Regulation by fatty acids of angiotensinogen gene expression in preadipose cells

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INTRODUCTION
Angiotensinogen (AT), the unique substrate of renin in the renin–angiotensin system (RAS) and precursor of angiotensin II (A-II), plays an important role in the regulation of blood pressure, fluid and electrolyte homeostasis [1]. AT is known to be mainly produced by the liver [2]. However, it has been shown that AT mRNA is present in several tissues [3], including brown and white adipose tissue [4–6]. Recent studies have shown that cells of 3T3-L1 and 3T3-F442A preadipocyte clonal lines synthesize and secrete AT which is increased during differentiation into adipocytes [7,8]. A strong correlation between visceral fat accumulation and hypertension has been observed in epidemiological studies [9] but, in this respect, the physiological and pathological significance of AT gene expression in adipose cells is not completely understood. Furthermore, the involvement of RAS in organogenesis has been proposed, as all components of RAS are active during fetal development and A-II receptor type 2 is transiently expressed at high levels in rodent, primate and human fetuses [10–12]. With respect to adipose tissue, components of RAS have been implicated in its development [13] whereas A-II via the A-II receptor type 2 has been implicated in the formation of new fat cells by a paracrine/autocrine mechanism [14].

Fatty acids have been reported to activate in preadipose cells the expression of various differentiation-dependent genes at the transcriptional level [15,16] and to promote terminal differentiation of Ob1771 preadipose to adipose cells [17]. These effects appear to be mediated by members of the nuclear steroid receptor family, i.e. peroxisome proliferator-activated receptors (PPARs), reported to be activated by fatty acids [18–21]. Indeed, two members of the PPAR family have been cloned from preadipose and adipose cells respectively and shown to be activated by fatty acids, namely a fatty acid-activated receptor, also termed PPARδ or mNUC1 [22,23], and PPARγ2 [24,25].

In the present study, the effect of fatty acids on the expression of the AT gene has been investigated during terminal differentiation of preadipose to adipose cells and compared with that of other differentiation-related genes. The late induction of AT mRNA appears to take place at the transcriptional level, and the effect of fatty acids on AT gene expression is reversible. Secretion of AT is also observed but appears mainly as a late differentiation-dependent phenomenon. Thus the AT gene appears to be a fatty acid-responsive gene; this regulation provides a potential link between the flux of fatty acids and the potential of adipose tissue to produce AT and possibly A-II.

MATERIALS AND METHODS

Cell culture
Ob1771 [26] cells were plated at a density of 2 \times 10^4/cm^2 and grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 8 \% bovine serum, 200 units/ml penicillin, 50 \mu g/ml streptomycin, 33 \mu M biotin and 17 \mu M pantothenate (standard medium). Confluence (day 0) was reached within 5 days. Differentiation of Ob1771 cells was obtained after confluence by chronic addition to the standard medium of 17 nM insulin and 2 nM tri-iodothyronine (T_3) (defined as the standard differentiation medium). In some experiments, 8 \% bovine serum was used in lieu of 8 \%, fetal bovine serum. Medium was changed every other day. Fatty acids and peroxisome proliferators were dissolved in ethanol at a concentration of 100 mM and BRL49653 was dissolved in DMSO at a concentration of 50 mM. Portions were added to the standard medium to obtain the final concentrations indicated. This medium was prewarmed at 37 °C for 15 min and then added to the cells after removal of the previous cul-
ture medium. Concentrations of ethanol and DMSO in the culture medium did not exceed 0.2%.

RNA analysis
RNA was prepared by the guanidinium thiocyanate technique [27]. For Northern-blot analysis, 20 µg of total RNA was electrophoresed on 1.2% (w/v) agarose gel containing 1.1 M formaldehyde and transferred to a Hybond-N membrane (Amersham, Les Ulis, France). Poly(A)-containing RNA was isolated by oligo(dT)-cellulose chromatography as described by Aviv and Leder [28]. Hybridization was performed for 24 h at 65°C in 0.5 M phosphate buffer, pH 7.2, containing 5% SDS and 106 c.p.m./ml of randomly primed 32P-labelled cDNA probes. The autoradiographs were quantified by densitometry with an LKB-XL laser photodensitometer (Rockville, MD, U.S.A.). All results were normalized to β-actin signals.

AT protein assay
Ob1771 cells maintained in the standard differentiation medium (2 cm2 culture wells) were washed at 37 °C with DMEM (2 × 30 s, then 1 h) at the indicated times during the differentiation process and incubated in 1 ml of DMEM containing 5 µg/ml insulin and 10 µg/ml transferrin. After 24 h, culture medium was collected. BSA and anti-proteolytic agents were then added to give final concentrations of 1%, BSA, 1 mM PMSF, 5 mM EDTA, 1 mM hydroxyquinoline and 10 µM captopril. Cells were scraped off in the presence of 0.2 ml of 50 mM phosphate buffer, pH 7.4, containing antiproteolytic agents and BSA as above and homogenized by sonication. The amount of AT was determined by measuring angiotensin I (A-I) after conversion with exogenous renin followed by RIA of A-I by the method of Sealey and Laragh [29]. Renin from mouse submaxillary gland was prepared by the method of Jacobsen and Poulsen [30].

Materials
Culture media were obtained from Gibco (Cergy-Pontoise, France). Fetal bovine serum was a product of Seromed. [2-32P]dCTP, random priming kit, Hybond membranes and products for AT assays were purchased from Amersham. Restriction enzymes were from Eurogentec. Bovine serum and other chemical products were obtained from Sigma Chimie (Saint-Quentin, France). BRL49653 was obtained from SmithKline Beecham Pharmaceuticals (Welwyn, Kent, U.K.). Wy-14,643 and LY171883 were obtained from Biomol Research Laboratories.

RESULTS
Gene expression and secretion of AT during adipose cell differentiation
AT mRNA became detectable at confluence (day 0), began to accumulate 3 days later and continued to increase until day 12 (Figure 1). When compared with the expression of the A2COL6/pOb24 gene as an early marker and that of adipocyte fatty acid-binding protein (aFABP) as a late marker of differentiation, the expression of the AT gene was similar to that of aFABP whereas, in agreement with previous studies [31], that of the A2COL6/pOb24 gene became significant after growth arrest, peaked at day 3 and decreased thereafter.

In order to determine whether increased levels of AT mRNA were accompanied by increased rate of AT synthesis and secretion, AT was measured in both homogenates and conditioned media of Ob1771 cells. As shown in Figure 2, the amount of cellular AT was slightly increased as a function of differentiation. In contrast, AT secretion, which was weakly enhanced until day 6, showed a dramatic increase at day 7 and thereafter, reaching a plateau at day 15. From day 11 to day 18, more than 90% of the AT produced by the cells was recovered in the secretion medium.

Induction by fatty acids of the AT gene in committed lipid-free preadipocytes
In order to study the effects of fatty acids on the activation of the AT gene, Ob1771 preadipose cells were treated at day 0 with 100 µM α-bromopalmitate. These conditions were chosen as this non-metabolized fatty acid was shown to be more potent than natural long-chain fatty acids in activating the aFABP gene in Ob1771 preadipose cells, and a concentration of 100 µM was shown to be optimal [16]. At day 0, preadipose cells were committed and expressed early markers only (Figure 1) [31]. Exposure to 100 µM α-bromopalmitate for 24 and 48 h led to a 4.8- and 17.6-fold increase in AT mRNA content respectively.
The effect of α-bromopalmitate is a fatty acid effect per se distinct from a mere accelerating effect on the differentiation process, as it did not require the presence of additional adipogenic factors such as T₃ and growth hormone, which are known to trigger terminal differentiation of preadipose cells in serum-supplemented medium (not shown). As expected from previous data [31], A2COL6/pOb24 mRNA content remained unchanged under these conditions. As expected also, α-bromopalmitate stimulated aFABP gene expression 15±4- and 30±1-fold after 24 and 48 h respectively. After a 48 h exposure to α-bromopalmitate, the content of AT and aFABP mRNAs (lane d) was similar to that obtained in differentiated Ob1771 cells exposed for 17 days to differentiation medium only. Within 48 h, the levels of AT and aFABP mRNAs reached those observed in fully differentiated Ob1771 cells. This effect of α-bromopalmitate appears to be quite specific since other late markers such as glyceraldehyde-3-phosphate dehydrogenase mRNA could not be detected (not shown). The reversibility of the fatty acid effect was examined next. After 48 h exposure to 100 µM α-bromopalmitate, its removal resulted in a decrease in AT mRNA content (half-life approx. 8 h) to the level observed in untreated cells whereas cells further exposed for 24 h continued to show an increase in AT mRNA level, reaching 84% of the maximal value (Figure 4). Thus, in preadipose cells, the optimal activation of the AT gene was dependent on the continuous presence of the fatty acid.

Transcriptional activation of the AT gene and half-life of AT mRNA

As shown in Figure 5, the accumulation of AT mRNA in preadipocytes exposed to 100 µM α-bromopalmitate for 24 h was abolished in the presence of actinomycin D, suggesting that α-bromopalmitate regulates the expression of the AT gene by means of transcriptional activation. The half-life of AT mRNA was estimated after pretreatment of preadipose cells for 48 h with 100 µM α-bromopalmitate. After removal of the fatty acid and simultaneous addition of actinomycin D, the disappearance of
Activation of fatty acid translocase mRNA expression in PPAR family members

Natural and non-metabolized fatty acids, peroxisome proliferators and BRL49653, an antidiabetic thiazolidinedione, have been reported to be activators of the PPAR family members, i.e. PPAR\(\alpha\), PPAR\(\delta\) and PPAR\(\gamma\). \(\alpha\)-Bromopalmitate has been reported to be the most potent activator of PPAR\(\delta\) in the activation of fatty acid translocase mRNA expression in PPAR\(\delta\)-expressing fibroblasts [23]. The effect of various activators on the expression of AT and aFABP genes was therefore compared with that of \(\alpha\)-bromopalmitate (Table 1). Eicosatrienoic acid (ETYA), which is another non-metabolized fatty acid, proved to be a more potent activator than \(\alpha\)-bromopalmitate and was thus taken as the reference compound. In agreement with previous data [23], \(\alpha\)-bromo-octanoate was not very active, and natural long-chain fatty acids, mono- or poly-unsaturated, were less active than \(\alpha\)-bromopalmitate when present at a concentration of 100 \(\mu\)M. The cytotoxic effect of 300 \(\mu\)M \(\alpha\)-bromopalmitate prevented any comparison with natural fatty acids when present at that concentration. The reason for the decrease in AT mRNA content and the increase in aFABP content, when the concentration of arachidonate was raised from 100 to 300 \(\mu\)M, remains unclear but was reproducibly observed. BRL49653, which binds PPAR\(\gamma\), and promotes adipose conversion of PPAR\(\gamma\)-expressing fibroblasts [32,33], was as potent as ETYA in up-regulating the expression of AT and aFABP genes. LY171883, another potent activator of PPAR\(\gamma\), was less active than and as active as ETYA in activating AT and aFABP genes respectively. Wy14,563, the most potent activator of PPAR\(\alpha\) [20], was 2-fold less active than ETYA. Clofibrate, also a very potent activator of PPAR\(\alpha\) and a weak activator of PPAR\(\delta\) and PPAR\(\gamma\), was only marginally effective at activating the AT and aFABP genes.

Table 1 Effect of various PPAR activators on AT and aFABP mRNA expression in Ob1771 preadipocytes

<table>
<thead>
<tr>
<th>Conditions</th>
<th>AT mRNA signal (μg/mg protein)</th>
<th>aFABP mRNA signal (μg/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>10.0 ± 3.5</td>
<td>4.8 ± 1.3</td>
</tr>
<tr>
<td>(\alpha)-Bromo-octanoate 100 (\mu)M</td>
<td>20.7 ± 4.0</td>
<td>15.4 ± 1.6</td>
</tr>
<tr>
<td>(\alpha)-Bromopalmitate 100 (\mu)M</td>
<td>51.5 ± 8.8</td>
<td>74.3 ± 7.4</td>
</tr>
<tr>
<td>Oleate 300 (\mu)M</td>
<td>51.2 ± 3.3</td>
<td>65.4 ± 13.2</td>
</tr>
<tr>
<td>Linoleate 100 (\mu)M</td>
<td>61.5 ± 13.2</td>
<td>47.9 ± 8.8</td>
</tr>
<tr>
<td>(\gamma)-Linolenate 300 (\mu)M</td>
<td>45.9 ± 0.5</td>
<td>14.0 ± 1.4</td>
</tr>
<tr>
<td>Arachidonate 300 (\mu)M</td>
<td>74.0 ± 8.3</td>
<td>31.3 ± 6.5</td>
</tr>
<tr>
<td>USF 100 (\mu)M</td>
<td>64.0 ± 15.0</td>
<td>48.3 ± 3.5</td>
</tr>
<tr>
<td>ETYA 100 (\mu)M</td>
<td>69.0 ± 14.0</td>
<td>21.3 ± 3.0</td>
</tr>
<tr>
<td>BRL49653 100 (\mu)M</td>
<td>42.9 ± 4.8</td>
<td>42.3 ± 3.5</td>
</tr>
<tr>
<td>Clofibrate 100 (\mu)M</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Wy14,563 100 (\mu)M</td>
<td>100</td>
<td>100</td>
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<tr>
<td>LY171883 100 (\mu)M</td>
<td>100</td>
<td>100</td>
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Figure 6 Half-life of AT mRNA after induction by \(\alpha\)-bromopalmitate

Ob1771 cells were maintained from confluence for 48 h in DMEM containing 8% bovine serum and supplemented with 100 \(\mu\)M \(\alpha\)-bromopalmitate (time zero). At time zero, 5 \(\mu\)g/ml actinomycin D was added for the indicated times. mRNA was prepared and analysed as described in the Materials and methods section. The results are given as means ± S.E.M. for three independent experiments.

Figure 7 PPAR expression in Ob1771 preadipocytes

Cells 1 day after confluence were maintained for 24 h in DMEM containing 8% bovine serum (lanes a and c). Differentiated Ob1771 cells were maintained for 14 days after confluence in the standard differentiation medium (lanes b and d). Total RNA (lanes a and b) and poly(A)-containing RNA (lanes c and d) were prepared and analysed with cDNA probes of PPAR\(\delta\), PPAR\(\gamma\), and \(\beta\)-actin. The autoradiogram is representative of three independent experiments that gave similar results.

Expression of various PPAR mRNAs during expression of the AT gene

In order to gain some insights into the ability of preadipose cells to respond to various activators of PPARs, PPAR expression was analysed by Northern blotting using RNAs and poly(A)-containing RNAs extracted from Ob1771 preadipocytes 1 day after confluence and from differentiated Ob1771 adipocytes 14 days after confluence. As shown in Figure 7, in both preadipocytes (lanes a and c) and adipocytes (lanes b and d) PPAR\(\delta\) mRNA was detectable at similar levels using either total RNA (lanes a and b) or poly(A)-containing RNA (lanes c and d). In contrast, PPAR\(\gamma\) mRNA was only detectable in preadipocytes when poly(A)-containing RNA was used (lane c compared with lane a) and accumulated in adipocytes (lanes b and d). Both the PPAR\(\delta\) and PPAR\(\gamma\) mRNA content was increased approx. 2-fold in preadipocytes maintained in the presence of 100 \(\mu\)M \(\alpha\)-bromopalmitate whereas PPAR\(\alpha\) mRNA could not be detected (not shown).
DISCUSSION

During adipose cell differentiation, the time course of AT gene expression observed in Ob1771 cells is similar to that reported for 3T3-L1 and 3T3-F442A cells [7,8], and confirms on AT the status of a late marker [15]. The increase in AT synthesis and secretion is clearly delayed compared with the accumulation of AT mRNA, whereas the steady-state levels of AT within differentiating cells increase with time in culture to a small extent only. The difference observed between the high levels of AT mRNA and the low levels of secreted AT in early differentiating cells compared with differentiated cells (Figures 1 and 2) appears to be very reproducible, but its significance remains unclear. It could be due to changes in translational efficiency of AT mRNA and/or to changes in post-translational events related to the secretion process itself. We have reported that fatty acid treatment only of preadipocytes induces the expression of several genes that encode proteins involved in fatty acid metabolism. These include lipoprotein lipase [34], aFABP [15], a long-chain acyl-CoA synthetase and a membrane protein involved in fatty acid binding and transport [16]. To our knowledge the AT gene is the first example of a gene not directly related to fatty acid metabolism that nevertheless appears to be regulated by fatty acids. As in the case of lipid-related genes, the expression of the AT gene is regulated by fatty acids at the transcriptional level. Induction of the AT gene is reversible and does not require fatty acid metabolism since it is observed with α-bromopalmitate which is not metabolized by preadipocytes [16]. Moreover, fatty acids are effective in the absence of adipogenic factors which are otherwise needed to trigger the terminal differentiation of preadipose into adipose cells [26], excluding the possibility that differentiation per se is responsible for the effects of fatty acids. The aFABP gene was the first to be shown to respond to long-chain natural fatty acids, peroxisome proliferators and BRL49653 has been reported [34,69] or to changes in post-translational events related to the secretion process itself.

It might also be that fatty acids act indirectly by activating PPARδ and/or PPARγ, which in turn modulates the expression or the activity of proteins able to bind to other cis-acting elements which remain to be characterized in the AT promoter.

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REFERENCES