation of macrophages and monocytes, which result in secreting increased levels of TNFα and IL-6 (Filleur et al., 2009). More importantly, anti-PEDF antibody attenuates PEDF-induced inflammation accompanied by reduced levels of TNFα and IL-6 (Chavan et al., 2012). Interestingly, PEDF also attenuates endotoxin-mediated TNFα and monocyte chemotactic protein 1 release (even though endotoxin does not induce PEDF secretion by the macrophages) and improves survival in murine endotoxemia.

PEDF also induces activation of p38 mitogen activated protein (MAP) kinase and extracellular signal-regulated kinases (ERK 1/2) in macrophages in an ATGL-mediated, concentration-dependent manner (Yang et al., 2010). Inhibition of the kinases or IRS blocked the inflammatory effect of PEDF. PEDF increases the phosphorylation of MAPK p38 and JNK in HUVECs as early as within 30 minutes in a concentration-dependent way (Chen et al., 2006), and PEDF-mediated effects were abrogated upon blocking these kinases in HUVECs. PEDF reduces the PI3K-AKT pathway in HUVECs and this reduction in AKT activity leads to mitogen-activated protein kinase 2 (MEKK2) activation (Gratton et al., 2001). This activation of MEKK2 in turn activates p38, suggesting a clear cross-talk between the PI3-AKT and the p38 MAPK pathways in a PEDF-dependent manner. These studies, whether in the context of IR or not, confirm PEDF-mediated activation of these kinases. Studies have shown that PEDF blocked phosphorylation/activation of p38 MAP kinase and ERK comparable with the effects of the kinase inhibitors in the context of the anti-permeability functions of PEDF in bovine retinal endothelial cells (Yang et al., 2010). These discrepancies may be attributed to the difference in cell types or experimental conditions involved.

Above all, PEDF has been suggested to be a potential therapeutic target for all types of inflammation in general, irrespective of the etiology (Tracy et al., 2013). It is apparent that PEDF activates Ser/Thr kinases: P38MAPK, IκKβ, JNK, and ERK1/2, which induces inflammatory signalling in an autocrine and paracrine manner, resulting in the recruitment and activation of macrophages and monocytes that thrive on the inflammatory cascade with mediators such as TNFα, IL-6, and MCP-1. The elevated levels of cytokines and chemokines resulting from PEDF activity also end up activating the serine/threonine kinases. The IRS proteins have emerged as the kinase-mediated PEDF target for Ser/Thr phosphorylation-based negative regulation. This uncouples the IRS from its upstream receptors and downstream effectors, thereby attenuating insulin signalling. Thus, PEDF-induced insulin resistance is a combinatorial effect of several kinases that act in concert to result in inhibitory Ser/Thr phosphorylation (Halfon and Zick, 2009). Hence, the PEDF stimulus is adequate to initiate, sustain, and progress the kinase-mediated inhibitory array of Ser/Thr phosphorylation cascade that attenuates insulin signalling in peripheral tissues (Table 2).

Table 2
Summary of the pathologic process of PEDF-induced insulin resistance.

Proposed etiology	Proposed mechanisms	Mediators of the pathogenesis	Downstream mediators	End result of signalling cascade	Refs.
PEDF	Inflammation	Cytokines: TNFα, IL-1, IL-6, MCP-1	P38 MAPK, JNK, IKK, ERK 1/2, PKC's	Ser/Thr phosphorylation of IRS	(Borg et al., 2011; Kim et al., 2008; Tracy et al., 2013; Yoshida et al., 2008)
	Mitochondrial dysfunction	Reactive oxygen species			
	Lipolysis	Free fatty acids, triglycerides and other lipid ligands			

6. Conclusion

In summary, the studies reviewed in this paper have extended the understanding of PEDF to include being a protein involved in metabolic inflammation promoting insulin resistance. There have been considerable advances made in understanding the genesis of insulin resistance, and potential triggering mechanisms such as lipolysis, inflammation, and mitochondrial dysfunction have been proposed and supported by experimental and observational data. PEDF is identified as a molecule whose autocrine and paracrine effects converge to the activation of Ser/Thr kinases, which causes the phosphorylation-based uncoupling of insulin signalling. This has focussed research attention to the IRS as the key target of PEDF action in the context of IR. PEDF-provoked kinase-mediated inhibitory Ser/Thr phosphorylation cascade of IRS is thus identified as the key mechanism of PEDF-induced insulin resistance in peripheral tissues. Further studies of physiological and clinical relevance are warranted for a deeper understanding of this issue, and PEDF may well be a potential therapeutic target to combat the pandemic of IR.

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References

Aguirre, V., et al., 2002. Phosphorylation of Ser 307 in insulin receptor substrate-1 blocks interactions with the insulin receptor and inhibits insulin action. J. Biol. Chem. 277, 1531–1537.
Ahmadian, M., et al., 2013. PPARγ signaling and metabolism: the good, the bad and the future. Nat. Med. 19, 557–566.
Arimura, T., et al., 2011. Association between plasma levels of pigment epithelium-derived factor and renal dysfunction in patients with coronary artery disease. Cardiol. J. 18, 515–520.
Becerra, S.P., Notario, V., 2013. The effects of PEDF on cancer biology: mechanisms of action and therapeutic potential. Nat. Rev. Cancer 13, 258–271.
Bernard, A., et al., 2009. Laminin receptor involvement in the anti-angiogenic activity of pigment epithelium-derived factor. J. Biol. Chem. 284, 10480–10490.
Boden, G., 2002. Interaction between free fatty acids and glucose metabolism. Curr. Opin. Clin. Nutr. Metab. Care 5, 545–549.
Borg, M.L., et al., 2011. Pigment epithelium-derived factor regulates lipid metabolism via adipose triglyceride lipase. Diabetes 60, 1458–1466.
Bouzakri, M., et al., 2011. Bimodal effect on pancreatic β-cells of secretory products from normal or insulin-resistant human skeletal muscle. Diabetes 60, 1111–1121.
Bruce, C.R., et al., 2006. Endurance training in obese humans improves glucose tolerance and mitochondrial fatty acid oxidation and alters muscle lipid content. Am. J. Physiol. Endocrinol. Metab. 291, E99–E107.
Cai, D., et al., 2005. Local and systemic insulin resistance resulting from hepatic activation of IKK-beta and NF-kappa B. Nat. Med. 11, 183–190.
Cai, J., et al., 2006. Pigment epithelium-derived factor inhibits angiogenesis via regulated intracellular proteolysis of vascular endothelial growth factor receptor 1. J. Biol. Chem. 281, 3604–3613.
Chavan, S., et al., 2012. Pigment epithelium-derived factor (PEDF) is an endogenous inflammatory factor. Mol. Med. 18, 1161–1168.
Chen, C., et al., 2010. Plasma level of pigment epithelium-derived factor is independently associated with the development of the metabolic syndrome in Chinese men: a 10-year prospective study. J. Clin. Endocrinol. Metab. 95, 5074–5081.
Chen, L., et al., 2006. PEDF induces apoptosis in human endothelial cells by activating p38 MAP kinase dependent cleavage of multiple caspases. Biochem. Biophys. Res. Commun. 348, 1288–1295.
Cheng, Q., et al., 2013. Association of serum pigment epithelium-derived factor with high-sensitivity C-reactive protein in women with polycystic ovary syndrome. J. Endocrinol. Invest. 36, 632–635.
Choi, K.M., et al., 2012. C1q/TNF-related protein-3 (CTRP-3) and pigment epithelium-derived factor (PEDF) concentrations in patients with type 2 diabetes and metabolic syndrome. Diabetes 61, 2932–2936.
Chung, C., et al., 2008a. Anti-angiogenic pigment epithelium derived factor regulates hepatocyte triglyceride content through adipose triglyceride lipase (ATGL). J. Hepatol. 48, 471–478.
Chung, C., et al., 2008b. Pigment epithelium-derived is an angiogenesis and lipid regulator that activates peroxisome proliferator-activated receptor alpha. Adv. Exp. Med. Biol. 617, 591–597.
Clevers, H., et al., 2012. Wnt/β-catenin signalling and disease. Cell 149, 1192–1205.
Crawford, S.E., et al., 2013. The many facets of PEDF in drug discovery and disease: a diamond in the rough or split personality disorder? Expert Opin. Drug Discov. 8, 769–792.
Crowe, S., et al., 2009. Pigment epithelium-derived factor contributes to insulin resistance in obesity. Cell Metab. 10, 40–47.
Dawson, D.W., et al., 1999. Pigment epithelium-derived factor: a potent inhibitor of angiogenesis. Science 285, 245–248.
Elahy, M., et al., 2012. The emerging role of PEDF in stem cell biology. J. Biomed. Biotechnol. doi:10.1155/23909.
Famulla, S., et al., 2011. Pigment epithelium-derived factor (PEDF) is one of the most abundant proteins secreted by human adipocytes and induces insulin resistance and inflammatory signaling in muscle and fat cells. Int. J. Obes. 35, 762–772.
Filleur, S., et al., 2009. Characterization of PEDF: a multi-functional serpin family protein. J. Cell. Biochem. 106, 769–775.
Gaetano, C., et al., 2007. Pedf, ppar-gamma, p53: deadly circuits arise when worlds collide. Cardiovasc. Res. 76, 195–196.
Gao, C.L., et al., 2010. Mitochondrial dysfunction is induced by high levels of glucose and free fatty acids in 3T3-L1 adipocytes. Mol. Cell. Endocrinol. 320, 25–33.
Gattu, A.K., et al., 2013. Determination of mesenchymal stem cell fate by pigment epithelium-derived factor (PEDF) results in increased adiposity and reduced bone mineral content. FASEB J. 27, 4384–4394.
Gattu, A.K., et al., 2014. Pigment epithelium-derived factor (PEDF) suppresses IL-1β-mediated c-JUN N-terminal (JNK) activation to improve hepatocyte insulin signalling. Endocrinology 155, 1373–1385.
Grant, S.F., et al., 2006. Variant of transcription factor 7-like 2 (TCF7L2) gene confers risk of type 2 diabetes. Nat. Genet. 38, 320–323.
Gratton, J.P., et al., 2001. Akt down-regulation of p38 signaling provides a novel mechanism of vascular endothelial growth factor-mediated cytoprotection in endothelial cells. J. Biol. Chem. 276, 30359–30365.
Griffin, M.E., et al., 1999. Free fatty acid-induced insulin resistance is associated with activation of protein kinase C theta and alterations in the insulin signaling cascade. Diabetes 48, 1270–1274.
Haemmerle, G., et al., 2006. Defective lipolysis and altered energy metabolism in mice lacking adipose triglyceride lipase. Science 312, 734–737.
Halfon, B.S., Zick, Y., 2009. Phosphorylation of IRS proteins, insulin action, and insulin resistance. Am. J. Physiol. Endocrinol. Metab. 296, E581–E591.
Ho, T.C., et al., 2007. PEDF induces p53-mediated apoptosis through PPAR gamma signalling in human umbilical vein endothelial cells. Cardiovasc. Res. 76, 213–223.
Holloway, G.P., et al., 2007. Skeletal muscle mitochondrial FAT/CD36 content and palmitate oxidation are not decreased in obese women. Am. J. Physiol. Endocrinol. Metab. 292, E1782–E1789.
Hotamisligil, G.S., et al., 1993. Adipose expression of tumor necrosis factor-alpha: direct role in obesity-linked insulin resistance. Science 259, 87–91.
Jenkins, A., et al., 2008. Increased serum pigment epithelium derived factor levels in type 2 diabetes patients. Diabetes Res. Clin. Pract. 82, e5–e7.

Joham, A.E., et al., 2012. Pigment epithelium-derived factor, insulin sensitivity, and adiposity in polycystic ovary syndrome: impact of exercise training. Obesity 20, 2390–2396.
Karin, M., 1999. The beginning of the end: I kappaB kinase (IKK) and NF-kappaB activation. J. Biol. Chem. 274, 27339–27342.
Kawaguchi, T., et al., 2009. Branched-chain amino acids and pigment epithelium derived factor: novel therapeutic agents for hepatitis C virus associated insulin resistance. Curr. Med. Chem. 16, 4843–4857.
Kawaguchi, T., et al., 2010. Structure-function relationships of PEDF. Curr. Mol. Med. 10, 302–311.
Kim, F., et al., 2007. Toll-like receptor-4 mediates vascular inflammation and insulin resistance in diet-induced obesity. Circ. Res. 100, 1589–1596.
Kim, J.A., et al., 2008. Role of mitochondrial dysfunction in insulin resistance. Circ. Res. 102, 401–414.
Kim, J.K., et al., 2004. PKC knockout mice are protected from fat-induced insulin resistance. J. Clin. Invest. 114, 823–827.
Kratchmarova, I., et al., 2002. A proteomic approach for identification of secreted proteins during the differentiation of 3T3-L1 preadipocytes to adipocytes. Mol. Cell. Proteomics 1, 213–222.
Lakeland, T.V., et al., 2014. Augmented expression and secretion of adipose-derived pigment epithelium-derived factor does not alter local angiogenesis or contribute to the development of systemic metabolic derangements. Am. J. Physiol. Endocrinol. Metab. 306, E1367–E1377.
Lang, C.H., et al., 1992. Tumor necrosis factor impairs insulin action on peripheral glucose disposal and hepatic glucose output. Endocrinology 130, 43–52.
Matsui, T., et al., 2014. Pigment epithelium-derived factor improves metabolic derangements and ameliorates dysregulation of adipocytokines in obese type 2 diabetic rats. Am. J. Pathol. 184, 1094–1103.
Matsumoto, K., et al., 2004. Antiangiogenic property of pigment epithelium-derived factor in hepatocellular carcinoma. Hepatology 40, 252–259.
Moreno-Navarrete, J.M., et al., 2013. Liver, but not adipose tissue PEDF gene expression is associated with insulin resistance. Int. J. Obes. 37, 1230–1237.
Motomiya, Y., et al., 2006. Increased serum concentrations of pigment epithelium-derived factor in patients with end-stage renal disease. Clin. Chem. 52, 1970–1971.
Nakamori, Y., et al., 2006. Myosin motor Myo1c and its receptor NEMO/IKK-gamma promote TNF-alpha-induced serine 307 phosphorylation of IRS-1. J. Cell Biol. 173, 665–671.
Nakamura, K., et al., 2009. Serum levels of pigment epithelium-derived factor (PEDF) are positively associated with visceral adiposity in Japanese patients with type 2 diabetes. Diabetes Metab. Res. Rev. 25, 52–56.
Nakamura, K., et al., 2010. Serum levels of pigment epithelium-derived factor (PEDF) are an independent determinant of insulin resistance in patients with essential hypertension. Int. J. Cardiol. 143, 96–98.
Norheim, F., et al., 2011. Proteomic identification of secreted proteins from human skeletal muscle cells and expression in response to strength training. Am. J. Physiol. Endocrinol. Metab. 301, E1013–E1021.
Notari, L., et al., 2010. Pigment epithelium derived factor binds to cell-surface F (1)-ATP synthase. FEBS J. 277, 2192–2195.
Park, K., et al., 2011. Identification of a novel inhibitor of the canonical wnt pathway. Mol. Cell. Biol. 31, 3038–3051.
Richards, J.C., et al., 2010. Short-term sprint interval training increases insulin sensitivity in healthy adults but does not affect the thermogenic response to beta-adrenergic stimulation. J. Physiol. 588, 2961–2972.
Rychli, K., et al., 2009. Pigment epithelium-derived factor (PEDF) as a therapeutic target in cardiovascular disease. Expert Opin. Ther. Targets 13, 1295–1302.
Sabater, M., et al., 2010. Circulating pigment epithelium-derived factor levels are associated with insulin resistance and decrease after weight loss. J. Clin. Endocrinol. Metab. 95, 4720–4728.
Shi, H., et al., 2006. TLR4 links innate immunity and fatty acid-induced insulin resistance. J. Clin. Invest. 116, 3015–3025.
Sparks, L.M., et al., 2005. A high-fat diet coordinately down regulates genes required for mitochondrial oxidative phosphorylation in skeletal muscle. Diabetes 54, 1926–1933.
Subramanian, P., et al., 2013. Pigment epithelium-derived factor receptor (PEDF-R): a plasma membrane-linked phospholipase with PEDF binding affinity. J. Biol. Chem. 288, 23928–23942.
Sun, Z., et al., 2000. PKC-theta is required for TCR-induced NF-kappaB activation in mature but not immature T lymphocytes. Nature 404, 402–407.
Sunderland, K.L., et al., 2012. Pigment epithelium-derived factor (PEDF) varies with body composition and insulin resistance in healthy young people. J. Clin. Endocrinol. Metab. 97, E2114–E2118.
Tombran-Tink, J., et al., 1991. PEDF: a pigment epithelium-derived factor with potent neuronal differentiative activity. Exp. Eye Res. 53, 411–414.
Tracey, K.J., 2002. The inflammatory reflex. Nature 420, 853–859.
Tracy, K., et al., 2013. The Feinstein Institute for Medical Research, Methods for treatment of inflammatory diseases, US 2013/0071389 A1.
Wang, M., et al., 2009. Pigment epithelium-derived factor suppresses adipogenesis via inhibition of the MAPK/ERK pathway in 3T3-L1 preadipocytes. Am. J. Physiol. Endocrinol. Metab. 297, E1378–E1387.
Yabe, T., et al., 2005. Pigment epithelium-derived factor induces pro-inflammatory genes in neonatal astrocytes through activation of NF-kB and CREB. Glia 50, 223–234.
Yamagishi, S., Matsui, T., 2011. Advanced glycation end products (AGEs), oxidative stress and diabetic retinopathy. Curr. Pharm. Biotechnol. 12, 362–368.
Yamagishi, S., et al., 2006. Elevated serum levels of pigment epithelium-derived factor in the metabolic syndrome. J. Clin. Endocrinol. Metab. 91, 2447–2450.
Yamagishi, S., et al., 2008. Pigment epithelium derived factor (PEDF): its potential therapeutic implications. Curr. Drug Targets 9, 1025–1029.
Yamagishi, S.I., et al., 2010. Pathophysiological role of pigment epithelium-derived factor (PEDF) in hepatic disorders. Curr. Med. Chem. 17, 1995–2000.
Yang, J., et al., 2010. Antipermeability function of PEDF involves blockade of the MAP kinase/GSK/-catenin signaling pathway and uPAR expression. Invest. Ophth. Vis. Sci. 51, 3273–3280.
Yang, S., et al., 2011. Serum pigment epithelium-derived factor is elevated in women with polycystic ovary syndrome and correlates with insulin resistance. J. Clin. Endocrinol. Metab. 96, 831–836.
Yang, S.L., et al., 2010. Pigment epithelium-derived factor induces interleukin-10 expression in human macrophages by induction of PPAR gamma. Life Sci. 87, 26–35.
Yoshida, T., et al., 2008. Pigment epithelium-derived factor (PEDF) ameliorated advanced glycation end product (AGE) – induced hepatic insulin resistance in vitro by suppressing rac-1 activation. Horm. Metab. Res. 40, 620–625.
Yoshida, Y., et al., 2009. Protective role of pigment epithelium derived factor (PEDF) in early phase of experimental diabetic retinopathy. Diabetes Metab. Res. Rev. 25, 678–686.
Yu, C., et al., 2002. Mechanism by which fatty acids inhibit insulin activation of insulin receptor substrate-1 (IRS-1)-associated phosphatidylinositol 3-kinase activity in muscle. J. Biol. Chem. 277, 50230–50236.
Yuan, M., et al., 2001. Reversal of obesity and diet-induced insulin resistance with salicylates or targeted disruption of Ikk beta. Science 293, 1673–1677.
Zhang, S.X., et al., 2006. Pigment epithelium-derived factor (PEDF) is an endogenous anti-inflammatory factor. FASEB J. 20, 323–325.
Zhou, Y., et al., 2013. PEDF expression is inhibited by insulin treatment in adipose tissue via suppressing 11β-HSD1. PLoS ONE 8, e84016.
Zimmermann, R., et al., 2004. Fat mobilization in adipose tissue is promoted by adipose triglyceride lipase. Science 306, 1383–1386.

Graphical abstract

Chapter 4: PEDF attenuates insulin-dependent molecular pathways of glucose homeostasis in skeletal myocytes

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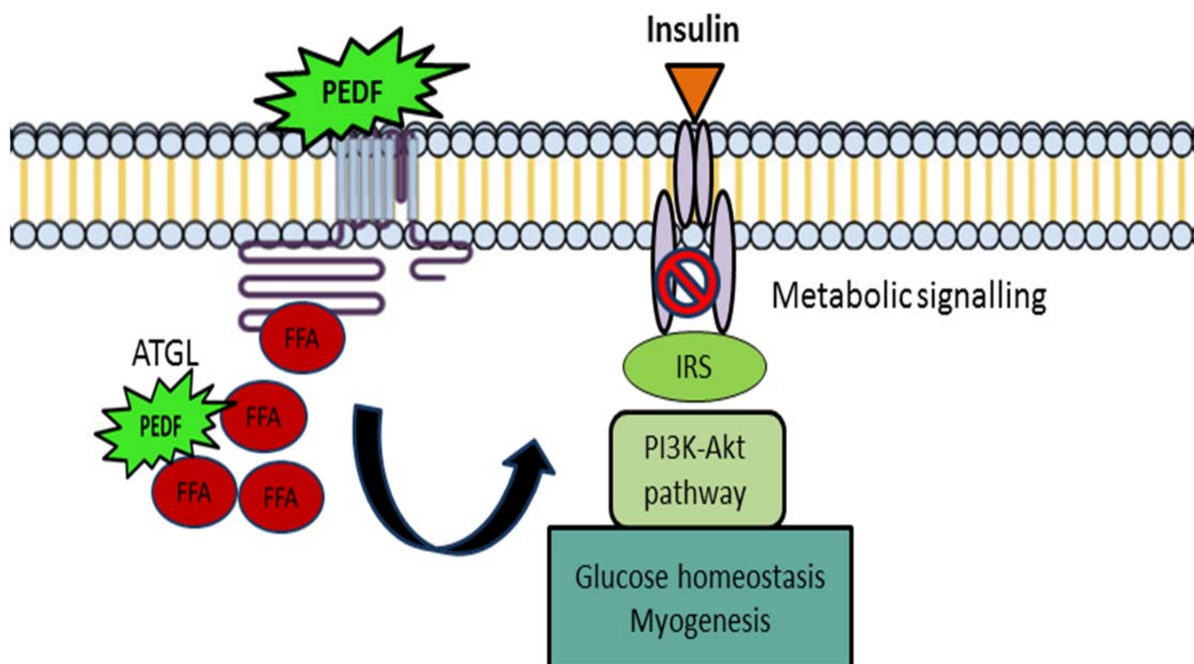

PEDF inhibits the entire metabolic insulin signalling: insulin-dependent tyrosine phosphorylation of insulin receptor β (IRβ) and insulin receptor substrate (IRS) that kick starts the insulin signalling governing glucose homeostasis - this results in sequential attenuation of the entire downstream insulin-dependent molecular mechanisms regulating glucose metabolism in skeletal muscle such as phosphorylation-inactivation of GSK 3β and GLUT4 translocation.

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PEDF attenuates insulin-dependent molecular pathways of glucose homeostasis in skeletal myocytes

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ABSTRACT

Pigment epithelium-derived factor (PEDF) is an anti-angiogenic serpin associated with insulin resistance in metabolic disorders such as diabetes, metabolic syndrome, obesity and polycystic ovarian syndrome. While the mechanism of PEDF induced-insulin resistance of metabolic disorders has been attributed to its inflammatory and lipolytic effects, little evidence exists to support a direct role of PEDF in mediating insulin resistance. Here, we seminally provide evidence that PEDF can inhibit insulin signal transduction governing glucose homeostasis from the receptor to the effector phosphorylation through Akt/PKB-dependent and -independent pathways in mouse and human skeletal muscle cell lines. PEDF attenuates the insulin-dependent molecular axes of glucose metabolism. Exposure of skeletal myocytes to PEDF attenuates insulin-dependent insulin receptor autophosphorylation, tyrosine phosphorylation of insulin receptor substrate 1, and dual loop phosphorylation-activation of Akt. PEDF significantly inhibits the downstream effector - glycogen synthase kinase (and thereby the glycogenic axis of insulin signalling). PEDF turned off both the molecular switches of GLUT4 translocation: IRS-Akt/PKB-AS160 mediated and IR-pCbl-dependent GLUT4 translocation (the molecular axis of glucose uptake). These findings implicate a direct effect of PEDF on multiple insulin-dependent molecular mechanisms of glucose homeostasis in skeletal muscle cells, thereby enabling it to contribute to peripheral insulin resistance at the cellular level.

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1. Introduction

Insulin resistance is the reduced capacity of endogenous insulin to elicit its response of glucose uptake and metabolism in target tissues such as liver, fat and skeletal muscle (Moller and Flier, 1991). It is also a ubiquitous correlate of obesity and a central component of metabolic syndrome (DeFronzo and Ferrannini, 2001). Skeletal muscle is the predominant insulin-responsive peripheral site of glucose metabolism and plays a crucial role in maintaining systemic glucose homeostasis through interactive cross-talk with hepatic and adipose tissues (DeFronzo and Tripathy, 2009). Skeletal muscle insulin resistance impacts whole body glucose homeostasis and is a consequence of impaired signalling events - defective post-receptor mechanisms that mediate various events of glucose metabolism and precedes the clinical diagnosis of metabolic diseases (Vaag et al., 1992).

The signalling pathways involved in insulin-dependent glucose metabolism are well characterised in skeletal muscle and can be dissected into two molecular axes: one governing glycogenesis and the other governing GLUT4 translocation (Carnagarin et al., 2015a, b). The stimulation of glycogen synthesis from glucose involves phosphatidylinositol-3-kinase (PI3K) activation through association with insulin receptor substrate 1 (IRS1), tyrosine phosphorylated by the activated insulin receptor (IR) (White et al., 1998). IRS1 is the predominant metabolic signal adaptor molecule involved in muscle (Yamauchi et al., 1996). PI3K catalyses the formation of phosphatidylinositol-3,4,5-trisphosphate which leads to the activation of Akt (v-akt murine thymoma viral oncogene homolog, also known as PKB or RAC kinase), through sequential phosphorylation on Thr-308 and Ser-473 by PDK1 and PDK2 (mammalian target of rapamycin complex 2 (MTORC2) in complex with Rictor and Sin),

Abbreviations: AS-160, akt substrate-160; Cbl, casitas B-lineage lymphoma; GSK, glycogen synthase kinase; IR, insulin receptor; IRS, insulin receptor substrate; PEDF, pigment epithelium-derived factor; PKB, protein kinase B.

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respectively (Alessi and Cohen, 1998). Akt in turn phosphorylates and inhibits glycogen synthase kinase (GSK), the most important kinase regulating the activity of glycogen synthase through phosphorylation-inhibition (Skurat and Roach, 1996).

The other rate-limiting step determining insulin resistance is glucose transport, mediated by increased translocation of GLUT4 glucose transporter levels (the predominant GLUT isoform in skeletal muscle). Two independent signalling pathways - tyrosine phosphorylation of Cbl (casitas b-lineage lymphoma) directly by the activated IR (Ribon et al., 1998), along with Akt-dependent phosphorylation-inactivation of AS160 (Akt substrate 160), have evolved as the predominant pathways of insulin-dependent glucose uptake mediated by GLUTs (Saltiel and Kahn, 2001). Thereby, defective glycogen synthesis or GLUT4 translocation play a dominant role in insulin resistance. A schematic overview of insulin-dependent molecular events involved in glucose homeostasis of skeletal muscle is depicted in Fig. 1.

PEDF is associated with insulin resistance in major metabolic disorders, endocrine, cardiovascular, hepatic and inflammatory disorders (Carnagarin et al., 2015a, b). PEDF is a multifunctional serpin with therapeutic potential due to its antiangiogenic property (Dawson et al., 1999; Tombran-Tink et al., 1991). PEDF levels are elevated in metabolic disorders and in insulin-resistant states in humans such as type 2 diabetes mellitus (Nakamura et al., 2010), obesity (Crowe et al., 2009), metabolic syndrome (Chen et al., 2010; Yamagishi et al., 2006), polycystic ovarian syndrome (PCOS) (Yang et al., 2011) and hepatic disorders (Yamagishi et al., 2010). Clinically, PEDF is positively correlated with several metabolic risk factors such a high body mass index (BMI), waist circumference, elevated triglycerides, glucose, and insulin, and is negatively correlated with circulating high density lipoprotein levels (Sabater et al., 2010). PEDF is an independent determinant of insulin resistance in patients with essential hypertension (Nakamura et al., 2010) and is associated with cardiovascular mortality and morbidity in humans (Rychli et al., 2009).

In the context of PEDF-mediated insulin resistance, the role of PEDF is evolving and is identified as a result of its lipolytic and inflammatory effects. While studies have shown that PEDF impairs insulin-stimulated glucose uptake and disposal in skeletal muscle and reduced the whole-body insulin sensitivity as measured by hyperinsulinemic-euglycemic clamps (Crowe et al.), investigations into molecular signalling mechanisms responsible for these metabolic responses of skeletal muscle are yet be studied. There is no evidence to date suggesting a direct role of PEDF in cellular insulin resistance at the molecular level. The present study examines the effect of PEDF on insulin signal transduction in skeletal muscle cells and demonstrates that PEDF can cause cellular insulin resistance across species. PEDF is shown to exert inhibitory effect right from IR autophosphorylation to the effector response of GSK3β phosphorylation and GLUT4 translocation in an AKT/PKB-dependent and -independent manner. These data are the first characterisation of PEDF-induced insulin resistance at the cellular level.

2. Methods

2.1. Materials

Recombinant human PEDF and PEDF polyclonal rabbit antibody were purchased from MD Bioproducts (Bethesda, MD, USA) and PEDF was dissolved in water to a 0.5 mg/ml stock solution. Fetal calf serum was purchased from Gibco (Fort Worth, TX, USA), human recombinant insulin, Dulbecco's Modified Eagle's Medium, penicillin/streptomycin and anti-GLUT4 antibody were obtained from Sigma–Aldrich (St Louis, MO, USA). The universal DNA ladder kit was purchased from KAPA Biosystems (Wilmington, MA, USA) and PCR master mix, Alexa Fluor® 594 F(ab')2 goat anti-rabbit IgG, Pierce 660 nm protein assay from Thermo Fisher Scientific (Waltham, Massachusetts, USA). PEDF siRNA (m), siRNA dilution buffer, control siRNA-A and siRNA-B and all RT-PCR primers and antibodies: IRS-1, pIRS-1(tyr 632) and insulin Rβ, p-insulin Rβ (tyr 1162/1163) and pCBL (Tyr 700)-R were purchased from Santa Cruz Biotechnology, Inc. (Dallas, Texas, USA). PNPLA2 from EMD Millipore Corporation (Temecula, CA, USA). Pan-Akt, phospho-Akt(Ser473), phospho-Akt(Thr308), phospho-Gsk-3β(Ser9) and LY294002 (PI3K inhibitor) and secondary anti-rabbit IgG, HRP-linked antibody and phospho-AS160 (Thr642) from Cell Signaling

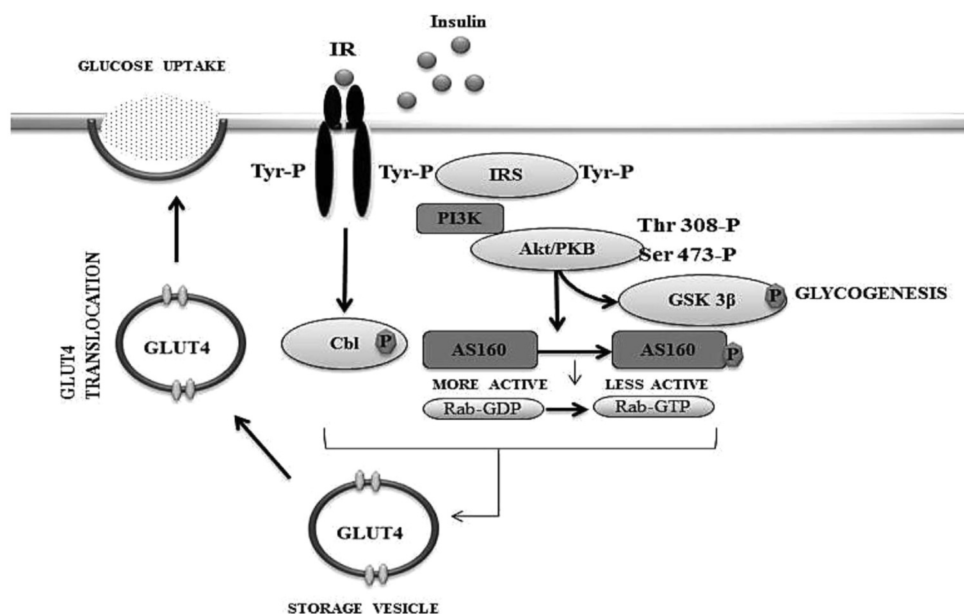


Fig. 1. Schematic overview of the insulin mediated molecular events involved in glucose homeostasis. Insulin binds and activates IR which tyrosine-phosphorylates IRS to initiate the dual critical axes of glycogenesis – IR/IRS/Akt/GSK3β and GLUT4 translocation-IR/IRS/Akt/As160/GLUT4 and IR/Cbl/GLUT4.

Technology (Beverly, MA, USA). Trizol (Invitrogen, Carlsbad, CA, USA) and ECL TM and ECL Plus immunoblot detection reagents were purchased from GE Healthcare (Piscataway Township, NJ, USA).

2.2. Cell culture

Human Skeletal Muscle Myoblasts (HSMMs) and Skeletal Muscle Myoblast Cell Media - Clonetics™ SkBM™-2 BulletKit™ were purchased from Lonza (Walkersville, MD, USA) and were used within 5 passages from receipt. C2C12 purchased from ATCC (Manassas, VA, USA) and used within passage 14. Cells were cultured under an atmosphere of 5% CO_2 at 37 °C in a humidified incubator and when confluent, cells were switched to differentiation with 2% serum and myotubes were used for experiments following 72 h of differentiation.

2.3. Knockdown of PEDF with siRNA

The silencing experiments and PEDF gene expression knockdown-PCR analysis were performed as per manufacturer's protocol. For each transfection 6 μl (60pmols) of siRNA duplex (scrambled and PEDF siRNA) was used for a transfection period of 72 h. To determine the efficiency of siRNA transfection, mRNA and protein were collected from the cells after transfection with siRNA (Scrambled and PEDF SiRNA); RT-PCR and immunoblot analyses were performed to detect PEDF protein levels.

2.4. RNA isolation and RT-PCR analysis

Total RNA from silenced cells was extracted using Trizol according to manufacturer's instructions. First-stranded cDNA synthesis was performed from 1 μg total RNA using Super-Script reverse transcriptase. cDNA was amplified in a 20-μl PCR mixture containing 10 μl master mix, 1 μl of forward and reverse PEDF primers (10 μM concentration). The conditions in the logarithmic phase of PCR amplification were as follows: 5 min initial denaturation at 95 °C, 30 s denaturation at 95 °C, 30 s annealing at 55 °C, and 1.5 min extension at 72 °C for 35 cycles. GAPDH was used as the internal control.

2.5. Cell viability assay

C2C12 and HSMM cells were seeded in 96-well plates in triplicates (400 cells/well), and were treated with 100 nM PEDF for 24 h, relative numbers of viable cells were measured in comparison to the untreated control and the solvent control using the fluorimetric, resazurin-based Cell-Titre Blue assay (Promega, Madison, WI, USA) according to the manufacturer's instructions at Ex_{560nm}/Em_{590nm} in a multilabel counter (PerkinElmer, Germany). Cell growth was expressed as percentage of the untreated medium control. A physiological (100 nM) concentration of PEDF chosen based on published data for circulating PEDF levels (Petersen et al., 2003) was added to the cells for 24 h.

2.6. Enzyme-linked immunosorbent assay (ELISA)

PEDF production in human skeletal muscle cells was determined using ChemiKineTM Pigment Epithelium-Derived Factor (PEDF) Sandwich ELISA Kit (Chemicon International, USA). The assays were performed in duplicates according to the manufacturer's instructions.

2.7. Preparation of cell lysates for immunoblotting

The whole-cell lysates of C2C12 and HSMM were exposed to 50 nM, 100 nM and 200 nM PEDF for 24 h and then subjected to insulin stimulation at 0, 100 nM and 200 nM concentrations to check the effects of PEDF on the various critical insulin signalling nodes. Further analysis was carried out with the optimal concentration of 100 nM PEDF with 100 nM insulin stimulation. To identify the effect of PEDF on PI3K, the cells were treated with LY294002 (PI3K inhibitor) for 1 h and stimulated with 100 nM insulin for 10 min. The cells were lysed with RIPA buffer containing protease and phosphatase inhibitors (1 mM phenylmethylsulfonyl fluoride, 2 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM Na_3VO_4; Sigma) and quantified using Pierce 660 nm assay as per the manufacturer's instructions. Samples (20 μg of protein) were fractionated at 150 V for 1 h by SDS-PAGE (Invitrogen) and electrotransferred to PVDF membranes. Membranes were blocked with 5% skimmed milk for 1 h and incubated with the indicated primary antibodies overnight at 4 °C, followed by 1 h incubation with horseradish peroxidase-conjugated secondary antibody.

All primary antibodies were used at 1:1000 dilution in 5% BSA prepared in TBS containing 0.1% Tween-20 (TBST). This was followed by HRP-conjugated secondary antibody incubation at 1:25,000 prepared in 5% skim milk in TBST for 1 h at room temperature, followed by chemiluminescence detection (Western Lightning Plus ECL kit, Perkin Elmer) of target bands. Intermittent washes following blocking, primary and secondary antibody incubation steps were performed using transfer buffer containing 0.05% Tween-20 (TBST). Densitometry of the detected bands was used to quantify the band intensities with Image Lab 5.2.1 software (Bio Rad), and the resulting band intensities were normalised to β-actin.

2.8. Fluorescence immunocytochemistry

Immunofluorescence experiments were performed using both mouse and human skeletal muscle cells using the following protocol. Cells in 96-well plates were fixed with 4% paraformaldehyde in PBS for 15 min at room temperature (RT) and then washed three times with 1X PBS. Subsequently the cells were permeabilised with 0.3% saponin in PBS for 10 min and rinsed with 1X PBS. Nonspecific binding sites were blocked with 0.1% saponin (Sigma; St Louis, MO) in PBS containing 0.25% BSA and 2% normal serum for 30 min at RT and rinsed with 1X PBS. The antibody solution contained 0.2% saponin and 0.5% BSA in PBS. The cells were then incubated with the primary antibodies at 1:250 dilution and left in the dark overnight at 4 °C. Wells were rinsed twice with PBS and incubated with 1:2000 secondary antibody at RT for 30 min in the dark. Wells were rinsed twice with PBS and nuclei counterstained with DAPI (50 μg/mL) for 10 min at RT or left unstained. Following a rinse with PBS, 2–3 drops of 100% glycerol was added to each well and observed under Olympus IX51 fluorescent microscope (Olympus Life Science Solutions, Pennsylvania, USA). The GLUT4 translocation images were analysed by measuring the fluorescent intensity in the perinuclear region using Fiji/Image J2 software, Madison, USA.

2.9. Statistical analysis

All experiments were performed in triplicates and the mean values ± SD were calculated. Comparisons between groups were performed with one-way ANOVA followed by post-hoc tests. $P < 0.05$ was considered statistically significant. Densitometric analyses of the immunoblots were performed using GS-670 Imaging Densitometer (Bio-Rad) and Molecular Analyst software (version 5.2.1, Bio-Rad). Giving an arbitrary value of 1.0 to the control samples, the relative values of the samples were then

determined.

3. Results

3.1. Skeletal muscle is both a source and target of PEDF

The expression of PEDF and it receptor in skeletal muscle indicate the possibility that skeletal muscle are subject to both autocrine and paracrine effects of PEDF. PEDF signalling is predominantly through a patatin-like phospholipase domain-containing 2 (PNPLA2) receptor and to a lesser extent through laminin receptor and we could detect the expression of PNPLA2 receptors and PEDF in C2C12 and HSMM (Fig. 2a) and the PEDF produced by the HSMM was quantified using ELISA (Fig. 2b).

3.2. PEDF inhibits insulin-induced glycogenesis in Akt/PKB-dependent manner in skeletal muscle

The effect of PEDF was studied on the critical phosphorylation nodes such as Akt and GSK3β in a concentration-dependent manner. As expected insulin stimulated phosphorylation of Akt and GSK3β and PEDF inhibited these critical nodes with increasing concentration (Fig. 3a). PEDF knockdown in C2C12 (Fig. 3b) was performed to minimise or ablate the endogenous PEDF and to identify its effect on critical insulin signalling nodes when treated with a physiological concentration of 100 nM. Akt/PKB is a survival factor and the possibility that this PKB inhibition was associated with cell death was ruled out by assessing the cell viability and metabolic capacity following 24 h exposure to PEDF (Fig. 3c). To determine the effect of PEDF on PI3K and Akt activation, we investigated the level of Ser-9 phosphorylation of GSK-3β and dual loop phosphorylation-Thr308 and Ser-473 phosphorylation along with total PKB in lysates of non-silenced and PEDF-silenced C2C12 (Fig. 3d), prepared after 100 nM PEDF treatment and 100 nM insulin stimulation, using phospho-specific antibodies. PEDF pretreatment of myocytes resulted in inhibition of the insulin-stimulated dual loop phosphorylation-activation of Akt/PKB, and also affected phosphorylation-inhibition of GSK-3β, no significant difference in PI3K activity in the presence or absence of PI3K inhibitor was observed. Similar results were obtained using phospho-specific antibodies directed against the PKB and GSK3β in HSMMs (Fig. 3e). These results indicate that PEDF inhibits insulin-stimulated glycogen synthesis through modulation of the Akt/PKB signalling pathway.

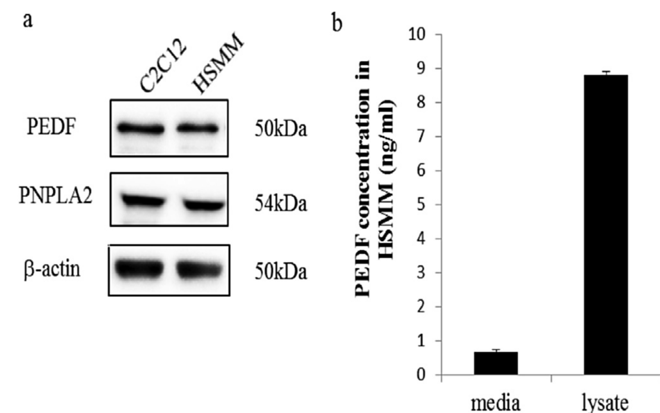


Fig. 2. Expression of PEDF and the dominant PEDF-receptor. (a) Immunoblot analysis of cell lysates of C2C12 and HSMM show PEDF and PNPLA2 expression and (b) PEDF secretion in human skeletal muscle myoblast was measured by ELISA.

3.3. PEDF inhibits insulin-induced GLUT4 translocation

The molecular mechanism by which PEDF produces insulin resistance by repressing glycogenesis is far from clear and prompted us to investigate the other critical endpoint of insulin signalling - GLUT4 translocation in skeletal muscle. Non-silenced and PEDF-silenced C2C12 cells were not treated and pre-treated with 100 nM PEDF for 24 h followed with and without 100 nM insulin stimulation for 5 min and then immediately fixed with 4% paraformaldehyde. Insulin stimulation significantly increased the basal GLUT4 translocation, but this effect was not produced when cells were pre-treated with PEDF. PEDF markedly attenuated GLUT4 translocation which was observed as perinuclear accumulation with C2C12 and were consistent with HSMM (Fig. 4). Thus, PEDF also inhibits insulin-induced GLUT4 translocation in skeletal muscle cells.

3.4. PEDF abrogates the molecular axes of insulin-induced GLUT4 translocation in Akt/PKB-dependent and -independent manner in skeletal muscle

To delineate the effect of PEDF on the molecular axes involved in GLUT4 translocation, we then planned to identify the effect of PEDF on the critical downstream molecular switches of insulin-dependent GLUT4 translocation which includes phosphorylation-inactivation of AS160 which is dependent on the tyrosine phosphorylation of IRS and Akt/PKB activation and identify if there is involvement of any Akt/PKB-independent mechanisms such as tyrosine phosphorylation of Cbl, directly activated by the insulin receptor. PEDF inhibited both the molecular axes of GLUT4 translocation: Akt/PKB-AS160-GLUT4axis; PEDF inhibited tyrosine phosphorylation of IRS with attenuation of phosphorylation-inactivation of AS160 and IR-pCbl-GLUT4 axis; PEDF inhibited IR autophosphorylation and also abrogated the tyrosine phosphorylation of Cbl (Fig. 5).

4. Discussion

Metabolic disorders and their associated morbidity are major health and economic challenges. Identifying and understanding mediators of the involved pathogenesis such as insulin resistance in humans may give important insights into disease outcomes and treatment of these conditions. PEDF is endogenously produced by the skeletal myocytes and predominantly interacts with cell surface patatin-like phospholipase domain containing 2 (PNPLA2) to generate cellular signal to exert its multimodal effects in various cell systems (Subramanian et al., 2010). Among the several cytokines that induce insulin resistance, PEDF has emerged as a novel protein of metabolic dysregulation with a causal role in insulin resistance but this has not been fully investigated. Studies have shown that PEDF impairs glucose uptake and reduces insulin sensitivity (Crowe et al., 2009) and we have focussed on the effect of PEDF on the molecular events involved in insulin signalling. . In this study we explored specifically the impact of PEDF on the critical nodes of insulin-dependent glucose metabolism such as glycogenesis and GLUT4 translocation in mice and human skeletal muscle cells.

The association between PEDF and insulin resistance has been established with elevated levels of circulating PEDF observed in metabolic disorders, in young as well as old patients, and is positively correlated with cardiometabolic risk factors (Sunderland et al., 2012). While the normal plasma concentration of PEDF is 100 nM (5 μg/ml) (Petersen et al., 2003), PEDF levels are found to be elevated by more than two-fold in diabetes mellitus and increased by several-fold with increasing components of metabolic syndrome

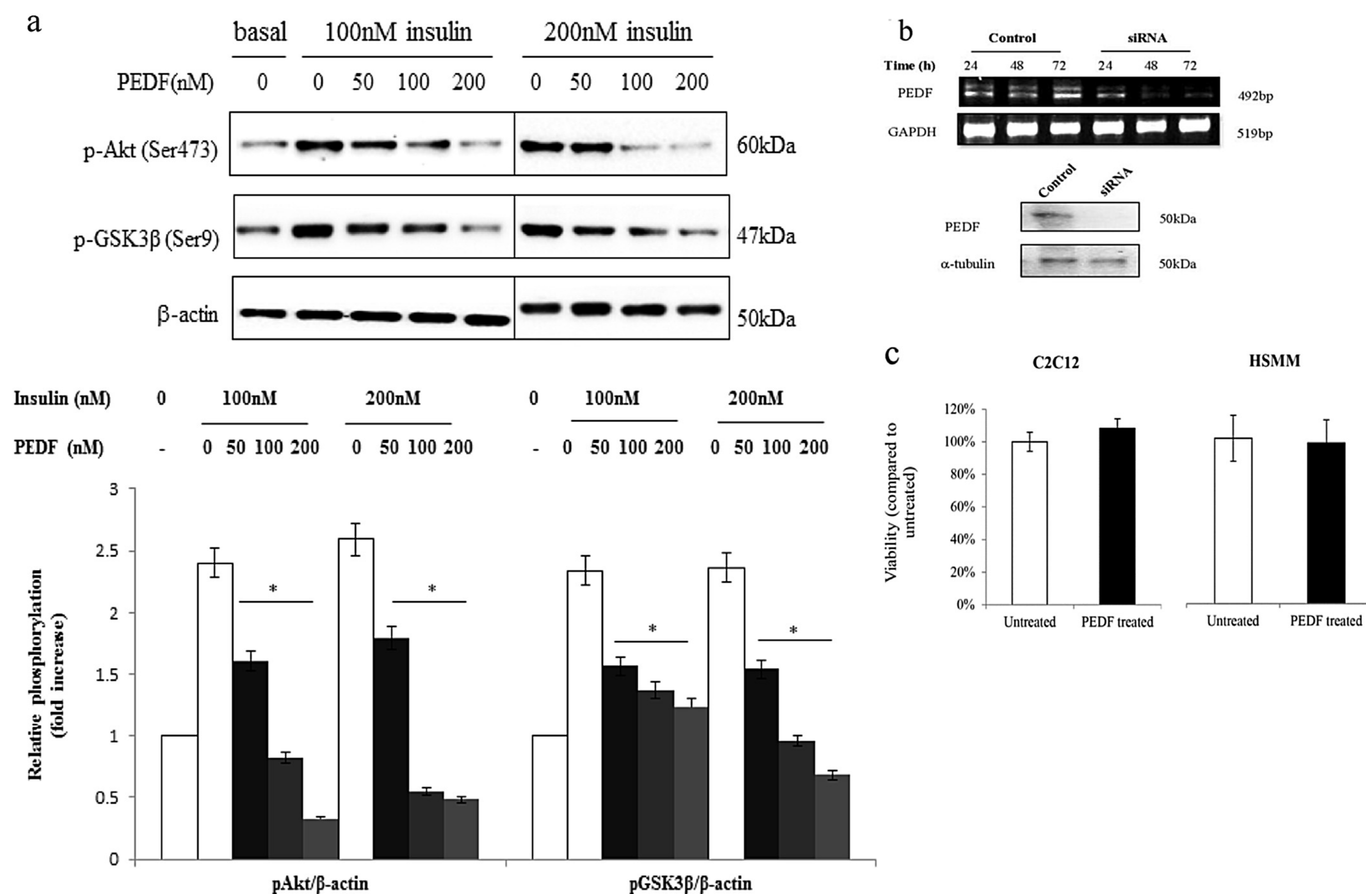
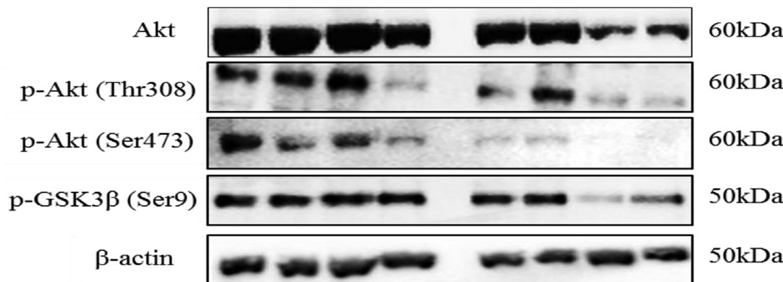


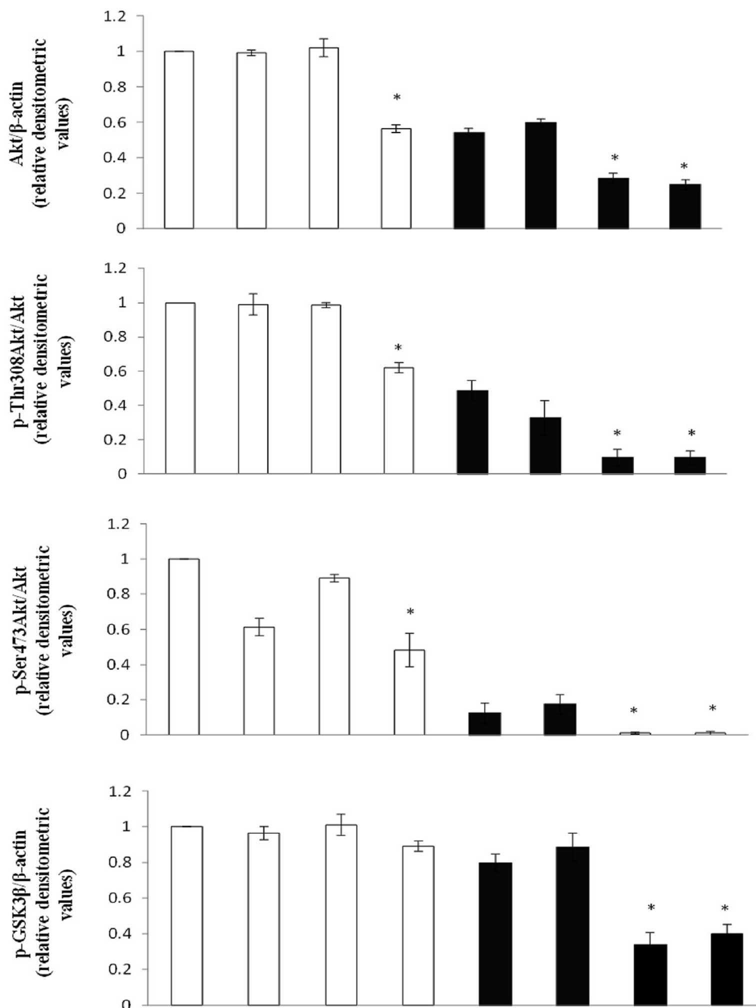
Fig. 3. Effect of PEDF on Akt/PKB signalling in skeletal myocytes. C2C12 cells were treated with 50 nM, 100 nM and 200 nM PEDF under 100 nM and 200 nM insulin stimulation initially which showed a concentration-dependent inhibitory effect of PEDF which was not allayed even by doubling the insulin stimulation (a) Endogenous PEDF production was attenuated by silencing to clearly identify the effect of PEDF on critical insulin signalling nodes. PEDF protein expression was completely attenuated at 72 h by siRNA transfection as confirmed by RT-PCR analysis and immunoblot. Transfection of control and PEDF siRNA at 24, 48 and 72 h and the corresponding mRNA and protein levels at various timepoints (b) GAPDH and α-tubulin were used as housekeeping controls. Further investigations were performed with an optimal concentration of 100 nM PEDF after confirming that cell viability remains unaltered by CT-Blue assay following 24 h PEDF exposure. (c) The effect of PEDF on PI3K and Akt activation was studied in C2C12 under non-silenced (scrambled siRNA) and silenced (PEDF siRNA) conditions during the last 24 h of silencing, followed by treatment with or without LY294002 (a PI3K inhibitor) 1 h before insulin stimulation, then finally stimulated with insulin (100 nM) for 5 min Cell lysates were subjected to immunoblotting and probed with specific antibodies and representative blots are shown in (d) The results obtained with C2C12 were consistent with HSMM (e). All data are presented after normalising with β-actin expression and are expressed relative to the control. Values are mean ± SD (n = 3), $^{*}p < 0.05$, PEDF-treated versus untreated.

(Yamagishi et al., 2006). Animal studies demonstrate that PEDF infusion causes insulin resistance while PEDF inhibition restores insulin sensitivity (Crowe et al., 2009). The causal role of PEDF in the context of insulin resistance was attributed to its influence on the systemic fatty acid metabolism promoting extensive lipolysis (Borg et al., 2011) and by the initiation of inflammatory signalling mediated by kinases such as JNK (Crowe et al., 2009), IKK and p38 MAPK in adipocytes, skeletal and vascular muscle cells. On the other hand adipocyte-generated PEDF-enhanced lipolysis did not contribute to the development of systemic metabolic derangements, but instead improved metabolic derangements in obese type 2 diabetic rats (Lakeland et al., 2014; Matsui et al., 2014). PEDF also mitigated IL-1β-induced JNK activation and suppressed metabolic stress-induced IL-1β release and improved hepatocyte insulin signalling playing a homeostatic role in PEDF KO mice (Gattu et al., 2013). Thereby, the role of this multifaceted protein in the context of metabolic homeostasis is controversial and warrants further studies for better understanding.

Skeletal muscle is the predominant site of insulin-dependent glucose metabolism and is both a potential source (Norheim et al., 2011) and target of PEDF with expression of its receptors (Famulla et al., 2011) and is subject to its autocrine and paracrine effects. The seminal work described here is a study on the molecular mechanism of PEDF-induced insulin resistance focussing on the critical nodes governing insulin-dependent glucose metabolism-glycogenesis and GLUT4 translocation in skeletal muscle cells, addressing the effects of PEDF on the signalling pathway from insulin receptor to GSK, as well as on GLUT4 translocation. While we have shown that a 24 h PEDF pre-incubation can inhibit insulin-stimulated molecular component of glycogenesis and GLUT4 translocation, our results indicate that this takes place *via* Akt/PKB-dependent and -independent pathways.

PEDF did inhibit immediate signalling *via* the insulin receptor such as tyrosine phosphorylation of IR and IRS, but PI3K recruitment remained unaltered with subtle changes in total IR levels in the presence of PEDF. A similar effect is produced by the suppressor of cytokine signaling proteins and proinflammatory cytokines such as TNF-α but these have been associated with impairment in PI3K activity (Ueki et al., 2004). However, PEDF did significantly reduce the activation of downstream Akt/PKB pathway which is a major regulatory input for GSK and GLUT4 translocation. We have established that PEDF acts at the level of Akt/PKB, reducing the phosphorylation-activation at both Thr-308 and Ser-473, though it remains to be determined if this could be a result of enhanced





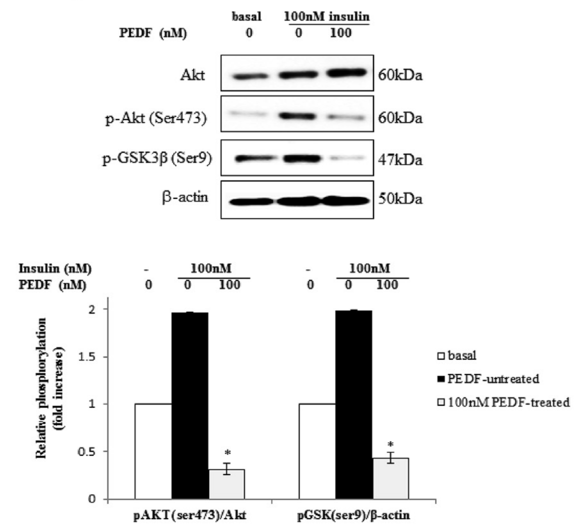


Fig. 3. (*continued*).

dephosphorylation. Such an effect is seen with hyperosmotic stress-mediated PKB inhibition that involves both inhibition of phosphorylation of these regulatory sites along with rapid dephosphorylation by protein phosphatase 2A, while PI3K activity remains unperturbed (Meier et al., 1998).

The effects of PEDF on insulin-dependent GLUT4 translocation through Akt/PKB-dependent and -independent mechanisms were also characterised. The two independent signalling pathways, (Akt) pathway and the Cbl pathway, are known to be involved in the insulin-stimulated GLUT4 translocation (Chang et al., 2004). PEDF

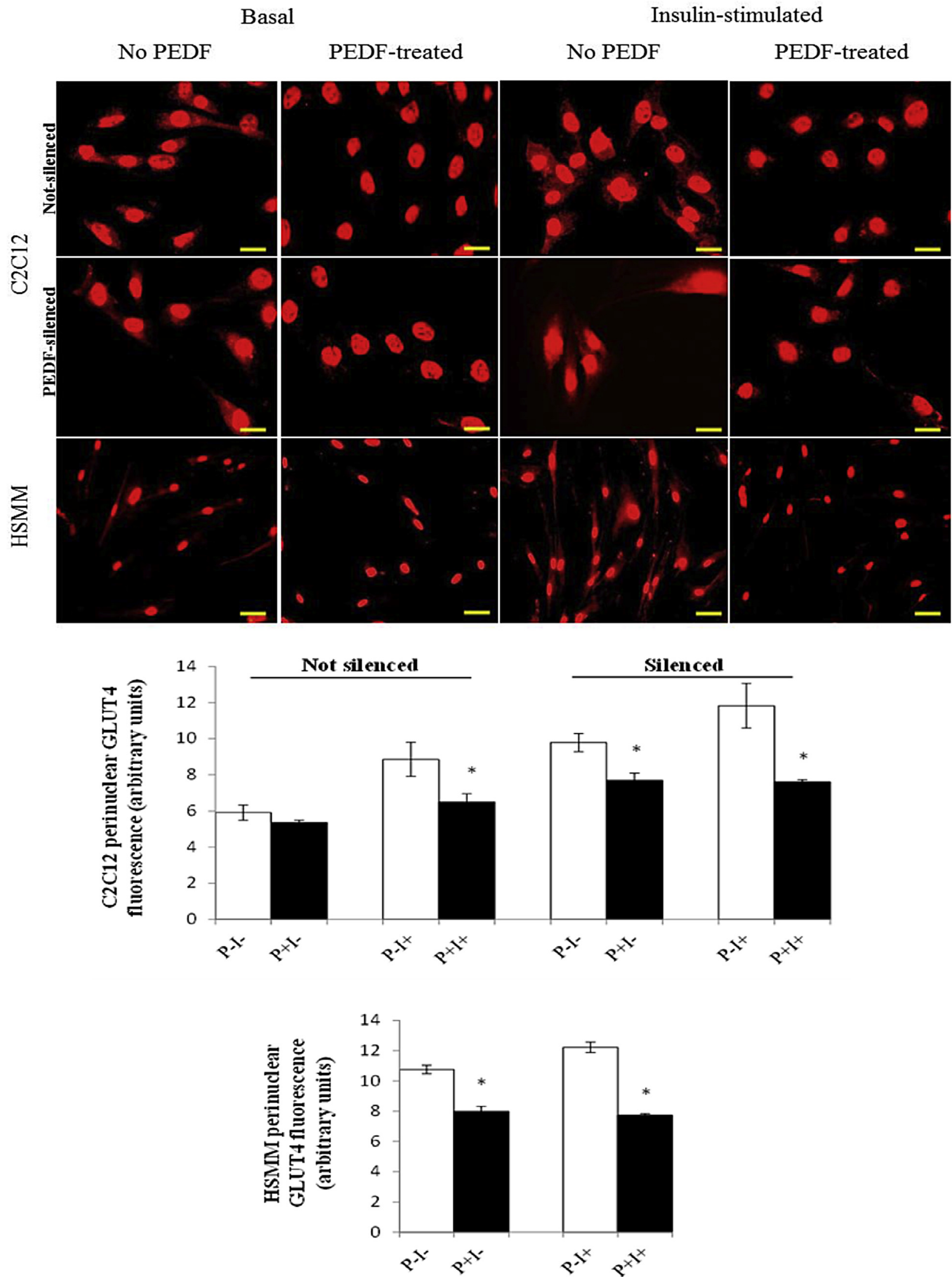


Fig. 4. Effect of PEDF on insulin-stimulated GLUT4 translocation in C2C12 and HSMM cells. C2C12 cells were treated with 100 nM PEDF under non-silenced (scrambled RNA) and silenced (PEDF siRNA) conditions during the last 24 h of silencing, followed with and without 100 nM insulin stimulation for 5 min prior to fixation and fluorescence immunocytochemistry with GLUT4 antibody. The representative images (Fig. 4) indicate the effect of PEDF on GLUT4 translocation in C2C12, PEDF-silenced C2C12, and HSMM cells. *Scale bar* = 50 μm and the bar represents semiquantitative densitometric analysis of the fluorescent intensity of GLUT4 at the perinuclear region analysed with Fiji software. *Abbreviations*; no insulin, I-, insulin, I+, not silenced (scrambled RNA), silenced with PEDF siRNA, S, no PEDF, P-, and PEDF, P+. Values are mean ± SD ($n = 3$), $^*p < 0.05$, PEDF-treated versus untreated.

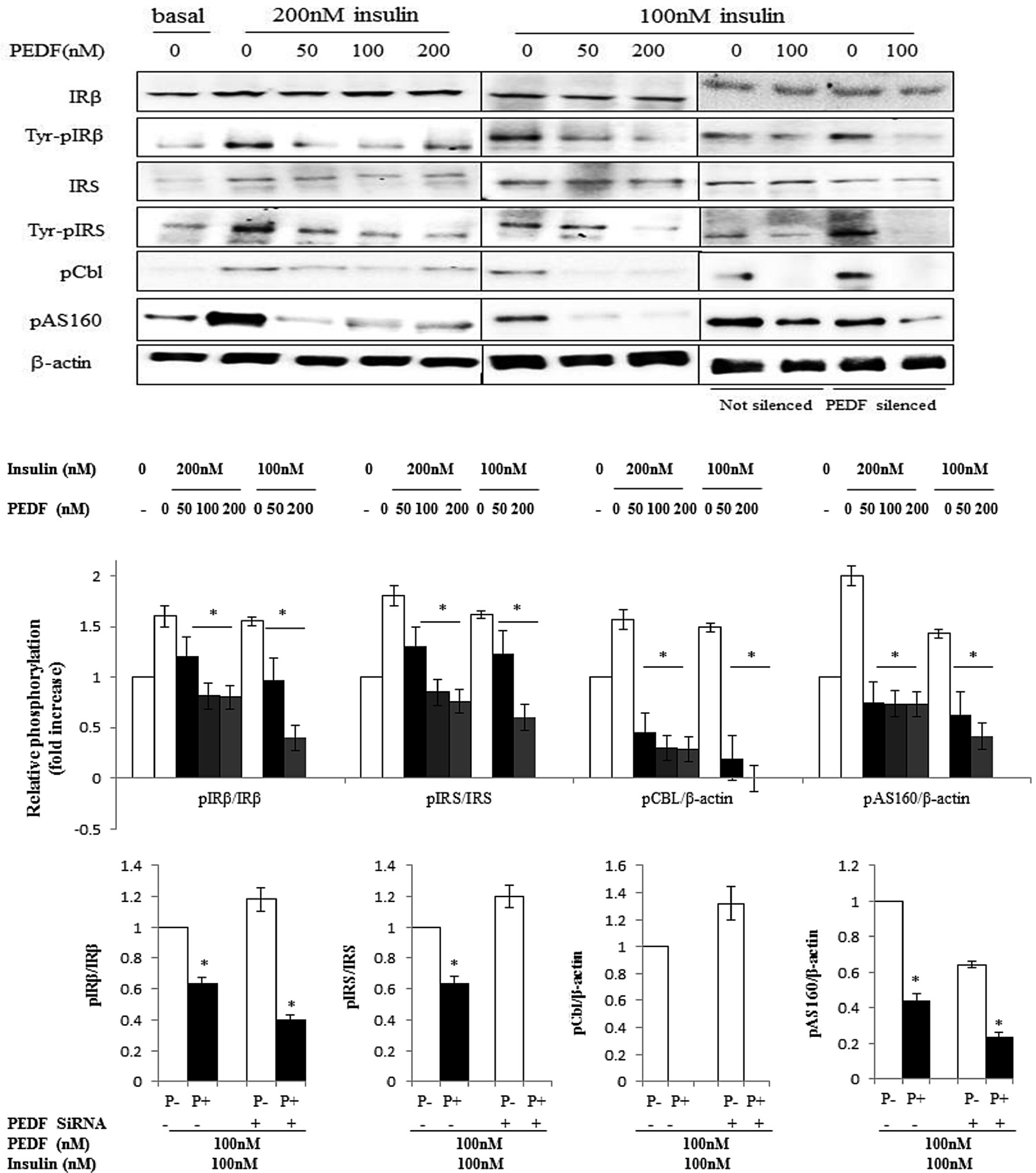


Fig. 5. Effect of PEDF on the Akt-dependent and independent molecular axes of insulin-stimulated GLUT4 translocation in C2C12 cells. C2C12 cells were treated with 50 nM, 100 nM and 200 nM PEDF under 100 nM and 200 nM insulin stimulation. Not-silenced and PEDF-silenced C2C12 cells were treated with 100 nM PEDF or left untreated for 24 h before stimulation with 100 nM insulin for 5 min, followed by immunoblotting with specific antibodies. PEDF inhibited tyrosine phosphorylation of IRS and also inhibited the phosphorylation-inactivation of AS160, thereby the Akt-dependent molecular axis of GLUT4 translocation: IRS-AKT/PKB-AS160-GLUT4. PEDF inhibited tyrosine phosphorylation of IRβ and also inhibited tyrosine phosphorylation of Cbl, thereby the Akt–independent molecular axis of GLUT4 translocation as well (IR-pCbl-GLUT4 axis). Column graphs represent relative densitometric analyses of the various proteins after normalising with β-actin levels and are expressed relative to the control. Values are mean ± SD ($n = 3$), *$p < 0.05$, PEDF-treated versus untreated.

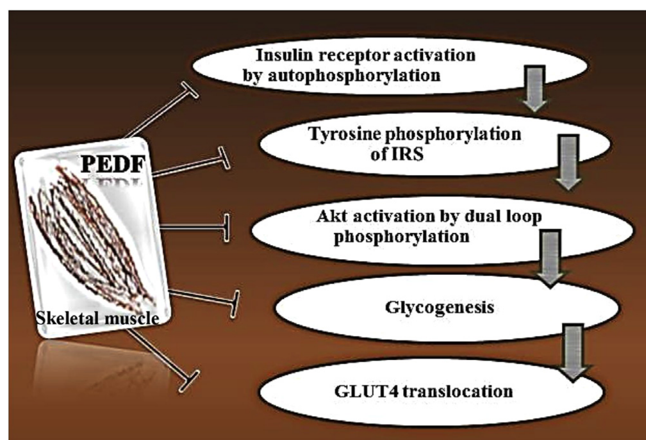


Fig. 6. Summary of the effect of PEDF on the insulin-mediated molecular events involved in glucose homeostasis. PEDF inhibits insulin-dependent IRβ autophosphorylation, IRS tyrosine phosphorylation, the dual loop phosphorylation-activation of Akt and phosphorylation-inactivation of GSK3β, thereby the glycogenic axis. PEDF inhibits insulin-dependent molecular axes of GLUT4 translocation, Akt-dependent phosphorylation-inactivation of As160 and Akt-independent tyrosine phosphorylation of Cbl pathway of GLUT4 translocation.

inhibited phosphorylation-inactivation of AS160. Phosphorylation of AS160, a protein containing Rab GAP (GTPase-activating protein) domain (Kane et al., 2002), is the most proximal step of the insulin signalling cascade identified to date and it promotes exocytosis of GLUT4 to the plasma membrane to facilitate glucose uptake and metabolism (Sano et al., 2003; Zeigerer et al., 2004). Insulin-stimulated AS160 phosphorylation is also impaired in skeletal muscle of type 2 diabetics (Karlsson et al., 2005) and such an effect is also produced by TNF-α (Plomgaard et al., 2005). In addition to its marked effect on the Akt/PKB signalling, PEDF abrogated tyrosine phosphorylation of Cbl, which is PKB-independent and completes the insulin-dependent GLUT4 translocation. Such an effect is observed in the skeletal muscle (Bernard et al., 2006) and visceral adipose tissue (Jun et al., 2008) of high-fat fed Sprague–Dawley rats. Taken together, these results suggest that PEDF might have relevance to insulin resistance both by the inhibition of glycogenesis and GLUT4 translocation. Our data provide insights into the more complex mechanisms underlying insulin resistance as pertinent to PEDF function (Fig. 6).

5. Conclusion

PEDF is a potential cancer therapeutic due to its anti-angiogenic and direct anticancer effects, hence characterising the other effects of PEDF becomes important in its drug development pathway. In summary, this study demonstrated for the first time the inhibitory effect of PEDF on insulin-dependent molecular mechanisms of glucose homeostasis, and suggests that PEDF could be a specific target in the management of metabolic disorders. This could develop better control over metabolic syndrome, obesity and related metabolic diseases such as type 2 diabetes, polycystic ovarian syndrome and hepatic disorders wherein PEDF levels are shown to be elevated. The findings from our study provide a detailed account of the effect of PEDF and antagonism of the insulin signalling pathway linked with insulin resistance. Overall, our study facilitates a better understanding of the direct cellular effect of a novel marker of metabolic dysregulation, PEDF on the insulin-dependent molecular mechanisms governing glucose homeostasis. Such understanding is essential to develop new therapeutic strategies to manage metabolic disorders, in attempts to allay the associated morbidity and mortality which is most preventable and definitely reversible and may result in improved disease outcome.

Conflict of interest

The authors declare no conflict of interest.

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References

Alessi, D.R., Cohen, P., 1998. Mechanism of activation and function of protein kinase B. Curr. Opin. Genet. Dev. 8, 55–62.
Bernard, J.R., et al., 2006. High-fat feeding effects on components of the CAP/Cbl signaling cascade in Sprague-Dawley rat skeletal muscle. Metabolism 55, 203–212.
Borg, M.L., et al., 2011. Pigment epithelium-derived factor regulates lipid metabolism via adipose triglyceride lipase. Diabetes 60, 1458–1466.
Carnagarin, R., et al., 2015a. PEDF-induced alteration of metabolism leading to insulin resistance. Mol. Cell. Endocrinol. 401, 98–104.
Carnagarin, R., et al., 2015b. Molecular aspects of glucose homeostasis in skeletal muscle - a focus on the molecular mechanisms of insulin resistance. Mol. Cell. Endocrinol. 417, 52–62. http://dx.doi.org/10.1016/j.mce.2015.09.004. S0303–7207(15)30070-8.
Chang, L., et al., 2004. Insulin signaling and the regulation of glucose transport. Mol. Med. 10, 65–71.
Chen, C., et al., 2010. Plasma level of pigment epithelium-derived factor is independently associated with the development of the metabolic syndrome in Chinese men: a 10-year prospective study. J. Clin. Endocrinol. Metab. 95, 5074–5081.
Crowe, S., et al., 2009. Pigment epithelium-derived factor contributes to insulin resistance in obesity. Cell Metab. 10, 40–47.
Dawson, D.W., et al., 1999. Pigment epithelium-derived factor: a potent inhibitor of angiogenesis. Science 285, 245–248.
DeFronzo, R.A., Ferrannini, E., 2001. Insulin resistance: a multifaceted syndrome responsible for NIDDM, obesity, hypertension, dyslipidemia, and atherosclerotic cardiovascular disease. Diabetes Care 14, 173–194.
DeFronzo, R.A., Tripathy, D., 2009. Skeletal muscle insulin resistance is the primary defect in type 2 diabetes. Diabetes Care 32, S157–S163.
Famulla, S., et al., 2011. Pigment epithelium-derived factor (PEDF) is one of the most abundant proteins secreted by human adipocytes and induces insulin resistance and inflammatory signaling in muscle and fat cells. Int. J. Obes. 35, 762–772.
Gattu, A.K., et al., 2013. Determination of mesenchymal stem cell fate by pigment epithelium-derived factor (PEDF) results in increased adiposity and reduced bone mineral content. FASEB J. 27, 4384–4394.
Jun, H., et al., 2008. High-fat diet alters pp2a, tc10, and cip4 expression in visceral adipose tissue of rats. Obesity 16, 1226–1231.
Kane, S., et al., 2002. A method to identify serine kinase substrates: akt phosphorylates a novel adipocyte protein with a Rab GTPase-activating protein (gap) domain. J. Biol. Chem. 277, 22115–22118.
Karlsson, H.K., et al., 2005. Insulin-stimulated phosphorylation of the Akt substrate AS160 is impaired in skeletal muscle of type 2 diabetic subjects. Diabetes 54, 1692–1697.
Lakeland, T.V., et al., 2014. Augmented expression and secretion of adipose-derived pigment epithelium-derived factor does not alter local angiogenesis or contribute to the development of systemic metabolic derangements. Am. J. Physiol. Endocrinol. Metab. 306, E1367–E1377.
Matsui, T., et al., 2014. Pigment epithelium-derived factor improves metabolic derangements and ameliorates dysregulation of adipocytokines in obese type 2 diabetic rats. Am. J. Pathol. 184, 1094–1103.
Meier, R., et al., 1998. Inactivation and dephosphorylation of protein kinase B alpha (PKB alpha) promoted by hyperosmotic stress. EMBO J. 17, 7294–7303.
Moller, D.E., Flier, J.S., 1991. Insulin resistance - mechanisms, syndromes, and implications. N. Engl. J. Med. 325, 938–948.
Nakamura, K., et al., 2010. Serum levels of pigment epithelium-derived factor (PEDF) are an independent determinant of insulin resistance in patients with essential hypertension. Int. J. Cardiol. 143, 96–98.
Norheim, F., et al., 2011. Proteomic identification of secreted proteins from human skeletal muscle cells and expression in response to strength training. Am. J. Physiol. Endocrinol. Metab. 301, E1013–E1021.
Petersen, S.V., et al., 2003. Pigment-epithelium-derived factor (PEDF) occurs at a physiologically relevant concentration in human blood: purification and characterization. Biochem. J. 374, 199–206.
Plomgaard, P., et al., 2005. Tumor necrosis factor-α induces skeletal muscle insulin resistance in healthy human subjects via inhibition of Akt substrate 160

phosphorylation. Diabetes 54, 2939–2945.
Ribon, V., et al., 1998. A novel, multifunctional c-Cbl binding protein in insulin receptor signaling in 3T3-L1 adipocytes. Mol. Cell. Biol. 18, 872–879.
Rychli, K., et al., 2009. Pigment epithelium-derived factor (PEDF) as a therapeutic target in cardiovascular disease. Expert Opin. Ther. Targets 13, 1295–1302.
Sabater, M., et al., 2010. Circulating pigment epithelium-derived factor levels are associated with insulin resistance and decrease after weight loss. J. Clin. Endocrinol. Metab. 95, 4720–4728.
Saltiel, A.R., Kahn, C.R., 2001. Insulin signalling and the regulation of glucose and lipid metabolism. Nature 414, 799–806.
Sano, H., et al., 2003. Insulin-stimulated phosphorylation of a Rab GTPase-activating protein regulates GLUT4 translocation. J. Biol. Chem. 278, 14599–14602.
Skurat, A.V., Roach, P.J., 1996. Multiple mechanisms for the phosphorylation of C-terminal regulatory sites in rabbit muscle glycogen synthase expressed in COS cells. Biochem. J. 313, 45–50.
Subramanian, P., et al., 2010. Pigment epithelium-derived factor receptor (PEDF-R): a plasma membrane-linked phospholipase with PEDF binding affinity. Adv. Exp. Med. Biol. 664, 29–37.
Sunderland, K.L., et al., 2012. Pigment epithelium-derived factor (PEDF) varies with body composition and insulin resistance in healthy young people. J. Clin. Endocrinol. Metab. 97, E2114–E2118.
Tombran-Tink, J., et al., 1991. PEDF: a pigment epithelium-derived factor with potent neuronal differentiative activity. Exp. Eye. Res. 53, 411–414.
Ueki, K., et al., 2004. Suppressor of cytokine signaling 1 (socs-1) and socs-3 cause insulin resistance through inhibition of tyrosine phosphorylation of insulin receptor substrate proteins by discrete mechanisms. Mol. Cell. Biol. 24, 5434–5446.
Vaag, A., et al., 1992. Decreased insulin activation of glycogen synthase in skeletal muscles in young nonobese Caucasian first-degree relatives of patients with non-insulin-dependent diabetes mellitus. J. Clin. Invest. 89, 782–788.
White, M.F., et al., 1998. The IRS-signalling system: a network of docking proteins that mediate insulin action. Mol. Cell. Biochem. 182, 3–11.
Yamagishi, S., et al., 2006. Elevated serum levels of pigment epithelium-derived factor in the metabolic syndrome. J. Clin. Endocrinol. Metab. 91, 2447–2450.
Yamagishi, S.I., et al., 2010. Pathophysiological role of pigment epithelium-derived factor (PEDF) in hepatic disorders. Curr. Med. Chem. 17, 1995–2000.
Yamauchi, T., et al., 1996. Insulin signalling and insulin actions in the muscles and livers of insulin-resistant, insulin receptor substrate 1-deficient mice. Mol. Cell. Biol. 16, 3074–3084.
Yang, S., et al., 2011. Serum pigment epithelium-derived factor is elevated in women with polycystic ovary syndrome and correlates with insulin resistance. J. Clin. Endocrinol. Metab. 96, 831–836.
Zeigerer, A., et al., 2004. Insulin stimulation of GLUT4 exocytosis, but not its Inhibition of endocytosis, is dependent on RabGAP AS160. Mol. Biol. Cell 15, 4406–4415.

Graphical abstract

Chapter 5: Pigment epithelium-derived factor stimulates skeletal muscle glycolytic activity through NADPH oxidase-dependent reactive oxygen species production – the mechanism of PEDF-induced metabolically inflexibility in skeletal myocytes

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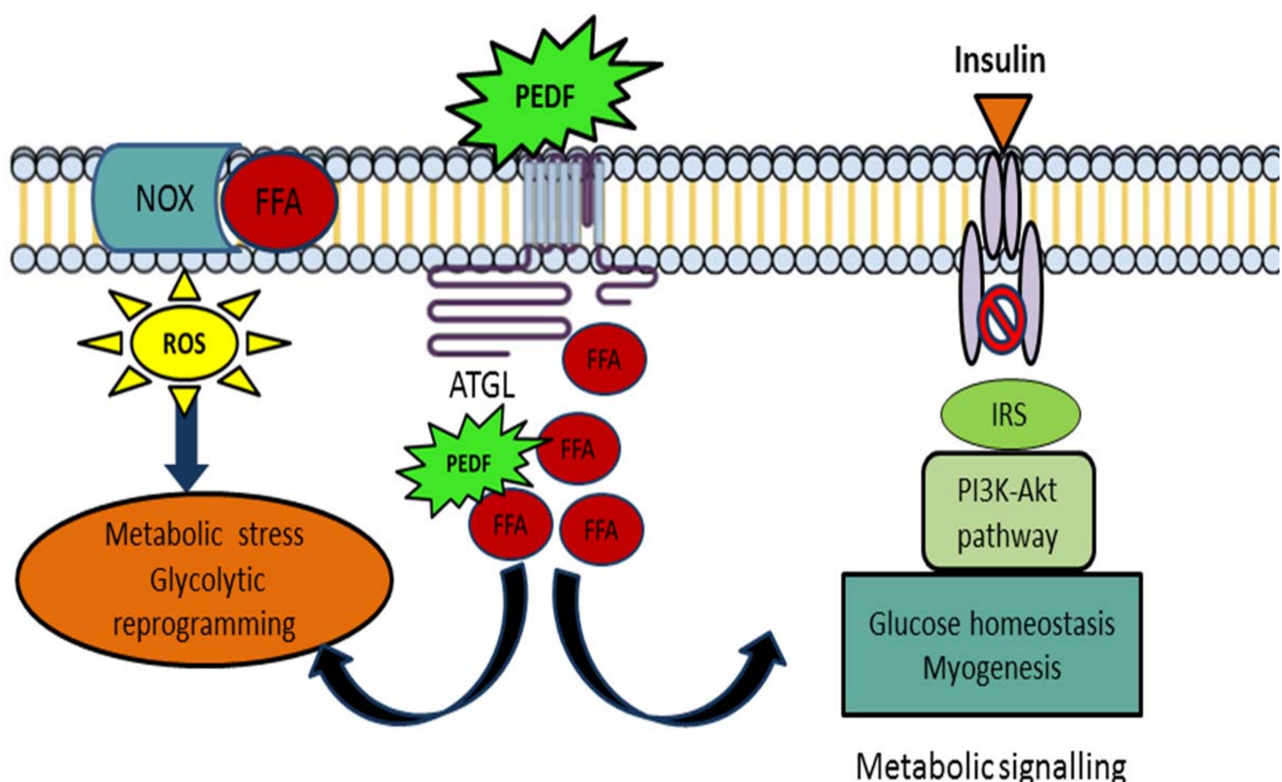

The skeletal myocytes express a PEDF-inducible oxidant generating system via nicotinamide adenine dinucleotide phosphate (NADPH) oxidase. The PEDF-induced NADPH-dependent reactive oxygen species (ROS) induced metabolic stress and triggered a shift towards glycolysis.

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Pigment epithelium-derived factor stimulates skeletal muscle glycolytic activity through NADPH oxidase-dependent reactive oxygen species production

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ABSTRACT

Pigment epithelium-derived factor is a multifunctional serpin implicated in insulin resistance in metabolic disorders. Recent evidence suggests that exposure of peripheral tissues such as skeletal muscle to PEDF has profound metabolic consequences with predisposition towards chronic conditions such as obesity, type 2 diabetes, metabolic syndrome and polycystic ovarian syndrome. Chronic inflammation shifts muscle metabolism towards increased glycolysis and decreased oxidative metabolism. In the present study, we demonstrate a novel effect of PEDF on cellular metabolism in mouse cell line (C2C12) and human primary skeletal muscle cells. PEDF addition to skeletal muscle cells induced enhanced phospholipase A2 activity. This was accompanied with increased production of reactive oxygen species in a nicotinamide adenine dinucleotide phosphate (NADPH) oxidase-dependent manner that triggered a shift towards a more glycolytic phenotype. Extracellular flux analysis and glucose consumption assays demonstrated that PEDF treatment resulted in enhanced glycolysis but did not change mitochondrial respiration. Our results demonstrate that skeletal muscle cells express a PEDF-inducible oxidant generating system that enhances glycolysis but is sensitive to antioxidants and NADPH oxidase inhibition.

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1. Introduction

Pigment epithelium-derived factor (PEDF) is a 50 kDa multifunctional glycoprotein implicated in the pathogenesis of metabolic dysregulation and insulin resistance involved in endocrine, cardiovascular, hepatic, and metabolic disorders (Carnagarin et al., 2015). PEDF is an extracellular serine protease inhibitor (Serpin) with demonstrable anti-angiogenic and anti-tumorigenic properties that contribute to its potential in cancer therapy (Dawson et al., 1999; Alcantara and Dass 2014). Identified originally in the retinal pigment epithelial cells, PEDF is endogenously produced by various cell types such as adipocytes, hepatocytes, skeletal and smooth muscle cells (Famulla et al., 2011) and exists at a physiological concentration of 100 nM in human plasma (Petersen et al., 2003). PEDF levels are elevated in patients with type 2 diabetes mellitus (Nakamura et al., 2009), obesity (Crowe et al., 2009), metabolic syndrome (Chen et al., 2010; Yamagishi et al., 2006), polycystic ovarian syndrome (PCOS) (Yang et al., 2011), hepatic disorders (Yamagishi et al., 2010). PEDF circulating levels positively correlates with metabolic syndrome risk factors including high body mass index (BMI) and waist-hip ratio, elevated triglycerides and negatively correlates with circulating high density lipoprotein levels (Sabater et al., 2010). PEDF is also a determinant of cardiovascular mortality and morbidity in humans (Rychli et al., 2009) and insulin resistance in metabolic disorders (Carnagarin et al., 2016) and essential hypertension (Nakamura et al., 2010).

The link between PEDF and reactive oxygen species (ROS) is an emerging topic. ROS act as endogenous signalling molecules to regulate skeletal muscle metabolism. ROS exhibit divergent functions: when generated during physiological processes at low concentration, they can have positive effects, however accumulating evidence suggests that metabolic stress-derived ROS could be

Abbreviations: PEDF, pigment epithelium-derived factor; NADPH, nicotinamide adenine dinucleotide phosphate.

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detrimental to skeletal muscle. The major ROS-producing machinery in skeletal muscle includes the mitochondria (Di Meo and Venditti 2001; Herrero and Barja 1997; Kozlov et al., 2005) and nicotinamide adenine dinucleotide phosphate (NADPH) oxidases (NOXs) located within the endoplasmic reticulum (Piao et al., 2005; Powers and Jackson 2008). Whilst the skeletal muscle mitochondrial ROS production is associated with its contractile function (Jackson et al., 2007), inflammatory responses trigger NOX-dependent ROS production which could be potentially amplified in a feed-forward manner by inflammatory cytokines (Li et al., 2007).

Another significant source of intracellular ROS production includes phospholipase A2 (PLA2) (Zhao et al., 2002), the activation of which results in lipolysis releasing free fatty acids such as arachidonic acid and glycerol in skeletal muscle. This particular phospholipase specifically recognizes and catalytically hydrolyses the sn-2acyl bond of the phospholipids releasing arachidonic acid and lysophosphatidic acid. Arachidonic acid is a potent NOX activator resulting in the induction of oxidative stress. (Matono et al., 2006). Recently, PEDF has emerged as a likely factor that could contribute to ROS homeostasis and ovarian oxidative stress (Kampfer et al., 2014). In this study, we sought to explore if PEDF could impact ROS production and ROS-dependent metabolic alterations in skeletal muscle cells.

2. Methods

2.1. Materials

Recombinant PEDF was purchased from MD Bioproducts (Bethesda, MD, USA). Fetal calf serum was purchased from Gibco (Fort Worth, TX, USA), human recombinant insulin, Dulbecco's Modified Eagle's Medium, penicillin/streptomycin and trypsin were obtained from Life Technologies. Skeletal Muscle Myoblast Cell Media – Clonetics™ SkBM™-2 BulletKit™ was purchased from Lonza (Walkersville, MD, USA). The patatin-like phospholipase domain-containing 2/adipose triglyceride lipase (PNPLA2/ATGL) antibody was purchased from EMD Millipore Corporation (Temecula, CA, USA), and ECL™ and ECL Plus reagents from GE Healthcare (Piscataway Township, NJ, USA). Catalase (1000U), superoxide dismutase (150U), ML171 (5 μM) and all other reagents were obtained from Sigma–Aldrich unless otherwise stated.

2.2. Cell culture

Human skeletal muscle myoblasts (HSMMs) were purchased from Lonza (Walkersville, MD, USA) and were used within 5 passages from receipt. C2C12 mice myoblasts were purchased from ATCC (Manassas, VA, USA) and used within passage 14. Cells were cultured under an atmosphere of 5% CO_2 at 37 °C in a humidified incubator and when confluent, cells were channelled towards differentiation with 2% serum and myotubes were used for experiments following 72 h of differentiation.

2.3. Immunoblot analysis

Cells were lysed in radioimmunoprecipitation (RIPA) buffer containing protease and phosphatase inhibitors (Cell Signaling Technology, Beverly, MA, USA) and quantified using bicinchoninic acid (BCA) assay (Pierce) as per the manufacturer's instructions. Samples were fractionated by SDS-PAGE (Invitrogen) at 150 V for an hour and electrotransferred to polyvinylidene fluoride (PVDF) membranes. Membranes were blocked with 5% skimmed milk for an hour and incubated overnight at 4 °C with the indicated primary antibodies, followed by 1 h incubation with horseradish peroxidase-conjugated secondary antibody (Dako). All primary antibodies were used at 1:1000 dilution in 5% BSA prepared in TBS containing 0.1% Tween-20 (TBST). This was followed by HRP-conjugated secondary antibody incubation at 1:20,000 prepared in 5% skim milk in TBST for 1 h at room temperature, followed by chemiluminescent detection of target bands. Intermittent washes following blocking, primary and secondary antibody incubation steps were performed using washing buffer containing 0.05% Tween-20 (TBST). All the primary antibodies were obtained from Cell Signaling Technology unless otherwise stated.

2.4. Fluorescence immunocytochemistry

Cells were seeded in 96-wells plate with 5000 cells/well. After 24 h treatment, immunofluorescence was conducted following our previously optimised protocol (Carnagarin et al., 2016). Cells were fixed with 4% paraformaldehyde and then permeabilised with 0.3% saponin in PBS. Nonspecific binding sites were blocked by 0.1% saponin in PBS with 0.25% BSA and 2% goat serum. Rabbit anti rat ATGL antibody (1:1000) was incubated with cells overnight at 4 °C. After wash with PBS, Alexa Fluor 488 conjugated anti-rabbit IgG (1:2000, Cell Signaling) was incubated with cells for 30 min. Before mounting with glycerol, nuclei of cells were counterstained with DAPI (Cell Signaling), and images were acquired with an Olympus IX51 fluorescent microscope (Olympus Life Science Solutions, USA).

2.5. Measurement of $PNPLA_2$-dependent glycerol secretion

Following 24 h PEDF treatment, the cell culture media from C2C12 and HSMM were collected for glycerol measurement using AdipoLyze™ Lipolysis Detection Assay (Lonza, USA) according to manufacturer's instructions. Fluorescence was detected at 595 nm after excitation at 570 nm using a multilabel counter (Enspire Multimode Plate Reader, PerkinElmer, USA).

2.6. ROS production

Intracellular ROS generation was measured using 2′,7′-dichlorodihydrofluorescein diacetate (chloromethyl-H_2DCFDA) probe (Invitrogen, USA). The cells were harvested and pre-stained with 10 μM CM-H2DCFDA at 37 °C for around 30 min in phenol red and serum-free DMEM media. The fluorescent intensity following various treatments was measured by flow cytometry (Attune Acoustic Focusing Cytometer, Applied Biosystems, USA) and analysed using FlowJo V10 software (TreeStar, USA).

2.7. Cell viability assay

Viability of C2C12 and HSMM cells following PEDF and other treatments was assessed using the Cell Titre Blue assay (Promega, Madison, WI, USA). Relative numbers of viable cells were measured in comparison to the untreated control and are expressed as percentage using the, resazurin-based, fluorometric assay according to the manufacturer's instructions at Ex560 nm/Em590 nm using a multimode Plate Reader (Enspire PerkinElmer, USA).

2.8. Extracellular flux analysis

The protocol for C2C12 extracellular flux analysis was performed using XFe96 Flux analyser (Seahorse Bioscience, Billercia, MA, USA) based on previously published work (Nicholls et al., 2010) using the Mito Stress Test kit from Seahorse Bioscience for the assessment of cellular bioenergetics. Following treatment with 100 nM PEDF for 24 h, C2C12 media was switched to serum-free DMEM, without bicarbonate but containing sodium pyruvate (1 mM) and d-glucose (2.5 mM final), followed by an

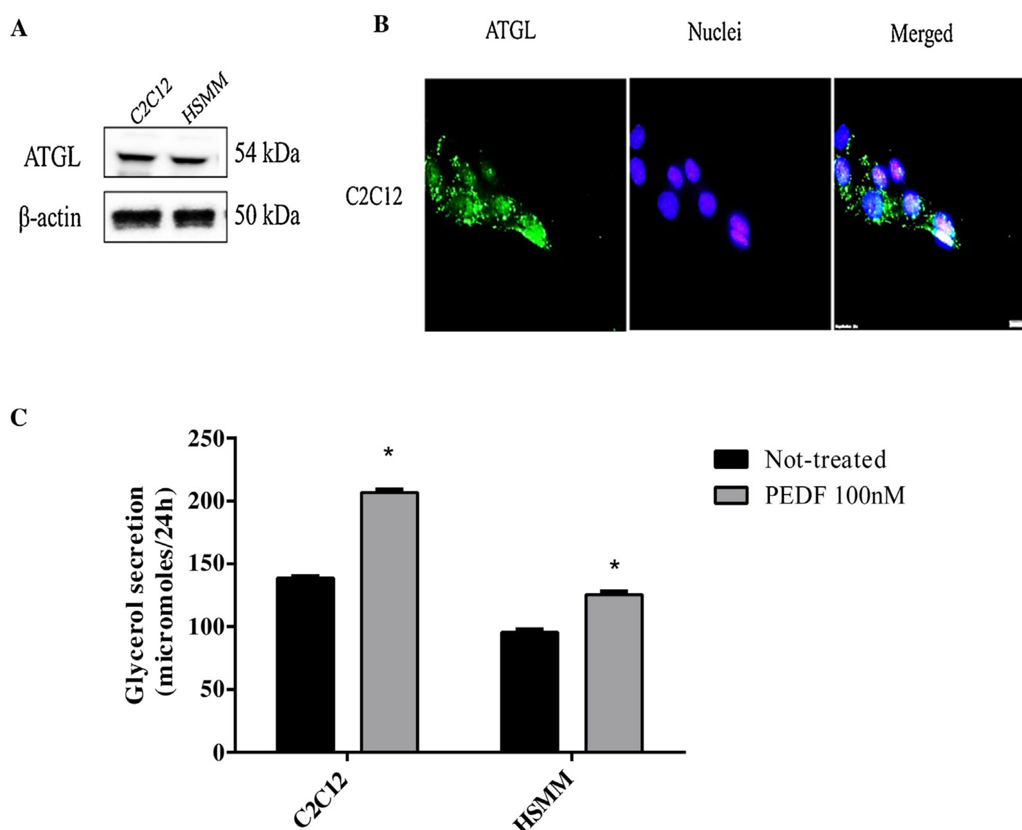

Fig. 1. PEDF-receptor expression in skeletal myocytes. (A) Immunoblot analysis shows the expression of ATGL/PNPLA2 in C2C12 and HSMM. (B) Immunofluorescence: C2C12 cells were subjected to analysis of ATGL/PNPLA2 expression by using rabbit anti-rat ATGL primary antibody, followed by goat anti-rabbit secondary antibody conjugated with Alexa fluor 488 (green). Nuclei were stained with DAPI (blue). Images are representative of replicates in one experiment. *Abbreviations; ATGL – adipose triglceride lipase, PEDF – pigment epithelium-derived factor, PNPLA2- patatin-like phospholipase domain-containing 2.* (C) Glycerol secretion assay: Following treatment of C2C12 and HSMMs with 100 nM PEDF for 24 h, the glycerol concentration was determined. Values are mean ± SE (n = 3), **p* < 0.05, PEDF-treated versus untreated (for interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

hour incubation at 37 °C in a non-CO_2 incubator. After recording the basal measurements, the injections via a pre-hydrated XF^{e}96 sensor cartridge were carried out consecutively as follows: insulin (100 nM final), oligomycin (2 μM final), carbonyl cyanide-4-trifluoromethoxy phenyhydrazone (FCCP) (0.4 μM final), and finally rotenone/antimycinA combination (1 μM final). O_2 consumption rate (OCR), which indicates mitochondrial respiration, was measured. Extracellular acidification rate (ECAR), the glycolytic index was measured following sequential injections of insulin (100 nM final), glucose (25 mM), oligomycin and 2-deoxy-glucose (2-DG) and measurements were normalized to total protein content per well using the BCA assay.

2.9. Glucose consumption assay

The glucose consumption of C2C12 and HSMM was determined using the Amplex® Red reagent (Invitrogen, USA) by measuring glucose levels of the corresponding media before and after 24 h incubation with or without PEDF in DMEM containing 11.1 mM and SkBM containing 5.55 mM of d-glucose respectively. The red fluorescence was detected at $Ex_{571\,nm}/Em_{585\,nm}$ using the Enspire Multimode Plate Reader. The glucose consumed over the 24 h period was calculated by subtracting the glucose concentration at 24 h from that at 0 h.

2.10. Statistical analysis

The results are presented as means ± S.E.M. The statistical differences were analysed using the two-tailed unpaired parametric *t*-test or one-way ANOVA with Tukey post-hoc tests. The statistical significance was defined as $p < 0.05$.

3. Results

3.1. PEDF induces ROS in a NOX-dependent manner in skeletal muscle in the absence of overt oxidative stress or damage

Initially we investigated the expression of PEDF receptor (ATGL/PNPLA2) in skeletal myocytes. The expression of PEDF-R was confirmed by immunoblot analysis in C2C12 and HSMM (Fig. 1A). When labelled with anti-ATGL antibody, a discrete positive punctate pattern, typical of membrane receptors was noticed at the C2C12 surface (Fig 1B).The stimulation with PEDF significantly increased PNPLA2-associated glycerol release (Fig. 1C) indicating PEDF-dependent receptor activation in the skeletal muscle system.

In the next experiment, we determined the effect of PEDF exposure on ROS production both in mouse (C2C12) and human (HSMM) skeletal muscle cells. PEDF caused the generation of ROS in both cell types within minutes and co-treatment with antioxidants such as catalase (1000U) and superoxide dismutase (150U) abolished ROS induction in C2C12 (Fig. 2A,B) and HSMM (Fig. 2C,D).

Interestingly, PEDF exposure (100 nM for 24 h) did not induce overt oxidative stress or damage and had no effect on cellular viability despite elevated ROS levels. PEDF treatment did not alter the viability of C2C12 cells, neither when the cells were treated alone with PEDF nor in combination with antioxidants and inhibitors (Fig. 3A,B).

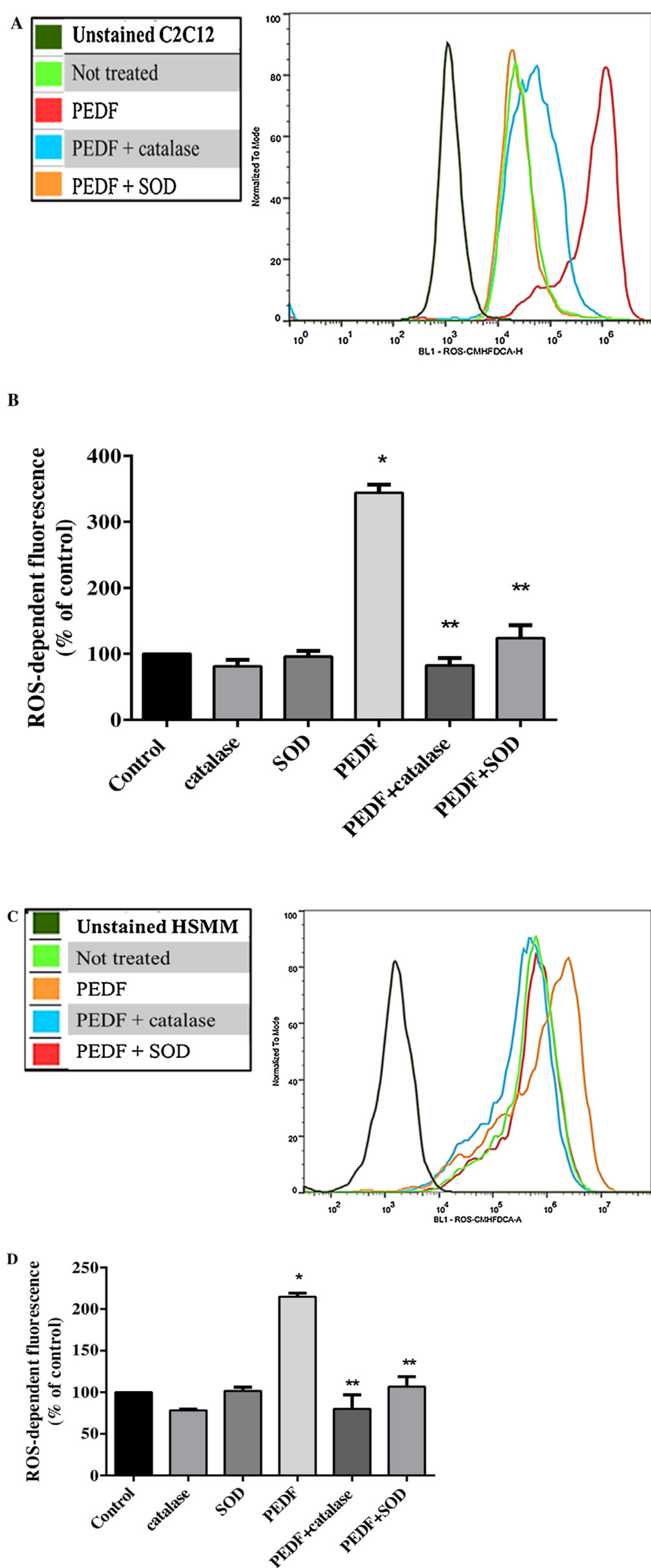

Fig. 2. The effect of PEDF on skeletal muscle ROS production. PEDF-induced ROS was determined by flow-cytometric detection with chloromethy-H2DCFDA probe and the effect of co-treatment with antioxidants such as catalase (1000U) and superoxide dismutase (150U) was assessed to confirm ROS in C2C12 (A&B) and HSMM (C&D). All experiments were repeated 3 times. *Abbreviations; PEDF – pigment epithelium-derived factor, ROS – reactive oxygen species, SOD – superoxide dismutase.* The data are presented as mean ± SE (n = 3), *$p < 0.05$, PEDF-treated versus untreated, **$p < 0.05$, PEDF treated with antioxidants versus PEDF treatment alone.

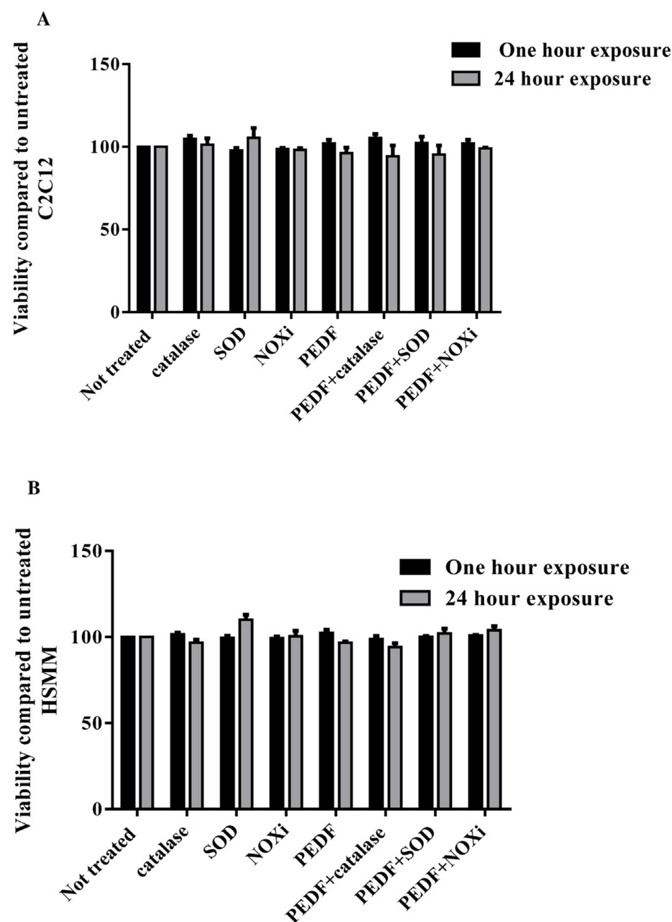

Fig. 3. PEDF does not induce any overt oxidative stress in skeletal muscle. Cell viability was analysed via a resazurin-based assay following various treatments – neither PEDF exposure nor did any treatment cause any overt oxidative stress or damage in C2C12 and HSMM. *Abbreviations; PEDF – pigment epithelium-derived factor, ROS – reactive oxygen species, SOD – superoxide dismutase, NOXi- NADPH oxidase inhibitor, ML171.* Values are mean ± SE (n = 3), *$p < 0.05$, PEDF-treated versus untreated.

Subsequently, mitochondrial bioenergetics was analysed by extracellular flux analysis in C2C12 following exposure to 100 nM PEDF for 24 h both in the presence and absence of 100 nM insulin. The vital mitochondrial parameter of basal OCR was measured following perturbation with three different compounds in succession. Proton leak was calculated by subtracting the minimum OCR obtained following rotenone/antimycin A injection (non-mitochondrial respiration) from the minimum OCR after injecting oligomycin, an adenosine triphosphate (ATP) synthase inhibitor and ATP production was calculated by subtracting proton leak from basal respiration. The addition of FCCP, an exogenous uncoupler increased OCR of the entire cell system to a higher level. Reserve respiration capacity well-defined as the quantitative difference between maximal and the initial OCR reflects the influence of any pathophysiological stimulus on the mitochondrial bioenergetic capacity. In the current study, PEDF exposure did not significantly change C2C12 mitochondrial bioenergetics (Fig 4).

In the next set of experiments, the other major ROS producer in skeletal muscle, the NADPH-oxidase system was investigated. We identified that PEDF treatment along with a specific NOX inhibiter such as ML171 (5 μM) significantly reduced the PEDF-induced ROS production in C2C12 cells (Fig 5A,B) and similar results were obtained with HSMMs (Fig 5C,D). Control experiments were performed with the antioxidants and NOX inhibitor alone did not result in ROS generation, ruling out any non-specific action.

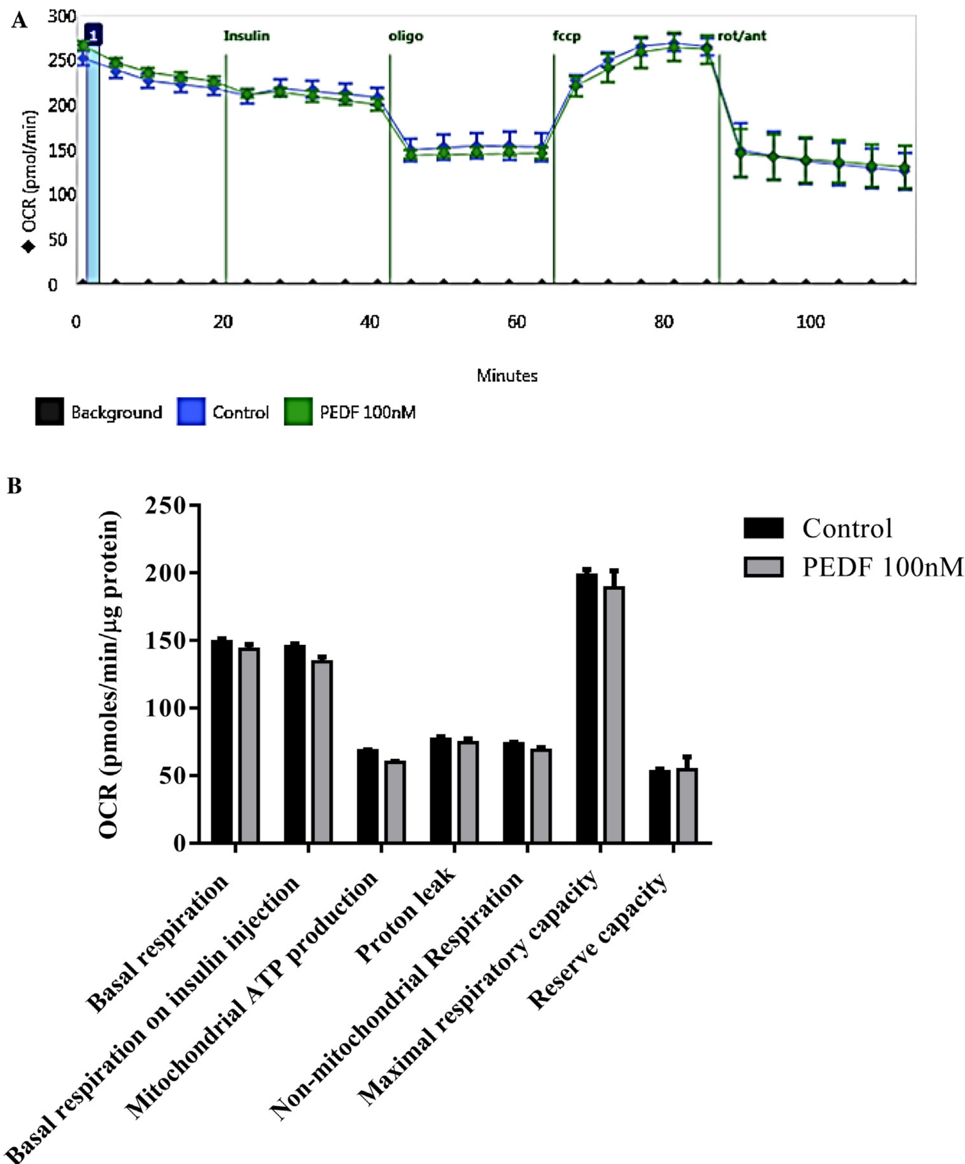

Fig. 4. Effect of PEDF on C2C12 bioenergetics. O_2 consumption rate (OCR) after 24 h pre-treatment of C2C12 with PEDF was measured using Seahorse flux analyser 96 in response to 100 nM insulin, 2 μM oligomycin, 0.5 μM FCCP and 1 μM rotenone/antimycin A (n = 3 per group). *Abbreviations; PEDF – pigment epithelium-derived factor, FCCP* – carbonyl *cyanide p-trifluoromethoxyphenylhydrazone, OCR – Oxygen consumption rate.* Basal respiration before and after insulin injection, ATP production, proton leak non-mitochondrial respiration, maximal respiratory and reserve capacity were the parameters calculated from OCR and the data are presented as mean ± SE, $^*p < 0.05$, in comparison with control. PEDF did not impact C2C12 bioenergetics.

3.2. PEDF induces ROS-dependent glycolytic reprogramming in skeletal muscle metabolism

PEDF-induced alteration in glycolytic activity was investigated by using a glycolytic stress test which non-invasively measures glycolysis in real time determining the rate of lactate production from glucose. C2C12 cells were treated with 100 nM PEDF for 24 h and glycolysis was measured by monitoring the ECAR of cells in culture (normalised to 10,000 cells). Baseline glycolysis measurements were taken at the initial 20 min followed by the addition of insulin, D-glucose, oligomycin and 2-DG. Insulin enhanced glycolytic capacity of the cell system and the ECAR increased when the cells were acutely given D-glucose which may be attributable to glucose metabolism through the glycolytic pathway. Oligomycin, a mitochondrial ATPase inhibitor, forced the cells to maximise their dependence on glycolysis so as to sustain ATP production and the 2-deoxy glucose competitively inhibited hexokinase that catalyses the first reaction of the glycolytic pathway and eliminates any residual ECAR signal that could be attributed to glucose metabolism, and the resulting ECAR decrease is defined as glycolytic reserve (Foresti et al., 2015). PEDF enhanced lactate production as indicated by the elevated ECAR. Interestingly, PEDF treatment significantly increased both baseline glycolysis (pre-oligomycin injection) and peak glycolytic capacity (post-oligomycin injection) in C2C12 (Fig 6A,B).

To further validate the PEDF-induced glycolytic reprogramming, we measured glucose consumption following 24 h of 100 nM PEDF treatment. The results showed enhanced glucose consumption by PEDF-treated C2C12 and HSMMs. However this effect was ablated by antioxidant treatment. C2C12 and HSMM were treated with catalase and superoxide dismutase at the same concentration that rescued cells from PEDF-induced oxidative stress. The antioxidant treatment significantly decreased the PEDF-induced increase in glucose consumption in both C2C12 and HSMM skeletal muscle cells indicating that ROS mediates PEDF-induced glycolytic switch (Fig 6C,D).

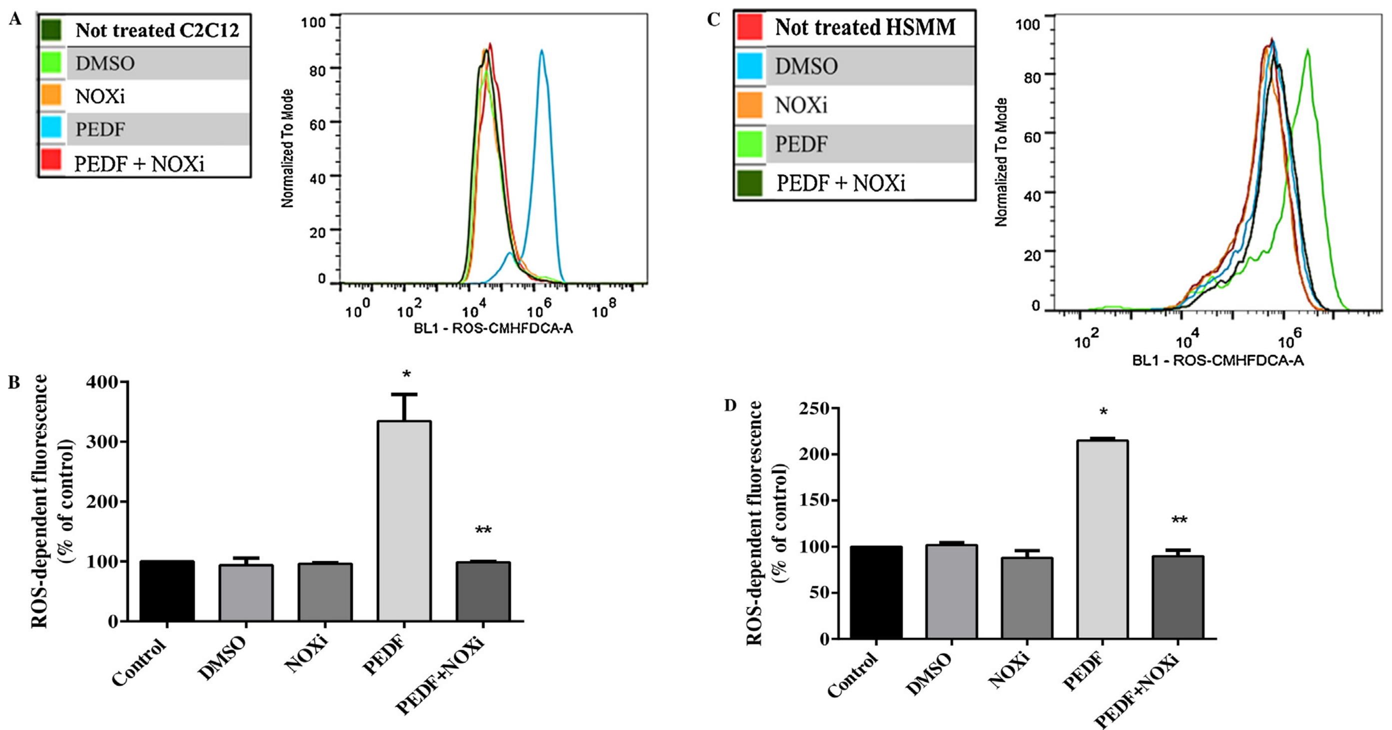

Fig. 5. Effect of PEDF on ROS production following NOX inhibition in C2C12 and HSMM cells. Representative images showing ROS levels with and without NOX-inhibition assessed using CM-H2DCFDA by flow cytometry. *Abbreviations*; *DMSO – dimethyl sulfoxide, NOXi – NOX inhibitor, ML171*. Values are mean ± SE (n = 3), **p* < 0.05, PEDF-treated versus untreated, ***p* < 0.05, PEDF treated with NOX inhibitor, Ml171 (5 μM), versus PEDF treatment alone.

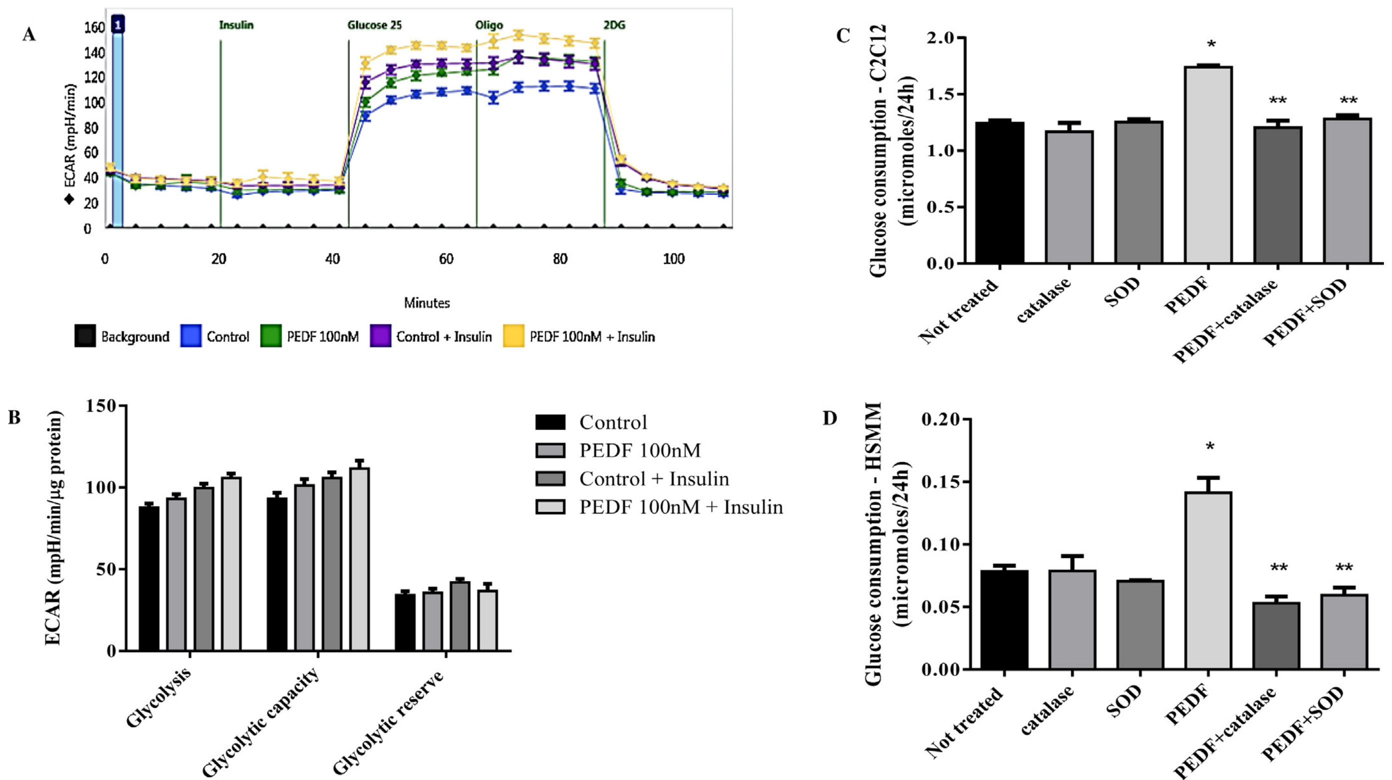

Fig. 6. PEDF induces ROS-dependent glycolytic reprogramming in skeletal myocytes. (A&B) Extracellular acidification rate (ECAR) following 24 h pre-treatment of C2C12 with PEDF was measured in response to 100 nM insulin, 25 mM glucose, 2 μM oligomycin, and 90 mM 2-deoxy-glucose (2-DG) (n = 3 per group). PEDF caused a glycolytic shift enhancing glycolysis, glycolytic capacity and glycolytic reserve of C2C12. The PEDF-induced glycolytic reprogramming was further validated by measuring glucose consumption following PEDF treatment along with antioxidant treatments. (C&D) PEDF enhanced glucose consumption in C2C12 and HSMMs, but this effect was significantly reduced when treated along with catalase and superoxide dismutase. *Abbreviations; PEDF – pigment epithelium-derived factor, ECAR – Extracellular acidification rate, SOD – superoxide dismutase.* The data are presented as mean ± SE (n = 3), **p* < 0.05, PEDF-treated versus untreated, ***p* < 0.05, PEDF treated with antioxidants versus PEDF treatment alone.

4. Discussion

It is known that pro-inflammatory cytokines induce metabolic stress, but the role of PEDF in inducing metabolic dysregulation in skeletal muscle was unknown. Accumulating evidence suggests that PEDF is a stress inducing protein that induces inflammatory signalling in peripheral tissues, increases adipocyte lipolysis and promotes lipid accumulation in muscle and liver (Famulla et al., 2011; Crowe et al., 2009). Recently, PEDF emerged as an ovarian factor involved in the generation of ROS (Kampfer et al., 2014). Hence, we intended to explore if PEDF impacts ROS generation and any associated metabolic effects in skeletal muscle, which is the major site of peripheral glucose and amino acid metabolism. In the present work, we have uncovered that skeletal muscle expresses PEDF-inducible oxidant-generating system sensitive to antioxidants and NOX inhibition, that enhances muscle glycolytic metabolism.

PEDF exerts its biologic actions by binding to its dominant cell surface receptor that possesses phospholipase activity (Filleur et al., 2005) and this interaction is responsible for PEDF-dependent metabolic dysregulation (Borg et al., 2011). Identified originally in retinal pigment epithelial cells (Notari et al., 2006), this receptor belongs to the family of patatin-like phospholipase domain-containing 2 (PNPLA2) proteins, a highly conserved triacylglycerol lipase (ATGL) critical in the maintenance of systemic lipid and glucose homeostasis (Haemmerle et al., 2006; Zimmermann et al., 2004; Grönke et al., 2005; Jenkins et al., 2004; Smirnova et al., 2006; Villena et al., 2004). The PEDF-ATGL interacts with high affinity resulting in potent phospholipase A2 activity following its cytosolic translocation (Notari et al., 2006) and catalyses the PEDF-induced lipolysis to yield free fatty acids including arachidonic acid and glycerol (Chung et al., 2008; Subramanian et al., 2010). We observed that skeletal myocytes express ATGL/PNPLA2, the PEDF receptor and PEDF induced lipolysis in skeletal myocytes determined by glycerol release as in various other cell models (Chung et al., 2008) confirming PEDF activity in the skeletal muscle system.

PEDF has emerged as a potent inducer of ROS in human ovary (Kampfer et al., 2014). This is in agreement with our data where PEDF exposure induced ROS generation in skeletal myocytes and such an effect was ablated by co-treatment with antioxidants such as catalase and superoxide dismutase. Surprisingly PEDF had no effect on cellular viability despite elevated ROS levels suggesting that PEDF-induced muscle ROS accumulation did not provoke any overt oxidative stress or damage. Furthermore, we identified the source of PEDF-induced ROS. Employing the mito-stress test in C2C12 using extracellular flux analysis, PEDF did not cause any measurable mitochondrial stress in C2C12 and our results are in agreement with studies conducted by Borg et al., 2011; where PEDF did not impact mitochondrial function when isolated from mouse muscle. This prompted us to look into the other major ROS sources in skeletal muscle which includes NOX, the inhibition of which attenuated PEDF-induced ROS and this is similar to the effect of PEDF in human ovarian cells where PEDF-induced ROS production was demonstrated to be NOX-dependent (Kampfer et al., 2014). Other studies have shown that PLA2 regulates NOX (Zhao et al., 2002) and increased PLA2 activity triggers NOX to produce ROS as seen in cardiac fibroblasts (Colston et al., 2005) and such an effect is also shown by TNFα and phorbol esters in endothelial cells (Li et al., 2002). We have shown that PEDF enhances phospholipase activity in the skeletal muscle system which could potentially fuel the NOX-dependent ROS production.

We next examined the effect of PEDF-induced ROS accumulation in muscle on glycolytic activity (Shi et al., 2015). Augmented muscle glycolytic activity has been reported following treatment with inflammatory cytokines such as TNFα (Remels et al., 2015; Benigni et al., 2000),in disorders such as endotoxemia (Spitzer et al., 1989) and mouse models of cancer cachexia associated with a pro-inflammatory state (Der-Torossian et al., 2013). ROS provokes glycolytic reprogramming in cancer cells (Chandel et al., 1998) to generate a chemically reduced milieu to combat ROS-induced damage (Brand and Hermfisse, 1997). Treatment of C2C12 with H_2O_2 induced a rapid increase in ECAR and such an effect was abolished by treatment with a superoxide dismutase-mimetic and the free radical scavenger tempol confirming ROS-induced glycolytic activation in peripheral metabolic tissues (Shi et al., 2015). PEDF-induced glycolytic activation was completely ablated by antioxidants such as catalase and superoxide dismutase in both C2C12 and HSMM confirming the role of ROS in PEDF-induced glycolytic reprogramming. Taken together, in concert with current literature, our data illustrate that PEDF-induced overproduction of ROS results in stimulation of glycolysis.

5. Conclusion

PEDF is a pluripotent protein with multimodal effects in various tissues. It is an emerging inducer of metabolic stress, which in addition to inflammatory and lipolytic actions, has a role in ROS generation in skeletal muscle cells which may involve the NADPH oxidase system. PEDF can contribute to ROS production and possibly oxidative stress in peripheral metabolic tissues such as skeletal muscle, altering metabolic status.

Conflict of interest

The authors declare no conflict of interest.

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References

Alcantara, M.B., Dass, C.R., 2014. Pigment epithelium-derived factor as a natural matrix metalloproteinase inhibitor: a comparison with classical matrix metalloproteinase inhibitors used for cancer treatment. J. Pharm. Pharmacol. 66, 895–902.

Benigni, F., et al., 2000. The proinflammatory mediator macrophage migration inhibitory factor induces glucose catabolism in muscle. J. Clin. Invest. 106, 1291–1300.

Borg, M.L., et al., 2011. Pigment epithelium-derived factor regulates lipid metabolism via adipose triglyceride lipase. Diabetes 60, 1458–1466.

Brand, K.A., Hermfisse, U., 1997. Aerobic glycolysis by proliferating cells: a protective strategy against reactive oxygen species. FASEB J. 11, 388–395.

Carnagarin, R., et al., 2015. PEDF-induced alteration of metabolism leading to insulin resistance. Mol. Cell. Endocrinol. 401, 98–104.

Carnagarin, R., et al., 2016. PEDF attenuates insulin-dependent molecular pathways of glucose homeostasis in skeletal myocytes. Mol. Cell. Endocrinol. 422, 115–124.

Chandel, N.S., et al., 1998. Mitochondrial reactive oxygen species trigger hypoxia-induced transcription. Proc. Natl. Acad. Sci. U.S.A. 95, 11715–11720.

Chen, C., et al., 2010. Plasma level of pigment epithelium-derived factor is independently associated with the development of the metabolic syndrome in Chinese men: a 10-year prospective study. J. Clin. Endocrinol. Metab. 95, 5074–5081.

Chung, C., et al., 2008. Anti-angiogenic pigment epithelium derivedfactor regulates hepatocyte triglyceride content through adipose triglyceride lipase (ATGL). J. Hepatol. 48, 471–478.

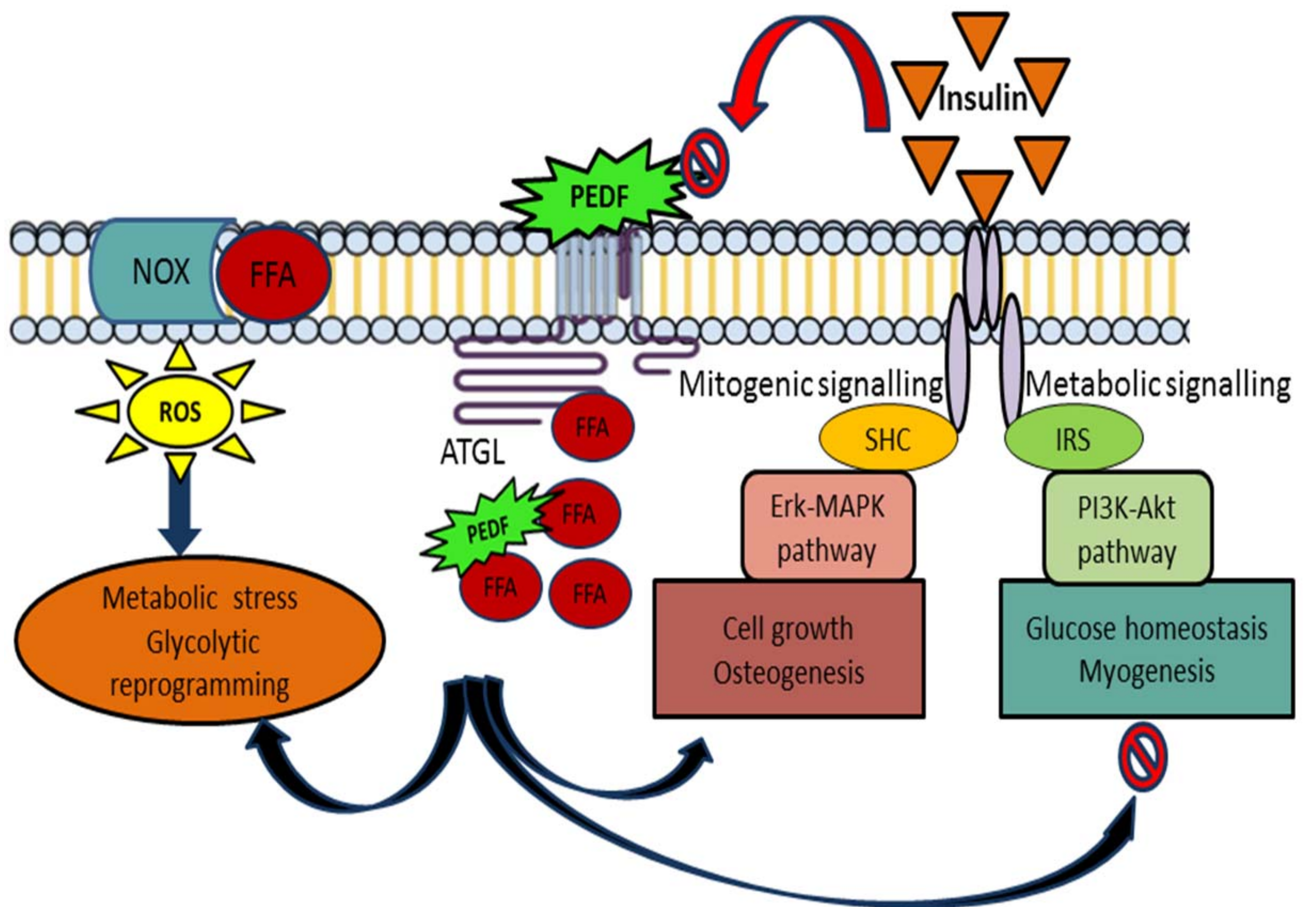
Colston, J.T., et al., 2005. H2O2 activates Nox4 through PLA2-dependent arachidonic acid production in adult cardiac fibroblasts. FEBS Lett. 579, 2533–2540.

Crowe, S., et al., 2009. Pigment epithelium-derived factor contributes to insulin resistance in obesity. Cell Metab. 10, 40–47.

Dawson, D.W., et al., 1999. Pigment epithelium-derived factor: a potent inhibitor of angiogenesis. Science 285, 245–248.
Der-Torossian, H., et al., 2013. Metabolic derangements in the gastrocnemius and the effect of Compound A therapy in a murine model of cancer cachexia. J. Cachexia Sarcopenia Muscle 4, 145–155.
Di Meo, S., Venditti, P., 2001. Mitochondria in exercise-induced oxidative stress. Biol. Signals Recept. 10, 125–140.
Famulla, S., et al., 2011. Pigment epithelium-derived factor is one of the most abundant proteins secreted by human adipocytes and induces insulin resistance and inflammatory signaling in muscle and fat cells. Int. J. Obes. (Lond.) 35, 762–772.
Filleur, S., et al., 2005. Two functional epitopes of pigment epithelial-derived factor block angiogenesis and induce differentiation in prostate cancer. Cancer Res. 65, 5144–5152.
Foresti, C., et al., 2015. Nrf2 activators modulate oxidative stress responses and bioenergetic profiles of human retinal epithelial cells cultured in normal or high glucose conditions. Pharmacol. Res. 99, 296–307.
Grönke, S., et al., 2005. Brummer lipase is an evolutionary conserved fat storage regulator in Drosophila. Cell Metab. 1, 323–330.
Haemmerle, G., et al., 2006. Defective lipolysis and altered energy metabolism in mice lacking adipose triglyceride lipase. Science 312, 73734–73737.
Herrero, A., Barja, G., 1997. ADP-Regulation of mitochondrial free radical production is different with complex I- or complex II-linked substrates: implications for the exercise paradox and brain hypermetabolism. J. Bioenerg. Biomembr. 29, 241–249.
Jackson, M.J., et al., 2007. The production of reactive oxygen and nitrogen species by skeletal muscle. J. Appl. Physiol. 102, 1664–1670.
Jenkins, C.M., et al., 2004. Identification, cloning, expression, and purification of three novel human calciumin dependent phospholipase A2 family members possessing triacylglycerol lipase and acylglycerol transacylase activities. J. Biol. Chem. 279, 48968–48975.
Kampfer, C., et al., 2014. Pigment-Epithelium Derived Factor (PEDF) and the human ovary: a role in the generation of ROS in granulosa cells. Life Sci. 97, 129–136.
Kozlov, A.V., et al., 2005. Skeletal muscles, heart, and lung are the main sources of oxygen radicals in old rats. BBA Mol. Basis Dis. 1740, 382–389.
Li, J.M., et al., 2002. Essential role of the NADPH oxidase subunit p47phox in endothelial cell superoxide production in response to phorbol ester and tumor necrosis factor-(alpha). Circ. Res. 90, 143–150.
Li, W., et al., 2007. Muscle-derived but not centrally derived transgene GDNF is neuroprotective in G93A-SOD1 mouse model of ALS. Exp. Neurol. 203, 457–471.
Matono, R., et al., 2006. Arachidonic acid induces direct interaction of the p67phox-Rac complex with the phagocyte oxidase Nox2, leading to superoxide production. J. Biol. Chem. 289, 24874–24884.
Nakamura, K., et al., 2009. Serum levels of pigment epithelium-derived factor (PEDF) are positively associated with visceral adiposity in Japanese patients with type 2 diabetes. Diabetes Metab. Res. Rev. 25, 52–56.
Nakamura, K., et al., 2010. Serum levels of pigment epithelium-derived factor (PEDF) are an independent determinant of insulin resistance in patients with essential hypertension. Int. J. Cardiol. 143, 96–98.
Nicholls, D.G., et al., 2010. Bioenergetic profile experiment using C2C12 myoblast cells. J. Vis. Expt. 46, 2511.
Notari, L., et al., 2006. Identification of a lipase linked cell membrane receptor for pigment epithelium-derived factor. J. Biol. Chem. 281, 38022–38037.
Petersen, S.V., et al., 2003. Pigment-epithelium-derived-factor (PEDF) occurs at a physiologically relevant concentration in human blood: purification and characterization. Biochem. J. 374, 199–206.
Piao, Y.J., et al., 2005. Nox 2 stimulates muscle differentiation via NF-κB/iNOS pathway. Free Radic. Biol. Med. 38, 989–1001.
Powers, S.K., Jackson, M.J., 2008. Exercise-induced oxidative stress: cellular mechanisms and impact on muscle force production. Physiol. Rev. 88, 1243–1276.
Remels, A.H.V., et al., 2015. TNF-α-Induced NF-κB activation stimulates skeletal muscle glycolytic metabolism through activation of HIF-1α. Endocrinology 156, 1770–1781.
Rychli, K., et al., 2009. Pigment epithelium-derived factor (PEDF) as a therapeutic target in cardiovascular disease. Expert Opin. Ther. Targets 13, 1295–1302.
Sabater, M., et al., 2010. Circulating pigment epithelium-derived factor levels are associated with insulin resistance and decrease after weight loss. J. Clin. Endocrinol. Metab. 95, 4720–4728.
Shi, S.Y., et al., 2015. DJ-1 links muscle ROS production with metabolic reprogramming and systemic energy homeostasis in mice. Nat. Commun. 6.
Smirnova, E., et al., 2006. ATGL has a key role in lipid droplet/adiposome degradation in mammalian cells. EMBO Rep. 7, 106–113.
Spitzer, J.J., et al., 1989. Altered control of carbohydrate metabolism in endotoxemia. Prog. Clin. Biol. Res. 286, 145–165.
Subramanian, P., et al., 2010. Pigment epithelium-derived factor receptor (PEDF-R): a plasma membrane-linked phospholipase with PEDF binding affinity. Adv. Exp. Med. Biol. 664, 29–37.
Villena, J.A., et al., 2004. Desnutrin, an adipocyte gene encoding a novel patatin domain-containing protein, is induced by fasting and glucocorticoids: ectopic expression of desnutrin increases triglyceride hydrolysis. J. Biol. Chem. 279, 47066–47075.
Yamagishi, S., et al., 2006. Elevated serum levels of pigment epithelium-derived factor in the metabolic syndrome. J. Clin. Endocrinol. Metab. 91, 2447–2450.
Yamagishi, S.I., et al., 2010. Pathophysiological role of pigment epithelium-derived factor (PEDF) in hepatic disorders. Curr. Med. Chem. 17, 1995–2000.
Yang, S., et al., 2011. Serum pigment epithelium-derived factor is elevated in women with polycystic ovary syndrome and correlates with insulin resistance. J. Clin. Endocrinol. Metab. 96, 831–836.
Zhao, X., et al., 2002. Cytosolic phospholipase A2 (cPLA2) regulation of human monocyte NADPH oxidase activity: cPLA2 affects translocation but not phosphorylation of p67phox and p47phox. J. Biol. Chem. 277, 25385–25392.
Zimmermann, R., et al., 2004. Fat mobilization in adipose tissue is promoted by adipose triglyceride lipase. Science 306, 1383–1386.

Graphical abstract

Chapter 6: Pigment epithelium-derived factor (PEDF)-induced modulation of lineage commitment of myocytes and heterotrophic ossification – an effect antagonised by insulin



PEDF attenuated another aspect of signalling in myocytes, namely their transdifferentiation into cells of the osteogenic lineage. Insulin blocked this transdifferentiation in vitro and intramuscular heterotrophic ossification in vivo.

Insulin antagonises pigment epithelium-derived factor (PEDF)-induced modulation of lineage commitment of myocytes and heterotrophic ossification

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Running title: Insulin attenuates PEDF-induced osteogenic differentiation in skeletal myocytes

Keywords: insulin, PEDF, skeletal muscle, bone, osteogenesis

Abstract

Extensive bone defects arising as a result of trauma, infection and tumour resection and other bone pathologies necessitate the identification of effective strategies in the form of tissue engineering, gene therapy and osteoinductive agents to enhance the bone repair process. PEDF is a multifunctional glycoprotein which plays an important role in regulating osteoblastic differentiation and bone formation. PEDF treatment of mice and human skeletal myocytes at physiological concentration inhibited myogenic differentiation and activated Erk1/2 MAPK-dependent osteogenic transdifferentiation of myocytes. In mice, insulin, a promoter of bone regeneration, attenuated PEDF-induced expression of osteogenic markers such as osteocalcin, alkaline phosphatase and mineralisation for bone formation in the muscle and surrounding adipose tissue. These results provide new insights into the molecular aspects of the antagonising effect of insulin on PEDF-dependent modulation of the differentiation commitment of musculoskeletal environment into osteogenesis, and suggest that PEDF may be developed as an effective clinical therapy for bone regeneration as its heterotopic ossification can be controlled via co-administration of insulin.

Abbreviations:

PEDF, pigment epithelium-derived factor, *Erk*, extracellular signal related kinase, *MAPK,* mitogen activated protein kinase, *ALP,* alkaline phosphatase, *ARS*, alizarin red, *BMP-2*, bone morphogenetic protein-2, *HO*, heterotrophic ossification, *OCN*, osteocalcin, *OI*, osteogenesis imperfecta, *MSC*, mesenchymal stem cell, *PTH*, parathyroid hormone.

1. Introduction

Extensive bone defects arising as a result of trauma, infection and tumour resection necessitates the identification of effective strategies in the form of tissue engineering and osteoinductive agents to enhance the bone repair process. The current anabolic management strategies are limited to bone morphogenetic protein-2 (BMP-2) and parathyroid hormone (PTH). BMP-2, predominantly a differentiation factor, terminally differentiates and induces apoptosis in osteoblasts [Hyzy et al 2012]. Moreover, BMP-2 enhances the metastasising capacity of prostate tumour cells [Spanjol et al 2010] and is associated with prolonged serous wound drainage post-surgery in acute traumatic and posttraumatic reconstructive extremity surgeries [Chan et al 2014]. In the case of PTH, reaching an effective therapeutic index in bone involves daily subcutaneous administration at supra-physiological doses which potentially results in side-effects in other PTH target tissues such as kidneys and placenta [Mannstadt et al 1999] and is associated with increased risk of osteosarcoma [Vahle et al 2002].

Pigment epithelium derived-factor (PEDF) is a 50kDa multifunctional serpin, originally isolated from the pigment epithelial cells of human foetal retina. The *serpinf1* gene located on chromosome 17p13 encodes for PEDF. PEDF is expressed by a wide variety of tissues and exerts varied effects such as regulation of angiogenesis [Dawson et al 1999, Cai et al 2006], neuronal differentiation [Tombran-Tink et al 1991], metabolism in peripheral tissues [Crowe et al 2009, Carnagarin et al 2016] and anti-tumorigenesis [Chandolu and Dass 2012]. PEDF plays an important role in regulating osteoblastic differentiation and bone formation. Bone pathologies such as osteogenesis imperfecta (OI) [Becker et al 2011] and osteosarcoma [Ek et al 2007] have absent or low levels of PEDF. Moreover, PEDF has shown potent anti-osteosarcoma efficacy as gene therapy [Dass et al 2007] or protein therapy [Broadhead et al 2011].

Studies from our lab and others have demonstrated the potency of PEDF to regulate osteogenic genes and induce osteogenesis in mesenchymal stem cells (MSCs). Exogenous PEDF induces the expression of osteoblastic genes such as osteocalcin, osteopontin and alkaline phosphatase and enhances mineralisation in mice and human MSCs [Elahy et al 2016, Li et al 2013]. PEDF suppresses sclerostin, an endogenous inhibitor of Wnt signalling that inhibits bone formation and enhances β-catenin levels [Li et al 2015]. PEDF localisation within the proliferative and hypertrophic epiphyseal growth plate zones and in the bony spicule is indicative of its role in

osteogenesis [Quan et al 2005]. PEDF restoration improves bone mineralisation in murine models of OI type VI and normalises mineralisation defects in pluripotent stem cells derived from PEDF null patients [Belinsky et al 2016]. Moreover, defects in the *serpinf1* gene, which encodes PEDF, results in OI IV characterised by bone mineralisation defects, which are also associated with neurotrophic and anti-angiogenic defects [Belinsky et al 2016, Tombran-Tink et al 2003, Dawson et al 1999].

Insulin is an anabolic hormone of the musculoskeletal system that regulates energy metabolism. Produced by the pancreatic β islets, it promotes glucose uptake and storage in the form of glycogen in muscle, adipose or liver cells [Del Prato et al 1987]. Insulin enhances osteoblast anabolism and impacts bone development and physiology [Pun et al 1989]. Pathologic insulin deficient states such as type-1 diabetes mellitus develop early onset osteopenia [Kemink et al 2000] or osteoporosis and are characterised by impaired bone healing and regeneration [Janghorban et al 2006, Nicodemus & Folsom 2001, Loder 1988]. Insulin resistance in type 2 diabetes is also associated with deleterious bone health [Thrailkill et al 2005]. Insulin regulates proliferation, migration, differentiation, relaxation and hypertrophy of skeletal muscle [Bolster et al 2004] and attenuates muscle heterotopic ossification induced by the potent inducer BMP-2 in a mouse embryonic myoblast cell line C2C12 and in the tibialis anterior muscle of C57BL/6 mice [Zhang et al 2014].

In the present study, PEDF treatment of mice and human skeletal myocytes activated Erk1/2 MAPK-dependent osteogenic transdifferentiation of skeletal myocytes. We were able to demonstrate bone formation in murine gastrocnemius muscle pockets and the surrounding adipose tissue of the PEDF group. The results of this study provide new insights into the molecular mechanisms underpinning PEDF's ability to modulate the differentiation commitment of musculoskeletal cells towards osteogenesis. Such activity has not been observed with any other bone-forming factors, not even BMP-2. We infer that this could pave the way for new strategies such as PEDF-incorporated muscle grafts and flaps, which could overcome the limitations of existing therapies to accelerate bone regeneration, or to address skeletal disorders. On the other hand, these results also indicate that diffusion of PEDF into muscle tissues around bone injury sites may lead to heterotopic ossification, which would be a limitation for PEDF use in treating bone defects in patients. We found insulin, which in itself is a positive regulator for bone regeneration, inhibited PEDF-induced muscle heterotopic ossification by suppressing osteogenic transdifferentiation of myocytes. This study is a step

towards developing PEDF as an effective clinical therapy for bone regeneration whilst inhibiting muscle heterotopic ossification.

2. Materials and methods

2.1. Materials

Recombinant human PEDF was purchased from MD Bioproducts (Bethesda, MD, USA). Insulin, fetal bovine serum was purchased from Gibco (Fort Worth, TX, USA), Dulbecco's Modified Eagle's Medium, penicillin/streptomycin and trypsin were obtained from Life Technologies. Skeletal Muscle Myoblast Cell Media - Clonetics™ SkBM™-2 BulletKit™ was purchased from Lonza (Walkersville, MD, USA). The Myo D(C-20), myogenin (M-225), osteocalcin (V-19) and alkaline phosphatase (H-300) antibodies were purchased from Santa Cruz Biotechnology, Inc. (Dallas, Texas, USA). The other antibodies such as Akt (pan)(C67E7), p-Akt(Ser473), α- actinin, p-pErk1/2 MAPK(Thr202/Tyr204), p-p38 MAPK(Thr180/Tyr182) and PD98059 inhibitor and secondary anti-rabbit IgG, HRP-linked antibody were purchased from Cell Signaling Technology (Beverly, MA, USA). ECL Prime immunoblotting detection reagent kit was purchased from GE Healthcare Amersham Melbourne, Australia. All other reagents were obtained from Sigma-Aldrich unless otherwise stated.

2.2. Cell culture

Human skeletal muscle myoblasts (HSMMs) were purchased from Lonza (Walkersville, MD, USA) and were used within 5 passages from receipt. C2C12 murine myoblasts were purchased from ATCC (Manassas, VA, USA) and used within passage 14. Cells were cultured under an atmosphere of 5% CO2 at 37 °C in a humidified incubator and when confluent, cells were channelled towards myogenic differentiation with 2% serum for a period of six days. MEK1,2-ERK1/2 (PD98059) signal transduction inhibitor was used at 25 μM and PEDF and insulin were used up to a concentration of 100nM.

2.3 Proliferation assay

The proliferation of C2C12 and HSMM cells following PEDF treatment was assessed using the Cell Titre Blue assay (Promega, Madison, WI, USA) according to the manufacturer's instructions at Ex560nm/Em590nm using a multimode Plate Reader (Enspire PerkinElmer, USA).

2.4 Scratch assay

Myocellular motility was analysed by an *in vitro* scratch assay [Kocić J et al 2012]. Cells were seeded in a 24-well plate and when fully confluent, a scratch was made using a sterile 200 microlitre pipette tip in the monolayer over the total diameter of each well and cells were allowed to grow for 24 hours in the same media with or without 100 nM PEDF. The cells were then fixed with 4% paraformaldehyde (PFA). Migration of the cells into the scratch area was visualised by light microscopy and quantified using Fiji/Image J2 software, Madison, USA.

2.5 Immunoblot analysis

Cells were lysed in RIPA (radioimmunoprecipitation) buffer with protease and phosphatase inhibitors (Cell Signaling Technology, Beverly, MA, USA) and quantified using the Pierce 660nm Protein Assay (Thermo Fisher Scientific, Waltham, MA, USA) as per the manufacturer's instructions. Samples were fractionated by SDS-PAGE (Invitrogen, Carlsbad, CA, USA) at 150V for an hour and electrotransferred to PVDF (polyvinylidene fluoride) membranes. Membranes were blocked with 5% skim milk for an hour and incubated overnight at 4°C with the indicated primary antibodies. All primary antibodies were used at 1:1000 dilution in 5% BSA prepared in transfer buffer (TBS) containing 0.1% Tween-20 (TBST). This was followed by HRP-conjugated secondary antibody incubation at 1:20,000 prepared in 5% skim milk in TBST for 1 hour at room temperature, followed by chemiluminescent detection of target bands. Intermittent washes following blocking, primary and secondary antibody incubation steps were performed using washing buffer containing 0.05% Tween-20. Densitometric analyses of the immunoblots were performed using GS-670 Imaging Densitometer (Bio-Rad) and Molecular analyst software (version 5.2.1, Bio-Rad).

2.6 Von Kossa staining

The monolayer culture of murine and human myoblasts following the various treatments – untreated, 100nM PEDF, 100nM PEDF + Erk inhibitor, and 100nM PEDF + insulin were subjected to von kossa staining [Dass et al 2006]. The monolayer culture plates were fixed with 4% paraformaldehyde for 30 min, stained with 5% silver nitrate and the plate placed under UV light for 30 min. Wells were rinsed once with water, treated with 5% sodium thiosulphate solution for 5 min when necessary. Following another rinse with water, 2 – 3 drops of 100% glycerol was added to each well and observed under Olympus IX51 fluorescent microscope

(Olympus Life Science Solutions, Pennsylvania, USA) and images analysed with Fiji/Image J2 software.

2.7 Alizarin red staining

The monolayer cultures of both murine and human myoblasts were evaluated for formation of calcium deposits at day 6 following various treatments by staining with 4% Alizarin red solution (ARS). Cells were seeded in 48-well plates with the mentioned treatments and at day 6, the cells were stained with ARS after fixation with 4% paraformaldehyde and images were taken. Then following acetic acid extraction, the samples were heated to 85°C and centrifuged (18000*g*). Supernatants were adjusted to within range 4.1-4.5 with 10% ammonium hydroxide. The supernatants were removed to a 96-well plate and read at OD405. A standard curve was prepared using ARS [Gregory et al 2004, Elahy et al 2016].

2.8 In vivo osteogeneration study

Approval was obtained from the Curtin University Animal Ethics Committee [# 2016/35] prior to experimentation. Six weeks old female Balb/c mice were anaesthetised with isoflurane. Under aseptic conditions, a lateral skin incision was made on the lateral aspect of each thigh and equally-sized gelfoam (Pfizer, NSW, Australia) discs were implanted into the surgically-created gastrocnemius muscle pockets [Elahy et al 2016]. Each group (n = 6 mice/group) were implanted with resorbable gelfoam discs of radius 2.5mm per implant site consisting of gelfoam disc impregnated with water as control, 100nM PEDF, or 100nM PEDF + 100nM insulin. Muscle and skin layers were sutured; subcutaneous (sc) metacam 1.5mg/kg was given after surgery and then buprenorphine 0.075mg/kg sc, twice daily for 72 hours post-surgery. Mice had unrestricted access to food and water, and were on 12h light/dark cycle and monitored closely after surgery until full recovery, then left for 8 weeks with daily monitoring. A pilot (go/no go) study with 6 mice was used to confirm that ossification occurred around 4 weeks. At the end of the study, mice were euthanised and the muscle pockets and surrounding adipose tissues were harvested and processed histologically post-fixation in 10% buffered formalin. Sections of tissues were prepared at standard 5 micron width and put on to Superfrost Plus slides (Thermo Fisher Scientific, Waltham, MA, USA).

2.9 Immunohistochemistry

Tissue sections were deparaffinised with 100% xylene and rehydrated with 100, 70, 30 and 0% ethanol. Antigen retrieval was achieved with incubation of sections in sodium citrate buffer (10mM) at pH 6 for 12 min at a temperature of 85-95°C [Clark et al 2011]. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide in phosphate buffered saline (PBS, pH 7.4). Tissues sections were blocked with BSA and incubated overnight with 1:250 dilution alkaline phosphatase antibody at 4°C in a humidified chamber, followed by another overnight incubation with secondary antibody at 1:1000 dilution (Dako) in a similar manner. Tissue sections were treated with biotinylated link (Dako), followed by streptavidin-HRP (Dako). Staining was achieved with DAB and counterstaining with haematoxylin and briefly with Scott's alkaline tap water (Sigma-Aldrich). Tissues were dehydrated in a series of ethanol washes in increasing concentrations (30, 70 and 100%) prior to a xylene rinse. Mounting was achieved with Depex solution (Sigma-Aldrich) and observed under light microscopy.

2.10 Statistical analysis

Data were analysed with GraphPad Prism software Prism version 6.00 for Windows, La Jolla, California, USA. The results are presented as means ± SD. The statistical significance was determined using one-way ANOVA analysis, with the correction of Bonferroni for multiple comparisons. Difference with $p < 0.05$ was considered statistically significant.

3. Results

3.1 PEDF inhibits migration without affecting the proliferation of skeletal myocytes

We have previously studied the expression of PEDF receptor, ATGL and its activation upon PEDF treatment in C2C12 and HSMM [Carnagarin et al 2016b] and now we determined the effect of PEDF on the proliferation and migration of skeletal myocytes. As determined by CT-Blue assay [Ahmed et al 1994], PEDF treatment did not alter the proliferation of the skeletal myocytes **(Fig 1a)** but impaired their motility when tested in the *in vitro* scratch assay. C2C12 and HSMM displayed high migration capacity in the growth media and almost completely colonised the scratch after a period of 24 hours but treatment with 100nM PEDF inhibited migration during the same period of time **(Fig 1b)**.

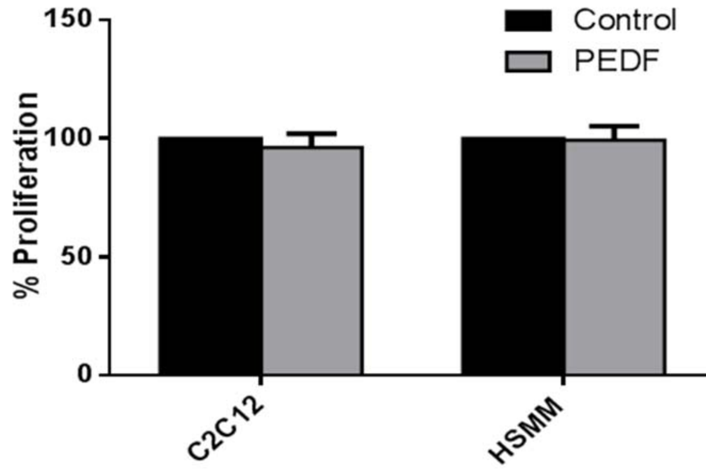

Fig1(a) Proliferation of C2C12 and HSMM - determined by CT-Blue assay after the cells were cultured in 100nM PEDF for 24h.

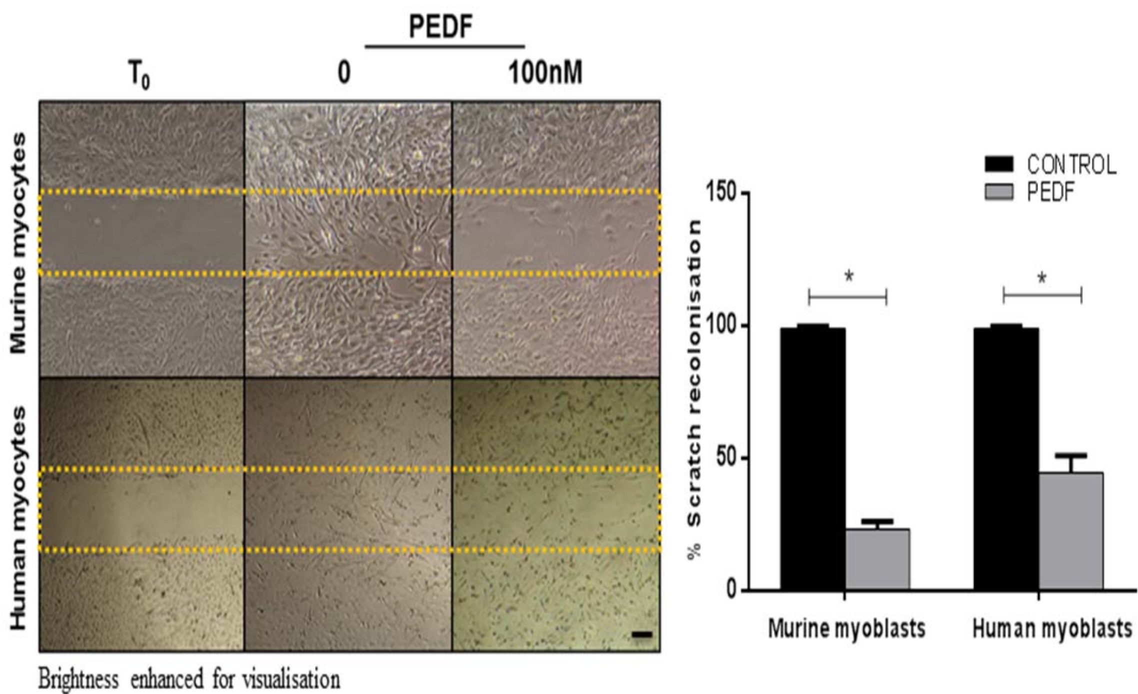

Fig1(b) Migration of skeletal myocytes were analysed by scratch assay: a scratch was made in the confluent monolayer of C2C12 and HSMM followed by treatment with 100nM PEDF. The recolonisation of the scratch was documented after 24h; T0, scratch made in the monolayer at zero time, *Scale bar = 10µm.*

3.2. PEDF attenuates Akt–MyoD-myogenin axis in skeletal myocytes during myogenic differentiation.

C2C12 and HSMM were induced to differentiate by cultivation in the differentiation medium for six days, and the effect of PEDF on myogenic signalling was analysed by immunoblot. PEDF impairs Akt signalling and was associated with the downregulation of myogenic differentiation markers such as myo D and myogenin **(Fig 2)**.

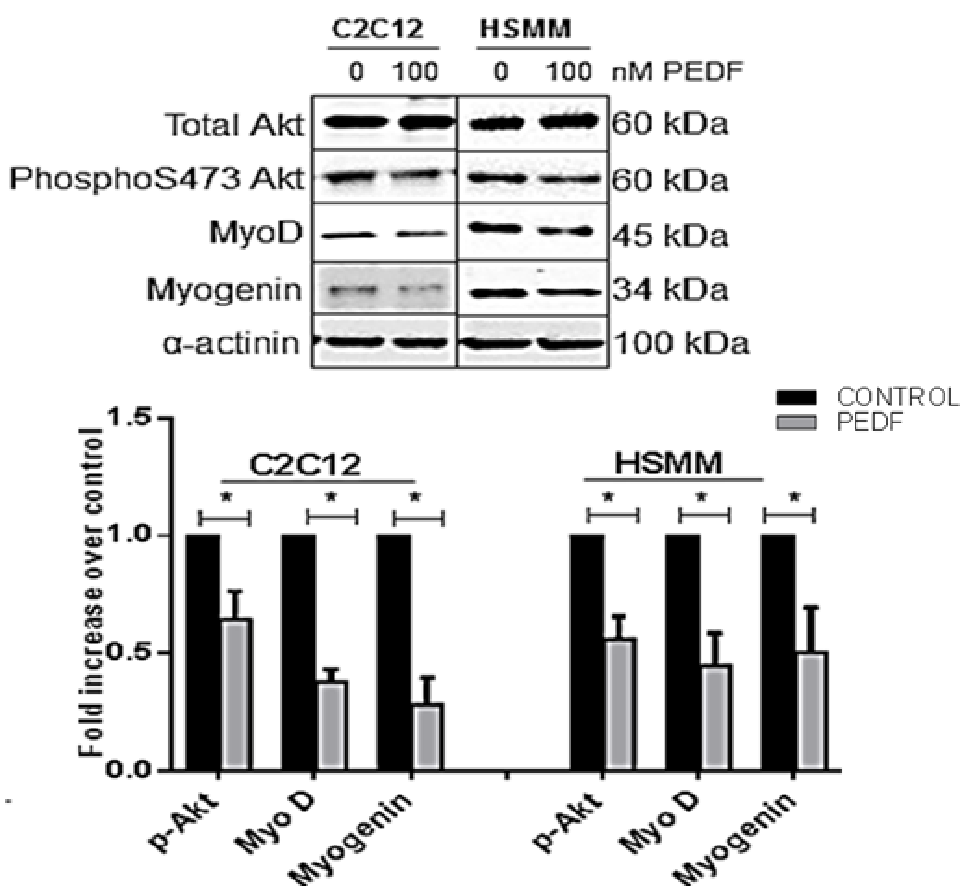

Fig2 PEDF attenuates Akt-MyoD-myogenin axis. Immunoblot analysis of myogenic markers in C2C12 and HSMM cells cultivated in myogenic differentiation medium in the presence of 100nM PEDF determined by immunoblot assay; α-actinin was used as a loading control. Values are mean ± SD (n = 3), *p < 0.05, PEDF-treated versus untreated.

3.3 PEDF activates ERK1/2 MAPK signalling in C2C12 cells.

To determine the effect of PEDF on MAPKs, growth-arrested skeletal myocytes were incubated with PEDF and the cell lysates were analysed for the expression of phosphorylated

p44/42 (Erk 1/2) MAPKs and the results are shown in **Fig 3**. PEDF significantly increased phospho ERK1/2 kinase activation, with peak activation at 60 minutes after PEDF treatment.

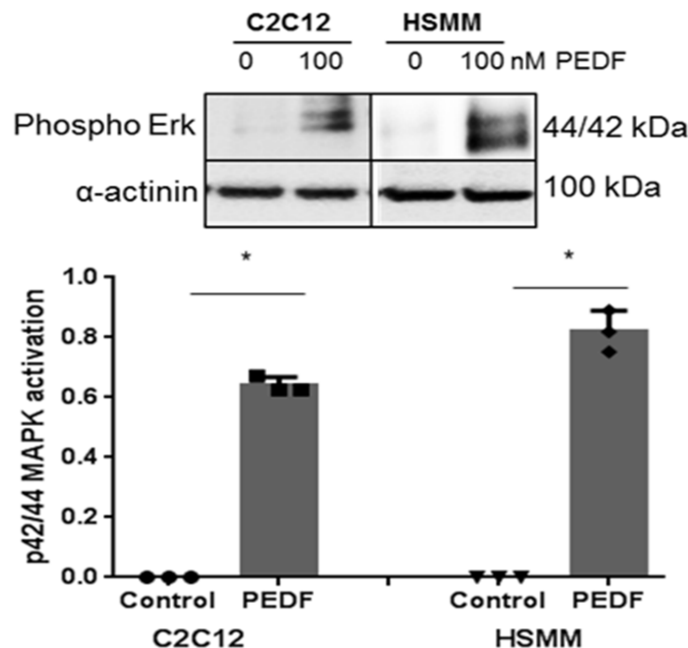

Fig3. PEDF activates Erk1/2 MAPK signalling. Activation of Erk 1/2 MAPK in C2C12 and HSMM cultured in serum-free medium and treated with 100nM PEDF was determined by immunoblot. The Erk 1/2 MAPK phosphorylation is normalised to the control and values are presented as mean ± SD ($n = 3$), $*p < 0.05$.

3.4 PEDF induces phospho 42/44 MAPK/ Erk1/2-dependent osteogenic induction.

Since the myogenic signalling was attenuated by PEDF in skeletal myocytes, we sought to determine whether the cells were directed to differentiate into another cell lineage. Considering recent research which demonstrated that PEDF induces osteogenic differentiation of MSCs both *in vitro* [Li et al 2013] and *in vivo* [Elahy et al 2016], we analysed the involvement of PEDF in osteogenic transdifferentiation of the skeletal myocytes. In the latter study, PEDF was able to induce osteogenesis even in the absence of MSCs, in the encapsulating alginate beads implanted in muscle pockets [Elahy et al. 2016], which prompted us to investigate whether myoblasts could be transdifferentiated by PEDF.

In this study, cells were cultured with or without 100nM PEDF for 6 days. The specific activation of the Erk-MAPK signalling by PEDF prompted us to analyse the involvement of the Erk1/2 MAPK in PEDF-induced osteogenesis. Hence the effect of Erk 1/2 kinase activation

in PEDF-induced osteogenic transdifferentiation of myocytes was analysed using its pharmacological inhibitor, PD98059. When the cells were treated with PEDF along with Erk inhibitor, the osteogenic markers were attenuated **(Fig 4a)** and this effect was confirmed by diminished PEDF-induced mineral deposition when co-treated with the Erk inhibitor indicating the significance of Erk 1/2 MAPK activation in PEDF-induced osteogenesis. Immunoblot analysis of osteogenic markers such as osteocalcin (OCN) and alkaline phosphatase (ALP) were performed parallel to mineral deposition assays such as von kossa and alizarin red staining. PEDF potently induced osteogenic transdifferentiation and increased mineral deposition in culture free of osteogenic supplements in myogenic conditions (**Fig 4b**).

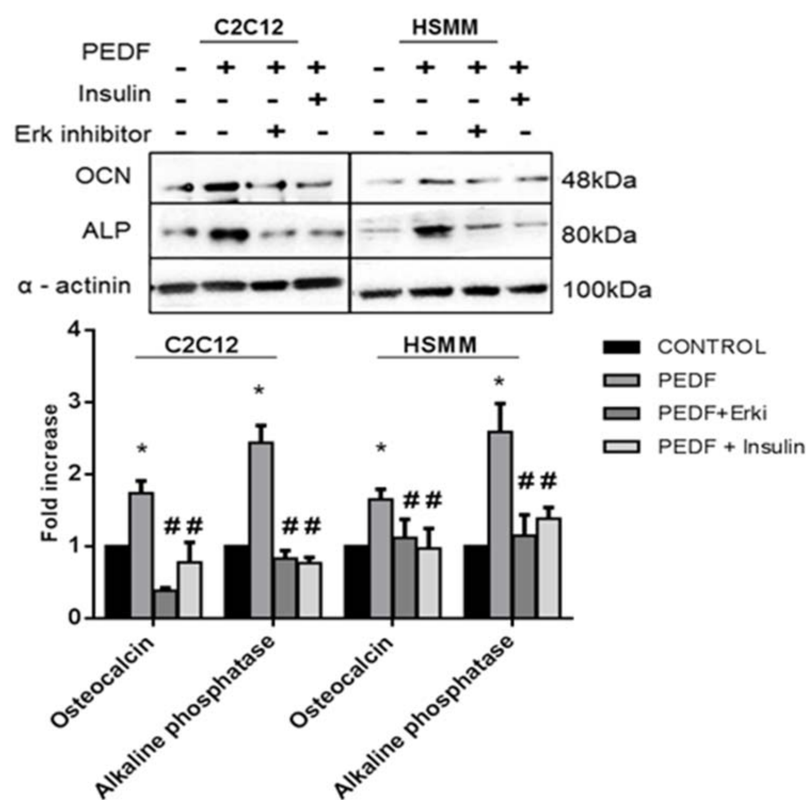

Fig4(a) The expression of osteogenic markers: Osteocalcin and alkaline phosphatase in C2C12 and HSMM cultivated in the presence of 100nM PEDF and when co-treated with Erk inhibitor and insulin, determined by immunoblot assay; α-actinin was used as a loading control. Values are mean ± SD ($n = 3$), $*p < 0.05$.
Abbreviations: OCN, osteocalcin; ALP, alkaline phosphatase; Erki, Erk inhibitor; PEDF, pigment epithelium-derived factor.

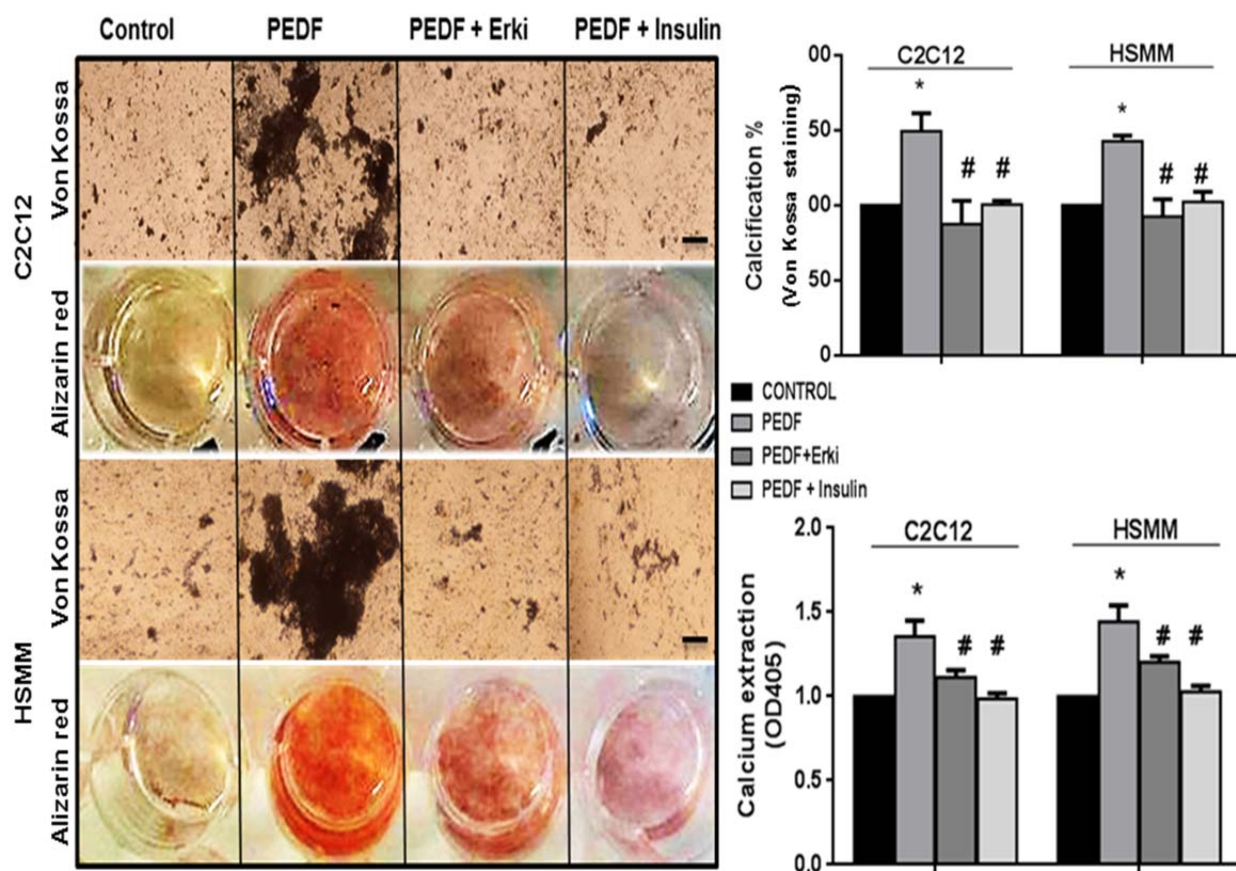

Fig4(b) PEDF potently induced Erk 1/2 MAPK-dependent mineral deposition in skeletal myocytes attenuated by insulin

Mineral deposition in C2C12 and HSMM were assessed by von kossa and alizarin red (ARS) staining at day 6 following various treatments. PEDF treatment enhanced mineral deposition in the skeletal myocytes compared to untreated cells and this effect was suppressed by inhibiting Erk activation and by co-treatment with insulin.

Images of representative von kossa staining. *Scale bar = 10µm*, in monolayer culture of C2C12 and HSMM at day 6. The bar represents % calcification, with the intensity in the control designated to 100%, analysed with Imagej2 software. Values are mean ± SD, *(n = 4), *p < 0.05*.

Mineral concentration in skeletal myocytes at day 6 in monolayer culture measured by alizarin red assay. The bar represents calcium extracted represented as a fold increase over the control. Values are mean ± SD, *(n = 4), *p < 0.05*.

Abbreviations: Erki, Erk inhibitor; PEDF, pigment epithelium-derived factor.

3.2 Insulin attenuates PEDF-induced osteogeneration *in vitro* and *in vivo*

Previous research indicates that insulin and PEDF share an antagonistic relationship [Carnagarin et al 2016a, Crowe et al 2007]. Insulin, a positive regulator for bone regeneration inhibited BMP2-induced muscle heterotopic ossification (HO) without interrupting its

osteoinductive activity [Zhang et al 2014]. As my study shows, PEDF is emerging with superior properties than the current anabolic therapeutic strategies for chronic bone ailments such as BMP-2 [Chan et al 2014] and PTH [Vahle et al 2002].

To further analyse the effects of insulin on heterotopic ossification (HO) of skeletal muscle *in vivo*, we implanted resorbable gelfoam sponges without any drug (control), with PEDF and PEDF + insulin in gastrocnemius muscle pockets. After 8 weeks, the ectopic bone, muscle and the surrounding adipose tissue were harvested and subjected to DAB staining for alkaline phosphatase and Von Kossa for mineralisation. HO was visible in PEDF group, and insulin significantly reduced PEDF-induced HO (**Fig. 5a**). PEDF induced significant bone formation which was characterised by increased alkaline phosphatase expression and enhanced mineralisation of tissues (**Figs. 5b and c**). Insulin attenuated PEDF-induced ossification due to the significant decrease of alkaline phosphatase and calcium deposition (**Fig. 5a - c**). These results demonstrated the inhibitory role of insulin on PEDF-induced heterotopic ossification in the gastrocnemius muscle *in vivo*.

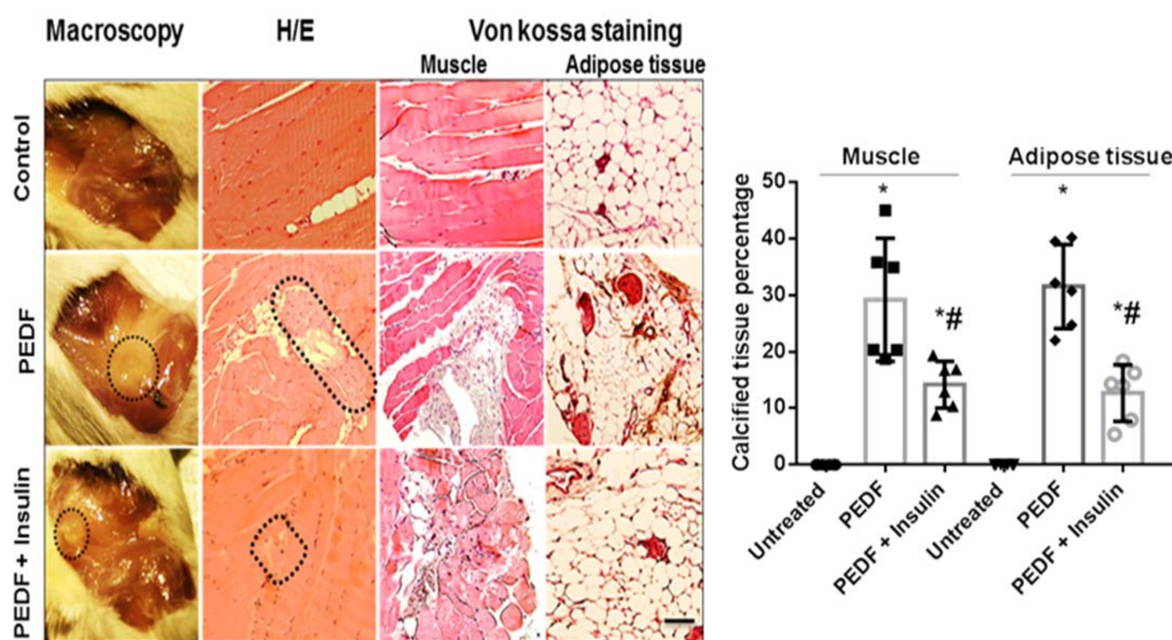

Fig5(a) Bone formation in the PEDF-incorporated gastrocnemius muscle pocket and the surrounding adipose tissue – attenuated by insulin.
Von Kossa staining of muscle heterotopic ossification after gelfoam implantation of water (control), PEDF or PEDF and insulin. *Scale bar = 100μm*. Quantification of calcified tissue area percentage from Von Kossa staining images. *$p < 0.05$ vs. control group, #$p < 0.05$ vs. PEDF group. $n = 6$ mice/group.

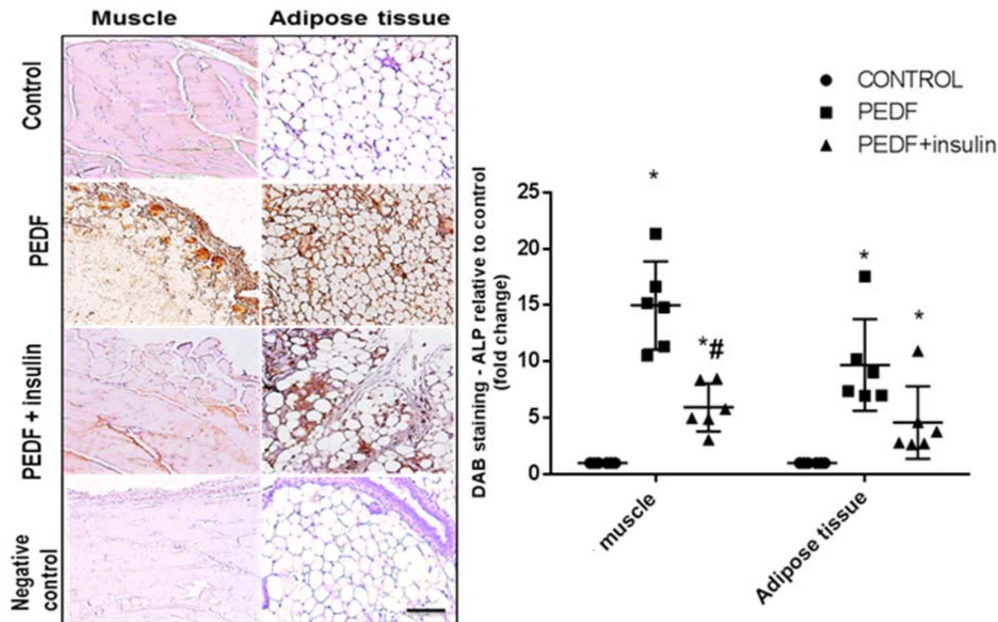

Fig5(b) Insulin attenuated PEDF-induced heterotopic ossification of gastrocnemius muscle and surrounding adipose tissue.

DAB staining for alkaline phosphatase - gastrocnemius muscle and surrounding adipocytes harvested after 8 weeks following gelfoam implantation: water (control), PEDF or PEDF and insulin. *Scale bar = 100μm.* Quantification of osseous area percentage from DAB staining for ALP images. *$p < 0.05$ vs. control group, #$p < 0.05$ vs. PEDF group. $n = 6$ mice/group.

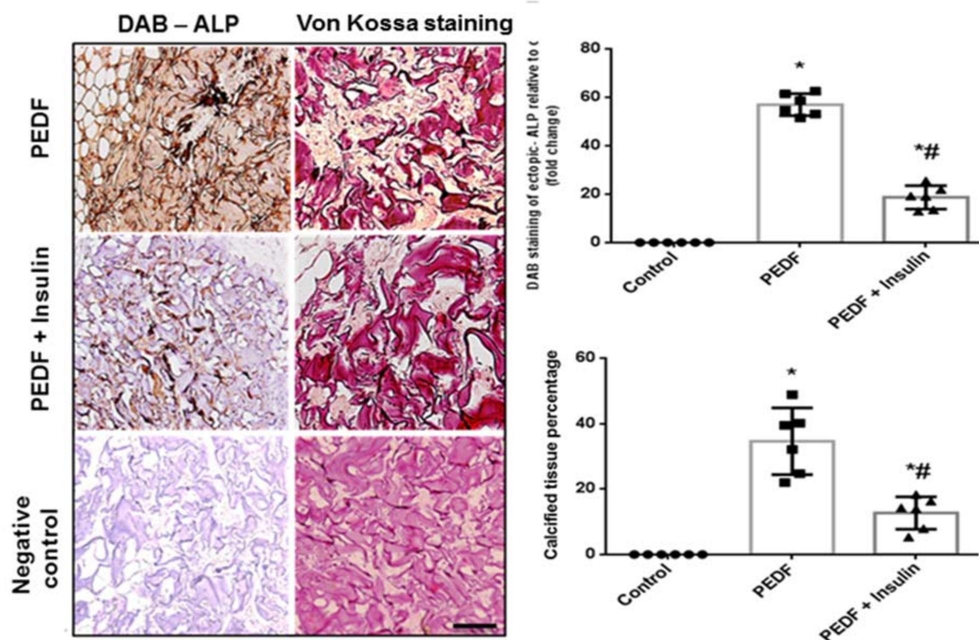

Fig5(c) PEDF-induced bone formation characterised by increased alkaline phosphate expression and enhanced mineralisation was attenuated by insulin.

The images represent DAB staining for alkaline phosphatase and von Kossa of the ectopic bone calcification and quantification; PEDF or PEDF and insulin. *Scale bar = 100μm.* *$p < 0.05$ vs. control group, #$p < 0.05$ *vs.* PEDF group. $n = 6$ mice/group.

4. Discussion

Skeletal muscle and bone originate from the mesoderm and are closely linked in development and growth [Brotto 2011, Karasik 2011]. Muscle bears a dependent association with bone and decreased muscle function with age or disease directly correlates with diminished bone function. Muscle-bone functional interaction is critical for skeletal regeneration and traumatic fractures associated with soft tissue damage are more prone for non-union or delayed union in clinical scenarios [Sen and Miclau 2007]. Muscle supports bone repair by means of osteogenic factors, vascularisation and satellite and post-natal stem cells and regulates inflammation during skeletal regeneration [Relaix & Zammit 2012, Murphy et al 2011, Abou-Khalil et al 2014]. Bone healing is a coordinated outcome of skeletal stem cell activation and muscle-derived satellite cells [Wright et al2002, Bosch et al 2000], which are capable of differentiating into osteoblasts and chondrocytes both *in vitro* and *in vivo*. Tumour necrosis factor α (TNFα) induces differentiation of muscle stromal cells into osteogenic and chondrogenic lineage to enhance bone healing [Glass et al 2011]. Studies have revealed that satellite cells adjacent to the fracture site are activated and serve as a source of cells that generate molecular signals for bone regeneration, and musculoskeletal regeneration is the outcome of coordinated concomitant activation of stem cells within both muscle and bone [Abou-Khalil et al 2015].

Muscle plays a very important role in the bone repair process, satellite stem cells (myoblasts), resident non-myogenic and non-satellite stem cells act as osteochondro progenitors and also as sources of growth factors required for bone regeneration. There is a need to search for growth factors that could exploit the potential of muscle cells to aid bone formation. PEDF is a potent cytokine that regulates diverse biological activities. In the current study, we analysed the involvement of PEDF in transdifferentiating myoblasts to the osteogenic lineage. PEDF enhanced mineral deposition in myoblasts, attenuated myogenic signalling and upregulated the expression of osteogenic markers. Skeletal myocellular differentiation is critically regulated by the Akt-myoD-myogenin axis and PEDF-induced attenuation of this axis suggests a negative action of PEDF on myogenic differentiation. The inhibitory effect of PEDF on the migration of skeletal myocytes further indicates that PEDF prevents alignment and fusion of myoblasts into myotubes by its inhibitory effect on myocellular migration capacity which impairs myogenic differentiation [Leloup et al 2007].

PEDF is a potent osteokine and the osteogenic effects on MSCs were demonstrated both by us and others [Elahy et al 2016, Li et al 2013]. In the current study, PEDF potently induces an osteogenic phenotype in skeletal myoblasts in the absence of any additional osteogenic signal. We can postulate that PEDF attenuates the myogenic signalling program and transdifferentiates the skeletal myoblasts towards an osteogenic lineage. Cell commitment to a particular lineage is governed by the interplay between cell signalling and transcriptional regulation in response to extracellular signals. In this perspective, the mitogen-activated protein kinase (MAPK) pathway is the most studied pathway [Jaiswal et al 2000, Gaffen et al 2009] that plays a critical role in muscle development [Keren et al 2006] and induction of osteogenic differentiation [Leloup et al 2007]. The Erk1/2 MAPK signalling pathway is activated by basic fibroblast growth factor and TGF-β to inhibit myogenic differentiation, and Erk activation determines the lineage differentiation fate of MSCs towards the osteogenic or adipogenic phenotype [Gallea et al 2001, Yang et al 2006]. Erk1/2 MAPK is a determinant of MSC fate following PEDF treatment to induce osteogenesis [Li et al 2013] and regulates interleukin-17-mediated inhibition of myogenesis and induction of osteogenesis in murine myoblasts, C2C12 [Kocic et al 2012]. PEDF activated Erk1/2 MAPK signalling, which acted as the key signal in determining PEDF-induced osteogenesis in skeletal myoblasts; and our results indicate that PEDF, through Erk 1/2 MAPK, opens a new signalling mechanism in murine and human primary skeletal myoblasts responsible for transdifferentiation to the osteogenic lineage. Such an effect was seen in C2C12 cells, which transdifferentiated towards the osteogenic lineage upon BMP2 treatment [Ramazzotti et al 2016].

Both skeletal muscle and bone are target tissues subject to the anabolic effects of insulin at physiological doses; insulin enhances glucose uptake, collagen synthesis and alkaline phosphatase [Pun et al 1989, Ituarte et al 1989]. Bone abnormalities have been observed in insulin-deficient conditions such as type-1 diabetes mellitus animal models and insulin treatment accelerated healing in these models [Gandhi et al 2005, Verhaeghe et al 1990]. Insulin promotes osteoblastogenesis and forms the molecular link between energy metabolism, bone development and bone remodeling [Ferron et al 2010]. In our study, we found that insulin inhibited the PEDF-induced osteogenic differentiation of skeletal muscle cells which was different to the positive role of insulin on osteoblasts. Insulin potently prevented heterotopic ossification while not affecting *in situ* bone healing. This is because of specific anabolic myocellular effects of insulin such as protein synthesis and glucose metabolism [Kimball et al 2002]. Moreover, it was also indicative of the involvement of different signaling pathways

in the transdifferentiation process compared to osteogenesis. Insulin is potentially myogenic [Conejo et al 2001] but could not rescue PEDF-dependent Akt inhibition [Carnagarin et al 2016a], resulting in the inhibition of the key myogenic transcription factors such as MyoD1 and myogenin significantly. These findings are also suggestive of the fact that insulin–induced attenuation of osteogenic differentiation is achieved by specific blockade of osteogenic signalling rather than inducing myogenic differentiation.

In conclusion, our study indicates that PEDF plays a role in the dynamic cross-talk between skeletal muscle and bone. PEDF inhibits myogenic differentiation through downregulation of the Akt-MyoD-myogenin signalling axis, and promotes Erk 1/2 MAPK-mediated transdifferentiation to an osteogenic lineage by upregulating osteogenic markers such as osteocalcin and alkaline phosphatase and enhancing mineral deposition in skeletal myocytes. Insulin blocked this PEDF-induced osteogenic signalling and thereby the skeletal myocellular transdifferentiation, and these results were confirmed *in vivo* (**Fig 6**). These findings provide insight into the role of PEDF in regulating cellular signalling during musculoskeletal regeneration via modulation of the multilineage commitment potential of skeletal myoblasts, and identify insulin as a potentially beneficial agent for the control of PEDF-mediated heterotrophic ossification.

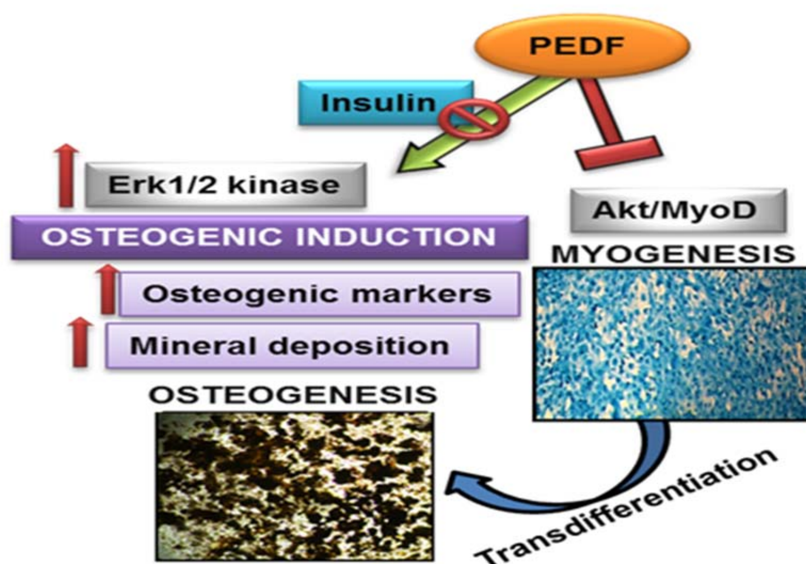

Fig6. PEDF modulates the lineage commitment of skeletal myoblasts.
PEDF attenuates myogenic signalling by downregulating the Akt-MyoD-Myogenin axis and induces Erk 1/2 MAPK-dependent osteogenic transdifferentiation of skeletal myocytes. Insulin selectively blocked PEDF-induced osteogeneration *in vitro* and *in vivo*.

Acknowledgements

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Conflict of interest

The authors declare no conflict of interest.

References

1. Abou-Khalil R et al. Delayed bone regeneration is linked to chronic inflammation in murine muscular dystrophy. J Bone Miner Res 2014; 29: 304-315.

2. Ahmed SA et al. A new rapid and simple non-radioactive assay to monitor and determine the proliferation of lymphocytes: An alternative to [3H] thymidine incorporation assays. J Immunol Meth 1994; 170: 211-224.

3. Becker J et al. Exome sequencing identifies truncating mutations in human SERPINF1 in autosomal-recessive osteogenesis imperfecta. Am J Hum Genet 2011; 88: 362-371.

4. Belinsky GS et al. Pigment epithelium-derived factor (PEDF) normalizes matrix defects in iPSCs derived from Osteogenesis imperfecta Type VI. Rare Dis 2016; 4: e1212150/doi:10.1080/21675511.2016.1212150.

5. Bolster DR et al. Regulation of protein synthesis associated with skeletal muscle hypertrophy by insulin, amino acid and exercise-induced signalling. Proc Nutr Soc 2004; 63: 351-356.

6. Borg ML et al. Pigment epithelium-derived factor regulates lipid metabolism via adipose triglyceride lipase. Diabetes 2011; 60: 1458-1466.

7. Bosch P et al. Osteoprogenitor cells within skeletal muscle. J Orthop Res 2000; 18: 933-944.

8. Broadhead ML et al. Systemically administered PEDF against primary and secondary tumours in a clinically relevant osteosarcoma model. Br J Cancer 2011; 105: 1503-1511.

9. Brotto M. Aging, sarcopenia and store-operated calcium entry: A common link? Cell Cycle 2011; 10: 4201-4202.

10. Cai J et al. Pigment epithelium-derived factor inhibits angiogenesis via regulated intracellular proteolysis of vascular endothelial growth factor receptor 1. J Biol Chem 2006; 281: 3604-3613.

11. Carnagarin R et al. PEDF attenuates insulin-dependent molecular pathways of glucose homeostasis in skeletal myocytes. Mol Cell Endocrinol 2016; 422: 115-124.

12. Carnagarin R et al. Pigment epithelium-derived factor stimulates skeletal muscle glycolytic activity through NADPH oxidase-dependent reactive oxygen speciesproduction. Int J Biochem Cell Biol 2016; 78: 229-236.

13. Chan DS et al. Wound complications associated with bone morphogenetic protein-2 in orthopaedic trauma surgery. J Orthop Trauma 2014; 28: 599-604.

14. Chandolu V and Dass CR. Cell and molecular biology underpinning the effects of PEDF on cancers in general and osteosarcoma in particular. J Biomed Biotechnol 2012; doi: 10.1155/2012/740295.

15. Conejo R et al. Insulin produces myogenesis in C2C12 myoblasts by induction of NF-kappaB and downregulation of AP-1 activities. J Cell Physiol 2001; 186: 82-94.

16. Crowe S et al. Pigment epithelium-derived factor contributes to insulin resistance in obesity. Cell Metab 2009; 10: 40-47.

17. Dass CR et al. A novel orthotopic murine model provides insights into cellular and molecular characteristics contributing to human osteosarcoma. Clin Exp Metastas. 2006; 23: 367-380.

18. Dass CR et al. Chitosan microparticles encapsulating PEDF plasmid demonstrate efficacy in an orthotopic metastatic model of osteosarcoma. Biomaterials 2007; 28: 3026-3033.

19. Dawson DW et al. Pigment epithelium-derived factor: a potent inhibitor of angiogenesis. Science 1997; 285: 245-248.

20. Del Prato S et al. Hyperglucagonemia and insulin-mediated glucose metabolism. J Clin Invest 1987; 79: 547-556.

21. Ek ETH et al. Pigment epithelium-derived factor overexpression inhibits orthotopic osteosarcoma growth, angiogenesis and metastasis. Cancer Gene Ther 2007; 14: 616-626.

22. Elahy M et al. Alginate bead-encapsulated PEDF induces ectopic bone formation in vivo in the absence of co-administered mesenchymal stem cells. Current Drug Targets 2016; 17: 1-12.

23. Ferron M et al. Insulin signaling in osteoblasts integrates bone remodeling and energy metabolism. Cell 2010; 142: 296-308.

24. Gaffen SL. Structure and signalling in the IL-17 receptor family. Nat Rev Immunol 2009; 9: 556–567.

25. Gallea S et al. Activation of mitogen-activated protein kinase cascades is involved in regulation of bone morphogenetic protein-2-induced osteoblast differentiation in pluripotent C2C12 cells. Bone 2001; 28: 491–498.

26. Gandhi A et al. The effects of local insulin delivery on diabetic fracture healing. Bone 2005; 37: 482-490.

27. Glass GE et al. TNF-alpha promotes fracture repair by augmenting the recruitment and differentiation of muscle-derived stromal cells. Proc Natl Acad Sci USA 2011; 108: 1585-1590.

28. Gregory CA et al. An Alizarin red based assay of mineralisation by adherent cells in culture: Comparison with cetylpyridinium chloride extraction. Anal Biochem 2004; 329: 77-84.

29. Hyzy SL et al. BMP2 induces osteoblast apoptosis in a maturation state and noggin-dependent manner. J Cell Biochem 2012; 113: 3236-3245.

30. Ituarte EA et al. Glucose transport system in UMR-106-01 osteoblastic osteosarcoma cells: regulation by insulin. Calcif Tissue Int 1989; 45: 27-33.

31. Jaiswal RK et al. Adult human mesenchymal stem cell differentiation to the osteogenic or adipogenic lineage is regulated by mitogen-activated protein kinase. J Biol Chem 2000; 275: 9645-9652.

32. Janghorbani M et al. Prospective study of diabetes and risk of hip fracture: the nurses' health study. Diabetes Care 2006; 29: 1573-1578.

33. Karasik D. How pleiotropic genetics of the musculoskeletal system can inform genomics and phenomics of aging. Age 2011; 33: 49-62

34. Kemink SA et al. Osteopenia in insulin-dependent diabetes mellitus; prevalence and aspects of pathophysiology. J Endocrinol Invest 2000; 23: 295-303.

35. Keren A et al. The p38 MAPK signaling pathway: a major regulator of skeletal muscle development. Mol Cell Endocrinol 2006; 252: 224-230.

36. Kimball SR et al. Role of insulin in translational control of protein synthesis in skeletal muscle by amino acids or exercise. J Appl Physiol 2002; 93:1168-1180.

37. Kocić J et al. Interleukin 17 inhibits myogenic and promotes osteogenic differentiation of C2C12 myoblasts by activating ERK1,2. Biochimica et Biophysica Acta 2012; 1823: 838-849.

38. Leloup L et al. Involvement of the ERK/MAP kinase signalling pathway in milli-calpain activation and myogenic cell migration. Int J Biochem Cell Biol 2007; 39: 1177-1189.

39. Li F et al. Pigment epithelium- derived factor suppresses expression of sost/sclerostin by osteocytes: implication for its role in bone matrix mineralisation. J Cell Physiol 2015; 230: 1243-1249.

40. Li F et al. Pigment epithelium-derived factor enhances differentiation and mineral deposition of human mesenchymal stem cells. Stem Cells 2013; 31: 2714-2723.

41. Loder RT. The influence of diabetes mellitus on the healing of closed fractures. Clin Orthop Relat Res 1988; 232: 210-216.

42. Mannstadt M et al. Receptors for PTH and PTHrP: their biological importance and functional properties. Am J Physiol 1999; 277: F665-F675.

43. Murphy MM et al. Satellite cells, connective tissue fibroblasts and their interactions are crucial for muscle regeneration. Development 2011; 138: 3625–3637.

44. Nicodemus KK & Folsom AR. Type 1 and type 2 diabetes and incident hip fractures in postmenopausal women. Diabetes Care 2001; 24:1192-1197.

45. Petersen SV et al. Pigment epithelium-derived factor (PEDF) occurs at a physiologically relevant concentration in human blood: purification and characterization. Biochem J 2003; 374: 199-206.

46. Pun KK et al. The characterization, regulation, and function of insulin receptors on osteoblast-like clonal osteosarcoma cell line. J Bone Min Res 1989; 4: 853-862.

47. Quan GM et al. Localization of pigment epithelium-derived factor in growing mouse bone. Calcif Tissue Int 2005; 76: 146-153.

48. Ramazzotti et al. BMP-2 induced expression of PLC beta1 that is a positive regulator of osteoblast differentiation. J Cell Physiol 2016; 231: 623-629.

49. Rauch F et al. Lack of circulating pigment epithelium-derived factor is a marker of osteogenesis imperfecta type VI. J Clin Endocrinol Metab 2012; 97: E1550-E1556.

50. Relaix F & Zammit PS. Satellite cells are essential for skeletal muscle regeneration: The cell on the edge returns centre stage. Development 2012; 139: 2845-2856.

51. Sen MK & Miclau T. Autologous iliac crest bone graft: Should it still be the gold standard for treating nonunions? Injury 2007; 38: S75-S80.

52. Spanjol J et al. Role of bone morphogenetic proteins in human prostate cancer pathogenesis and development of bone metastases: immunohistochemical study. Coll Antropol 2010; 34: S119-S125.

53. Thrailkill KM et al. Is insulin an anabolic agent in bone? Dissecting the diabetic bone for clues. Am J Physiol Endocrinol Metab 2005; 289: E735-E745.

54. Tombran-Tink J & Barnstable CJ. PEDF: a multifaceted neurotrophic factor. Nat Rev Neurosci 2003; 4: 628-636.

55. Tombran-Tink J et al. PEDF: a pigment epithelium-derived factor with potent neuronal differentiative activity. Exp Eye Res 1991; 53: 411-414.

56. Vahle JL et al. Skeletal changes in rats given daily subcutaneous injections of recombinant human parathyroid hormone (1-34) for 2 years and relevance to human safety. Toxicol Pathol 2002; 30: 312-321.

57. Verhaeghe J et al. Bone and mineral metabolism in BB rats with long-term diabetes. Decreased bone turnover and osteoporosis. Diabetes 1990; 39: 477- 482.

58. Walker B et al. A consensus statement regarding the utilization of BMP in spine surgery. Curr Rev Musculoskelet Med 2014; 7: 208-219.

59. Wang M et al. Pigment epithelium-derived factor suppresses adipogenesis via inhibition of the MAPK/ERK pathway in 3T3-L1 preadipocytes. Am J Physiol Endocrinol Metab 2009 Dec; 297: E1378-E1387.

60. Wright V et al. BMP4-expressing muscle-derived stem cells differentiate into osteogenic lineage and improve bone healing in immunocompetent mice. Mol Ther 2002; 6:169-178.

61. Yang W et al. Extracellular signal regulated kinase 1/2 mitogen-activated protein kinase pathway is involved in myostatin-regulated differentiation repression. Cancer Res 2006; 66: 1320-1326.

62. Zhang J et al. The inhibition effects of insulin on BMP2-induced muscle heterotopic ossification. Biomaterials 2014; 35: 9322-9331.

Chapter 7: General discussion

This chapter gives an overview of the studies undertaken to understand the role of PEDF and insulin crosstalk in skeletal muscle biology. I started my research by reviewing the existing literature to identify the critical nodes of insulin signaling [Carnagarin et al 2015a] which could be a probable target for the metabolic alterations induced by PEDF in skeletal muscle, in order to understand the mechanism of PEDF-induced insulin resistance [Carnagarin et al 2015b].

Pigment epithelium-derived factor (PEDF) is an anti-angiogenic serpin associated with the central pathogenesis of insulin resistance in metabolic disorders. Skeletal muscle is the predominant site of insulin-dependent glucose metabolism [Norheim et al 2011] and is both a potential source and target of PEDF with expression of its receptors and is subject to its autocrine and paracrine effects [Famulla et al 2011]. The insulin signal transduction was inhibited right from the phosphorylation activation of the insulin receptor to its effector through Akt/PKB- dependent and -independent pathways in skeletal myocytes. The fact that insulin receptor is a cognitive molecule capable of regulating its own expression based on cells requirement indicates that the effect of PEDF is in proximity to receptor to attenuate the entire transduction such as the cell membrane. PEDF attenuated the metabolic axes of insulin signaling: downstream signalling events involved in glycogenesis and GLUT4 translocation. These findings implicate a direct effect of PEDF on multiple insulin-dependent metabolic molecular mechanisms in skeletal muscle cells [Carnagarin et al 2016a].

Next, I sought to identify the effect of PEDF on skeletal myocellular metabolism using a murine myocyte cell line (C2C12) and human primary skeletal myocytes. The skeletal myocytes expressed a PEDF-inducible oxidant-generating system that was sensitive to antioxidants and NOX inhibition, which rendered the skeletal muscle metabolically inflexible. PEDF enhanced phospholipase A2 activity in skeletal myocytes to yield free fatty acids such as arachidonic acid and glycerol [Chung et al 2008, Subramanian et al 2010], which potently stimulated NADPH oxidase that induced oxidative stress and triggered a shift towards a more glycolytic phenotype. A major limitation of this study is the methodology used for the quantification of ROS – DCFDA as certain papers question the robustness of probe for measuring ROS and the that it can be redox cycled in the cells could make it prone to artefacts.

However the PEDF-dependent ROS production was an acute process which circumvented the problem of redox recycling of the probe. Furthermore the control of ROS with the use of the antioxidants such as SOD and catalase as controls are certainly supportive of ROS production. Extracellular flux analysis demonstrated that PEDF treatment resulted in a glycolytic shift, which was further confirmed with glucose consumption assays demonstrating no change in mitochondrial respiration. PEDF emerged as a potent inducer of metabolic stress through NADPH oxidase-dependent ROS generation in skeletal muscle cells [Carnagarin et al 2016a].

PEDF treatment resulted in sequential attenuation of all the insulin-dependent molecular metabolic mechanisms in skeletal myocytes. However, PEDF selectively ameliorated the metabolic signalling and did not have any effect on the mitogenic signalling evident from unaltered myocellular viability, in fact caused hyperactivation of p42/44 MAPK. The combinatorial effect of PEDF: metabolic stress, attenuated insulin signaling, Erk-activation with no effect on cell viability instigated me to explore what exactly was the role of PEDF pertaining to skeletal muscle biology and functioning. Erk1/2 MAPK activation is a critical determinant of MSC fate following PEDF treatment to induce osteogenesis [Li et al 2013] and regulates interleukin-17-mediated inhibition of myogenesis and induction of osteogenesis in C2C12 [Kocic et al 2012]. The MAPK pathway is one of the most studied pathways [Jaiswal et al 2000, Gaffen et al 2009], and is crucial for development of muscle [Keren et al 2006], as well as bone [Leloup et al 2007]. Growth factors such as fibroblast growth factor and TGF-β activate Erk MAPK to inhibit myogenesis and determine the differentiation fate of MSCs towards either osteogenesis or adipogenesis [Gallea et al 2001, Yang et al].

PEDF is a potent osteokine and its osteogenic effects on MSCs were demonstrated both by us and others [Elahy et al 2016, Li et al 2013]. PEDF potently induced osteogenesis in skeletal myoblasts by attenuating the myogenic signalling program and transdifferentiating the skeletal myoblasts towards an osteogenic lineage. PEDF activated Erk1/2 MAPK which acted as the key signal in modulating the lineage commitment of skeletal myoblasts to induce osteogenesis. PEDF, through Erk 1/2 MAPK activation combined with its antagonism towards insulin, opens up a new signalling mechanism in skeletal myocytes in inducing transdifferentiation to an osteogenic lineage. Such an effect was seen in C2C12 treated with BMP2 [Ramazzotti et al 2016].

However this effect was rescued by insulin. Insulin blocked PEDF-induced expression of osteogenic markers such as osteocalcin and alkaline phosphatase, and mineralisation for bone formation the muscle and surrounding adipose tissue in mice. This identifies the therapeutic potential of PEDF-insulin crosstalk in skeletal disorders. Whilst the PEDF-dependent modulation of the differentiation commitment of musculoskeletal environment into osteogenesis could result in heterotrophic ossification, the combinatorial effect of insulin administered with PEDF may be developed as an effective clinical therapy for bone regeneration.

Major limitations and future directions:

PEDF causes a wide range of cellular events vitally important for the organism such as survival and differentiation, migration and invasion, lipid metabolism and stem cell maintenance through its four receptors and more are discovered. Focusing on receptor based approaches is by itself a vast area and all my studies were with focus on the PEDF molecule and its metabolic receptor The various cellular processes are deregulated in multiple pathological conditions, including cancer, metabolic and cardiovascular disease. With ROS as the downstream effector od PEDF signalling, detailed exploration of stress- related signalling cascades such as ER stress, Nrf2, NF-κB, AP-1, ATF3 or alterations in the ratio of GSH:GSSG could further shed light in this area. PEDF has been successfully used in various preclinical models of these conditions and human correlates suggest a wide utility of PEDF-based drugs. However the most significant clinical application of PEDF, to date, is limited to its use in age-related macular degeneration. This is due to gaps in the knowledge and further research required to fill in the gaps with the discovery of the new PEDF receptors. However, PEDF-based gene therapy has advanced to early stage clinical trials with mapping of active PEDF fragments to design short peptide mimetics conferring distinct functions of PEDF, which may address specific clinical problems and become prototype drugs.

Chapter 8: Conclusions

PEDF is a multifaceted molecule which influences various cellular functions such as survival, proliferation, migration, differentiation and metabolism. It is yet worth exploring whether PEDF has a direct role when it comes to skeletal muscle metabolism. Our study facilitates a better understanding of PEDF signaling, which is likely to be an important regulator of skeletal muscle metabolism, in particular the insulin–dependent metabolic processes and the PEDF-insulin antagonistic crosstalk involved in the modulation of skeletal muscle biology. The attenuation of PEDF signalling is an effective approach to manage and develop better control over metabolic disorders associated with elevated PEDF levels such as metabolic syndrome, obesity, type 2 diabetes, polycystic ovarian syndrome and hepatic diseases allaying the morbidity associated with PEDF-induced metabolic inflexibility and insulin resistance. Furthermore, this study has also explored the positive aspect of the PEDF-insulin antagonism in the context of osteoregeneration which could pave the way for effective therapeutic strategies to achieve controlled bone formation. PEDF is a potent osteokine that is able to transdifferentiate skeletal myocytes to the osteogenic lineage. In future, this knowledge may be used in conjunction with the potential role of insulin as therapy for PEDF-induced heterotrophic ossification.

Chapter 9: References

1. Alberdi *et al*. Binding of pigment epithelium-derived factor (PEDF) to retinoblastoma cells and cerebellar granule neurons. Evidence for a PEDF receptor. *J Biol Chem* 1999; **274**: 31605-12
2. Alberdi *et al*. Pigment epithelium-derived factor (PEDF) binds to glycosaminoglycans: analysis of the binding site. *Biochemistry* 1998; **37**: 10643-52
3. Arnhold *et al*. Adenovirally transduced bone marrow stromal cells differentiate into pigment epithelial cells and induce rescue effects in RCS rats. *Invest Ophthalmol Vis Sci* 2006; **47**: 4121-9
4. Aymerich *et al*. Evidence for pigment epithelium-derived factor receptors in the neural retina. *Invest Ophthalmol Vis Sci* 2001; **42**: 3287–93
5. Becerra and Notario. The effects of PEDF on cancer biology: mechanisms of action and therapeutic potential. *Nat Rev Cancer* 2013; **13**: 258-71
6. Becerra SP. Focus on molecules: pigment epithelium-derived factor (PEDF) *Exp. Res.* 2006; **82**: 739-40
7. Becerra *et al*. Overexpression of fetal human pigment epithelium-derived factor in Escherichia coli. A functionally active neurotrophic factor. *J Biol Chem* 1993; **268**: 23148-56
8. Belinsky *et al.* Pigment epithelium-derived factor (PEDF) normalizes matrix defects in iPSCs derived from Osteogenesis imperfecta Type VI. *Rare dis* 2016; **4:** e1212150/doi: 10.1080/21675511.2016.1212150.
9. Bernard *et al*. Laminin receptor involvement in the anti-angiogenic activity of pigment epithelium-derived factor. *J Biol Chem* 2009; **284**: 10480-90
10. Bilak *et al.* Identification of the neuroprotective molecular region of pigment epithelium-derived factor and its binding sites on motor neurons. *J Neurosci* 2002; **22**: 9378-86
11. Borg *et al*. Pigment epithelium-derived factor regulates lipid metabolism via adipose triglyceride lipase. *Diabetes* 2011; **60**: 1458-66
12. Bouck N. PEDF: anti-angiogenic guardian of ocular function. *Trends Mol Med* 2002; **8**: 330-4
13. Broadhead *et al*. the applied biochemistry of PEDF and implications for tissue homeostasis. *Growth Factors* 2010;**28**: 280-85

14. Cai *et al*. Pigment epithelium-derived factor inhibits angiogenesis via regulated intracellular proteolysis of vascular endothelial growth factor receptor 1. *J Biol Chem* 2006; **281**: 3604-13
15. Carnagarin R *et al*. Pigment epithelium-derived factor stimulates skeletal muscle glycolytic activity through NADPH oxidase-dependent reactive oxygen species production. *Int J biochem & cell biol* 2016; **78**: 229-36
16. Chen C *et al*. Plasma level of pigment epithelium-derived factor is independently associated with the development of the metabolic syndrome in Chinese men: a 10-year prospective study. *J Clin Endocrinol Metab* 2010; **95**: 5074–81
17. Chung *et al.* Anti-angiogenic pigment epithelium-derived factor regulates hepatocyte triglyceride content through adipose triglyceride lipase (ATGL). *J Hepatol* 2008; **48**: 471-8
18. Crowe *et al.* Pigment epithelium-derived factor contributes to insulin resistance in obesity. *Cell Metab* 2009; **10**: 40-7
19. Dawson *et al*. Pigment epithelium-derived factor: a potent inhibitor of angiogenesis. *Science* 1999; **285**: 245-8
20. Doll et al. Pigment epithelium-derived factor regulates the vasculature and mass of the prostate and pancreas. *Nat Med* 2003; **9**: 774-80
21. Doyon *et a*l. Nuclear receptor co-repressor is required to maintain proliferation of normal intestinal epithelial cells in culture and down-modulates the expression of pigment epithelium-derived factor. *J Biol Chem* 2009; **284**: 25220-9
22. Ek *et al*. PEDF-derived synthetic peptides exhibit antitumor activity in an orthotopic model of human osteosarcoma. *J Orthop Res* 2007; **25**: 1671-80
23. Elahy M *et al*. Alginate bead-encapsulated PEDF induces ectopic bone formation in vivo in the absence of co-administered mesenchymal stem cells. *Curr Drug Targets* 2016; **17**: 1-12
24. Famulla *et al*. Pigment epithelium-derived factor (PEDF) is one of the most abundant proteins secreted by human adipocytes and induces insulin resistance and inflammatory signaling in muscle and fat cells. *Int J Obes (Lond)* 2011; **35**: 762-72
25. Filleur *et al*. Two functional epitopes of pigment epithelial-derived factor block angiogenesis and induce differentiation in prostate cancer. *Cancer Res* 2005; **65**: 5144-52
26. Gao *et al*. Human mesenchymal stem cells overexpressing pigment epithelium-derived factor inhibit hepatocellular carcinoma in nude mice. *Oncogene* 2010; **29**: 2784-94

27. Halin *et al*. Decreased pigment epithelium-derived factor is associated with metastatic phenotype in human and rat prostate tumors. *Cancer Res* 2004; **64**: 5664-71
28. Ho *et al*. PEDF-derived peptide promotes skeletal muscle regeneration through its mitogenic effect on muscle progenitor cells. *Am J Physiol Cell Physiol* 2015; **309**: C159–C168
29. Holekamp *et al*. Pigment epithelium-derived factor is deficient in the vitreous of patients with choroidal neovascularization due to age-related macular degeneration. *Am J Ophthalmol* 2002; **134**: 220-7
30. Hou *et al*. A therapeutic strategy for choroidal neovascularization based on recruitment of mesenchymal stem cells to the sites of lesions. *Mol Ther* 2010; **18**: 1837-45
31. Huang *et al.* PEDF deficient mice exhibit an enhanced rate of retinal vascular expansion and are more sensitive to hyperoxia-mediated vessel obliteration. *Exp Eye Res* 2008; **87**: 226-41
32. James *et al*. TGFbeta/activin/nodal signaling is necessary for the maintenance of pluripotency in human embryonic stem cells. *Development* 2005; **132**: 1273-82
33. Kampfer C et al. Pigment epithelium-derived factor (PEDF) and the human ovary: a role in the generation of ROS in granulosa cells. *Life Sci* 2014; **97**: 129-36.
34. Kawaguchi *et al.* Structure-function relationships of PEDF. *Curr Mol Med* 2010;**10**: 302-11
35. Li and Xie. Stem cell niche: structure and function. *Annu Rev Cell Dev Biol* 2005; **21**: 605-31
36. Li *et al*. Pigment epithelium derived factor suppresses expression of Sost/Sclerostin by osteocytes: Implication for its role in bone matrix mineralization. *J Cell Physiol* 2015; **230**: 1243-49
37. Li *et al*. Pigment epithelium-derived factor enhances differentiation and mineral deposition of human mesenchymal stem cells. *Stem Cells* 2013; **31**: 2714-23
38. Liang *et al*. Increased expression of pigment epithelium-derived factor in aged mesenchymal stem cells impairs their therapeutic efficacy for attenuating myocardial infarction injury. *Eur Heart J* 2011; **34**: 1681-90
39. Liu *et al*. Identification of the antivasopermeability effect of pigment epithelium-derived factor and its active site. *Proc Natl Acad Sci* 2004; **101**: 6605–10
40. Liu *et al*. Stroke induces gene profile changes associated with neurogenesis and angiogenesis in adult *subventricular* zone progenitor cells. *J Cereb Blood Flow Metab* 2007; **27**: 564-74

41. Lu *et al*. Long-term safety and function of RPE from human embryonic stem cells in preclinical models of macular degeneration. *Stem Cells* 2009; **27**: 2126-35
42. Maik-Rachline *et al*. Variable phosphorylation states of pigment-epithelium-derived factor differentially regulate its function. *Blood.* 2006;**107**:2745-52
43. Menon *et al*. Differential gene expression associated with migration of mesenchymal stem cells to conditioned medium from tumor cells or bone marrow cells. *Stem Cells* 2007; **25**: 520-8
44. Meyer *et al*. Mapping the type I collagen-binding site on pigment epithelium-derived factor. Implications for its antiangiogenic activity. *J Biol Chem* 2002**; 277**: 45400–7
45. Marzo *et al*. PEDF promotes retinal neurosphere formation and expansion in vitro. *Adv Exp Med Biol* 2010**; 664**: 621-30
46. Nakamura *et al*. Serum levels of pigment epithelium-derived factor (PEDF) are positively associated with visceral adiposity in Japanese patients with type 2 diabetes. *Diabetes Metab Res Rev* 2009; **25**: 52-6
47. Nakamura *et al*. Serum levels of pigment epithelium-derived factor (PEDF) are an independent determinant of insulin resistance in patients with essential hypertension. Int *J Cardiol* 2010; **143**: 96-8
48. Notari *et al*. Identification of a Lipase-linked cell membrane receptor for Pigment epithelium-derived Factor. *J Biol Chem* 2006**; 281**: 38022-37
49. Notari *et al.* Pigment epithelium-derived factor binds to cell-surface F(1)-ATP synthase. *FEBS J* 2010; **277**: 2192-205
50. Ohno-Matsui *et al*. Novel mechanism for age-related macular degeneration: an equilibrium shift between the angiogenesis factors VEGF and PEDF. *J Cell Physiol* 2001; **189**: 323-33
51. Orgaz *et al.* Loss of pigment epithelium-derived factor enables migration, invasion and metastatic spread of human melanoma. *Oncogene* 2009; **28**: 4147-61
52. Otto and Wright. Mesenchymal stem cells: from experiment to clinic. *Fibrog Tiss Rep* 2011; **4**: 20, doi: 10.1186/1755-1536-4-20
53. Park *et al.* Identification of a novel inhibitor of the canonical wnt pathway. *Mol Cell Biol* 2011**; 31**: 3038-51
54. Pignolo *et al*. Putative role for EPC-1/PEDF in the G0 growth arrest of human diploid fibroblasts. *J Cell Physiol* 2003; **195**: 12-20
55. Pumiglia *et al*. PEDF: bridging neurovascular interactions in the stem cell niche. *Nat Neurosci* 2006; **9**: 299-300

56. Qin *et al*. Mesenchymal stem cells: Mechanisms and role in bone regeneration. *Postgrad Med J* 2014; **90**: 643-47
57. Quan *et al*. Localization of pigment epithelium-derived factor in growing mouse bone. *Calcif Tissue Int* 2005; **76**: 146-153
58. Ramírez-Castillejo *et al*. Pigment epithelium-derived factor is a niche signal for neural stem cell renewal. *Nat Neurosci* 2006; 9: 331-9
59. Ross *et al.* Inhibition of adipogenesis by Wnt signaling. *Science* 2000; **289**: 950-3
60. Rychli *et al*. Pigment epithelium-derived factor (PEDF) as a therapeutic target in cardiovascular disease. *Expert Opin Ther Targets* 2009; **13:**1295–1302
61. Saishin *et al*. Periocular gene transfer of pigment epithelium-derived factor inhibits choroidal neovascularization in a human-sized eye. *Hum Gene Ther* 2005; **16**: 473-8
62. Sarojini *et al.* PEDF from mouse mesenchymal stem cell secretome attracts fibroblasts. *J Cell Biochem* 2008; **104**: 1793-802
63. Shao *et al*. Secretion of pigment epithelium-derived factor - Mutagenic study. *Eur J Biochem* 2003; **270**: 822-31
64. Shen *et al*. Endothelial cells stimulate self-renewal and expand neurogenesis of neural stem cells. *Science* 2004, **304**: 1338-40
65. Simonovic *et al*. Crystal structure of human PEDF, a potent anti-angiogenic and neurite growth-promoting factor. *Proc Natl Acad Sci* 2001; **98**: 11131–5
66. Smith AG. Embryo-derived stem cells: of mice and men. *Annu Rev Cell Dev Biol* 2001; **17**: 435-62
67. Steele *et al*. Pigment epithelium-derived factor: neurotrophic activity and identification as a member of the serine protease inhibitor gene family. *Proc Natl Acad Sci* 1993; **90**: 1526-30
68. Toloza *et al*. Comparison of plasma pigment epithelium-derived factor (PEDF), retinol binding protein 4 (RBP-4), chitinase-3-like protein 1 (YKL-40) and brain-derived neurotrophic factor (BDNF) for the identification of insulin resistance. *J Diabetes Complicatio*ns. https://doi.org/10.1016/j.jdiacomp.2017.06.002
69. Tombran-Tink *et al*. PEDF: a pigment epithelium-derived factor with potent neuronal differentiative activity. *Exp Eye Res* 1991; **53**:411- 414
70. Tombran-Tink *et al*. Localization of the gene for pigment epithelium-derived factor (PEDF) to chromosome 17p13.1 and expression in cultured human retinoblastoma cells. *Genomics* 1994; **19**:72

71. Tombran-Tink et al. PEDF and the serpins: phylogeny, sequence conservation, and functional domains J Struct Biol 2005; 151:130-50
72. Uehara *et al*. Expression of pigment epithelium-derived factor decreases liver metastasis and correlates with favorable prognosis for patients with ductal pancreatic adenocarcinoma. *Cancer Res* 2004**; 64**: 3533-7
73. Volpert *et al*. Inducer-stimulated Fas targets activated endothelium for destruction by anti-angiogenic thrombospondin-1 and pigment epithelium-derived factor. *Nat Med* 2002**; 8:** 349-57
74. Wang *et al*. Decreased Expression of Pigment Epithelium-Derived Factor Is Involved in the Pathogenesis of Diabetic Nephropathy. *Diabetes* 2005; **54:** 243-50
75. Wang *et al*. Late outgrowth endothelial cells derived from Wharton jelly in human umbilical cord reduce neointimal formation after vascular injury: involvement of pigment epithelium-derived factor. *Arterioscl Thromb Vasc Biol* 2009; **29**: 816-22
76. Yamagishi *et al*. Elevated serum levels of pigment epithelium-derived factor in the metabolic syndrome. *J Clin Endocrinol Metab* 2006; **91**: 2447-50
77. Yamagishi *et al.* Pathophysiological role of pigment epithelium-derived factor (PEDF) in hepatic disorders. *Curr Med Chem* 2010; **17:** 1995-2000
78. Yang *et al*. Serum pigment epithelium-derived factor is elevated in women with polycystic ovary syndrome and correlates with insulin resistance. *J Clin Endocrinol. Metab* 2011; **96**: 831-36
79. Yasui *et al.* Dual-site recognition of different extracellular matrix components by antiangiogenic/neurotrophic serpin, PEDF. *Biochemistry* 2003; **42**: 3160-7
80. Zhou *et al*. Evaluation of protein pigment epithelium-derived factor (PEDF) and microvessel density (MVD) as prognostic indicators in breast cancer. *J Cancer Res Clin Oncol* 2010**; 136**: 1719-27
81. Zhu *et al*. Polarized secretion of PEDF from human embryonic stem cell-derived RPE promotes retinal progenitor cell survival. *Invest Ophthalmol Vis Sci* 2011; **52:** 1573-85
82. Zolochevska *et al.* Pigment epithelial-derived factor and melanoma differentiation associated gene-7 cytokine gene therapies delivered by adipose-derived stromal/mesenchymal stem cells are effective in reducing prostate cancer cell growth. *Stem Cells Dev* 2011: **21**; 1112-23

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Title: Molecular aspects of glucose homeostasis in skeletal muscle – A focus on the molecular mechanisms of insulin resistance

Author: Revathy Carnagarin,Arun M. Dharmarajan,Crispin R. Dass

Publication: Molecular and Cellular Endocrinology

Publisher: Elsevier

Date: 5 December 2015

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Title:	PEDF-induced alteration of metabolism leading to insulin resistance
Author:	Revathy Carnagarin,Arunasalam M. Dharmarajan,Crispin R. Dass
Publication:	Molecular and Cellular Endocrinology
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Title: PEDF attenuates insulin-dependent molecular pathways of glucose homeostasis in skeletal myocytes

Author: Revathy Carnagarin,Arun M. Dharmarajan,Crispin R. Dass

Publication: Molecular and Cellular Endocrinology

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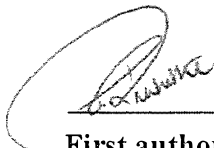
I, Revathy Carnagarin, as the first author of the publication entitled **"PEDF attenuates insulin-dependent molecular pathways of glucose homeostasis in skeletal myocytes"**, declare that this work was primarily designed, experimentally executed, interpreted, and written by the first author of this manuscript.



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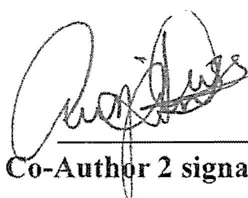

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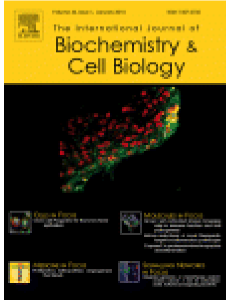

Title: Pigment epithelium-derived factor stimulates skeletal muscle glycolytic activity through NADPH oxidase-dependent reactive oxygen species production

Author: Revathy Carnagarin,Rodrigo Carlessi,Philip Newsholme,Arun M. Dharmarajan,Crispin R. Dass

Publication: The International Journal of Biochemistry & Cell Biology

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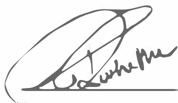

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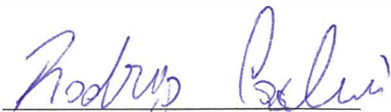

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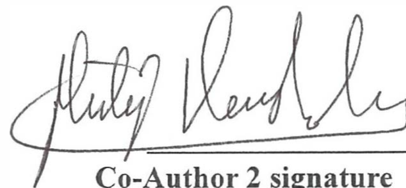

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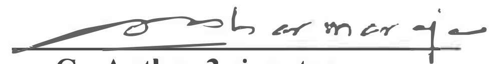

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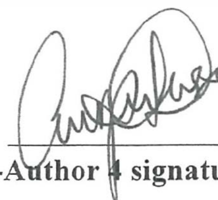

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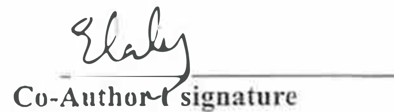
Mina Elahy	
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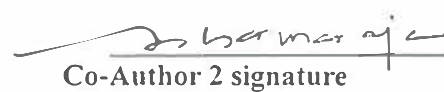
Arun M Dharmarajan	
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