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In vitro alanine utilization by rat interscapular brown adipose tissue

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The in vitro oxidation to CO_2 and tissue incorporation of alanine label by pieces of rat interscapular brown adipose tissue (IBAT) has been investigated. Insulin increased both uptake and oxidation of alanine, as well as the incorporation of alanine label into tissue. This effect only was observed in the presence of glucose in the incubation medium. Noradrenaline hampered alanine incorporation, not affecting its rate of oxidation. IBAT from 4-h cold-exposed rats showed a higher alanine utilization than that of controls; however, IBAT pieces from both 36-h starved and 30-day cold-exposed rats presented lower rates of alanine utilization. The main fate of alanine taken up by the IBAT pieces was its oxidation to CO_2. Part of the label was also incorporated into the fatty acid fraction of lipids. The results obtained in this study agree with a possible role of alanine as alternative energetic substrate for IBAT.

Introduction

Brown adipose tissue function has been directly related with adaptative thermogenesis [1], being recognized as the main site of nonshivering thermogenesis induced by cold [2] or diet [3]. Brown adipose tissue shows a very high capability for the oxidation of different metabolic substrates. Fatty acids, either from its intracellular triacylglycerol stores [4], or released through lipolysis from circulating lipoproteins [5], are considered as the main fuel for heat generation. Glucose is also a very important fuel for thermogenesis, as found in studies both in vivo [6] and in vitro [7]. Moreover, brown adipose tissue thermogenesis can be fueled by other substrates, such as ketone bodies [8] or amino acids [9].

IBAT has an important amino acid metabolism enzyme machinery, the activities of which are comparable to those of liver and muscle [10]. The changes observed in these activities, as well as in the amino acids pool composition under certain situations, indicate that amino acids can be eventually important energetic substrates. Amino acids are used by the IBAT under conditions of exposure to cold temperature [11], and their utilization is decreased in situations of diminished thermogenesis as in starvation [12]. The quantitative importance of IBAT amino acid metabolism under situations of short-term cold-exposure has been recently demonstrated in vivo [9].

Alanine can be used as substrate by the IBAT because this tissue can incorporate alanine hydrocarbon skeletons both into fat and proteins [13]. The IBAT contains a high alanine transaminase activity [10], and alanine concentration is deeply altered under the effects of cold exposure [11]. It has also been postulated that the utilization of this amino acid by the tissue can help to prevent glucose wastage in postprandial situations [14].

In the present work we have studied the in vitro utilization of alanine by pieces of IBAT of animals subjected to a short-term starvation period as well as by the tissue fragments from rats subjected to short- or long-term cold exposure. The effects of insulin and noradrenaline on the amino acid utilization under these situations has also been studied.

Materials and Methods

Male Wistar rats weighing 240–250 g were used. They were fed ad libitum a standard pellet diet (type A04 from Panlab, Barcelona). The rats were maintained

Abbreviation: IBAT, interscapular brown adipose tissue.

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in a temperature (21–22° C), humidity (75–85%) and light-period (lights on from 08:00 to 20:00 h) controlled animal room; they were housed individually in polypropylene-bottomed cages with wood shavings as absorbing material. Four groups of animals were studied: group A (cold-exposed rats) were left in a cold environment (4° C) for 4 h before death; group B (cold-acclimated rats) were left for 30 days at 4° C; group C (starved rats) had all food removed from their cages 36 h prior to death; group D (control rats) were maintained under the basal conditions outlined.

The animals were killed by beheading at 09:00–10:00 h; immediately, the interscapular brown adipose tissue was exposed and dissected, weighed and sliced into pieces of about 5 mg. The pieces were introduced into 20 ml glass vials containing 1 ml Krebs-Ringer bicarbonate buffer (pH 7.4) with one half of the original calcium concentration [15], 10 g/l fatty acid-free bovine serum albumin (Sigma), 0.75 mM L-alanine, 5 mM D-glucose and 18.5 kBq/ml of L-[U-^{14}C]alanine (Amersham). The medium was supplemented with either 0.2 U/l bovine insulin (Novo) or 0.5 mg/l noradrenaline bitartrate (Sigma).

The vials were sealed with rubber stoppers equipped with hanging wells; the preparations were gassed (10 min) with O_2/CO_2 (95:5%, v/v), and then they were incubated for 20 min at 37° C in a shaking (1.33 Hz) water bath. At the end of the incubation period, 0.2 ml of 1 M hyamine hydroxide (Amersham) in methanol was injected through the rubber stopper into the hanging wells, and the reaction was stopped by injecting 0.5 ml of 3 M perchloric acid into the reaction medium. The vials were then left standing 1 h at room temperature, in order to allow enough time to fix most of the evolved $^{14}CO_2$. The plastic wells were then removed and the radioactivity of the vial contents was estimated. The pieces of tissue were fished out, washed once with fresh medium, and used for tissue lipids extraction [16], purification and fractionation [17]. The radioactivity in the fatty acid and glyceride glycerol fractions was estimated. The lipid extract, as well as the insoluble residue were washed with 9 g/l NaCl; the washings were then dried and the residue (water-soluble) radioactivity was estimated. The delipidated dry residue was solubilized with NCS (Amersham) and its radioactivity was measured.

IBAT alanine concentration was estimated with an automatic amino acid analyzer (Rank-Hilger) using *o*-phthalaldehyde as amino group reagent [18]. Tissue protein was determined with the Folin phenol reagent [19]. All results have been expressed as per tissue protein in order to render the different experimental groups more comparable.

Statistical comparisons between groups were established with Student's *t*-test, as well as analysis of variance (ANOVA, BMDP Statistical Software) programs.

Results

The net production of $^{14}CO_2$ and the incorporation of alanine carbon in the different tissue fractions with respect to the time of incubation (up to 60 min), showed linear regression coefficients greater than 0.98 in all cases.

There were not important changes between groups in the IBAT alanine concentration. The values obtained were 1.66 ± 0.11, 1.34 ± 0.07, 1.62 ± 0.19 and 1.48 ± 0.07 μmol/g tissue for control, starved, cold-exposed and cold-acclimated animals, respectively.

In Table I the values corresponding to the utilization of alanine by pieces of IBAT of control animals incubated with different concentrations of insulin and noradrenaline, in the presence or absence of 5 mM glucose, are presented. Insulin induced an increase in both oxidation of alanine to CO_2 and its incorporation to the tissue fractions at concentrations from 0.2 U/l. However, the effect was only observed in the presence of glucose. Noradrenaline did not affect the rate of oxidation of alanine, but induced an important decrease of its incorporation into the tissue fractions. In all cases,

TABLE I

Effect of insulin and noradrenaline on in vitro alanine utilization by rat interscapular brown adipose tissue in the presence of absence of glucose in the incubation medium.

The results are expressed in μmol alanine oxidized to CO_2 or incorporated to the tissue/h per g protein. The incubations ($n = 5$–6) were done in two different media: with 5 mM glucose or without glucose. The concentrations of the different hormones correspond to the final concentrations in the incubation vial. Statistical significance of the differences between groups: [a] = $P < 0.05$ versus basal conditions; [b] = $P < 0.05$ between the incubations made with and without glucose. Two-way ANOVA P values are also shown.

	Medium with glucose		Medium without glucose	
	oxidation	incorporation	oxidation	incorporation
Insulin (U/l)				
basal	9.78 ± 0.48	5.87 ± 0.36	8.06 ± 0.97	2.19 ± 0.36 [b]
0.05	11.76 ± 1.10	6.90 ± 1.22	10.96 ± 1.05	2.61 ± 0.76 [b]
0.2	13.28 ± 0.55 [a]	8.42 ± 0.67 [a]	10.38 ± 0.36 [a,b]	2.72 ± 0.34 [b]
1.0	13.19 ± 0.67 [a]	8.83 ± 1.01 [a]	11.36 ± 0.95 [a]	2.73 ± 0.69 [b]
Noradrenaline (mg/l)				
basal	10.65 ± 0.51	5.87 ± 0.38	8.06 ± 0.75 [b]	2.19 ± 0.32 [b]
0.1	11.60 ± 0.55	2.17 ± 0.39 [a]	8.07 ± 0.70 [b]	0.60 ± 0.07 [a,b]
0.5	10.66 ± 0.85	1.12 ± 0.13 [a]	8.05 ± 0.56 [b]	0.43 ± 0.05 [a,b]
1.0	10.99 ± 0.41	1.30 ± 0.15 [a]	7.22 ± 0.40 [b]	0.41 ± 0.05 [a,b]

ANOVA	Oxidation	incorporation
Insulin	0.001	0.026
Glucose	0.003	0.000
Ins/Glc	0.642	0.129
Noradrenaline	0.683	0.000
Glucose	0.000	0.000
NA/Glc	0.674	0.000

the presence of glucose in the incubating medium determined an increase in the incorporation of alanine label into the tissular components, but in the presence of insulin or noradrenaline. This effect of glucose was less patent on the rates of alanine oxidation.

Table II shows the metabolic fate of the labelled alanine in the different IBAT fractions from control, 36-h starved, 4-h cold-exposed and 30-day cold-acclimated rats under basal conditions, as well as in the presence of insulin or noradrenaline. The oxidation of alanine to CO_2 was higher in the animals exposed to cold than in controls, but the values corresponding to starved or cold-acclimated animals were lower. The presence of insulin in the incubation medium determined increases in this parameter in all the experimental groups, except in the cold exposed animals. Noradrenaline did not affect this parameter except for an increased oxidation of the amino acid in the cold-acclimated animals.

The incorporation of alanine carbon into the fatty acids fraction was higher in control and cold-exposed animals than in the other experimental groups. The presence of insulin increased the synthesis of fatty acids from alanine, especially in the cold-acclimated group, whereas noradrenaline diminished that incorporation.

Alanine carbon was also incorporated in the glyceride glycerol fraction, with a pattern similar to that of fatty acids under basal conditions. Insulin did not affect this trend, inducing only small increases except in the control group. Noradrenaline determined a significant decrease in the control group, a pattern similar (but with less marked changes) to that in the other situations studied.

The presence of alanine label in the water-soluble fraction was very similar in all the situations tested. The most important feature was the higher activity found in the cold-exposed rats, which was maintained in the presence of both insulin and noradrenaline. The incorporation of the label in the delipidated water-extracted residue followed the pattern already described. The addition of insulin did not change the tissue response to alanine; noradrenaline induced a significant decrease in alanine label incorporation into this fraction for all groups except the cold-acclimated rats.

Discussion

It is well known that insulin enhances glucose utilization by IBAT, increasing its uptake [20], oxidation [7] and the incorporation of glucose carbon into fatty acids [21]. Insulin also clearly stimulated alanine utilization by IBAT, but this effect was only apparent when glucose was present in the incubation medium. Alanine degradative pathway is initiated with its transamination

TABLE II

In vitro alanine utilization by interscapular brown adipose tissue of control, 36-h starved, 4-h cold-exposed and 30-day cold-acclimated rats in the presence of insulin or noradrenaline

The results are expressed in μmol of alanine oxidized to CO_2 or incorporated to the different tissue fractions/h per g protein. Fractions: I = alanine oxidized to CO_2; II = alanine incorporated into the fatty acids of the lipid fraction; III = alanine incorporated into the glycerol of the lipid fraction; IV = alanine label present in the tissue in water-soluble form; V = alanine label present in the delipidated water-extracted residue of the tissue. The incubations ($n = 5–6$) were made in a medium containing 0.75 mM L-alanine, 5 mM D-glucose and either 0.2 mU/ml insulin (+INS) or 0.5 μg/ml noradrenaline (+NA). Statistical significance of the differences between groups: [a] $P < 0.05$ versus control rats; [b] = $P < 0.05$ versus basal conditions. Two-way ANOVA P values are also shown.

Group	Hormone	Fraction				
		I	II	III	IV	V
Control	basal	10.10 ± 0.68	4.36 ± 0.32	0.47 ± 0.08	0.27 ± 0.04	0.31 ± 0.03
	+INS	13.72 ± 0.68 [b]	5.87 ± 0.06 [b]	0.31 ± 0.08	0.33 ± 0.05	0.31 ± 0.04
	+NA	8.72 ± 0.81	1.64 ± 0.16 [b]	0.13 ± 0.02 [b]	0.21 ± 0.06	0.19 ± 0.01 [b]
36-h starved	basal	4.50 ± 0.30 [a]	0.36 ± 0.10 [a]	0.05 ± 0.01 [a]	0.15 ± 0.04	0.14 ± 0.02 [a]
	+INS	5.53 ± 0.42 [a]	0.59 ± 0.17 [a]	0.09 ± 0.01 [a,b]	0.25 ± 0.05	0.13 ± 0.02 [a]
	+NA	4.70 ± 0.44 [a]	0.10 ± 0.02 [a,b]	0.03 ± 0.01 [a]	0.18 ± 0.04	0.10 ± 0.01 [a,b]
4-h cold-exposed	basal	14.38 ± 1.40 [a]	3.41 ± 0.57	0.33 ± 0.07	0.45 ± 0.04 [a]	0.40 ± 0.07
	+INS	14.21 ± 1.07	4.54 ± 0.51 [a]	0.43 ± 0.04	0.44 ± 0.03	0.26 ± 0.04
	+NA	11.83 ± 0.78 [a]	1.10 ± 0.14 [a,b]	0.18 ± 0.04	0.43 ± 0.04 [a]	0.19 ± 0.03 [b]
30-day cold-acclimated	basal	3.89 ± 0.66 [a]	0.45 ± 0.13 [a]	0.08 ± 0.02 [a]	0.33 ± 0.08	0.13 ± 0.02 [a]
	+INS	6.48 ± 0.98 [a]	2.04 ± 0.46 [a,b]	0.19 ± 0.04 [b]	0.38 ± 0.04	0.17 ± 0.03 [a]
	+NA	5.73 ± 0.42 [a,b]	0.69 ± 0.18 [a]	0.08 ± 0.01 [a]	0.36 ± 0.04	0.14 ± 0.01 [a]
ANOVA Group		0.000	0.000	0.000	0.000	0.000
Hormone		0.000	0.000	0.000	0.224	0.000
Group/hormone		0.016	0.000	0.002	0.822	0.012

into pyruvate by means of alanine transaminase, an enzyme found at a high activity in the IBAT [10]. The increase in alanine utilization induced by insulin can be related to its activation of pyruvate dehydrogenase [22]. On the other hand, insulin activates acetyl-CoA carboxylase [22], which can explain the enhanced incorporation of the amino acid label into fatty acids. The presence of glucose in the incubation medium enhanced the effects of insulin in a similar way to that described for white adipose tissue in vitro, in which the activation of pyruvate dehydrogenase requires the presence of an exogenous source of carbohydrate [23].

Insulin stimulated amino acid uptake by different tissues [24] as well as their incorporation into proteins [25]. Although the amino acid transport mechanisms have not yet been fully characterized in brown adipose tissue, insulin increased the Na^+-dependent uptake of 2-aminoisobutyric acid [26]; the synergic effect of glucose can also be related to the energetic cost of this transport.

Noradrenaline decreased the incorporation of alanine into tissue components. This can be due in part to the catecholamine inhibition of glucose oxidation [27] which reduces the synthesis of fatty acids from glucose through a decrease in the acetyl-CoA carboxylase activity [28]. Different studies realized in vitro have shown that the IBAT is very sensitive to the lipolytic action of noradrenaline [24,29]. Its action results in higher availability of acetyl-CoA originating in the metabolization of intracellular triacylglycerols. The reduced oxidation of alanine can be a consequence of increased availability of fat-lipolysis-derived acetyl-CoA.

The main fate of the alanine taken up by IBAT was its oxidation to CO_2, in a proportion (with respect to all alanine taken up) ranging from 87% in the starved group to 65% in the control group. Most of the label not oxidized, but incorporated into the tissue, found its way into fatty acids, in accordance with the active IBAT lipogenesis described [8].

The incorporation of alanine label into the glycerol moiety of glycerides suggests that, in the IBAT, pyruvate is not solely a source of acetyl-CoA, but can also be utilized in part for the triose-phosphate synthesis. The alanine label present in the water-soluble fraction represents mainly amino acids (i.e., alanine), intermediate metabolites and glycogen. On the other hand, the delipidated water-extracted residue was essentially composed of protein and nucleic acids. The amount of radioactivity found in these two fractions was small, and for that reason the actual contribution of their different components was not evaluated. The relative lack of incorporation of alanine into protein, despite the high metabolic relevance of this tissue strengthens the suggested role of alanine as eventual substrate. It is also indicative of the high turnover of the tissue alanine pool, since the radioactivity found in the water soluble fraction was very small, despite the size of the cellular alanine pool [11].

The utilization of alanine by the IBAT of the starved rats was reduced with respect to controls. This is in agreement with the diminished IBAT thermogenic capability described under food deprivation conditions [30]. In this situation, incorporation of label into the fatty acid fraction was most deeply affected, with values of about one-tenth of those observed in the control rats.

Starvation inhibits fatty acid synthesis [31] and the in vivo incorporation of alanine to IBAT lipids is grossly reduced in the starved rat [13]. IBAT amino acid metabolism decreases during starvation, with important changes in the activities of some enzymes as well as in the amino acid pool composition [12]. The decrease of tissue alanine levels has been related to the net release of this amino acid into blood in the post-prandial and starved states [32], a situation comparable to that of white adipose tissue [33]. The alanine efflux agrees with the observed decrease in alanine utilization by the IBAT in this situation.

The IBAT from short-term cold-exposed rats had the highest rates of alanine utilization; however, when the duration of the cold-temperature stimulus was prolonged (cold-acclimation) the use of alanine as fuel was very much reduced. Amino acids can play a significant role as energetic substrates for IBAT under conditions of cold-exposure, as suggested by the changes observed in enzymes involved in their metabolism [11] and the in vivo amino acid uptake evaluations [9]. During long-term exposition to the cold (cold-acclimation) other substrates seem to play a more important role, such as glucose [9] and fatty acids from lipoprotein triacylglycerols [5]. Under all these other situations there is a net IBAT efflux of alanine and reduced amino acid oxidation [9].

In conclusion, the present results agree with a putative role of alanine as energetic substrate for IBAT under emergency conditions such as short-term cold-exposure; however, this role does not seem to have a special quantitative importance under other situations such as starvation or cold acclimation. The modulation of this utilization by hormones and the availability of glucose agree with this role of transient emergency substrate postulated for alanine in the IBAT.

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