

Effect of Norepinephrine on the Krebs Cycle Intermediates of Brown Adipose Tissue in *Mesocricetus Auratus* Hamster

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Abstract

This study was carried out to determine the rate-limiting and/or norepinephrine-stimulated step in the tricarboxylic acid cycle of hamster (*Mesocricetus Auratus*) brown adipocytes. For this determination, citrate, malate and α -ketoglutarate in interscapular brown fat (IBAT) were measured spectrofluorometrically before and after stimulation with norepinephrine. Norepinephrine infusion increased both IBAT and colonic temperature which are respectively $1.6 \pm 0.2^\circ\text{C}$, $0.4 \pm 0.1^\circ\text{C}$. The concentration and the total amount of citrate and malate were significantly greater in norepinephrine-stimulated than in non stimulated IBAT. In contrast, the concentration and the total amount of α -ketoglutarate were significantly less in stimulated as compared to nonstimulated IBAT. The direction and magnitude of these changes support the view that α -ketoglutarate dehydrogenase is the rate-limiting step in TCA cycle of unstimulated brown adipose tissue; this enzyme is activated when brown fat is stimulated by norepinephrine, and that isocitrate dehydrogenase is most likely the rate-limiting step in the norepinephrine-stimulated brown fat.

Key words: Brown fat thermogenesis, catecholamines, isocitrate dehydrogenase, citrate synthase, α -ketoglutarate dehydrogenase.

ملخص

أجريت هذه الدراسة لتحديد المرحلة المحددة لسرعة و/أو المرحلة التي يتم تنشيطها بواسطة هرمون النورإبينفرين في دورة كريس لحيوان الهامستر (*Mesocricetus Auratus*) ولتحديد ذلك تم تقدير حمض citric malate, ketoglutarate α - في النسيج الدهني الرمادي بين الكتفي (IBAT) بواسطة جهاز spectrofluorometer قبل و بعد حقن الحيوان بواسطة ن. يؤدي ن إلى زيادة في كل من درجة حرارة IBAT ($1.6 \pm 0.2^\circ\text{C}$) وكذلك درجة الحرارة الشرجية ($0.4 \pm 0.1^\circ\text{C}$). لوحظ زيادة ذات معنى في تركيز و الكمية الكلية ل citrate و malate في دورة كريس للنسيج الدهني المنبهة مقارنة بتلك في النسيج الدهني الغير منبه وبالعكس فإن التركيز و الكمية ل α -ketoglutarate أقل بصورة معنوية في النسيج الدهني المنبه مقارنة بتلك الموجودة في النسيج الدهني الغير منبه. يدل أو تدعم هذه الاختلافات الرأي بأن α -ketoglutarate dehydrogenase يعتبر هو المرحلة المحددة لسرعة دورة كريس في النسيج الدهني الرمادي بواسطة ن بينما الأنزيم isocitrate dehydrogenase هو المرجح لأن يكون المرحلة المحددة لسرعة دورة كريس في النسيج الدهني (IBAT) المنبه بهرمون نورإبينفرين.

1. Introduction

Brown adipose tissue is a major site of heat production in mammalian neonates and in various mammalian adults [1, 2, 3]. Increased oxidative metabolism (thermogenesis) of brown fat cells is initiated when during cold exposure (nonshivering thermogenesis) [4] and in some cases when animals

are fed a palatable diet (diet-induced thermogenesis) [5]. Thus, brown fat heat production is important during arousal from hibernation [3], as a defence against hypothermia [6], for retardation of obesity in mammals eating an unbalanced diet [7, 8] and during acclimation to cold.

Brown fat cells have an unique uncoupling protein UCP1 and UCP2, subtypes of UCP [9], in the inner mitochondria membrane which serves as a channel for proton re-entry into the mitochondrial matrix independent of ATP synthetase [10]. This results in heat production rather than ATP i.e., uncoupling [11, 12, 13]. The primary substrate for this heat production is fatty acids which are provided to the mitochondria by norepinephrine-stimulated lipolysis. Brown fat cells are highly enervated by the sympathetic nervous system [14]. Norepinephrine binds primarily to β_3 -adrenergic receptors [15], and the transduction of this signal is mediated through a second messenger pathway involving adenylate cyclase; and most of the heat is produced via the β -adrenergic pathway although is an α -adrenergic contribution [16, 17]. Since substrate oxidation under uncoupled conditions is dependent on substrate, rather than ADP, availability, the rate-limiting step in the tricarboxylic acid cycle may be an important point of regulation. Norepinephrine promotes the synthesis of brown fat-specific mitochondrial UCP [18, 19]. The determination of the rate limiting step can be used as an index of heat production which is easy to practice in vitro than the determination of guanosine 5-diphosphate (GDP) binding to isolated brown adipose tissue mitochondria [20].

The present study was designed to determine this rate-limiting step in the TCA cycle in brown adipocytes. For this determination, the concentration of α -ketoglutarate, citrate, and malate were measured before and during stimulation with norepinephrine. These three metabolites were chosen because of the likelihood that the rate-limiting step was either citrate synthase, isocitrate dehydrogenase or ketoglutarate dehydrogenase [21].

2. Materials and methods:

1. Experimental animals:

Seven male hamsters (*Mesocricetus Auratus*), weighing 150.75 ± 7.4 g (mean $\pm$ SEM) were housed individually at a photoperiod of 10 hours light, 14 hours dark. The animals were exposed to cold ($9 \pm 2^\circ\text{C}$) for two weeks. On the day of the experiment, hamsters were anesthetised with sodium pentobarbital (60 mg/kg, intraperitoneally), and the jugular vein was cannulated with polyethylene-10 tubing filled with saline (0.9% NaCl). This cannula was used for maintenance of anaesthesia and addition of norepinephrine and the vehicle (ascorbic acid, 1 mg/ml, and pH 7.4). An incision was made in the dorsal mid-line, and both interscapular brown fat pads were dissected free of connective tissue. Immediately after a haemostat was attached to the vascular of one pad, the pad was removed and freeze clamped in liquid nitrogen. A YSI thermistor was positioned under the remained pad, and the skin was placed over the pad and thermistor. Ascorbic acid was then

infused for 6 minutes while brown fat and colonic temperature were measured (the latter with a YSI probe inserted 6 cm into the rectum). The infusion was then switched to norepinephrine (3.5 mg/kg/min) for an additional 6 to 8 minutes. Brown fat responsiveness was assessed from the increased tissue temperature induced by norepinephrine. After the temperature increased, the pad was immediately removed and freeze clamped in liquid nitrogen. The clamped tissue was weighed on a piece of tarred aluminium foil, wrapped, and stored in a biological freezer at -70°C .

2. Extraction and homogenisation of tissues

The foil-wrapped sample was removed from the freezer, placed on a rigid surface and hit with a large wooden mallet. The pulverised tissue was quickly put into a glass-glass homogenizer containing 3.5 (v/v) of 8 % perchloric acid in 40 % ethanol. The empty aluminium foil was weighed and compared to the tare to account for any remaining brown fat. This remainder was subtracted from the brown fat weight obtained at the time of sacrifice and used in the calculation. The tissue was homogenized and prepared for determination of citrate, α -ketoglutarate and malate according to the technique by Bergmeyer and al [22].

3. Analytical procedures

Citric acid, α -ketoglutaric acid, and malic acid concentrations were determined by spectrofluorometry based on the method of Bergmeyer and al [22]. This technique was developed and modified for measurements of the low concentrations of these metabolites in brown adipose tissue. Fluorescence was measured with an Aminco-Bowman spectrofluorometer at room temperature ($22 \pm 2^\circ\text{C}$). The instrument was fitted with an Osram XBO-150high pressure Xenon-Arc Lamp and an RCA IP28 multiplier phototube. The slit arrangement for NADH and NADPH had widths of 1 and 0.5 mm for excitation and emission, respectively. NADH and NADPH are excited at 340nm and emit maximally at 460 nm. For all assays, the reaction buffers were prepared fresh daily in a final volume of 100 ml and kept in amber bottles at room temperature ($22 \pm 2^\circ\text{C}$).

Citric acid concentration was determined fluorometrically in the presence of citrate lyase and malate dehydrogenase. The reaction buffer contained: tris hydroxymethyl-aminomethane (tris, 50mM, pH 8), reduced nicotinamide-adenine dinucleotide (NADH Na_2 , 0.0005 mM), magnesium chloride (MgCl_2 , 2 mM), ethylenediaminetetra-acetate ($\text{EDTA-Na}_2\text{H}_2$, 0.1 mM), and malate dehydrogenase (MDH, 288mU/mL) from pig heart suspensions in

3.2 M ammonium sulphate solution. Other solutions used in the assay included citrate standard (1mM stock) and citrate lyase (0.5 mg protein/ml; 8 u/mg protein at 25°C) from *Aerobacter aerogenes*. A standard curve for citrate was obtained by pipetting the following: 1 ml buffer, citrate standard (varying volumes + distilled water for a total of 10 µl), and 100 µl triethanolamine base (0.5 M, pH 7.4). The contents of the cuvette were mixed, fluorescence was recorded, and then 10µl citrate was added to the cuvette and mixed. After the reaction had gone to completion (5 to 6 minutes), fluorescence was again recorded. The difference (ΔF) between the two fluorescence readings was correlated with the concentration of citrate standard added.

For determination of the citrate concentration in brown adipose tissue, cuvettes were prepared as for the standard but 10 µl of distilled water and 100 µl of the tissue supernatant were used instead of the standard solution and the triethanolamine buffer respectively. The time required for completion of the reaction was 12 to 13 minutes. Before the first reading of the experimental cuvette (i.e., the cuvette containing the standard solution or the tissue sample) and after the second reading of the same cuvette, the fluorescence of a control cuvette (containing solvent rather than standard or tissue sample) was measured. Any change in fluorescence in the control was subtracted from the change in fluorescence of the experimental cuvette (ΔF).

α -Ketoglutaric acid concentration was determined fluorometrically in the presence of glutamate dehydrogenase (GDH) [22]. The reaction buffer contained dipotassium hydrogen phosphate, K_2HPO_4 and sodium hydrogen phosphate, $NaHPO_4$ (phosphate buffer, 50 mM, pH 6.8) ammonium acetate (40mM), $EDTA-Na_2H_2$ (0.1 mM), adenosine-5-diphosphate ($ADP-Na_2$, 0.1 mM), and $NADH-Na_2$ (0.01 mM). Other solutions used in the assay included α -ketoglutarate standard solution (1 mM) and glutamate dehydrogenase (10 mg protein/ml; 90 U/mg at 25°C) from bovine liver, solution in 50% glycerol. Construction of the standard curve for α -ketoglutarate and the measurement of brown fat α -ketoglutarate followed a procedure analogous to that described above for citrate. For the α -ketoglutarate assay, fluorescence was recorded before and after addition of glutamate dehydrogenase (9-10 minutes after GDH for the standard curve, 12-13 minutes after GDH for the homogenate samples).

For malate determination in brown adipose tissue samples, cuvettes were prepared as for the standard, but 6 µl of distilled water and 54 µl of the tissue supernatant were used instead of the standard and triethanolamine base buffer respectively. The time required for the completion of the reaction was 17 to 18 minutes. A control cuvette was used as for citric acid and α -ketoglutaric acid assays.

4. Statistical analysis

Values (mean $\pm$ SEM) of interscapular brown fat weight recovered after pulverization, concentration and changes in fluorescence (Δf) for malate, citrate and α -ketoglutarate, and brown fat weight before and during stimulation with norepinephrine were analyzed by a paired t test.

3. Results:

Figure 1 and figure 2 show the changes in interscapular brown adipose tissue temperature and colonic temperature before and during norepinephrine infusion. Colonic temperature remained relatively constant prior to norepinephrine infusion (i.e., during infusion of saline) but began to slowly increase within 2 minutes after norepinephrine infusion began. After 6 minutes of norepinephrine infusion, colonic temperature had increased $0.4 \pm 0.1^\circ C$ ($35.6^\circ C$ to $36^\circ C$).

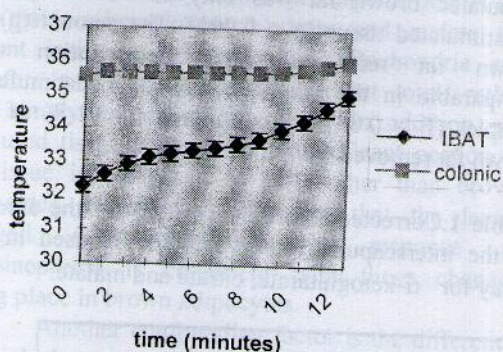


Fig 1. Colonic and interscapular brown adipose tissue temperature before and after stimulation with norepinephrine. Norepinephrine (NE) was infused after 6 minutes of temperature measurements. (Values are mean $\pm$ SEM, $n=7$)

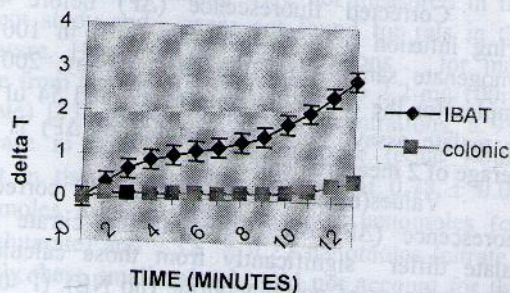


Fig. 2. Changes in interscapular brown fat (IBAT) and colonic temperatures during infusion of saline (0 to 6 min.) and during subsequent infusion of

norepinephrine (NE, 3.5 mg/kg min). At zero time, the colonic temperature averaged $35.5 \pm 0.6^{\circ}\text{C}$; and the IBAT averaged $32.3 \pm 0.4^{\circ}\text{C}$. (Values are mean $\pm$ SEM, n=7)

Interscapular brown fat temperature increased rapidly during the first 3 minutes of saline infusion, levelling off to a much reduced rate of temperature rise. Since initiation of saline infusion occurred immediately after the exposed fat pad was recovered with skin, this initial warming most likely reflects decreased heat loss to the air. Within 1 min after norepinephrine infusion began, brown fat temperature began to rise, increasing $1.6 \pm 0.2^{\circ}\text{C}$ (33.4°C to 35°C) after 6 min of infusion. This increase in brown fat temperature began before any measurable change in colonic temperature occurred.

Table 1 shows the corrected fluorescence (ΔF) and Table 2, the corresponding concentrations for citrate, α -ketoglutarate, and malate in interscapular brown fat and during stimulation with norepinephrine. The concentration of citrate and malate were significantly greater in norepinephrine-stimulated brown fat than they were in the nonstimulated tissue (table 1). In contrast, the concentration of α -ketoglutarate in norepinephrine-stimulated brown fat was only 53 % that in the nonstimulated tissue ($p < 0.001$). The amount (g) of brown fat recovered after pulverisation was comparable in the stimulated versus nonstimulated samples ($P > 0.05$) and accounted for 91 % of the brown fat removed from the animal.

Table 1.Corrected fluorescence (ΔF) for the amount of the interscapular brown fat (IBAT) used in the assay for α -ketoglutarate, citrate and malate.

Fluorescence (ΔF)			
	α -ketoglutarate	Citrate	Malate
no NE	0.0725 ± 0.0081	0.0412 ± 0.0063	0.0073 ± 0.001
NE	0.0448 ± 0.005	0.0875 ± 0.012	0.0295 ± 0.002
P value	<0.01	<0.002	<0.001

Corrected fluorescence (ΔF) before and during infusion of norepinephrine (NE) in 100 ul homogenate samples for the citrate assay, 200 ul samples for the α -ketoglutarate assay, and 54 ul for the malate assay. For each metabolite (ΔF) is the average of 2 measurements.

Values(mean $\pm$ SEM, n=7) of corrected fluorescence (ΔF) of α -ketoglutarate, citrate and malate differ significantly from those calculated before infusion of norepinephrine (no NE). ($P<0.01$, $P<0.002$, and $P<0.001$ respectively

Table 2. Interscapular brown fat (IBAT) weight and TCA cycle metabolite concentration before and during norepinephrine (NE) infusion

	IBAT Weight (g)	nanomoles/g wet weight recovered#		
		α -ketoglutarate	Citrate	Malate
no NE	0.231 ± 0.029	55.9 ± 8.3	50.0 ± 9.0	42.8 ± 6.0
NE	0.251 ± 0.028	29.6 ± 4.1	106.6 ± 15.7	125.4 ± 11.3
P value	NS	<0.001	<0.001	<0.001

Values (mean $\pm$ SEM, n=7) of nanomoles of α -ketoglutarate, citrate and malate per gram calculated differ significantly from those calculated before infusion of norepinephrine (no NE). ($P<0.001$). #This weight is that of IBAT recovered after pulverisation of the frozen tissue. Values of these weights (mean $\pm$ SEM, n=7) are not significantly (NS) different before and during infusion of norepinephrin

The same pattern was seen when metabolite amount rather than concentration was considered (table3). That is, the total number of nanomoles of α -ketoglutarate present in interscapular brown fat pads stimulated with norepinephrine was 44 % less than that present in nonstimulated tissue ($p < 0.001$). On the other hand, the levels of citrate and malate (nanomoles) in the norepinephrine-stimulated brown fat were more then twice those in the nonstimulated tissue ($p < 0.01$ and $p < 0.001$ respectively). Stimulated interscapular brown fat depots were slightly (9 %) but significantly heavier than those taken before infusion with norepinephrine (Table2), most likely reflecting increased blood volume.

Table 3. Interscapular brown fat (IBAT) weight and TCA cycle metabolite levels before and during stimulation with norepinephrine (NE).

	IBATWeight (g)	nanomoles		
		α -ketoglutarat	Citrate	Malate
no NE	0.253 ± 0.023	14.1 ± 2.2	12.7 ± 2.4	10.3 ± 1.1
NE	0.276 ± 0.029	7.9 ± 1.0	29.8 ± 6.2	33.4 ± 2.8
P value	<0.05	<0.001	<0.01	<0.001

Values (mean $\pm$ SEM) of interscapular brown fat weight and the total number of nanomoles of α -ketoglutarate, citrate and malate differ significantly from those measured before norepinephrine stimulation (no NE) ($P<0.05$, $P<0.001$, $P<0.01$ and 0.001 respectively).

4. Discussion

The levels of citrate and malate increased while that of α -ketoglutarate decreased in the norepinephrine-stimulated vs the nonstimulated brown fat pads. These changes were accompanied by significant norepinephrine-induced increases in brown fat temperature, reflecting enhanced substrate oxidation and associated heat production [11, 12, 13]. Thus, under conditions in which brown fat thermogenesis was stimulated [1, 3], and the metabolism rate was increased [23] both the concentration and the absolute amount of TCA metabolites were altered.

The nature of these alterations suggests that α -ketoglutarate dehydrogenase, rather citrate synthase or isocitrate dehydrogenase, is a rate-limiting step of the TCA cycle in unstimulated brown fat. The reasoning underlying this suggestion is as follows. During norepinephrine stimulation, flux through the TCA cycle increase (as measured independently by increased brown fat oxygen consumption). This increase is due to mass action (i.e., increased input of acetyl CoA resulting from norepinephrine stimulation of lipolysis and beta oxidation of fatty acids) and in part to specific activation of at least one enzyme of the TCA cycle—namely, α -ketoglutarate dehydrogenase. That is, under conditions in which flux through the TCA cycle is stimulated, one would not expect the level of a particular metabolite to fall (relative to the unstimulated state) unless it were being converted to product at a faster rate or unless the enzyme producing the metabolite were inhibited. Hence, the fact that α -ketoglutarate was decreased in the stimulated vs nonstimulated brown fat pads while citrate and malate were increased, implies that α -ketoglutarate dehydrogenase activity was stimulated to a greater extent than was citrate synthase or isocitrate dehydrogenase. This, in turn, is consistent with the hypothesis that α -ketoglutarate dehydrogenase catalyses a flux controlling step in the TCA cycle under nonstimulated conditions, this step being activated during catecholamine stimulation of brown adipose tissue.

After norepinephrine stimulation, isocitrate dehydrogenase appears to become a flux controlling step. This is indicated the fact that in the norepinephrine-stimulated state, levels of α -ketoglutarate are considerably lower than those of malate, citrate and presumably isocitrate, suggesting that the oxidation of isocitrate to α -ketoglutarate is lower than the generation of citrate from oxaloacetate and acetyl CoA. Although brown fat levels of isocitrate dehydrogenase have not been measured, rat heart isocitrate dehydrogenase activity is less than α -ketoglutarate dehydrogenase activity quantitative index of the maximal flux through the TCA cycle in rat heart [21, 24]. However, the *in vitro* in

dehydrogenase and α -ketoglutarate dehydrogenase may differ due to allosteric controls and the fact that α -ketoglutarate dehydrogenase occurs in both an active and inactive form. More detailed information about the activities of isocitrate dehydrogenase and α -ketoglutarate dehydrogenase in brown adipose tissue is needed to determine if α -ketoglutarate dehydrogenase activity can be used as an index of oxidative capacity.

The mechanism underlying norepinephrine activation of α -ketoglutarate dehydrogenase is not known. The short time frame involved—within 6 minutes of norepinephrine administration—makes synthesis of new enzyme an unlikely explanation. One possible short-term regulator is Ca^{++} which has been shown to activate α -ketoglutarate dehydrogenase in rat brown fat and in heart by increasing the enzyme's affinity for α -ketoglutarate [25]. Although norepinephrine stimulation has been postulated to increase free cytosol Ca^{++} in brown fat [26], and an effect of α -adrenergic receptors was demonstrated in brown adipose tissue [27] and in preadipocytes [28]. Norepinephrine's effect on intramitochondrial levels of free cytosol Ca^{++} ions has not yet been investigated.

One difficulty in interpreting our data is the fact that α -ketoglutarate, citrate and malate are present inside and outside the mitochondria and therefore our measurements represent total, rather than simply mitochondrial values. In addition, we measured tissue values, and a significant portion of the tissue is composed of cells other than brown adipocytes. Nonetheless, the fact that the levels changed so dramatically in response to norepinephrine is consistent with these changes taking place in brown adipocytes.

Another confounding factor is the difference in weight between the stimulated and non stimulated brown fat pads. This difference is most likely due to the presence of more blood. During norepinephrine stimulation of brown fat, blood flow and vascularity increases dramatically in adipose tissue [1, 29, 30]. Moreover norepinephrine has also been shown to stimulate proliferation of brown adipocytes in culture [31, 32]. Plasma concentration of citrate, α -ketoglutarate, and malate were not measured in the present study and were not found for rats in the literature. However, the concentrations for man range from 0.5 mg/100 ml (malate) to 2.2 mg/100ml (citrate) [33]. Based on these concentrations, a 9% increase in blood in brown adipose tissue would result in tissue metabolite changes of 0.78 ± 0.08 nanomoles (malate), 1.45 ± 0.07 nanomoles (α -ketoglutarate) and 2.32 ± 0.27 nanomoles (citrate). Clearly these small values could not account for the observed tissue metabolite changes [21 (malate), 6.19 (α -ketoglutarate) and 17.12 (citrate)].

α -ketoglutarate dehydrogenase activity has been shown to be an index of maximal flux through heart muscle [21, 24].

Newsholme [34] have compared the activity of this enzyme to oxygen consumption data obtained from cold-acclimated rats [1] and lean (ob/+) mouse [35] and have suggested that the activity of α -ketoglutarate dehydrogenase could be used as an index of the thermogenic (oxidative) capacity of the tissue. While this possibility is intriguing, the fact that this comparison involved different groups of animals (oxygen consumption and enzyme activity were not measured in the same animals or by the same investigators) makes it likely that the animals were housed under different conditions of temperature, photoperiod, and diet – all conditions that can affect thermogenic capacity of rodents. Thus, the degree to which α -ketoglutarate dehydrogenase activity is correlated with brown fat thermogenic capacity remains uncertain.

5. Conclusion

In conclusion, the results from this study indicate that neither citrate synthase nor isocitrate dehydrogenase was rate-limiting enzymes in the TCA cycle of unstimulated brown adipose tissue. Rather, they support the view that the rate limiting step in the cycle is catalyzed by α -ketoglutarate dehydrogenase, and this enzyme is activated when brown fat is stimulated by norepinephrine. Following such stimulation, isocitrate dehydrogenase appears to become rate limiting. This suggests that isocitrate dehydrogenase activity may be a more valid index of brown fat thermogenic capacity than α -ketoglutarate dehydrogenase activity in the norepinephrine-stimulated state. This hypothesis needs further evaluation.

6. References

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