Preferential expression of PAPPA in human preadipocytes from omental fat

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Abstract

Fat distribution differs between individuals, and those with visceral fat predominance develop metabolic profiles that increase the risk of adverse cardiovascular events. This is due, in part, to the proinflammatory state associated with visceral obesity as well as depot-specific adipogenesis. The IGF system is important in adipose tissue development and metabolic function. Pregnancy-associated plasma protein A (PAPPA) is a novel zinc metalloproteinase that regulates local IGF availability. The first aim of this study was to characterize PAPPA mRNA and protein expression in primary cultures of human preadipocytes isolated from omental, mesenteric, and subcutaneous depots. PAPPA expression was significantly increased in omental preadipocytes compared with mesenteric and subcutaneous preadipocytes. The second aim of this study was to investigate the factors regulating PAPPA expression, focusing on proinflammatory cytokines and resveratrol that have been shown to have negative and positive effects, respectively, on metabolism and diet-induced obesity. Treatment of cultured primary human preadipocytes with tumor necrosis factor α and interleukin 1 β led to significant increases in PAPPA expression. Activated pathways mediating cytokine-induced PAPPA expression include the nuclear factor κ B pathway and the MAPK family, particularly c-Jun NH₂-terminal kinase and p38 MAPK. Resveratrol, a polyphenolic compound with beneficial cardiometabolic effects, significantly downregulated PAPPA expression under basal and stimulated conditions. Effects of resveratrol on PAPPA appeared to be mediated through pathways independent of silent mating type information regulation 2 homolog 1 (SIRT1) and AMP kinase activation. Depot-specific PAPPA expression in human preadipocytes may contribute to a depot-specific function.

Key Words

- ▶ PAPPA
- ▶ adipose tissue
- ▶ resveratrol
- ▶ cytokines
- ▶ inflammation

Journal of Endocrinology
(2014) 222, 87–97

Introduction

The prevalence of obesity is increasing, particularly in westernized countries, and is now considered an epidemic. Distribution of fat may differ between individuals, and metabolic functions of adipose tissue are inherently distinct between their fat depots (Caserta *et al.* 2001). This is due, in part, to regional variation of preadipocytes with respect to

adipocyte development and metabolic function. Obesity is considered a low-grade proinflammatory state with increased circulating cytokines, chemokines, and growth factors (Lacasa *et al.* 2007). This adipose tissue inflammation leads to a higher likelihood of adverse metabolic profiles, including diabetes and atherosclerosis, particularly

in subjects with visceral fat predominance (Coppack 2001, Tchkonina *et al.* 2010). Little is known about the regulatory factors leading to a depot-specific function.

Genome-wide expression profiles of primary preadipocytes from human fat depots identified pregnancy-associated plasma protein A (PAPPA) as one of the most distinctive genes expressed, with levels in preadipocytes from omental fat greatly exceeding those from subcutaneous fat (Tchkonina *et al.* 2007). PAPPA is a zinc metalloproteinase that enhances local insulin-like growth factor (IGF) action through cleavage of inhibitory proteins that bind IGFs with high affinity, thereby freeing the IGFs in the pericellular environment to bind and activate receptors (Conover 2012). Elevated PAPPA level has been implicated in aging and age-related disease, while *Pappa* knockout mice have a 30% longer lifespan than WT mice, with resistance to atherosclerotic plaque development (Harrington *et al.* 2007, Conover *et al.* 2010, Boldt *et al.* 2013) and visceral fat accumulation on high-fat diet (Conover *et al.* 2013).

In this study, we follow up on the DNA microarray data and characterize PAPPA mRNA and protein expression in primary cultures of human preadipocytes from omental, mesenteric, and subcutaneous fat depots. Furthermore, we investigate the factors that regulate PAPPA expression in human preadipocytes and the underlying mechanisms for their regulation, including proinflammatory cytokines and resveratrol, the latter being a polyphenolic compound shown to have beneficial cardiometabolic effects.

Materials and methods

Materials

Tumor necrosis factor alpha (TNF α) was purchased from Research Diagnostics, Inc. (Flanders, NJ, USA). Interleukin 1 β (IL1 β), IL6, IL6 soluble receptor, and EX-527 were purchased from R&D Systems (Minneapolis, MN, USA). Antibodies against total and phosphorylated ERK1/2, c-Jun NH₂-terminal kinase (JNK), p38 MAPK, nuclear factor κ B (NF κ B) (including inhibitory κ B α and β (I κ B α and I κ B β)), phospho-Hsp27, and IGFBP4 were purchased from Abcam (Cambridge, MA, USA). Antibodies against total and phosphorylated AKT were purchased from Novus Biologicals (Littleton, CO, USA). SB203580, SP600125, rolipram, and splitomycin were purchased from Enzo Life Sciences (Farmingdale, NY, USA). BAY11-7082 was purchased from Calbiochem (San Diego, CA, USA). Resveratrol was purchased from Sigma-Aldrich.

Reagents for SDS-PAGE, mini gels, and blocking buffer were purchased from Bio-Rad Laboratories and tissue culture supplements and fetal bovine serum were purchased from Life Technologies. A769662 was purchased from Santa Cruz Biotechnology.

Cell culture

Human adipose tissue was obtained under Mayo Clinic IRB-approved protocols. Human preadipocytes isolated from subcutaneous, mesenteric, and omental fat depots were cultured in alpha MEM containing 10% fetal bovine serum, 100 units/ml penicillin, and 100 mg/ml streptomycin as described previously (Tchkonina *et al.* 2002, 2007). Donor characteristics are summarized in Table 1. Cells of the third to ninth passage were used in experiments. Preliminary dose-response and time-course experiments were carried out to determine the effective concentrations and appropriate duration of treatments. For some studies, cells were pretreated for 60 min with resveratrol before the addition of cytokines. At the end of the incubation,

Table 1 Human preadipocytes: donor characteristics. Numbers in parentheses on the left indicate the number of donors for which information was available. Numbers for specific diseases and medications on the right indicate the number of donors. Some donors will have more than one

Gender (M/F) (15)	3/12
Age (years) (15)	41 $\pm$ 2.5
BMI (kg/m ²) (15)	51 $\pm$ 3.7
Concomitant diseases (9)	
Diabetes	2
Degenerative joint disease	3
Obesity hypoventilation syndrome	3
Gastroesophageal reflux disease	2
Sleep apnea	2
Others ^a	
Medications	
Diabetes	2 ^b
Heart/blood pressure	5 ^c
Heartburn	5 ^d
Depression	4 ^e
Inflammation/pain	3 ^f
Others ^g	

^aOne each hypothyroidism, hyperlipidemia, asthma, hypertension, and recurrent sinus infection.

^bAvandia, Metformin, and Prandin.

^cSotalol, Lasix, Digoxin, Avapro, Hydrochlorothiazide, Lovenox, Lipitor, Lopid, Verapamil, and Norvasc.

^dPrilosec.

^ePaxil, Zoloft, Celexa, Trazodone, Prozac, and Lorazepam.

^fFlonase, Vioxx, Nasacort, Zyrtec, and Vicodin.

^gOne each Carbidopa/Levodopa, Unithroid, Ortho-Novum, and Intracozole.

conditioned media were collected and cells were harvested for RNA and stored at -80°C .

Real-time PCR

Upon harvest, the preadipocytes were immediately suspended in TRIzol (Life Technologies). Total RNA was then isolated, reverse transcribed with the SuperScript III First-Strand Synthesis System (Life Technologies), and evaluated by quantitative real-time PCR using the iCycler iQ5 Detection System with iQ SYBR Green PCR Master Mix (Bio-Rad). Amplification plots were analyzed with iQ5 Optical System Software version 2.1 (Bio-Rad). Primer sequences for specific detection and amplification of PAPPa as well as assay validations were described previously (Conover *et al.* 2006).

Western blotting

Following experimental treatments, cells were lysed, subjected to SDS-PAGE, and transferred onto PVDF membranes. Filters were blocked with 5% non-fat dry milk in Tris-buffered saline/0.1% Tween 20 or blocking buffer (Bio-Rad) and probed with a primary antibody at the recommended dilution. After the reaction, secondary antibody was applied (fluorescent labeled where applicable (LI-COR, Lincoln, NE, USA)). Blots were visualized using enhanced chemiluminescence reagents and autoradiography or captured with LI-COR Odyssey Imagers.

PAPPa ELISA

Conditioned medium (CM) from human preadipocytes was collected, cellular debris was removed by centrifugation, and the supernatant was stored at -80°C before assay. Protein was quantified using the Pierce BCA Protein assay kit (Thermo Scientific, Waltham, MA, USA). PAPPa protein in the CM was quantified by picoPAPP-A ELISA (generously provided by AnshLabs, Webster, TX, USA), a high-sensitivity assay designed to detect PAPPa levels as low as 100 pg/ml with observed CV values routinely <5.0 . Some of the early experiments with conditioned media were carried out using the Ultra-sensitive PAPP-A ELISA kit kindly provided by Diagnostic Systems Laboratories, Inc. (Webster, TX, USA) as described previously (Boldt & Conover 2011).

Miscellaneous biochemistries

TNF α and IL1 β levels in CM were assayed by the Mayo Clinic Immunochemical Core Laboratory.

Statistical analysis

Results are expressed as mean $\pm$ S.E.M. for the indicated number of experiments. The number of experiments noted in figure legends is based on primary cultured cells from different donors. We used ANOVA for pairwise comparisons and the Bonferroni correction for multiple comparisons. We used two-sample *t*-tests to compare two groups. Significance was set at $P < 0.05$.

Results

Depot-specific PAPPa expression

PAPPa mRNA and protein expression were determined in the primary cultures of human preadipocytes collected from subcutaneous, mesenteric, and omental adipose tissue depots by real-time PCR and PAPPa ELISA respectively. As shown in Fig. 1A, PAPPa mRNA expression was

Figure 1

PAPPa expression in human preadipocytes. (A) PAPPa mRNA and (B) PAPPa protein expression in preadipocytes from omental (Om), mesenteric (Mes), and subcutaneous (Sc) depots were determined by real-time PCR and PAPPa ELISA, respectively, as described in the Materials and methods section. Results are expressed as mean $\pm$ S.E.M., $n = 6-10$. * $P < 0.05$.

significantly higher (fourfold) in omental preadipocytes compared with subcutaneous preadipocytes. Although abdominal in origin, mesenteric fat is distinct from omental fat with respect to cellular and gene expression properties. In our experiments, *PAPPa* mRNA expression in omental fat was twofold higher than that in mesenteric fat. Levels of PAPPa protein were also significantly higher in CM from omental preadipocytes compared with both mesenteric and subcutaneous preadipocytes (Fig. 1B). Although these primary cultures of preadipocytes came from severely obese donors (Table 1), PAPPa protein in extracts of fat depots from subjects with a BMI <30 kg/m² showed the same significant difference (four- to sixfold) between omental and subcutaneous depots (data not shown). Furthermore, concomitant diseases or the variety of medications taken by the donors (Table 1) did not influence this differential expression. Thus, depot-specific PAPPa expression is highest in preadipocytes from human visceral fat.

Regulation of PAPPa expression by cytokines

The effects of proinflammatory cytokines, TNF α , IL1 β , and IL6, on PAPPa expression were determined in subcutaneous, mesenteric, and omental preadipocytes. Cells were treated with both IL6 and the IL6 soluble receptor to provide more reliable activation of the IL6 signaling protein, gp130, in the preadipocytes (Franchimont *et al.* 1997, Resch *et al.* 2006). PAPPa mRNA levels increased in response to TNF α and IL1 β after 6 h and remained increased for 24 h based on time-course experiments (data not shown). The effects of these cytokines on PAPPa mRNA expression are shown in Fig. 2. TNF α and IL1 β led to a significant threefold increase in PAPPa mRNA expression in the omental and mesenteric preadipocytes with no significant increase observed in the subcutaneous preadipocytes. No significant effect was observed on PAPPa expression after treatment with IL6 and IL6 soluble receptor. PAPPa protein levels in CM were also increased by TNF α and IL1 β , with no effect after treatment with IL6 (data not shown).

To determine the intracellular pathways mediating TNF α and IL1 β stimulation of PAPPa expression in human preadipocytes, we focused on those commonly associated with stress, apoptosis, and proliferation. These included MAPK family, phosphatidylinositol 3-kinase (PI3K), and NF κ B pathways.

The MAPK family has three main subgroupings: Erk1/2, p38 kinase, and JNK. Western blot analyses were performed using antibodies specific for these pathways. Stimulation with TNF α and IL1 β had little effect on Erk1/2

Figure 2

Effect of cytokines on PAPPa expression. Preadipocytes were stimulated with TNF α (1 nM), IL1 β (1 nM), and IL6 (50 ng/ml) with soluble receptor (100 ng/ml) for 24 h. Upon harvest, RNA was isolated from preadipocytes, reverse transcribed, and evaluated by quantitative real-time PCR. Results (mean $\pm$ s.e.m., $n = 3-4$) are expressed relative to control (represented by the dotted line). * $P < 0.05$.

phosphorylation but induced p38 phosphorylation at 10 and 30 min with a decrease toward basal levels at 60 and 120 min. JNK phosphorylation was induced 10 and 30 min after stimulation with IL1 β but returned to baseline at 60 min. IL1 β appeared to be more effective than TNF α in activating the JNK pathway (Figs 3A and 4B).

Treatment of human preadipocytes with TNF α and IL1 β for 10, 30, 60, or 120 min did not result in detectable AKT phosphorylation, a downstream substrate for PI3K. Reprobing with an antibody against total AKT indicated that the protein was present (data not shown).

NF κ B is usually sequestered in the cytoplasm bound to the I κ Bs. I κ B α and I κ B β are prototypes of the inhibitory I κ B family. NF κ B is activated through phosphorylation of the I κ Bs, which leads to translocation of NF κ B into the nucleus where the regulation of gene expression occurs (Resch *et al.* 2006). Western blotting after stimulation with TNF α and IL1 β led to a rapid loss of I κ B α within 10 min, which returned to detectable levels by 60 min. TNF α and IL1 β had little or no effect on I κ B β (Fig. 3B).

Inhibitors of the NF κ B (BAY11-7082), JNK (SP600125), and p38 (SB203580) pathways were evaluated by western blotting to establish effectiveness (Fig. 4). To determine which pathways mediated cytokine-stimulated PAPPa gene expression, real-time RT-PCR was carried out on RNA isolated from human preadipocytes treated with the inhibitors BAY11-7082, SB203580, and SP600125 and then stimulated with IL1 β and TNF α . BAY11-7082 (NF κ B inhibitor) significantly inhibited IL1 β - and TNF α -stimulated PAPPa gene expression (Fig. 5A). SB203580

Figure 3

TNF α and IL1 β regulation of (A) MAPK and (B) NF κ B signaling. Human preadipocytes from omental fat were treated with TNF (1 nM) or IL1 β (1 nM) for the indicated times. Western blotting was performed for

phosphorylated (P) Erk1/2, p38 and JNK, total (T) p38 and ERK1/2, and I κ B α and I κ B β as described in the Materials and methods section. GAPDH was used to quantify loading for P-JNK.

(p38 inhibitor) showed similar inhibition, but did not reach statistical significance (Fig. 5B). SP600125 (JNK inhibitor) significantly inhibited IL1 β -induced, but not TNF α -induced, PAPP α expression (Fig. 5C).

In summary, the intracellular pathways mediating PAPP α upregulation by IL1 β and TNF α are commonly associated with stress, including NF κ B, p38, and JNK, although IL1 β and TNF α seem to have differing effects on activation of the JNK pathway in human preadipocytes.

Regulation of PAPP α expression by resveratrol

Resveratrol is found in multiple plant derivatives such as grapes and grape products (Baur *et al.* 2012). There has been much interest in resveratrol over the last decade, due to its potential protective metabolic effects. Pretreatment of human preadipocytes with resveratrol at a concentration of 50 μ M led to a significant reduction in both PAPP α mRNA and protein expression under basal conditions (Fig. 6A). The effect of resveratrol on PAPP α expression was observed in preadipocytes from all three depots. Resveratrol also markedly inhibited IL1 β - and TNF α -stimulated PAPP α mRNA expression (Fig. 6B). The effects of

stimulating PAPP α expression in preadipocytes (IL1 β treatment) and inhibiting PAPP α expression (resveratrol treatment) on PAPP α activity, i.e., IGFBP4 proteolysis, are shown in Fig. 7. IL1 β increased IGFBP4 fragmentation and

Figure 4

Efficacy of inhibitor concentrations. Human preadipocytes from omental fat were treated with BAY11-7082 (5 μ M), SP600125 (20 μ M) or SB203580 (2.5 μ M), and cytokines TNF α (1 nM) and IL1 β (1 nM) for 6 h before cell lysis for western blotting. (A) Effect of BAY11-7082 on cytokine-induced NF κ B activation (I κ B α phosphorylation and degradation). (B) Effect of SP600125 on cytokine-induced JNK activation (phosphorylation of JNK). (C) Effect of SB203580 on cytokine-induced p38 kinase activation (phosphorylation of Hsp27). C, control.

Figure 5

Effect of pathway inhibitors on PAPPa expression. Real-time PCR was carried out on RNA isolated from omental preadipocytes treated with (A) NFκB inhibitor BAY11-7082 (5 μM) (B) p38 inhibitor SB203580 (2.5 μM), and (C) JNK inhibitor SP600125 (20 μM), and then stimulated with IL1β (1 nM) and TNFα (1 nM) for 24 h. Results (mean ± s.e.m., *n* = 6) are expressed relative to control (represented by dotted line). **P* < 0.05. Inhib, inhibitor.

resveratrol inhibited the appearance of fragments. Addition of a neutralizing PAPPa antibody confirmed the specificity of PAPPa-mediated IGFBP4 proteolysis.

Resveratrol is thought to act through multiple pathways, but primarily through activation of silent mating type information regulation 2 homolog 1 (SIRT1), a mammalian sirtuin that is thought to extend lifespan in a variety of species (Howitz *et al.* 2003, Wood *et al.* 2004). Other resveratrol targets include the activation

of AMP kinase (AMPK), part of a nutrient-sensing pathway, which may also activate SIRT1 (Park *et al.* 2012). To determine whether resveratrol exerts its effects on PAPPa expression through SIRT1, preadipocytes were treated with the SIRT1 inhibitor, EX-527, plus resveratrol. SIRT1 is a deacetylase enzyme with p53 as a native substrate. We confirmed acetylation of p53 in the presence of EX-527 by western blotting (Fig. 8A). Resveratrol effectively downregulated PAPPa mRNA expression in the presence of EX-527 (Fig. 8B). We confirmed our findings using two other SIRT1 inhibitors, splitomycin and nicotinamide (data not shown). To determine whether resveratrol exerts its effects on

Figure 6

Effect of resveratrol on PAPPa expression. (A) Preadipocytes were treated with resveratrol (Res; 50 μM) for 24 h. (B) Omental preadipocytes were treated with resveratrol (50 μM) alone, resveratrol plus cytokines IL1β (1 nM) and TNFα (1 nM), and cytokines IL1β and TNFα alone for 24 h. Preadipocytes were then harvested for real-time PCR. Results (mean ± s.e.m., *n* = 3–10) are expressed relative to control (represented by dotted line). **P* < 0.05.

Figure 7

Effect of IL1 β and resveratrol on PAPPa activity. Omental preadipocytes were incubated with IL1 β (1 nM), resveratrol (50 μ M), or appropriate vehicle for 24 h. Conditioned medium was collected and incubated with IGFBP4 and IGF2 without or with a PAPPa inhibitory antibody (i). Arrows indicate intact and N- and C-terminal fragments of IGFBP4. frag, positive control for PAPPa-mediated IGFBP4 proteolysis; Res, resveratrol.

PAPPa expression through AMPK, preadipocytes were treated with resveratrol or the AMPK activator, rolipram (Park *et al.* 2012). AMPK phosphorylation analyzed by western blotting was used to confirm the activity of rolipram before experiments (Fig. 8C). Treatment with rolipram did not reduce PAPPa mRNA or protein

expression (Fig. 8D). We confirmed our findings using a second AMPK activator, A769662, with similar results (data not shown).

In summary, resveratrol leads to downregulation of PAPPa expression in human preadipocytes under both basal and cytokine-stimulated conditions. The effects of

Figure 8

Effect of SIRT1 inhibition and AMPK activation on PAPPa expression. (A) Omental preadipocytes were treated with resveratrol (Res) (50 μ M) and EX-527 (10 μ M) for 24 h. Western blotting was performed for p53 and acetylated p53. (B) Omental preadipocytes were treated with resveratrol alone, resveratrol plus SIRT1 inhibitor EX-527 (10 μ M), and EX-527 alone for 24 h. Preadipocytes were then harvested for real-time PCR. Results (mean $\pm$ S.E.M., $n=3$) are expressed relative to control (represented by dotted line).

(C) Omental preadipocytes were treated with rolipram (25 (L) and 50 (H) μ M) for 24 h. Western blotting was performed for AMPK and phosphorylated AMPK (P-AMPK). (D) Omental preadipocytes were treated with resveratrol or the AMPK activator, rolipram (25 μ M), for 24 h. Preadipocytes were then harvested for real-time PCR. Results (mean $\pm$ S.E.M., $n=4$) are expressed relative to control (represented as dotted line). * $P<0.05$.

resveratrol on PAPPa are not mediated through the AMPK or SIRT1 pathway.

Discussion

PAPPa is preferentially expressed in human preadipocytes from visceral fat depots compared with preadipocytes from subcutaneous depots

Obesity is commonly associated with health risks, particularly cardiovascular disease. Individuals may preferentially accumulate fat in specific anatomical fat depots including the upper-body subcutaneous fat depot, the lower-body fat depot (intramuscular fat, the subcutaneous leg fat, and the gluteal fat), and the intra-abdominal fat depot (mesenteric and omental fat, or visceral fat) (Tchkonia *et al.* 2010). Adipose tissue in these fat depots may behave differently. The typical 'pear' shape of fat distribution (peripheral fat distribution) may lead to fewer cardiovascular risk factors than the typical 'apple' shape of fat distribution (central fat accumulation). Omental fat accumulation, in particular, is associated with adverse metabolic risk profiles including diabetes, insulin resistance, dyslipidemia, and atherosclerosis with dysregulation of fatty acid storage and release. Although abdominal in origin, mesenteric fat is distinct from omental fat with respect to cellular and gene expression properties (Tchkonia *et al.* 2013). Our studies indicate that PAPPa is preferentially expressed and secreted in human preadipocytes from omental fat compared with subcutaneous fat, despite the variability in donor characteristics. Thus, PAPPa expression in preadipocytes may be an intrinsic property of the fat depots and may play a role in depot-specific adipogenesis (Tchkonia *et al.* 2002).

Proinflammatory cytokines IL1 β and TNF α are potent stimulators of PAPPa expression in human preadipocytes

The proinflammatory state associated with visceral obesity is thought to be a contributing factor to adverse metabolic profiles (Shah *et al.* 2008). The stromal vascular fraction of adipose tissue contains many cell types including macrophages, lymphocytes, and preadipocytes. Activated macrophages secrete cytokines that can have paracrine effects in adipose tissue (Tchkonia *et al.* 2013). Omental adipose tissue has been shown to have elevated macrophage infiltration compared with subcutaneous fat, even in lean subjects (Harman-Boehm *et al.* 2007).

Upregulation of PAPPa mRNA and protein expression in preadipocytes was stimulated by cytokines known to be

secreted by activated macrophages. (It should be noted that these cultures are essentially pure preadipocytes (Tchkonia *et al.* 2002, 2007).) IL1 β and TNF α stimulated a threefold increase in PAPPa expression in preadipocytes from the omental and mesenteric depots. Cytokine-induced PAPPa expression is not unique to preadipocytes. Cytokines have also upregulated the expression of PAPPa in human coronary artery smooth muscle cells (Conover *et al.* 2006), fibroblasts (Resch *et al.* 2006), and osteoblasts (Conover *et al.* 2004). Previous experiments in human coronary artery smooth muscle cells indicate that the effects of IL1 β and TNF α on PAPPa gene expression are at the level of transcription (Conover *et al.* 2008). PAPPa expression in human preadipocytes was not stimulated by IL6. There appears to be cell-specific regulation of PAPPa expression by IL6. PAPPa expression was upregulated by IL6 in vascular smooth muscle cells (Boldt & Conover 2007), but not in vascular endothelial cells or osteoblasts (Conover *et al.* 2004, 2008). This is not due to the lack of receptor activation, as the IL6 soluble receptor was added. IL6 exhibits both pro- and anti-inflammatory properties.

To determine the intracellular signaling pathways mediating cytokine-induced PAPPa expression, we focused on pathways that are commonly stimulated by cytokines in other cell types (Landry & Huot 1995, Waskiewicz & Cooper 1995, Duronio *et al.* 1998, Young 1998, Li & Karin 1999, Schaeffer & Weber 1999). PAPPa upregulation was mediated through pathways typically associated with stress, including the NF κ B, p38, and JNK pathways. TNF α was not as effective as IL1 β in stimulating the JNK pathway, suggesting differing effects of these cytokines in adipose tissue. However, our data using pathway inhibitors (BAY11-7082, SB203580, and SP600125) do not rule out interactive pathways, as there are issues with specificity (Bain *et al.* 2007). It is known that the NF κ B pathway is not activated in isolation during a stress response, and activation of the p38 and JNK pathways may occur simultaneously (Herlaar & Brown 1999).

Resveratrol potently inhibits PAPPa expression in human preadipocytes, independent of its known effects on SIRT1 and AMPK activation

Resveratrol is associated with positive metabolic effects through modulation of cell signaling pathways and has been shown to inhibit PAPPa expression in vascular smooth muscle cells (Conover *et al.* 2006). Many of the beneficial effects of resveratrol are thought to occur through activation of SIRT1 and/or AMPK. Activation of

these pathways has been shown to protect mice against diet-induced obesity and insulin resistance leading to clinical interest in specific SIRT1 analogs (Lagouge *et al.* 2006). However, not all data convincingly show that action of resveratrol occurs through the sirtuins alone (Baur *et al.* 2012). Based on our studies using SIRT1 inhibitors, downregulation of PAPPa expression by resveratrol does not seem to be mediated through the activation of SIRT1. Metabolic benefits of resveratrol may also depend on the activation of alternative pathways, as mice with tamoxifen-induced deletion of *Sirt1* still show a beneficial glucose response to resveratrol (Price *et al.* 2012). Another target of resveratrol is to inhibit the c-AMP-specific phosphodiesterases. Rolipram, a phosphodiesterase inhibitor that leads to activation of AMPK and SIRT1, is thought to mimic resveratrol in that it can reproduce its favorable metabolic effects in mice (Park *et al.* 2012). In our studies, rolipram did not mimic the effect of resveratrol on PAPPa. Thus, resveratrol not only downregulates PAPPa expression in human preadipocytes under basal conditions, but it is also able to overcome upregulation of PAPPa induced by cytokines IL1 β and TNF α . The effect of resveratrol to inhibit TNF α - or IL1 β -induced PAPPa expression is unlikely to explain the effect on basal PAPPa expression, as there was no detectable TNF α or IL1 β in the media under these conditions. This striking effect of resveratrol on PAPPa expression suggests that downregulation of PAPPa may be an additional mechanism by which resveratrol exerts its beneficial effects on metabolic profiles. The mechanism behind the ability of resveratrol to downregulate PAPPa remains to be elucidated, but possibilities include inhibition of NF κ B activation and attenuation of cell senescence (Holmes-McNary & Baldsin 2000).

Possible role of PAPPa in depot-specific adipogenesis

PAPPa proteolyzes inhibitory binding proteins, particularly IGFBP4 (see Fig.7), allowing increased pericellular IGF availability for activation of the IGF receptor (Boldt & Conover 2007). Therefore, we would expect upregulation of PAPPa to increase local IGF-stimulated differentiation, proliferation, and survival. However, preadipocytes from human omental fat have increased PAPPa but are relatively IGF resistant in terms of proliferation and differentiation (Caserta *et al.* 2001). Our study did not address the specific role of preadipocyte-derived PAPPa in adipogenesis. However, we speculate that PAPPa is secreted by the preadipocytes or endothelial cells in the stromal vascular fraction (PAPPa is not secreted by

macrophages (Conover *et al.* 2007)) with subsequent paracrine effects on stromal vascular cells as well as mature adipocytes. This is similar to a model proposed for PAPPa in atherosclerotic plaque progression (Conover 2010). A recent study on mice has suggested a novel interactive *in vivo* setting, whereby PAPPa regulates visceral adipose tissue response to insulin (Conover *et al.* 2013).

Conclusion

PAPPa is highly expressed in preadipocytes from omental fat depots. PAPPa expression in human preadipocytes is upregulated by proinflammatory cytokines, particularly IL1 β and TNF α . This upregulation is mediated through pathways commonly associated with stress, including the JNK, p38, and NF κ B pathways. PAPPa mRNA and protein expression is downregulated by resveratrol that has protective metabolic effects. This downregulation by resveratrol appears to be independent of SIRT1 and AMPK. Thus, reduction in PAPPa expression may be another mechanism responsible for the beneficial effects of resveratrol. Further evaluation of the specific role of PAPPa in depot-specific adipogenesis is needed, as these findings in human preadipocytes in conjunction with the work on mice (Conover *et al.* 2013) suggest the potential for PAPPa to be a therapeutic target in the treatment of visceral obesity.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This work was supported by the NIH grant AG028141 (to C A C) and the Minnesota Obesity Center Grant DK50456.

Author contribution statement

C D-P: designed and performed the experiments, analyzed the data, and wrote the manuscript. C J E: contributed to the immunoblotting experiments. C A C: helped with the design of the experiments, discussed the data, and reviewed the manuscript.

Acknowledgements

The authors thank Dr James Kirkland and Nino Giorgadze for providing the human preadipocyte cultures and Dr Michael Jensen for providing the human fat samples. All samples were collected under IRB-approved protocols.

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Received in final form 14 April 2014

Accepted 22 April 2014

Accepted Preprint published online 29 April 2014