Regulation by fatty acids of angiotensinogen gene expression in preadipose cells

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Adipocytes represent an important source of angiotensinogen (AT). Angiotensin II (A-II) stimulates *in vitro* and *in vivo* the formation and release of prostacyclin which acts as a potent adipogenic signal in triggering the terminal differentiation of preadipocytes into adipocytes [Darimont, Vassaux, Gaillard, Ailhaud and Nègre (1994) *Int. J. Obes.* **18**, 783–788]. Since fatty acids have been reported to activate in preadipose cells the expression of various differentiation-dependent genes, the role of fatty acids in the regulation of AT gene expression was investigated. Long-chain natural and non-metabolized fatty acids as

well as peroxisome proliferators behave as activators of AT gene expression. Accumulation of AT mRNA parallels that of the adipocyte fatty acid-binding protein gene and is primarily due to transcriptional activation of the AT gene. AT mRNA decreases after fatty acid removal (half-life approx. 8 h). 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.

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 fibrates and 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 PPAR δ or mN1 [22,23], and PPAR γ [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. The results support PPAR δ and/or PPAR γ being the mediators of the effects of fatty acids.

MATERIALS AND METHODS

Cell culture

Ob1771 [26] cells were plated at a density of $2 \times 10^3/\text{cm}^2$ and grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 8 % bovine serum, 200 units/ml penicillin, 50 $\mu\text{g}/\text{ml}$ streptomycin, 33 μM biotin and 17 μM 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-

Abbreviations used: aFABP, adipocyte fatty acid-binding protein; AT, angiotensinogen; A-I, angiotensin I; A-II, angiotensin II; C/EBP α , CAAT/enhancer-binding protein α ; DMEM, Dulbecco's modified Eagle's medium; ETYA, eicosatrienoic acid; PPAR, peroxisome proliferator-activated receptors; RAS, renin–angiotensin system; T_3 , tri-iodothyronine.

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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 μ g 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 10^6 c.p.m./ml of randomly primed 32 P-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 $\times$ 30 s, then 1 h) at the indicated times during the differentiation process and incubated in 1 ml of DMEM containing 5 μ g/ml insulin and 10 μ g/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 μ M 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. [α - 32 P]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

Figure 1 Kinetics of AT gene expression in Ob1771 cells

Cells were grown in the standard medium and transferred at confluence in the standard differentiation medium as described in the Materials and methods section. At the indicated times, mRNA was isolated and Northern-blot analysis performed. The values were normalized to β -actin signals. The results are given as means $\pm$ S.E.M. for three independent experiments. $\circ$, A2COL6/pOb24; $\bullet$, AT; $\triangle$, aFABP.

Figure 2 Kinetics of AT synthesis and secretion in Ob1771 cells

Cells were grown in the standard medium and transferred at confluence in the standard differentiation medium as described in the Materials and methods section. At the indicated times, cells were washed as described in the Materials and methods section, and DMEM containing 5 μ g/ml insulin and 10 μ g/ml transferrin was added. After 24 h, AT was measured in cellular homogenates ($\circ$) and conditioned media ($\bullet$). The results are given as means $\pm$ S.E.M. for three independent experiments.

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 μ M 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 μ M was shown to be optimal [16]. At day 0, preadipose cells were committed and expressed early markers only (Figure 1) [31]. Exposure to 100 μ M α -bromopalmitate for 24 and 48 h led to a 4.8- and 17.6-fold increase in AT mRNA content respectively

Figure 3 Effect of α -bromopalmitate on AT, A2COL6/pOb24 and aFABP mRNAs in Ob1771 preadipocytes

Cells 1 day after confluence were maintained for 24 h (lanes a and b) and 48 h (lanes c and d) in the medium containing 8% bovine serum in the absence (lanes a and c) or presence (lanes b and d) of 100 μ M α -bromopalmitate. Differentiated Ob1771 cells were obtained after maintenance in the standard differentiation medium for 17 days after confluence (lane e). mRNA was prepared and analysed as described in the Materials and methods section. (A) Northern-blot analysis of various mRNAs; (B) the results of (A) are normalized to β -actin mRNA signals, taking as 100% the maximal signal obtained for each probe, and represent means $\pm$ S.E.M. for three independent experiments.

(Figure 3). 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 T_3 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 glycerol-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 μ M 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 Time course and reversibility of induction of AT mRNA by α -bromopalmitate

Cells were maintained for the indicated times in DMEM containing 8% bovine serum without (○) or with (●) 100 μ M α -bromopalmitate. α -Bromopalmitate was removed by two washes and further incubation in DMEM with 8% bovine serum (△). mRNA was prepared and analysed as described in the Materials and methods section. The results are given as means $\pm$ S.E.M. for three independent experiments.

Figure 5 Effect of actinomycin D on the induction of AT mRNA by α -bromopalmitate

(A) Ob1771 cells 1 day after confluence were maintained for 24 h in DMEM containing 8% bovine serum (lanes a and b), supplemented with 100 μ M α -bromopalmitate (lanes c and d) and exposed (lanes b and d) or not (lanes a and c) to 5 μ g/ml actinomycin D. The results are representative of three independent experiments. (B) Values of mRNA signals obtained from (A) are given as means $\pm$ S.E.M. for three independent experiments.

(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 μ M 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 μ M α -bromopalmitate. After removal of the fatty acid and simultaneous addition of actinomycin D, the disappearance of

Figure 6 Half-life of AT mRNA after induction by α -bromopalmitate

Ob1771 cells were maintained from confluence for 48 h in DMEM containing 8% bovine serum and supplemented with 100 μ M α -bromopalmitate (time zero). At time zero, 5 μ 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 $\pm$ S.E.M. for three independent experiments.

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

Cells 1 day after confluence were maintained for 24 h in DMEM containing 8% bovine serum and supplemented or not with various PPAR activators. RNA was prepared and analysed with AT and aFABP cDNA probes. The values were normalized to β -actin signals. The results are presented as means $\pm$ S.E.M. for two independent experiments.

Conditions	AT mRNA signal (% of maximum)	aFABP mRNA signal (% of maximum)
Control	10.8 $\pm$ 3.9	4.8 $\pm$ 1.3
α -Bromo-octanoate 100 μ M	20.7 $\pm$ 4.0	15.4 $\pm$ 1.6
α -Bromopalmitate 100 μ M	51.5 $\pm$ 8.8	74.3 $\pm$ 7.4
Oleate 100 μ M	51.2 $\pm$ 3.3	24.0 $\pm$ 1.2
300 μ M	61.5 $\pm$ 13.2	47.9 $\pm$ 8.8
Linoleate 100 μ M	45.9 $\pm$ 0.5	14.0 $\pm$ 1.4
300 μ M	74.0 $\pm$ 8.3	31.3 $\pm$ 6.5
γ -Linolenate 100 μ M	29.5 $\pm$ 5.5	10.5 $\pm$ 1.4
300 μ M	64.0 $\pm$ 15.0	48.3 $\pm$ 3.5
Arachidonate 100 μ M	69.0 $\pm$ 14.0	21.3 $\pm$ 3.0
300 μ M	42.9 $\pm$ 4.8	42.3 $\pm$ 3.5
ETYA 100 μ M	100	100
BRL49653 100 μ M	89.7 $\pm$ 10.3	102.8 $\pm$ 17.3
Clofibrate 100 μ M	22.5 $\pm$ 5.8	17.3 $\pm$ 4.8
Wy-14,653 100 μ M	52.7 $\pm$ 7.4	50.0 $\pm$ 12.7
LY171883 100 μ M	65.3 $\pm$ 11.2	98.5 $\pm$ 17.4

AT mRNA was determined as a function of time. The results in Figure 6 allow an estimation of the half-life of AT mRNA of approx. 8 h, a value similar to that estimated after fatty acid removal in the absence of actinomycin D (Figure 4).

Effect of various activators on the expression of AT and aFABP genes

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 α , PPAR δ and PPAR γ_2 . α -Bromopalmitate has been reported to be the most potent activator of PPAR δ in the activation of fatty acid translocase mRNA expression in PPAR δ -expressing fibroblasts [23]. The effect of various activators on the expression of AT and aFABP genes was therefore compared with that of α -bromopalmitate (Table 1). Eicosatrienoic acid (ETYA), which is another non-metabolized fatty acid, proved to

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 δ , PPAR γ_2 and β -actin. The autoradiogram is representative of three independent experiments that gave similar results.

be a more potent activator than α -bromopalmitate and was thus taken as the reference compound. In agreement with previous data [23], α -bromo-octanoate was not very active, and natural long-chain fatty acids, mono- or poly-unsaturated, were less active than α -bromopalmitate when present at a concentration of 100 μ M. The cytotoxic effect of 300 μ M α -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 μ M, remains unclear but was reproducibly observed. BRL49653, which binds PPAR γ_2 and promotes adipose conversion of PPAR γ_2 -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 γ , was less active than and as active as ETYA in activating AT and aFABP genes respectively. Wy14,653, the most potent activator of PPAR α [20], was 2-fold less active than ETYA. Clofibrate, also a very potent activator of PPAR α and a weak activator of PPAR δ and PPAR γ_2 , was only marginally effective at activating the AT and aFABP genes.

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 δ 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 γ_2 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 δ and PPAR γ_2 mRNA content was increased approx. 2-fold in preadipocytes maintained in the presence of 100 μ M α -bromopalmitate whereas PPAR α 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 confers 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 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 and non-metabolized fatty acids [15]. Therefore the expression of both the AT gene and the aFABP gene was followed as a function of time, and the nature of the inducers was examined. The time course of induction (Figure 1) and the response to various inducers (Table 1) indicate important similarities between the two responding genes, although some quantitative differences are observed, particularly in the case of polyunsaturated fatty acids. Taken together, the specificity profiles implicate PPARs in the mediation of the transcriptional effects on both genes. Differential activation of the PPAR family members by fatty acids, peroxisome proliferators and BRL49653 has been reported by many investigators [32,33,35,36] but a comparison of trans-activation data indicates that the specificity of PPAR α , PPAR δ and PPAR γ_2 for a given inducer depends to a large extent on the experimental design, i.e. the nature of the recipient cells and/or the expression vectors and their levels of expression [23,32,33,35,36]. As PPAR α is not detected in Ob1771 preadipose cells, it is likely that PPAR δ and/or PPAR γ_2 , which are present at high and low levels respectively in preadipose cells, are responsible for the specificity of the observed responses (Figure 7). So far, within the promoter region of the mouse AT gene, no peroxisome proliferator-response element or CAAT/enhancer-binding protein (C/EBP)-response element has been reported. However, since AT and aFABP mRNAs emerge in parallel (Figure 1), and since aFABP possesses a functional peroxisome proliferator-response element in addition to a functional C/EBP-response element [37], it cannot be excluded that a similar situation is present within the AT promoter, despite the fact that the *cis*-acting elements so far described do not share any homology with the consensus binding motifs of C/EBP α [38,39].

It might also be that fatty acids act indirectly by activating PPAR δ and/or PPAR γ_2 , 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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