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ADRENERGIC REGULATION OF THE UNCOUPLING PROTEIN EXPRESSION IN FOETAL RAT BROWN ADIPOCYTES IN PRIMARY CULTURE

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SUMMARY: The adrenergic and T_3 modulation of UCP expression in non-proliferative foetal brown adipocyte primary cultures were studied. The UCP in the cultured cells was determined by immunological detection of the protein and by quantification of the mitochondrial GDP-binding. Our results showed a relative increase of 65-75% in UCP levels and 60-80% in the mitochondrial GDP-binding capacity under β-adrenergic stimulatory conditions, while neither α_1-adrenergic agonists nor T_3 showed an effect.

The main function of brown adipose tissue (BAT) is to generate heat in a process generally called "non-shivering thermogenesis". This tissue is specially involved in heat production in newborn mammals and hibernating animals and its thermogenic capacity can be increased in some adult animal species by cold-acclimation and overfeeding (1). The nature of heat production lies on brown fat mitochondria having a highly specialized protein called "uncoupling protein" (UCP) which is a major component of the inner membrane and is only present in this tissue. This protein allows the regulated reentry of protons extruded by the respiratory chain, thus, dissipating the proton-electrochemical gradient, as heat. Binding of purine nucleotides, such as GDP, to this protein inhibits this pathway, hence the quantification of the mitochondrial GDP binding capacity is a valid measure of the functional uncoupling protein content (1). Synthesis of the uncoupling protein "in vivo" is at least partially controlled by the sympathetic fibers, releasing noradrenaline after the stimulation either by decreased environmental temperature (neonatal hypothermia or cold-acclimation) or by nutritional status (1). The accute cold effect is mimicked by the injection of a selective β-agonist (BRL 26830A), which increases uncoupling protein mRNA (2). Thyroid hormones have also been reported to exert certain control on BAT

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Abbreviations: BAT, brown adipose tissue; DTT, ditiothreitol; Iso, isoproterenol; NA, noradrenaline; Phe, phenylephrine; Pro, propranolol; SDS, sodium dodecil sulphate; T_3, triiodothyronine; T_4, tiroxine; UCP, uncoupling protein; Yoh, yohimbine.

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function (1,3); on one hand, there is a reduced BAT responsiveness to sympathetic stimulation in hypothyroid animals and this can be restored by T_4 administration (4) and on the other hand, large T_4 doses reduced the functional UCP content measured by its capacity to bind GDP (5). In addition, BAT contains a type II T_4 5'deiodinase which is stimulated via α_1-adrenergic receptors and provides a large proportion of T_3 from circulating T_4 (6), amplifying noradrenaline (NA) stimulation of UCP gene transcription (7). However, the role of thyroid hormone and its relationship with the noradrenergic stimulation is still poorly understood. The use of brown adipose tissue primary cultures should allow us to work out under several hormonal conditions in a separate manner. In this paper, we report on the use of primary cultures of brown adipocytes from rat foetuses in the last day of gestation to study the adrenergic and T_3 regulation of uncoupling protein expression. The UCP was quantified by immunodetection and its functional activity was evaluated from the mitochondrial GDP-binding capacity.

MATERIAL AND METHODS

Isolation and culture of brown adipocytes: Interscapular brown adipose tissue was taken from 22-day foetuses of Wistar rats under sterile conditions. The tissue was finely chopped and brown adipocytes were isolated according to Lorenzo et al. (8). Routinely, the number of cells obtained was approx. 25-35x10^6 cells per g of brown adipose tissue. Cell viability, determined by Trypan Blue exclusion, was over 95%.

The isolated cells were plated in 6 cm-diam. plastic dishes, inoculating 2x10^6 cells/dish resuspended in 2.5 ml of Eagle's medium modified with Earle's salts, glutamine and 20 mM HEPES supplemented with 10% foetal-calf serum and antibiotics (120 µg penicillin, 100 µg streptomycin, 50 µg gentamicin and 25 µg nistatin/ml). Cells were maintained with this medium during the first 6 h to provide good cellular attachment. The medium was then replaced by a new one containing 0.5% foetal-calf, 100 µM ascorbate (to avoid oxidation of adrenergic agents) and with various combinations of agonists or antagonists: 10 µM isoproterenol (Iso), 10 µM triiodothyronine (T_3), 10 µM isoproterenol + 10 µM triiodothyronine, 10 µM phenylephrine (Phe) + 100 µM propranolol (Pro), 10 µM noradrenaline (NA), 10 µM noradrenaline + 10 µM yohimbine (Yoh). Culture medium was changed every 12 h to assure the maintenance of the adrenergic compounds concentrations; in fact, it has been already reported that after 24 h in culture the concentration of NA decreased only by 25% in the presence of ascorbate (9). Cells were maintained in a 5% CO_2 atmosphere at 37 °C during 48 h under the different treatments. Cells were removed with a Ca^{+} and Mg^{2+} free Hank's solution containing 200 mM EDTA and pelleted by centrifugation at 9000 g for 10 min at 4 °C.

Mitochondria isolation: Mitochondria were isolated from culture cells, basically, as described by Cannon and Lindberg (10).

Protein content was determined by Lowry method (11) modified by Petterson (12)

GDP-binding capacity: GDP-binding capacity was essentialy performed as previously described (13). 50 µg of mitochondrial protein were used per assay and ^{3}H-GDP concentrations ranged between 0:15-3.15 µM.

Western blot analysis of mitochondrial proteins: Mitochondrial proteins were separated by vertical slab-gel elecphoresis in a SDS-11% polyacrilamide gel according to Laemmli (14). Proteins were transferred to nitrocellulose overnight (16 h at 250 mA) according to Towbin et al. (15). After transfer, nitrocellulose filters were processed for immunological detection.

Dot-blot analysis of mitochondrial proteins: Mitochondrial fractions were resuspended in a medium containing 10 mM Tris-HCl, 150 mM NaCl, 0.1% SDS and 20 mM DTT and applied (6 µg/50 µl) to nitrocellulose membrane in a 96 well Bio-Dot

Microfiltration Apparatus (Bio-Rad Laboratories). Nitrocellulose filters were then removed and processed for immunological detection of the uncoupling protein.

Immunological detection of the uncoupling protein: The uncoupling protein retained by the nitrocellulose sheets from either Western or Dot-blot analysis was immunologically detected by using a rabbit anti-(uncoupling protein) serum (16). Bound antibody was visualized by using a goat anti-(rabbit Ig G) conjugated with peroxidase, developed with 4-chloronaphtol and scanned in a Bio-Rad Model 620 densitometer.

RESULTS AND DISCUSSION

The adrenergic and T_3 modulation of UCP expression in non-proliferative foetal brown adipocyte primary cultures is reported in this paper. Cells were maintained in culture in a medium supplemented with the minimum foetal serum concentration (0.5%) required to assure cell viability and attachment. Under these conditions, cells did not proliferate as revealed by protein and DNA content (data not shown). To investigate the effects of different agents, cells were maintained for 48 h under various conditions: 10 μM Isoproterenol (β-agonist); 10 μM Triiodothyronine, 10 μM Isoproterenol + 10 μM Triiodothyronine to investigate the potentiating effect of T_3 on β-adrenergic stimulation; 10 μM Phenylephrine (α_1-agonist) + 100 μM Propranolol (β-antagonist) to block the possible β effect of Phenylephrine at high doses; 10 μM Noradrenaline (α-β-agonist); 10 μM Noradrenaline + 10 μM Yohimbine (α_2-antagonist) to study the β-adrenergic effect of noradrenaline.

The content of UCP in the cultured cells were studied by immunological detection of the protein in Western-blot and Dot-blot analysis as well as by measuring the mitochondrial GDP-binding capacity.

The Western analysis of UCP (Fig. 1A) revealed only one band at about 32 KD which is absent in controls performed with liver mitochondria. In addition, the immunoblot revealed that there is expression of uncoupling protein after treatment of cells with Iso, Iso plus T_3, NA plus Yoh, while T_3 or Phe plus Pro had no effect. These results were confirmed and quantified by Dot-blot analysis and its densitometric scanning (Fig. 1B), showing a relative increase of 65-75% in UCP levels in the presence of Iso, Iso + T_3, NA and NA + Yoh. The stimulatory effect observed was similar in the four treatments since there were not statistically significant differences among them. Furthermore, neither α_1-adrenergic agonist nor T_3 had an effect on uncoupling protein content.

The functional activity of the UCP was studied, in terms of mitochondrial GDP-binding capacity. The original assay (13) had to be modified due to the practical problems derived from the low amount of mitochondria available from the small yield of foetal cells maintained in culture under non-proliferative conditions. The modification included lowering of the protein content per assay (50 μg) and the use of low GDP concentrations (0.15-3.15 μM). The results (Fig. 2A, 2B and Table 1) confirmed those from the Western and Dot-blot analysis.

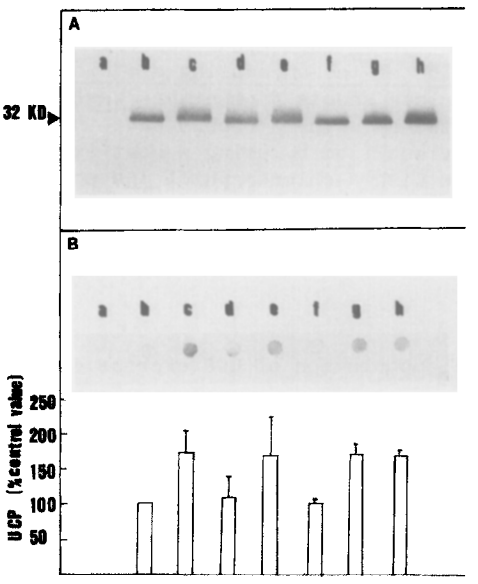

Figure 1. **Effect of adrenergic agents and T_3 on UCP content in cultured brown adipocytes.** A/ Representative Western-blot analysis of mitochondrial proteins (10 µg) after immunological detection of the UCP as described in Material and Methods. B/ Representative Dot-blot analysis of mitochondrial proteins (6 µg) after immunological detection of the UCP. Histograms represent the relative quantification of the UCP obtained by densitometric scanning of four dot-blot series.
Mitochondrial proteins of liver (a), control brown adipocytes (b) and brown adipocytes cultured in the presence of isoproterenol (c); triiodothyronine (d); isoproterenol + triiodothyronine (e); phenylephrine (e); phenylephrine + propranolol (f); noradrenaline (g) and noradrenaline + yohimbine (h).

Fig. 2A shows the direct equilibrium binding curves and Fig. 2B the corresponding Scatchard plots of some representative experiments. The mean values of the GDP-binding capacity (Bmax) and the apparent Kd, both obtained from Scatchard analysis

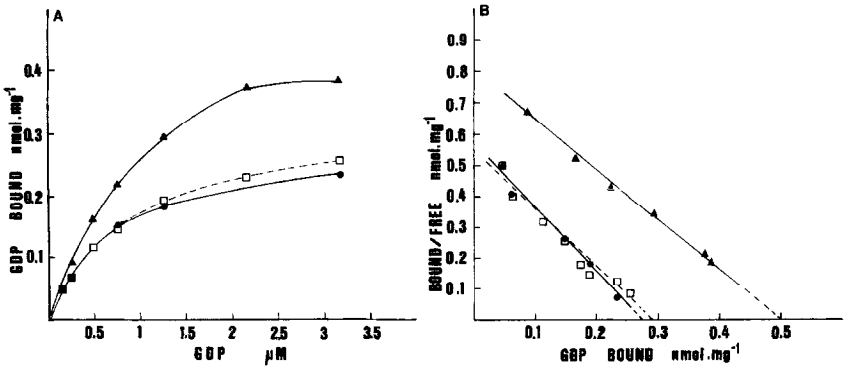

Figure 2. **(^{3}H)-GDP specific binding to brown adipocyte mitochondria.**
A/ Equilibrium binding curves from representative experiments performed as described in Material and Methods. B/ Scatchard plots derived from the representative binding curves shown in A/.
(^{3}H) GDP-binding to mitochondria from control brown adipocytes (●) and brown adipocytes cultured in the presence of triiodothyronine (□) and isoproterenol (▲).

TABLE 1. Effect of adrenergic agents and T_3 on ^{3}H-GDP binding capacity to mitochondria from brown adipocytes in culture

	Bmax (GDP bound nmol/mg prot)	Kd (μM GDP)
Control	0.28 ± 0.02	0.8 ± 0.2
Isoproterenol	0.49 ± 0.03**	0.6 ± 0.1
T_3	0.35 ± 0.02	0.8 ± 0.1
Isoproterenol + T_3	0.45 ± 0.04**	0.6 ± 0.2
Phenylephrine + Propranolol	0.31 ± 0.02	0.9 ± 0.2
Noradrenaline	0.50 ± 0.02**	0.5 ± 0.1
Noradrenaline + Yohimbine	0.46 ± 0.04**	0.5 ± 0.1

Values represent means ± S.E.M. of five experiments. **p < 0.01 compared with control values (Student's paired t test).

of five separate experiments, are shown in Table 1. The apparent Kd was not affected by any treatment. However, GDP-binding capacity (Bmax) increases by 60-80% after treatment with Iso, Iso plus T_3, NA and NA plus Yoh, without statistically significant differences among these four conditions, and α_1-agonist or T_3 had no effect.

These results clearly indicate the adrenergic stimulation of UCP expression was produced in a β-adrenergic manner. The β-adrenergic regulation was previously described "in vivo" (2,17,20) after treatment with NA (17) or with long-acting β-agonist (18) to the same extent as chronic cold exposure. Furthermore, chronic administration of NA to animals mimicked the increase in UCP mRNA observed in cold exposed animals or animals bearing pheochromocytoma (19). That effect was inhibited by treatment with Pro (20). Similarly, an increase in UCP mRNA levels were also induced by administration of a β-selective agonist, BRL 26830 A, to rats (3). These studies indicate a β-adrenergic regulation of UCP synthesis "in vivo". However, it has been suggested (21) that a simultaneous α_1 and β stimulation is needed to increase UCP mRNA levels to the same extent as induced by chronic cold exposure.

In order to get a better understanding of this regulation primary cultures of brown adipocytes have already been used by other authors but they failed to prove any adrenergic regulation (22). However, a stimulatory effect of Iso on the UCP expression has been reported in mature brown adipocytes from mouse, cultured under proliferative conditions, although Iso was not able to induce the expression of UCP in undifferentiated cells (23). The positive effect of Iso is in agreement with our results even though both culture conditions were rather different.

With regard to thyroid hormones, we did not see any effect of T_3, alone or combined with Iso, on UCP expression in non-proliferative foetal brown adipocyte cultures. These results seem to differ from "in vivo" studies in adult rats, where it was reported a requirement of T_3 for the optimal UCP expression in cold exposed animals by conversion of T_4 to T_3 via the activation of the brown adipose tissue T_4 5'deiodinase (24,25). After T_3 and T_4 treatments a parallel increase in UCP and UCP mRNA levels was observed (24), being necessary a saturation of nuclear T_3 receptors for a full expression of UCP in response to cold (25). It was also suggested a concerted regulation of UCP gene by NA and T_3, being NA the primary signal increasing the rate of UCP mRNA transcription and T_3 amplifying 4-5 folds the transcriptional response to NA, while T_3 failed to stimulate the gene in the absence of adrenergic input (7). This lack of T_3 effect in the absence of adrenergic input could explained why T_3 failed to increase UCP in our culture system but not the lack of effect in the presence of Iso. However, this could be due to either a possible lack of T_3 receptors, a lack of saturation of these receptors or a failure in the postreceptor mechanisms due to the inmaturity of these foetal brown adipocytes. On the other hand, these foetal cells could have a different regulation of UCP expression than adult cells. In fact, brown fat T_4 5'deiodinase activity during the perinatal period is dissociated from the development of the thermogenic activity of the tissue and seems to exclude a direct role of the locally produced T_3 in the modulation of the UCP gene expression (26). However, a lower amount of UCP mRNA was found in the rat foetuses and newborns from dams treated with methimazole to induce hypothyroidism (27).

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