Human Adipose Tissue Cells Keep Tight Control on the Angiotensin II Levels in Their Vicinity*

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Petra Schling§§ and Thorsten Schäfer¶

From the Institute for Clinical Chemistry and Laboratory Medicine, University of Regensburg, Franz-Josef-Strauß-Allee 11, 93053 Regensburg and Biochemie-Zentrum (BZH), University of Heidelberg, Im Neuenheimer Feld 328, 69120 Heidelberg, Germany

Human adipose tissue expresses all components necessary for the local production of angiotensin II, which has multiple functions in adipose tissue, ranging from regulation of local blood flow to complex influences on tissue homeostasis. Still, the mechanisms controlling human adipose tissue angiotensin II concentrations are not yet known. We investigated whether angiotensin II is degraded by human primary cultured preadipocytes and adipocytes and which enzymes are responsible for its metabolism. Distinct but transient angiotensin II production was limited by degradation due to consecutive proteolytic cleavage by endopeptidase and aminopeptidase activities. The endopeptidase could be identified as neprilysin expressed on the surface of both preadipocytes and adipocytes. Degradation of angiotensin II was preceded by a lag phase that was considerably longer in preadipocytes. This time span could not be explained by an induction of neprilysin nor by an increase in its surface localization. Following the lag phase, adipocytes showed a higher degradation activity than preadipocytes as mirrored by increased neprilysin levels and activity measured in their membrane fractions. Our findings demonstrate that human preadipocytes and adipocytes differentially express functional neprilysin and aminopeptidase activity involved in the regulation of angiotensin II concentrations in human adipose tissue.

Angiotensin (Ang) peptides are the active products of the renin-angiotensin system (RAS), a peptidergic hormone system formerly thought to reside in the circulation only. Research over last decades has uncovered that the evolutionary origin of the RAS is the central nervous system, where Ang peptides control electrolyte and water balance in species as diverse as leeches and mammals (1). During the evolution of vertebrates the RAS was adapted to function in the circulation and other tissues as well, although with modifications.

Adipose tissue is one of those tissues to possess a local RAS. Angiotensinogen, the sole precursor for all Ang peptides, is synthesized and secreted by adipocytes from all species tested so far. Its production is up-regulated during the differentiation of preadipocytes and can therefore be considered a late marker of adipose conversion (2). Experiments with transgenic mice engineered to express angiotensinogen only in adipose tissue show that adipose tissue-angiotensinogen is released from adipose tissue into the bloodstream and plays a role in the circulating RAS as well (3). Next to angiotensinogen adipose tissue possesses proteases able to cleave angiotensinogen to Ang I or Ang II, namely renin (EC 3.4.23.15) (4–6), kallikrein (EC 3.4.21.34/35) (7), cathepsin D (EC 3.4.23.5) (4, 8), and cathepsin G (EC 3.4.21.20) (4). Interconversion of Ang I, which is physiologically inactive, to Ang II and alternative fragments can be achieved by aminopeptidases (AP), carboxypeptidases, and endopeptidases. AP that have been demonstrated to act on Ang peptides and to be present in adipose tissue are membrane alanine AP (EC 3.4.11.2) (9), adipocyte-derived leucine AP (EC 3.4.11.7) (10), and cystinyl-AP (EC 3.4.11.3) (11). Next to kallikrein and cathepsin G, which can act on Ang I in addition to angiotensinogen, dipeptidyl-dipeptidase A (EC 3.4.15.1), better known as angiotensin-converting enzyme (ACE), and chymase (3.4.21.39) both cleave Ang I to Ang II (12) and can be found in adipose tissue (5, 13). Ang II seems to be a hyperplastic and hypertrophic factor for murine adipose tissue (14) but inhibits adipose conversion of human preadipocytes (15).

Once created, signals also have to be switched off for effective signal transduction. Peptidergic hormones can be degraded by extracellular peptides or internalized by ligand-mediated receptor endocytosis to be hydrolyzed intracellularly. No information is yet available on the control of Ang II levels in adipose tissue. Still this issue is of vital importance to Ang II signal transduction in human adipose tissue, where it is thought to take part in the development and maintenance of diseases clustered in the metabolic syndrome (16).

In the present study we therefore investigated the production and degradation of Ang II by human preadipocytes and in vitro differentiated adipocytes. Human adipose tissue was chosen because of severe species differences to rodent models concerning the adipose tissue RAS. Renin, for example, cannot be detected in mouse adipocytes (8), but when human renin is expressed ectopically in mice, high expression levels of the transgene are found in white adipose tissue (17). ACE, on the other hand, is only present in the stromal vascular fraction of rat adipose tissue (18), although it is expressed in both preadipocytes and adipocytes in humans (5, 19). Therefore, only results obtained with human adipose tissue cells can be expected to be valid for the in vivo situation in man. By using primary isolated preadipocytes, secondarily differentiating them in vitro, we were able to disclose individual sequences of...
events avoiding the overall complexity of adipose tissue. The present study characterizes for the first time the enzymes responsible for Ang II degradation by human adipose tissue cells and defines their importance in regulating the extracellular Ang II concentration.

**EXPERIMENTAL PROCEDURES**

**Tissue Preparation and Cell Culture**—Human preadipocytes were isolated from subcutaneous adipose tissue obtained during abdominal or breast plastic reductive surgery from healthy women aged 17–59 years as described (5, 15). The basal serum-free defined medium (SDa medium) used in all cell culture experiments consisted of Dulbecco’s modified Eagle’s Medium/F-12 (3:1) without phenol red supplemented with 100 units/ml penicillin, 0.1 mg/ml streptomycin, 30 mM NaHCO3, 1 g/ml bovine, 17 mM pantothenate, 2 g/ml transferrin, and 1 g/ml insulin. For all experiments isolated, preadipose cells were placed into culture dishes in SDa medium supplemented with 10% fetal calf serum (FCS) to allow for overnight attachment. Three culture regimens were used to obtain preadipocytes, differentiating cells, and adipocytes. Preadipocytes were propagated to confluence in SDa medium supplemented with 10% FCS. On the day of confluence the medium was changed to SDa medium alone and the incubation continued for another 14 days. To obtain differentiating cells, the serum-containing medium was removed 1 day after cell preparation, and propagation was achieved in SDa medium supplemented with 1% (w/v) bovine insulin. During the entire culture period, media changes were conducted three times weekly and cells incubated in a humidified atmosphere with 5% CO2 at 37°C. Lysate identity was monitored by cell morphotype contrast microscopy.

**Cell Fractionations**—For the enzymatic and Ang II degradation measurements, preadipocytes and adipocytes were lysed by sonication (homogenate) followed by centrifugation at 4°C and 140,000 × g for 1 h. The resulting supernatant was collected (soluble cell fraction), and the pellet, corresponding to a crude membrane fraction, was resuspended in either enzyme assay buffer or SDa medium (membranes). For Western blots N1 fibroblast homogenates were fractionated by sequential centrifugation at 4°C as follows: 15 min at 1,000 × g, 15 min at 16,000 × g, 30 min at 100,000 × g. The supernatant of the last centrifugation step was referred to as “soluble cell fraction.” As sonication was the means of initial cell breakage, both the plasma membrane and organelles will have been ruptured into fragments of variable size and might therefore be contained in the 16,000 × g pellet as well as the 140,000 × g pellet. Protein concentrations were determined by the method of Bradford (20).

**Specific Enzyme Activity Measurements**—14 days after having reached confluence, on the day of the experiment, preadipocytes and adipocytes were harvested and cell fractions subsequently diluted to 200 μg/ml. All enzyme assays were performed at room temperature. The glycerol-3-phosphate dehydrogenase (GPDH) assay was performed essentially as described (21). AP activity was determined with five different amino acid-para-nitroanilides (pNA) as artificial substrates. 200 μl of reaction mixture in assay buffer (0.01 μM NaCl, 0.05 μM Tris-HCl, pH 7.4) contained the indicated concentrations of substrate (0.1–8 μM) and 25 μl of diluted cell fractions. An increase in absorbance at 405 nm was followed for 60 min and ΔA/min determined from the linear part of the curve. Neprilysin activity was determined as described (22), with Suc-Ala-Ala-Phe-pNA as substrate (23). To cleave the resulting Phe-pNA 5 μl of 0.5 mg/ml membrane alanine AP (Sigma) was included in the test, which had been shown earlier to be well above the expected activity of neprilysin. Because the AP itself slowly liberates pNA from Suc-Ala-Ala-Phe-pNA, a control reaction was always included in the measurement to be subtracted from the experimental measurements.

**Ang II Determination by Enzyme Immunoassay**—Per 10-ccm cell culture dish, 2 ml of SDa medium with or without 0.5 nM Ang II were added either 14 (preadipocytes/adipocytes) or 2 days (differentiating cells) post-confluence, and 125- (degradation measurements) or 400-μl aliquots (Ang II secretion measurements) were taken at the time points indicated under “Results.” The aliquots were mixed with 4 volumes of ice-cold ethanol and centrifuged at 4°C, 2000 × g for 15 min, and the supernatant was lyophilized. The lyophilisate was dissolved in EIA buffer, and Ang II concentrations were determined with the commercially available Ang II EIA kit (Bachem, Germany) according to the manufacturer’s instructions. Medium controls were always included and subtracted from each sample.

**High Performance Liquid Chromatography Detection of Ang II Fragments**—Medium aliquots were prepared as described for Ang II measurements by EIA, with the exception of Ang II concentration, which was 0.5 nM instead of 0.5 nM. Lyophilisates were dissolved in 125 μl of high performance liquid chromatography (HPLC) solution A (30 mM formic acid/50 mM ammonium formate/2 mM thiourea) immediately before injection. For reverse phase HPLC separation of Ang II and its fragments, a C18 column and a linear gradient of acetonitrile at 0.5 μl/min were used. The detector was set to read the absorbance at 232 nm. The injection of the sample at 100% solution A was followed by a linear gradient, increasing solution B (100% acetonitrile) from 0% to 20% over 15 min. This ratio was maintained for 10 min, in which the last peak, Ang II, eluted. The column was washed by increasing solution B to 100% within 15 min and maintaining this concentration for 10 min. No prominent peaks were ever observed during the regeneration of the column.

**Neprilysin Detection by Western Blots**—Preadipocytes, adipocytes, N1 fibroblasts, and rat kidney were lysed by sonication. Cell fractions were centrifuged with Suc-Ala-Ala-Phe-pNA as described. These preliminary results with N1 cells showed no necessity for membranes preparations (Fig. 5A). The protein concentration of cell lysates was determined by the method of Bradford (20), and equal loading was verified by Coomassie staining of a second gel run in parallel (not shown). 20 μg of N1 lysates, 1 μg of rat kidney lysates, and 2 μg of adipocyte and preadipocyte lysates were routinely used for SDS-PAGE (24). The separated proteins were electroblotted onto nitrocellulose, followed by incubation in blocking solution (PBS containing 1% (w/v) bovine serum albumin and 0.2% Nonidet P-40) for 2 h at room temperature, overnight incubation at 4°C in blocking solution containing either mouse monoclonal antibody NCL-CD10-270 (Novoceastra, UK), diluted 1:200, or rabbit polyclonal antibody sc-9149 (Santa Cruz Biotechnology), diluted 1:100. Washed membranes were incubated with the corresponding horseradish peroxidase-linked secondary antibodies at 4°C for 1 h (Sigma). Membranes were washed again before treatment with enhanced chemiluminescence reagent and exposure to x-ray film.

**Neprilysin Detection by Immunohistochemistry**—14 days after reaching confluence on collagen I-coated coverslips, cells were incubated for 0.5 h in SDa medium containing 0.5 nM Ang II, washed with physiological NaCl solution, and fixed overnight at 4°C in 4% formaldehyde in PBS. All further incubation steps were performed at room temperature in 1% (w/v) bovine serum albumin in PBS if not specified otherwise. Formaldehyde was washed off with PBS, and cells were incubated with 0.005% (w/v) saponin in PBS for 30 min for permeabilization with PBS alone to be used for immunostaining of the plasma membrane was not desired. Unspecific binding was blocked for 30 min, followed by incubation of the cover slips with the primary antibody (NCL-CD10-270, 1:5; Novoceastra, UK) for 2 h, a 5-min wash repeated twice, incubation with the secondary antibody (IgG fluorescein isothiocyanate-conjugated, 1:5; Sigma), and washing three times for 10 min. Cells were mounted with the slow fade light anti-fade kit (Molecular Probes) and photographed.

**Data Analysis**—Calculations of arithmetic means, S.E., and Student’s tests were performed with the SPSS software from SPSS, Inc. (Chicago). The level of significance was set to 5%. “n” depicts individual repeats of the respective experiment, each with cells isolated from a different tissue preparations. Graphic representation of data was achieved with the software program Sigma Plot from SPSS, Inc. (Chicago). Densitometric scanning of Western blots was performed with the Bio 1D densitometric system from Vilbert Lourmat (Marne la Vallee, France).

**RESULTS**

**In Vitro Differentiation of Human Preadipocytes**—Human preadipocytes were kept in a fibroblast-like cell morphology by propagation in SDa medium containing 10% FCS. The same cells, cultured in basal serum-free defined medium and induced with IBMX and cortisol for 3 days after reaching confluence, differentiated into adipocytes as monitored by intracellular lipid accumulation (not shown) and increased specific GP DH
activity. Specific GPDH activity was highest in the soluble fraction of cell lysates and was significantly elevated in adipocytes (Table I), as expected for a cytosolic adipogenic differentiation marker.

**Human Adipose Tissue Cells Secrete Ang II—** Ang II concentrations were determined in basal serum-free defined cell culture medium conditioned by preadipocytes, adipocytes, and cells stimulated for 2 days with cortisol and IBMX. Concerning the first 9 h of observation, an accumulation of Ang II was found that was followed by a gradual decline in the concentration of the hormone. This finding holds true for all cell types tested (Fig. 1A). Whereas maximal Ang II levels were comparable in preadipocytes and adipocytes, they were significantly lower in cells treated with IBMX and cortisol for 2 days post-confluence. This is in contrast to results obtained previously with 3T3-L1 cells (25). In an additional experiment confluent preadipocytes and adipocytes were incubated in SD6 medium containing no additives, cortisol, IBMX, or both for 2 days. Two hours after addition of fresh media, no alteration in Ang II levels was found (data not shown).

**Ang II Disappears from Cell Culture Medium—** Preadipocytes as well as adipocytes metabolized Ang II when exogenously added to the cell culture medium (Fig. 1B). 0.5 nM Ang II was diminished to basal levels within 5 h on differentiating cells and within 10 h upon addition to adipocytes, while it took 24–36 h with preadipocytes. There are three possible explanations for this discrepancy. First, confluent differentiating cells and adipocytes possess a higher specific metabolizing activity toward Ang II. Second, differentiating cells and adipocytes have a higher overall protein content than preadipocytes at confluence. Third, differentiating cells and adipocytes start the degradation prior to preadipocytes. Two of these possibilities were shown to apply, although the specific Ang II degrading activity was only slightly elevated in differentiating cells/adipocytes (23.05 ± 6.3 nanounits/g in differentiating cells and 21.1 ± 3.5 nanounits/g in adipocytes versus 15.2 ± 2.8 nanounits/g in preadipocytes; not significant (p = 0.341/0.676))²; the whole cell protein content was elevated in differentiating cells and significantly higher in adipocytes (0.18 ± 0.04 mg/dish in differentiating cells and 0.21 ± 0.02 mg/dish in adipocytes versus 0.13 ± 0.02 mg/dish in preadipocytes; p = 0.291/0.005). This consequently led to an increased absolute metabolization rate in differentiating cells and adipocytes (0.23 ± 0.02 nmol/h in differentiating cells and 0.24 ± 0.04 nmol/h in adipocytes versus 0.08 ± 0.006 nmol/h in preadipocytes; p = 0.0077/0.003).² Next, the lag phase (Fig. 1B, inset) preceding the onset of Ang II degradation was much longer for preadipocytes than for differentiating cells and adipocytes (<1 h for differentiating cells and 3.35 ± 0.39 h for adipocytes versus 8.20 ± 1.07 h for preadipocytes; p < 0.001). Therefore, Ang II disappearance from the culture medium began earlier and proceeded

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² The measurement of Ang II degrading activity was done right after termination of the lag phase, when the degradation rate was maximal. The units are used as follows: nanounits/g, 10⁻¹² mol of Ang II degraded per min per g of cellular protein; nmol/h, 10⁻⁶ μmol Ang II degraded within 1 h.

**Table I**

<table>
<thead>
<tr>
<th>Cell fraction</th>
<th>Preadipocytes</th>
<th></th>
<th>Adipocytes</th>
<th></th>
<th>Difference (p)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Specific activity</td>
<td>% maximum activity</td>
<td>Specific activity</td>
<td>% maximum activity</td>
<td></td>
</tr>
<tr>
<td>Homogenate</td>
<td>61.5 ± 38.8</td>
<td>53.9</td>
<td>1622.6 ± 457.4</td>
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</tr>
<tr>
<td>Membranes</td>
<td>17.7 ± 9.7</td>
<td>15.6</td>
<td>452.2 ± 184.4</td>
<td>15.6</td>
<td>0.149</td>
</tr>
<tr>
<td>Soluble fraction</td>
<td>113.7 ± 83.2</td>
<td>100</td>
<td>2899.5 ± 793.4</td>
<td>100</td>
<td>0.007</td>
</tr>
</tbody>
</table>

**Fig. 1.** Ang II production and degradation by human adipose tissue cells. A, time course of Ang II accumulation in cell culture media. Ang II-free SD6 medium was added onto preadipocytes, adipocytes, and onto differentiating cells that had been stimulated for 2 days post-confluence with 100 nM cortisol and 0.5 mM IBMX. B, time course of degradation of externally added Ang II (0.5 nM). Inset, same data as in main figure but with different scaling of the x axis. Ang II concentrations were quantified by EIA. (n = 4 for preadipocytes and adipocytes and n = 3 for differentiating cells, mean ± S.E.; exceptions: #, p < 0.05 versus preadipocytes and adipocytes.)
FIG. 2. Ang II disappearance from the cell culture medium can be prevented by metalloprotease inhibitors and is cell membrane-associated. A and B, preadipocytes/adipocytes were treated with 0.5 nM Ang II and either protease inhibitors or receptor blockers in the concentrations indicated for 12/8 h, respectively. (n = 2, mean ± S.E.) C and D, preadipocytes/adipocytes were treated with 0.5 nM Ang II and either phosphoramidon or thiorphan in the concentrations indicated for 9/4.5 h, respectively. Ang II concentrations were quantified by EIA. E and F, cells were preincubated for 9 (preadipocytes) or 4.5 h (adipocytes), or not, with 0.5 nM Ang II. One 10-cm² dish of preadipocytes/adipocytes was then again supplemented with 0.5 nM Ang II (intact cells), whereas a second parallel dish was fractionated into the 2-day conditioned medium, whole cell homogenate, crude membrane fraction, and the soluble fraction. Care was taken to adjust the respective volumes so to be comparable with the 2 ml/dish ratio of intact cells. Incubations were performed at 37 °C for 2 h and values expressed as % of the starting concentration of Ang II. (n = 3, mean ± S.E.)
at a higher rate with differentiating cells and adipocytes (Fig. 1B).

The Disappearance of Ang II Is Due to Extracellular Proteolytic Degradation—Ang II disappearance from the cell culture medium can either be caused by ligand-mediated receptor endocytosis or by extracellular degradation catalyzed by secreted or membrane-based ectopeptidases. To distinguish between these two possibilities Ang II was added in combination with diverse protease inhibitors and AT1- and AT2-receptor blockers. Although the receptor blockers losartan (AT1-specific) and PD 123319 (AT2-specific) alone or in combination had no effect on the disappearance rate of Ang II, protease inhibitors stabilized Ang II levels in preadipocyte and adipocyte supernatants to a variable degree (Fig. 2, A and B). The two metalloprotease inhibitors phosphoramidon and thiorphan were especially effective, even at nanomolar concentrations (Fig. 2, C and D). Nevertheless, a receptor-independent uptake of Ang II as well as intracellular effects of the protease inhibitors used here cannot be ruled out completely. To address the question if proteolysis occurs in the extracellular space or at the cell surface, the Ang II-degrading capability of different cell fractions, conditioned medium alone, and intact cells was compared (Fig. 2, E and F). As expected, neither SD6 medium alone nor the soluble cell fraction showed considerable degradation of the added Ang II. Instead, intact cells and cell homogenates are both active toward Ang II and degrade 40–60% of the added peptide within 2 h. Unexpectedly, 2 h after mixing the crude membrane fraction with SD6 medium containing 0.5 mM Ang II, 0.55–0.7 mM Ang II was measured. Still this value dropped to 0.1–0.2 mM Ang II when membranes were incubated for 12 h instead (data not shown).

Identity of the Ang II Fragments—The absorbance of Ang II and its fragments can be detected at 232 nm following separation by HPLC. To obtain fragment concentrations well above the detection limit, a supraphysiological Ang II concentration of 0.5 mM had to be used. With these experimental settings, four additional peaks could be detected next to the expected Ang II signal and the three medium peaks (Fig. 3). Peaks 4, 5, and 8 were identified by mass spectrometry and Edman protein sequencing as Ang(1–4)/Asp-Arg-Val-Tyr, Ang(6–8)/His-Pro-Phe, and Ang II, respectively. Peaks 2 and 6 had identical retention times as Ang(3–4)/Val-Tyr and Ang(5–8)/Ile-His-Pro-Phe, respectively. Ile eluted together with peak 1 and is therefore not displayed here.

Chronological Order of Individual Degradation Steps—When interpreting the time course of appearance and disappearance of the Ang II fragments (Fig. 4, A and B), it was found for both preadipocytes and adipocytes, that Ang(1–4) and Ang(5–8) appeared first, followed by Ang(3–4) and Ang(6–8). Ang II was thus first cleaved centrally to yield two tetrapeptides, which were secondarily degraded themselves. Ang(1–4) was cleaved to Ang(3–4) and either Asp and Arg or the dipeptide Ang(1–2)/Asp-Arg. Ang(5–8) was cleaved to Ile and Ang(6–8).
The turnover rates of Ang(5–8) in preadipocytes and adipocytes and of Ang(6–8) in preadipocytes approximately equaled their rates of synthesis, as hardly any accumulation of these peptides was found. Formation and degradation of the other fragments was about three times slower in preadipocytes than in adipocytes. In preadipocytes Ang(1–4) and Ang(5–8) were maximal 36 h after Ang II had been added to the medium, followed by the Ang(3–4) peak another 12 h later (Fig. 4A). In adipocytes Ang(1–4) and Ang(5–8) were maximal ~7 h after Ang II addition to the medium, followed by the Ang(3–4) and Ang(6–8) peak 5 h later (Fig. 4B).

**Functional Neprilysin Is Present on the Plasma Membrane of Human Preadipocytes and Adipocytes**—Four metalloendopeptidases are known to cleave Ang II into the fragments Ang(1–4) and Ang(5–8) observed here; neprilysin (EC 3.4.24.11), thimet oligopeptidase (EC 3.4.24.15), neurolysin (EC 3.4.24.16), and meprin A (EC 3.4.24.18) (26). Whereas thimet oligopeptidase and neurolysin are intracellular enzymes (27), meprin A is insensitive to phosphoramidon (28) and thiorphan (29). We therefore examined if neprilysin is expressed in human preadipocytes and in *in vitro* differentiated adipocytes. We detected high levels, comparable with those of rat kidney and about 10 times higher than those found in fibroblasts, of neprilysin in whole cell lysates by Western blot (Fig. 5, C and D). On the contrary, the NCL-C10-270 antibody showed no immunoreactivity toward the soluble cell fraction of fibroblasts (Fig. 5A), preadipocytes, and adipocytes (data not shown). A second antibody against neprilysin (sc-9149, Santa Cruz Biotechnology) also did detect a protein in the expected molecular weight range, although with far less specificity, as two additional bands were observed in the soluble cell fraction (Fig. 5B). The band believed to be immunoreactive neprilysin is seen in the cell homogenate (lane 1), the 16,000 × g fraction (lane 3), and the 140,000 × g fraction (lane 5). For further studies only the NCL-C10-270 antibody was used. Adipocytes expressed twice as much neprilysin per whole cell protein than preadipocytes. Neither in adipocytes nor in preadipocytes did neprilysin levels alter upon exposure to 0.5 nM Ang II.

By immunohistochemistry neprilysin was found in punctate intensities on the cell surface of preadipocytes and adipocytes (Fig. 6, A–C), similar to the pattern previously described for human neutrophils (30). The pattern was not influenced by permeabilization of the cell membrane with saponin, indicating that no additional intracellular neprilysin stores exist that could serve as reservoirs for rapid alterations in neprilysin membrane levels. Neither was any difference seen in cells pretreated with 0.5 nM Ang II (data not shown).

Neprilysin activity was highest in the membrane fraction of preadipocytes as compared with whole cell lysates and the soluble cell fraction (Table II) and inhibited by nanomolar concentrations of phosphoramidon and thiorphan (Table III). Neprilysin activity in preadipocyte membranes was only 70%
Angiotensin II Metabolism of Human Adipose Cells

Preadipocytes were grown to confluence in SD₆ medium containing 10% FCS and then kept in basal serum-free defined medium. Adipocytes were obtained by growing preadipocyte cells of the same tissue preparations in SD₆ medium supplemented with bFGF and cortisol for proliferation and with IBMX and cortisol for induction of differentiation. Both cell populations were harvested, fractionated, and specific peptidase activities measured 14 days post-confluence. Substrate concentrations were 50 μM Suc-Ala-Ala-Phe-NA for neprilysin- and 4 mM Leu-NA for AP measurements. For % maximum activity, absolute values were also related to the cell fraction of highest activity, i.e. the membrane fraction, and accordingly expressed in percent as well.

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<th>Cell fraction</th>
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<th>Aminopeptidase</th>
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<tr>
<td></td>
<td>Specific activity</td>
<td>% maximum activity</td>
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<tr>
<td></td>
<td>milliunits/mg</td>
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<tr>
<td>Preadipocyte</td>
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<td>Homogenate</td>
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<td>Preadipocyte membranes</td>
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<tr>
<td>Preadipocyte soluble fraction</td>
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<td>Adipocyte membranes</td>
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n = 8, mean ± S.E.

TABLE III

Inhibition of neprilysin and aminopeptidase activity

Preadipocytes were grown to confluence in SD₆ medium containing 10% FCS and then kept in basal serum-free defined medium. Adipocytes were obtained by growing preadipocyte cells of the same tissue preparations in SD₆ medium supplemented with bFGF and cortisol for proliferation and with IBMX and cortisol for induction of differentiation. Both cell populations were harvested, fractionated, and specific peptidase activities measured 14 days post-confluence. Substrate concentrations were 50 μM Suc-Ala-Ala-Phe-NA for neprilysin measurements (n = 3, mean ± S.E.) and 1 mM Leu-NA for AP measurements (n = 4, mean ± S.E.). For % maximum activity, absolute values were also related to the samples of highest activity, i.e. without inhibitors, and accordingly expressed in percent as well.

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<td>4.17 ± 0.52</td>
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<td>Thiorphan</td>
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<td>Phosphoramidon</td>
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<td>8.1 ± 1.6</td>
<td>17.5</td>
<td></td>
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<tr>
<td>1000</td>
<td>1.5 ± 0.3</td>
<td>3.3</td>
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<tr>
<td>Leucinethiol</td>
<td>μM</td>
<td>milliunits/mg</td>
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<tr>
<td>0</td>
<td>51.1 ± 10.1</td>
<td>100</td>
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<tr>
<td>0.01</td>
<td>38.1 ± 8.9</td>
<td>74.4</td>
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<tr>
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<td>6.3 ± 0.9</td>
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<tr>
<td>1</td>
<td>1.6 ± 0.4</td>
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</table>

that found in adipocyte membranes (Table II), which correlates well with the Western blot data.

Characterization of the Membrane AP Activity—Isoleucine was found to be cleaved off from Ang(5–8) both in preadipocyte and adipocyte samples (Fig. 4). Neprilysin, being an endopeptidase, cannot be responsible for this amino-terminal degradation, however. We therefore tested different cell fractions for AP activity toward five different synthetic amino acid-NA substrates and compared the results with those for commercially available membrane alanine AP. Asp-NA and Ile-NA were very poor substrates for the tested AP activities (Fig. 7). Ala-, Arg-, and Leu-NA were cleaved in the following rank order: Ala-NA ≈ Arg-NA > Leu-NA for membrane alanine AP (Fig. 7A) and Ala-NA > Leu-NA > Arg-NA for membrane and soluble neutral AP of preadipocytes (Fig. 7, B and C). Neutral AP activity was highest for membrane fractions but very low in the soluble fraction (Table II). Preadipocyte membranes exhibited nearly three times more AP activity than adipocyte membranes (Table II). This activity was inhibited by micromolar concentrations of bestatin and by nanomolar concentrations of leucine thiol (Table III). To test the effect of AP inhibitors on the accumulation of Ang II fragments, HPLC measurements were performed with cells exposed to 0.5 mM Ang II with or without 100 μM bestatin and 10 mM leucine thiol, respectively. As expected, neither inhibitor affected the turnover of Ang II. Its fragments, however, were all stabilized to different degrees by AP inhibition, with leucine Thiokol being more effective than bestatin; 24 h after addition of 0.5 mM Ang II and 10 mM leucine thiol to the preadipocyte medium Ang(1–4)/Asp-Arg-Val-Tyr levels were 125%, Ang(5–8)/Ile-His-Pro-Phe levels 160%, Ang(3–4)/Val-Tyr levels 140%, and Ang(6–8)/His-Pro-Phe levels 160% of those found upon addition of Ang II...
alone. The effect of AP inhibition was thus most clearly seen for the two fragments of lowest accumulation, i.e., synthesis to degradation rates ratio (Fig. 4A). These results confirm AP degradation for Ang(5–8) and suggest a major contribution of AP in the cleavage of Ang(6–8) and Ang(3–4). Ang(1–4) levels are only marginally higher during AP inhibition, suggesting a different degradation route for this peptide.

DISCUSSION

Adipose tissue has long lost its image as passive, inactive storage tissue. It is now viewed as an organ that takes part in energy regulation, inflammation, and cardiovascular function via endocrine, paracrine, and autocrine signals. Research of the past decade has pointed to the existence of a functional RAS in human adipose tissue. Although expression of angiotensinogen and proteases for its cleavage to Ang II has been confirmed by multiple independent work groups (4, 5, 13), the regulation of Ang II levels is still obscure. After initial observations of the secretion of Ang II by human preadipocytes (5), we here confirm production as well as degradation of this peptide by human adipose tissue cells (Fig. 8).

Maximal Ang II concentrations produced in our cell culture system were in the range of 7 to 20 pM depending on the state of cell differentiation (Fig. 1A). These levels are close to those shown previously to elicit effects on 3T3-L1 and human primary adipocytes (31) and are in the range of normal plasma levels (32). Yet they are 20–50 times lower than concentrations in interstitial fluid of intact brown adipose tissue in rats when calculated from the minimal data available; the extracellular water space of adipose tissue was calculated to be 11 ± 1.1% (33), and 38 fmol/g Ang II were detected in whole tissue homogenates, which amounts to roughly 360 pM Ang II in the interstitial fluid. A direct comparison of our cell culture data to intact tissue is difficult, however, not only because of the much higher amount of extracellular fluid in our in vitro system (2 ml of medium on 10-cm² monolayer of cells) but also because of the continuous exchange of interstitial fluid in vivo due to high average resting blood flow and capillary filtration coefficient (34). An interesting, yet to date unexplained, finding is the surplus of Ang II found in the crude membrane fraction after 2 h of incubation with 0.5 nM Ang II-containing SD₈ medium (Fig. 2, E and F). We will examine the possibility of Ang II production by the membrane fraction under these experimental settings in future experiments. Differentiating fat cell precursors showed significantly lower levels of Ang II formation than both preadipocytes and adipocytes. Ang II levels are therefore probably transiently down-regulated in the vicinity of differentiating preadipocytes in adipose tissue. In this article we demonstrate how Ang II is effectively degraded by neprilysin and AP, present in the plasma membrane of both human preadipocytes and adipocytes.

![Fig. 7. Comparison of preadipocyte membrane and soluble aminopeptidase activity with membrane alanine aminopeptidase (EC 3.4.11.2). The specific activities of commercially available membrane alanine AP (EC 3.4.11.2) (A), AP activity present in membrane preparations of human preadipocytes (B), and soluble AP activity of human preadipocyte lysates (C) toward five different amino acid-pNAs are shown. Soluble and membrane AP from human preadipocytes have a comparable substrate selectivity, whereas membrane alanine AP behaves differently in that it shows a higher preference for arginine than for leucine. Note the different spread of the y axes. (n = 3, mean ± S.E.)](http://www.jbc.org/)

![Fig. 8. Formation and degradation of Ang II by human adipose tissue cells. Ang II can be synthesized from angiotensinogen by extracellular (renin, ACE, and chymase) and intracellular (cathepsins) enzymes found in human adipose tissue. In this article we demonstrate how Ang II is effectively degraded by neprilysin and AP, present in the plasma membrane of both human preadipocytes and adipocytes.](http://www.jbc.org/)
tensinases and angiotensinogen (15). An elevated production of active angiotensinases in adipocytes as compared with preadipocytes could account for the unchanged Ang II concentration in the adipocyte culture medium despite elevated angiotensinogen expression in these cells.

Endogenously produced Ang II as well as exogenously added Ang II is degraded by primary human preadipocytes, differentiating cells, and adipocytes (Fig. 1, A and B). Still no functional angiotensinase had been described for human adipose tissue cells to date. Neprilysin, also called neutral endopeptidase (NEP), common acute lymphoblastic leukemia antigen (CALLA), or CD 10 (23), was here shown to be the enzyme primarily responsible for preadipocyte and adipocyte Ang II degradation. As expected, both its protein level and activity were higher in adipocytes as compared with preadipocytes. Neprilysin activity toward the artificial substrate Suc-Ala-Ala-Phe-pNA resides in the membrane fraction of preadipocytes and adipocytes (Table II), and neprilysin immunoreactivity is detected exclusively on the plasma membrane of intact cells (Fig. 6). As degrading activity toward Ang II was not detected in conditioned medium and the soluble cell fraction (Fig. 2, E and F), a release of the active protein from the plasma membrane into the interor intracellular space can be ruled out. Although not seen after 2 h of incubation (Fig. 2, E and F), the membrane fraction of preadipocytes as well as adipocytes does degrade Ang II to a similar degree as the respective cell homogenate within 12 h. Neprilysin is active in this cell fraction (Tables II and III); therefore less than 0.5 nM Ang II, not more, was expected to be seen with this experimental setting after 2 h of incubation. The initially elevated Ang II concentrations must point to a countering phenomenon, like Ang II synthesis by a yet unexplained mechanism. This Ang II-generating activity, masking active degradation by neprilysin, is exhausted somewhere between 2 and 12 h after preparation of the membrane fraction so that the activity of neprilysin is revealed. Clarifying the cellular localization of possible Ang II pools and the synthesis machinery might lead to a greater understanding of this phenomenon.

Human preadipocytes and adipocytes are most likely lacking significant amounts of glutamyl AP (also known as angiotensinase A (EC 3.4.11.7)), as Ang III could not be detected by HPLC. Instead, the AP activity measured was dependent on the prior cleavage of Ang II to Ang(5–8) by neprilysin. Although Ile-pNA was not cleaved by either membrane alanine AP or the AP activity of preadipocyte membranes, this specificity cannot be true for peptides with amino-terminal isoleucine; otherwise Ang(5–8) would not have been degraded to Ang(6–8). The clear preference of preadipocyte membrane AP activity for Ala-pNA distinguishes it from membrane alanine AP, which acts equally well on Arg-pNA in our enzyme assay. Two additional APs have been found to be expressed in human adipose tissue, namely cystinyl AP (EC 3.4.11.3) (9) and adipocyte-derived leucine AP (EC 3.4.11.7) (10). A major contribution of adipocyte-derived leucine AP is unlikely, however, as this enzyme was shown to release aspartate from Ang II to yield Ang III (10), an activity clearly not seen with our cells. This leaves cystinyl AP as an attractive candidate for the observed conversion of Ang(5–8) to Ang(6–8). Cystinyl AP has also been named oxtocinase, human placental leucine AP (35), glycoprotein of molecular weight 160,000 (gp160) (36), vesicle protein of 165 kDa (vp165) (37), insulin-responsive AP (IRAP) (38), and Ang IV receptor (AT4) (39). Cystinyl AP was shown to act on Ang III and Ang IV, whereas Ang I and Ang II were poor substrates (11). We cannot, however, exclude the possibility that more than one AP is responsible for the activity described here. Differences in substrate specificity between commercial membrane alanine AP and preadipocyte/adipocyte cell membranes could also be explained by interference of additional APs present in the membrane preparations, for example cystinyl AP.

Between the addition of Ang II to the cell culture medium and the first measurable decrease in its concentration lay a lag phase of ∼3 h with adipocytes and 8 h with preadipocytes, in which Ang II levels remained fairly stable. We therefore expected to find an increase in neprilysin protein, activity, or cell surface expression induced by Ang II, but neither whole neprilysin content of the cell lysates (as detected by Western blot), nor membrane enzyme activity (as measured by colorimetric enzyme assay), nor surface expression (as detected by immunohistochemistry) showed any sign of change when the cells were preincubated with 0.5 nM Ang II. Still a 4.5-h preincubation with 0.5 nM Ang II increased the Ang II degradation of intact adipocytes by ∼40% (Fig. 2F). However, this difference did not reach statistical significance and was not seen with preadipocytes (Fig. 2E). This leads us to presume that neprilysin is constitutively expressed on the cell surface of preadipocytes and adipocytes, but that it might not be constitutively active. Its activity could be regulated by cytosolic or membrane proteins that associate with it. Dissociation of these complexes during membrane preparation might have disabled the regulatory influence on neprilysin and led to an unchanged activity in our in vitro assay. More detailed studies of the lag phase will show if the neprilysin activity of intact cells can be induced by Ang II. Nevertheless, the idea of neprilysin activity regulation by protein-protein interactions is intriguing. The cytoplasmic tail of neprilysin contains two consensus recognition sequences for casein kinase II and can be phosphorylated by this kinase (40). Neprilysin has been shown to associate with tyrosine-phosphorylated Lyn kinase, which then binds to the p85 subunit of phosphatidylinositol 3-kinase (PI3-K) resulting in a neprilysin-Lyn-PI3-K protein complex. This competitively blocks the interaction of PI3-K with other signaling molecules (41). In fibroblast-like synoviocytes, neprilysin was localized to caveolae/lipid rafts (42), which (if also true for preadipocytes and adipocytes) would explain the punctate pattern observed on the cell surface in this study. Thus there can be no doubt about the complex protein-protein interactions involving neprilysin, but effects on the peptidase activity of the enzyme by phosphorylation or interactions with other proteins have not been published yet. We propose a putative negative feedback loop on the extracellular Ang II concentration, in which Ang II binds to one of its receptors and activates neprilysin, maybe by signaling through casein kinase II. This view is supported by the fact that the AT1 receptor associates directly with caveolin upon ligand binding (43) and thus shares the same membrane microdomain as neprilysin after binding of Ang II, and that caveolin kinase II is discussed as the enzyme responsible for the phosphorylation of the rRNA transcription factor upstream binding factor following Ang II stimulation of vascular smooth muscle cells (44).

Tissue RAS have so far been defined concerning the expression of angiotensinogen, renin, and ACE only. Few articles looked at other potential Ang peptide generating enzymes and even fewer determined possible degradation pathways. The experimental outcome of this study clearly shows that in the RAS at least as much emphasis has to be put on peptide metabolism as is put on peptide synthesis. Human adipose tissue cells control the Ang II concentration in their vicinity mainly by regulating angiotensinase, i.e. neprilysin, activity, which interferes with de novo Ang II measurements. Specific inhibition of Ang II degradation will therefore allow a more
detailed examination of its synthesis pathways and their regulation.

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