Angiotensin II Increases Leptin Secretion by 3T3-L1 and Human Adipocytes via a Prostaglandin-Independent Mechanism1,2

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ABSTRACT We previously reported that angiotensin II (Ang II) increases adipocyte fatty acid synthesis and triglyceride content. Triglyceride stores or adiposity correlate positively with the amount of circulating leptin. Ang II was proposed to increase adipocyte differentiation and growth by promoting prostaglandin (PG) production. The purpose of this study was to determine whether Ang II increases leptin secretion via a PG-dependent mechanism. Physiologic doses of Ang II significantly increased leptin secretion by 3T3-L1 adipocytes and human adipocytes. Elevation of PG secretions was elicited at physiologic concentrations of Ang II (P < 0.05). Secretions of 6-keto PGF1α, a stable derivative of PGI2, and PGE2 were induced by physiologic concentrations of Ang II in a time-responsive fashion (P < 0.05). Inhibition of PG synthesis by indomethacin and aspirin significantly suppressed basal as well as Ang II-induced PG levels, but did not significantly affect basal and Ang II-induced leptin secretion. In conclusion, although Ang II stimulates both leptin and PG secretion by adipocytes, regulation of leptin secretion by Ang II in adipocytes is not mediated by a PG-dependent mechanism.

KEY WORDS: adipocytes  •  angiotensin II  •  leptin  •  prostaglandins

Angiotensin II (Ang II) is a vasoactive peptide hormone that regulates blood pressure as well as fluid and electrolyte balance (1). Ang II is classically synthesized by two enzymatic steps in which renin and angiotensin-converting enzyme cleave the precursor angiotensinogen (AGT) produced by the liver (1). Local synthesis of the renin angiotensin system (RAS) has been documented in several tissues including liver, kidney, heart and adipose tissue (1). Adipose tissue represents the potentially largest source of the AGT, especially in obese models (2–4). We showed previously that the AGT gene is expressed in human and rodent adipose tissue where it is regulated nutritionally as well as hormonally, providing important insights into the function of adipocyte-derived Ang II in adipocyte metabolism (5). In support of this hypothesis, data from cultured murine adipocytes indicate an adipogenic role of Ang II in adipocyte growth and development (6,7). Further, this effect of Ang II on adipocyte development has been shown to be mediated by prostaglandins (PG) (8,9).

Adipocytes are endocrine cells that secrete several hormones including leptin (2). Leptin is the protein product of the ob gene cloned by Friedman’s group in 1994 and is expressed primarily in adipose tissue (10). Leptin circulates in the blood and acts on the central neural network that regulates body weight and energy stores (11). Defects in the ob gene as well as leptin receptor gene cause obesity in ob/ob and db/db mice, respectively, and rare mutations have been reported in humans (10–13). Most human obesity is positively correlated with elevated serum concentrations of leptin as well as decreased sensitivity to leptin (12–14). Leptin administration leads to decreased food consumption and increased energy expenditure, both of which result in loss of adipose mass in lean, obese and diet-induced obesity models, indicating a weight-reducing and antiobesity effect of leptin (15,16). Previous studies from our laboratory demonstrated that Ang II activates ob gene expression (6). Mechanistically, Ang II enhances adipocyte differentiation by an AT2 receptor-dependent generation of prostanycin (8). Studies by Borglum et al. (17) demonstrated that Ang II increases expression of prostaglandin endoperoxide H synthase (PGHS)-1 and PGHS-2 mRNA in mature adipocytes, leading to PG production. Prostacyclin and PGE2 have been shown to exert their roles as paracrine or auto-


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4 Abbreviations used: Ang II, angiotensin II; AGT, angiotensinogen; COX, cyclooxygenase; DMEM, Dulbecco’s modified Eagle’s medium; FBS, fetal bovine serum; 6-keto-PGF1α, 6-keto prostaglandin F1α; PG, prostaglandin; PGE2, prostaglandin E2; PGHS, prostaglandin endoperoxide H synthase; PGI2, prostaglandin I2; PPARγ, peroxisome proliferator activated receptor γ; RAS, renin angiotensin system.
crine adipogenic effectors in adipocyte growth and development (18–20). A more recent study illustrated that exogenous PGE2 stimulates both leptin release and leptin mRNA accumulation by explants of human adipose tissue in primary culture (25). However, the mechanism of Ang II regulation of adiposity or leptin and the role of PG in this regulation are unknown.

We hypothesized that Ang II regulates leptin in adipocytes via a PG-mediated mechanism. We report that Ang II increases leptin and PG secretions in a dose- and time-responsive manner. However, stimulation of leptin secretion by Ang II in adipocytes is independent of PG synthesis.

**MATERIALS AND METHODS**

**3T3-L1 adipocytes.** 3T3-L1 adipose cell lines are derived from mouse embryo (21). Cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA) and were grown in 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (regular media). 3T3-L1 cells were plated (~20 × 10⁵ cells/100 mm dish; 0.5 × 10⁵ cells/35 mm dish) and grown to confluence. At confluence, the media were supplemented with 25 mM/L dexamethasone and 0.5 mM/L isobutyl methylxanthine for 72 h, after which cells were cultured with regular media for an additional 2 d (21). Differentiation was considered to be complete at 5 d postconfluence. All studies were conducted using differentiated cells (~80–95%). Before any treatment with Ang II or inhibitors, cells were incubated for 24 h in serum-free media containing 1% bovine serum albumin.

**Human adipose tissue.** Subcutaneous abdominal adipose tissue was taken from female patients, 30–50 y of age, undergoing elective cosmetic surgery. Samples were obtained in compliance with a protocol approved by the Institutional Review Board for Human subjects and by the Committee for Research Protocols at the University of Tennessee, Knoxville. All patients were nondiabetic and nonhypertensive, with no known metabolic abnormalities. Three patients with the range of body mass index (25–26 kg/m²) were included in this study. Isolated human adipose tissue was maintained in DMEM supplemented with 1% FBS for 2 d and incubated with serum-free media for 24 h before treatment. Human adipose tissue was treated for 48 h with serum-free media supplemented with 10 mM/L Ang II or without Ang II as controls. The culture media were collected at 3, 6, 12, 24, and 48 h and leptin concentration in the culture media was measured by RIA.

**Experimental design.** Optimal responses of secreted leptin and cyclooxygenase (COX)-derived eicosanoids (6-keto PGF1α and PGE2) to Ang II treatment were established by determining an optimal dose-response (0.1 mM/L-1 mM/L) and time-dependence (0–96 h) curve. 3T3-L1 adipose cells were cultured with serum-free media overnight in 100-mm culture dishes or 35-mm plates and then treated with Ang II (Sigma Chemical, St. Louis, MO) in serum-free media. In dose-response studies, cells were incubated for 48 h with or without Ang II. After 48 h of culture, culture media were collected to measure leptin and PG levels. To analyze the time response, culture media of 3T3-L1 adipocytes were treated in presence or in absence of 10 mM/L Ang II were used to measure the levels of leptin, 6-keto PGF1α, and PGE2, at the indicated time points. Cell extracts were collected for cellular protein contents and used to normalize the levels of leptin and eicosanoids. To determine the possible mediators of Ang II-induced PG and leptin secretion in 3T3-L1 adipocytes, indomethacin (reversible) and aspirin (irreversible) (Sigma Chemical) inhibitors of both COX-1 and COX-2 were used (22). For inhibitor studies, adipocytes were preincubated with serum-free media in the absence or presence of various concentrations (1–100 μM/L) of indomethacin and aspirin dissolved in ethanol (0.2%, v/v) for 6 h. The same volume of carrier was used for control cells. After pretreatment with inhibitors, 10 mM/L Ang II or vehicle alone was added and cells were maintained for an additional 24 h. Cells and media were then collected for PG and leptin secretion. These experiments were repeated 3–5 times.

**Quantitation of leptin in the media.** Leptin secreted in the media was determined by RIA. The mouse leptin RIA kit used in this study was purchased from Linco Research (St. Charles, MO). The medium (100 mL) collected was used and all samples were measured in duplicate. Leptin level was corrected to milligrams of cellular protein content or grams of tissue.

**Prostaglandin assay.** Prostaglandins were determined as described previously (23). Briefly, culture media were treated with cold acetic acid methanol solution (MeOH/formic acid, 9:1, v/v, pH 3.5) followed by isolation of PG using C18 solid-phase columns (Burdick & Jackson, Muskegon, MI) and eluted with 100% methanol. The methanol was evaporated under an atmosphere of nitrogen and the extracts were resuspended with 250 μL of 0.1 mM/L PBS plus 1 g/L gelatin, pH 7.4. The resuspended PG were assayed via RIA according to manufacturer’s instructions (PerSeptive Diagnostics, Cambridge, MA). PGE2 and 6-keto PGF1α antiserum were obtained from PerSeptive Diagnostics. PGE2 and 6-keto PGF1α standards were purchased from Cayman Chemical (Ann Arbor, MI), and [3H] PGE2 and [3H] 6-keto PGF1α were obtained from New England Nuclear (Boston, MA). All experiments were repeated at least twice. Data are expressed as picograms or picomoles of PGE2 and 6-keto PGF1α per milligram protein.

**Protein assay.** The cellular protein contents were measured by the method of Bradford (24). Adipocytes were harvested in 500 μL of 0.5 mol/L sucrose (pH 7.4), 1 mM/L dithioeritol, 1 mM/L EDTA and 100 mM/L phenylmethylsulfonyl fluoride. Cell homogenates were sonicated for 5 s after 1 h of ultracentrifugation (12,000 × g) at 4°C. The protein content of the supernatant was determined. All samples were assayed in duplicate.

**Statistics.** Student’s t test (Figs. 2b, 4a, 4b) and ANOVA (Figs. 1, 2a, 3a, 3b, 5a, 5b) were used to compare overall group means; two-way ANOVA (Fig. 6) was performed for comparing the interactions of two factors. The Bonferroni test for multiple comparison (SAS, Cary, NC) was used to compare differences among group means after a significant F-test (Figs. 1, 2a, 3a, 3b, 5a, 5b). All values were expressed as means ± SEM. Values of P < 0.05 were considered significant.

**RESULTS**

**Effect of Ang II on leptin secretion in 3T3-L1 and human adipocytes.** Consistent with the increase in transcription rate of ob gene upon Ang II treatment, Ang II at concentrations of 1 and 10 mM/L significantly increased leptin secretion by 3T3-L1 adipocytes at 48 h by 24 ± 9.8 and 57 ± 6.7%, respectively (Fig. 1). A significant effect of Ang II on leptin

![FIGURE 1](https://example.com/figure1.png)
secretion was observed at 12, 24, 36 and 48 h by 10 nmol/L of Ang II treatment when leptin secretion was threefold the control levels (Fig. 2a, *P < 0.01). Similarly, physiologic concentrations of Ang II significantly stimulated leptin secretion from human adipose tissue in a time-responsive manner (Fig. 2b).

**Effect of Ang II on PG secretions in 3T3-L1 adipocytes.** Consistent with stimulation of leptin secretion by Ang II, PG secretions were significantly induced by physiologic doses of Ang II (Fig. 3a and b). A significant effect of Ang II on 6-keto PGE\(_{1\alpha}\) secretion was present at 48 h of treatment with 1 and 10 nmol/L (Fig. 3a). In concordance, 3T3-L1 adipose cells cultured with 1 and 10 nmol/L Ang II also increased PGE\(_2\) secretion at 48 h (Fig. 3b). Ang II treatment (10 nmol/L) markedly and acutely stimulated 6-keto PGE\(_{1\alpha}\) secretion from 3T3-L1 adipose cells at 3 h, and this stimulatory effect by Ang II was sustained for an additional 72 h (Fig. 4a). Ang II significantly elevated PGE\(_2\) secretion at 6 h and this level was maintained through 96 h (Fig. 4b). Consistent with significant inductions of PG secretions by Ang II (10 nmol/L) after 24 h, Ang II at concentration of 1 nmol/L significantly enhanced secretions of both PG after 24 h (data not shown). In summary, Ang II at physiologic concentrations increased PG secretion from 3T3-L1 adipose cells in a time-dependent manner. These results are consistent with the previously reported induction of PGHS gene expression by Ang II in murine adipose cell lines (17) and with induction of 6-keto PGE\(_{1\alpha}\) secretion by an Ang II stimulus (8).

**Effect of indomethacin and aspirin on Ang II-induced PG and leptin secretions in 3T3-L1 adipocytes.** To investigate further the mechanisms that mediate increased leptin secretion by Ang II in adipocytes, the effect of PG synthesis inhibition on Ang II-induced PG and leptin secretions was conducted as follows. Prostaglandins. We focused on the effects of two inhibitors, indomethacin and aspirin, on Ang II-induced prostacyclin and leptin secretions. As anticipated, pretreatment (6 h before 10 nmol/L of Ang II treatment) with indomethacin (Fig. 5a) and aspirin (Fig. 5b) at concentrations of 1, 10, 50 and 100 \(\mu\)mol/L significantly inhibited both basal and Ang II-induced 6-keto PGE\(_{1\alpha}\) secretions in a dose-responsive manner (*P < 0.05). The concentration of 10 \(\mu\)mol/L of indomethacin maximally reduced 6-keto PGE\(_{1\alpha}\) synthesis in both vehicle- and Ang II-treated cells by 49 and 58%, respectively. Similarly, aspirin at a concentration of 10 \(\mu\)mol/L maximally inhibited basal and Ang II-induced 6-keto PGE\(_{1\alpha}\) levels by 45 and 65%, respectively (Fig. 5a). These similar reductions (~35–60%) in basal and Ang II-induced 6-keto PGE\(_{1\alpha}\) levels were also noted when
either inhibitor was added with Ang II for 24 h (data not shown).

Leptin. The effects of increasing doses of indomethacin and aspirin on basal as well as Ang II-stimulated leptin secretions were evaluated. Inhibition of Ang II-induced PG synthesis by indomethacin or aspirin did not modify basal leptin secretion (Fig. 6). Neither aspirin nor indomethacin prevented increased leptin secretion induced by Ang II; rather, this secretion was significantly enhanced by the combination of indomethacin and Ang II, indicating that inhibition of PG synthesis does not prevent leptin secretion.

DISCUSSION

In this study, we investigated the biochemical mechanisms by which Ang II regulates leptin secretion in adipocytes. Previous investigations indicated that Ang II may function as an adipogenic factor in adipocyte growth and development through interaction with its receptors (6–8). We demonstrated that Ang II increases leptin expression in cultured adipocytes (6). These cumulative findings suggest a strong link between Ang II and leptin in the adipogenic functions of Ang II. Our results clearly demonstrate that Ang II increases leptin secretion from 3T3-L1 and human adipocytes. Accordingly, our results support the idea that Ang II may play a role as a regulator of leptin in adipocytes. To date, the signaling mechanisms by which Ang II increases leptin secretion and promotes adiposity remain to be elucidated. A previous study by Darimont et al. (8) suggested that prostacyclin synthesized by mature adipocytes functions as a mediator of Ang II action on adipocyte growth. Additionally, preliminary research has shown that PGE2 and PG12, produced mainly from rodent and human adipocytes, function as hypertrophic as well as hyperplastic effectors in adipocyte differentiation and development through paracrine/autocrine effects (19,20). Recently, Fain et al. (25) demonstrated that exogenous PGE2 increases leptin secretion and leptin mRNA in adipose tissue from obese individuals and inhibits lipolysis. Interestingly, heterozygous but not wild-type or COX-2 knockout mice became obese in response to a high fat diet, suggesting the potential role of cyclooxygenase or its downstream metabolites such as PGE2 in obesity (26).

Consistent with previous reports (8,17), our study demonstrates that Ang II at physiologic concentrations produces time-dependent stimulations of 6-keto PGF1α and PGE2 secretions in adipocytes. Ang II treatment stimulated PG secretion from 3T3-L1 adipocytes both acutely (within 3 h), possibly due to a post-translational effect of Ang II and chronically (up to 72 h), possibly as a result of a transcriptional or
translational effect of Ang II on PG synthesis. These results suggest that these PG may mediate the effect of Ang II on leptin secretion. Accordingly, we investigated whether Ang II enhances leptin stimulation via a PG-mediated mechanism. Both inhibitors (aspirin and indomethacin) significantly decreased basal and Ang II-induced 6-keto PGF_1α secretion from adipose cells. However, COX inhibition by aspirin did not modify basal or Ang II-induced leptin releases from 3T3-L1 cells. Interestingly, although indomethacin did not modify basal leptin secretion, it significantly potentiated Ang II effects on leptin secretion. Thus, results from this study clearly demonstrate that Ang II stimulates leptin secretion but this effect does not appear to be mediated through PG. In addition, we compared the effects of Ang II on leptin secretion from the pretreated adipocytes to that of simultaneous addition of inhibitors with Ang II. Our results were consistent whether the cells were treated with inhibitors before hormone addition or added simultaneously with the inhibitor and the hormone (data not shown). These results further support our findings that Ang II regulates leptin secretion in adipocytes via a PG-independent mechanism. Ang II induction of leptin was potentiated by indomethacin; this inhibitor was indeed shown to play a critical role as a ligand of the adipogenic transcription factor, peroxisome proliferator activated receptor γ (PPARγ), rather than as an inhibitor in PG biosynthesis. Recent research has shown that indomethacin at high concentrations (μmol/L range) binds and activates PPARγ, thus inducing adipogenesis, but at low concentrations (nmol/L range), it exhibits an inhibitory effect on PG synthesis (27). More recently, a study by Sinha et al. (28) illustrated the inhibitory effect of indomethacin on leptin secretion at the concentration at which it activates PPARγ in adipocytes. In the present study, high concentrations of indomethacin that induced reduction of PG secretion did not modify leptin secretion, suggesting that it may affect predominately COX inhibition vs. PPARγ activation under the present experimental conditions.

In summary, our data indicate that Ang II increases leptin secretion from adipocytes via a PG-independent mechanism, suggesting that alternative or additional mechanisms are involved in Ang II-induced leptin secretion. In addition to stimulation of fatty acid and triglyceride synthesis by Ang II, this hormone also increases leptin secretion. Therefore, this study provides a potential mechanism by which Ang II may regulate adiposity. Understanding of the adipocyte RAS and Ang II paracrine effects in adipocytes may provide insights into the development of new therapeutic approaches to prevent obesity and its associated disorders such as hypertension. Further work is required to characterize the mechanism of Ang II regulation of the ob gene and identify mechanism of Ang II signaling in adipocytes leading to fatty acid synthesis and leptin secretion.

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LITERATURE CITED


