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HORMONAL REGULATION OF MALIC ENZYME EXPRESSION IN PRIMARY CULTURES OF FOETAL BROWN ADIPOCYTES

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SUMMARY: Insulin increased malic enzyme activity and protein content in foetal brown adipocyte primary cultures through a two fold increase in the rates of enzyme synthesis. Conversely, noradrenaline prevented the induction caused by insulin on enzyme expression through an accelerate rate of malic enzyme degradation. Tri-iodothyronine had no effect on the expression of malic enzyme in cultured foetal brown adipocytes.

Differentiated brown adipose tissue is a major site for lipid metabolism. Thus the rates of fatty acid synthesis increased during the last days of foetal development in the rat (1). During the foetal-neonatal transition, the rates of lipogenesis sharply decreased (2), carnitine palmitoyl -transferase I sensitive to malonyl-CoA increased (3) and mitochondrial fatty acid oxidation required for non-shivering thermogenesis ocurred (4). Malic enzyme (L-malate-$NADP^+$ oxidoreductase decarboxilating) is one of the enzymes involved in the regulation of fatty acid synthesis, a NADPH-doner for lipogenesis. The genetic expression of malic enzyme is regulated by nutritional and hormonal factors (5) as well as by the state of differentiation and development in mammals. Its activity in brown adipose tissue is maximal prior to birth, and slowly decreases during the neonatal life in the rat (1). Foetal brown adipocyte primary cultures has proved to be a suitable system for lipid synthesis studies (6). Thus insulin increased lipogenesis at 24 h related to changes in malic enzyme and fatty acid synthase activities (7). None effect was found on lipogenesis by glucagon in isolated adipocytes in suspension (8). Conversely, noradrenaline produced an inhibition on the rates of lipogenesis in isolated brown adipocytes in suspension (8) and in culture at 3 h (7). These results are consistent with the noradrenergic stimulation of thermogenesis in brown adipose tissue at birth (4). Finally, tri-iodothyronine (a major thermogenic hormone) increased lipogenesis related to a parallel increase in fatty acid synthase activity, but without any change in malic

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enzyme (7). Accordingly, the aim of this work was to study the hormonal regulation of malic enzyme expression in primary cultures of foetal brown adipocytes.

MATERIAL AND METHODS

Brown adipocytes were isolated from 22 days foetuses of Wistar rats and plated ($2.5x10^6$-$3x10^6$/60 mm-diam plastic dish) as described previously (7). After 4 h of culture in the presence of 10% foetal calf serum, the attached monolayer was washed twice with Hanks' balanced salt solution and cells were cultured for a further 42 h in a serum-free Eagle's medium modified with Earle's salts and supplemented with 0.2% (w/v) albumin, in the presence or absence of hormones. The culture medium was changed every 12 h. After washing with 20 mM-EDTA in Ca^{2+}/Mg^{2+}-free Hanks' balanced salt solution, the cell monolayer was scrapped off, sonicated for 30 s at 1.5 mA, in 1 mM EDTA/1 mM-dithiothreitol/0.25 M-sucrose/ 25 mM Tris/HCl (pH 7.5) and centrifuged at 12000 g for 4 min. Malic enzyme activity was measured espectrophotometrically (9) from the cytosolic supernatants. Protein were determined by (10) with bovine serum albumin as standard. Enzyme activity was expressed as nmol of NADPH formed/min per mg of protein.

For Western Blot analysis of protein, proteins from cytosolic supernatants were submitted to 7.5% poliacrylamide gel under non-denaturing conditions (11) and transferred to nitrocellulose overnight (16 h at 250 mA). The transfer buffer contained 25 mM Tris/HCl, 190 mM glycine, 20% (v/v) methanol pH 8.3. Filters were analysed by immunodetection with anti-malic enzyme rabbit serum generously provided by (12), after being blocked by incubation for 1 h with 10 mM Tris/HCl, 150 mM NaCl, 3% (w/v) albumin, pH 7.3. Bound antibody was detected by sheep-anti-(rabbit IgG) conjugated with peroxidase, developed in 10 mM Tris/HCl, 150 mM NaCl pH 7.6, with 120 µg 4-chloronaphtol/ml in the presence of H_2O_2. Duplicate gels were transferred as described above and filters were analysed for detection of malic enzyme activity by staining in 0.1 M Tris/HCl pH 7.4, 1 mM $MnCl_2$, 0.25 mM $NADP^+$, 1.5 malate, 25 µg nitro-blue tetrazolium/ml and 25 µg phenazine metasulphate/ml at 37 °C in the dark. Filters were analysed by densitometric scanning of the bands.

The measurement of relative rate of enzyme synthesis was as followed. After 38 h of culture in the presence or absence of hormones, cells were pulse labelled with 25 µCi of L-[4.5-^{3}H] leucine (0.25 µCi/nmol) for 4 h. At the end of the labelling period, cells were washed four times with fresh medium and cytosols were prepared as described above. Malic enzyme was immunoprecipitated with anti-malic enzyme rabbit serum in a constant volume adjusted with 2 mM leucine-150 mM NaCl and Triton X-100 to a final concentration of 1.7% (w/v). Immunoprecipitation of malic enzyme was carried out with 50 munits of enzyme activity and sufficient antiserum to precipitate 120% of the activity present, as described (13). Then, the immunoprecipitate was dissociated and subjected to electrophoresis in SDS/ 10% poliacrylamide gels (11), and the malic enzyme band was cut out of the gel and counted (13). Duplicate aliquots were used for measuring the incorporation of radioactive aminoacid into cytosolic protein, after precipitation with ice-cold 10% (w/v) trichloroacetic acid in the presence of 1 mg of albumin as carrier (14). Relative rates of synthesis were expressed as percentages of cpm incorporated into malic enzyme/cpm incorporated into cytosolic protein.

The measurement of enzyme degradation was as followed. After 15 h of culture in the presence or absence of hormones, cells were pulse labelled with L-[4.5-^{3}H] leucine for 4 h and washed as described above. After removal of the radioactive label, cells were cultured for a further 0, 6, 20 and 27 h, being the medium replenished every 6 h. Harvesting of cells, incorporation of radioactivity into cytosolic protein and immunoprecipitation and subsequent analysis of malic enzyme were performed as described above for the relative rates of synthesis. Rates of degradation were calculated as percentages of cpm incorporated at the indicated times/cpm incorporated at the end of the pulse (t = 0).

RESULTS AND DISCUSSION

Hormonal effect on malic enzyme in brown adipocyte primary cultures.

Malic enzyme specific activity in foetal brown adipocytes did not change along the period of 42 h culture in a serum-free medium (Table 1). In addition, we have studied the effect of several hormones, involved in the physiological and metabolical control of brown adipose tissue, on the regulation of malic enzyme specific activity under the same experimental conditions (Table 1). The presence of insulin (40 nM) in the culture medium increased malic enzyme specific activity by 120% as compared with its control in the absence of hormone (Table 1). Tri-iodothyronine (10 μM) had no effect on the specific activity of malic enzyme, and the presence of insulin plus tri-iodothyronine did not produce any further increase in the stimulatory effect described for insulin alone (Table 1). Noradrenaline (10 μM) did not change malic enzyme specific activity as compared with brown adipocytes cultured in the presence of hormones (Table 1). However, the presence of noradrenaline in the culture medium totally prevented the stimulatory effect of insulin on malic enzyme specific activity observed in its absence (Table 1). In addition, noradrenaline produced the same effect on malic enzyme activity in cultured brown adipocytes submitted to insulin plus tri-iodothyronine (Table 1).

In order to establish if the changes induced by hormones in malic enzyme specific activity were related to changes in the amount of enzyme protein, we submitted cytosolic extracts from brown adipocyte primary cultures to Western blot analysis (Fig. 1). Malic enzyme was immunodetected with anti-malic enzyme rabbit serum (Fig. 1 b) as well as developed for specific activity (Fig. 1 a) as described in Material and Methods. Densitometric scanning of bands showed that the amount of malic enzyme protein from cells extracts treated with insulin or insulin plus tri-iodothyronine was 120% higher than the enzyme content found in cells cultured in the absence of hormones. No differences in the level of malic enzyme protein

Table 1. HORMONAL EFFECT ON MALIC ENZYME SPECIFIC ACTIVITY IN BROWN ADIPOCYTE PRIMARY CULTURES

Hormone added	None	Insulin (40 nM)	Tri-iodothyronine (10 μM)	Insulin (40 nM) + Tri-iodothyronine (10 μM)
None	24.6 ± 1.2	54.0 ± 1.6*	26.3 ± 3.8	54.7 ± 3.1*
Noradrenaline (10 μM)	23.5 ± 1.7	27.3 ± 1.8	22.8 ± 1.9	23.1 ± 2.0

Cells were cultured for 42 h in the presence of several hormones as indicated in the table. For details see Materials and Methods. Enzyme activity is expressed as nmol/min per mg of protein. Results are means ± S.E.M. from 10-12 separate experiments. Values that are significantly by the Student's t test from those none hormones added in the presence or in the absence of noradrenaline are shown by: *p 0.001. Malic enzyme activity prior to 42 h culture in serum-free medium was 25.7 ± 1.1.

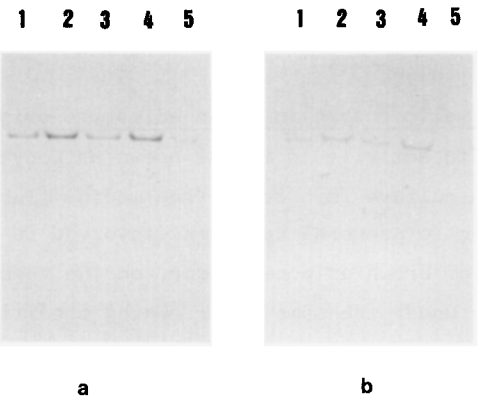

Figure 1. **Western blot analysis of malic enzyme in cytosols from brown adipocyte primary cultures.**

Cells were cultured as described in Table 1. Protein from cytosols (10 µg) were electrophoresed, blotted to nitrocellulose and analysed for malic enzyme activity (a) and enzymic protein with anti-malic enzyme rabbit serum (b) as described in Material and Methods. Lanes represent cells cultured with none hormone added (1), insulin (2), Tri-iodothyronine (3), insulin + Tri-iodothyronine (4) and insulin + noradrenaline (5).

were found between cells treated with tri-iodothyronine or insulin plus noradrenaline as compared with their control (Fig. 1 b). In addition, our results showed that there was a good correlation between the corresponding changes in malic enzyme specific activity and in the amount of protein found in the presence of the several hormones studied (Fig. 1).

The stimulatory effect of insulin on malic enzyme activity has been previously described in brown adipocytes cultured for 5 days in 10% calf-serum medium and a further 24 h in a serum-free medium supplemented with the hormones, related to an increase in the lipogenic flux (7). In other systems as cultured adult rat hepatocytes, insulin present 20 h increased malic enzyme as well as other lipogenic enzymes (15). The lack of effect of tri-iodothyronine on the expression of malic enzyme in foetal adipocytes contrasts with its stimulatory effect on the expression of this enzyme reported in adult rat liver (16,17) and in cultured hepatocytes (15) Moreover, a tissue-specific regulation of malic enzyme by tri-iodothyronine has been described (18), being liver, heart and kidney tri-iodothyronine responsive tissues in contrast with lung or brain where malic enzyme is not inducible by tri-iodothyronine. Whether the lack of effect of tri-iodothyronine on malic enzyme expression in cultured foetal brown adipocytes could be due to the absence of receptors or to the fail of the post-receptor mechanism, remains to be established. Finally, noradrenaline was able to totally prevent the stimulatory effect of insulin or insulin plus tri-iodothyronine on the genetic expression of the malic enzyme. This effect has not been previously described, but it is plausible since noradrenaline has an important role in cultured foetal brown adipocytes inhibiting the lipogenic flux (7).

Table 2. EFFECT OF INSULIN AND NORADRENALINE ON THE RELATIVE RATES OF SYNTHESIS OF MALIC ENZYME IN BROWN ADIPOCYTE PRIMARY CULTURES

Hormone added	Enzyme activity (nmol/min per mg of protein)	Relative rate of enzyme synthesis (%)
None	24.6 ± 1.2	0.097 ± 0.005
Insulin (40 nM)	54.0 ± 1.6*	0.190 ± 0.02*
Insulin (40 nM) + Noradrenaline (10 μM)	27.3 ± 1.8	0.220 ± 0.04*

Cells were cultured, labelled and analysed for the determination of radioactive leucine incorporation into cytosolic and malic enzyme protein as described in Material and Methods. Results are means ± S.E.M. from 4 separate experiments. Relative rates of synthesis are expressed as percentages of cpm incorporated into malic enzyme/cpm incorporated into cytosolic protein. Values that are significantly different by Student's t test from those for none hormone added are shown by: *$p < 0.001$.

Relative rates of protein synthesis and rates of degradation of malic enzyme in foetal brown adipocyte primary cultures: Effect of insulin and noradrenaline.

In order to test whether the enhancement of malic enzyme protein content by insulin was due to an increase in enzyme synthesis and/or to a decrease of enzyme degradation, the relative rates of synthesis (Table 2) and degradation (Fig. 2) of malic enzyme were determined by the incorporation of ^{3}H-leucine and immunoprecipitation of the enzyme with anti-malic enzyme rabbit serum, as described in Material and Methods. The relative rates of synthesis for malic enzyme represented over 0.1% of protein synthesis "de novo" observed in foetal brown adipocyte primary cultures in the absence of hormones (Table 2). Insulin increased the relative rates of synthesis for malic enzyme by 100% as compared with their controls in the absence of hormones (Table 2). However, noradrenaline in the presence of insulin did not produce any significant change in the relative rates of synthesis for malic enzyme as compared with those values observed in its absence, but produced a two fold increase as compared with values obtained in the absence of noradrenaline and insulin (Table 2). Thus noradrenaline was not able to prevent the increase caused by insulin on the relative rate of enzyme synthesis.

On the other hand, we have measured the rates of degradation of malic enzyme and cytosolic protein in the presence of insulin and insulin plus noradrenaline in foetal brown adipocyte primary cultures (Fig. 2). Cells were pulse labelled with ^{3}H-leucine and chased up to 27 h in the presence of insulin (Fig. 2 b). No differences were found between the rates of degradation of malic enzyme and cytosolic protein, either in the presence or in the absence of insulin (results not shown). The apparent half-lives for malic enzyme and cytosolic protein were over 20 h under this experimental conditions. However, in the presence of insulin

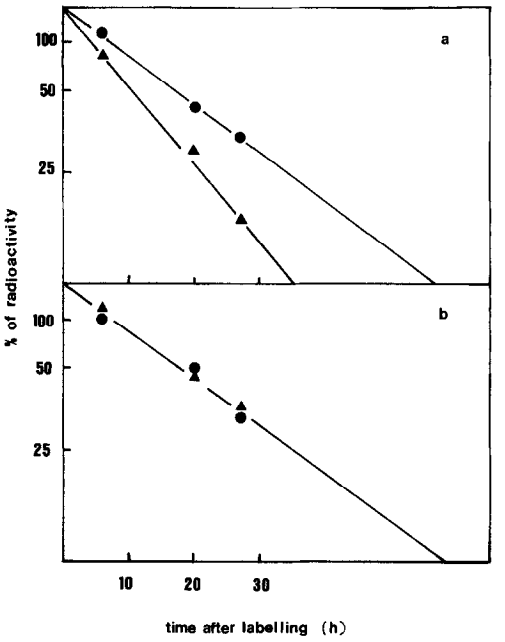

Figure 2. **Effect of insulin and noradrenaline on the degradation of cytosolic and malic enzyme protein in brown adipocyte primary cultures.**
Cells were cultured and labelled as described in Material and Methods. After removal of the radioactive label, the decrease of radioactivity in malic enzyme (▲) and cytosolic protein (●) was determined in cells cultured in the presence of insulin plus noradrenaline (a) and in the presence of insulin (b). Results are expressed as percentages of cpm incorporated at the indicated times/ cpm incorporated at the end of the plus (t = 0). A representative experiment is shown.

plus noradrenaline in the culture medium (Fig. 2 a) the rates of degradation for malic enzyme was higher than that observed for cytosolic protein. Thus noradrenaline in the presence of insulin did not change the rate of degradation for cytosolic proteins (apparent half-life around 20 h), but produced a significant change in the rate of degradation for malic enzyme (apparent half-life around 11 h), as compared with those values found in its absence but with insulin present (Fig. 2 a, b). Therefore, noradrenaline in the presence of insulin accelerated the rates of degradation of malic enzyme observed in the presence of insulin alone. Accordingly, our results indicate that insulin increased the specific activity and the amount of malic enzyme in foetal brown adipocytes in culture through a mechanism that involves an increase by two fold in the relative rate of enzyme synthesis. These results are consistent with those found in adult rat liver where insulin is involved in malic enzyme expression at transcriptional (17) and post-transcriptional levels (19). Conversely, noradrenaline prevented the stimulatory effect of insulin on malic enzyme content in brown cell in culture, accelerating the rates of enzyme degradation without any change in the relative rates of enzyme synthesis. These results have not been previously described, and the mechanism involved remains to be established.

In conclusion, we have reported a tissue-specific hormonal regulation for malic enzyme expression in foetal brown adipocyte primary cultures. Insulin induced the enzyme through an increase in the relative rate of synthesis. Noradrenaline prevented the induction caused by insulin through an accelerate rate of enzyme degradation. Tri-iodothyronine had no effect on the expression of the enzyme in this system.

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REFERENCES

1. Pillay, D. and Bailey, E. (1982). Int. J. Biochem., 14, 511-517.
2. Benito, M., Lorenzo, M. and Caldés, T. (1984). Biochem. J., 224, 823-828.
3. Peñas, M. and Benito, M. (1986). Biochem. Biophys. Res. Commun., 135, 589-596.
4. Benito, M. (1985). Biol. Neonate, 48, 245-249.
5. Mariash, C.N., McSwigan, C.R., Towle, H.C., Schwart, H.L. and Oppenheimer, J.M. (1981). J. Clin. Invest., 68, 1485-1490.
6. Lorenzo, M., Fabregat, I., Roncero, C. and Benito, M. (1988a). Biochem. Soc. Trans., 16, 274-175.
7. Lorenzo, M., Roncero, C., Fabregat, I. and Benito, M. (1988b). Biochem. J., 251, 617-620.
8. Roncero, C., Lorenzo, M. and Benito, M. (1987). Biochem. J., 243, 617-620.
9. Ochoa, S., Mehler, A.H. and Kornberg, A. (1948). J. Biol. Chem., 174, 979-1000.
10. Bradford, M. (1976). Anal. Biochem., 72, 248-254.
11. Maizel, J.V. Jr. (1971). Methods Virol., 5, 180-244.
12. Ayala, A., Lobato, M.F. and Machado, A. (1986). FEBS Letters. 202, 102-106.
13. Lobato, M.F., Ross, M., Moreno, F.J. and Garcia-Ruiz, J.P. (1986). Biochem. J., 236, 441-445.
14. Mans, R.J. and Novelli, G.D. (1960). Biochem. Biophys. Res. Commun., 3, 540-543.
15. Spence, J.T. and Pitot, H.C. (1982). Eur. J. Biochem., 128, 15-20.
16. Dozin, B., Magnuson, M.A. and Nikodem, V.M. (1986). J. Biol. Chem. 261, 10290-10292.
17. Katsurada, A., Iritani, N., Fukuda, H., Noguchi, T. and Tanaka, T. (1988). Biochim. Biophys. Acta., 950, 113-117.
18. Dozin, B., Magnuson, M.A. and Nikodem, V.M. (1985). Biochemistry, 24, 5581-5586.
19. Davis, B.B., Magge, S., Mucenski, C.C. and Drake, R.L. (1988). Biochem. Biophys. Res. Commun., 154, 1081-1087.