Microcalorimetric measurements of heat production in brown adipocytes from control and cafeteria-fed rats

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1. The effects of the sequential addition of glucose, noradrenaline, propranolol and oleic acid on the rates of O₂ consumption and heat production by isolated interscapular brown adipocytes from control and cafeteria-fed rats were compared. 2. Although the chemical agents produced very similar changes in oxidative metabolism, the actual rates of O₂ uptake and heat output in adipocytes from the cafeteria-fed rats, when expressed per g dry wt. of cells, were approx. 65% less than those obtained with cells from the control rats. 3. However, when the same results were expressed per 10⁸ multilocular brown adipocytes, rather than gravimetrically, rates of O₂ consumption and heat production were equivalent. 4. Further interpretation of these data is complicated, because the average volume of multilocular brown adipocytes from cafeteria-fed rats was 2.5 times that for multilocular cells from control animals.

INTRODUCTION

Depots of brown adipose tissue are located around the kidneys, the aorta and the thoracic region of newborns, hibernants and cold-acclimated adults of a large number of mammalian species. The individual cells are characterized by many small, discrete, lipid droplets, a centrally located nucleus, coarse granular cytoplasm and numerous, densely packed, spherical mitochondria (Nedergaard & Lindberg, 1982). It is now generally accepted that these adipocytes, because of their ability to increase greatly their rate of fatty acid oxidation (Prusiner *et al.*, 1968) and hence cellular heat production, play a major thermoregulatory role in adaption to cold (Smith, 1961), arousal from hibernation (Smith & Hock, 1963) and warming of mammalian newborns (Dawkins & Hull, 1964).

More recently it has also been suggested that there is an inverse relationship between the activity of brown adipose tissue and the development of obesity (Himms-Hagen, 1979; Rothwell & Stock, 1979). In some strains of rodents it appears that total body energy balance includes a mechanism which can increase energy expenditure in response to overfeeding; this energy-dissipating process, once known as 'luxusconsumption', is now called 'diet-induced thermogenesis' (Rothwell & Stock, 1979). Brown fat, which hypertrophies during overfeeding of a highly palatable 'cafeteria' diet, is considered, by some, to be the main effector of this adaptive mechanism (Rothwell & Stock, 1979, 1981).

Since diet-induced thermogenesis, but more especially the role of brown adipose tissue in this mechanism, is not universally accepted (see Hervey & Tobin, 1983), it was decided to compare the effects of glucose, noradrenaline, propranolol and non-esterified fatty acids (Bukowiecki *et al.*, 1981) on the rates of O₂ consumption and heat production in isolated brown adipocytes from control and cafeteria-fed rats.

MATERIALS AND METHODS

Animals

Male Hooded Wistar rats, initially 100–120 g body wt., were housed at 23 ± 1 °C and were offered a pelleted commercial stock diet (Milling Industries Ltd., Adelaide, South Australia, Australia) together with a varied choice of highly palatable human food items (Sclafani & Springer, 1976) for at least 4 weeks. This dietary regime has been termed 'cafeteria' feeding by Rothwell & Stock (1979). Body weights and total food intake were measured daily (Rothwell & Stock, 1979; Berry *et al.*, 1985). Rats (260–320 g body wt.) were anaesthetized by intraperitoneal injection of sodium pentobarbitone (60 mg/ml: 100 µl/100 g body wt.) before surgery.

Preparation of adipocytes

Isolated fat-cells were prepared from the interscapular brown-adipose-tissue depot (1.6–2.0 g wet wt.) of anaesthetized cardiotoxized rats by the method of Bukowiecki *et al.* (1981), except that the adipocytes were prepared and washed in Krebs-Henseleit (1932) bicarbonate buffer containing 7 mM-glucose and 1% (w/v) defatted bovine serum albumin, and two different populations of cells were prepared; the first after incubation of the cubed tissue with collagenase (3 mg/ml) for 10 min, and the second after an additional 30 min digestion of the non-filterable fat tissue in fresh collagenase. The isolated brown adipocytes received their ultimate wash and final resuspension in the same buffer (9.0 ml), but with 4% (w/v) defatted bovine serum albumin (Bukowiecki *et al.*, 1981).

Samples of both adipocyte suspensions were diluted with an equal volume of 0.9% (w/v) NaCl containing Trypan Blue (0.3%, w/v), and the numbers of unilocular, multilocular and damaged cells were counted in an improved Neubauer haemocytometer.

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Measurement of O₂ consumption and heat production

O₂ consumption was determined polarographically in a Yellow Springs Instruments model 53 O₂-electrode assembly, and heat production was measured in a modified LKB model 10700 batch-type microcalorimeter as described previously (Clark *et al.*, 1979; Jarrett *et al.*, 1979), except that 8 ml rather than 12 ml of cell suspension was introduced into the calorimeter vessel and the remaining space (4 ml) was filled with O₂/CO₂ (19:1) in order to avoid hypoxia.

Resting O₂ consumption *in vivo* was measured simultaneously in four control and four cafeteria-fed rats (initially 1130–1180 g total body wt.) in a modified open-circuit respirometer (Glatz & Polkinghorne, 1981) between 09:30 and 15:30 h on 9 days. The rats were housed in two air-tight Perspex cylinders (300 mm × 300 mm) at a temperature of 25 °C. Air passing through each chamber (flow rate of 3.5 ml/min) was sampled for comparative analyses by a Beckman model 755 paramagnetic O₂ analyser.

Preparation of brown adipose tissue for morphological studies

The interscapular brown-adipose-tissue depot was rapidly removed from four control and four cafeteria-fed rats, and a 1.5 mm-wide longitudinal midline slice was placed in 20 vol. of 10% neutral-buffered formalin and cut into 1.5 mm cubes. Material for light-microscopy was dehydrated in graded alcohols and embedded in Araldite; 2 µm serial sections were stained with haematoxylin and eosin (Haynes *et al.*, 1983). Cell diameters were measured on a micro-projector by using an automatic caliper system, and the numbers of unilocular and multilocular cells were determined, in fields selected at random, by using the standard grid system. At least 1200 cells were evaluated in each section.

Analytical determinations

The dry weights of the adipocyte suspensions were determined gravimetrically.

Purification of bovine serum albumin

Fatty acids present in bovine serum albumin were removed by the acid charcoal method of Chen (1967), and the albumin was dialysed against 20 vol. of water at 4 °C. After four changes of water over 4 days, the protein was freeze-dried.

Statistical methods

The statistical significance of differences between experimental groups was determined by a one-way analysis of variance (Brownlee, 1949). A *P* value of 0.05 or less was taken as the criterion of statistical significance.

Materials

Collagenase (type II) was purchased from Worthington Biochemical Corp., Freehold, NJ, U.S.A. Bovine serum albumin (fraction V powder) noradrenaline hydrochloride and DL-propranolol hydrochloride were obtained from the Sigma Chemical Co., St. Louis, MO, U.S.A.

RESULTS AND DISCUSSION

Cafeteria-fed rats

The cafeteria-fed rats, in addition to having free access

Fig. 1. Growth rates for rats maintained on a cafeteria and/or a stock diet fed *ad libitum* or as meals

Five groups of rats were housed and maintained as described in the Materials and methods and Results and discussion sections. Total food intake and body weights were measured each day. The data are expressed as means $\pm$ S.E.M. for rats ($n = 6$) fed on stock diet (□, ■), cafeteria diet (△) or cafeteria plus stock diet (○, ●) *ad libitum* (■, ●) or as a meal from 09:00 to 12:00 h (□, △, ○) each day.

to a pelleted stock diet, drinking water and 33% (v/v) sweetened condensed milk, also received four of the following food items each day in a sequential manner: peanut butter and sugar sandwiches, savoury biscuits, chocolate, cornflakes, mettwurst, cheese, 'cocopops', fruit cake, jelly sweets and chocolate-chip biscuits. After 1 week on this diet it became apparent that the rats had developed preferences for some of the new foodstuffs. The favourite items, sandwiches, cornflakes and 'cocopops', were rapidly devoured; second-choice foodstuffs (condensed milk, mettwurst, biscuits and fruit cake) were then started. Consumption of stock diet occurred mainly at night and was decreased to approx. 25% of that of the control rats.

In our hands the cafeteria-feeding regime resulted in the production of rats with meal-feeding rather than '*ad libitum*' feeding characteristics. In an attempt to obtain suitable control animals for subsequent experiments, groups of rodents were maintained on regular stock, cafeteria or cafeteria plus stock diet as a meal from 09:00 to 12:00 h daily. Weight gains in these rats and rats fed on the stock or cafeteria (fresh at 09:00 h daily) plus stock diet *ad libitum* are shown in Fig 1. The meal-fed animals took 5 days to adapt to the different feeding regime (Fig. 1) but then gained weight in a linear manner for the next 25 days. Weight gains averaged 3 g/day in rats meal-fed on either the stock or cafeteria diet alone, 4.2 g/day when meal-fed on both diets together, and 5.5 g/day when fed with stock or cafeteria plus stock diet *ad libitum* (Fig. 1). Although the feeding of the cafeteria diet as meals doubled the mass of the interscapular brown adipose tissue, epididymal and perirenal fat-pads, it was decided not to use the meal-fed animals, as their rate of weight gain on the stock diet (and the cafeteria diet

Fig. 2. Representative traces of heat output (a) and O_2 consumption (b) rates by isolated brown adipocytes from cafeteria-fed rats after the sequential addition of glucose, noradrenaline, propranolol and oleic acid

Isolated brown adipocytes (2.65×10^6 cells/ml) were prepared from two cafeteria-fed rats as described in the Materials and methods section. Noradrenaline, propranolol and oleic acid were prepared in Krebs–Henseleit bicarbonate-buffered saline, equilibrated at 37°C for 3.0 min, and added in a concentrated form at the times indicated.

alone) was 45% less than that for the rats fed on the stock diet *ad libitum* (Fig. 1).

To overcome partially some of the problems of this pseudo-meal-feeding regimen, the rats on the cafeteria diet were fed at 17:00, rather than 09:00 h, and were used for the preparation of adipocytes between 09:00 and 10:00 h after a minimum of 4 weeks on the diet. Weight gains in these rats were the same as those for rats fed on the stock diet *ad libitum*, although their total energy intake was increased by approx. 45% during the first week and by 20–30% during the subsequent weeks of feeding. After 4 weeks on the palatable diet, rates of O_2 consumption *in vivo*, between 09:30 and 15:30 h, were determined in four control and four cafeteria-fed animals on 9 days. O_2 uptake by the control rats (11.5 ± 0.18 ml/min per wt.^{0.75}) on days 5–9 inclusive was 20% less ($P < 0.001$) than that for the cafeteria-fed

animals (13.98 ± 0.21 ml/min per wt.^{0.75}), although their mean body weights (311 ± 5 g for controls; 323 ± 5 g for cafeteria-fed rats) were not significantly different. Days 1–4 were used to acclimatize the rats to the respirometer. These results suggest that the rats fed on the cafeteria diet were exhibiting diet-induced thermogenesis (Rothwell & Stock, 1979).

Heat production and O_2 consumption in isolated brown adipocytes

Representative traces of heat production and O_2 consumption after the sequential addition of noradrenaline, propranolol and oleic acid to a suspension of isolated brown adipocytes are shown in Fig. 2.

Consumption of O_2 by adipocytes from control rats, in the presence of 7 mM-glucose, was stimulated more than 11-fold by the addition of 100 nM-noradrenaline: 2 μM -

Table 1. Effect of sequential addition of noradrenaline, propranolol and oleic acid on heat production and O₂ consumption by isolated brown adipocytes from control and cafeteria-fed rats

Isolated brown adipocytes were prepared from control and cafeteria-fed rats (286 ± 2 and 314 ± 4 g body wt. respectively) as described in the Materials and methods section. The cells [$2.58 (\pm 0.03) \times 10^6$ and $7.89 (\pm 0.02) \times 10^6$ cells/ml for cafeteria-fed and control rats respectively] in Krebs-Henseleit (1932) bicarbonate-buffered saline containing 7 mM-glucose and 4% (w/v) defatted bovine serum albumin were diluted with 10 vol. of Krebs-Henseleit bicarbonate-buffered saline containing 7 mM-glucose (Bukowiecki *et al.*, 1981). After pre-equilibration of the cell suspension at 37 °C for 3.0 min, 8.0 ml was transferred to the microcalorimeter vessel and 8.0 ml to the O₂-electrode assembly. Values are shown as means $\pm$ S.E.M. for five cell preparations from cafeteria-fed and eight preparations from control rats. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ for adipocytes from control versus adipocytes from cafeteria-fed rats (N.S., not significant).

Addition to medium	Rats...	Heat production				O ₂ consumption			
		(J/min per g dry wt.)		(mW/10 ⁸ cells)		Control		(μmol/min per g dry wt.)	
		Control	Cafeteria	Control	Cafeteria	Control	Cafeteria	Control	Cafeteria
None		0.77 $\pm$ 0.08	*** 0.34 $\pm$ 0.10	16 $\pm$ 2	N.S. 20 $\pm$ 1	3.7 $\pm$ 0.5	*** 1.3 $\pm$ 0.5	4.5 $\pm$ 0.6	N.S. 5.0 $\pm$ 1.3
100 nM-noradrenaline		23.4 $\pm$ 2.4	*** 9.30 $\pm$ 2.7	509 $\pm$ 86	N.S. 587 $\pm$ 31	41.5 $\pm$ 5.5	** 17.9 $\pm$ 5.4	52.5 $\pm$ 7.4	N.S. 66.3 $\pm$ 3.0
2 μM-propranolol		3.9 $\pm$ 0.6	*** 1.0 $\pm$ 0.2	85 $\pm$ 19	N.S. 72 $\pm$ 14	4.1 $\pm$ 0.7	*** 1.1 $\pm$ 0.4	6.1 $\pm$ 1.5	N.S. 3.9 $\pm$ 0.8
1 mM-oleic acid		10.5 $\pm$ 1.5	** 3.9 $\pm$ 1.2	221 $\pm$ 38	N.S. 241 $\pm$ 15	20.2 $\pm$ 4.5	*** 3.3 $\pm$ 1.1	26.8 $\pm$ 6.8	N.S. 12.2 $\pm$ 0.8

propranolol, a β -adrenergic antagonist, almost abolished this effect, but the succeeding addition of 1 mM-oleic acid resulted in a 5-fold stimulation of O₂ uptake (Table 1). On the assumption that 1 g of brown adipose tissue contains 10⁸ cells (Nedergaard & Lindberg, 1982), these values for O₂ uptake are nearly identical with those reported by Bukowiecki *et al.* (1981). Simultaneous measurements of cellular thermogenesis are also compared in Table 1. In control cells heat production in the presence of 7 mM-glucose was increased more than 30-fold after stimulation with noradrenaline. The addition of 2 μM-propranolol decreased cellular heat production by more than 80% (Table 1), but with this antagonist heat output was still 5-fold greater than that observed with glucose alone. Subsequent injection of oleic acid resulted in a marked stimulation of adipocyte thermogenesis (Table 1).

Similar changes in O₂ consumption and heat production were observed with isolated brown adipocytes from cafeteria-fed rats, but with these cells both basal and stimulated metabolism was 55–75% less than that observed with the control adipocytes (Table 1).

Adipocyte morphometry

In our procedure for the preparation of isolated brown adipocytes, the cubed tissue was incubated with fresh collagenase twice during each isolation. The initial 10 min preincubation was included to lessen contamination by superficial unilocular cells. Heat production in these preparations (> 95% unilocular, < 5% multilocular cells), in the presence of 7 mM-glucose, proceeded at 0.08 ± 0.02 ($n = 8$) J/min per g dry wt. of cells, and was only increased by 50–100% after the addition of 100 mM-noradrenaline.

Light-microscope examination of adipocytes isolated after an additional 30 min incubation with collagenase revealed that the preparations from control rats ($n = 8$) were composed of $64 \pm 2\%$ multilocular cells (the remainder being unilocular cells or fat droplets), whereas the preparations from cafeteria-fed rats ($n = 5$) contained only $35 \pm 7\%$ ($P < 0.001$) multilocular cells. These proportions of multilocular and unilocular cells are very similar to those observed in 2 μm sections of formalin-fixed interscapular brown adipose tissue: intact tissue from control rats contained $67 \pm 3\%$ multilocular cells and $33 \pm 7\%$ unilocular cells, and that from cafeteria-fed rats $41.5 \pm 2.5\%$ ($P < 0.001$) multilocular cells and $58 \pm 4\%$ ($P < 0.001$) unilocular cells. As the unilocular adipocytes from brown adipose tissue, like white adipocytes from human subcutaneous fat (Sorbis *et al.*, 1979), have very low rates of oxidative metabolism, the rates of heat production and O₂ consumption in the second adipocyte preparations (Table 1) have also been expressed per 10⁸ multilocular brown adipocytes (Table 1). The heat-production results are expressed as mW/10⁸ cells to comply with the recommendations of the Interunion Commission on Biothermodynamics (IUPAC, IUB, IUPAB). On a cell-number basis the rates of heat production and O₂ consumption by isolated multilocular brown adipocytes from cafeteria-fed rats, in the presence of 7 mM-glucose and 100 nM-noradrenaline, instead of being approx. 65% less, were approx. 15% greater (not significant) than the corresponding values for multilocular brown adipocytes from control rats (Table 1). Although these results indicate that isolated brown adipocytes from cafeteria-fed

Fig. 3. Size-distribution curves for interscapular multilocular brown adipocytes from control and cafeteria-fed rats

Isolated brown adipocytes were prepared from control and cafeteria-fed rats as described in the Materials and methods section, diluted with an equal volume of Krebs–Henseleit physiological saline containing Trypan Blue (0.3%) and placed in an improved Neubauer haemocytometer. Cell diameters were measured on a micro-projector by using an automated caliper system. At least 500 cells were evaluated in each cell preparation. The data are shown as means $\pm$ S.E.M. for five different cell preparations from control rats ($\square$) and six different cell preparations from cafeteria-fed rats ($\circ$).

rats behave in a very similar manner to those from control rats in the resting and stimulated states, there is an additional confusing factor. Fig. 3 demonstrates that the diameters of the multilocular cells from the cafeteria-fed rats are considerably greater than those from the control rats: the average diameter of cells from the control animals was 28.4 μm , whereas that from the cafeteria-fed rats was 38.8 μm .

If it is assumed that these adipocytes are spherical (see Nedergaard & Lindberg, 1982), then the average volume of the multilocular cells from cafeteria-fed rats (245000 μm^3) is 2.5 times that of the control cells (95000 μm^3). This difference in cell diameter does not appear to be an isolation artifact, as the average diameter of multilocular cells in intact sections of interscapular brown adipose tissue from cafeteria-fed rats was 35.6 μm . A smaller average diameter for cells from intact tissue was expected, as only a few cells would be sectioned at their widest point (Tulp, 1981). The multilocular cells from control rats were considerably smaller than those from the cafeteria-fed rats, but, as these cells were very densely packed, it was not possible to measure their diameters accurately.

The difference in volume of the multilocular (and unilocular) cells from cafeteria-fed rats corresponds to the difference in the weight of this tissue between the two

groups of animals. The average weight of the interscapular brown-adipose-tissue depot in cafeteria-fed rats (0.94 ± 0.05 g; $n = 10$) was nearly 2.4 times ($P < 0.001$) that from the controls (0.40 ± 0.05 ; $n = 32$).

Heat production in brown adipose tissue

Nedergaard & Lindberg (1982) have demonstrated that the rates of O_2 consumption in preparations of isolated brown adipocytes approach those measured by arterio-venous differences in O_2 content across brown adipose tissue *in vivo*. For this reason it would be expected that measurements of the rates of brown-fat-cell heat production *in vitro* should be comparable with those occurring in the intact tissue *in situ*. Chinnet *et al.* (1977) reported that the basal rate of heat production in 20 mm $\times$ 1 mm thread-like strips of rat interscapular brown adipose tissue was 52 mcal/min per g wet wt. (approx. 0.22 J/min per g wet wt.). Noradrenaline-stimulated rates have been measured as 820 mcal/10⁶ cells in 60 min (approx. 5.75 J/min per g wet wt.) in adipocytes isolated from the interscapular brown-adipose-tissue depot of rats (Yahata & Karoshima, 1982) and at 820 $\mu\text{W}/10^6$ cells (approx. 4.9 J/min per g wet wt.) in the same type of cells, but from hamsters (Nedergaard *et al.*, 1977). These results are similar to those obtained with adipocytes from the cafeteria-fed rats (Table 1), but are approx. 75% less than the corresponding values for brown-fat cells from the control animals (Table 1). This difference might result from the inclusion of purified bovine serum albumin in the adipocyte preparation and incubation buffers in the more recent studies (see the Materials and methods section; Bukowiecki *et al.*, 1981).

Use of isolated brown adipocytes in metabolic studies

A major disadvantage of isolated brown-fat-cell preparations for metabolic studies is the low yield of cells. For hamsters the yield is only 1.6–16 million cells per g wet wt. of tissue (1–10%; Nedergaard & Lindberg, 1982). Our procedure produced approx. 5.6×10^6 cells/g from the interscapular brown-adipose-tissue depot of control rats, but only approx. 1.3×10^6 cells/g from cafeteria-fed-rats. It can be argued therefore that the reason why oxidative metabolism was not significantly increased in isolated brown adipocytes from cafeteria-fed rats was that most of the active brown adipocytes, perhaps as a result of a fragility problem, did not survive the vigorous isolation procedure. This criticism cannot be answered satisfactorily until these adipocytes can be produced in yields approaching 100%. Nevertheless, as our isolation procedure yielded adipocytes with nearly the same proportion of unilocular and multilocular cells as that found in the intact tissue, and the size distribution of the isolated cells was very similar to that of the adipocytes *in situ*, it would appear that this investigation was performed with an adipocyte population representative of intact interscapular brown adipose tissue. If this premise is correct, the total heat production of the intact interscapular brown-adipose-tissue depot of control rats (0.40 g) amounts to 5 mW in the basal state and 156 mW during hormonal stimulation (Table 1). The corresponding values for brown adipose tissue (0.94 g) from the cafeteria-fed animals are 5 and 146 mW respectively.

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