IKK β and the anti-adipogenic effect of platelet-derived growth factor in human abdominal subcutaneous preadipocytes

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Abstract

To clarify how anti-adipogenic factors act on preadipocytes to inhibit their differentiation, we compared preadipocyte signaling responses generated by platelet-derived growth factor (PDGF; anti-adipogenic) versus insulin (pro-adipogenic). PDGF, but not insulin, stimulated the phosphorylation of inhibitor of κ B kinase β (IKK β) in a time-dependent manner. This PDGF-dependent phosphorylation event was inhibited by 60% ($P < 0.05$) when the cells were pretreated with wortmannin, indicating a requirement for the phosphatidylinositol (PI) 3-kinase/AKT pathway. IKK β phosphorylation by PDGF was neither accompanied by I κ B α

degradation nor NF- κ B activation. PDGF inhibited human adipocyte differentiation, assessed by triacylglycerol accumulation (75% reduction; $P < 0.01$) and by fatty acid synthase protein expression (60% reduction; $P < 0.05$); these responses were no longer apparent in the presence of sc-514, a selective inhibitor of IKK β . Our data describe a novel PDGF response in human preadipocytes that involves the pro-inflammatory kinase IKK β and demonstrate that it is required for the inhibition of adipogenesis.

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Introduction

Preadipocytes are mesenchymal fibroblast-like cells within the stromal compartment of adipose tissue that are committed to the adipocyte lineage (Scadden 2007). Healthy adipose tissue growth and functionality are maintained when differentiation of preadipocytes into adipocytes is coordinated with lipid storage within mature adipocytes.

Obesity, commonly defined as an excess of adipose tissue mass, can also be characterized by the functional status of adipocytes. In particular, insulin resistance and inflammation are features of hypertrophied adipocytes (Schenk *et al.* 2008). Their prominence in adipose tissue may be a compensatory response for reduced adipogenic capacity when energy balance is chronically positive (Danforth 2000, Heilbronn *et al.* 2004, Dubois *et al.* 2006). Preventing the decrease in adipocyte differentiation is therefore a potential therapeutic target to restore insulin sensitivity, and learning more about signaling pathways activated by anti-adipogenic factors may be informative.

Platelet-derived growth factor (PDGF) is a negative regulator of adipogenesis (Hauner *et al.* 1995, Krieger-Brauer & Kather 1995, Artemenko *et al.* 2005). PDGF receptor (PDGFR) mRNA and protein levels fall during 3T3-L1 adipocyte differentiation (Vaziri & Faller 1996, Summers *et al.* 1999, Whiteman *et al.* 2003), and a correlation between PDGFR expression and adipocyte size has also been observed

(Blucher *et al.* 2004). Recent reports have further highlighted the physiological relevance of PDGF in adipose tissue inflammation (Nishimura *et al.* 2008, Pang *et al.* 2008).

To identify anti-adipogenic preadipocyte signaling networks activated by PDGF, we compared preadipocyte responses elicited by PDGF versus insulin, a pro-adipogenic factor (Gagnon *et al.* 1999). Our data demonstrate that PDGF, but not insulin, activates the inhibitor of κ B kinase β (IKK β) in human preadipocytes. Inhibition of this event with an IKK β inhibitor curtails the anti-adipogenic action of PDGF, highlighting its importance as a key PDGF signaling response in these cells.

Materials and Methods

Culture of 3T3-L1 preadipocytes and screening of activated kinases

3T3-L1 preadipocytes were grown to confluence in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% calf serum, 100 U/ml penicillin, and 0.1 mg/ml streptomycin (all from Invitrogen). Cells were placed in serum-reduced condition overnight prior to 5-min stimulation with 1 μ M insulin or 10 ng/ml PDGF-BB (Calbiochem, San Diego, CA, USA). Cellular lysates were prepared by solubilizing proteins in Laemmli buffer (Laemmli 1970) supplemented with 1 mM sodium orthovanadate,

5 mM sodium pyrophosphate, 50 mM sodium fluoride (NaF), and 5 mM EGTA. Samples were sent to Kinexus for analysis of phosphorylated kinases with a Kinetworks phosphosite screen (KCPS-1.0). Alternatively, cellular lysates were processed for immunoblot analysis, as described below.

Isolation and culture of human preadipocytes

Subcutaneous adipose tissue was obtained from 19 patients (15 women and 4 men) undergoing elective abdominal surgery (approved by The Ottawa Hospital Research Ethics Board). Mean age was 51 ± 12 years, and mean body mass index (BMI) was 30 ± 6 kg/m² ($\pm$ S.D.; range from 22 to 48 kg/m²). We did not detect any obvious effects on IKK β -related cellular responses associated with this variation in BMI. Preadipocytes were isolated as previously described (Artemenko *et al.* 2005). Briefly, tissue was separated from connective tissue and capillaries by dissection, then digested with collagenase CLS type I (600 U/g of tissue; Worthington, Lakewood, NJ, USA). The digested tissue was subjected to progressive size filtration and centrifugation, followed by incubation in erythrocyte lysis buffer. Preadipocytes were seeded at a density of 3×10^4 cells/cm² and grown to confluence in DMEM supplemented with 10% fetal bovine serum (Invitrogen), 100 U/ml penicillin, 0.1 mg/ml streptomycin, and 50 U/ml nystatin (Calbiochem). Confluent cultures were used either for stimulation or differentiation studies, as described below.

Stimulation of human preadipocytes

Prior to stimulation, confluent preadipocytes were maintained in DMEM supplemented with 0.5% calf serum and antibiotics for 16–20 h. Cells were placed in Krebs–Ringer–HEPES (KRH) buffer (Gagnon *et al.* 1999), and stimulated at 37 °C with 1 μ M insulin, 5–20 ng/ml PDGF-BB or vehicle (2 mg/ml BSA in KRH), for up to 30 min, as indicated. For some experiments, cells were pretreated for 1 h with 100 μ M sc-514 (Calbiochem), for 15 min with 100 nM wortmannin (Kamiya Biochemical Company, Seattle, WA, USA), or vehicle (0.1% DMSO) prior to stimulation, as indicated. Proteins were solubilized in Laemmli buffer (Laemmli 1970) supplemented with 1 mM sodium orthovanadate, 5 mM sodium pyrophosphate, 50 mM NaF, and 5 mM EGTA.

Differentiation of human preadipocytes

Confluent human preadipocytes were pretreated for 15 min with 100 μ M sc-514 (Calbiochem) or 0.1% DMSO prior to induction of differentiation. Differentiation was induced as previously described (Artemenko *et al.* 2005) by supplementing the medium with 5 μ g/ml insulin, 100 μ M indomethacin, 0.5 μ M dexamethasone, and 0.25 mM isobutylmethylxanthine in the presence or absence of 10 ng/ml PDGF-BB, and either 100 μ M sc-514 or 0.1% DMSO. After 12 to 15 days, cultures were photographed with a Nikon Coolpix 995 digital

camera mounted on a Nikon Eclipse TS100 microscope. Cells were washed, and triacylglycerol (TG) was extracted and quantified spectrophotometrically (Gagnon *et al.* 1999). Proteins were solubilized in Laemmli buffer (Laemmli 1970) and used in immunoblot analysis.

Immunoblot analysis

Equal amounts of protein (5–25 μ g depending on the experiment) were resolved by SDS–PAGE and transferred to a nitrocellulose membrane. Non-specific binding sites were blocked and membranes were incubated with antibodies specific for actin (0.4 μ g/ml; Santa Cruz Biotech., Santa Cruz, CA, USA), AKT (clone C-20; 2 μ g/ml; Santa Cruz Biotech.), fatty acid synthase (FAS; 1.0 μ g/ml; BD Biosciences, Mississauga, ON, Canada), inhibitor of κ B α (IkB α ; 1:1000), IKK β (1:500), phospho-AKT Ser473 (pAKT; 1:1000), or phospho-IKK (pIKK; 1:500), all from Cell Signaling (Danvers, MA, USA).

Preparation of nuclear extracts and NF- κ B DNA-binding assay

Human abdominal s.c. preadipocytes were placed in DMEM supplemented with 1% calf serum and antibiotics. Cells were pretreated with either 100 μ M sc-514 or 0.1% DMSO for 1 h, then treated with or without 10 ng/ml PDGF for 30 min. Cells were washed twice with ice-cold PBS/phosphatase inhibitor buffer, then placed in 20 mM Hepes, pH 7.5, 5 mM NaF, 10 μ M Na₂MoO₄, and 0.1 mM EDTA, and allowed to swell. Nonidet P-40 was added to a final concentration of 0.5%, then the homogenates were centrifuged at 14 000 *g* for 1 min at 4 °C. Nuclear pellets were then processed for NF- κ B DNA-binding activity using the TransAM NF- κ B kit (Active Motif, Carlsbad, CA, USA), according to the manufacturer's instructions. A nuclear fraction prepared from activated Jurkat cells, provided with the kit, was used as a positive control for the assay.

Statistical analyses

ANOVA was performed, followed by the Newman–Keul's test to assess differences between means (Instat, version 3.05; GraphPad, San Diego, CA, USA) *P* values < 0.05 were considered significant.

Results

IKK β phosphorylation occurs in response to PDGF, but not insulin, in 3T3-L1 and human preadipocytes

In a search for intracellular targets differentially regulated by insulin and PDGF, 3T3-L1 preadipocytes were stimulated for 5 min with insulin or PDGF. A commercial phosphokinase screen of cellular lysates by Kinexus was conducted. Whereas the phosphorylation of some kinases in response to PDGF

versus insulin was similar, the phosphorylation of IKK β was 12.4-fold higher in PDGF- versus insulin-treated 3T3-L1 preadipocytes (Fig. 1A). We confirmed this large differential signaling response in 3T3-L1 preadipocytes, and demonstrated that it also occurred in primary human abdominal s.c. preadipocytes (Fig. 1B). Treatment with 10 ng/ml PDGF for 5 min increased IKK β phosphorylation by 20-fold ($P<0.001$) and 7.6-fold ($P<0.01$), compared with basal, in 3T3-L1 and human preadipocytes respectively. Treatment with 1 μ M insulin failed to increase IKK β phosphorylation.

IKK β phosphorylation by PDGF depends on phosphatidylinositol (PI) 3-kinase

To further characterize the regulation of IKK β by PDGF, human preadipocytes were treated with insulin or PDGF for the indicated times. IKK β phosphorylation was transiently stimulated by PDGF, with a 16-fold increase within 5 min of exposure ($P<0.001$), which returned to basal levels within 30 min (Fig. 2A). IKK β phosphorylation in response to insulin

was not observed at any of the times tested. Maximal IKK β phosphorylation was observed at a dose of 5 ng/ml PDGF (Fig. 2B). Pretreatment with wortmannin, a specific PI3-kinase inhibitor, significantly reduced PDGF-induced IKK β phosphorylation by 60% ($P<0.05$), implicating the PI3-kinase/AKT pathway in this regulation (Fig. 2C). Insulin, like PDGF, also stimulated AKT phosphorylation, but did not have a significant effect on IKK β phosphorylation.

PDGF-induced IKK β phosphorylation does not lead to the degradation of I κ B α and activation of NF- κ B

To investigate downstream signaling events beyond PDGF-stimulated IKK β phosphorylation, we assessed the degradation of I κ B α and activation of NF- κ B, known consequences of IKK β activation. However, the levels of I κ B α were unchanged in PDGF-treated human preadipocytes. Consistent with this observation, PDGF failed to activate NF- κ B, assessed by DNA binding to an NF- κ B-specific response element.

Figure 1 PDGF, but not insulin, induces IKK β phosphorylation in 3T3-L1 and human abdominal s.c. preadipocytes. Confluent 3T3-L1 or human preadipocytes were placed in medium supplemented with 0.5% calf serum overnight prior to stimulation. Cells were stimulated for 5 min with 1 μ M insulin (Ins), 10 ng/ml PDGF, or vehicle control (Ctrl). (A) Cellular lysates from 3T3-L1 preadipocytes were processed for phosphokinase analysis as described. The intensity of the bands was quantified and is reported as counts per minute (cpm) for the indicated kinases. N.D., not detectable. (B) Equal amounts of solubilized protein from 3T3-L1 or human preadipocytes were immunoblotted with the indicated antibodies. Representative immunoblots from one experiment or patient sample are shown. Densitometric data from three independent experiments or three separate patient samples are expressed as means $\pm$ s.e.m. Statistical significance is indicated as *** $P<0.001$ or ** $P<0.01$ compared with control and insulin-treated conditions. IOD, integrated optical density.

Figure 2 PDGF induces IKK β phosphorylation in human abdominal s.c. preadipocytes via activation of PI3-kinase. Confluent human preadipocytes were placed in medium supplemented with 0.5% calf serum overnight prior to stimulation. (A) Cells were stimulated with 1 μ M insulin, 10 ng/ml PDGF, or vehicle control for the indicated times. (B) Cells were stimulated with 0–20 ng/ml PDGF for 5 min. (C) Cells were pretreated with 100 nM wortmannin or vehicle (0.1% DMSO) for 15 min, then stimulated for 5 min with 1 μ M insulin or 10 ng/ml PDGF. Equal amounts of solubilized protein were immunoblotted with the indicated antibodies. Representative immunoblots from a single patient sample are shown. Densitometric data from three separate patient samples are expressed as means $\pm$ S.E.M. Statistical significance is indicated as *** P < 0.001, ** P < 0.01, or * P < 0.05 compared with control and insulin-treated conditions, or as indicated. IOD, integrated optical density.

Sc-514 reverses the anti-adipogenic effect of PDGF on human adipogenesis

We examined the functional role of IKK β with respect to PDGF-mediated inhibition of adipogenesis. Human abdominal s.c. preadipocytes were treated with sc-514, a selective inhibitor of IKK β (Kishore *et al.* 2003), for 15 min, followed by induction of differentiation, either in the presence or absence of 10 ng/ml PDGF. As previously reported (Artemenko *et al.* 2005), PDGF treatment strongly reduced the morphological changes that characteristically occur during adipocyte differentiation, including the accumulation of lipid droplets (Fig. 3A). The inhibitory effect of PDGF on lipid droplet accumulation was nullified by sc-514. These morphological effects were quantified by measuring cellular TG content (Fig. 3B). TG accumulation was reduced by 75% (P < 0.01) in response to PDGF, but when sc-514 was present, the inhibition by PDGF was only 28%, and was no longer significant. Consistent with this response, the protein expression of FAS, an adipogenic marker, was reduced by 60% (P < 0.05) in the presence of PDGF, but with sc-514 present, the inhibition by PDGF was attenuated to 23%, and no longer reached significance (Fig. 3C). There was no significant effect on either FAS expression or TG accumulation with sc-514 alone.

Discussion

PDGF is a growth factor that is known to be anti-adipogenic (Hauner *et al.* 1995, Krieger-Brauer & Kather 1995, Artemenko *et al.* 2005). PDGFR expression is regulated according to the stage of adipocyte differentiation as well as by adipocyte cell size (Vaziri & Faller 1996, Summers *et al.* 1999, Whiteman *et al.* 2003, Bluher *et al.* 2004). Here, we demonstrate that IKK β is activated by PDGF in human preadipocytes, and that it is required for the inhibition of adipogenesis by PDGF. IKK β was phosphorylated by PDGF, but not by insulin, a pro-adipogenic agent.

Only a few other studies have linked the IKK β /NF- κ B pathway to PDGF signaling and responses in other cell types. NF- κ B participates in the regulation of PDGF-inducible genes and cell transformation in mouse fibroblasts (Olashaw *et al.* 1992). In addition, anti-apoptotic and proliferative responses by human skin fibroblasts and rat synoviocytes induced by PDGF depend on IKK β /NF- κ B (Romashkova & Makarov 1999). To our knowledge, this is the first study reporting the regulation of this pro-inflammatory pathway in human adipose cells.

The phosphorylation of IKK β observed in response to PDGF treatment of human preadipocytes was not accompanied by degradation of I κ B α or activation of NF- κ B. Although infrequent, activation of IKK β , without concomitant NF- κ B activation was recently shown to occur with respect to the regulation of interferon- γ -dependent genes (Tudhope *et al.* 2007). IKK β has also been reported to directly phosphorylate substrates such as IRS-1 (on serine residue 307/312; rodent/human), as well as FOXO3, and 14-3-3b (Perkins 2007); these proteins have been implicated in the regulation of adipose tissue function (Miki *et al.* 2001, Hong *et al.* 2005, Luo *et al.* 2008). Further research will be needed to evaluate the possible roles of each of these candidate proteins with respect to the PDGF-mediated anti-adipogenic effect we have observed here.

The role of the PI3-kinase/AKT pathway in the activation of IKK β has been noted in other cells (Parhar *et al.* 2007, Dan *et al.* 2008). It has been demonstrated that AKT and IKK β associate in response to PDGF (Romashkova & Makarov 1999). However, it is unclear why insulin, which also activates AKT in human preadipocytes, does not lead to IKK β phosphorylation. We have previously shown that anti-adipogenic PDGF generates both PI(3,4)P2 and PI(3,4,5)P3, while pro-adipogenic insulin leads to accumulation of only PI(3,4,5)P3 in preadipocytes (Sorisky *et al.* 1996, Gagnon *et al.* 1999). This differential generation of 3-phosphoinositides by these two agonists may alter the activation characteristics of AKT or its cellular compartmentalization, leading to the activation/phosphorylation of distinct arrays of substrates that may differentially modulate the activation of IKK β . It is also possible that other, as yet unidentified, upstream regulatory molecules play a role in PDGF-stimulated IKK β phosphorylation.

Figure 3 Sc-514 reverses the anti-adipogenic action of PDGF on human s.c. abdominal preadipocyte differentiation. Human preadipocytes were pretreated for 15 min with 100 μM sc-514 prior to induction of differentiation in the presence or absence of 10 ng/ml PDGF for 14 days. (A) Cultures were photographed at 400× magnification. Representative microphotographs from a single patient sample are shown. (B) TG was extracted, quantified, and normalized to protein content. Results are expressed as the mean ± s.d. of five patient samples. ** indicates $P < 0.01$ compared with other differentiated conditions. (C) Solubilized proteins were immunoblotted with antibodies against FAS or actin (used as a loading control). Representative immunoblots from a single patient sample are shown. (D) Densitometric data from the five patient samples are expressed as mean ± s.d. * indicates $P < 0.05$ compared with other differentiated conditions. IOD, integrated optical density.

Previous studies have examined PDGF and preadipocytes to determine which downstream signaling events are involved in its ability to inhibit adipogenesis. Conventional protein kinase C (PKCs) have been implicated in studies investigating the anti-adipogenic effect of PDGF on human preadipocytes (Artemenko *et al.* 2005), and conventional PKCs, ERK1/2, and NADPH-dependent H₂O₂ generation have been reported using 3T3-L1 preadipocytes (Krieger-Brauer & Kather 1995, Camp & Tafuri 1997, Artemenko *et al.* 2005). At present, the precise relationship between these signaling events in preadipocytes and the activation of the IKK β pathway is unknown, but merits attention.

Our studies with the pharmacological inhibitor sc-514 suggest that IKK β participates in the anti-adipogenic effects of PDGF. Sc-514 has been shown to be a potent inhibitor of IKK β , with ten times more selectivity for IKK β than many other kinases stimulated by PDGF, including ERKs, p38 MAPK, and conventional PKCs (Kishore *et al.* 2003). Further studies, such as RNA silencing, would nonetheless complement these findings.

In summary, our data reveal a pro-inflammatory signaling response that is a required element for the anti-adipogenic effect of PDGF on human preadipocytes.

Declaration of interest

The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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