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Glucose metabolism in isolated brown adipocytes under β-adrenergic stimulation

Quantitative contribution of glucose to total thermogenesis

Dorothee ISLER, Hans-Peter HILL and Marcel K. MEIER
Department of Pharmaceutical Research, F. Hoffmann–La Roche and Co., Grenzacherstrasse 124, CH-4002 Basle, Switzerland

To quantify the potential of brown adipose tissue as a target organ for glucose oxidation, O_2 consumption and glucose metabolism in isolated rat brown adipocytes were measured in the presence and absence of insulin, by using the β-agonists isoprenaline or Ro 16-8714 to stimulate thermogenesis. Basal metabolic rate (278 μmol of O_2/h per g of lipid) was maximally stimulated with isoprenaline (20 nM) and Ro 16-8714 (20 μM) to 1633 and 1024 μmol of O_2/h per g respectively, whereas insulin had no effect on O_2 consumption. Total glucose uptake, derived from the sum of [U-^{14}C]glucose incorporation into CO_2 and total lipids and lactate release, was enhanced with insulin. Isoprenaline and Ro 16-8714 had no effect on insulin-induced glucose uptake, but promoted glucose oxidation while inhibiting insulin-dependent lipogenesis and lactate production. A maximal value for glucose oxidation was obtained under the combined action of Ro 16-8714 and insulin, which corresponded to an equivalent of 165 μmol of O_2/h per g of lipid. This makes it clear that glucose is a minor substrate for isolated brown adipocytes, fuelling thermogenesis by a maximum of 16%.

INTRODUCTION

Heat production in brown adipose tissue, the major thermogenic site in rodents, is elicited by the release of noradrenaline from sympathetic nerve terminals. Fatty acids released by β-adrenergic mechanisms are presumed to be the primary fuel (Bukowiecki, 1985). However, brown adipose tissue, known to be insulin-sensitive (Czech *et al.*, 1974), is also optimally equipped for glucose metabolism, by its high activity of glycolytic enzymes (Cooney & Newsholme, 1982) and its substantial capacity for fatty acid synthesis from glucose (McCormack & Denton, 1977; Trayhurn, 1979, 1981; McCormack, 1982). It has been postulated that brown adipose tissue may even play a role in glucose homoeostasis (Cooney & Newsholme, 1982; McCormack, 1982). Under conditions of activated thermogenesis by cold exposure, noradrenaline injection or treatment with β-adrenergic agonists, 2-deoxyglucose uptake was preferentially elevated in brown adipose tissue as compared with other tissues (Mercer & Trayhurn, 1984; Cooney *et al.*, 1985*a*,*b*; Young *et al.*, 1985). However, in none of these studies were the methods adequate to quantify glucose utilization, since correction factors for 2-deoxyglucose discrimination in glucose pathways were not assessed for different tissues. More direct evidence for glucose uptake being increased in brown adipose tissue after noradrenaline infusion came from two studies *in vivo* (Portet *et al.*, 1974; Ma & Foster, 1986). However, the fate of glucose within brown adipose tissue is at present not clear. β-Adrenergic agonists have potent anti-diabetic effects in hyperglycaemic rodents (Meier, 1982; Meier *et al.*, 1984; Carroll *et al.*, 1985). In *ob*/*ob* mice, isoprenaline and Ro 16-8714 decreased glucosuria and hyperglycaemia by an overall increase in glucose oxidation and produced a parallel activation of thermogenic function in brown adipose tissue (Meier, 1982; Isler *et al.*, 1984).

The purpose of this study was to quantify the contribution of glucose oxidation to total thermogenesis in brown adipose tissue. We therefore examined overall glucose metabolism and the thermogenic response in isolated brown adipocytes under β-adrenergic stimulation with isoprenaline and Ro 16-8714.

MATERIALS AND METHODS

Animals

Male albino rats (10–12 weeks old; 210–240 g) of the Füllinsdorf strain, maintained on chow diet (6% fat, 41% carbohydrate and 25% protein) and water *ad libitum*, were kept in pairs in wire-mesh cages in a room with 12 h light and 12 h dark cycles at 23 ± 1 °C for at least 5 days before being killed, which was done at 08:00 h.

Cells

Brown-fat cells were prepared from the interscapular tissues (2–3 g of cleaned tissue, collected from eight to twelve rats) by the method of Fain (1975) with minor modifications. Buffer used was Krebs–Ringer–Hepes (15 mM-$NaHCO_3$/10 mM-Hepes, pH 7.4) containing 5 mM-glucose and 4% (w/v) fatty-acid-free bovine serum albumin (Sigma no. A-6003). For digestion, 4 mg of collagenase (Cooper Biomedical no. 4196)/ml and 0.3 mg of trypsin inhibitor (Sigma no. T 9003)/ml were added to approx. 1 g of minced tissue/3 ml. This was gently stirred with a loosely fitting rod (50 shakes/min) for 30 min at 37 °C, under O_2/CO_2 (19:1). The digests were washed three times as described by Bukowiecki *et al.* (1980). The adipocytes were counted in a Neubauer's haemocytometer and stored under O_2/CO_2 (19:1) at about 10 mg of lipid/ml for 1–2 h at 22 °C. Before use, the cells were diluted further to their final concentration (1–3 mg of lipid/ml, about 1×10^5–3×10^5 cells/ml) with fresh buffer.

Incubation procedure

Preincubation (8 min) and incubation (60 min, if not indicated otherwise) of cells was performed under O_2/CO_2 (19:1) at 37 °C with constant shaking in silicone-treated glass Erlenmeyer flasks capped with rubber seals and equipped with disposable plastic wells hanging over the solution. The incubation volume was generally 2.5 ml for assessment of glucose metabolism and 3 ml for O_2-consumption measurements.

Determination of glucose metabolism

After preincubation, agents of interest and [U-^{14}C]-glucose were added to give a final specific radioactivity of 16 nCi/μmol (40 nCi/μmol for measurement of glucose incorporation into fatty acids). To absorb CO_2, 0.4 ml of 1 M-KOH was injected into the hanging plastic well, the reaction was immediately stopped by acidifying the medium with 0.15 ml of 2.3 M-HCl, and the vials were shaken for a further 90 min. Alkaline $K_2{}^{14}CO_3$ was counted directly for radioactivity in a Supertron (Kontron). Lactate was measured enzymically (Bachmann, 1978) after precipitation of a sample of cell suspension with trichloroacetic acid (final concn. 2%). Total lipids and fatty acids were extracted from further samples (Folch *et al.*, 1957; Stansbie *et al.*, 1976) and their ^{14}C incorporation was measured. Total lipids were determined gravimetrically. Calculation of glucose conversion into CO_2, total lipids and fatty acids was based on the initial specific radioactivity of the glucose in the medium and the quantity of label in the products. Lactate production was considered to result entirely from metabolism of exogenous glucose, since no glycogen could be detected in freshly isolated cells (Van Handel, 1965; Johnson & Fusaro, 1966).

Determination of O_2 consumption

Measurements were performed at the beginning and the end of the incubation period (60 min), with a Yellow Springs oxygen monitor Model 53. As a basis for calculation, buffer saturated with O_2/CO_2 (19:1) at 37 °C was considered to contain 0.95 μmol of O_2/ml.

Cell number, lipid content and cell concentration

On average, 1 g of lipid extract corresponded to 10^8 multilocular cells (unilocular cells, amounting to about 20% of the total, were disregarded). Initial experiments had shown that varying the cell concentration from 6×10^4 to 10^6 cells/ml (0.6–10 mg of lipid extract/ml) did not influence the cellular responsiveness with regard to O_2 consumption and glucose metabolism.

Statistical analysis

Means ± S.E.M. for n experiments are indicated. Each determination in an experiment was performed in duplicate. Statistical significance was assessed with a two-dimensional analysis of variance, including treatment and the experimental day as factors (Statistical Analysis System: SAS-Institute, Cary, NC, U.S.A.). Logarithms of the values were used for calculation. The α-error for multiple statistical comparison was controlled by the Bonferroni correction ($\alpha_{Bonferroni} = 0.05$/number of comparisons) (Miller, 1966).

Compounds

DL-Isoprenaline was purchased from Fluka (no. 59650), and insulin Actrapid MC (40 units/ml) from Novo. DL-Propranolol was kindly provided by ICI, Macclesfield, Cheshire, U.K. Ro 16-8714/002 {*p*-[(*R*)-3-{bis-[(*R*)-β-hydroxyphenethyl]amino}butylbenzamide maleate} was prepared in these laboratories by Dr. M. Müller.

RESULTS AND DISCUSSION

Effects of isoprenaline, Ro 16-8714 and insulin on O_2 consumption

Isoprenaline dose-dependently increased the metabolic rate of brown adipocytes (50% of maximal effect with 2.5 nM). The maximal rate, at 20 nM-isoprenaline (1633 ± 232 μmol of O_2/h per g of lipid), corresponded to a 6-fold increase above basal rates (278 ± 52 μmol/h per g). Ro 16-8714 is a partial β-adrenergic agonist. Its maximal effect, being 55% of the maximal stimulation caused by isoprenaline, was seen at 20–30 μM (1024 ± 39 μmol of O_2/h per g of lipid) (data summarized in Table 2).

Incubation with 80 μunits of insulin/ml had no effect on basal, isoprenaline- or Ro 16-8714-stimulated O_2 consumption (results not shown). Inhibitory effects of insulin on thermogenesis have previously been reported only at much higher insulin concentrations (Fain & Reed, 1970; Bukowiecki, 1985).

Effects of insulin, isoprenaline and Ro 16-8714 on glucose metabolism

Fig. 1 shows the time course of effects on glucose metabolism in brown-fat cells for insulin and isoprenaline at maximal thermogenic concentration (20 nM). Isoprenaline stimulated basal glucose conversion into CO_2 2-fold, leaving glucose incorporation into total lipids and lactate unaffected. Insulin alone stimulated glucose conversion into CO_2, lipids and lactate. The two agents showed an additive effect on glucose conversion into CO_2, whereas isoprenaline decreased insulin-induced lipogenesis and lactate accumulation.

Isoprenaline and Ro 16-8714 elicited essentially the same changes in glucose metabolism in the presence of insulin, when investigated at equally thermogenic concentrations (about 50% of the maximal thermogenic capacity; results not shown). Furthermore, incubations with 1 μM-DL-propranolol prevented all effects of isoprenaline and Ro 16-8714 (results not shown). Therefore the effects of both compounds are mediated by β-adrenergic receptors, and their similar effects on glucose utilization suggest a connection between the two activities.

Glucose incorporation into total lipids and fatty acids

Basal fatty acid synthesis was suppressed by isoprenaline, and insulin (80 μunits/ml)-stimulated fatty acid synthesis was dose-dependently decreased by isoprenaline and Ro 16-8714 (Table 1). The observed inhibition of fatty acid synthesis by both β-adrenergic agents is in line with investigations by Gibbins *et al.* (1985), which showed that acetyl-CoA carboxylase and fatty acid synthesis in brown adipose tissue were

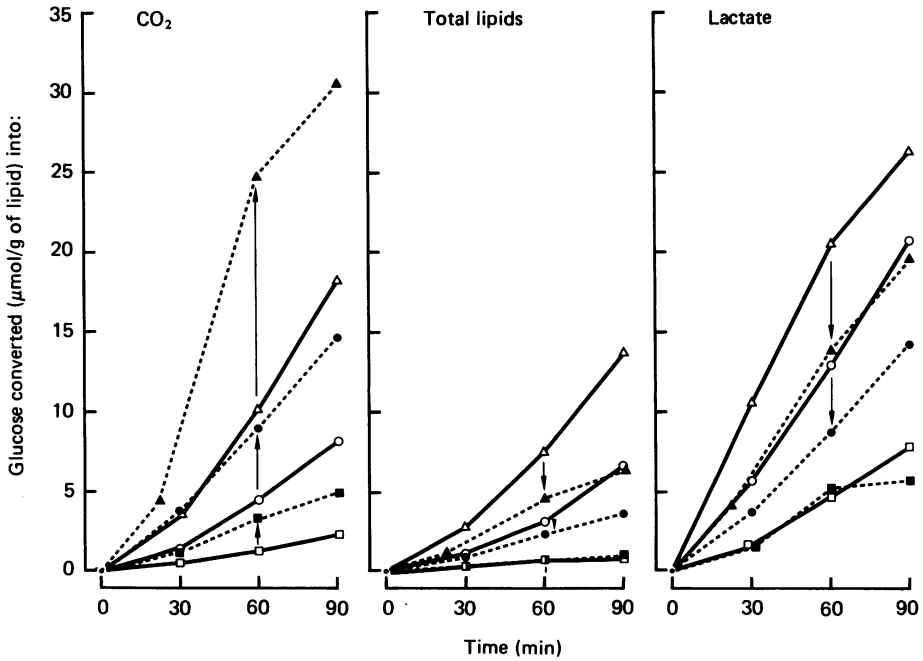

Fig. 1. Time course of the effects of isoprenaline (20 nM) on conversion of glucose into CO_2, total lipids and lactate in the presence and absence of insulin

Brown adipocytes were incubated for the time indicated: basal (□); + isoprenaline (20 nM) (■); +insulin (40 μunits/ml), without (○) and with (●) isoprenaline; +insulin (80 μunits/ml), without (△) and with (▲) isoprenaline. Arrows indicate the direction of isoprenaline action. The data are means of duplicates from a representative experiment.

inhibited by noradrenaline. In spite of the marked decrease in fatty acid synthesis, insulin-stimulated incorporation of labelled glucose into total lipids was decreased only at high isoprenaline concentrations (> 20 nM). However, it remained unchanged in the presence of Ro 16-8714 and lower doses of isoprenaline, indicating stimulated re-esterification of liberated fatty acids by glucose-derived α-glycerophosphate. The decrease in labelled acylglycerol glycerol at high isoprenaline concentrations may indicate a decreased rate of re-esterification or increased loss of labelled glycerol by cellular release or oxidation.

Effects of insulin, isoprenaline and Ro 16-8714 on glucose utilization and total glucose uptake

Whether in the presence or the absence of insulin, at their maximal activation of thermogenesis isoprenaline (20 nM) and Ro 16-8714 (20 μM) similarly stimulated glucose conversion into CO_2 (Table 2). However, the partial β-adrenergic agonist Ro 16-8714 elicited no significant inhibition of glucose incorporation into total lipids, in contrast with isoprenaline. Furthermore, the decrease in insulin-dependent lactate formation was smaller for Ro 16-8714. This decrease in lactate release may reflect increased oxidation of pyruvate, since β-adrenoceptor-mediated activation of pyruvate dehydrogenase has been demonstrated (Gibbins *et al.*, 1985).

CO_2, triacylglycerols and lactate are the main products of glucose metabolism. Hence an approximation to the total glucose uptake can be made by summing up the glucose converted into CO_2, lipids and lactate (Table 2). As the isolated cells were glycogen-depleted (see the Materials and methods section), all lactate must have been formed from added glucose. Pyruvate efflux was not measured, but makes only a small contribution, amounting at most to one-fifth of total lactate release (Kather *et al.*, 1972; Ma & Foster, 1986). The estimations in Table 2 show that the total glucose uptake in the presence and absence of insulin was not changed by the β-agonists isoprenaline and Ro 16-8714. In brown adipose tissue of cold-adapted rats, glucose uptake increases upon noradrenaline infusion, from 0.22 and 0.02 to 0.67 and 1.9 μmol/min per g of tissue in the studies of Portet *et al.* (1974) and Ma & Foster (1986) respectively. In our study, 1 g of brown adipose tissue corresponded to about 250 mg of cellular lipid extract. Hence the recalculated basal glucose uptake *in vitro* increases from 0.06 to a maximum of 0.25 μmol/min per g of tissue in the presence of insulin and Ro 16-8714. The data *in vitro* and *in vivo* on basal glucose uptake are in good agreement, but the maximal glucose uptake observed in isolated cells is definitely smaller than that *in vivo*. The difference may partly be explained by the fact that, in the studies *in vivo*, cold-adapted rats were used, which have increased glycolytic enzymes (Cooney & Newsholme, 1982) and therefore increased capacity for glucose metabolism. The failure of the β-adrenergic agonists to increase glucose uptake under basal conditions *in vitro* may be due to the absence of insulin (permissive effect). When combined with insulin (80 μunits/ml), the maximal capacity for glucose uptake may have already been reached with insulin alone.

In line with another study *in vitro* (Ebner *et al.*, 1986), we showed that β-agonists did not affect basal lactate production, but decreased insulin-induced lactate release.

Table 1. Effect of isoprenaline on the conversion of glucose into total lipids and fatty acids in the presence and absence of insulin: comparison with Ro 16-8714

Brown adipocytes were incubated as described in the Materials and methods section. Total lipid and fatty acid extractions were performed from samples of identical incubations assayed in duplicate. Results are means ± S.E.M. of n (3–5) individual experiments. Values in the same column with no common letters in their superscript differ significantly ($P < 0.05$) as assessed with two-dimensional analysis of variance after Bonferroni correction as described in the Materials and methods section.

Additions				Glucose converted (μmol/h per g of lipid) into:		Glucose conversion ratio (fatty acids/total lipids)
Insulin (80 μunits/ml)	Isoprenaline (nM)	Ro 16-8714 (μM)	n	Total lipids	Fatty acids	
–	–	–	5	1.51 ± 0.27^{a}	0.97 ± 0.15^{ae}	0.64 ± 0.02
–	20	–	3	1.34 ± 0.44^{a}	0.29 ± 0.75^{b}	0.22 ± 0.01
+	–	–	5	7.33 ± 0.78^{bc}	5.42 ± 0.60^{c}	0.74 ± 0.02
+	1	–	4	8.50 ± 1.03^{b}	5.35 ± 1.10^{c}	0.63 ± 0.06
+	5	–	5	6.84 ± 0.96^{bc}	2.05 ± 0.46^{d}	0.30 ± 0.03
+	20	–	5	4.60 ± 0.71^{bc}	0.94 ± 0.14^{ae}	0.20 ± 0.01
+	100	–	3	3.72 ± 1.20^{c}	0.82 ± 0.24^{a}	0.23 ± 0.01
+	–	2.5	3	7.33 ± 0.76^{bc}	3.82 ± 0.62^{cf}	0.52 ± 0.06
+	–	5.0	3	7.29 ± 1.57^{bc}	3.13 ± 0.89^{cdf}	0.43 ± 0.06
+	–	10.0	3	6.01 ± 1.32^{bc}	1.79 ± 0.49^{def}	0.30 ± 0.03
+	–	20.0	3	5.67 ± 1.38^{bc}	1.63 ± 0.44^{abd}	0.29 ± 0.02

Table 2. Effects of β-adrenergic agents and insulin on glucose metabolism and the contribution of glucose oxidation to overall thermogenesis

Brown adipocytes were treated and the statistics evaluated as described in the Materials and methods section. Results are means ± S.E.M. derived from eight individual experiments each in duplicate. Values in the same column with no common letters in their superscript differ significantly ($P < 0.05$, after Bonferroni correction). Values for glucose-derived fatty acids were calculated from glucose incorporation into total lipids by using the ratios of fatty acids/total lipids in Table 1. The following equations were used for further calculations:

$$9C_6H_{12}O_6 + 8O_2 \rightarrow 2C_{16}H_{32}O_2 + 22CO_2 + 22H_2O \quad (1)$$

$$C_6H_{12}O_6 + 6O_2 \rightarrow 6CO_2 + 6H_2O \quad (2)$$

Eqn. (1) gives the amount of CO_2 obligatorily formed by conversion of glucose into palmitate. Thus the carbon ratio for CO_2/fatty acids is 0.69. This value can be used for the estimation of glucose conversion into CO_2 owing to fatty acid synthesis. By subtracting this amount from the total $^{14}CO_2$, an assessment of the contribution of glucose oxidation to overall O_2 consumption can be achieved by using Eqn. (2). O_2 consumed during fatty acid synthesis (Eqn. 1) is negligible, and overall O_2 consumption was not corrected for.

Additions	n	Glucose converted (μmol/h per g of lipid) into:			Total glucose uptake (μmol/h per g of lipid)	O_2 consumed (μmol/h per g of lipid)	
		CO_2	Total lipid	Lactate		Total	Owing to glucose oxidation
Basal	8	3.0 ± 0.4^{a}	2.0 ± 0.3^{a}	9.5 ± 1.3^{a}	14.5 ± 2.0^{a}	278 ± 52^{a}	12.6 ± 1.7^{a} (4.5%)
Isoprenaline (20 nM)	8	6.6 ± 0.7^{b}	1.7 ± 0.3^{a}	9.2 ± 1.5^{a}	17.5 ± 2.3^{a}	1633 ± 232^{b}	37.8 ± 4.2^{b} (2.3%)
Ro16-8714 (20 μM)	3	5.5 ± 0.5^{b}	1.8 ± 0.03^{a}	8.2 ± 1.5^{a}	15.5 ± 1.5^{a}	1024 ± 39^{b}	–
Insulin (80 μunits/ml)	8	14.5 ± 1.3^{c}	13.4 ± 1.6^{b}	26.5 ± 3.1^{b}	54.4 ± 5.5^{b}	278 ± 52^{a}	46.2 ± 3.7^{b} (16.6%)
Insulin + isoprenaline	8	23.7 ± 1.6^{d}	7.3 ± 0.9^{c}	14.3 ± 1.4^{c}	45.3 ± 3.2^{b}	1633 ± 232^{b}	136.2 ± 9.2^{c} (8.3%)
Insulin + Ro 16-8714	3	29.9 ± 2.7^{d}	12.6 ± 1.8^{bc}	17.4 ± 4.6^{bc}	59.9 ± 1.6^{b}	1024 ± 39^{b}	165.0 ± 14.1^{c} (16.1%)

In contrast, in Ma & Foster's (1986) study *in vivo*, more than two-thirds of the noradrenaline-stimulated glucose uptake was accounted for by increased lactate formation. Lactate produced *in vivo* may, however, have resulted in part from noradrenaline-stimulated glycogenolysis (Gibbins & Denton, 1986), whereas isolated cells used *in vitro* were glycogen-depleted.

Contribution of glucose oxidation to overall thermogenesis

The reductive synthesis of fatty acids from glucose is obligatory paralleled by glucose conversion into CO_2 and O_2 consumption, according to the general equation given in the legend to Table 2. In order to estimate the quantitative contribution of glucose oxidation to overall

O_2 consumption, total glucose conversion into CO_2 must be corrected for CO_2 produced during fatty acid synthesis (Table 2).

Under basal conditions (5 mM-glucose), glucose contributed 4.5% of the total metabolic rate (Table 2), and this proportion was increased to 16.6% by insulin (80 μunits/ml). Isoprenaline increased both the basal and the insulin-stimulated glucose oxidation, but decreased glucose fuel contribution to overall oxidation 2-fold. Ro 16-8714 increased glucose oxidation over that observed with insulin alone, but did not further increase the percentage glucose contribution to overall thermogenesis. Therefore, even in the presence of insulin, thermogenesis in brown adipocytes is mainly fuelled by fatty acids. Our data show that, in the presence of 80 μunits of insulin/ml alone, glucose oxidation accounts for one-sixth of total O_2 consumption, and that glucose is also incorporated into fatty acids. Under conditions *in vivo*, the carbohydrate may be oxidized either directly or via lipogenic pathways, followed by catabolism of newly synthesized fatty acids. Both pathways thereby contribute to overall glucose oxidation. Hence, under these conditions glucose may become an important substrate for basal energy requirement.

Treatment of *ob/ob* mice with the β-adrenergic agonists isoprenaline and Ro 16-8714 decreased urinary glucose excretion to virtually zero, and normalized blood glucose concentrations (Meier, 1982; Meier *et al.*, 1984). Since these effects could be attributed to stimulation of glucose oxidation (Isler *et al.*, 1984), it was of interest to estimate whether brown adipose tissue may have contributed significantly. After 1 day of treatment with Ro 16-8714, the obese mice had increased their overall glucose oxidation by about 1 g/day per mouse. Interscapular and subscapular tissue of these mice amounted to 200 mg, which would correspond to a total tissue mass of maximally 1 g (Thurlby & Trayhurn, 1980). The maximal glucose oxidation in brown adipocytes isolated from rats was 27.5 μmol/h per g of lipid in the presence of insulin and Ro 16-8714 (165 μmol of O_2 consumed owing to glucose oxidation, divided by 6; Table 2), which corresponds to 30 mg of glucose/day per g of tissue (250 mg of cellular lipid extract/g of tissue). With reservation, and assuming that no major species difference exists between brown adipose tissue of mice and rats, then glucose oxidation by brown adipose tissue can contribute only minimally to the overall anti-diabetic effect of β-adrenergic agents in obese mice.

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