Adrenergic stimulation of lipoprotein lipase gene expression in rat brown adipocytes differentiated in culture: mediation via β_3- and α_1-adrenergic receptors

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In order to investigate whether the positive effect of adrenergic stimulation on lipoprotein lipase (LPL) gene expression in brown adipose tissue is a direct effect on the brown adipocytes themselves, the expression of the LPL gene was investigated by measuring LPL mRNA levels in brown adipocytes, isolated as precursors from the brown adipose tissue of rats and grown in culture in a fully defined medium before experimentation. Addition of noradrenaline led to an enhancement of LPL gene expression; the mRNA levels increased as a linear function of time for at least 5 h and were finally approx. 3 times higher than in control cells, an increase commensurate with that seen *in vivo* in both LPL mRNA levels and LPL activity during physiological stimulation. The increase was dependent on transcription. The effect of noradrenaline showed simple Michaelis–Menten kinetics with an EC_{50} of approx. 11 nM. β_3-Agonists (BRL-37344 and CGP-12177) could mimic the effect of noradrenaline; the β_1-agonist dobutamine and the β_2-agonist salbutamol could not; the α_1-agonist cirazoline had only a weak effect. The effect of noradrenaline was fully inhibited by the β-antagonist propranolol and was halved by the α_1-antagonist prazosin; the α_2-antagonist yohimbine was without effect. An increase in LPL mRNA level similar to (but not significantly exceeding) that caused by noradrenaline could also be induced by the cAMP-elevating agents forskolin and cholera toxin, and 8-Br-cAMP also increased LPL mRNA levels. The increase in LPL gene expression was not mediated via an increase in the level of an intermediary proteinaceous factor. It is concluded that the physiologically induced increase in LPL gene expression is a direct effect of noradrenaline on the brown adipocytes themselves, mediated via a dominant β_3-adrenergic pathway and an auxillary α_1-adrenergic pathway which converge at a regulatory point in transcriptional control.

INTRODUCTION

Owing to the pivotal role of lipoprotein lipase (LPL) in the regulation of lipid import into the various tissues of the body, its regulation has attracted much attention [1]. After the isolation of cDNA clones corresponding to LPL mRNA [2–5], the focus of attention was directed to the regulation of LPL gene expression.

In a series of studies, performed *in vivo* in rats, we obtained evidence indicating that the regulation of LPL activity in brown adipose tissue occurs mainly at the pretranslational level, i.e. at the mRNA level, and that gene expression is positively regulated not only by insulin (as in white adipose tissue) but also by noradrenaline, working through β-adrenergic receptors and presumably with cAMP as intracellular mediator [6–10]. In order to understand better the control of the expression of the LPL gene, we decided to investigate this issue in a rat brown adipocyte culture system [11–14]. We have therefore examined here the control of gene expression at the pretranslational level. To what extent alterations in LPL mRNA levels lead to alterations in LPL activity has not been directly addressed in this investigation; rather we have examined whether the action of noradrenaline is a direct effect on the brown adipocyte, and have investigated the intracellular mediation of the adrenergic signal.

MATERIALS AND METHODS

Cell culture

Brown preadipocytes were isolated as the stromal–vascular fraction of the interscapular and cervical brown adipose tissue pads from 4-week-old male Sprague–Dawley rats, principally as described previously [11–14]. Pads from 12 rats were routinely pooled. Minced tissue pieces were incubated with 0.2 % (w/v) collagenase (Sigma, type II) in 24 ml of isolation buffer [in mM: Na^+ 123, K^+ 5, Ca^{2+} 1.3, Cl^- 131, glucose 5, Hepes 100 (pH 7.45 with NaOH), 1.5 % crude serum albumin (Fraction V, Boehringer-Mannheim; sterile-filtered with the collagenase)] in 50 ml polypropylene tubes at 37 °C for 30 min with vortexing every 5th min. The tissue remnants were removed by filtration of the incubation mixture through a 250 μm nylon screen into 10 ml polystyrene test tubes. The filtrate was left for 30 min on ice to allow fat-replete cells to float; the infranatant was collected and slowly filtered through a 25 μm nylon screen, and the cells were pelleted by centrifugation at 700 ***g*** for 10 min. The pellet was resuspended and washed in 10 ml of Dulbecco's modified Eagle's medium (DMEM) (Flow; cat. no. 12-323) at room temperature and re-centrifuged at 700 ***g*** for 10 min. This final pellet was suspended in 500 μl of culture medium (see below) per animal originally used. The isolated preadipocytes were inoculated into culture wells prefilled with medium (well area 9 cm^2; Corning) at a density corresponding to 2.5 wells per animal.

During the first 6 days, the preadipocytes were grown in 2 ml of a medium consisting of DMEM supplemented with 10 % newborn calf serum (not heat-inactivated; Flow; cat. no. 29-121), 4 nM insulin, 4 mM glutamine, antibiotics (50 IU of penicillin/ml and 50 μg of streptomycin/ml), 10 mM Hepes (Flow) and 25 μg/ml sodium ascorbate [13]. This medium containing serum was changed on days 1, 3 and 5. On day 6, the medium was discarded, the cells were washed with DMEM at

Abbreviations used: LPL, lipoprotein lipase; UCP, uncoupling protein; CRE, cAMP response element; CREB, CRE-binding proteins; DMEM, Dulbecco's modified Eagle's medium.

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room temperature, and new medium, identical except for the absence of serum, was added. This medium without serum was changed on days 8 and 10.

On day 12, the medium was discarded and the cultures were carefully rinsed with DMEM and then preincubated for 30 min with 2 ml of fresh medium (still without serum); thereafter the agents were added to the wells (normally in 10 μl solutions). The incubation time was 4 h (except for the time-dependence experiments). After this, the medium was discarded, the cultures were rinsed with saline, and the cells were harvested for isolation of RNA.

RNA isolation

The RNA isolation procedure was similar to that used previously [15]. The cells were dissolved in 2×0.5 ml of boiling extraction buffer (8 M guanidinium chloride, 0.1 M Tris/HCl, 10 mM dithiothreitol, 1 % *N*-lauroylsarcosine and 10 mM EDTA). The combined solution was transferred to a 3 ml tube, 100 μl of 2 M sodium acetate, pH 5.6 was added, and 550 μl of ice-cold ethanol was slowly added with constant vortex-mixing. After precipitation for 2 h at -20 °C, the tubes were centrifuged for 30 min at 15400 ***g*** (14000 rev./min). Then 300 μl of extraction buffer/ethanol (2:1, v/v) was added to the pellet. The tube was centrifuged for 5 min and the pellet resuspended in 100 μl of 15 % formaldehyde. After the addition of 10 μl of 2 M sodium acetate and 275 μl of ice-cold ethanol, the precipitate was collected by a 30 min centrifugation, washed with 300 μl of 70 % ethanol, and the tube centrifuged for 5 min. The pellet was extracted with 100 μl of 10 mM EDTA at 70 °C for 10 min with intermittent vortex-mixing (2 min intervals), and the pellet repelleted by 10 min of centrifugation. After this final centrifugation, the total RNA concentration in the final supernatant was spectrophotometrically determined (Gentech) at 260 nm (mean yield per well was 40 ± 1 μg; mean of 68 wells) and the A_{260}/A_{280} ratio determined (mean ratio 1.79).

Northern-blotting procedure

LPL mRNA levels were evaluated by a Northern-blot technique, as described [16] with minor modifications. A volume corresponding to 5 μg of total RNA was vacuum-dried (Speedvac) for 5 min; 18 μl of RNA cocktail [50 % $1 \times$ Mops solution (20 mM Mops, 10 mM EDTA, 50 mM sodium acetate) with 6.6 % formaldehyde + 50 % formamide] and 2 μl of loading buffer (50 % glycerol + 50 % 1 mM EDTA with 2.5 mg/ml Bromophenol Blue) were added, and the tube heated to 70 °C for 5 min and quenched on ice for 5 min. The samples were loaded on a denaturation gel (1.2 % agarose in $1 \times$ Mops solution with 6.6 % formaldehyde and 0.1 μg/ml ethyl bromide). As a positive control on every blot, 5 μg of total RNA isolated from brown adipose tissue of mice that had been exposed to 4 °C for 24 h was run; as a negative control, 5 μg of total RNA of mouse brain isolated from the same animals was used (brain has low expression of LPL mRNA [3]).

The $1 \times$ Mops solution was used as buffer in the electrophoresis chamber (Pharmacia) equipped with a buffer-circulation pump (MasterFlex). The samples were run into the gel at 100 V for 30 min, and thereafter routinely run overnight at 25–30 V. After the electrophoresis, the gel was photographed under UV light and checked for RNA integrity.

Blotting was performed in $10 \times$ SSC (1.5 M NaCl, 0.15 M sodium citrate, adjusted to pH 7.0 with HCl), with the gel placed between moistened 3MM Whatman filter papers and a moistened Hybond-N membrane (Amersham), with dry Whatman papers on the top, for approx. 12 h. The blotted Hybond membrane was photographed under UV light and dried overnight before hybridization.

In some experiments, as indicated, a slot-blot procedure [9] was used instead of the Northern-blot procedure.

Hybridization

Prehybridization was performed in buffer composed of 50 % formamide, $5 \times$ SSC, 5 mM sodium phosphate, pH 6.5, $5 \times$ Denhardt's and 0.5 % SDS, with 200 μg of salmon sperm DNA, 500 μg of poly(A) (Sigma) and 500 μg of poly(C) (Sigma) per ml for 4 h in tubes in a Hybaid hybridization oven at 45 °C.

Hybridization was performed in an identical solution (except that Denhardt's was reduced to $2 \times$) with a random-prime-labelled (random prime DNA labelling kit; Boeringer-Mannheim) LPL cDNA [3] probe with a specific radioactivity of about 10^8 c.p.m./mg [$(1.5–5) \times 10^6$ c.p.m./ml of hybridization solution] for 24 h in tubes in a hybridization oven at 45 °C.

The filters were washed twice with $2 \times$ SSC/0.2 % SDS at room temperature for 15 min, and twice with $0.1 \times$ SSC/0.2 % SDS for 30 min, and dried between Whatman paper sheets overnight. The blots were exposed on PhosphorImager screens and analysed with a Molecular Dynamics PhosphorImager and an image analysis program (ImageQuant).

cAMP analysis

Before determination of cAMP, the cells were incubated for 20 min with the indicated agents, then the culture medium was discarded and 0.8 ml of 95 % ethanol was added to each well and the suspension was collected; 0.8 ml of ethanol was then again added to the wells. The combined ethanol extracts were dried in a Speedvac centrifuge for 4 h at 55 °C. The dried samples were then dissolved in 350 μl of the Tris/EDTA buffer (buffer 1) provided with the cAMP 3H Assay System from Amersham, sonicated for approx. 5 s and centrifuged in an Eppendorf centrifuge at 15400 ***g*** (14000 rev./min) for 15 min. Aliquots of the supernatants (25 μl) were analysed as described in the assay system.

Chemicals

The following agents were used, dissolved in water if not otherwise indicated: noradrenaline (L-Arterenol bitartrate, Sigma); isoprenaline (Sigma); BRL-37344 (gift from SmithKline Beecham Laboratories); ICI-D7114 (gift from ICI-Zeneca Pharmaceuticals); CGP-12177A (Ciba-Geigy); salbutamol (Ventoline; Glaxo; 2 mg/ml; infusion concentrate); dobutamine (hydrochloride; Dobutrex; Lilly, 12.5 mg/ml; infusion concentrate); cirazoline (L.E.R.S., Paris, France); yohimbine (Sigma); prazosin [Pfizer; dissolved in 95 % ethanol/water (1:1, v/v) and added 20 μl/2 ml, or in DMSO/water (1:1, v/v) similarly added, as indicated]; propranolol (ICI); Br-cAMP (8-bromo-cAMP; Sigma); cholera toxin (1 mg/ml; Sigma); forskolin (Sigma; dissolved in ethanol as for prazosin); actinomycin D (Cosmogen; Merck-Sharp & Dohme); cycloheximide (Sigma).

RESULTS

For the present experiments, we used a primary cell culture system for which precursors of brown preadipocytes were isolated from the brown adipose tissue pads of young rats and grown in culture [11–14]. In this system, the brown preadipocytes proliferate rapidly and come to confluence at 5–8 days. At the same

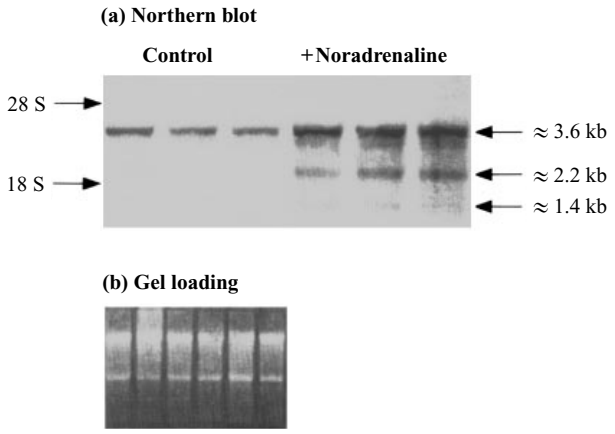

Figure 1 Effect of noradrenaline on LPL mRNA levels in cultured brown adipocytes

Brown adipocyte precursor cells were isolated from young rats and grown in culture for a total of 12 days, the last 6 days in a fully defined medium (no serum addition), as detailed in the Materials and methods section. The cells were then incubated for 7 h in the absence (Control) or presence (+Noradrenaline) of 10 μM noradrenaline. Total RNA was then isolated as described and 5 μg electrophoresed and hybridized with the ^{32}P-labelled LPL cDNA probe. (**A**) Northern blot. Results from three parallel wells from each condition are shown. The positions of the 28 and 18 S ribosomal bands are indicated, as well as the estimated lengths of the three bands observed. (**B**) Ethidium bromide staining of the same gel demonstrating equal loading.

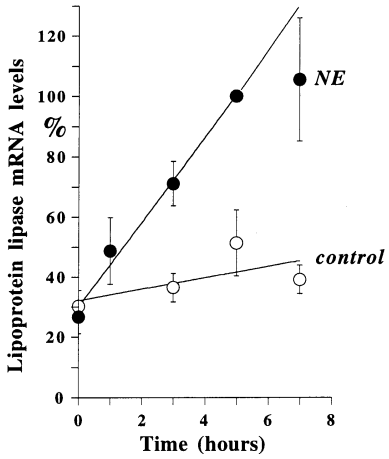

Figure 2 Time course of the effect of noradrenaline on LPL gene expression in cultured brown adipocytes

Brown adipocyte precursor cells were isolated and grown in culture, as in Figure 1. The cells were then incubated for the indicated time in the absence (control) or presence of 10 μM noradrenaline (NE). Total RNA was then isolated and 5 μg electrophoresed and hybridized with the LPL cDNA probe. Values are means $\pm$ S.E.M. from two or three experiments, each performed in duplicate or triplicate wells; in each experiment, the mean value in the noradrenaline-treated cells after 5 h was set to 100%. The lines were drawn for best fit to the points; the 7 h point for noradrenaline-stimulated cells was excluded for this fit. The correlation coefficient was 0.64 for the control cells and 0.99 for the noradrenaline-treated cells.

time they acquire several of the characteristics of mature brown adipose tissue, e.g. they start to accumulate triacylglycerols in a multilocular way, and they obtain a high complement of mitochondria, at least compared with precursor cells obtained from white adipose tissue and grown in parallel [11].

In the routine cell culture procedure referred to above, newborn calf serum is included in the culture medium, principally to promote cell proliferation. However, as proliferation as such may suppress the progress of the cells into more differentiated states, and as enhanced LPL gene expression is part of the differentiation programme in adipocytes [17], serum may have negative effects on LPL gene expression. Furthermore, serum contains other factors (fatty acids, hormones) that may influence LPL gene expression [18]. It was therefore decided to perform the present experiments in a fully defined medium, i.e. in the absence of serum. Thus in the present experiments we used cultures that had grown for the first 6 days in medium containing serum but which during the next 6 days, up to the 12th day, only had fully defined (serum-free) medium. Even under these conditions, the cells retained morphological characteristics of differentiation (multilocular fat droplets) and could persist in culture for an extended time.

Noradrenaline enhances the expression of the LPL gene in rat brown adipocyte cultures

To investigate if noradrenaline is able to directly affect LPL gene expression in cultured brown adipocytes, it was added to the cell cultures and LPL mRNA levels were measured 7 h later.

On the Northern blot (Figure 1a), a band hybridizing with the LPL cDNA probe is present in non-stimulated cells. Addition of noradrenaline induced a large increase in the level of LPL mRNA (Figure 1a), indicating that the previously observed effect of noradrenaline on LPL mRNA levels in the brown adipose tissue of intact rats [9] was a direct effect on the brown adipocytes themselves.

The major transcript of LPL mRNA, detected in all samples, had a size of 3.6 kb. This is similar to that observed in the intact rat, in both brown adipose tissue [9,19,20] and other tissues [3,19,21,22], as well as in other rat cell culture systems [23,24]. Thus the transcription process in the intact rat and in these cultured cells is the same.

Two shorter bands were also visible for the RNA from the noradrenaline-stimulated cells; these bands could also be observed in the control cells (only weakly seen in Figure 1a). The nature of the two shorter mRNA species is not known; they may represent different polyadenylation sites, as has been discussed for other species [3]. The major band constituted $81 \pm 12\%$ of total hybridizing RNA in control and $71 \pm 4\%$ in noradrenaline-stimulated cells; the 2.2 kb band was 13 ± 2 and $21 \pm 1\%$, and the 1.4 kb band 7 ± 1 and $8 \pm 1\%$ respectively. Thus noradrenaline caused no significant change in distribution. The presence of similar short LPL mRNAs has also been observed by others (in rat heart tissue) [25], but there was some variation in relative amount. Only the major (3.6 kb) band was routinely analysed in further studies.

Dependence on time of noradrenaline stimulation of LPL gene expression

To determine the time course of the effect of noradrenaline on LPL gene expression, the cell cultures were incubated with and without noradrenaline for time periods up to 7 h.

In control cultures, LPL mRNA levels remained fairly stable with time (Figure 2), although a tendency to increase was observed; this may be related to the addition of fresh medium 30 min before the start of the experiment. However, in the noradrenaline-stimulated cells, there was a potent increase in LPL mRNA levels. This increase was linear with time for at least the first 5 h, and the mRNA levels remained elevated for at

Table 1 Effect of inhibition of transcription on LPL mRNA levels in cultured rat brown adipocytes

Brown adipocyte precursor cells were isolated and grown in culture, as in Figure 1. Actinomycin (1 μg/ml) was then added as indicated, and 1–2 min later 10 μM noradrenaline was added. The cell cultures were incubated for 4 h; total RNA was then isolated and LPL mRNA levels estimated by the slot-blot technique (see the Materials and methods section). Results are means $\pm$ S.E.M. from two experimental series (two to four replicate wells per series); in each experimental series, the expression level obtained with noradrenaline was set to 100%.

Treatment	LPL mRNA levels
Control	45 $\pm$ 4
Actinomycin	55 $\pm$ 10
Noradrenaline	100 $\pm$ 0
Noradrenaline + actinomycin	58 $\pm$ 3

least 7 h after the addition of noradrenaline. In the following experiments, a 4 h time point was therefore used.

The time course may be compared with previous observations in intact rats. After a single injection of noradrenaline into rats, an increase in LPL mRNA levels was seen, but the increase was not stable with time for such a long time; it started to decrease after 4 h [9]. The difference in time course is probably due to elimination of the injected noradrenaline from the animal, where e.g. analysis of thermogenesis indicates that the noradrenaline concentration only remains elevated for about 40 min [26]. In contrast, the noradrenaline seems to persist in the cell cultures.

Adrenergic stimulation of LPL gene expression is a transcription-dependent process

The noradrenaline-induced increase in LPL mRNA level could be due to either an increased rate of gene transcription or stabilization of pre-existing mRNA. In order to investigate whether the increase was independent of transcription, we inhibited transcription with actinomycin D at a concentration (1 μg/ml) that was previously shown to inhibit fully noradrenaline-induced gene expression in brown adipocyte cultures [27].

As seen in Table 1, actinomycin treatment did not in itself have any significant effect on LPL mRNA levels in control cells after 4 h of incubation. The fact that no decrease in LPL mRNA level was observed in these cells after this amount of time would indicate that the half-life of LPL mRNA under these conditions is very long. This is in good agreement with previous observations in intact rats where both indirect [7] and direct [9] estimates of LPL mRNA stability in brown adipose tissue have indicated very long half-lives in the unstimulated situation (approx. 33 and 44 h respectively).

The absence of an effect of actinomycin on LPL mRNA levels makes it unlikely that mRNA stabilization is the explanation for the rapid increase in LPL mRNA level observed after noradrenaline addition. In agreement with this, the increase in LPL mRNA level observed after noradrenaline addition was fully abolished by the presence of actinomycin (Table 1).

Thus the noradrenaline-induced increase in LPL mRNA levels is dependent on transcription and is not due, to any appreciable extent, to prolongation of the half-life of mRNA. This conclusion is also in agreement with experiments in intact rats, both when LPL gene expression was studied as enzyme activity in brown adipose tissue [7] and when LPL mRNA levels were followed directly in the tissue [9].

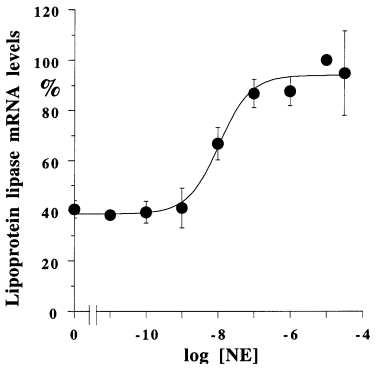

Figure 3 Dose–response curve for the effect of noradrenaline on LPL gene expression

Brown adipocyte precursor cells were isolated and grown in culture, as in Figure 1. The indicated concentrations of noradrenaline (NE) were then added, and 4 h later, the cultures were harvested and LPL mRNA levels determined. Results are means $\pm$ S.E.M. from six to ten experiments, each performed in duplicate to quadruplicate wells; in each series, the mean value at 10 μM noradrenaline was set to 100%. The curve was drawn for best fit of the indicated mean values to Michaelis–Menten kinetics, i.e. for the equation $E_{NE} = E_{basal} + E_{max}[NE/(NE + EC_{50})]$ where E_{NE} is the mRNA level at a given noradrenaline (NE) concentration, E_{basal} the initial mRNA level and E_{max} the maximal increase in mRNA level induced by noradrenaline. Curve fitting was performed by the general curve-fitting procedure of the KaleidaGraph application for Macintosh. The resulting parameters were $E_{basal} = 39 \pm 2$, $E_{max} = 55 \pm 3$ and $pEC_{50} = -7.96 \pm 0.13$, corresponding to an estimated EC_{50} of 11 nM (8–15 nM); the correlation coefficient was 0.99.

Adrenergic sensitivity of LPL gene expression

To investigate the adrenergic sensitivity of LPL gene expression in the cultured brown adipocytes, dose–response experiments were undertaken. Different noradrenaline concentrations were added and the levels of LPL mRNA were analysed after 4 h incubation.

As seen in the resulting dose–response curve (Figure 3), the response followed Michaelis–Menten saturation kinetics. There was no indication of interacting sites (Hill coefficient ≈ 1, not shown). The EC_{50} of 11 nM was similar to that observed for the stimulation of uncoupling protein (UCP) gene expression in cultured mouse brown adipocytes [28]. However, in contrast with noradrenaline-induced UCP gene expression in the mouse cells, the dose–response curve was simple, not bell-shaped. Thus any noradrenaline concentration above about 0.1 μM would be expected to give at least 90% of the full effect; in the following experiments we have used concentrations between 0.1 and 10 μM noradrenaline (as indicated).

Pharmacological characterization of the adrenoceptors that mediate the noradrenaline effect

To identify the type of adrenergic receptors involved in the activation of LPL gene expression in brown adipocyte cultures, we tested the ability of adrenergic subtype-selective agonists to enhance LPL gene expression, as well as the ability of subtype-selective antagonists to inhibit the noradrenaline effect on LPL gene expression.

Agonists

The agonist results are summarized in Table 2(A). We again observed a 2.5-fold higher level of LPL mRNA in noradrenaline-stimulated cells than in control cells. The α_1-adrenoceptor-selective agonist cirazoline (even at a 10-fold higher concen-

Table 2 Effect of (A) adrenergic agonists and (B) antagonists on LPL gene expression in cultured brown adipocytes

Brown adipocyte precursor cells were isolated and grown in culture, as in Figure 1. The indicated adrenergic agonists (added to a concentration of 0.1 μM if not otherwise indicated) and antagonists (all at 10 μM, added 1 min before the agonists) were then added; where indicated, ethanol and DMSO (which were used to dissolve prazosin) were added in equal amounts to the parallel cultures (see the Materials and methods section). After 4 h the cultures were harvested and LPL mRNA levels determined. The data are means $\pm$ S.E.M. from the indicated (n) number of experimental series, each based on duplicate–quadruplicate wells; in each experiment, the mean mRNA level observed after addition of 0.1 μM noradrenaline was set to 100%. (*)$P < 0.1$, *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$, statistically significant effects of antagonist addition on the agonist effect; Student's paired t test.

(A) Adrenergic agonists

Treatment	Receptor types activated	LPL mRNA levels (%)	n
Control	–	40 $\pm$ 2	12
Noradrenaline	β_1 β_2 β_3 α_1 α_2	100	12
Cirazoline (1 μM)	α_1	67 $\pm$ 11	3
Isoprenaline	β_1 β_2 β_3	110 $\pm$ 10	3
Cirazoline (1 μM) + isoprenaline	β_1 β_2 β_3 + α_1	126 $\pm$ 8	3
Dobutamine	β_1	49 $\pm$ 4	3
Salbutamol	β_2	50 $\pm$ 3	3
BRL-37344	β_3	103 $\pm$ 9	4
ICI-D7114	β_3	116 $\pm$ 8	4
CGP-12177A	β_3	70 $\pm$ 22	4

(B) Adrenergic antagonists

Agonist	Solvent	Antagonist	Receptor types activated	LPL mRNA levels (%)	n
α_1			–		
Control	EtOH			45 $\pm$ 19	2
Noradrenaline	EtOH		β_1 β_2 β_3 α_1 α_2	100	4
Noradrenaline	EtOH	Prazosin	β_1 β_2 β_3 α_2	70 $\pm$ 7*	4
Control	DMSO		–	38 $\pm$ 16	2
Noradrenaline	DMSO		β_1 β_2 β_3 α_1 α_2	100	2
Noradrenaline	DMSO	Prazosin	β_1 β_2 β_3 α_2	70 $\pm$ 5(*)	2
β					
Control			–	42 $\pm$ 1	6
Noradrenaline			β_1 β_2 β_3 α_1 α_2	100	6
Noradrenaline		Propranolol	α_1 α_2	30 $\pm$ 3***	6
Noradrenaline (1 μM)			β_1 β_2 β_3 α_1 α_2	85 $\pm$ 6	6
Noradrenaline (1 μM)		Propranolol	α_1 α_2 (β_3)	51 $\pm$ 7***	6
α_2					
Control			–	43 $\pm$ 5	6
Noradrenaline			β_1 β_2 β_3 α_1 α_2	100	6
Noradrenaline		Yohimbine	β_1 β_2 β_3 α_1	95 $\pm$ 7	6
Isoprenaline			β_1 β_2 β_3	94 $\pm$ 6	6
Isoprenaline		Yohimbine	β_1 β_2 β_3	109 $\pm$ 11	6

tration) had a much smaller effect than noradrenaline, whereas the β-adrenoceptor-selective agonist isoprenaline was equipotent with noradrenaline. This indicated a predominantly or exclusive β-adrenergic pathway for stimulation. However, when isoprenaline + cirazoline were present together, a larger effect was seen than with isoprenaline alone. This indicated that not only β-receptors may be involved.

To investigate the β-adrenergic subtype involved, we used subtype-selective agonists. The β_1-agonist dobutamine and the β_2-agonist salbutamol were practically without effect on LPL gene expression. In contrast, the β_3-adrenoceptor-selective agonists BRL-37344 and ICI-D7114 were equipotent with noradrenaline. At the concentration used here (0.1 μM) the β_3-specific agonist CGP-12177 [29] did not induce the mRNA level to the same extent as noradrenaline. However, as a thermogenic drug, CGP-12177 has a significantly higher EC_{50} than has noradrenaline [30], and when a higher dose of CGP-12177 (10 μM) was used, it was found to be at least equipotent with noradrenaline in enhancing LPL gene expression (133 $\pm$ 42% of the effect of 0.1 μM noradrenaline, $n = 2$).

Thus, from these experiments, it was concluded that the effect of noradrenaline was mainly mediated via β_3-adrenoceptors, perhaps with some positive influence of α_1-receptors.

Antagonists

We performed experiments with antagonists to the three main classes of adrenergic receptors: α_1, β and α_2 (Table 2B).

The α_1-adrenergic antagonist prazosin was able to significantly inhibit the effect of noradrenaline, irrespective of diluent. In order to exclude the possibility that this inhibition was secondary to (unexpected) effects of prazosin on cAMP levels, we investigated the effect of prazosin on cAMP levels. Addition of 0.1 μM noradrenaline led, as expected, to a large increase in cAMP levels (from 12 $\pm$ 6 to 828 $\pm$ 53 pmol of cAMP per well), but there was no effect of 10 μM prazosin on the noradrenaline-induced increase in cAMP levels (842 $\pm$ 173 pmol; prazosin was dissolved in ethanol which was also added to the parallel incubations; measurements 20 min after noradrenaline addition). We also investigated whether prazosin would influence noradrenaline-stimulated thermogenesis in isolated brown adipocytes from rat; this was found not to be the case (oxygen electrode experiments with 1 μM noradrenaline $\pm$ 5 μM prazosin and 0.1 μM noradrenaline $\pm$ 10 μM prazosin; not shown). Thus the prazosin effect was not mediated via an unspecific effect on the β-adrenergic/cAMP system. The relationship between the concentration of prazosin and noradrenaline was such (100-fold excess of prazosin which also has a 10000-fold higher affinity than noradrenaline for the α_1-receptors in the tissue [31]) that it must be considered very unlikely that only a partial inhibition of the α_1-adrenergic pathway had occurred. Thus whereas the experiment indicated that stimulation of α_1-receptors was able to positively influence the expression of the LPL gene in cultured brown adipocytes, it was also clear that this was not the only adrenergic pathway that was utilized.

The β-adrenergic antagonist propranolol was able to inhibit fully the effect of noradrenaline, when noradrenaline was added at a concentration of 0.1 μM (Table 2B). When a higher concentration (1 μM) was used, propranolol seemed less efficient. The somewhat poorer inhibitory effect of propranolol against higher noradrenaline concentrations is in accordance with its relatively poor affinity for β_3-receptors, as compared with its affinity for classical β_1/β_2-receptors [32]. The inhibitory effect of propranolol could be fully explained by its ability to inhibit the noradrenaline-induced increase in cAMP levels: in unstimulated cells, 5 $\pm$ 5 pmol of cAMP was found per well; in cells stimulated with 0.1 μM noradrenaline, the level was 1097 $\pm$ 97; and in cells to which 10 μM propranolol was added before the noradrenaline, the cAMP level was reduced to 26 $\pm$ 12 pmol.

Thus the β-blocker experiments indicated that the adrenergic effect was fully mediated via β-receptors and are compatible, with β_3-receptors being the active receptor type.

The α_2-adrenergic antagonist yohimbine was without effect on noradrenaline- (or isoprenaline-) stimulated LPL gene expression (Table 2B). This was unexpected, as previous indications were that blockade of α_2-receptors may enhance noradrenaline-induced lipolysis in these cells (although under somewhat different conditions) [12]. A possible interpretation is that an

Table 3 Effect of (A) cAMP-elevating agents and (B) protein-synthesis inhibition on LPL gene expression in cultured brown adipocytes

Brown adipocyte precursor cells were isolated and grown in culture, as in Figure 1. The indicated agents were then added and the cell cultures harvested 4 h later and LPL mRNA levels determined. Data are means ± S.E.M. from three series, each performed with duplicate–quadruplicate wells; in each series, the mean level of LPL mRNA observed in the noradrenaline-treated cultures was set to 100%.

(A) cAMP-elevating agents

Agent	LPL mRNA levels (%)
Control	39 ± 2
Noradrenaline (0.1 μM)	100
Cholera toxin (1 μg/ml)	91 ± 14
Forskolin (10 μM)	101 ± 8

(B) Protein-synthesis inhibition

Treatment	LPL mRNA levels (%)
Control	44 ± 5
Cycloheximide (50 μM)	53 ± 9
Noradrenaline (10 μM)	100
Noradrenaline + cycloheximide	109 ± 30

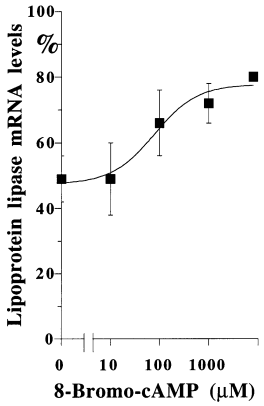

Figure 4 Dose–response curve for the effect of Br-cAMP on LPL gene expression

Brown adipocyte precursor cells were isolated and grown in culture, as in Figure 1. The indicated concentrations of Br-cAMP were then added, and 4 h later the cultures were harvested and LPL mRNA levels determined. Results are means ± S.E.M. from three experiments (1 for 8 mM Br-cAMP), each performed in duplicate–quadruplicate wells; in each series, the mean value at 10 μM noradrenaline was set to 100%. The curve was drawn for best fit of the indicated mean values to Michaelis–Menten kinetics, as in Figure 3. The resulting parameters were $E_{basal} = 47 \pm 3\%$, $E_{max} = 30 \pm 4\%$ and $EC_{50} = 77 \pm 48$ μM; the correlation coefficient was 0.98.

increase in cAMP in excess of the level obtained with 0.1 μM noradrenaline is unable to stimulate LPL gene expression further, whereas it is able to stimulate lipolysis further.

Taken together, the adrenergic agonist and antagonist experiments reveal a somewhat complex picture of the receptors involved, in that β_3-receptors are clearly able to account fully for the stimulation of gene expression but nevertheless α_1-adrenergic pathways also have a positive influence.

Intracellular mediation of the adrenergic effect

The implication from the adrenergic receptor studies was that LPL gene expression is enhanced mainly through β_3-receptors and that this enhancement is thus most likely mediated via an increase in cAMP levels. To test this possibility, we increased intracellular cAMP levels in three ways: by stimulating the $G_S\alpha$ protein with cholera toxin, by stimulating the adenylate cyclase directly with forskolin, and by addition of the cAMP analogue 8-Br-cAMP.

As seen in Table 3A, both cholera toxin and forskolin increased LPL gene expression to a level similar to that observed with noradrenaline. It has previously been observed that forskolin is able to increase cAMP levels in cultured brown adipocytes to a much higher level than that seen with noradrenaline [33]; thus the present result again indicates that it is not possible to increase LPL gene expression above that induced with a maximal noradrenaline concentration.

Br-cAMP was also able to enhance LPL gene expression in the cultured brown adipocytes (Figure 4). However, the stimulated level did not approach that observed with noradrenaline stimulation. That Br-cAMP is less effective than noradrenaline or forskolin in stimulating cAMP-dependent processes in brown adipocytes has been observed previously [34]. The simplest interpretation is that it permeates the plasma membrane relatively slowly and is susceptible to phosphodiesterase activity [35]; thus the intracellular concentrations actually reached may be lower than after noradrenaline treatment.

Taken together, these experiments with agents that increase functional intracellular cAMP levels indicate that the enhanced LPL gene expression seen after noradrenaline treatment can be fully mimicked by increasing cAMP levels.

Is the adrenergic effect secondary to an adrenergically induced synthesis of mediatory proteins?

The observations above on the significance of both β_3- and α_1-adrenoceptors can be linked to both earlier observations on the control of gene expression in cultured brown adipocytes and to the regulation of LPL in similar systems.

We have previously observed that a strong synergistic interaction is observed between α_1- and β-adrenergic stimulation (and corresponding intracellular pathways) in the adrenergic control of c-*fos* expression in cultured brown adipocytes [33]. Further, in certain cultured adipocyte-like cell lines (Ob1771), it has been demonstrated that the stimulatory effect of growth hormone on LPL gene expression is mediated via an increase in c-*fos* expression, i.e. by the increased level of Fos protein [36]; in agreement with this, an AP1 consensus sequence is found in the promoter of the LPL gene [37]. It could therefore be suggested that Fos mediates the action of adrenergic agents on LPL gene expression in cultured brown adipocytes. If this is the case, the noradrenaline effect should be dependent on protein synthesis. In order to test this possibility, brown adipocyte cultures were treated with the protein synthesis inhibitor cycloheximide at a concentration (50 μM) that has been demonstrated to inhibit completely UCP synthesis in brown adipocyte cultures [38].

As seen in Table 3B, the addition of cycloheximide itself had no effect on LPL gene expression; thus basal LPL gene expression is not under the control of a short-lived transcription factor. When cycloheximide was added together with noradrenaline, a reduction in the noradrenaline-induced level was not observed;

thus the adrenergically stimulated level of LPL gene expression was not dependent on the synthesis of c-Fos protein or any other protein mediator with the characteristics of a transcription factor. This experiment also eliminated the possibility that the adrenergic effect was secondary to induction of the synthesis of an autocrine factor of the type that has been shown to induce LPL gene expression in long-term experiments in heart cell cultures [39] (but it did not eliminate the possibility of the release of a prestored long-lived autocrine factor).

DISCUSSION

In the present investigation we have examined the regulation of the expression of the LPL gene in a brown adipocyte culture system. We have established that the stimulatory effect of adrenergic stimulation on LPL gene expression, implied from previous *in vivo* experiments, occurs directly on the brown adipocytes themselves and that it occurs mainly through β_3-adrenergic receptors and via an increase in cAMP levels. The adrenergic effect is transcription-dependent but is not mediated via the synthesis of an intermediary protein.

For the present study, we chose to use brown adipocyte cultures from rats because the implications from *in vivo* investigations may be tested in this species: experiments from different laboratories imply that the increase in LPL activity observed in brown adipose tissue when the thermogenic activity of the tissue is physiologically stimulated results from increased transcription of the LPL gene, induced by noradrenaline released from the sympathetic nervous system and mediated via β-receptors and increases in cAMP levels [6,7,9,10,20,40–49]. However, that this is really what occurs in the brown adipocyte has not been demonstrated until now.

The precursor cells used for the present study, isolated from brown adipose tissue depots of rats, develop differently from precursor cells isolated from white adipose tissue grown in culture under identical conditions [11]. Although the cultured brown adipocytes demonstrate several characteristics of differentiated brown adipocytes, we have not observed noradrenaline-induced expression of UCP (thermogenin) in these cells. However, other groups [50,51] have demonstrated UCP expression in cultured rat brown adipocytes under similar conditions, indicating that these cells can reach full differentiation in culture; in such cells a positive effect of noradrenaline on LPL gene expression was also noted [51].

Pretranslational versus (post)translational regulation of LPL activity

In the present study we have only examined LPL gene expression at the mRNA level. On the basis of our initial studies *in vivo*, we suggested that the regulation of LPL activity in brown adipose tissue is mainly at this level, i.e. the pretranslational level [7], and in later studies *in vivo* we confirmed reasonably good agreement between relative mRNA levels and activity [9]. Also the relative effects observed here (2.5-fold after 4 h) are in good agreement with the relative increases in LPL activity observed *in vivo*. Other investigators have also observed good agreement between LPL mRNA levels and LPL activity in brown adipose tissue of rats [20]. However, such a simple relation has been reported to not exist during virgin/pregnancy/lactation transitions in the rat [19]. The reason for this is unknown; the conclusion from the present series of experiments, in connection with our previous observations, is that regulation of expression is an adequate explanation for alterations in LPL activity in this tissue. Thus, even though post-translational steps are essential for LPL activity [37,52], these steps do not seem to have a regulatory role in brown adipose tissue under the conditions studied.

Adrenergically induced LPL gene expression in brown adipocytes is primarily mediated via β_3-receptors and an increase in cAMP

There is a basal level of LPL gene expression in cultured brown adipocytes in the absence of added adrenergic agents. To some extent, this level is probably maintained by the insulin present in the defined medium used here. However, adrenergic stimulation increased this level markedly, mainly via β-receptors.

That expression of the LPL gene is mainly enhanced via β-receptors is in good agreement with studies *in vivo* [6,7,9,10,48]; so is the observation that it is the β_3-receptor that is the coupled subtype [20]. The positive α_1-adrenergic effect observed here is somewhat more difficult to relate to observations *in vivo*. Both increases and decreases in LPL activity by α_1-adrenoceptor blockade with prazosin have been observed in intact rats [53,54].

Regulation of LPL gene expression in rat brown adipocytes compared with regulation in other systems

Difference between cultures from rat brown and white adipose tissue

The positive effect of adrenergic stimulation and cAMP observed here in rat brown adipocyte primary cultures is directly opposite to that observed in rat white adipocyte primary cultures where adrenaline leads to decreased LPL activity which may be mediated via a decrease in mRNA levels [55,56]. This also means that the brown adipocytes studied here, despite not expressing UCP, are clearly differentiated from white adipocyte cells to the extent that they show different regulation of LPL gene expression.

In the mouse white adipocyte-like cell line 3T3-F442A both isoprenaline and forskolin also decrease LPL gene expression [57]. cAMP has also been shown to inhibit LPL gene expression in mouse macrophages [58].

α_1-Adrenergic stimulation of cultured brown adipocytes is known to result in an increase in intracellular Ca^{2+} levels [33]. Thus the tendency to a positive effect of α_1-adrenergic stimulation on LPL gene expression found here is in contrast with the results for the mouse preadipocyte cell line Ob1771, where an increase in Ca^{2+} leads to inhibition of expression [59].

Taken together, these data indicate that the regulation of LPL gene expression in cultured brown adipocytes is qualitatively different from that in white adipocytes or white adipocyte-like cell lines in culture.

Similarity between brown adipose tissue and heart

Although the regulation observed here is qualitatively different from that observed in white adipocytes, there are *in vitro* systems that demonstrate similar regulatory properties. For instance, positive effects of cholera toxin on LPL mRNA levels have been observed in heart cell cultures [23] but no direct effects of noradrenaline on LPL gene expression have been reported in heart cell cultures, despite the fact that noradrenaline is expected to be the physiological regulator of LPL activity (and thus gene expression) also in that tissue. Also in a hepatoma cell line ($BWTG_3$) in which LPL gene is expressed (although LPL is not expressed in normal mature liver cells), this expression is under the positive control of cAMP [60].

Comparison with mouse brown adipocytes in culture

Modest positive effects of adrenergic stimulation on LPL activity have been reported in primary cell cultures obtained from brown adipose tissue of mice, as well as in the mouse brown adipocyte-

like cell line BFC-1 [61,62], and the results presented here are therefore principally in agreement. However, concerning the level of LPL mRNA in our cultured mouse brown adipocytes, we have not observed positive effects of adrenergic stimulation [63] (P. Kuusela, S. Rehnmark, A. Jacobsson, B. Cannon and J. Nedergaard, unpublished work). This is surprising, since LPL gene expression *in situ* can be induced by noradrenaline, even in mice ([64]; P. Kuusela, S. Rehnmark, A. Jacobsson, B. Cannon and J. Nedergaard, unpublished work).

In another mouse cell line of brown adipocyte character, HIB 1B, inconsistent effects of adrenergic stimulation on LPL gene expression have been observed [65]. Even when clear positive effects of isoprenaline and dibutyryl-cAMP on UCP gene expression were observed, no systematic positive effect of these agents on LPL gene expression was observed; if anything, the effect tended to be negative [66].

Thus there may be a basic difference between the response of cultured brown adipocytes from mouse and those from rat.

Regulation of LPL gene expression

The present observations highlight the interesting question of the tissue-specific adrenergic regulation of the expression of the LPL gene, with the contrasting effects of cAMP-elevating agents that occur in different tissues. There is only one gene for the enzyme [37,67], although tissue variants may exist [68]; thus the differential expression must be regulated in the promoter region of the gene. The promoter is still not fully analysed and no functional cAMP response element (CRE) has been identified, although a CRE consensus sequence may occur at approx. −400 in the promoter in certain species [37]. However, the regulatory sequences extend much further: experiments with transgenic mice indicate that within the first 1824 bases there are sequences that lead to very high levels of expression in brown adipose tissue [69].

From what is known today it may be concluded that this promoter contains tissue-specific element(s) that in some way interact with CRE-binding proteins (CREB), or CREB-like factors, in such a way that the CREB signal is interpreted oppositely in different tissues, i.e. as an inhibitory signal for transcription in e.g. white adipose tissue and as a stimulatory signal in e.g. brown adipose tissue. The fact that two otherwise similar tissues must contain factors that alter the response so dramatically makes the study of LPL gene expression a promising model for developing a greater understanding of differentiation processes.

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