The anti-adipogenic effect of angiotensin II on human preadipose cells involves ERK_{1,2} activation and PPAR γ phosphorylation

Paula Fuentes, María José Acuña, Mariana Cifuentes and Cecilia V Rojas

Institute of Nutrition and Food Technology, Universidad de Chile, Casilla 138-11, Santiago 6650606, Chile

(Correspondence should be addressed to C V Rojas; Email: crojas@inta.cl)

Abstract

Despite the importance of adipocyte formation for adipose tissue physiology, current knowledge about the mechanisms that regulate the recruitment of progenitor cells to undergo adipogenic differentiation is limited. A role for locally generated angiotensin II emerged from studies with human and murine cells. Preadipose cells from different human fat depots show reduced response to adipogenic stimuli when exposed to angiotensin II. This investigation sought to gain an insight into the intracellular mechanisms involved in the anti-adipogenic response of human preadipose cells from omental fat to angiotensin II. Its effect was evaluated on cells stimulated to adipogenic differentiation *in vitro*, by assessment of glycerol-3-phosphate dehydrogenase activity and expression of early markers of adipogenesis. Extracellular signal-regulated kinase_{1,2} (ERK_{1,2}) pathway activation was inferred from the phosphorylated to total ERK_{1,2} ratio determined by western blot. Exposure to angiotensin II

throughout the 10-day differentiation period resulted in a reduced adipogenic response. A similar anti-adipogenic effect was observed when this hormone was present during the first 48 h of induction to differentiation. Angiotensin II treatment had no consequences on CCAAT/enhancer-binding protein β and peroxisome proliferator-activated receptor γ (PPAR γ) induction, but increased the phosphorylated form of the key adipogenic regulator PPAR γ . Upon angiotensin II exposure, a raise of phosphorylated ERK_{1,2} was determined, which was more prominent 8–20 h after induction of adipogenesis (when controls reached negligible values). Chemical inhibition of ERK_{1,2} phosphorylation prevented angiotensin II-dependent reduction in adipogenesis. These results support the participation of the mitogen-activated protein kinase/ERK_{1,2} pathway in the anti-adipogenic effect of angiotensin II on preadipose cells from human omental adipose tissue.

Journal of Endocrinology (2010) **206**, 75–83

Introduction

New adipocyte formation (adipogenesis) from precursor cells residing within the adipose tissue appears to be tightly regulated by positive and negative effectors that are delivered via circulation or locally generated. Classical studies based on 3T3-L1 and 3T3-F442 cells unveiled a complex differentiation program, which involves coordinate expression of genes encoding for master regulators of adipogenesis, such as peroxisome proliferator-activated receptor γ (PPAR γ) and members of the CCAAT/enhancer-binding protein (C/EBP or CEBPA as listed in the HUGO Database) family of transcription factors. These proteins participate in a transcriptional cascade that controls the expression of a number of gene products with essential functions in the mature lipogenic and insulin-sensitive adipocyte (Rangwala & Mitchell 2000, Rosen & Spiegelman 2000). Molecules that serve as positive signals for adipogenesis have been subject of extensive characterization (Gregoire *et al.* 1998, Rosen & Spiegelman 2000, Farmer 2006). On the contrary, signals which operate as negative regulators that preclude new adipocyte formation are less understood.

A functional renin–angiotensin system (RAS) is expressed in adipose tissue (Thatcher *et al.* 2009). Early studies reported that angiotensin II stimulated triglyceride accumulation in the 3T3-L1 cell line, promoting cell hypertrophy (Jones *et al.* 1997). A role for angiotensin II in the control of new fat cell recruitment emerged from studies carried on angiotensinogen-deficient mice. Overexpression in adipose tissue of the gene for angiotensinogen led to adipocyte hypertrophy (Yvan-Charvet *et al.* 2009) and also, to a significant reduction in adipose cell number in these mice (Massiéra *et al.* 2001). Gene knockout and pharmacological studies have collectively supported a role for angiotensin II in regulating adipose tissue mass and function. In effect, rodents with genetic deficiency of RAS components involved in angiotensin II production or intracellular signaling show reduced body fat and improvement of those metabolic parameters commonly linked with obesity (Massiéra *et al.* 2001, Kouyama *et al.* 2005, Yvan-Charvet *et al.* 2009). Furthermore, inhibition of the RAS by prolonged administration of angiotensin-converting enzyme (ACE) inhibitors, angiotensin II receptor blockers, or a renin inhibitor to several murine models of obesity and diabetes

also produces adipose tissue mass reduction, restitution of the normal adipokine profile, and recovery of glucose and lipid metabolism indicators (Mathai *et al.* 2008, Kudo *et al.* 2009, Santos *et al.* 2009, Stucchi *et al.* 2009, Weisinger *et al.* 2009). These features associate with increased abundance of small functional adipocytes in visceral adipose tissue (Furuhashi *et al.* 2004, Lee *et al.* 2008) and expression of molecular markers of adipogenesis (Tomono *et al.* 2008) in treated animals. In agreement with these findings, different research groups, including our own, have shown that angiotensin II is able to reduce *in vitro* adipogenic differentiation of precursor cells from human fat depots (Schling & Löffler 2001, Janke *et al.* 2002, Brücher *et al.* 2007). Thus, a large body of literature suggests that angiotensin II is a negative regulator for new adipocyte formation; however, the mechanism for the anti-adipogenic effect remains to be understood.

Angiotensin II type 1 and type 2 receptors are members of the seven-transmembrane domain class of receptors, whose activation is typically transduced via heterotrimeric G proteins and downstream second messenger molecules (Rockman *et al.* 2002). However, over the years, it has become apparent that angiotensin II can also turn on the mitogen-activated protein kinases (MAPKs)/extracellular signal-regulated kinases (ERKs; Lefkowitz & Shenoy 2005, Zhai *et al.* 2005). As a consequence of upstream activation of the MAPK/ERK pathway, the dual-specificity kinase MEK1 phosphorylates ERK₁ and ERK₂. Typically, activated ERK_{1,2} kinases translocate into the nucleus to regulate the activity of diverse transcription factors involved in growth and differentiation processes. Participation of MAPK/ERK_{1,2} signaling in adipocyte differentiation has been demonstrated in murine cell line models by using chemical MEK1 inhibitors (Tang *et al.* 2003), and *Erk* gene knockout in mice (Bost *et al.* 2005a,b). The collective evidence from studies in the 3T3-L1 cell line reveals that adipogenesis is positively or negatively regulated by the MEK/ERK_{1,2} pathway, depending on the time of its activation. Shortly, after exposure to the adipogenic inductor, ERK_{1,2} activation promotes differentiation (Tang *et al.* 2003); whereas, their delayed activation causes inhibition of adipogenesis in this cell line. In the latter case, the underlying mechanism appears to involve phosphorylation of PPAR γ and, consequently, inactivation of this key adipogenic transcription factor (Hu *et al.* 1996, Chan *et al.* 2001, Tanabe *et al.* 2004). At present, it is not clear whether ERK_{1,2} activation plays a similar role on adipogenic differentiation of human adipocyte precursor cells.

Given that ERK_{1,2} activation is reportedly involved in the inhibition of adipogenesis by distinct molecules (Bhattacharya & Ullrich 2006, Constant *et al.* 2008), this work was aimed to investigate the implication of the MEK1/ERK_{1,2} pathway in the anti-adipogenic response of human preadipose cells from omental fat to angiotensin II.

Materials and Methods

Isolation and culture of preadipose cells

Human omental fat was obtained from nonobese subjects that underwent elective abdominal surgery. The protocol was approved by the Institutional Review Board at INTA, University of Chile, and informed consent was signed by the donors. Fat tissue was transported to the laboratory in sterile saline solution and processed promptly after collection. Adipose tissue was minced into 2–3 mm² pieces in Hanks' balanced salt solution (HBSS), after removal of all visible connective tissues, blood clots, and vessels. Preadipose cells were isolated using a method based on Zuk's (Zuk *et al.* 2002). Briefly, adipose tissue was dissociated with 1 g/l collagenase type I (Worthington Biochemical Corp., Lakewood, NJ, USA) in HBSS, with continuous mixing at 37 °C for 60 min. The cell suspension was filtered through a sterile gauze pad, and allowed to stand for a few minutes to aspirate and discard floating adipocytes. Cells were sedimented by centrifugation at 800 g for 10 min, resuspended in culture medium (DMEM/Ham's F12 (1:1), containing penicillin and streptomycin (Invitrogen Corp.)), supplemented with 10% v/v fetal bovine serum (FBS, Invitrogen Corp.), and grown on plastic culture dishes at 37 °C in a humidified atmosphere with 5% CO₂ until confluence was reached. Media were replaced every 3 days. Cultures at second passage were used for differentiation experiments. Cell count was determined under a light microscope using a hemocytometer.

Adipogenic differentiation

Adherent cells were seeded at 2.5 to 3.5 × 10⁴ cells/cm² in DMEM/Ham's F12 (1:1) supplemented with 10% v/v FBS. Before induction of adipogenic differentiation, cells were incubated overnight in DMEM/Ham's F12 without FBS (basal medium) to prevent confounding effects of mitogens. For long-term differentiation experiments (lasting up to 10 days), adipogenesis was induced with a mixture of effectors consisting of 1 × 10^{−6} M human insulin (Eli Lilly & Co., SA de CV, México), 2.5 × 10^{−7} M dexamethasone (Sigma), and 5 × 10^{−4} M 3-isobutyl-1-methylxanthine (IBMX, Sigma) in DMEM/Ham's F12 (1:1). When studying the effect of angiotensin II on ERK_{1,2} phosphorylation (experiments lasting up to 24 h), dexamethasone and IBMX were the only inducers included in the differentiation mixture to avoid MAPK/ERK pathway activation as the result of insulin or insulin-like growth factor 1 receptor signaling. Preadipose cell response to dexamethasone and IBMX alone was verified in long-term differentiation experiments. These cells exhibited typical cytoplasmic lipid droplets, but their size was smaller than those in cells stimulated with the complete adipogenic mixture. Moreover, the former also showed a slower time course of adipogenic differentiation measured by glycerol-3-phosphate dehydrogenase (G3PDH) activity (see below).

Angiotensin II was added 60 min prior to the differentiation mixture and maintained thereafter, except when specified. Angiotensin II concentration in the differentiation media was 1.5×10^{-5} M (to counteract its rapid decline due to high *in situ* degradation activity). The specific chemical MEK₁ inhibitor U0126 was used to address the participation of ERK_{1,2} activation in the response to angiotensin II. Because U0126 at concentrations above 2×10^{-6} M affected the survival of preadipose cells in long-term experiments, it was replaced by 1×10^{-5} M PD98059 in these adipogenic assays.

Assessment of adipogenesis by G3PDH activity

Adipogenic differentiation was evaluated by measuring the activity of G3PDH (Tchkonia *et al.* 2002), which catalyzes a rate-limiting reaction for triglyceride production in adipose tissue, given that this is the sole source of the glycerol-3-phosphate required for glycerol backbone synthesis in this tissue. Its use as an adipocyte marker is supported by the upregulation of its mRNA and activity in the course of the adipogenic differentiation (Moustaid *et al.* 1996, Rumberger *et al.* 2003). G3PDH activity in cell homogenates was measured according to Sottile & Seuwen (2001), by monitoring NADH oxidation at 340 nm in a microplate reader (EL-808, BioTek Instruments, Winooski, VT, USA) as described before (Brücher *et al.* 2007). To calculate G3PDH-specific activity, protein concentration in cell homogenates was measured according to the assay by Bradford (1976). G3PDH-specific activity in cells stimulated to adipogenic differentiation was expressed relative to the value determined in parallel cultures maintained in basal nonadipogenic medium. In order to compare the effect of angiotensin II on cells from different donors (with largely differing absolute enzymatic activity values), G3PDH-specific activity with angiotensin II was expressed as the percentage of the corresponding controls (in the absence of this hormone). No effect of angiotensin II was detected on the small basal G3PDH activity.

Western blot analysis

Cells were washed three times with cold HBSS and scraped with 15 µl of RIPA buffer (consisting of 50 mM Tris-HCl, 150 mM NaCl, 1% v/v Nonidet P-40, 0.5% w/v sodium deoxycholate, 0.21% w/v SDS, pH 8.0, supplemented with Complete protease inhibitors cocktail, Pepstatin A, and Phosphatase Inhibitor Cocktail Set III (Calbiochem-Merck)) per cm² of culture dish area. Cell lysates were centrifuged at 4 °C for 15 min at 5000 g, and supernatants were kept at -80 °C until analysis. Protein concentration was determined using the bicinchoninic acid method (Pierce, Thermo Scientific, Waltham, MA, USA). In total, 7–30 µg protein per sample were separated by 10% PAGE under denaturing conditions and transferred to 0.45 µm Immobilon-P polyvinylidene difluoride membranes

(Millipore Corp., Billerica, MA, USA). After protein transfer, nonspecific-binding sites on the membranes were blocked with BSA and probed with the following primary antibodies specific for phosphorylated ERK_{1,2}, C/EBPβ, PPARG2 (Cell Signaling Technology, Inc., Danvers, MA, USA), and phosphorylated PPARG (Upstate-Millipore Corp.), according to the manufacturers' instructions. Detection of the immune complexes was performed with appropriate HRP-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA, USA). Immunoreactivity was detected by enhanced chemiluminescence (Millipore Corp.) and exposure to light sensitive films (BioMax, Eastman Kodak Co). Intensity of specific bands was determined by the Digital Science Image Analysis Software (Eastman Kodak Co.) after digitalization of film images.

Data analysis

Data represent means $\pm$ S.E.M., and pair wise differences were analyzed by Student's *t*-test. One-way ANOVA was used for multiple treatment analysis, followed by Tukey's *post-hoc* comparison test. A probability (*P*) < 0.05 was considered significant. All experiments were performed in duplicate, and the number of samples from different donors (*n*) included in each case is indicated in figure legends.

Results

Angiotensin II reduces adipogenesis when provided to preadipose cells during the first 48 h of exposure to differentiation medium

The anti-adipogenic effect of angiotensin II was evidenced by decreased adipogenic marker activity in preadipose cell cultures that were maintained in differentiation conditions for 10 days. The G3PDH-specific activity was reduced $45.2 \pm 2.8\%$ with respect to the corresponding controls not exposed to the hormone (*n* = 13, *P* < 0.05) in cell cultures treated with 1.5×10^{-5} M angiotensin II throughout the assay period (not shown).

Given that the observed anti-adipogenic effect could arise from the mitogenic properties of angiotensin II, as reported for hematopoietic, cardiac, and vascular smooth muscle cells (McEwan *et al.* 1998, Rodgers *et al.* 2000, Min *et al.* 2005), this possibility was investigated in human preadipose cells. Despite their ability to proliferate in culture medium supplemented with 10% v/v FBS, preadipose cell cultures showed negligible growth (similar to vehicle treated cells) when exposed to angiotensin II, at concentrations used in the differentiation experiments (data not shown). Therefore, diminished adipogenic differentiation by angiotensin II is unlikely a consequence of stimulation of preadipose cell proliferation.

As shown in Fig. 1, G3PDH activity was reduced to a similar extent when angiotensin II was continuously present throughout the 10-day assay period or during the first 48 h after the addition of the adipogenic differentiation medium

Figure 1 Window of susceptibility to the anti-adipogenic effect of angiotensin II (All). Preadipose cell cultures were exposed to the adipogenic medium during 10 days with or without angiotensin II (All). When added, All was provided during a 48-h period beginning at days 0 or 3, and compared with exposure to the hormone during the entire experiment. G3PDH-specific activity, expressed as a percentage of the control maintained solely with adipogenic medium, was 44.3 ± 1.7 , 77.8 ± 3.8 , and 44.7 ± 4.1 after treatment with All during 0–2, 3–5, and 0–10 days respectively. Different letters denote significantly different values, $P < 0.01$, $n = 3$ (one-way ANOVA followed by Tukey's *post-hoc* test).

(44.7 ± 4.1 and $44.3 \pm 1.7\%$ of controls without this hormone respectively). In contrast, an attenuated effect was observed when angiotensin II was provided for the same period (48 h), but 3 days post induction of adipogenesis. In the latter conditions, G3PDH activity was $77.8 \pm 3.8\%$ of the controls (in the absence of angiotensin II).

Angiotensin II effect on early adipogenic markers C/EBP β and PPARG

Angiotensin II appears to exert a larger anti-adipogenic effect when provided during the first 2 days that follow the stimulation of preadipose cell differentiation. Therefore, induction of the early adipogenic markers C/EBP β and PPARG were investigated. C/EBP β was barely detectable in preadipose cell cultures maintained under basal conditions. Increased expression of this protein was observed as early as 2 h after stimulation with adipogenic differentiation; however, C/EBP β induction was not affected by treatment with angiotensin II (Fig. 2A).

PPARG protein, which was measurable in preadipose cells under basal culture conditions, showed a two- to threefold increase after induction of adipogenesis, with a maximum 3.5 ± 1.1 -fold ($P < 0.01$) and 3.4 ± 1.2 -fold ($P < 0.05$) increase at day 2 in the absence or in the presence of angiotensin II respectively (Fig. 2B and C). Treatment with angiotensin II did not result in a significant change in the relative abundance of PPARG protein when compared with the controls maintained without this hormone (Fig. 2C).

Interestingly, phosphorylated PPARG increased in cell cultures exposed to angiotensin II throughout the differentiation assay (Fig. 2D). For example, in angiotensin II-treated cells, the ratio p-PPARG/PPARG increased 2.9 ± 0.2 -fold ($P = 0.01$) under basal culture conditions and 3.2 ± 0.1 -fold ($P = 0.03$) at day 9 of adipogenesis (versus the corresponding control without hormone).

Inhibition of MEK1 activity prevents the anti-adipogenic effect of angiotensin II

To gain an insight into the participation of the ERK_{1,2} pathway in the anti-adipogenic effect of angiotensin II, the outcome of ERK_{1,2} activation blockade with the MEK1 inhibitor PD98059 was explored. Preadipose cell cultures were maintained under adipogenic conditions in the presence of angiotensin II and treated with 1×10^{-5} M PD98059 throughout the 10-day differentiation period. In cells treated with angiotensin II, G3PDH-specific activity was $50.9 \pm 2.7\%$ of controls (kept in adipogenic medium); whereas, the specific activity of this adipogenic marker was significantly higher ($90.6 \pm 16.2\%$, $P < 0.01$) in those cells treated with angiotensin and PD98059. Thus, prevention of the anti-adipogenic effect of angiotensin II by PD98059 suggested the participation of MEK and ERK_{1,2} activities in this response to the hormone.

To further uphold ERK_{1,2} activation in response to angiotensin II, changes in phosphorylated ERK_{1,2} (p-ERK_{1,2}) were assessed by western blotting. In agreement with studies in the 3T3-L1 cell line (Bost *et al.* 2005a,b), p-ERK_{1,2} displayed a biphasic response in human preadipose cell cultures induced to adipogenic differentiation. P-ERK_{1,2} levels showed a transient increase (lasting 4–6 h), followed by a progressive decline (during 3–4 h), and reached a value below the basal level thereafter (Fig. 3A and B).

Supplementation of the adipogenic medium with angiotensin II led to an increase in p-ERK_{1,2} levels with respect to the controls without this hormone. Duration of angiotensin II treatment was assessed, and the largest response was observed after a 2-h exposure (not shown). Angiotensin II effect on p-ERK_{1,2} levels was evaluated at different time points after the stimulation of adipogenesis. As illustrated in Fig. 3C and D, a prominent increase in p-ERK_{1,2} levels by angiotensin II treatment was detected in the period comprising 8–20 h after the addition of the adipogenic medium. A twofold rise of the ratio p-ERK_{1,2}/ERK_{1,2} (2.4 ± 0.3 , $P = 0.002$, $n = 4$) was determined at 16-h post induction of adipogenesis, compared with the corresponding controls without angiotensin II (Fig. 3D). A fairly lower increase in p-ERK_{1,2}/ERK_{1,2} was found after 8 h (the ratio was 1.6 ± 0.2 -fold, $P = 0.02$, $n = 5$). A modest raise of p-ERK was apparent under basal conditions and early (1–6 h) after induction of adipogenesis, when control levels were high (data not shown).

Moreover, pre-exposure of cell cultures to the MEK1 inhibitor U0126 prevented the angiotensin II-dependent increase in p-ERK_{1,2} (Fig. 4). In cell cultures maintained in

the adipogenic medium supplemented with angiotensin II alone, the ratio p-ERK_{1,2}/ERK_{1,2} (normalized with respect to the value determined solely in adipogenic medium) was 1.75 ± 0.24 ; whereas, in the presence of U0126, the value was 0.07 ± 0.06 ($P < 0.001$, $n = 5$). Under the same conditions, exposure of cells to adipogenic medium supplemented with U0126 also reduced p-ERK_{1,2} to barely detectable levels (data not shown).

Discussion

In line with previously reported findings (Brücher *et al.* 2007), in the present study, we observed that adipocyte precursor cells isolated from the omental adipose tissue of nonobese

human subjects showed reduced adipogenic response when stimulated to differentiation in the presence of angiotensin II. The adipocyte marker enzyme G3PDH showed $45.2 \pm 2.8\%$ reduction in specific activity ($P < 0.05$, $n = 13$) in angiotensin II-treated cells with respect to the controls maintained without the hormone. The extent of the reduction in adipogenic differentiation was similar when cell cultures were treated with angiotensin II throughout the experiment or during the first 2 days of exposure to the differentiation mixture. In contrast, a weaker response was found when cells were treated with angiotensin II after the third day in adipogenic conditions (Fig. 1). A similar critical period of sensitivity to unidentified negative effectors of adipogenesis that were present in macrophage-conditioned medium was reported in the 3T3-L1 cell line (Constant *et al.* 2008).

Given that the largest response to angiotensin II was observed shortly after the start of adipogenesis, we investigated angiotensin II effect on the induction of the transcription factors *C/EBPβ* and *PPARG*, which play a central role at the beginning of the adipogenic differentiation program. In 3T3-L1 cells, expression of the *C/EBPβ* protein is rapidly induced during the first hours after the stimulation of differentiation (Christy *et al.* 1991, Lane *et al.* 1999), then it is activated by sequential phosphorylation (Tang *et al.* 2005) and imported into the nucleus. Activated *C/EBPβ* directly triggers transcriptional expression of the *PPARG* gene (Salma *et al.* 2004, Tang *et al.* 2005), and indirectly promotes the expression of *C/EBPα* (Zuo *et al.* 2006). In our study with preadipose cell cultures from human omental fat, increased expression of the *C/EBPβ* protein was first detected after 2–4 h of exposure to dexamethasone and IBMX, both in the

Figure 2 *C/EBPβ*, *PPARG* and p-*PPARG* in angiotensin II-treated preadipose cells. Preadipose cell cultures induced to adipogenic differentiation in the presence or in the absence of angiotensin II (All), which was added 1 h before the adipogenic stimuli and maintained until cell lysis. Western blot analysis of *C/EBPβ*, *PPARG*, p-*PPARG* and β-actin was carried out in cell extracts obtained at indicated periods. (A) Representative immunodetection of *C/EBPβ* and β-actin in extracts from cells maintained under adipogenic conditions for 0, 3, or 18 h in the presence (+) or in the absence (–) of All. Top and bottom panels show *C/EBPβ* induction in preadipose cell cultures from two donors. The *C/EBPβ*/β-actin ratio after 3 and 18 h in adipogenic conditions with angiotensin II was 0.82 ± 0.24 - and 1.05 ± 0.07 -fold the corresponding control (without the hormone) respectively. (B) Illustrative western blot analyses of *PPARG* and β-actin during the differentiation assay of cells solely with adipogenic medium (top panel), and in a different cell culture maintained for 4, 8, and 11 days in the presence (+) or in the absence (–) of All (bottom panel). (C) Relative abundance of *PPARG* at indicated time points after stimulation of adipogenic differentiation in the presence (empty triangles) or in the absence (filled squares) of All. The *PPARG*/β-actin ratio at each time point was determined in independent experiments and normalized to the basal level. Points are the means $\pm$ standard error of measurements in different cell cultures from 3 to 7 subjects. (D) Representative western blot analysis of p-*PPARG* compared with total *PPARG* and β-actin in extracts from cells maintained under basal or adipogenic culture medium or during 8 or 11 days in the presence (+) or in the absence (–) of All.

Figure 3 Angiotensin II increases p-ERK_{1,2}. Immunodetection of phosphorylated and total ERK_{1,2} in extracts from cells stimulated to adipogenic differentiation up to 24 h. (A) Illustrative western blot analysis of p-ERK_{1,2} and ERK_{1,2} at different time points (0, 2, 4, 6, 9, 20, and 24 h) after stimulation of adipogenic differentiation. Top and bottom panel images were obtained after different periods of exposure to the film to compensate for differences in signal intensities. For comparison purposes, the sample corresponding to time zero was included in both panels. (B) The ratio p-ERK_{1,2} to total ERK_{1,2} proteins, at each time point after the addition of the adipogenic medium, was determined in independent experiments and normalized to the corresponding basal level. Each point represents the mean $\pm$ standard error of measurements in cell cultures from 3 to 7 adipose tissue samples. Distinct letters denote statistically different values ($P < 0.05$). (C) Western blot images illustrate an increase in p-ERK_{1,2} by treatment with angiotensin II (All) in cell cultures maintained for 8 and 20 h in adipogenic medium. The symbols + and – denote the presence or the absence of All respectively. To compensate for differences in p-ERK_{1,2} signal intensities, the image corresponding to the bottom panel (20 h) was obtained after a longer exposure to the film than the one in the top panel (8 h). (D) The p-ERK_{1,2}/ERK_{1,2} ratio was determined from western blot analyses of cell cultures maintained for 16 h in adipogenic medium and supplemented with All during the last 3 h. After 16 h under adipogenic conditions, the ratios normalized to the basal value were 0.5 ± 0.04 and 1.2 ± 0.09 without and with All respectively (* $P < 0.002$, $n = 4$).

presence or in the absence of angiotensin II (Fig. 2A). Thus, indicating that C/EBP β induction was not prevented by pretreatment of the cells with angiotensin II.

In agreement with a previous report (Tchkonina *et al.* 2002), we found that PPARG was present in preadipose cells from omental fat maintained under basal culture conditions. Upon stimulation of adipogenesis, a 3.5 ± 1.1 -fold ($P < 0.01$) increase in PPARG protein was detected. Supplementation of the differentiation medium with angiotensin II did not result in a significant change in the relative abundance of PPARG when compared with the controls maintained in the absence of this hormone (Fig. 2C). These results were consistent with a small reduction in relative PPARG2 mRNA level (0.73 ± 0.04 ,

$P < 0.001$, $n = 3$) found in preadipose cells maintained for 4 days in adipogenic medium supplemented with angiotensin II, normalized with respect to those in the absence of the hormone (data not shown). Therefore, it seems unlikely that the anti-adipogenic effect of angiotensin II involves a major change in PPARG protein abundance. This is in agreement with previous observations in 3T3-L1 cells, in which a moderate reduction (29%) of PPARG was reported after treatment with a macrophage-conditioned medium that caused 59% inhibition of differentiation (Constant *et al.* 2008). Besides, it is known that PPARG function is modulated by posttranslational modifications, such as phosphorylation at a MAPK consensus sequence within the amino-terminal domain that results in reduction of its activity as a transcriptional factor, irrespective of ligand binding (van Beekum *et al.* 2009). Interestingly, our observations show an increase in phosphorylated PPARG upon exposure of omental preadipose cells to angiotensin II. The transcriptional consequences of increased levels of phosphorylated PPARG remain to be investigated in these cells.

As mentioned above, studies conducted in murine cell line models have shown that activated ERK_{1,2} regulate key

Figure 4 MEK inhibitor U0126 abolishes the angiotensin II-dependent increase in p-ERK_{1,2}. Phosphorylated ERK_{1,2} levels were determined in preadipose cell cultures maintained in adipogenic medium during 8 h and supplemented with angiotensin II for two additional hours. When indicated, 1×10^{-5} M U0126 was provided 1 h prior to angiotensin II addition. The p-ERK_{1,2}/ERK_{1,2} ratio was normalized with respect to the value measured after 10 h solely in adipogenic conditions. Top panel: illustrative western blot analysis of phosphorylated and total ERK_{1,2} under the experimental conditions that are indicated above. Immunodetection of β -actin was also included. * Denotes value significantly different from that without inhibitor, $P < 0.001$, $n = 5$.

components of the transcriptional machinery that underlie adipocyte differentiation process (Bost *et al.* 2005a,b), and exert stimulatory or inhibitory effects depending on the timing of activation. In agreement, chemical inhibitors of ERK_{1,2} activation decreased adipogenic differentiation when cells were treated shortly after the stimulation of adipogenesis (Prusty *et al.* 2002). Conversely, delayed inhibition of ERK_{1,2} activity enhanced adipogenesis because it prevented phosphorylation of PPAR γ , thus averting inactivation of this master regulator of adipogenic differentiation (Hu *et al.* 1996, Camp & Tafuri 1997, Hosooka *et al.* 2008). Hence, sustained ERK_{1,2} activation appears to inhibit new adipocyte formation (Prusty *et al.* 2002), at least in part, via PPAR γ phosphorylation.

In the present work, we found that pre-exposure of human preadipose cell cultures to the MEK1 inhibitor PD98059 precluded the reduction in adipogenesis elicited by angiotensin II. This initial evidence for MAPK/ERK pathway participation in the anti-adipogenic response to angiotensin II prompted us to analyze the p-ERK_{1,2} status. In agreement with prior findings in the 3T3-L1 cell line, a biphasic change of phosphorylated ERK_{1,2} was found in human preadipose cells stimulated to adipogenic differentiation; however, some differences were noticed. In contrast with negligible basal p-ERK_{1,2} levels in 3T3-L1 cells (Prusty *et al.* 2002, Bhattacharya & Ullrich 2006, Li *et al.* 2007), a substantial proportion of phosphorylated ERK_{1,2} was detected in human preadipose cells before stimulation of adipogenic differentiation. Upon addition of the differentiation mixture, phosphorylated ERK_{1,2} rapidly augmented. Levels above the basal value were sustained for ~5 h. It is worth to note that before supplementation with adipogenic inducers (dexamethasone and IBMX), primary cell cultures were FBS-deprived overnight to prevent the confounding effect of mitogens on MAPK/ERK pathway activity. The initial rise of p-ERK_{1,2} was followed by a progressive decline to a level below the basal value in a period that comprised 8–16 h post-induction (Fig. 3A and B), and remained barely detectable after 24 h. The results reported here showed that exposure to angiotensin II led to a raise of the p-ERK_{1,2}/ERK_{1,2} ratio, which was prominent after ~8 h of exposure to adipogenic conditions, when control levels below the basal value. Moreover, inhibition of MEK1 activity with U0126 prevented the angiotensin II-induced increase in p-ERK_{1,2} (Fig. 4). A similar response to other anti-adipogenic effectors was reported in 3T3-L1 cells. The vasoconstrictor peptide endothelin-1 caused the reduction in G3PDH activity and a sustained increase in p-ERK_{1,2} (Bhattacharya & Ullrich 2006). Likewise, high p-ERK_{1,2} levels were also associated with the anti-adipogenic effect of the alkaloid evodiamine (Wang *et al.* 2008) and macrophage-conditioned medium (Constant *et al.* 2008). Thus, it is apparent that increased phosphorylation of ERK_{1,2} is a common response to molecules, diverse in chemical nature, which cause attenuation of adipogenic differentiation. Our previous investigations showed that the anti-adipogenic effect of angiotensin II

was prevented by the AT1 receptor inhibitor losartan, but not by the AT2 receptor antagonist CGP-42112A (Brücher *et al.* 2007). Additional investigations will be needed to determine the involvement of AT1 receptors in MAPK/ERK_{1,2} pathway activation on preadipose cells from omental fat exposed to angiotensin II.

As discussed above, increasing evidence supports the participation of RAS in modulating preadipose cell conversion into adipocytes, and thus, influencing adipocyte number and adipose tissue functionality. According to a currently accepted view, formation of new adipocytes is part of the physiological response to excessive calorie intake (Danforth 2000); thus, newly differentiated adipocytes are important in maintaining a healthy adipose tissue. On the contrary, a reduced capacity to form new adipose cells would result in adipocyte hypertrophy, because limited adipose cells are available for triglyceride storage, if positive energy balance conditions prevail. Adverse metabolic effects are likely to arise from predominance of enlarged adipocytes, which exhibit an abnormal secretory profile and altered response to insulin, and other regulatory signals (Kashiwagi *et al.* 1985, Weyer *et al.* 2000). This appears to be particularly relevant for visceral adipose tissue, whose association with the adverse consequences of obesity is extensively acknowledged. Angiotensinogen expression is prominent in adipocytes from visceral fat of overweight individuals (Giacchetti *et al.* 2002, Rahmouni *et al.* 2004). In addition, angiotensin II exerts a larger anti-adipogenic effect on preadipose cells from obese humans (Brücher *et al.* 2007). Therefore, a better understanding of the intracellular mechanisms involved in reduced new adipocyte formation by local angiotensin II will help unravel its contribution to the accumulation of large dysfunctional adipocytes in visceral fat from obese subjects. This would raise the possibility to design strategies to ameliorate the adverse consequences of visceral adipose tissue expansion.

Our data show that angiotensin II-triggered attenuation of adipogenic differentiation in human preadipose cell cultures involves an increased ratio of phosphorylated ERK_{1,2} to ERK_{1,2} proteins. Our observations also suggest that angiotensin II causes an increase in phosphorylated PPAR γ , which would likely reduce adipogenic differentiation of preadipose cells. Further investigations will be required to elucidate whether other molecular targets are involved in the anti-adipogenic response to angiotensin II.

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 study was supported by the 'Fondo de Desarrollo Científico y Tecnológico' (1070632).

Acknowledgements

We are grateful to Dr Miguel Ángel Celis at Luis Tisé Hospital and Dr Leonardo Rodríguez at Clínica INDISA for providing adipose tissue, as well as to Ms Marisol Blanco for technical assistance. We thank Drs Alejandra Villavicencio and Marcela Reyes for critical reading of the manuscript.

References

- van Beekun O, Fleskens V & Kalkhoven E 2009 Posttranslational modifications of PPAR- γ : fine-tuning the metabolic master regulator. *Obesity* **17** 213–219.
- Bhattacharya I & Ullrich A 2006 Endothelin-1 inhibits adipogenesis: role of phosphorylation of Akt and ERK $_{1/2}$. *FEBS Letters* **580** 5765–5771.
- Bost F, Aouadi M, Caron L & Binétruy B 2005a The role of MAPKs in adipocyte differentiation and obesity. *Biochimie* **87** 51–56.
- Bost F, Aouadi M, Caron L, Even P, Belmonte N, Prot M, Dani C, Hofman P, Pagès G, Pouyssegur J *et al.* 2005b The extracellular signal-regulated kinase isoform ERK $_1$ is specifically required for *in vitro* and *in vivo* adipogenesis. *Diabetes* **54** 402–411.
- Bradford MM 1976 A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* **72** 248–254.
- Brücher R, Cifuentes M, Acuña MJ, Albala C & Rojas CV 2007 Larger anti-adipogenic effect of angiotensin II on omental preadipose cells of obese humans. *Obesity* **15** 1643–1646.
- Camp HS & Tafuri SR 1997 Regulation of peroxisome proliferator-activated receptor gamma activity by mitogen-activated protein kinase. *Journal of Biological Chemistry* **272** 10811–10816.
- Chan GK, Deckelbaum RA, Bolivar I, Goltzman D & Karaplis AC 2001 PTHrP inhibits adipocyte differentiation by down-regulating PPAR gamma activity via a MAPK-dependent pathway. *Endocrinology* **142** 4900–4909.
- Christy RJ, Kaestner KH, Geiman DE & Lane MD 1991 CCAAT/enhancer binding protein gene promoter: binding of nuclear factors during differentiation of 3T3-L1 preadipocytes. *PNAS* **88** 2593–2597.
- Constant VA, Gagnon AM, Yarmo M & Sorisky A 2008 The antiadipogenic effect of macrophage-conditioned medium depends on ERK $_{1/2}$ activation. *Metabolism* **5** 465–472.
- Danforth E 2000 Failure of adipocyte differentiation causes type II diabetes mellitus? *Nature Genetics* **26** 13.
- Farmer SR 2006 Transcriptional control of adipocyte formation. *Cell Metabolism* **4** 263–273.
- Furuhashi M, Ura N, Takizawa H, Yoshida D, Moniwa N, Murakami H, Higashiura K & Shimamoto K 2004 Blockade of the renin-angiotensin system decreases adipocyte size with improvement in insulin sensitivity. *Journal of Hypertension* **22** 1977–1982.
- Giacchetti G, Faloia E, Mariniello B, Sardù C, Gatti C, Camilloni MA, Guerrieri M & Mantero F 2002 Overexpression of the renin-angiotensin system in human visceral adipose tissue in normal and overweight subjects. *American Journal of Hypertension* **15** 381–388.
- Gregoire FM, Smas CM & Sul HS 1998 Understanding adipocyte differentiation. *Physiological Reviews* **78** 783–809.
- Hosooka T, Noguchi T, Kotani K, Nakamura T, Sakaue H, Inoue H, Ogawa W, Tobimatsu K, Takazawa K, Sakai M *et al.* 2008 Dok1 mediates high-fat diet-induced adipocyte hypertrophy and obesity through modulation of PPAR- γ phosphorylation. *Nature Medicine* **14** 188–193.
- Hu E, Kim JB, Sarraf P & Spiegelman BM 1996 Inhibition of adipogenesis through MAP kinase-mediated phosphorylation of PPARgamma. *Science* **274** 2100–2103.
- Janke J, Engeli S, Gorzelniak K, Luft FC & Sharma AM 2002 Mature adipocytes inhibit *in vitro* differentiation of human preadipocytes via angiotensin type 1 receptors. *Diabetes* **51** 1699–1707.
- Jones BH, Standridge MK & Moustaid N 1997 Angiotensin II increases lipogenesis in 3T3-L1 and human adipose cells. *Endocrinology* **138** 1512–1519.
- Kashiwagi A, Mott D, Bogardus C, Lillioja S, Reaven G & Foley J 1985 The effects of short-term overfeeding on adipocyte metabolism in Pima Indians. *Metabolism* **34** 364–370.
- Kouyama R, Suganami T, Nishida J, Tanaka M, Toyoda T, Kiso M, Chiwata T, Miyamoto Y, Yoshimasa Y, Fukamizu A *et al.* 2005 Attenuation of diet-induced weight gain and adiposity through increased energy expenditure in mice lacking angiotensin II type 1a receptor. *Endocrinology* **146** 3481–3489.
- Kudo H, Yata Y, Takahara T, Kawai K, Nakayama Y, Kanayama M, Oya T, Morita S, Sasahara M, Mann DA *et al.* 2009 Telmisartan attenuates progression of steatohepatitis in mice: role of hepatic macrophage infiltration and effects on adipose tissue. *Liver International* **29** 988–996.
- Lane MD, Tang QQ & Jiang MS 1999 Role of the CCAAT enhancer binding proteins (C/EBPs) in adipocyte differentiation. *Biochemical and Biophysical Research Communications* **266** 677–683.
- Lee MH, Song HK, Ko GJ, Kang YS, Han SY, Han KH, Kim HK, Han JY & Cha DR 2008 Angiotensin receptor blockers improve insulin resistance in type 2 diabetic rats by modulating adipose tissue. *Kidney International* **74** 890–900.
- Lefkowitz RJ & Shenoy SK 2005 Transduction of receptor signals by beta-arrestins. *Science* **308** 512–517.
- Li X, Kim JW, Grønborg M, Urlaub H, Lane MD & Tang QQ 2007 Role of cdk2 in the sequential phosphorylation/activation of C/EBPbeta during adipocyte differentiation. *PNAS* **104** 11597–11602.
- Massiera F, Bloch-Faure M, Ceiler D, Murakami K, Fukamizu A, Gasc JM, Quignard-Boulange A, Negrel R, Ailhaud G, Seydoux J *et al.* 2001 Adipose angiotensinogen is involved in adipose tissue growth and blood pressure regulation. *FASEB Journal* **15** 2727–2729.
- Mathai ML, Naik S, Sinclair AJ, Weisinger HS & Weisinger RS 2008 Selective reduction in body fat mass and plasma leptin induced by angiotensin-converting enzyme inhibition in rats. *International Journal of Obesity* **32** 1576–1578.
- McEwan PE, Gray GA, Sherry L, Webb DJ & Kenyon CJ 1998 Differential effects of angiotensin II on cardiac cell proliferation and intramyocardial perivascular fibrosis *in vivo*. *Circulation* **98** 2765–2773.
- Min LJ, Mogi M, Li JM, Iwanami J, Iwai M, Horiuchi M 2005 Aldosterone and angiotensin II synergistically induce mitogenic response in vascular smooth muscle cells. *Circulation Research* **97** 434–442.
- Moustaid N, Jones BH & Taylor JW 1996 Insulin increases lipogenic enzyme activity in human adipocytes in primary culture. *Journal of Nutrition* **126** 865–870.
- Prusty D, Park BH, Davis KE & Farmer SR 2002 Activation of MEK/ERK signaling promotes adipogenesis by enhancing peroxisome proliferator-activated receptor gamma (PPARgamma) and C/EBPalpha gene expression during the differentiation of 3T3-L1 preadipocytes. *Journal of Biological Chemistry* **277** 46226–46232.
- Rahmouni K, Mark AL, Haynes WG & Sigmund CD 2004 Adipose depot-specific modulation of angiotensinogen gene expression in diet-induced obesity. *American Journal of Physiology: Endocrinology and Metabolism* **286** E891–E895.
- Rangwala SM & Mitchell AL 2000 Transcriptional control of adipogenesis. *Annual Review of Nutrition* **20** 535–559.
- Rockman HA, Koch WJ & Lefkowitz RJ 2002 Seven-transmembrane-spanning receptors and heart function. *Nature* **415** 206–212.
- Rodgers KE, Xiong S, Steer R & diZerega GS 2000 Effect of angiotensin II on hematopoietic progenitor cell proliferation. *Stem Cells* **18** 287–294.
- Rosen ED & Spiegelman BM 2000 Molecular regulation of adipogenesis. *Annual Review of Cell and Developmental Biology* **16** 145–171.
- Rumberger JM, Wu T, Hering MA & Marshall S 2003 Role of hexosamine biosynthesis in glucose-mediated up-regulation of lipogenic enzyme mRNA levels: effects of glucose, glutamine, and glucosamine on glycerophosphate dehydrogenase, fatty acid synthase, and acetyl-CoA carboxylase mRNA levels. *Journal of Biological Chemistry* **278** 28547–28552.

- Salma N, Xiao H, Mueller E & Imbalzano AN 2004 Temporal recruitment of transcription factors and SWI/SNF chromatin-remodeling enzymes during adipogenic induction of the peroxisome proliferator-activated receptor gamma nuclear hormone receptor. *Molecular and Cellular Biology* **24** 4651–4663.
- Santos EL, de Picoli Souza K, da Silva ED, Batista EC, Martins PJ, D'Almeida V & Pesquero JB 2009 Long term treatment with ACE inhibitor enalapril decreases body weight gain and increases life span in rats. *Biochemical Pharmacology* **78** 951–958.
- Schling P & Löffler G 2001 Effects of angiotensin II on adipose conversion and expression of genes of the renin-angiotensin system in human preadipocytes. *Hormone and Metabolic Research* **33** 189–195.
- Sottile V & Seuwen K 2001 A high-capacity screen for adipogenic differentiation. *Analytical Biochemistry* **293** 124–128.
- Stucchi P, Cano V, Ruiz-Gayo M & Fernández-Alfonso MS 2009 Aliskiren reduces body-weight gain, adiposity and plasma leptin during diet-induced obesity. *British Journal of Pharmacology* **158** 771–778.
- Tanabe Y, Koga M, Saito M, Matsunaga Y & Nakayama K 2004 Inhibition of adipocyte differentiation by mechanical stretching through ERK-mediated downregulation of PPARgamma2. *Journal of Cell Science* **117** 3605–3614.
- Tang QQ, Otto TC & Lane MD 2003 Mitotic clonal expansion: a synchronous process required for adipogenesis. *PNAS* **100** 44–49.
- Tang QQ, Grønborg M, Huang H, Kim JW, Otto TC, Pandey A & Lane MD 2005 Sequential phosphorylation of CCAAT enhancer-binding protein beta by MAPK and glycogen synthase kinase 3beta is required for adipogenesis. *PNAS* **102** 9766–9771.
- Tchkonina T, Giorgadze N, Pirtskhalava T, Tchoukalova Y, Karagiannides I, Forse RA, DePonte M, Stevenson M, Guo W, Han J *et al.* 2002 Fat depot origin affects adipogenesis in primary cultured and cloned human preadipocytes. *American Journal of Physiology: Regulatory, Integrative and Comparative Physiology* **282** R1286–R1296.
- Thatcher S, Yiannikouris F, Gupte M & Cassis L 2009 The adipose renin-angiotensin system: role in cardiovascular disease. *Molecular and Cellular Endocrinology* **302** 111–117.
- Tomono Y, Iwai M, Inaba S, Mogi M & Horiuchi M 2008 Blockade of AT1 receptor improves adipocyte differentiation in atherosclerotic and diabetic models. *American Journal of Hypertension* **21** 206–212.
- Wang T, Wang Y, Kontani Y, Kobayashi Y, Sato Y, Mori N & Yamashita H 2008 Evodiamine improves diet-induced obesity in a uncoupling protein-1-independent manner: involvement of antiadipogenic mechanism and extracellularly regulated kinase/mitogen-activated protein kinase signaling. *Endocrinology* **149** 358–366.
- Weisinger RS, Stanley TK, Begg DP, Weisinger HS, Spark KJ & Jois M 2009 Angiotensin converting enzyme inhibition lowers body weight and improves glucose tolerance in C57BL/6J mice maintained on a high fat diet. *Physiology and Behavior* **98** 192–197.
- Weyer C, Foley JE, Bogardus C, Tataranni PA & Pratley RE 2000 Enlarged subcutaneous abdominal adipocyte size, but not obesity itself, predicts type II diabetes independent of insulin resistance. *Diabetologia* **43** 1498–1506.
- Yvan-Charvet L, Massiera F, Lamandé N, Ailhaud G, Teboul M, Moustaid-Moussa N, Gasc JM & Quignard-Boulangé A 2009 Deficiency of angiotensin type 2 receptor rescues obesity but not hypertension induced by overexpression of angiotensinogen in adipose tissue. *Endocrinology* **150** 1421–1428.
- Zhai P, Yamamoto M, Galeotti J, Liu J, Masurekar M, Thaisz J, Irie K, Holle E, Yu X, Kupersmidt S *et al.* 2005 Cardiac-specific overexpression of AT1 receptor mutant lacking G alpha q/G alpha i coupling causes hypertrophy and bradycardia in transgenic mice. *Journal of Clinical Investigation* **115** 3045–3056.
- Zuk PA, Zhu M, Ashjian P, De Ugarte DA, Huang JL, Mizuno H, Alfonso ZC, Fraser JK, Benhaim P & Hedrick MH 2002 Human adipose tissue is a source of multipotent stem cells. *Molecular Biology of the Cell* **13** 4279–4295.
- Zuo Y, Qiang L & Farmer SR 2006 Activation of CCAAT/enhancer-binding protein (C/EBP) alpha expression by C/EBP beta during adipogenesis requires a peroxisome proliferator-activated receptor-gamma-associated repression of HDAC1 at the C/ebp alpha gene promoter. *Journal of Biological Chemistry* **281** 7960–7967.

Received in final form 31 March 2010

Accepted 6 May 2010

Made available online as an Accepted Preprint
7 May 2010