

Expression of β_1 - and β_3 -adrenergic-receptor messages and adenylate cyclase β -adrenergic response in bovine perirenal adipose tissue during its transformation from brown into white fat

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Possible modifications of the β -adrenergic effector system during the development of bovine perirenal brown adipose tissue (BAT) *in utero* and its transformation into white-like adipose tissue after birth were studied. The parameters assessed were the level of expression of β_1 -, β_2 - and β_3 -adrenergic receptor (AR) mRNAs and the response of the plasma-membrane adenylate cyclase to (–)-isoprenaline and to the β_3 -agonist BRL 37344. The β_3 -AR mRNA was found to be expressed very early *in utero*, i.e. before the third month of foetal life. Then it increased dramatically (9-fold) between month 6 of foetal life and birth. A high β_3 -AR mRNA level was maintained after birth up to an age of 3 months. After conversion of BAT into white-like adipose tissue, i.e. in the adult bovine, the β_3 -AR mRNA expression

became small or not detectable, and the β_1 -AR mRNA, which was expressed much less than the β_3 -AR mRNA in foetal life, became predominant. A response of the adenylate cyclase to (–)-isoprenaline was observed in foetal life (3.1-fold stimulation). It decreased after birth (1.8-fold stimulation) and then remained constant until adulthood. A response to BRL 37344 was also observed in foetal life (1.8-fold stimulation). It was maintained after birth, but disappeared in the adult. A possible relationship between the β -AR expression and the adenylate cyclase response to (–)-isoprenaline on the one hand and the uncoupling-protein expression on the other is discussed. The bovine might represent a good model to understand the transition from brown to white fat in the human.

INTRODUCTION

In rodents, the biological importance of brown adipose tissue (BAT) as a thermogenic effector throughout life has been clearly demonstrated. This tissue plays a crucial role in the thermoregulation of newborns [1] and contributes to the overall energy expenditure in the adults [2]. BAT is under control of the sympathetic nervous system, which mainly acts via β -adrenergic receptors (β -AR) [3]. One of the rate-limiting steps of BAT thermogenesis is a mitochondrial protein, unique to brown adipocytes, called uncoupling protein (UCP). The expression of this protein is regulated at the transcriptional level by catecholamines [4]. UCP can be used as a powerful marker to distinguish between brown and white adipocytes [5].

In large mammals, including ruminants and humans, the importance of BAT in adult life is still an open question. In these species BAT appears during foetal life and is present at birth in most fat-pads, as evidenced by specific BAT features, i.e. typical mitochondria, UCP expression and iodothyronine 5'-deiodinase activity [6–9]. BAT is involved in thermoregulation in newborns, as in rodents, and is then gradually converted into white-like adipose tissue with disappearance of BAT markers [5–9]. A recent study in bovine perirenal fat has shown that UCP mRNA does not appear before month 7 of gestation and disappears a few days after birth [10]. BAT reappearance in adult humans has been observed in increased sympathetic activity [11–13]. These observations question the mechanisms responsible for development *in utero* of brown fat at thermoneutral temperature and for its transformation after birth into white-like adipose tissue. The bovine was chosen as a good model for the understanding of adipose-tissue development in ruminants and also in the human.

Recently, a new β -AR subtype, called β_3 -AR, was cloned from

human and mouse genomic libraries [14,15] and from rat BAT cDNA libraries [16,17]. In rodents, the β_3 -AR seems to be the predominant β -AR subtype expressed in BAT, in white adipose tissue (WAT) and in 3T3-F442A adipocytes [16–18]. Its mRNA level was found to be higher in BAT than in WAT and was decreased in both tissues of obese rats, as compared with lean rats [17]. It was shown that the β -adrenergic stimulation of lipolysis and thermogenesis is mediated essentially in BAT by the β_3 -AR [19]. *In vivo*, chronic stimulation of adults dogs with a β_3 -adrenergic agonist ICI D7114 leads to a re-activation of UCP expression in WAT, associated with a decreased weight gain in the animal [20]. *In vitro*, many experiments demonstrate the efficiency of β_3 -adrenergic agonists in promoting differentiation and in increasing UCP mRNA expression in brown adipocytes in primary culture [21,22]. Taken together, these results strongly suggest possible relationships between the stimulation of β_3 -AR and BAT development.

In this study, we have investigated in the bovine how the development of BAT *in utero* and its transformation into white-like adipose tissue after birth is correlated with β -AR subtype expression. Due to the lack of available labelled β_3 -AR ligand, it is at present difficult to measure the amount of β_3 -AR protein. We therefore prepared DNA probes for the three β -AR subtypes and measured the level of the corresponding mRNAs as indexes of their expressions. We also studied the plasma-membrane adenylate cyclase response to β -adrenergic stimulation.

MATERIALS AND METHODS

Chemicals

All organic and inorganic chemicals were of analytical or molecular-biology grade and were purchased from Merck (Darm-

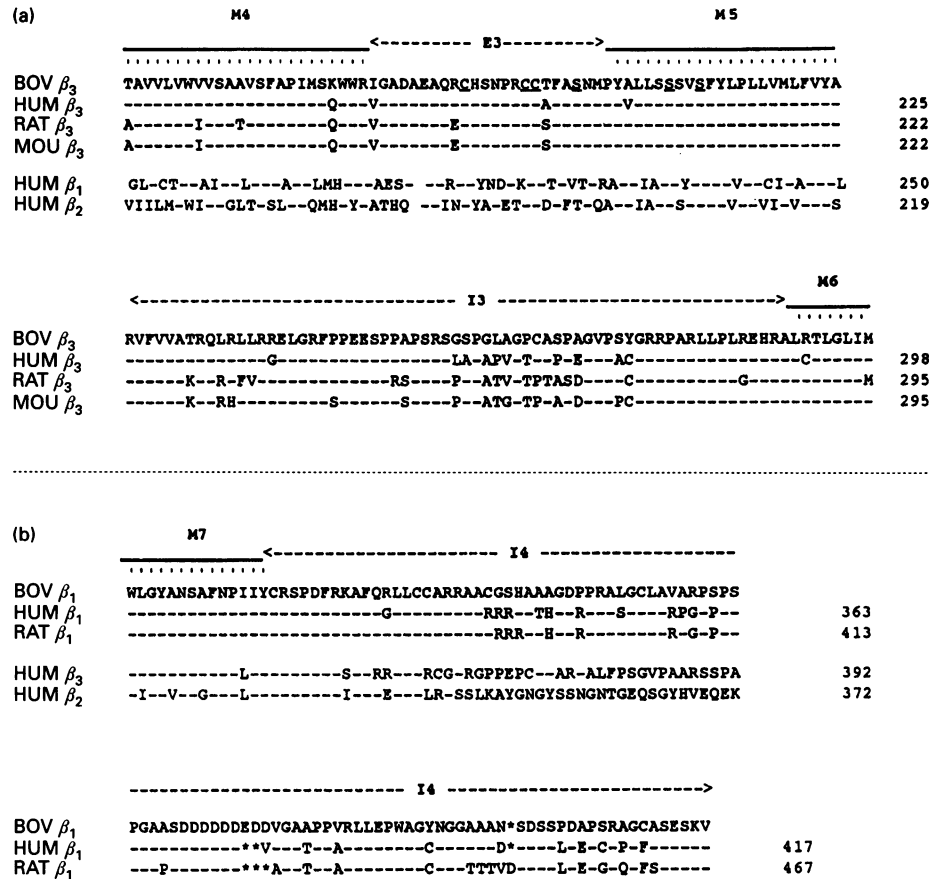


Figure 1 Amino acid sequences of (a) the bovine (BOV) β_3 -AR PCR fragment 501–978 (see the Materials and methods section) and (b) (BOV) β_1 -AR PCR fragment 1174–1614

In (a), alignments with the corresponding regions of the human (HUM) [18], rat (RAT) [21] and mouse (MOU) [19] β_3 -AR and with the human β_1 -AR [38] and β_2 -AR [32] are shown. In (b), alignments with the corresponding regions of the human (HUM) [38] and rat (RAT) [39] β_1 -AR and with the human β_3 -AR [18] and β_2 -AR [32] are shown. M4–M7, transmembrane domains 4–7; E3, extracellular loop 3; I3 and I4, intracellular loops 3 and 4.

stadt, Germany), Sigma (St. Louis, MO, U.S.A.), Fluke (Buchs, Switzerland), Gibco BRL (New York, NY, U.S.A.), Pharmacia (Uppsala, Sweden) or Boehringer (Mannheim, Germany). [α - 32 P]dCTP (3000 Ci/mmol) was purchased from Amersham International (Amersham, Bucks., U.K.). Duralon-UV membranes, First-Strand Synthesis Kit, *Pyrococcus furiosus* (*Pfu*) DNA polymerase and the Quik-Hyb hybridization mixture were from Stratagene (La Jolla, CA, U.S.A.), and pBluescript plasmids, T₇ polymerase kit and prepacked oligo(dT)–cellulose columns were from Pharmacia. BRL 37344 was a gift from SmithKline Beecham Pharmaceuticals (Epsom, Surrey, U.K.).

Tissue sampling

Bovine (Friesian) foetuses, calves and 2–5-year-old cows (referred to in the text as adult bovine) were killed in a slaughter-house. Perirenal adipose tissue was immediately dissected, and a sample was frozen in liquid N₂ and stored at –20 °C for RNA extraction or membrane preparation.

PCR

The PCR amplification was performed by adding 250 ng of bovine genomic DNA to 20 mM Tris/HCl, pH 8.8, 10 mM KCl, 6 mM (NH₄)₂SO₄, 1.5 mM MgCl₂, 3% (v/v) formamide, 0.1%

Triton X-100, 1 μ M of each PCR primer and 1.25 units of thermostable DNA polymerase (total volume 50 μ l).

To prepare the β_3 -AR gene fragment, a sense primer matched with the human β_3 -AR gene sequence (GenBank data bank: M29932) from positions 501 to 520 and an antisense primer from positions 958 to 978 were used. To prepare the bovine β_1 -AR gene fragment, a sense primer matched with the human β_1 -AR gene sequence (GenBank data bank: JO3019) from positions 1174 to 1193 and an antisense primer from positions 1595 to 1614 were used.

The reaction mixture was overlaid with 100 μ l of mineral oil, and the PCR amplification was performed using a Perkin-Elmer Cetus DNA Thermal Cycler. After an initial denaturation step of 3 min at 95 °C, 30 cycles were performed, which consisted of denaturation (1 min at 95 °C), annealing (1.5 min at 56 °C) and extension (1 min at 72 °C).

Cloning and sequencing

PCR products were analysed by electrophoresis on a 2%–agarose gel. Bands of interest were identified by ethidium bromide staining, eluted from the gel and cloned in pBluescript plasmids. Three independent clones of the PCR products obtained with

each pair of primers were sequenced by using the T₇ polymerase kit.

RNA extraction and Northern-blot analysis

Total RNA was isolated from the adipose tissue by the caesium/trifluoroacetic acid gradient method of Okayama et al. [23]. The poly(A)⁺ RNA was isolated from total RNA by the method described by Aviv and Leder [24], by using prepacked oligo(dT)-cellulose columns.

Total RNA (20 μ g) or poly(A)⁺ RNA (15 μ g) was electrophoresed on a 1% agarose gel containing formaldehyde, as described by Lehrach et al. [25], and transferred to Duralon-UV membranes by capillary blotting. PCR fragments of β_1 - and β_3 -AR obtained from bovine genomic DNA as described above and the entire coding region of the human β_2 -AR cDNA (gift from Dr. C. M. Fraser [26]) were labelled by random priming with [α -³²P]dCTP to a specific radioactivity of approx. 1×10^9 d.p.m./ μ g of DNA and used as probes. RNA blots were hybridized for 1 h at 68 °C in the Quik-Hyb hybridization mix, washed in a solution of 0.1 \times SSC (SSC = 0.15 M NaCl/0.15 M sodium citrate)/0.1% SDS at 65 °C for 15 min and exposed to Kodak X-AR films at -70 °C. Size estimates of the RNA species were established by comparison with an RNA ladder.

Adenylate cyclase studies

Adipose-tissue plasma membranes were prepared as described previously for rat BAT [27]. Protein concentrations were determined by the method of Lowry et al. [28]. The adenylate cyclase activity of the membranes was measured by the technique described by Cooper et al. [29] in the presence of 10 μ M GTP.

RESULTS

Characterization of the β_3 - and β_1 -AR probes

The amino acid sequence of the β_3 -AR fragment obtained by PCR amplification of bovine genomic DNA with appropriate primers is shown in Figure 1(a). This sequence shows a higher degree of similarity to the human [14] than to the rat [17] β_3 -AR corresponding sequence (89% and 82% respectively). It shows a low degree of similarity to the corresponding human β_1 -AR [30] and β_2 -AR [26] sequences (54% and 43% respectively). The amino acid sequence of the β_1 -AR PCR fragment was found to display the same degree of similarity to the corresponding human [30] and rat [31] β_1 -AR sequences (81%) (Figure 1b).

Developmental changes in the β -AR mRNA levels

In the bovine foetus, the largest single depot of adipose tissue is located around the kidney. As early as at month 3 of foetal life (time of gestation in the bovine is 9 months), a small but significant amount of β_3 -AR mRNA could be detected in perirenal fat by Northern-blot analysis (results not shown). Figure 2 shows that the β_3 -AR mRNA level starts to rise after month 6 of foetal life until birth (9-fold increase). From month 3 of postnatal life it decreases, and is either still detectable, but at a low level, or undetectable, in 3 and 2, respectively, of the 5 adult bovines tested.

As can be measured from Figure 3, the sizes of β_1 - and β_2 -AR mRNA are 3.1 and 2.4 kb respectively. The sizes of β_3 -AR mRNAs are 2.2 and 1.9 kb. β_1 -, β_2 - and β_3 -AR mRNA levels were

compared in foetal and adult bovine perirenal fat by Northern-blot analysis. To increase the sensitivity of the method, poly(A)⁺ RNA was used as the starting material. At month 8 of foetal life, the β_1 -AR mRNA level is lower than that of β_3 -AR mRNA (Figure 3). In the adult bovine, however, the β_1 -AR mRNA level is higher than that of β_3 -AR mRNA (Figure 3). In the foetus β_2 -AR mRNA can be detected, but at a much lower level than β_3 - or β_1 -AR mRNA. In the adult the β_2 - and β_3 -AR mRNA are at the same level.

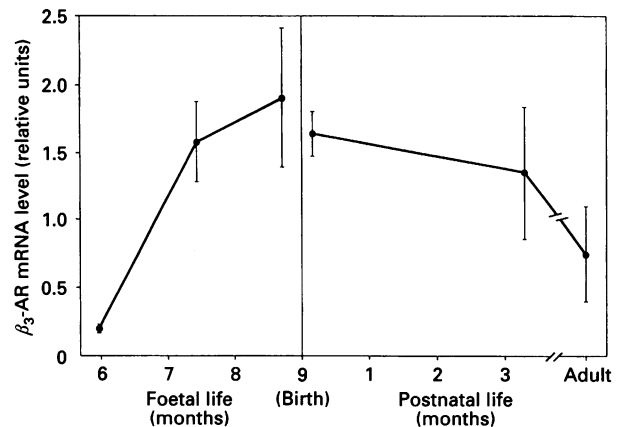


Figure 2 β_3 -AR mRNA levels in bovine perirenal fat at various times of foetal and postnatal life

Samples (30 μ g) of RNA were electrophoresed, transferred to membrane filters, hybridized with a ³²P-labelled bovine β_3 -AR genomic DNA probe, and the autoradiographic signals were quantified by densitometric analysis. The results are expressed in relative units. They are means \pm S.E.M. of 3–5 experiments performed on tissues from 3–5 different animals. The ages at which the samples were obtained are expressed in months.

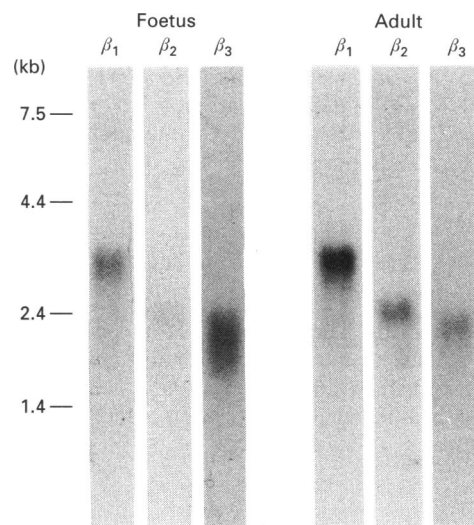


Figure 3 β -AR-subtype mRNA levels in foetal (month 8 of foetal life) and adult bovine perirenal fat

Samples (15 μ g) of poly(A)⁺ RNA were electrophoresed, transferred to membrane filters and hybridized with ³²P-labelled bovine β_1 - and β_3 -AR genomic DNA probes and the human β_2 -AR cDNA probe. The representative autoradiograms shown were obtained under identical conditions and with probes of similar specific radioactivities. The positions of the marker RNAs are shown in kb.

Table 1 Effect of (–)-isoprenaline and BRL 37344 on the adenylate cyclase activity of bovine perirenal-fat plasma membranes

The different times of bovine development chosen are: foetal life (month 8 of foetal life), neonatal life (10 ± 3 days) and adulthood. The results are means ± S.E.M. of the numbers of experiments in parentheses. They are expressed as percentages of the control value measured in the absence of β -adrenergic agonist. * P < 0.05, ** P < 0.005 versus the respective value in foetal life.

	Adenylate cyclase activity (% of control)	
	(–)-Isoprenaline	BRL 37344
Foetal life	311 ± 43 (4)	177 ± 19 (4)
Neonatal life	184 ± 6 (3)*	163 ± 7 (3)
Adulthood	202 ± 24 (4)*	98 ± 9 (4)**

Developmental changes in adenylate cyclase stimulation by (–)-isoprenaline and BRL 37344

The basal adenylate cyclase activity of perirenal-fat plasma membranes is 35.2 pmol/min per mg of protein, and NaF (10 mM) stimulates this activity 3.3-fold. Basal and NaF-stimulation values were found to be constant at all stages of the development studied.

The response of the adenylate cyclase activity to (–)-isoprenaline or BRL 37344 was studied. BRL 37344 is a potent agonist of all β_3 -ARs studied until now, including those of human [14] and dog [32]. As may be inferred from results obtained in the rat by Muzzin et al. [33,34], at the concentrations used, (–)-isoprenaline should act via β_1 -, β_2 - and β_3 -AR, whereas BRL 37344 should act via β_3 -AR. A stimulation of the adenylate cyclase activity by (–)-isoprenaline (1.6-fold; $n = 2$) and by BRL 37344 (1.3-fold; $n = 2$) is already observed at month 6 of foetal life. As shown in Table 1, the maximal response to (–)-isoprenaline (3.1-fold stimulation at month 8 of foetal life) decreases significantly after birth (1.8-fold stimulation) and remains practically unchanged until adulthood. The response to BRL 37344 (1.8-fold stimulation) at month 8 of foetal life remains constant after birth (1.6-fold stimulation), but is no longer detectable in adulthood.

DISCUSSION

In larger mammals, BAT is well developed prenatally and immediately after birth. Then it involutes and is replaced by white fat. It seemed interesting to test in ruminants if the development of brown fat and its transition to white fat during development might be related to possible changes in the β -adrenergic control.

Bovine β_3 - and β_1 -AR probes

The β_3 -AR probe encompasses a domain from trans-membrane region 4 (M4, Figure 1a) to the beginning of trans-membrane region 6 (M6). Its sequence includes cysteine-186, -192, -193 and serine-206 and -209, which play an important role in β -AR–ligand interaction and the third intracellular loop which confers the specificity of coupling to GTP-binding protein [35].

The β_1 -AR probe (Figure 1b) encompasses a domain from the

end of the 3' coding to the beginning of the 3' non-coding region. The amino acid sequence corresponds to the cytoplasmic tail of the receptor, which is rich in serine and threonine. These residues may serve as substrates for the β -AR kinase during the desensitization process [35].

Developmental changes in the β -AR levels

In foetal life, perirenal fat is characterized by a high expression of the β_3 -AR gene. At this stage of the development the β_1 -AR gene is expressed at a low level, and the β_2 -AR mRNA is undetectable. Accordingly, a high level of the β_3 -AR mRNA has been observed in rat [17] and human child brown fat [36]. It is noteworthy that the appearance of β_3 -AR mRNA during foetal life in perirenal fat precedes that of UCP mRNA, which occurs only at month 7 of gestation [10]. A high β_3 -AR mRNA level is maintained after birth up to the age of 3 months (Figure 2), at which UCP mRNA level has been reported to be practically undetectable [10]. It is known that, during the first 3 months of life, the number of adipocytes in bovine perirenal fat remains unchanged [37]. The disappearance of UCP mRNA can therefore be considered as an indication that brown adipocytes have been transformed into white-like adipocytes. The question can be asked whether the presence of the β_3 -AR mRNA is the witness of a previous brown-fat phenotype, or if the β_3 -AR is normally expressed in typical bovine white adipocytes.

The β_3 -AR mRNA expression is small or undetectable in the adult bovine (Figure 2). This is in contrast with the results obtained in rodent WAT, where the β_3 -AR was found to be highly expressed [17], but in agreement with the results obtained until now in adult human WAT [36,38]. Thus the brown-fat to white-like-fat transformation does not necessarily involve the same changes in rodents and in large mammals, and the bovine species might be a good model of what is the real scheme in human.

Developmental changes in adenylate cyclase stimulation by (–)-isoprenaline and BRL 37344

In this part of the study, (–)-isoprenaline was chosen as a general agonist for all β -adrenergic subtypes, and BRL 37344 as a specific β_3 -adrenergic agonist [14].

The developmental changes in the response of adenylate cyclase to BRL 37344 (Table 1) are rather well correlated with the changes in the β_3 -AR mRNA level (Figure 2).

After birth, the adenylate cyclase response to (–)-isoprenaline (Table 1) decreases significantly. At the same time, the UCP mRNA level decreases sharply [10]. These results agree with those of Klein et al. [39] who have reported that the catecholamine response of adipocyte respiration, which increases in ovine species during foetal life, disappears after birth. In the ruminant newborn, parturition is associated with a high neurosympathetic stimulation [40] which could induce a β -AR desensitization. The decrease in the response of adenylate cyclase to (–)-isoprenaline observed after birth may be due to β_1 -AR desensitization. It is noteworthy that there is no change in BRL 37344 response after birth. Granneman [41] reported that exposure of isolated white adipocytes to a concentration of (–)-isoprenaline that stimulates the adenylate cyclase via both β_1 - and β_3 -ARs induces an uncoupling of the β_1 -AR, but not of the β_3 -AR response. The results of the present study and those of Granneman [41] suggest that the β_1 - and β_3 -AR might not share the same desensitization process.

It is tempting to speculate that the increase in UCP expression in bovine perirenal fat during foetal life is under the control of

the β_3 -adrenergic system, and that the rapid decrease in UCP expression shortly after birth is related to a decrease in the (-)-isoprenaline response.

It is proposed that perirenal adipose tissue of the bovine is a good model for studying the brown-to-white adipocyte phenotype change in large mammals, including humans. Factors controlling this change are of importance to understand the development of whole adipose-tissue mass.

We thank Ms Françoise Kühne for excellent technical assistance, and Susi Klaus and Odette Champigny for her helpful discussion. This work was supported by a grant (no. 32-26331-89) from Swiss National Science Foundation, by a grant from INRA (no. 4588), and by grants-in-aid from the Ernst and Lucie Schmidheiny, Geigy-Jubiläums and Sandoz Foundations. L.C. has been supported by a grant from C.N.R.S.

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