

# Mature Adipocytes Inhibit In Vitro Differentiation of Human Preadipocytes via Angiotensin Type 1 Receptors

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Recent studies suggest that angiotensin II (Ang II) plays a role in the adipogenesis of murine preadipocytes. Here, we examined the role of Ang II for the differentiation of primary cultured human preadipocytes. Preadipocytes were isolated from human adipose tissue and stimulated to differentiate. Quantitation of gene expression during adipogenesis was performed for renin-angiotensin system (RAS) genes. The influence of the RAS on adipogenic differentiation was investigated by addition of either angiotensinogen (AGT), Ang II, or angiotensin receptor antagonists to the differentiation medium. We also examined the influence of adipocytes on adipogenesis by co-culture experiments. Expression of the RAS genes AGT, renin, angiotensin-converting enzyme, and Ang II type 1 receptor increased during adipogenesis. Stimulation of the Ang II type 1 receptor by Ang II reduced adipose conversion, whereas blockade of this receptor markedly enhanced adipogenesis. Adipocytes were able to inhibit preadipocyte differentiation in the co-culture, and this effect was abolished by blockade of the Ang II type 1 receptor. This finding points to a functional role of the RAS in the differentiation of human adipose tissue. Because AGT secretion and Ang II generation are characteristic features of adipogenesis, we postulate a paracrine negative-feedback loop that inhibits further recruitment of preadipocytes by maturing adipocytes. *Diabetes* 51:1699–1707, 2002

Obesity is well recognized as a major risk factor for the development of type 2 diabetes. Paradoxically, loss of adipose tissue (1) or impaired adipogenic differentiation (2) have been proposed as important mechanisms underlying the development of insulin resistance and type 2 diabetes (lipotoxicity hypothesis [3]). Adipogenesis results from a precise interplay of transcription factors, enabling preadipocytes to accumulate lipids and respond to insulin (4). Interestingly,

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AGT, angiotensinogen; Ang, angiotensin; DMEM, Dulbecco's modified Eagle's medium; HBSS, Hank's balanced salt solution; IBMX, isobutyl methyl xanthine; IRS-1, insulin receptor substrate 1; PI, phosphatidylinositol; PPAR- $\gamma$ , peroxisome proliferator-activated receptor- $\gamma$ ; RAS, renin-angiotensin system.

mature adipocytes secrete a host of vasoactive substances that influence adipogenesis (5), including the adipogenic vasodilators nitric oxide (6) and prostacyclin (7) and the antiadipogenic vasoconstrictor endothelin-1 (8), suggesting a paracrine role of vasoactive molecules in the regulation of adipocyte growth and differentiation.

Angiotensin II (Ang II), another vasoactive molecule, has also recently been implicated in the modulation of adipogenesis (9). Thus, mature adipocytes express all components of the renin-angiotensin system (RAS), including angiotensinogen (AGT), the sole precursor of Ang II, as well as the angiotensin peptide forming enzymes renin, ACE, and chymase. Adipocytes also express the type 1 (AT<sub>1</sub>) and type 2 (AT<sub>2</sub>) angiotensin receptor subtypes (10). AGT expression and secretion increases during adipogenesis (11–14), whereas suppression of the differentiation-specific element binding protein, the activator of differentiation-dependent AGT gene expression, suppressed AGT expression and adipogenesis in 3T3-L1 mouse clonal preadipocytes (15). Although previous studies in rodents pointed to adipogenic effects of Ang II by AT<sub>2</sub> receptor-mediated formation of prostacyclin by mature adipocytes (16,17), adipocyte-specific expression of the AGT gene in transgenic mice was accompanied by adipocyte hypertrophy and hypoplasia (18), suggesting that Ang II is a trophic factor for adipocytes. In contrast, human preadipocytes and adipocytes express almost exclusively the AT<sub>1</sub> receptor subtype (14,19), and Ang II was recently shown to reduce lipid accumulation in human preadipocytes committed to adipogenesis by insulin (20). Ang II (in the presence of FCS) also increased proliferation in human preadipocytes by stimulation of the cell cycle regulator cyclin D1 (21). Because several recent clinical studies suggest that pharmacological blockade of the RAS may prevent the development of type 2 diabetes (22,23), the intriguing hypothesis has emerged that these effects may be due (in part) to the influence of RAS blockade on human adipogenesis.

The aim of our study was to examine the effect of Ang II on adipogenic differentiation of human primary cultured preadipocytes. Because mature adipocytes secrete AGT and may thus serve as a local source of endogenous Ang II, we also examined the effect of mature adipocytes on the adipogenic differentiation of human preadipocytes.

## RESEARCH DESIGN AND METHODS

**Isolation of preadipocytes and mature adipocytes from human adipose tissue.** Human preadipocytes were isolated from subcutaneous adipose tissue obtained during breast reduction from healthy women (aged 20–60 years, BMI 22–35 kg/m<sup>2</sup>). After excision of blood vessels, adipose tissue was minced into small pieces, washed in Hank's balanced salt solution (HBSS) once, and

TABLE 1  
RT-PCR primers, probes, and templates

Gene	5' sequence 3'	Concentration (nmol/l)	cDNA (ng/ $\mu$ l)
AGT F	CTTCACTGAGAGCGCCTGC	900	
AGT R	GAGACCCTCCACCTTGTTCCA	900	2.5
AGT P	CTGATCCAGCCTCACTATGCCTCTGACC	225	
REN F	TTGGAGAGGTACGGAGATGC	50	
REN R	TGCCACAACCCCATCAAAC	900	2.5
REN P	CGCCTTACCCTTCATGCTGGCCG	100	
ACE F	CACCCAGGCCAGGAAGTTT	900	
ACE R	TGCCCGTTCTAGGTCCTGAA	900	1
ACE P	CCAGTTGCAGAACCACACTATCAAGCGG	175	
AT <sub>1</sub> F	ACCCAATGAAGTCCCGCCT	300	
AT <sub>1</sub> R	AGCAGCCAAATGATGATGCAG	900	1
AT <sub>1</sub> P	CGACGCACAATGCTTGATGCCAAAGTCA	175	
AT <sub>2</sub> F	CCAAGTCCTGAAGATGGCAGC	300	
AT <sub>2</sub> R	GGAAGGTGAGAATGGAAGGG	900	2.5
AT <sub>2</sub> P	TTGTTCTGGCCTTCATCATTTGCTGGC	100	
PPAR $\gamma$ F	TCAAACACATCACCCCTG	300	
PPAR $\gamma$ R	TGGCAGCCCTGAAAGATGC	900	1
PPAR $\gamma$ P	AGGAGCAGAGCAAAGAGGTGGCCATC	100	
LEP F	ACCAGGATCAATGACATTTACAC	50	
LEP R	CCAGGAATGAAGTCCAAACCG	300	2.5
LEP P	CGCAGTCAGTCTCCTCCAAACAGAAAGTCAT	250	
FAS F	TGGCAAATTCGACCTTTCTCA	50	
FAS R	GGACCCCGTGGAAATGTCAC	300	2.5
FAS P	ACCACCCGCTCGGCATGGC	250	

The cDNA concentration refers to the final concentration of cDNA equal to reverse-transcribed RNA in each PCR. F, forward primer; R, reverse primer; P, probe (labeled 5' FAM and 3' TAMRA).

centrifuged for 5 min at 380g. Tissue was then decanted into isolation medium (0.7 g tissue/ml HBSS supplemented with 25 mmol/l HEPES, 200  $\mu$ g/ml kanamycin, 100 units/ml penicillin, 100  $\mu$ g/ml streptomycin, 3% BSA, and 0.75 mg/ml collagenase) and digested for 1 h at 37°C with constant shaking. Suspended cells were then filtered through a 250- $\mu$ m nylon filter and spun for 10 min at 380g.

Adipocytes were decanted, washed, and spun (200g, 5 min) three times in isolation medium without collagenase. Then, 20 ml adipocytes were resuspended in 50 ml adipocyte medium overnight (Dubecco's modified Eagle's medium, [DMEM]/Ham's F12 [1:1], supplemented with 14 mmol/l NaHCO<sub>3</sub>, 16.5  $\mu$ mol/l biotin, 8.5  $\mu$ mol/l pantothenate, 15 mmol/l glucose, 2 mmol/l L-glutamin, 13.5 mmol/l HEPES, 200  $\mu$ g/ml kanamycin, 100 units/ml penicillin, 100  $\mu$ g/ml streptomycin, and 10% FCS). On the next day, adipocytes were spun (200g, 5 min), decanted, resuspended in adipocyte medium without FCS, washed three times, and cultured for 24 h in FCS-free adipocyte medium. Viability of isolated adipocytes was determined by acridine orange staining (24), revealing >90% metabolically active cells.

Stromavascular pellets were resuspended in 100 ml of erythrocyte lysis buffer (0.154 mol/l NH<sub>4</sub>Cl, 10 mmol/l KHCO<sub>3</sub>, 0.1 mmol/l EDTA, and 10% FCS) and allowed to settle for 10 min at room temperature, followed by a 10-min centrifugation at 380g. The cell pellet was resuspended in adipocyte medium. After filtration through a 20- $\mu$ m nylon mesh, cells corresponding to 50 g of adipose tissue were dispensed under constant stirring onto a 24-well plate (7  $\times$  10<sup>4</sup> cells per well) or two 24-cm<sup>2</sup> cell culture flasks (9  $\times$  10<sup>5</sup> cells), resulting in confluency. After an overnight rest, adherent preadipocytes were washed three times (short shaking) with FCS-free adipocyte medium and subsequently cultured for 24 h in the same medium. Repeated counting revealed equal cell numbers in the wells of multiwell plates.

Fluorescence-activated cell sorting analysis of isolated primary preadipocytes revealed <5% contamination with endothelial cells, thrombocytes, granulocytes, and B- and T-cells, and it also revealed ~10% contamination with monocytes. The antibodies used in this analysis were directed against CD62P (endothelial cells, activated thrombocytes), CD41 (thrombocytes), CD45 (leukocytes), CD19 (B-cells), CD3 (T-cells), and CD11B and CD14 (monocytes).

**Cell culture and differentiation assays.** Preadipocytes were incubated at 37°C in humidified atmosphere with 5% CO<sub>2</sub>. Cells were stimulated to differentiate over 8 or 12 days in differentiation medium (FCS-free adipocyte medium, supplemented with 1  $\mu$ mol/l insulin, 1 nmol/l triiodothyronine [T<sub>3</sub>], and 100 nmol/l hydrocortisone). Under these conditions, 60–80% of the cells were able to differentiate. Media were changed every 3 days. Cells for

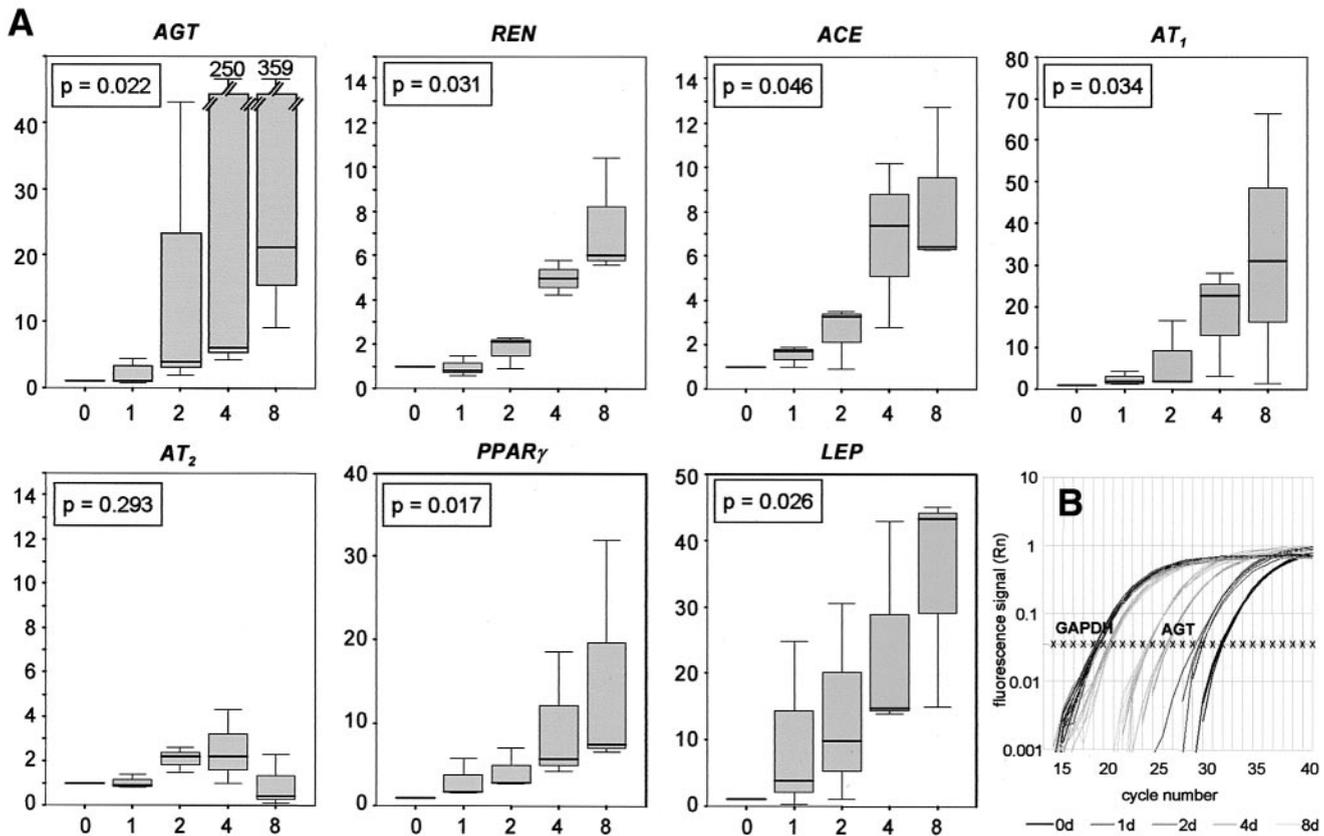
expression analysis were cultured in 24-cm<sup>2</sup> flasks, and cells for quantitation of lipid accumulation (see below) were cultured in 24-well plates. The influence of the RAS on adipogenesis was tested by supplementing the differentiation medium with Ang II, AGT, irbesartan (a specific AT<sub>1</sub> receptor antagonist [25]), PD-123,319 (a specific AT<sub>2</sub> receptor antagonist [26]), or captopril (a selective ACE inhibitor [27]) over the complete period of the experiment (four wells for each substance and concentration). Solvents like ethanol were without effect on adipogenesis (data not shown).

**Co-culture experiments.** Co-culture experiments were performed in 12-well dishes with polycarbonate membrane inserts (see Fig. 4A). The membrane pore size of 3.0  $\mu$ m permits free exchange of media between insert and well. Approximately 1.2  $\times$  10<sup>5</sup> preadipocytes/well were seeded under constant stirring, and 3  $\times$  10<sup>4</sup> adipocytes from the same subject were co-cultured in the insert (see Fig. 4A) under the already-described cell culture and differentiation conditions in FCS-free media. We also tested an alternative approach, in that equal cell numbers of undifferentiated or in vitro-differentiated preadipocytes (immature adipocytes) were cultured on the insert membrane. Unstimulated preadipocytes (background control), stimulated preadipocytes (differentiation control), stimulated preadipocytes co-cultured with adipocytes, and stimulated preadipocytes co-cultured with adipocytes in the presence of the AT<sub>1</sub>-receptor blocker irbesartan were compared at day 12.

**Gene expression analysis.** Preadipocytes were trypsinized, washed once with PBS, and pelleted (380g, 10 min) at 0, 1, 2, 4, and 8 days. RNA isolation, reverse transcription, and quantitation of gene expression analysis with the ABI 5700 sequence detection system for real time PCR (TaqMan) were performed as described previously (28).

Expression analysis was performed for the genes encoding AGT (AGT), ACE (ACE), renin (REN), AT<sub>1</sub>-receptor (AT<sub>1</sub>), and AT<sub>2</sub>-receptor (AT<sub>2</sub>) and also for the differentiation marker genes PPAR $\gamma$ , leptin (LEP), and fatty acid synthase (FAS). Human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was chosen as the endogenous control ("housekeeping gene") (29). Sequences and concentrations of primers, probes, and cDNA are given in Table 1.

**Quantitation of intracytoplasmic lipids.** Intracytoplasmic lipids were quantitated according to the method of Ramírez-Zacarias et al. (30). Immature adipocytes were fixed by the addition of glutaraldehyde to a final concentration of 3% for at least 2 h. Fixation solution was then replaced by 500  $\mu$ l of 60% isopropyl alcohol. After 5 min, isopropyl alcohol was evaporated and preadipocytes were stained in a working solution of 300  $\mu$ l Oil Red O for 2 h. The stain solution was removed, and cells were rinsed in 500  $\mu$ l of 60% isopropyl alcohol for 5 s. To extract dye, 700  $\mu$ l of 60% isopropyl alcohol was added per well and sealed plates were shaken for 2 h. The extracted dye was quantitated



**FIG. 1.** RNA expression analysis of RAS genes and adipogenic “marker-genes” *PPAR $\gamma$*  and *LEP* during human preadipocyte differentiation. **A:** Relative expression levels on days 1, 2, 4, and 8 after hormonal stimulation compared with unstimulated cells (day 0 = 1,  $n = 3$  for each gene). **B:** The amplification plot of *AGT* and the endogenous control *GAPDH* shown as an example. The threshold cycle ( $C_T$ ) indicates the fractional cycle number at which the amount of amplified target reached a fixed threshold (horizontal line) in the exponential amplification phase. One cycle difference ( $\Delta C_T = 1$ ) corresponds to a twofold increase or decrease of the mRNA template. The standard curves for *AGT* and *GAPDH* are not shown because they overlay the sample curves. The average of the quadruplicate determination of *AGT* and *GAPDH* expression in each sample was first aligned with their standard curve. After that, *AGT* expression was normalized by the expression of *GAPDH* (internal control).

spectrophotometrically at 510 nm. Lipid accumulation was compared between differentiated cells with and without RAS additives. Undifferentiated cells that served as background control were subtracted.

**Materials.** DMEM/Ham’s F12, HBSS, HEPES, PBS, FCS, collagenase, L-glutamin, penicillin, streptomycin, MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) proliferation kit, cell culture flasks, and 24-well plates were from Biochrom (Berlin). Insulin, hydrocortisone, triiodothyronine, pantothenate, biotin, glucose,  $\text{NH}_4\text{Cl}$ ,  $\text{KHCO}_3$ ,  $\text{NaHCO}_3$ , EDTA, glutaraldehyde, Oil Red O, isopropyl alcohol, AGT, Ang II, and PD-123,319 were from Sigma-Aldrich Chemie and Sigma RBI (Taufkirchen, Germany). PCR primers and kanamycin were from Life Technologies (Karlsruhe, Germany), BSA was from Biomol (Hamburg, Germany), nylon filters were from neoLab (Heidelberg, Germany), and co-culture 12-well plates were from Corning Costar (Bodenheim, Germany). Labeled oligonucleotides were from BioTez (Berlin). Irbesartan was provided by Sanofi Synthelabo (Berlin). Captopril was a gift from D.L. Crandall, Wyeth-Ayerst Research (Radnor, PA).

**Statistics.** The number of experiments was limited by the number of cells isolated from each subject. Consequently, primary cultures were established from different individuals, and all experiments were repeated at least three times. Interindividual differences in adipogenic potential were commonly observed, probably related to differences in body weight or age of the donors. Because of the large variation between experiments, data were analyzed by a nonparametric test for multiple related samples (Friedman’s test) and are presented as box and whisker plots (with median, 25th and 75th percentiles, and extremes). Statistically significant differences between the samples of each experiment were accepted at  $P$  values  $<0.05$ .

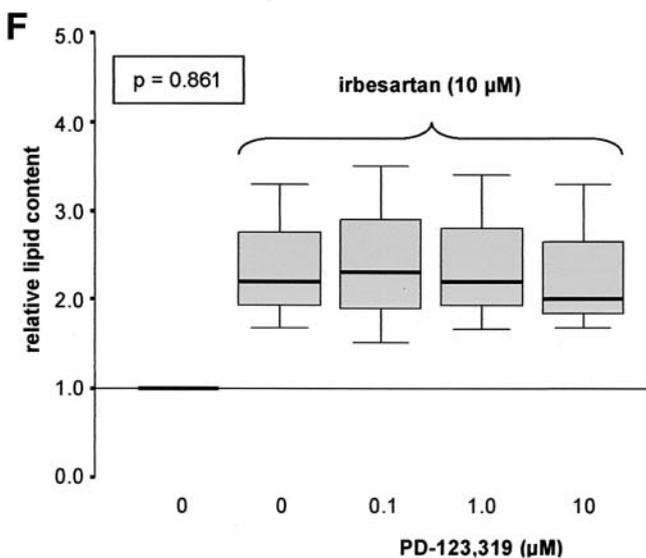
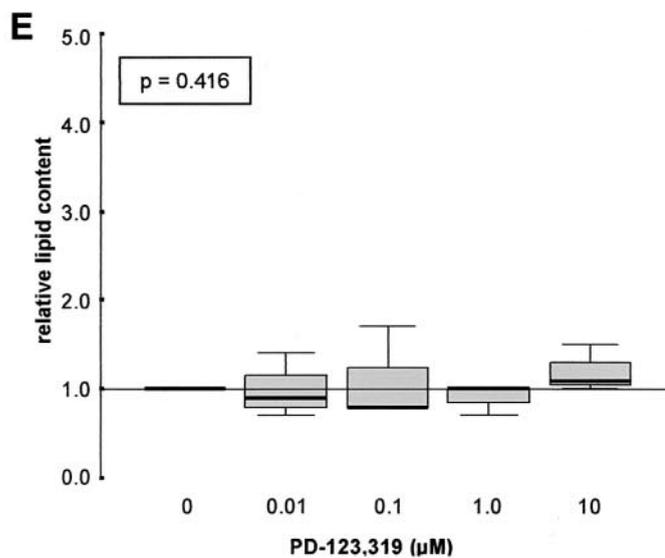
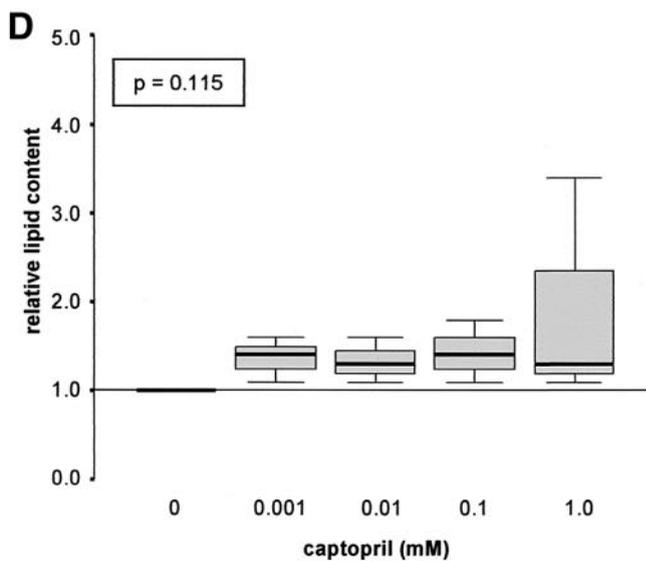
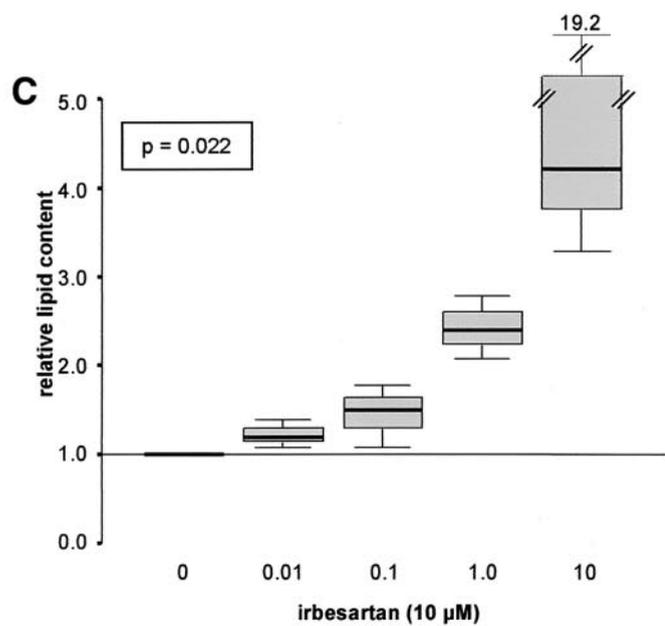
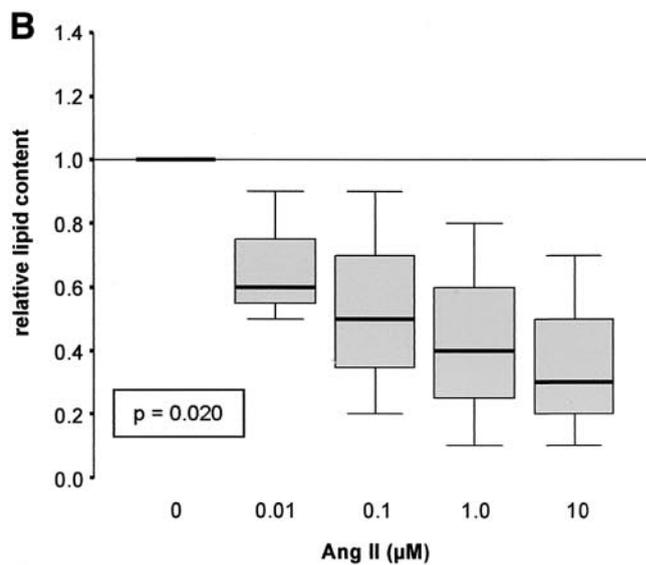
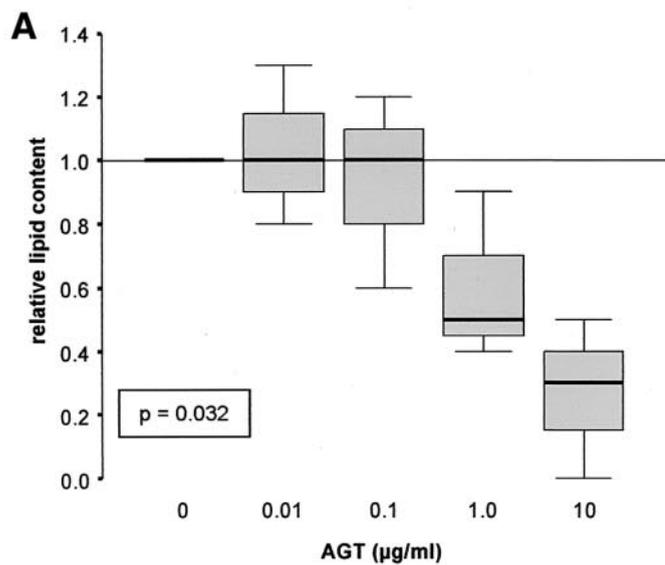
## RESULTS

### Influence of adipogenesis on RAS gene expression.

As expected, adipogenesis was accompanied by increased RNA expression of the early marker gene *PPAR $\gamma$*  and the

intermediate marker gene for leptin. Adipogenic differentiation was also associated with a marked increase in the RNA expression of *AGT*, *REN*, *ACE*, and *AT<sub>1</sub>* (Fig. 1). In contrast, *AT<sub>2</sub>* RNA expression was barely detectable in preadipocytes and increased only marginally during adipogenesis.

**Influence of Ang II on adipogenesis.** Incubation with Ang II or AGT resulted in a dose-dependent inhibition of adipogenic differentiation, despite concomitant stimulation with the differentiation cocktail (insulin + hydrocortisone +  $\text{T}_3$ ) (Fig. 2A and B). In contrast to stimulation with Ang II, *AT<sub>1</sub>* receptor blockade with irbesartan increased lipid accumulation (Fig. 2C). Increased lipid accumulation was attributable to both an increased number of differentiated cells and an increase in intracytoplasmic lipid accumulation (see also Fig. 4). Specific ACE blockade with captopril, although a strong adipogenic stimulus in some of the experiments, failed to reach statistical significance across all experiments. The antiadipogenic effect was dependent on the addition of the differentiation cocktail because *AT<sub>1</sub>* receptor blockade or ACE inhibition alone failed to induce lipid accumulation (data not shown). Inhibition of adipogenic differentiation by Ang II and stimulation by *AT<sub>1</sub>* receptor blockade was also accompanied by a respective decrease or increase in expression of the adipogenic marker genes *PPAR $\gamma$*  and *FAS* (Fig. 3).



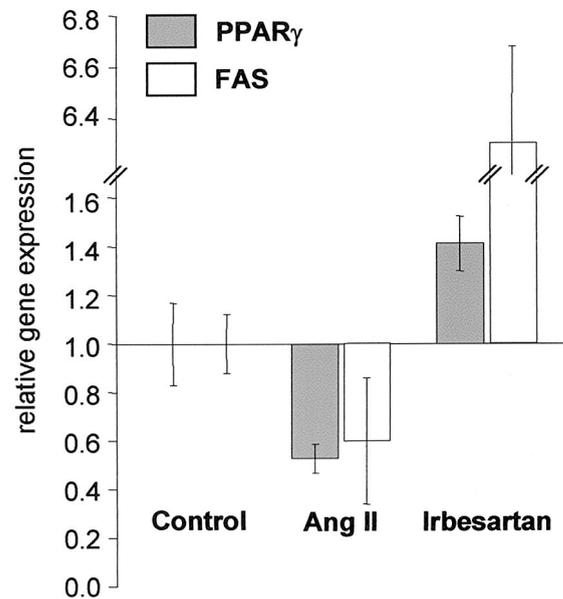
Specific  $AT_2$  receptor blockade with PD-123,319 had no effect on adipogenic differentiation and failed to counteract the effect of  $AT_1$  receptor blockade (Fig. 2E and F).

**Influence of mature adipocytes on adipogenesis (co-culture experiments).** Because Ang II inhibited in vitro adipogenesis and adipocytes are known to form Ang II, we tested the hypothesis that mature adipocytes inhibit adipogenesis via the  $AT_1$  receptor. In the presence of co-cultured mature adipocytes, adipogenic differentiation of preadipocytes was virtually halted, despite the presence of the differentiation cocktail (Fig. 4B). In contrast,  $AT_1$  receptor blockade abolished the inhibitory effect of mature adipocytes and resulted in a rapid and greater lipid accumulation than in controls. Similar results were obtained when in vitro differentiated immature adipocytes were used instead of mature adipocytes, but the inhibitory potency of these cells on adipogenesis was lower than that of mature adipocytes (Fig. 4C). Undifferentiated preadipocytes, co-cultured in the insert, did not influence in vitro adipogenesis, but rather underwent differentiation at rates comparable to those of seeded preadipocytes.

## DISCUSSION

This study demonstrates that Ang II inhibits adipogenic differentiation of primary cultured human preadipocytes. In addition, specific  $AT_1$  receptor blockade markedly augments lipid accumulation and differentiation of these cells. Importantly, co-culture with mature adipocytes resulted in inhibition of adipogenic differentiation, and this effect was again abolished by  $AT_1$  receptor blockade. Together with our observation that adipogenic differentiation is associated with increased expression of *AGT*, *REN*, and *ACE*, as well as the previous demonstration of increased Ang II formation during human preadipocyte differentiation by Schling et al. (14), our data clearly suggest that the ability of mature and maturing adipocytes to inhibit adipogenic differentiation was indeed mediated by Ang II. Combined, these findings support the hypothesis that the adipose tissue RAS plays a functional role in the regulation of adipogenesis in human adipose tissue. The fact that ACE inhibition was not as effective as  $AT_1$  receptor blockade in promoting adipogenesis may be attributable to the presence of other angiotensin peptide-forming enzymes, such as chymase or cathepsins (10), that may contribute to the endogenous formation of Ang II.

Interestingly, our results are in contrast to earlier findings in Ob1771 mouse adipocytes, where Ang II was shown to have indirect adipogenic properties, possibly mediated through activation of the  $AT_2$  receptor (16). Although the  $AT_2$  receptor has also been found in murine 3T3-L1 cells (31), our data do not support a role of this receptor in human preadipocyte differentiation. Thus,  $AT_2$  expression was barely detectable in these cells, and pharmacological inhibition had no effect on Ang II-induced inhibition or  $AT_1$  receptor blockade-induced stimulation of adipogenic differentiation. Whether these disparate findings represent species differences and/or differences between clonal cell

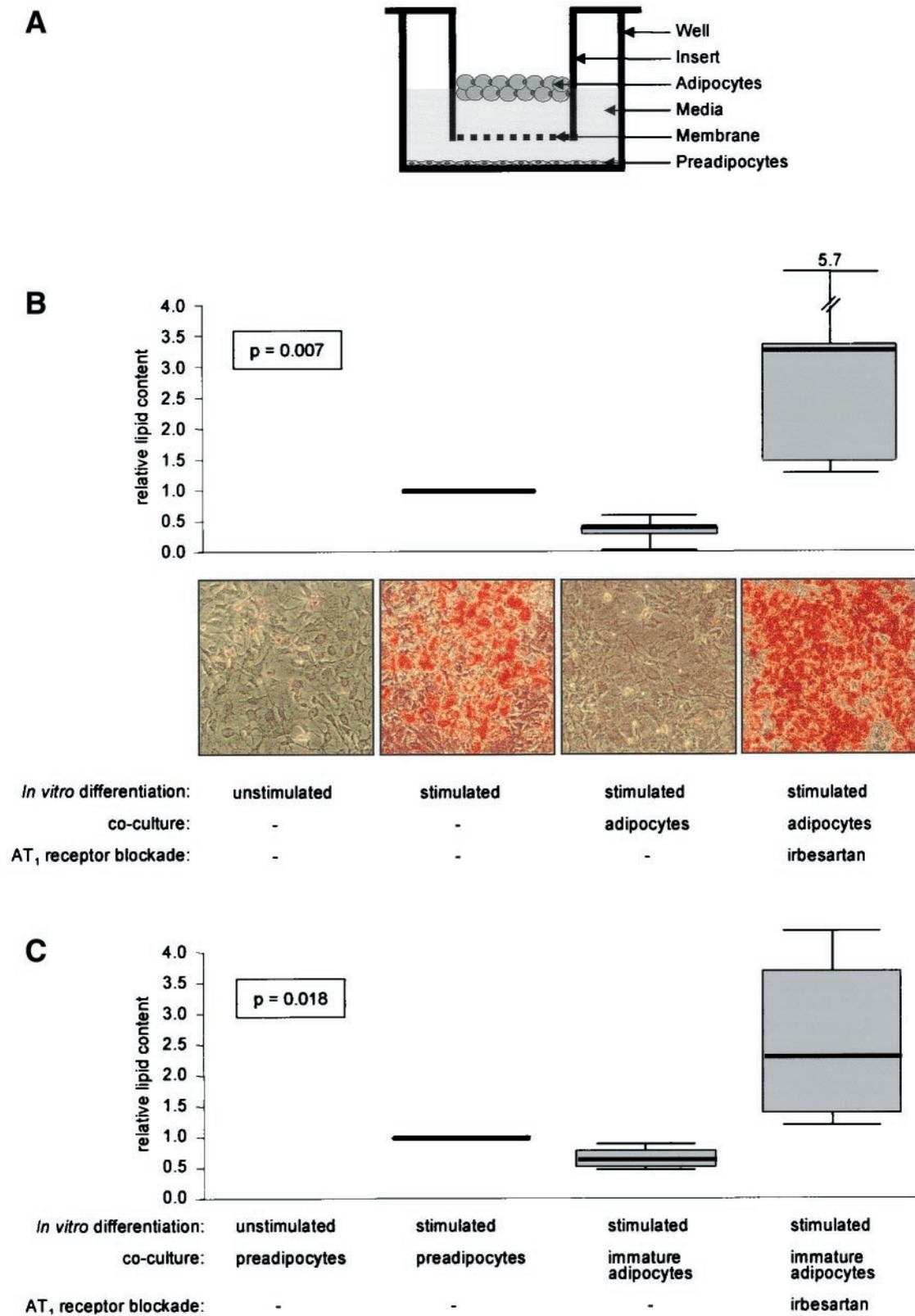


**FIG. 3.** Expression analysis of the adipogenic marker genes encoding *PPAR $\gamma$*  and *FAS* in preadipocytes at day 8 after induction of differentiation with insulin + hydrocortisone +  $T_3$ . Expression levels of cells differentiated in the presence of 10  $\mu\text{mol/l}$  Ang II ( $AT_1$  receptor agonist) or 10  $\mu\text{mol/l}$  irbesartan ( $AT_1$  receptor antagonist) were compared with those differentiated without irbesartan or Ang II (control). Data are means of quadruplicate determinations during a single experiment.

lines and primary cultured preadipocytes remains to be resolved. In human preadipocytes, Schling and Löffler (20) also described an inhibitory effect of Ang II on adipose conversion under similar conditions (differentiation with insulin), whereas differentiation in the presence of isobutyl methyl xanthine (IBMX) was not inhibited by Ang II. Thus, apparently IBMX, a strong inducer of intracellular cAMP concentrations, can “override” the inhibitory effects of Ang II. This is in line with the known differences between the adipogenic pathways induced by insulin and cAMP (32).

Despite the lucid nature of our observations, certain methodological issues deserve mention. The methods used in our study (isolation, in vitro stimulated differentiation, and Oil Red O staining of human primary preadipocytes) are based on well-established protocols (33). As shown by Ramírez-Zacarias et al. (30), quantitation of lipid accumulation by Oil Red O staining correlates well with the measurement of glycerophosphate dehydrogenase activity. Additionally, the antiadipogenic effects of Ang II (decreased expression of both markers compared with differentiating cells without Ang II) and the adipogenic effects of specific  $AT_1$  receptor blockade was demonstrated by expression analyses of the adipogenic marker genes *FAS* and *PPAR $\gamma$* . In this context, it may be of interest to note that 12–72 h of incubation with Ang II has been shown to directly influence *FAS* gene expression in differentiated 3T3-L1 cells and mature human adipocytes

**FIG. 2.** Influence of Ang II (A), AGT (B),  $AT_1$  receptor antagonist irbesartan (C), ACE inhibitor captopril (D),  $AT_2$  receptor antagonist PD-123,319 alone (E), and PD-123,319 combined with 10  $\mu\text{mol/l}$  irbesartan (F) on lipid accumulation in differentiating preadipocytes at day 12 (A, B, C, D, and F:  $n = 3$ ; E:  $n = 4$ ). Cells were stimulated to differentiate with insulin + hydrocortisone +  $T_3$ . The relative lipid content after in vitro differentiation with different concentrations of additives is compared with that of cells in pure differentiation medium. Statistical analysis was performed for the complete data set (A–E) and for the combination of irbesartan and PD-123,319 (F).



**FIG. 4.** Co-culture of differentiating preadipocytes and adipocytes. **A:** Schematic illustration of a co-culture well. The permeable membrane of the insert allows the free exchange of media between the two culture compartments. Preadipocytes were first seeded on the well bottom, and then the floating adipocytes were transferred into the insert (see **B**). Alternatively, preadipocytes were first allowed to differentiate to immature adipocytes on the insert membrane, and the insert was then placed into a well with undifferentiated preadipocytes seeded on the bottom (see **C**). **B:** Oil Red O lipid staining after 12 days. First column: unstimulated preadipocytes. Second column: differentiated preadipocytes after hormonal stimulation. Third column: preadipocytes cultured with 50  $\mu$ l of adipocytes failed to differentiate under the same conditions. Fourth column: the inhibitory influence of adipocytes was abolished by 10  $\mu$ mol/l of the AT<sub>1</sub> receptor blocker irbesartan ( $n = 5$ ). **C:** The same experimental conditions as reported for **B**, but with *in vitro* differentiated immature adipocytes in place of mature adipocytes. The inhibitory effect by immature adipocytes on lipid accumulation was less pronounced than that of mature adipocytes ( $n = 4$ ).

(34,35). It is thus important to distinguish between the induction of gene expression during adipogenesis (as measured in our study) and the hormonal modulation of a gene in mature cells that already express this gene.

Adipogenic conversion was observed in up to 80% of the isolated cells. To study the influence of the RAS on adipogenesis, we chose a cell culture model that allows the demonstration of both an increase as well as a decrease in adipogenic conversion. For this purpose, we used a hormonal cocktail that differs from that usually used in human preadipocytes (36) in that it did not include a xanthine derivative (see above) or a peroxisome proliferator-activated receptor- $\gamma$  (PPAR- $\gamma$ ) ligand. Such additives are often used to achieve maximum differentiation of preadipocytes and may thus override the effects of subtle modulators of adipogenic differentiation. Additionally, the use of only three naturally occurring hormones in the culture system minimizes interactions between the substances used to manipulate adipogenic stimulation *in vitro*. Importantly, a possible inhibitory influence of Ang II on PPAR- $\gamma$  activity is conceivable and may be confounded by the use of a strong PPAR- $\gamma$  agonist.

Although some contamination with other cells such as monocytes cannot be ruled out (see RESEARCH DESIGN AND METHODS), it is highly unlikely that the presence of such cells accounts for the dramatic effects of Ang II or AT<sub>1</sub> receptor blockade on adipogenic differentiation observed in our study. Furthermore, it is important to note that the adipogenic properties of primary cultured preadipocytes show considerable inter-individual variability. Nevertheless, all experiments were repeated at least three times with cells obtained from several different individuals and showed similar results in direction if not in magnitude.

Inhibition of adipogenic differentiation by mature adipocytes clearly suggests the presence of a paracrine negative feedback loop, by which increased production of AGT/Ang II results in the decreased recruitment of preadipocytes. This negative feedback loop can be overcome by AT<sub>1</sub> receptor blockade. We may thus speculate that although increased tissue activity of the RAS in adipose tissue can prevent the recruitment and adipogenic differentiation of preadipocytes, blockade of this system may result in the increased formation of fat cells. This notion is supported by a recent *in vivo* study demonstrating that selective overexpression of the *AGT* gene in adipocytes driven by the *aP2* promoter leads to adipocyte hypertrophy and hypoplasia, suggesting that Ang II has trophic rather than adipogenic effects (18). However, it is important to remember that weight gain can be caused by both an increase in the size of existing adipocytes and further recruitment of preadipocytes. Thus, changes in the formation of new fat cells must not necessarily be reflected in changes in total adipose mass, and the use of ACE inhibitors has occasionally been reported to be associated with decreased body weight (37–39).

Currently, we can only speculate on the mechanisms involved in the Ang II-mediated inhibition of adipogenic differentiation. Ang II has been demonstrated to decrease insulin-stimulated tyrosine phosphorylation of insulin receptor substrate 1 (IRS-1) and its docking with phosphatidylinositol 3 (PI-3) kinase in vascular smooth muscle

(40). Ang II has also been shown to increase serine phosphorylation of the insulin receptor, IRS-1, and PI-3 kinase, which in general has been shown to downregulate insulin receptor signaling (41). These effects, if also active in preadipocytes, may in part explain our findings because insulin is the key adipogenic substance in our assays. However, it is important to note that the crosstalk between Ang II, insulin receptors, and other proteins involved in growth and differentiation is complex and may involve a variety of pathways, including activator protein 1 (AP-1), signal transducer and activator of transcription (STAT), or nuclear factor- $\kappa$ B (42).

A possible clinical implication of our findings could well be that blockade of the RAS may increase the number of small, newly differentiated adipocytes, which are known to be more insulin-sensitive than older, larger adipocytes (43,44). This idea may indeed provide a novel explanation for the unexpected recent demonstration that RAS blockade may lower the risk for the development of type 2 diabetes (22,23). In fact, Danforth (2) recently proposed the intriguing idea that an impaired ability to differentiate new fat cells, possibly manifested as enlargement of existing ones, may in part play a role in the development of type 2 diabetes. The mechanisms behind this may be by promoting the storage of excess lipids in other organs like the liver, muscle, or pancreas (lipotoxicity hypothesis [3]). This hypothesis is also supported by the observation that a genetic form of lipodystrophy in the mouse can cause a syndrome of severe insulin resistance that can be largely reversed by implantation of adipose tissue (1). Furthermore, pharmacologically induced (45) or familial forms of partial lipodystrophy (46) have been associated with an increased risk of insulin resistance and the development of type 2 diabetes in humans, whereas induction of adipose tissue differentiation by thiazolidinediones is associated with increased insulin sensitivity (47). Finally, it has long been noted that the presence of large adipocytes, perhaps as a result of failure to produce new fat cells, is associated with the development of diabetes and the metabolic syndrome (48,49). Thus, as recently pointed out by Townsend (50), the adipose tissue RAS may serve more to regulate both regional blood flow to adipose tissue and the size and number of fat cells, rather than participate directly in the regulation of substrate utilization.

In conclusion, our data indicate that under our experimental conditions, adipocytes inhibit *in vitro* adipogenic differentiation of human preadipocytes via Ang II and the AT<sub>1</sub> receptor, whereas blockade of this receptor leads to a marked augmentation of adipogenesis. This finding points to a functional role of the RAS for the differentiation of adipose tissue. Future studies will be required to determine both the signaling pathways leading from the stimulation of the AT<sub>1</sub> receptor to the inhibition of human adipogenesis as well as the clinical implications of these findings for human health and disease.

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## REFERENCES

- Gavrilova O, Marcus-Samuels B, Graham D, Kim JK, Shulman GI, Castle AL, Vinson C, Eckhaus M, Reitman ML: Surgical implantation of adipose tissue reverses diabetes in lipoatrophic mice. *J Clin Invest* 105:271–278, 2000
- Danforth E: Failure of adipocyte differentiation causes type II diabetes mellitus? *Nat Genet* 26:13, 2000
- Unger RH: Lipotoxic diseases. *Annu Rev Med* 53:319–336, 2002
- Rosen ED, Spiegelman BM: Molecular regulation of adipogenesis. *Annu Rev Cell Dev Biol* 16:145–171, 2000
- Engeli S, Sharma AM: Role of adipose tissue for cardiovascular-renal regulation in health and disease. *Horm Metab Res* 32:485–499, 2000
- Nisoli E, Clementi E, Tonello C, Sciorati C, Briscini L, Carruba MO: Effects of nitric oxide on proliferation and differentiation of rat brown adipocytes in primary cultures. *Br J Pharmacol* 125:888–894, 1998
- Aubert J, Saint-Marc P, Belmonte N, Dani C, Negrel R, Ailhaud G: Prostacyclin IP receptor up-regulates the early expression of C/EBPbeta and C/EBPdelta in preadipose cells. *Mol Cell Endocrinol* 160:149–156, 2000
- Hauner H, Petruschke T, Gries FA: Endothelin-1 inhibits the adipose differentiation of cultured human adipocyte precursor cells. *Metabolism* 43:227–232, 1994
- Ailhaud G, Fukamizu A, Massiera F, Negrel R, Saint-Marc P, Teboul M: Angiotensinogen, angiotensin II and adipose tissue development. *Int J Obes Relat Metab Disord* 24 (Suppl. 4):S33–S35, 2000
- Engeli S, Negrel R, Sharma AM: Physiology and pathophysiology of the adipose tissue renin-angiotensin system. *Hypertension* 35:1270–1277, 2000
- Saye J, Lynch KR, Peach MJ: Changes in angiotensinogen messenger RNA in differentiating 3T3-F442A adipocytes. *Hypertension* 15:867–871, 1990
- Tamura K, Umemura S, Iwamoto T, Yamaguchi S, Kobayashi S, Takeda K, Tokita Y, Takagi N, Murakami K, Fukamizu A, Ishii M: Molecular mechanism of adipogenic activation of the angiotensinogen gene. *Hypertension* 23:364–368, 1994
- McGehee RE Jr, Habener JF: Differentiation-specific element binding protein (DSEB) binds to a defined element in the promoter of the angiotensinogen gene required for the irreversible induction of gene expression during differentiation of 3T3-L1 adipoblasts to adipocytes. *Mol Endocrinol* 9:487–501, 1995
- Schling P, Mallow H, Trindl A, Löffler G: Evidence for a local renin angiotensin system in primary cultured human preadipocytes. *Int J Obes Relat Metab Disord* 23:336–341, 1999
- Lyle RE, Habener JF, McGehee RE Jr: Antisense oligonucleotides to differentiation-specific element binding protein (DSEB) mRNA inhibit adipocyte differentiation. *Biochem Biophys Res Commun* 228:709–715, 1996
- Darimont C, Vassaux G, Ailhaud G, Negrel R: Differentiation of preadipose cells: paracrine role of prostacyclin upon stimulation of adipose cells by angiotensin-II. *Endocrinology* 135:2030–2036, 1994
- Saint-Marc P, Kozak LP, Ailhaud G, Darimont C, Negrel R: Angiotensin II as a trophic factor of white adipose tissue: stimulation of adipose cell formation. *Endocrinology* 142:487–492, 2001
- Massiera F, Bloch-Faure M, Ceiler D, Murakami K, Fukamizu A, Gasc JM, Quignard-Boulange A, Negrel R, Ailhaud G, Seydoux J, Meneton P, Teboul M: Adipose angiotensinogen is involved in adipose tissue growth and blood pressure regulation. *FASEB J* 15:2727–2729, 2001
- Engeli S, Gorzelnik K, Kreutz R, Runkel N, Distler A, Sharma AM: Co-expression of renin-angiotensin system genes in human adipose tissue. *J Hypertension* 17:555–560, 1999
- Schling P, Löffler G: Effects of angiotensin II on adipose conversion and expression of genes of the renin-angiotensin system in human preadipocytes. *Horm Metab Res* 33:189–195, 2001
- Crandall DL, Armellino DC, Busler DE, McHendry-Rinde B, Kral JG: Angiotensin II receptors in human preadipocytes: role in cell cycle regulation. *Endocrinology* 140:154–158, 1999
- Yusuf S, Sleight P, Pogue J, Bosch J, Davies R, Dagenais G: Effects of an angiotensin-converting-enzyme inhibitor, ramipril, on cardiovascular events in high-risk patients. *N Engl J Med* 342:145–153, 2000
- Hansson L, Lindholm LH, Niskanen L, Lanke J, Hedner T, Niklason A, Luomanmaki K, Dahlöf B, De Faire U, Morlin C, Karlberg BE, Wester PO, Björck JE: Effect of angiotensin-converting-enzyme inhibition compared with conventional therapy on cardiovascular morbidity and mortality in hypertension: the Captopril Prevention Project (CAPPP) randomised trial. *Lancet* 353:611–616, 1999
- Lorch E, Rentsch G: A simple method for staining and counting isolated adipose tissue fat cells. *Diabetologia* 5:356–357, 1969
- Markham A, Spencer CM, Jarvis B: Irbesartan: an updated review of its use in cardiovascular disorders. *Drugs* 59:1187–1206, 2000
- Bosse R, Servant G, Zhou LM, Boulay G, Guillemette G, Escher E: Sar1-p-benzoylphenylalanine-angiotensin, a new photoaffinity probe for selective labeling of the type 2 angiotensin receptor. *Regul Pept* 44:215–223, 1993
- Brogden RN, Todd PA, Sorkin EM: Captopril: an update of its pharmacodynamic and pharmacokinetic properties, and therapeutic use in hypertension and congestive heart failure. *Drugs* 36:540–600, 1988
- Janke J, Engeli S, Gorzelnik K, Luft FC, Sharma AM: Resistin gene expression in human adipocytes is not related to insulin resistance. *Obes Res* 10:1–5, 2002
- Gorzelnik K, Janke J, Engeli S, Sharma AM: Validation of endogenous controls for gene expression studies in human adipocytes and preadipocytes. *Horm Metab Res* 33:625–627, 2001
- Ramírez-Zacarias JL, Castro-Munozledo F, Kuri-Harcuch W: Quantitation of adipose conversion and triglycerides by staining intracytoplasmic lipids with Oil red O. *Histochemistry* 97:493–497, 1992
- Mallow H, Trindl A, Löffler G: Production of angiotensin II receptors type one (AT1) and type two (AT2) during the differentiation of 3T3-L1 preadipocytes. *Horm Metab Res* 32:500–503, 2000
- Hamun JK, Park BH, Farmer SR: A role for C/EBPbeta in regulating peroxisome proliferator-activated receptor gamma activity during adipogenesis in 3T3-L1 preadipocytes. *J Biol Chem* 276:18464–18471, 2001
- Strutt B, Khalil W, Killinger D: Growth and differentiation of human adipose stromal cells in culture. In *Human Cell Culture Protocols*. Jones GE, Ed. Totowa, NJ, Humana Press, 1996, p. 41–51
- Jones BH, Standridge MK, Moustaid N: Angiotensin II increases lipogenesis in 3T3-L1 and human adipose cells. *Endocrinology* 138:1512–1519, 1997
- Kim S, Dugail I, Standridge M, Claycombe K, Chun J, Moustaid-Moussa N: Angiotensin II-responsive element is the insulin-responsive element in the adipocyte fatty acid synthase gene: role of adipocyte determination and differentiation factor 1/sterol-regulatory-element-binding protein 1c. *Biochem J* 357:899–904, 2001
- Skurk T, Lee YM, Rohrig K, Hauner H: Effect of angiotensin peptides on PAI-1 expression and production in human adipocytes. *Horm Metab Res* 33:196–200, 2001
- McGrath BP, Matthews PG, Louis W, Howes L, Whitworth JA, Kincaid Smith PS, Fraser I, Scheinkestel C, MacDonald G, Rallings M: Double-blind study of dilevalol and captopril, both in combination with hydrochlorothiazide, in patients with moderate to severe hypertension. *J Cardiovasc Pharmacol* 16:831–838, 1990
- Enalapril in essential hypertension: a comparative study with propranolol. Enalapril in Hypertension Study Group (UK). *Br J Clin Pharmacol* 18:51–56, 1984
- Dominguez JR, de la Calle H, Hurtado A, Robles RG, Sancho-Rof J: Effect of converting enzyme inhibitors in hypertensive patients with non-insulin-dependent diabetes mellitus. *Postgrad Med J* 62 (Suppl. 1):66–68, 1986
- Velloso LA, Folli F, Sun XJ, White MF, Saad MJ, Kahn CR: Cross-talk between the insulin and angiotensin signaling systems. *Proc Natl Acad Sci U S A* 93:12490–12495, 1996
- Folli F, Saad MJ, Velloso L, Hansen H, Carandente O, Feener EP, Kahn CR: Crosstalk between insulin and angiotensin II signalling systems. *Exp Clin Endocrinol Diabetes* 107:133–139, 1999
- Brasier AR, Jamaluddin M, Han Y, Patterson C, Runge MS: Angiotensin II induces gene transcription through cell-type-dependent effects on the nuclear factor-kappaB (NF-kappaB) transcription factor. *Mol Cell Biochem* 212:155–169, 2000
- Hauner H, Röhrig K, Spelleken M, Liu LS, Eckel J: Development of insulin-responsive glucose uptake and GLUT4 expression in differentiating human adipocyte precursor cells. *Int J Obes Relat Metab Disord* 22:448–453, 1998
- Marin P, Andersson B, Ottosson M, Olbe L, Chowdhury B, Kvist H, Holm G, Sjöström L, Björntorp P: The morphology and metabolism of intraabdominal adipose tissue in men. *Metabolism* 41:1242–1248, 1992
- Vigouroux C, Gharakhanian S, Salhi Y, Nguyen TH, Chevenne D, Capeau J, Rozenbaum W: Diabetes, insulin resistance and dyslipidaemia in lipodystrophic HIV-infected patients on highly active antiretroviral therapy (HAART). *Diabetes Metab* 25:225–232, 1999
- Garg A: Gender differences in the prevalence of metabolic complications in familial partial lipodystrophy (Dunnigan variety). *J Clin Endocrinol Metab* 85:1776–1782, 2000

47. Okuno A, Tamemoto H, Tobe K, Ueki K, Mori Y, Iwamoto K, Umesono K, Akanuma Y, Fujiwara T, Horikoshi H, Yazaki Y, Kadowaki T: Troglitazone increases the number of small adipocytes without the change of white adipose tissue mass in obese Zucker rats. *J Clin Invest* 101:1354–1361, 1998
48. Leonhardt W, Hanefeld M, Haller H: Fat-cell volume in maturity-onset diabetes. *Lancet* 2:221, 1971
49. Weyer C, Foley JE, Bogardus C, Tataranni PA, Pratley RE: Enlarged subcutaneous abdominal adipocyte size, but not obesity itself, predicts type II diabetes independent of insulin resistance. *Diabetologia* 43:1498–1506, 2000
50. Townsend RR: The effects of angiotensin-II on lipolysis in humans. *Metabolism* 50:468–472, 2001