Introduction

The renin-angiotensin system expressed in adipose tissue has been implicated in the modulation of adipocyte formation, glucose metabolism, triglyceride accumulation, lipolysis, and the onset of the adverse metabolic consequences of obesity. As we investigated angiotensin II signal transduction mechanisms in human preadipose cells, an interplay of extracellular-signal-regulated kinases 1 and 2 (ERK1,2) and Akt/PKB became evident. Angiotensin II caused attenuation of phosphorylated Akt (p-Akt), at serine 473; the p-Akt/Akt ratio decreased to 0.5 ± 0.2-fold the control value without angiotensin II (p < 0.001). Here we report that the reduction of phosphorylated Akt associates with ERK1,2 activities. In the absence of angiotensin II, inhibition of ERK1,2 activation with U0126 or PD98059 resulted in a 2.1 ± 0.5 (p < 0.001) and 1.4 ± 0.2-fold (p < 0.05) increase in the p-Akt/Akt ratio, respectively. In addition, partial knockdown of ERK protein expression by the short hairpin RNA technique also raised phosphorylated Akt in these cells (the p-Akt/Akt ratio was 1.5 ± 0.1-fold the corresponding control; p < 0.05). Furthermore, inhibition of ERK1,2 activation with U0126 prevented the reduction of p-Akt/Akt by angiotensin II. An analogous effect was found on the phosphorylation status of Akt downstream effectors, the forkhead box (Fox) proteins O1 and O4. Altogether, these results indicate that angiotensin II signaling in human preadipose cells involves an ERK1,2-dependent attenuation of Akt activity, whose impact on the biological functions under its regulation is not fully understood.

Abstract

The renin-angiotensin system expressed in adipose tissue has been implicated in the modulation of adipocyte formation, glucose metabolism, triglyceride accumulation, lipolysis, and the onset of the adverse metabolic consequences of obesity. As we investigated angiotensin II signal transduction mechanisms in human preadipose cells, an interplay of extracellular-signal-regulated kinases 1 and 2 (ERK1,2) and Akt/PKB became evident. Angiotensin II caused attenuation of phosphorylated Akt (p-Akt), at serine 473; the p-Akt/Akt ratio decreased to 0.5 ± 0.2-fold the control value without angiotensin II (p < 0.001). Here we report that the reduction of phosphorylated Akt associates with ERK1,2 activities. In the absence of angiotensin II, inhibition of ERK1,2 activation with U0126 or PD98059 resulted in a 2.1 ± 0.5 (p < 0.001) and 1.4 ± 0.2-fold (p < 0.05) increase in the p-Akt/Akt ratio, respectively. In addition, partial knockdown of ERK protein expression by the short hairpin RNA technique also raised phosphorylated Akt in these cells (the p-Akt/Akt ratio was 1.5 ± 0.1-fold the corresponding control; p < 0.05). Furthermore, inhibition of ERK1,2 activation with U0126 prevented the reduction of p-Akt/Akt by angiotensin II. An analogous effect was found on the phosphorylation status of Akt downstream effectors, the forkhead box (Fox) proteins O1 and O4. Altogether, these results indicate that angiotensin II signaling in human preadipose cells involves an ERK1,2-dependent attenuation of Akt activity, whose impact on the biological functions under its regulation is not fully understood.

Citation: Dünner N, Quezada C, Berndt FA, Cánovas J, Rojas CV (2013) Angiotensin II Signaling in Human Preadipose Cells: Participation of ERK1,2-Dependent Modulation of Akt. PLoS ONE 8(10): e75440. doi:10.1371/journal.pone.0075440

Received March 21, 2013; Accepted August 14, 2013; Published October 2, 2013

Copyright: © 2013 Dünner et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: This study was supported by CONICYT, FONDECYT/Regular 1100126. http://www.conicyt.cl/fondecyt/. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: crojas@inta.uchile.cl

© 2013 Dünner et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.
systems, in which opposite physiological responses are triggered after binding type 1 (AT1) or type 2 (AT2) angiotensin II receptors. AT1 and AT2 receptors appear to participate in modulating adipocyte formation and function in mice and rats [19,20]. Transcripts for both angiotensin II receptors have been detected in human visceral preadipose cells [21]. However, binding studies in preadipose cells and mature adipocytes from human adipose tissue only demonstrated presence of AT1 receptors [22,23]. In addition, the AT1 receptor blocker irbesartan but not the AT2 receptor antagonist PD123319 overturned the inhibition of adipogenic differentiation by angiotensin II in preadipose cells from breast subcutaneous adipose tissue [14]. In agreement, our prior investigations showed that the AT1 receptor inhibitor losartan prevented angiotensin II reduction of adipogenesis in preadipose cells from human omental fat [13]. The canonical signaling mode of activated AT1 receptors involves heteromeric G proteins, namely, Gα11 or Gαi [24,25]. However, angiotensin II signal transduction has proved to be far more complex than initially considered. Alternate AT1 receptor signaling pathways have also been described [25]. One of these signal transduction mechanisms involves interaction of AT1 receptors with β arrestin and G protein-coupled receptor kinases, which regulate the activity of mitogen-activated protein kinases (MAPK), specifically, ERK1 and ERK2. In addition, AT1 receptors are able to transactivate several members of the tyrosine kinase receptor family (such as PDGF, IGF-1 and EGF) in different cell types [26]. Phosphorylation of ERK1/2 by MAPK/ERK kinase 1 (MEK1) typically promotes translocation of these proteins into the nucleus where they regulate the activity of several transcription factors involved in growth and differentiation processes. Moreover, the presence of renin-angiotensin system components in intracellular compartments also associates with transcriptional regulation of gene expression [27,28].

Participation of MAPK/ERK1_2 signaling in the response of human preadipose cell cultures to angiotensin II was previously reported [29]. We observed that exposure to angiotensin II increased phosphorylation of ERK1_2 and the key adipogenic transcription factor PPAR-gamma. An unanticipated attenuation of Akt phosphorylation was also detected, suggesting a connection between ERK1_2 and Akt activation. Integration of ERK1_2 and Akt signaling pathways has been found in numerous cell types in response to diverse stimuli, including angiotensin II [30,31,32,33,34,35,36], and can result in either positive or negative modulation of the pathway activity, depending on the biological context. Likewise, a cross-talk between ERK1_2 and Akt appears to operate in preadipose cells. Diverse inhibitors of adipogenesis, such as the polyphenol endothelium 1 [37] and the alkaloid evodiamine [38] have been reported to increase phosphorylated ERK1_2 and concurrently decrease phosphorylated Akt levels. Furthermore, chemical inhibition of ERK1_2 phosphorylation restores Akt activation; thus supporting the convergence of these signaling transduction pathways in preadipose cells.

Given that the phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathway plays an important role in preadipocyte processes [39,40,41,42], we sought to investigate the consequences of ERK1_2 activation by angiotensin II on Akt phosphorylation/activation in adipocyte precursor cell cultures obtained from human adipose tissue. This report shows that angiotensin II signaling involves an ERK1_2-dependent attenuation of Akt activity and reduced phosphorylation of Akt downstream effectors Fox O1 and Fox O4 in these cells.

### Materials and Methods

#### Isolation and Culture of Preadipose Cells from Human Omental Adipose Tissue

Omental fat was obtained from 14 women that underwent elective abdominal surgery (gynecological procedures) at the Gynecology and Obstetrics Unit, Dr Luis Tiné Hospital. Institutional ethical committees at INTA, University of Chile, and Metropolitan Health Service, Santiago, Chile, approved the protocol. The donors signed informed consent. Women with malignancies or taking medications known to influence adipose tissue mass or metabolism were excluded. The mean body mass index of the donors was 34±5.7 Kg/m². Routine blood analyses showed biochemical parameters within the normal range. Tolerance to glucose and sensitivity to insulin were not tested. Determination of basal phosphorylated Akt levels in this study provided no indication of altered sensitivity to insulin in cell cultures from any adipose tissue donor (the mean pAkt/Akt ratio in the whole group was 0.41±0.13; ranging from 0.36±0.10 to 0.43±0.11). Moreover, no association between the basal pAkt/Akt ratio and the donors’ BMI was found.

Fresh adipose tissue samples were immediately transported to the laboratory and the stromal-vascular cells were obtained as reported before [29]. Adherent cells were seeded in Dulbecco’s modified Eagle’s medium (DMEM)/Ham’s F12 (1:1), containing penicillin, streptomycin (Invitrogen Corp, Carlsbad, CA), and supplemented with 10% fetal bovine serum (FBS, Hyclone, Thermo Scientific, South Logan, UT). Cells were grown on plastic culture dishes at 37°C in a humidified atmosphere with 5% CO2 until confluence was reached. Culture medium was replaced every 3 days. Expanded cultures were stored in liquid nitrogen for later use. Experiments were carried out after the second or third cell passage. Cell count was determined under a light microscope by means of a hemocytometer. To assess the phosphorylation status of proteins, cryopreserved stocks were seeded at 3.0 to 3.5x10⁴ cells/cm². Given the variable yield of preadipose cells from different adipose tissue donors, the number of adipose cell samples included in each experiment is indicated in the figure legend.

### SGBS Cell Culture

The Simpson-Golabi-Behmel syndrome (SGBS) preadipose cells [43], generously provided by Dr Martin Wabitsch (University of Ulm, Germany), were used to assess the effect of angiotensin II on the phosphorylation status of FoxO1 and FoxO4 proteins. This clonal cell strain, obtained from a newborn with Simpson-Golabi-Behmel syndrome, has been validated as a model for human adipose cell studies [44]. These non-immortalized cells have the ability to proliferate for many generations, and to differentiate into cells with phenotypic and functional properties of mature adipocytes from healthy human subjects, including sensitivity to insulin. Their response to angiotensin II in adipogenic differentiation assays is similar to that of preadipose cell cultures from human adipose tissue (unpublished results). SGBS preadipocytes were expanded in DMEM/Ham’s F12 (1:1) supplemented with 33x10⁻⁵ M biotin, 17x10⁻⁴ M sodium pantothenate, 0.1 mg/ml streptomycin, 100 U/ml penicillin, and 10% FBS.

#### Determination of the Phosphorylation Status of ERK1_2, Akt, FoxO1 and FoxO4 Proteins

The relative level of phosphorylated ERK1_2 and Akt was evaluated to assess the signaling status of the corresponding intracellular pathways. To avoid a misleading effect of FBS components on the pathways’ activity, cultures were kept

---

PLOS ONE | www.plosone.org 2 October 2013 | Volume 8 | Issue 10 | e75440

---

ERK-Akt Crosstalk in Angiotensin II Signaling
overnight with low (2%) FBS in DMEM/Ham’s F12 (1:1), and maintained in FBS-free culture medium for 60 min before starting the experiments designed to test the effect of hormones or inhibitors. Given that phosphorylated Akt was barely detectable in this condition, the outcome of cell exposure to angiotensin II (alone or in combination with MEK inhibitors) was evaluated in FBS-free culture medium supplemented with human insulin. Hereafter, the basal culture medium contains 1.0×10⁻⁶ M insulin in DMEM/Ham’s F12 (1:1).

When studying the effect of angiotensin II on the status of phosphorylated Akt and ERK1,2 under adipogenic conditions, adherent cells were also seeded at 3.0 to 3.5×10⁴ cells/cm². Before stimulation of adipogenic differentiation, cells were incubated overnight with 2% FBS in DMEM/Ham’s F12 (1:1). Adipogenesis was induced with 2.5×10⁻⁵ M dexamethasone (Sigma-Aldrich, St. Louis, MO, USA), and 5×10⁻⁹ M 3-isobutyl-1-methylxanthine (Sigma-Aldrich) in DMEM/Ham’s F12 (1:1).

The dose-response to angiotensin II in human preadipose cells was previously determined [29]. Angiotensin was used at a supraphysiological concentration in order to compensate for the high rate of angiotensin II withdrawal detected in these cell cultures [29]. Angiotensin II concentration in all experiments was 1.5×10⁻⁵ M. Two cell-permeable MEK inhibitors, which prevent ERK1,2 activation through different mechanisms, were used to test participation of ERK1,2 in the response to angiotensin II. The compound 1,4-diamino-2,3-dicyano-1,4-bis (2-aminothio) butadiene (U0126; Calbiochem EMD Biosciences Inc., San Diego, CA) directly inhibits MEK, whereas the flavone derivative 2’-amino-3’-methoxy flavone (PD98059; Calbiochem EMD Biosciences Inc.) prevents MEK phosphorylation/activation [46,47]. When indicated, culture medium was supplemented with 1×10⁻⁵ M U0126 or PD98059. The cell-permeable PI3K inhibitor 2-(4-morpholinyl)-8-phenyl-4H-1-benzo[4-one (Ly294002; Calbiochem EMD Biosciences Inc.) was used at 5×10⁻⁵ M. Inhibitors were added to the culture medium 60 min before angiotensin II, with dimethyl sulfoxide used as vehicle.

Cell lysates for Western blot analyses were prepared with 0.015 ml of RIPA buffer (consisting of 5×10⁻² M Tris-HCl, 15×10⁻² M NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.21% SDS, pH 8.0, supplemented with Complete protease inhibitors cocktail, Pepstatin A (Roche Applied Science, Mannheim, Germany) and Halt Phosphatase Inhibitor (Pierce, Thermo Scientific, Rockford, IL) per cm² cell dish area. Cell lysates were centrifuged at 4°C for 15 min at 5,000×g and supernatants were kept at −80°C until analyzed. Protein concentration was determined using the bichinchoninic acid method (Pierce, Thermo Fisher Scientific). Twenty to 60 micrograms of protein per sample were separated by 10% polyacrylamide gel electrophoresis under denaturing conditions and transferred to 0.45 µm Immobilon-P polyvinylidene difluoride membranes (Merck Millipore, Darmstadt, Germany). After protein transfer, nonspecific binding sites on the membranes were blocked with bovine serum albumin and probed with corresponding primary antibodies. Phosphorylated proteins were detected with antibodies (Cell Signaling Technology Inc, Danvers, MA) that recognize the specified phosphorylated amino acid residues in the corresponding target (ERK1 Thr202/Tyr204, ERK2 Thr185/Tyr187, Akt Ser473, FoxO1 Ser256 and FoxO4 Ser262). Antibodies raised against a different epitope to determine abundance of total (phosphorylated and non-phosphorylated) matching proteins were also from Cell Signaling Technology Inc. The level of phosphorylated FoxO1 and FoxO4 proteins was expressed relative to β-tubulin because we were unable to immunodetect total FoxO proteins when blots were re-probed. The antibody specific for β-tubulin was purchased from Cell Signaling Technology Inc. The immune complexes were evidenced with appropriate horseradish peroxidase-conjugated secondary antibodies (Jackson Immunoresearch Laboratories, West Grove, PA) and enhanced chemiluminescence (Merck Millipore) was detected with the FOTO/Analyst Luminary/FX workstation (Fotodyne Inc., Hartland, WI). Phosphorylated to total protein ratios were calculated from digital images analyzed with the Total Lab TL100 software (Fotodyne Inc.) and expressed relative to the corresponding control in each experiment.

Knockdown of ERK Expression by Posttranscriptional Gene Silencing

Lentiviral vectors carrying sequences for the expression of short hairpin RNAs (shRNA) designed to target ERK1 synthesis were generated. Based on mouse ERK1 short hairpin RNA [48], the following oligonucleotides were synthesized: 5’-GATCCCC-GACCGGATTTAGTAA _GCTTTTATCTCTTGAAAGA-3’ and 5’-AGCTTTTTCGAAAAAGACCGGATTTAGCTTTTATCTGTATCCTTGAAATAAGGTCTACATCCGGTCCGG-3’. The sequences in the human ERK1 gene (GenBank ID: NM002746) that created the stem of the shRNA are underlined. These oligonucleotides were annealed, and ligated into the lentiviral vector pUGW61 [49]. Viral stocks were prepared in 293T cells after cotransfection with the HIV-1 packaging vector pCMV-ΔR8.91 and the vector pVSVG-G that expresses the envelope VSVG-glycoprotein [50], using the calcium phosphate method [51]. Viral particles in cell culture supernatants were harvested from 16 up to 84 h after transfection, concentrated by filtration through Ultracel-100 membranes (ULTRA-15™ filter units, Merck Millipore), and titrated by transducing HEK 293T cells with serial dilutions of the viral preparation. The percentage of GFP positive cells was determined 72 hours after infection by FACS analysis. The typical yield was 10⁶ viral particles per ml.

Transduction of primary preadipose cells was performed in 24-wells culture plates containing 8×10⁴ cells per well. Lentiviral particles (5.4×10⁶ per well) were added in 0.25 ml of DMEM/Ham’s F12 (1:1) containing 10% SFB. Cells were incubated at 37°C in a humidified 5% CO₂ atmosphere and the culture medium was replaced 16 h after viral infection. Transfection efficiency, monitored by green fluorescent protein expression, ranged from 70 to 80% in different experiments. To allow full expression of silencing shRNAs, cells were maintained in culture for 9 to 10 days, with renewal of the medium every three days. As indicated above, cells were kept overnight with 2% FBS in DMEM/Ham’s F12 (1:1) and incubated in serum-free culture medium for 60 min before experiments were started.

Data Analysis

All experiments were performed in duplicate and the number of samples from different adipose tissue donors included (n) is indicated in each case. Mean and standard deviation were determined. Pair wise comparisons were validated by Student’s t-test. To compare the response to parallel interventions one factor ANOVA was used, and followed by Tukey’s post hoc multiple comparison test. A probability (p) <0.05 was considered significant.
Results

Angiotensin II Reduces Phosphorylated Akt

We had previously demonstrated an increase in phosphorylated ERK\textsubscript{1,2} when primary cultures of preadipose cells maintained in adipogenic differentiation medium were exposed to angiotensin II [29]. A concurrent decrease of phosphorylated Akt at serine 473 was initially noticed in this condition (Figure 1 A and B). Further analyses evidenced that the reduction of phosphorylated Akt after treatment with angiotensin II also occurred in the absence of adipogenic inducers, when cells were maintained in culture medium supplemented with fetal bovine serum or insulin. Hence, the following studies on the effect of angiotensin II on the phosphorylation status of ERK\textsubscript{1,2} and Akt were carried out in the presence of insulin. Under this experimental condition, angiotensin II reduced the relative abundance of phosphorylated Akt, as illustrated in figure 1 (C and D). The p-Akt/Akt ratio was 0.5±0.2-fold that determined in the corresponding controls without angiotensin II (p<0.001, n=8 donors).

Inhibition of ERK\textsubscript{1,2} Activation Increases Phosphorylated Akt

Chemical inhibition of ERK\textsubscript{1,2} activation supported a connection with Akt phosphorylation in these cells. When ERK\textsubscript{1,2} phosphorylation was prevented by treatment of cell cultures with U0126, a 2.1±0.5-fold rise in phosphorylated Akt was found (p<0.001; n=9 donors), as illustrated in figure 2A. It is worth noting that Akt phosphorylation, which is undetectable in cells maintained without insulin, became apparent after exposure to U0126 in the absence of this hormone (data not shown). To rule out any nonspecific effect of U0126, the MEK inhibitor PD98059 was assessed in parallel. In consonance, both inhibitors of ERK\textsubscript{1,2} activation reduced the fraction of phosphorylated ERK\textsubscript{1,2}, as shown in figure 2A. The p-ERK\textsubscript{1,2}/ERK\textsubscript{1,2} ratio was 0.47±0.18 and 0.05±0.04 relative to the control (vehicle) in cells that were incubated with 1×10\textsuperscript{-5} M PD980059 or 1×10\textsuperscript{-5} M U0126, respectively. As depicted in figure 2B, an increase in phosphorylated Akt was noticed in cells treated with both inhibitors. After exposure to 1×10\textsuperscript{-5} M PD980059 or 1×10\textsuperscript{-5} M U0126, the p-

Figure 1. Reduction of phosphorylated Akt by angiotensin II. (A) Representative Western blot analysis of phosphorylated Akt (p-Akt), total Akt, phosphorylated ERK\textsubscript{1,2} (p-ERK) and total ERK\textsubscript{1,2} in extracts from cells incubated 8 h in adipogenic medium without (-) or with (+) 1.5×10\textsuperscript{-5} M angiotensin II (AII) during the last 2 h. (B) p-Akt/Akt ratios determined from Western blots in the conditions indicated in A and expressed relative to the value in the corresponding controls maintained in adipogenic medium without angiotensin II. The mean pAkt/Akt for the controls in these experiments was 1.56±0.27 (n=5 donors). (C) The Western blots illustrate phosphorylated Akt (p-Akt), total Akt, phosphorylated ERK\textsubscript{1,2} (p-ERK) and total ERK\textsubscript{1,2} in extracts from cells maintained in basal culture conditions and treated without (-) or with (+) angiotensin II during 2 h. (D) The p-Akt/Akt ratios were determined from Western blots and normalized with respect to the corresponding controls in the absence of angiotensin II. The mean pAkt/Akt value for the control condition in these experiments was 0.43±0.11 (n=8 donors). Blots in part A were overexposed to enhance low chemoluminescent signals for phosphoproteins at this time point. The symbol * denotes p<0.05. doi:10.1371/journal.pone.0075440.g001
Akt/Akt ratio respectively was 1.4±0.2-fold (p<0.05) and 1.9±0.3-fold (p<0.01) the control value.

**ERK Knockdown Results in Increased Akt Phosphorylation**

To further uphold ERK1,2 involvement in the reduction of Akt phosphorylation, as suggested by the results with chemical inhibitors of ERK1,2 activation, we undertook the post transcriptional gene silencing approach with shRNA methodology. Given that only ERK1 appears to play an essential role in adipogenic differentiation [52], lentiviral vectors carrying the sequences for intracellular expression of shRNAs were designed to target ERK1 synthesis and thus to reduce the endogenous level of the ERK1 protein. Lower levels of ERK1 in primary cell cultures that were transduced with the lentivirus bearing the shRNA ERK1 construct were verified by Western blot analysis (Figure 3A). Nine days after infection, the ERK1/ERK2 ratio was 0.5±0.1 (range 0.4 to 0.7, p<0.0001, n = 5 donors) and the ERK1/β-actin ratio was 0.4±0.1-fold (range 0.2 to 0.6, p<0.0001, n = 5) with respect to control cells transduced with a scrambled sequence (no specific target). The reduced level of phosphorylated ERK1 in cells transduced with shRNA ERK1 was verified when expressed relative to β-actin. The p-ERK1/β-actin ratio was 0.5±0.1 (range 0.3 to 0.6, p = 0.004, n = 4 donors) versus the value in control cells transduced with a scrambled shRNA (shRNA scr), whereas the p-ERK1/ERK1 ratio was 1.4±0.2 the control value (range 1.2 to 1.6, p = 0.005, n = 4 donors).

The cells expressing shRNA ERK1 maintained under basal culture conditions showed an increased fraction of phosphorylated Akt, as illustrated in figure 3B. The p-Akt/β-actin ratio was 1.5±0.1-fold the value determined in control cells transduced with scrambled shRNA (Figure 3C). This effect of ERK1 protein knockdown on the status of Akt phosphorylation was compared with that of chemical inhibition of ERK activation. To this end, the p-Akt/Akt ratio was determined in parallel in cells from the same donor, but transduced with shRNA scr and incubated in basal culture medium with or without the inhibitor U0126, as exemplified in figure 3B. The p-Akt/β-actin ratio was 2.1±0.1 in cells with shRNA scr and treated with U0126 (Figure 3C). It is worth mentioning that exposure of the shRNA scr expressing cells to U0126 decreased the p-ERK/β-actin ratio to 0.3-fold the value in the absence of inhibitor; whereas the ratio was reduced to 0.5-fold in shRNA ERK1 expressing cells, as indicated above.

**Inhibition of ERK1,2 Activation Prevents Angiotensin II Attenuation of Akt, FoxO1 and FoxO4 Phosphorylation**

As mentioned above, the p-Akt/Akt ratio was reduced in primary preadipose cell cultures treated with angiotensin II. To test whether ERK1,2 activation is involved in attenuation of insulin-mediated phosphorylation of Akt by angiotensin II we made use of U0126, because the decrease of phosphorylated ERK achieved with this potent inhibitor was larger than that attained with the shRNA methodology. Pre-incubation with U0126 prevented a reduction of phosphorylated Akt by angiotensin II (Figure 4A). In those cells exposed to angiotensin II the p-Akt/Akt ratio was 0.5±0.2-fold the control value determined in the absence of this hormone. When angiotensin II was added to cells that had been pre-treated with 1×10⁻⁵ M U0126, the p-Akt/Akt ratio was 1.7±0.4-fold that determined in the corresponding controls, which is comparable to the increased value (2.2±0.5-fold) found after treatment solely with U0126 (Figure 4B). Analogous results were observed when the phosphorylation status of downstream Akt effectors, FoxO1 and FoxO4, was assessed after angiotensin II treatment under the experimental conditions indicated above. Because of limited availability of cells from adipose tissue donors, assessment of FoxO1 and FoxO4 phosphorylation was carried out with the SGBS cell strain, which show similar response to angiotensin II, with increased p-ERK1,2 (Figure 5A) and attenuated p-Akt levels (Figure 5B). Treatment of SGBS cells with angiotensin II resulted in decreased phosphorylation of FoxO1 (Figure 5C) and FoxO4 (Figure 5D), and this effect was averted by pre-treatment with U0126.

Taken together, these results indicate that reduced phosphorylation of Akt by angiotensin II depends on ERK1,2 activation. In contrast, inhibition of PI3K activity with LY294002, which decreased downstream Akt phosphorylation to 0.3±0.1-fold the corresponding control, had no effect on basal phosphorylated ERK1,2 levels. Moreover, the previously reported increase in the p-ERK1,2/ERK1,2 ratio by angiotensin II was not significantly modified by preincubation of the cells with 5×10⁻⁵ M LY294002.
Thus suggesting that PI3K activity is not involved in modulating ERK1,2 activity, under the experimental conditions here described.

Discussion

Increasing evidence supports the complexity of angiotensin signal transduction. In this context, this study was designed to investigate a connection between ERK1,2 and Akt transduction pathways in the angiotensin II signaling mechanism, in preadipose cells from human omental fat. As shown above, inhibition of ERK1,2 activity by two structurally unrelated compounds (U0126 and PD98059) caused a 1.4 to 2-fold increase in the fraction of phosphorylated Akt protein (Figure 2), suggesting that ERK1,2 activities elicit a negative control over insulin-dependent Akt phosphorylation. It is noteworthy that phosphorylated Akt, which is barely detectable in cells maintained without insulin or SFB, became measurable after chemical inhibition of ERK1,2 activation under this condition (data not shown). The magnitude of the phosphorylated-Akt increase seems to relate to the extent of

Figure 3. Increased phosphorylated Akt by ERK1 knockdown. (A) The Western blots illustrate ERK1, ERK2, and β-actin levels in cell lysates obtained 9 days after infection with lentiviral vectors carrying shRNA specific for ERK1 (shRNA ERK1) or a scrambled sequence (shRNA scr). (B) Representative Western blots of phosphorylated Akt (p-Akt), total Akt, phosphorylated ERK1,2 (p-ERK), total ERK1,2, and β-actin in cell cultures transduced either with ERK1 or scrambled shRNA constructs. When indicated (+), nine days after transduction with shRNA scr, cell cultures were exposed for 2 h to 1 × 10⁻⁵ M U0126 in basal medium. (C) The p-Akt/Akt ratios were determined from Western blots of cells transduced with shRNA scr (white bars) or shRNA ERK1 (black bar), and treated under the experimental conditions specified in part B, (n = 3 donors). The mean pAkt/Akt ratio in the control condition was 0.39±0.14. The symbols * and ** denote significantly different mean values, at p<0.05 and p<0.01, respectively (One-way ANOVA followed by Tukey’s post-hoc test).

doi:10.1371/journal.pone.0075440.g003

Figure 4. The inhibitor U0126 abolishes angiotensin II-dependent reduction of phosphorylated Akt. (A) Representative Western blot analysis of phosphorylated and total Akt in extracts from cells treated with 1 × 10⁻⁵ M U0126 for 60 min before exposure to angiotensin II (All) for additional 60 min. Immunodetection of phosphorylated and total ERK1,2 illustrates p-ERK1,2 decrease after treatment with U0126. β-actin abundance was included as reference. The symbols ± and – respectively denote presence or absence of All or U0126 in the culture medium. (B) The p-Akt/Akt ratios were normalized with respect to the value measured in control cells without All or U0126 (U). The mean pAkt/Akt value in the control condition was 0.36±0.10 (white bar). Different letters denote significantly different values, p<0.01; one-way ANOVA followed by Tukey’s post-hoc test; n = 4 donors.

doi:10.1371/journal.pone.0075440.g004
p-ERK \(_{1,2}\) reduction by U0126 or PD98059 (Figure 2). In addition, a diminished level of phosphorylated ERK, achieved by shRNA-mediated ERK\(_{1}\) knockdown, also caused a rise in the relative amount of phosphorylated Akt (Figure 3). Overall, these results endorse the hypothesis that the reduced level of activated ERK\(_{1,2}\) releases a negative control exerted on Akt phosphorylation/activation. This study did not elucidate the mechanism by which ERK\(_{1,2}\) down-regulates Akt kinase activity in these cells. Conceivably, activated ERK\(_{1,2}\) may control the activity of kinases or phosphatases that modulate Akt [32,33,36,52]. Otherwise, ERK\(_{1,2}\) activities may determine the association of Akt with adaptor or scaffolding proteins, thus affecting its interaction with other regulators of intracellular signal transduction [53,54].

As reported here, angiotensin II reduces insulin-dependent Akt phosphorylation at serine 473 and this effect is abolished by inhibition of ERK\(_{1,2}\) activation with U0126 (Figure 4). A coherent effect was revealed on typical downstream Akt effectors. Reduced phosphorylation of the transcription factors FoxO1 and FoxO4 was found after exposure to angiotensin II, and this effect was also prevented by treatment with U0126 (Figures 5C and D). The FoxO transcription factors, whose activity is regulated by protein-protein interactions and post-translational modifications (such as phosphorylation) have been implicated in diverse cellular processes, including glucose metabolism, adipocyte and muscle differentiation, oxidative stress response and inflammation [55]. Therefore, the outcome of reduced FoxO phosphorylation by angiotensin II in preadipose cells remains a matter of conjecture and deserve to be addressed in future investigations.

We had previously shown that angiotensin II treatment increased phosphorylated ERK\(_{1,2}\) [29]. Here we report that angiotensin II modulation of Akt phosphorylation in human preadipose cells appears to depend, at least in part, on ERK\(_{1,2}\) activities. In these cells, angiotensin II signaling resembles that of the antiadipogenic alkaloid evodiamine in the 3T3-L1 cell line [38]. In the latter evodiamine caused increased ERK phosphorylation and reduced insulin-stimulated phosphorylation of Akt, which were prevented by the MEK inhibitor PD98059. In addition, the authors report that upstream signals within the insulin/IGF-I pathway were not affected by evodiamine. Likewise, when bone marrow-derived mesenchymal stems cells were stimulated to adipogenic differentiation and exposed to high glucose, an ERK\(_{1,2}\)-dependent increase in Akt phosphorylation was reported [56]. In these experimental conditions the PI3K inhibitor LY294002 did not affect the high glucose induced increase in ERK\(_{1,2}\) phosphorylation. Similarly, we found no evidence of PI3K activation upstream of angiotensin II-dependent ERK\(_{1,2}\) phosphorylation in human preadipose cells (data not shown). In contrast, MAPK-ERK and PI3K-Akt pathway

Figure 5. The inhibitor U0126 abolishes angiotensin II-dependent reduction of phosphorylated FoxO1 and FoxO4. SGBS cells were treated with 1x10\(^{-5}\) M U0126 (U) for 30 min before addition of angiotensin II (All) and incubated in culture medium with insulin for additional 60 min. The phosphorylated level of ERK\(_{1,2}\) (A), Akt (B), FoxO1 (C) and FoxO4 (D) in cells treated with U plus All were compared with those determined in cells treated solely with All or U. The p-ERK\(_{1,2}/\text{ERK}\_1,2\) ratio and the p-Akt/Akt ratio in each experimental condition were expressed relative to the value measured in control cells without All or U. Mean p-ERK\(_{1,2}/\text{ERK}\_1,2\), p-Akt/Akt values in the control condition were 0.87±0.17 and 0.40±0.12, respectively (white bars in A and B). Due to technical limitations, the p-FoxO1 and p-FoxO4 levels were expressed relative to β-tubulin abundance and normalized versus the corresponding value in the control condition specified above. Mean p-FoxO1/β-tubulin and p-FoxO4/β-tubulin ratios in the control condition were 0.14±0.04 and 0.37±0.07, respectively (white bars in C and D). Different letters denote significantly different values, p<0.05, n = 4 (One-way ANOVA followed by Tukey’s post-hoc test).

doi:10.1371/journal.pone.0075440.g005
interactions appear to involve PI3K-dependent ERK$_{1,2}$ phosphorylation in other cell types [32,35,36,57,50]. We did not investigate other intracellular mechanisms reportedly involved in angiotensin II signal transduction in different cell types. Therefore, we cannot rule out their participation in the inhibition of Akt activation reported here.

In addition to the acknowledged functions of angiotensin II as a regulator of vascular and renal homeostasis, attention has been lately paid to its putative role in the onset of resistance to insulin. Clinical studies have shown that angiotensin-converting enzyme inhibitors as well as angiotensin II receptor blockers improve whole body insulin sensitivity [59]. In accordance, angiotensin II appears to modulate insulin sensitivity in diverse cell types, such as endothelial [31], hepatic [32] smooth and skeletal muscle [60,61,62]. Moreover, in a model of fructose-induced insulin-resistant rats, administration of the AT1 receptor antagonist candesartan cilexetil led to improvement of the defects in dorsal root ganglia neurons associated with impaired AT2 receptor function [63]. On this ground, it is tempting to speculate that reduced phosphorylation at serine 473 has also been reported in adipocytes from subjects with insulin resistance [64]. Moreover, diminished Akt phosphorylation at serine 473 has been found from obese individuals [3,4,5,6,7] would result in a high local insulin resistance [63]. On this ground, it is tempting to speculate that reduced phosphorylation at serine 473 may predispose to impaired sensitivity to insulin in adipose tissue. Given the complexity of Akt interactions with many intracellular pathways that regulate a broad range of cellular processes it is unlikely that the outcome of impaired Akt phosphorylation by angiotensin II would be restricted to insensitivity to insulin.

In conclusion, we report an ERK$_{1,2}$-dependent attenuation of Akt phosphorylation/activity in the response of human adipose cells to angiotensin II. Given the involvement of angiotensin II in modulation of diverse processes, such as oxidative stress, inflammation, insulin sensitivity, and cellular differentiation, a comprehensive understanding of the underlying pathways in specific target cells should provide the bases for the design of new therapeutic strategies.

Acknowledgments

We are grateful to Dr Miguel Angel Celis at Luis Tisné Hospital for contributing adipose tissue samples and Dr Martin Walbich for providing the SGBS cells. We also thank Mariol Blanco for technical assistance and Karl A. P. Payne for editorial help.

Author Contributions

Conceived and designed the experiments: CVR. Performed the experiments: ND CQ FAB JC CVR. Analyzed the data: ND CQ FAB JC CVR. Contributed reagents/materials/analysis tools: CVR. Wrote the paper: CVR. Critical revision of the manuscript: ND CQ FAB JC.

References

1. Schling P, Mallow H, Trinell A, Löffler G (1999) Evidence for a local renin angiotensin system in primary cultured human preadipocytes. Int J Obes Relat Metab Disord 23: 316–314.
2. Dussere E, Moulin P, Vital D (2000) Differences in mRNA expression of the proteins secreted by the adipocytes in human subcutaneous and visceral adipose tissues. Biochem Biophys Acta 1500: 89–96.
3. Giacchetti G, Falopa E, Marinello B, Sardu C, Gatti C, et al. (2002) Overexpression of the renin-angiotensin system in human visceral adipose tissue in normal and overweight subjects. Am J Hypertens 15: 381–389.
4. Rahmouni K, Mark AL, Haynes WG, Sigmund CD (2004) Adipose depot-specific modulation of angiotensinogen gene expression in diet-induced obesity. Am J Physiol Endocrinol Metab 286: E891–E895.
5. Van Harmelen V, Elizalde M, Ariapart P, Bergstedt-Lindqvist S, Reynisdottir S, et al. (2000) Increased adipose angiotensinogen gene expression in human obesity. Obes Res 8: 337–341.
6. Van Harmelen V, Elizalde M, Ariapart P, Bergstedt-Lindqvist S, Reynisdottir S, et al. (2000b) The association of human adipose angiotensin gene expression with abdominal fat distribution in obesity. Int J Obes Relat Metab Disord 24: 673–676.
7. Yasue S, Masazaki H, Okada S, Ishii T, Kozuka C, et al. (2010) Adipose tissue-specific regulation of angiotensinogen in obese mice and man: impact of nutritional status and adipocyte hypertrophy. Am J Hypertens 23: 423–431.
8. Karlsson C, Lindell K, Orottos MSJ, Ström J, Carlsson B, et al. (1998) Human adipose tissue express angiotensinogen and enzymes required for its conversion to angiotensin II. J Clin Endocrinol Metab 83: 3925–3929.
9. Schling P, Schäfer T (2002) Human adipose tissue cells keep tight control on the angiotensin II level in their vicinity. J Biol Chem 277: 48066–48075.
10. Santos RA, Ferreira AJ, Verano-Braga T, Bader M (2013) Angiotensin-converting enzyme 2, angiotensin(-1–7) and Mas: new players of the renin-angiotensin system. J Endocrinol 216: R1–R17.
11. Kalupahana NS, Mountain-Mousa N (2012) The adipose tissue renin-angiotensin system and metabolic disorders: a review of molecular mechanisms. Crit Rev Biochem Mol Biol 47: 379–90.
12. Massiera F, Bloch-Faure M, Celler D, Murakami K, Fakazmiti A, et al. (2001) Adipose angiotensinogen is involved in adipose tissue growth and blood pressure regulation. FASEB J 15: 2727–2732.
13. Brucher R, Cifuentes M, Albala C, Rojas CV (2007) Angiotensin II reduces adipogenic differentiation of adipocytes precursor cells from human omental adipose tissue. Obesity 15: 1699–1701.
14. Janke J, Engel R, Gerecztiak K, Luth FC, Sharma A (2002) Mature adipocytes inhibit in vitro differentiation of human preadipocytes via angiotensin type 1 receptors. Diabetes 51: 1699–1701.
29. Fuentes P, Acuña MJ, Cifuentes M, Rojas CV (2010) The anti-adipogenic effect of angiotensin II on human preadipose cells depends on MEK/ERK1,2 activation and phosphorylation. J Endocrinol 206: 75–83.

30. Albinsson S, Hellstrånd P (2007) Integration of signal pathways for stretch-dependent growth and differentiation in vascular smooth muscle cells. Am J Physiol Cell Physiol 293: C772-C782.

31. Arellano-Plancharre A, Hernández-Aranda J, Catt KJ, Olivares-Reyes JA (2010) Angiotensin-induced EGFR receptor transactivation inhibits insulin signaling in C9 hepatic cells. Biochem Pharmacol 79: 735-745.

32. Khundmiri SJ, Amin V, Henson J, Lewis J, Ameen M, et al. (2007) Ouabain stimulates protein kinase B (Akt) phosphorylation in opossum kidney proximal tubule cells through an ERK-dependent pathway. Am J Physiol Cell Physiol 293: C1171-C1180.

33. Mergith S, Benini A, Miranda P, Gesi S, Varani K, et al. (2006) Modulation of the Akt/Ras/Raf/MEK/ERK pathway by A (3) adenosine receptor. Purinergic Signal 2: 627-632.

34. Sinha A, Bambergee N, Schwartz JH, Lieberthal W, Levine JS (2004) Inhibition of ligand-independent ERK1/2 activity in kidney proximal tubular cells deprived of soluble survival factors up-regulates Akt and prevents apoptosis. J Biol Chem 279: 10982-10972.

35. Yang SJ, Chang SC, Wen H-C, Chen C-Y, Liu J-F, et al. (2010) Plumbagin activates ERK1/2 and Akt via superoxide, Src and PI3-kinase in 3T3-L1 Cells. Eur J Pharmacol 630: 21-29.

36. Bhattacharya I, Ullrich A (2006) Endothelin-1 inhibits adipogenic role of phosphorylation of Akt and ERK1/2. FEBS Lett 580: 5763-5771.

37. Wang T, Wang Y, Kontani Y, Kobayashi Y, Sato Y, et al. (2008) Evodiamine improves diet-induced obesity in a uncoiling protein-l-independent manner: involvement of antiadipogenic mechanism and extracellularly regulated kinase/mitogen-activated protein kinase signaling. Endocrinology 149: 358-366.

38. Kohn AD, Summers SA, Birnbaum MJ, Roth RA (1996) Expression of a constitutively active form of protein kinase B (Akt) in 3T3-L1 preadipose cells causes spontaneous differentiation. Endocrinology 137: 3590-3593.

39. Yoshida D, Sato N, Torii T, Mori H, Yoshida R, et al. (2007) Adaptor protein SH2-B linking receptor-tyrosine kinase and Akt promotes adipocyte differentiation by regulating peroxisome proliferator-activated receptor gamma messenger ribonucleic acid levels. Mol Endocrinol 21: 1120-1131.

40. Mun C, Benamer M, Neve RL, Catt KJ, Lindahl P, et al. (2004) Perforin stimulates protein kinase B (Akt) phosphorylation in opossum kidney proximal tubule cells. Biochem Biophys Res Commun 371: 138-143.

41. Carvalho E, Eliasson B, Wesslau C, Smith U (2000) Impaired phosphorylation of Akt and ERK1/2 mitogen-activated protein kinases affect Ras-dependent cell signaling differentially. J Biol Chem 275: 14.

42. Leal-Ortiz S, Waites CL, Terry-Lorenzo R, Zamorano P, Gundellinger ED, et al. (2003) Piccolo modulation of Synapsin I dynamics regulates synaptic vesicle exocytosis. J Cell Biol 161: 831-846.

43. Zufferey R, Nagy D, Mandel RJ, Nadlinski L, Trono D (1997) Multiply attenuated lentiviral vector achieves efficient gene delivery in vivo. Nat Biotechnol 15: 871-875.

44. Salmon P, Trono D (2006) Production and titration of lentiviral vectors. Curr Protoc Neurosci Chapter 4: Unit 4.21.

45. Bost F, Aouadi M, Caron L, Even P, Belmonte N, et al. (2005) The extracellular signal-regulated kinase isoform ERK1 is specifically required for in vitro and in vivo adipogenesis. Diabetes 54: 402-411.

46. Fritsche L, Neumann KS, Lehmann K, Krennem J, Hennige AM, et al. (2011) Insulin-induced serine phosphorylation of IRS-2 via ERK1/2 and mTOR-studies on the function of Ser675 and Ser907. Am J Physiol Endocrinol Metab 300: E224-E236.

47. Vantaggiato C, Formentini C, Bondanza A, Bonini C, Nadlinski L, et al. (2006) ERK1 and ERK2 mitogen-activated protein kinases affect Ras-dependent cell signaling differentially. J Biol Chem 281: 14.

48. Leal-Ortiz S, Waites CL, Terry-Lorenzo R, Zamorano P, Gundellinger ED, et al. (2003) Piccolo modulation of SynapsinI dynamics regulates synaptic vesicle exocytosis. J Cell Biol 161: 831-846.

49. Leal-Ortiz S, Waites CL, Terry-Lorenzo R, Zamorano P, Gundellinger ED, et al. (2003) Piccolo modulation of SynapsinI dynamics regulates synaptic vesicle exocytosis. J Cell Biol 161: 831-846.