

# Angiotensin II Regulates Adipocyte Apolipoprotein E Expression

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**Context:** Obesity is increasing in prevalence and it is important to understand factors that regulate adipose tissue lipid metabolism. Recently, endogenous expression of apolipoprotein E (apoE) in adipose tissue has been shown to have important effects on adipocyte lipid flux and gene expression. Adipose tissue is also a physiological target of angiotensin II (AII).

**Objective:** The aim of the current study was to evaluate a potential regulatory effect for AII on adipose tissue apoE expression.

**Results:** Infusion of AII into mice for 3 d significantly reduced apoE expression in adipocytes from freshly isolated adipose tissue. ApoE expression was unchanged by the AII infusion in the stromovascular fraction. In isolated human adipocytes, treatment with AII significantly reduced cellular and secreted apolipoprotein E (by 20–60%). Suppression of apoE expression was observed in sc adipocytes obtained

from nonobese (body mass index < 30 kg/m<sup>2</sup>) donors, and in sc and omental adipocytes obtained from obese (body mass index > 30 kg/m<sup>2</sup>) donors. Evaluation of the effect of AII in matched sets of sc and omental adipocytes from three separate donors showed lower overall apoE expression in omental adipocytes in two of the donors, and a concordant down-regulation of apoE expression in sc and omental adipocytes from all three subjects. The specific AT<sub>1</sub> receptor blocker, valsartan, eliminated the effect of AII on adipocyte apoE expression.

**Conclusion:** Both apoE and components of the renin-angiotensin system are expressed in adipose tissue, and each has important effects on adipocyte lipid metabolism and gene expression. The regulatory interaction we have identified between these two pathways has important implications for a complete understanding of adipose tissue lipid homeostasis. (*J Clin Endocrinol Metab* 92: 4366–4372, 2007)

OBESITY IS INCREASING in prevalence in adults and in children (1–3). This presents a significant public health and clinical challenge because obesity is associated with increased morbidity and mortality, especially from cardiovascular diseases (1–4). Because of this, the impact of adipose tissue, especially excess adipose tissue, on the vascular wall has recently been the subject of heightened interest. Over recent years, it has become clear that the adipose tissue is a dynamic endocrine organ that modulates systemic substrate flux and metabolism by controlling the level of circulating substrates, and by secretion of factors that can modulate substrate metabolism in distant tissues (4–6). In addition, adipose tissue has been shown to secrete a number of factors that can directly impact the vessel wall and vessel wall cells. One of the factors highly expressed in the adipose organ is apolipoprotein E (apoE) (7–9).

ApoE mRNA and protein can be found in human and rodent adipose tissue, where it is expressed by both mature adipocytes and adipose tissue macrophages (7–9). In rodents, up to 80% of all apoE mRNA in adipose tissue can be ascribed to mature adipocytes (9). Recently, an important role has been demonstrated for endogenous adipocyte apoE expression, *in vivo* and *in vitro*, for modulating adipocyte lipid metabolism and adipocyte gene expression (9). Although most circulating apoE comes from the liver, the size of the

adipose organ suggests that it could be a significant source of extrahepatic apoE, especially in certain pathophysiological states (8). In animal models, extrahepatic apoE has been shown to have important effects on lipoprotein composition, systemic lipoprotein metabolism, and on the vessel wall directly (4, 10, 11). In view of the aforementioned observations, it is important to understand factors that regulate adipose tissue apoE expression. We have previously shown that adipose apoE expression responds to peroxisome proliferator-activated receptor  $\gamma$  agonists and TNF $\alpha$  (8). We have also recently demonstrated significant control of adipose tissue apoE expression by organismal nutritional balance (12). Adipose tissue expresses components of the renin-angiotensin system and expresses angiotensin II (AII) (13, 14) receptors. Furthermore, adipose tissue is an important physiological target of AII; AII has been shown to modulate adipocyte lipid metabolism, increase inflammatory gene expression, and decrease adiponectin expression (15–18). Given the emerging evidence regarding the potential significance of adipocyte apoE expression, the current studies were performed to evaluate the effect of AII on adipocyte apoE expression.

## Materials and Methods

### AII infusion

Eight-week-old C57BL/6 mice were purchased from the Jackson Laboratories (Bar Harbor, ME). All animal protocols were approved by the Institutional Animal Care and Use Committee of the University of Illinois, Chicago. Experimental mice received human AII infusion for 3 d via sc osmotic pump (Durect Corporation, Cupertino, CA) at 1 mg/kg·d rate. Control mice received a sc infusion of sterile PBS for the same period. After the infusion, intraabdominal fat pads were collected for experiments. Adipocytes were isolated in a floating fraction after di-

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Abbreviations: AII, Angiotensin II; apoE, apolipoprotein E; BMI, body mass index.

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gesting freshly obtained adipose tissue with 0.5 mg/ml collagenase in DMEM for 1 h at 37°C in a shaking water bath as previously described (9). Stromovascular cells were pelleted and washed twice with DMEM. After centrifugation, floating cells (freshly isolated adipocytes) and the cell pellet (stromovascular cells) were used for RNA isolation. Peritoneal macrophages were harvested by peritoneal lavage with sterile PBS, resuspended in DMEM with 10% FBS, and maintained in six-well plates for 2 h at 37°C, after which they were used for RNA isolation.

### Cell culture

Human sc and omental preadipocytes were obtained from Zen-Bio (Research Triangle Park, NC). Cells were maintained in preadipocyte media (PM-1 for sc cells, PM-OM for omental cells; Zen-Bio) until confluent. Cells were differentiated using differentiation media (DM-2 for sc cells; DMOM, differentiation media for omental cells; Zen-Bio) for 7 d. On d 7, cells were incubated overnight in SFAM, serum free adipocyte media for sc cells, and OMAM, omental adipocyte media (Zen-Bio) with 0.2% BSA alone or with following additions: AII  $10^{-5}$  M (Sigma, St. Louis, MO) alone or 1 h after pretreatment with valsartan  $10^{-4}$  M (obtained from Novartis, East Hanover, NJ). After treatment, media and/or cell lysates (as noted in the figures) were used for Western Blot or quantitative RT-PCR.

### Western blot analysis

Human adipocytes were lysed in 4% SDS containing radioimmunoprecipitation assay buffer. Media and cell lysates were resolved by 10% SDS-PAGE and electrotransferred to a nitrocellulose membrane. Goat antihuman apoE antibody (International Immunology Corporation, Murrieta, CA) was used to assess apoE expression (1:200). The apoE signal was developed using an antigoat horseradish peroxidase secondary antibody (1:1000; Pierce, Rockford, IL). A chemiluminescent detection system ECL (Amersham Pharmacia Biotech, Little Chalfont, UK) was used for visualization of bands, and intensity was quantitated using densitometry software (ImageQuant v2005; Amersham Biosciences, Piscataway, NJ). Flotillin expression was used for normalization of the apoE signal.

### Quantitative RT-PCR

Total RNA was extracted using Qiagen kits (Valencia, CA). First strand cDNA was synthesized from 1  $\mu$ g total RNA using random hexamer primers according to the manufacturer's instructions (Fermentas, Hanover, MD). Real-time PCR was performed on each mouse isolate in duplicate using the Mx3000p Quantitative PCR system (Stratagene, La Jolla, CA). Reactions were carried out in a total volume of 25  $\mu$ l using Brilliant SYBR Green QRT-PCR Master Mix (Stratagene). Relative quantification for each gene (expressed as fold increase over control) was calculated after normalization to  $\beta$ -actin RNA. Primer pairs (forward, reverse) used for each gene were: human  $\beta$ -actin, tcacctgaagtaccatcg, agaagcatttgcggtggacg; human apoE, cacaggcaggaagatgaagg, agcgag-gtaatcccaaaag; human adiponectin, ggcatgaccaggaaccac, tcaccgatgtctccttagg; mouse  $\beta$ -actin, ctgggacgacatggagaaga, agaggcatcaggacagca; mouse apoE, agtgcaaaacaaacc, ctccgtcatagtctctcca; mouse adiponectin, ggagatgcaggctcttctt, tctcaggctctcttctt; mouse CD36, caggctgacaactcacag, aggtacggaaccaactcaa; mouse CD68, acttcgggc-catgttctct, gctggtaggtgattgtcgt.

### Statistical analysis

Each experiment shown is representative of three to four experiments (each done in triplicate) with similar results. Statistical significance of observed differences was analyzed using Student's *t* test or ANOVA using SPSS (Chicago, IL).

## Results

The effect of AII *in vivo* on adipocyte apoE expression was examined in 8-wk-old C57BL/6 mice. Animals received a continuous sc infusion of AII (1 mg/kg·d) or PBS for 3 d. After the infusion, intraabdominal fat pads were harvested

and adipocytes were separated from the stromovascular fraction as described in *Materials and Methods*. The AII infusion did not produce a significant change in total body weight compared with the PBS control, nor did it alter total cholesterol or triglyceride levels (data not shown). The effect on the AII infusion on adipocyte mRNA levels of apoE, CD36, and adiponectin are shown in Fig. 1A. AII infusion significantly decreased apoE mRNA level, and the magnitude of change was similar to that produced in adiponectin mRNA level. There was no effect of AII on CD36 levels in freshly isolated adipocytes. Adipose tissue macrophages, found in the stromovascular fraction, also express apoE. Therefore, we evaluated the effect of the AII infusion on macrophage content and apoE expression in the adipose tissue stromovascular fraction. The AII infusion over 3 d did not produce an influx of macrophages into the adipose tissue stromovascular fraction as demonstrated by the absence of a significant increase in CD68 mRNA level; a marker for adipose tissue macrophage content (12). AII had no effect on apoE expression in the adipose tissue stromovascular fraction. Macrophages isolated from different tissues can be functionally heterogeneous, and, therefore, we evaluated the effect of the AII infusion on freshly isolated peritoneal macrophages. Different from both adipose tissue macrophages and mature adipocytes, the AII infusion increased apoE expression in mouse peritoneal macrophages.

AII can have pleiotropic effects *in vivo*, and, therefore, we next used an *in vitro* system to evaluate whether the effect of AII on apoE expression was mediated by a direct effect of AII on adipocytes. Human preadipocyte pools obtained from sc or omental adipose tissue depots were differentiated *in vitro* and treated with AII. Figure 2, A–C, shows the effect of the AII treatment on cellular and secreted apoE as measured by Western blot. Subcutaneous adipocytes obtained from pooled donors with body mass index (BMI) less than 30 kg/m<sup>2</sup>, and sc and omental adipocytes obtained from pooled donors with BMI more than 30 kg/m<sup>2</sup> each demonstrated a significant reduction in both cellular and secreted apoE in response to the AII treatment. Figure 2D shows the results of AII treatment on mRNA levels for apoE and adiponectin in pooled sc adipocytes from donors with BMI less than 30 kg/m<sup>2</sup>. Consistent with the results from the *in vivo* infusion, AII treatment significantly reduced both apoE and adiponectin mRNA levels.

Figure 3 presents the results of experiments in which we evaluated the effect of AII treatment on matched sets of sc and omental adipocytes from three individual subjects with BMI more than 30 kg/m<sup>2</sup>. In two of the three subjects, apoE expression was significantly lower in untreated omental compared with untreated sc adipocytes, with a trend toward lower expression in the third subject. In all three subjects, AII treatment produced significant reduction of cellular apoE in both sc and omental adipocytes of approximately the same magnitude. Medium apoE responded similarly to treatment with AII (data not shown).

AII is known to exert most effects via AT<sub>1</sub> and AT<sub>2</sub> receptors. The AT<sub>1</sub> receptor has been studied extensively and is highly expressed in adipose tissue. To evaluate whether AT<sub>1</sub> receptors are involved in the negative effect of AII on apoE expression in adipocytes, we treated human adipocytes

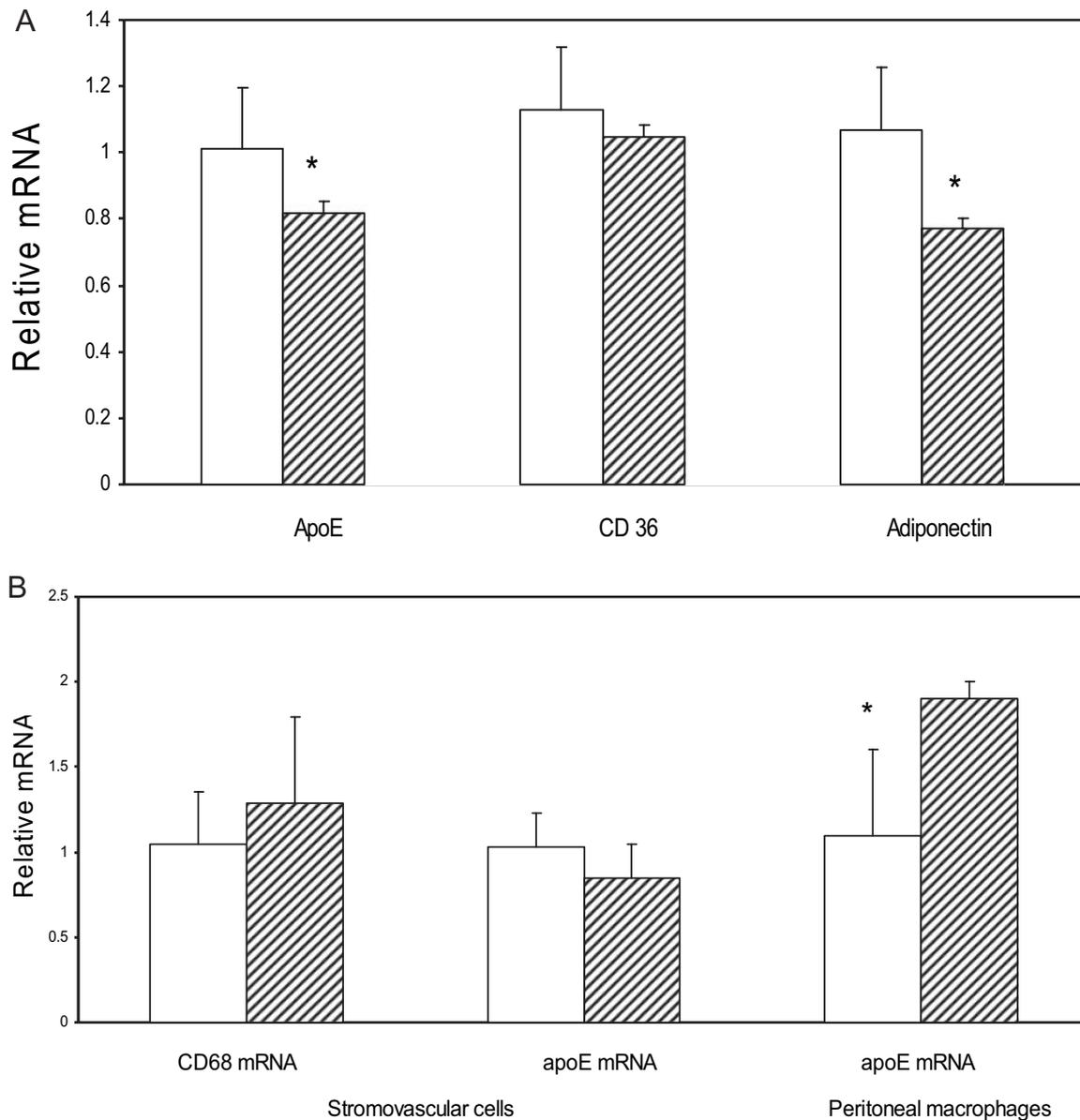


FIG. 1. Effect of AII infusion on adipose tissue apoE expression *in vivo*. Eight-week-old C57BL/6 mice were infused sc with either sterile PBS (untreated) or AII (1 mg/kg/d) for 3 d. A, Mature adipocytes were isolated from intraabdominal fat pads as described in *Materials and Methods*, and mRNA levels were measured by real-time PCR. Values shown are mean  $\pm$  SD from six mice. B, Stromovascular cells were isolated from the intraabdominal fat pad, and peritoneal macrophages were obtained by peritoneal lavage as described in *Materials and Methods*. \*,  $P < 0.05$  for difference in mRNA level comparing untreated to AII treated. Values shown are mean  $\pm$  SD from six mice. Open bars, Untreated; hatched bars, AII treated.

with AII with or without the AT<sub>1</sub> blocker, valsartan (Fig. 4). Treatment with AII alone reduced cellular apoE levels significantly. Treatment with valsartan alone had no effect on apoE levels; however, pretreatment with valsartan completely blocked the effect of AII on apoE expression. The results in Fig. 4 are from sc adipocytes obtained from pooled donors with BMI less than 30 kg/m<sup>2</sup>; however, similar results were obtained from sc and omental adipocytes obtained from donors with BMI more than 30 kg/m<sup>2</sup> (results not shown).

### Discussion

Our results demonstrate a significant effect of AII on adipose tissue apoE expression *in vivo*. Infusion of AII for

3 d reduced apoE expression exclusively in the mature adipocyte fraction of adipose tissue. ApoE expression in the stromovascular fraction of adipose tissue, which is primarily accounted for by macrophages, did not respond to AII infusion. The selective decrease in adipocyte apoE expression in response to the AII infusion *in vivo* is further demonstrated by the observation that apoE expression increased in freshly isolated peritoneal macrophages after 3 d of AII treatment. Because AII may affect multiple *in vivo* pathways with impact on adipose tissue, we used an *in vitro* model of human adipocytes to confirm a direct effect of AII on apoE expression in adipocytes. Subcutaneous adipocytes from nonobese humans and sc and

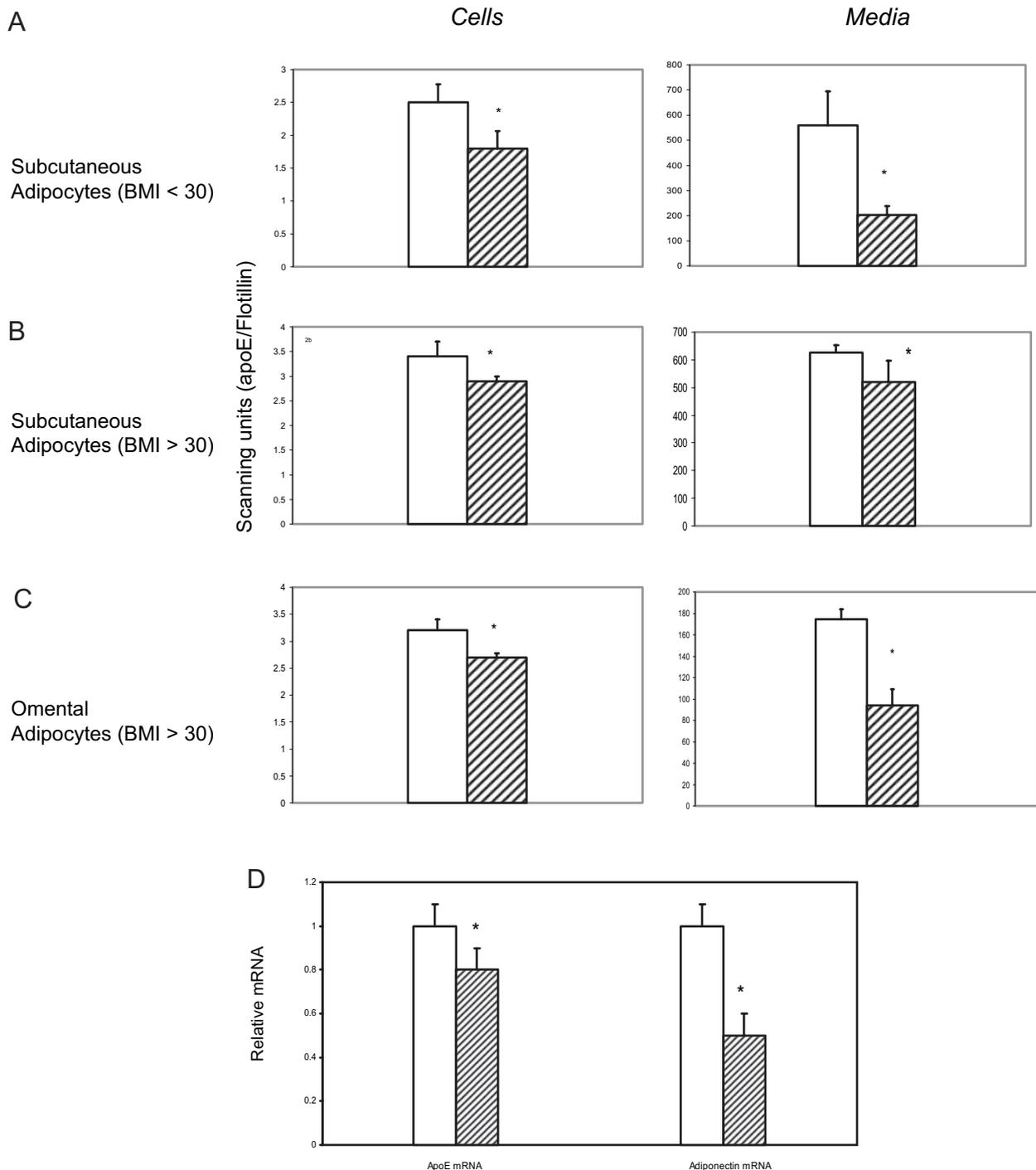


FIG. 2. AII decreases apoE expression in human sc and omental adipocytes. Cells from a pool of six donors with similar BMI were incubated overnight in serum-free media with 0.2% BSA with or without the addition of AII  $10^{-5}$  M. Media and cell lysates were collected, and apoE protein expression was measured by Western blot as described in *Materials and Methods*. A, Subcutaneous adipocytes from donors with BMI less than  $30 \text{ kg/m}^2$ . B, Subcutaneous adipocytes from donors with BMI more than  $30 \text{ kg/m}^2$ . C, Omental adipocytes from donors with BMI more than  $30 \text{ kg/m}^2$ . D, ApoE and adiponectin mRNA levels were measured in sc adipocytes from donors with BMI less than  $30 \text{ kg/m}^2$  after incubation with or without AII as described above. Values shown are mean  $\pm$  SD from triplicates of wells of cells. Open columns, Untreated; hatched columns, AII. \*,  $P < 0.05$  for the difference between untreated and AII-treated cells.

omental adipocytes from obese humans responded to AII with reduction of apoE expression. Adipose tissue has been shown to abundantly express  $AT_1$  receptors, and expression levels for this receptor are influenced by patho-

physiological states such as hypertension and obesity (14, 19). Using the specific  $AT_1$  receptor blocker, valsartan, we also showed that the effect of AII on adipocyte apoE expression was mediated by the  $AT_1$  receptor.

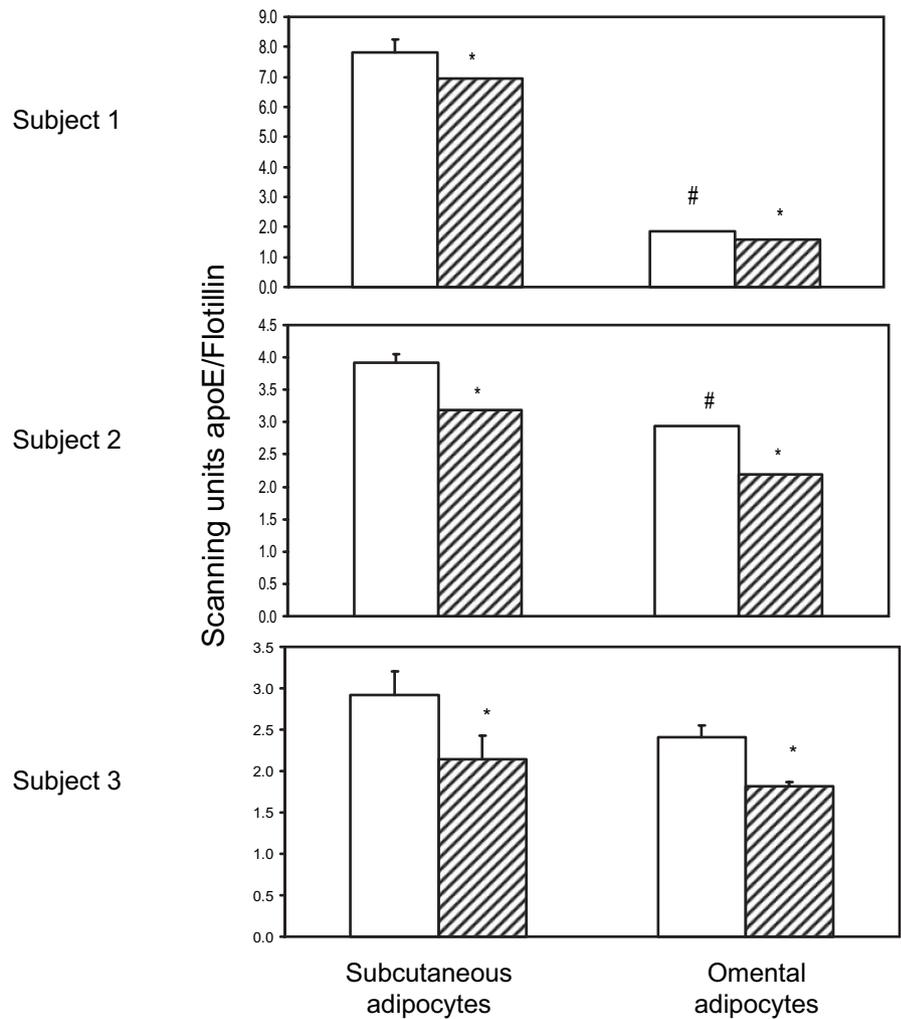


FIG. 3. Effect of AII on apoE expression in a matched set of sc and omental adipocytes from single donors. Subcutaneous and omental adipocytes from three subjects (BMI > 30 kg/m<sup>2</sup>) were incubated overnight in serum-free media with 0.2% BSA with or without addition of AII 10<sup>-5</sup> M. Cell lysates were collected and apoE protein expression was measured by Western blot as described in *Materials and Methods*. \*, *P* < 0.05 for the difference between untreated and AII-treated cells. #, *P* < 0.05 for the difference between untreated omental and sc adipocytes. Values shown are mean ± SD from triplicate wells of cells. Where error lines are not visible, they are contained within the bar. Open bars, Untreated; hatched bars, AII treated.

Several components of the renin-angiotensin system have been identified in freshly isolated adipose tissue from both rodents and humans. Adipose angiotensinogen expression is decreased by fasting and increased by refeeding (13, 14, 20). The expression of adipose tissue renin-angiotensin system components *in vivo* has also been shown to increase in obese states, and genetic variants of the renin-angiotensin system have been associated with obesity (21). AII has also been shown to directly regulate important aspects of adipocyte function. AII treatment of adipose tissue stimulates IL-6 and

IL-8 production from human adipocytes by a nuclear factor κB-dependent pathway and decreases adipose tissue adiponectin mRNA and circulating adiponectin levels in rodents (18, 22). Recent evidence suggests that AII may be involved in regulation of adipogenesis and fat pad mass; AII treatment of adipocytes stimulates lipogenesis and suppresses hydrolysis (22, 23).

The endogenous expression of apoE by adipose tissue from humans and rodents has been demonstrated, and an important effect of endogenous apoE on adipocyte size, tri-

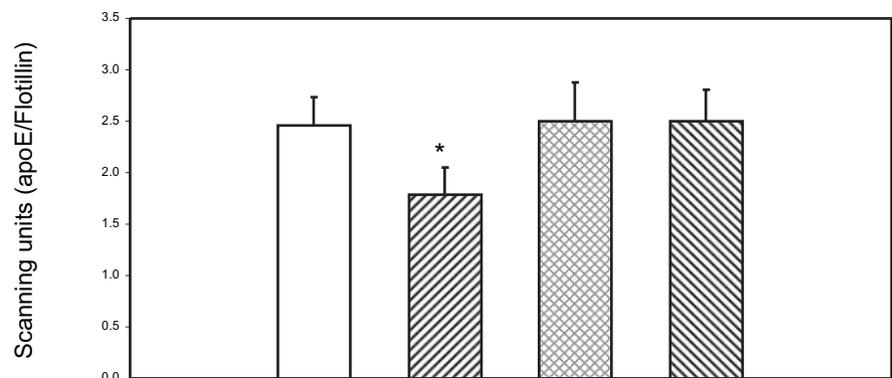


FIG. 4. AII effect on adipocyte apoE expression is mediated via the AT<sub>1</sub> receptor. Human sc adipocytes from pooled donors (BMI < 30 kg/m<sup>2</sup>) were incubated overnight with no addition, AII 10<sup>-5</sup> M, valsartan 10<sup>-4</sup> M, or both. Cell lysates were collected, and apoE protein expression was measured by Western blot. \*, *P* < 0.05 for the difference compared with the untreated control. Values shown are mean ± SD from triplicate wells of cells. □, Untreated; ▨, AII treated; ▩, valsartan and AII treated; ▧, valsartan treated.

glyceride synthesis, and triglyceride hydrolysis has been very recently demonstrated (7–9). Endogenous expression of apoE in adipocytes also impacts the expression of a number of adipocyte genes including those coding for enzymes in the fatty acid oxidation pathway (9). Physiologically relevant regulation of apoE has also been recently confirmed. ApoE expression in human adipose tissue and in 3T3-L1 cells is increased by treatment with peroxisome proliferator-activated receptor  $\gamma$  agonists, and in 3T3-L1 cells is decreased by treatment with TNF $\alpha$  (8). We have also recently shown that adipocyte apoE expression is modulated by organismal energy balance (12). Obesity induced by feeding high-fat diet, or by the hyperphagia attending leptin deficiency, reduces adipocyte apoE expression. Conversely, acute fasting or chronic hypocaloric intake increases adipocyte apoE expression. In isolated adipocytes, we have noted that absence of apoE expression suppresses triglyceride synthesis and increases hydrolysis, and these defects can be reversed by adenoviral-mediated expression of apoE (8). Therefore, we have interpreted the suppression of apoE expression in response to obesity as a homeostatic response to limit the further accumulation of triglycerides by adipocytes (12). AII treatment of adipocytes has been shown to stimulate triglyceride synthesis and suppress hydrolysis. Therefore, the suppression of apoE expression by AII could also be a homeostatic response to limit further triglyceride accumulation in adipocytes. Adipocytes are also direct targets of glucose and insulin, and these also may play a role in modulating adipose tissue apoE expression in response to nutritional manipulation (24, 25). Recently reported observations suggest that insulin and AII can work cooperatively to modulate aspects of adipocyte lipid metabolism including scavenger receptor B1 function (26).

AII has been shown to have negative effects on the vasculature and systemic oxidant stress (27), and its systemic proinflammatory effects can be blocked by treatment with AT $_1$  receptor blockers (28). Our current results establish a new regulatory target of AII, adipocyte apoE expression. The relationship between apoE, AII, and overall adipose tissue physiology is likely to be complex, and additional data will be needed before a comprehensive and integrated model of their roles in adipose tissue can be proposed. Both apoE and components of the renin-angiotensin system are expressed in adipose tissue, and each has important autocrine effects on adipose tissue lipid metabolism and gene expression. Further evaluation of how expression of these two pathways are coordinately regulated in adipose tissue, and the impact that this new regulatory interaction we have demonstrated has on adipose tissue lipid metabolism, will be important for a more complete understanding of adipose tissue lipid flux and the pathophysiology of obesity.

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