Changes in uncoupling protein and GLUT4 glucose transporter expressions in interscapular brown adipose tissue of diabetic rats: relative roles of hyperglycaemia and hypoinsulinemia

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We have studied the time course and relative effects of hypoinsulinemia and hyperglycaemia on concentrations of uncoupling protein (UCP) and glucose transporter (GLUT4) and their mRNAs in brown adipose tissue (BAT) during the early phase of diabetes induced by streptozotocin. Two days after intravenous injection of streptozotocin, plasma insulin concentration was at its lowest and glycaemia was higher than 22 mmol/l. After 3 days, a 60% decrease in BAT UCP mRNA concentration and a 36% decrease in UCP was observed. Concomitantly, there was an 80% decrease in GLUT4 mRNA and a 44% decrease in GLUT4 levels. When hyperglycaemia was prevented by infusing phlorizin into diabetic rats, BAT UCP mRNA and protein levels were further decreased (respectively 90% and 60%, lower than in control rats). In contrast, the marked decreases in GLUT4 mRNA and protein concentrations in BAT were similar in hyperglycaemic and normoglycaemic diabetic rats. Infusion of physiological amounts of insulin restored normoglycaemia in diabetic rats, and BAT UCP and GLUT4 mRNA and protein concentrations were maintained at the level of control rats. When insulin infusion was stopped, a 75% decrease in BAT UCP mRNA level and a 75% decrease in GLUT4 mRNA level were observed after 24 h, but UCP and GLUT4 concentrations did not decrease. This study shows that insulin plays an important role in the regulation of UCP and GLUT4 mRNA and protein concentrations in BAT. Hyperglycaemia partially prevents the rapid decrease in concentration of UCP and its mRNA observed in insulinopenic diabetes whereas it did not affect the decrease in GLUT4 mRNA and protein concentration. It is suggested that UCP is produced by a glucose-dependent gene.

INTRODUCTION

Brown adipose tissue (BAT) is specialized in heat production (Himms-Hagen, 1990). A 33 kDa protein, called uncoupling protein (UCP), is the key element of BAT thermogenic activity. This protein is uniquely expressed in the inner membrane of mitochondria of BAT where it dissipates the proton gradient generated by the respiratory chain (Nichols and Locke, 1984; Cannon and Nedergaard, 1985; Klaus et al., 1991b). In BAT, energy is essentially provided by the oxidation of non-esterified fatty acids (Nichols and Locke, 1984) and to a lesser extent by the oxidation of glucose (Ferre et al., 1985; Young et al., 1985). BAT is activated upon exposure of animals to cold or during hyperphagia (diet-induced thermogenesis). The major mechanism for activation of BAT is the sympathetic nervous system. Noradrenaline release at the nerve endings stimulates BAT lipolysis and fatty acid oxidation via β-adrenergic receptors (Girardier, 1983). Insulin has also been postulated to be a factor controlling thermogenesis and UCP synthesis in BAT (Jamal and Saggerson, 1988; Rothwell and Stock, 1988; Géloën and Trayhurn, 1990a). In diabetic rats, cold tolerance and noradrenaline-stimulated thermogenesis are markedly impaired (Poe and Davis, 1962), and this is due to a decreased concentration of UCP or GDP-binding sites in BAT (Seydoux et al., 1984; Jamal and Saggerson, 1988; Géloën and Trayhurn, 1990a). The maintenance of normal concentrations of UCP or GDP-binding sites in BAT has been shown to require intact sympathetic activity (Young et al., 1982; Yoshida et al., 1985) and normal plasma insulin levels (Géloën and Trayhurn, 1990a,b). However, these studies did not determine if insulin controlled BAT UCP at the transcriptional or post-transcriptional level. The aim of the present study was thus to investigate the time course of the effect of streptozotocin-induced diabetes on UCP mRNA concentration in BAT as well as the relative contributions of hyperglycaemia and hypoinsulinemia.

MATERIALS AND METHODS

Experimental procedures

Male Wistar rats (250 g) (IFFA Credo, L’arbresle, France) were housed at 24 °C with light from 07:00 to 19:00 h. They had free access to water and chow pellets (energy contributions: carbohydrate 65%, fat 11%, protein 24%). The studies were performed in conscious rats, 24 h after implantation of catheters into the two jugular veins, under pentobarbital anaesthesia (50 mg/kg body weight). Catheters were externalized on the back of the neck through a skin incision and anchored to the skull with a dental cement cap (Pennwald; Philadelphia, PA, U.S.A.). Immediately after surgery, rats were randomly distributed into five different groups and housed in individual cages.

Group 1 rats had free access to food and were killed at 08.00 h at day zero. This group was termed ‘fed’ control rats.

Group 2 rats were injected with streptozotocin (STZ; Sigma, St. Louis, MO, U.S.A.) through the jugular vein, at a dose of 65 mg/kg body weight (STZ was dissolved extemporaneously in cold 0.1 M sodium citrate buffer, pH 4.5). They were killed at 48 and 72 h after the STZ injection. They had free access to food during the experimental period. All rats injected with STZ became diabetic within 24 h. In order to check that diabetic rats were unable to secrete insulin, glucose (200 mg/100 g body weight) was injected through the left jugular vein 30 h after STZ

Abbreviations used: BAT, brown adipose tissue; UCP, uncoupling protein; STZ, streptozotocin; GLUT4, glucose transporter.
injection. Blood (300 μl) was sampled from the right jugular vein before and 5 min after glucose injection. Blood was immediately centrifuged and plasma was frozen until used for glucose and insulin determinations. Plasma insulin concentration in all diabetic rats remained at the low basal value despite very high plasma glucose concentration (22 mmol/l) (results not shown). This group was termed ‘diabetic’ rats.

Group 3 were a group of STZ-diabetic rats infused through the jugular vein with phlorizin (Sigma) at variable rates: 0.5-2.0 g/day per kg, from 18 h after STZ injection to 72 h. By inhibiting glucose reabsorption from the renal tubule, phlorizin normalized blood glucose level in diabetic rats, without altering plasma insulin concentration. These rats had free access to food during the experimental period. This group was termed ‘phlorizin-treated diabetic’ rats.

Group 4 were fed non-diabetic rats infused intravenously with phlorizin or vehicle (propylene glycol) as in group 3.

Group 5 were a group of STZ diabetic rats infused intravenously with insulin (Actrapid, Novo, Copenhagen, Denmark) at a constant rate: 0.25 unit/h per kg, from 18 h after STZ injection to 48 h, to maintain their blood glucose level in the normal range. They had free access to food during the experimental period. This group was termed ‘insulin-treated diabetic’ rats. In some of these insulin-treated diabetic rats, insulin infusion was stopped after 48 h. The rats were killed 6 or 24 h after insulin withdrawal.

Monitoring of blood glucose and plasma insulin concentrations

Blood glucose and plasma insulin were monitored daily throughout the experimental period in rats from groups 2 to 5. Blood glucose was routinely monitored by the glucose oxidase method using a Beckman glucose analyser (Beckman BGA2, Gallway, Ireland). Plasma insulin concentration was determined by radioimmunoassay as previously described (Girard et al., 1973).

Killing of animals and tissue sampling

Rats were anaesthetized by an intraperitoneal injection of pentobarbital (60 mg/kg body weight). Blood was sampled from the jugular vein and collected in the presence of 2000 units of aprotinin/ml of blood and EDTA (0.01 M). Blood was immediately centrifuged at 4 °C and the plasma frozen at −20 °C until insulin determination. After blood sampling, the interscapular BAT was rapidly removed, rinsed in 0.9 % NaCl, wiped and frozen at −80 °C.

Quantification of UCP and GLUT4 mRNAs

Total RNA was isolated from BAT using the guanidine thiocyanate method (Chomczynski and Sacchi, 1987). RNA concentration was determined by absorbance at 260 nm. All samples had a 260/280 absorbance ratio of about 2.0. For Northern-blot analysis, total RNA (20 μg) was denaturated in a solution containing 2.2 mM formaldehyde and 30 % (v/v) formamide by heating at 95 °C for 2 min, size-fractionated by electrophoresis and transferred to a nylon membrane (Hybond N, Amersham, Bucks., U.K.). The cDNA of rat GLUT4 mRNA was a 2.0 kb insert into the EcoRI site of plasmid Bluescript (James et al., 1989) and the pUCP36 cDNA of rat uncoupling protein mRNA was a 1.9 kb insert into the PsI site of plasmid pBR327 (Bouillaud et al., 1985). Probes were labelled with 32P[dCTP] using the Multiprime labelling system kit (Amersham, Bucks., U.K.). Hybridizations were performed in solutions containing 42 %, deionized formamide, 7.5 % dextran sulphate, 8 × Denhardt’s solution, 40 mM Tris/HCl, pH 7.5, 4 × SSC (1 × SSC = 0.15 M NaCl/15 mM trisodium citrate, pH 7.0), 0.4 % sodium pyrophosphate and 1 % SDS at 42 °C overnight. The membranes were washed twice for 30 min each, with 2 × SSC/0.1 % SDS at 42 °C and twice for 30 min each with 0.1 × SSC/0.1 % SDS at 55 °C and autoradiographed for 4-72 h at −80 °C with intensifying screens. Quantification of autoradiographs was performed by scanning densitometry using a densitometer (Hoeffer Scientific Instruments). Standardization of Northern blots was performed using an oligonucleotide representing the sequence 1047–1070 of rat 18S rRNA (Chan et al., 1984), labelled with 32P]ATP by bacteriophage T4 polymerase kinase, to verify the integrity of RNA and that each lane was loaded with the same amount of total RNA. Results of the UCP and GLUT4 probe analyses were corrected for RNA added by dividing by the 18S RNA values.

Quantification of UCP and GLUT4 by immunoblotting

BAT was homogenized in 10 vol. of Tris/EDTA/sucrose, pH 7.4. The proteins were quantified using the Bio-Rad assay (Munich, Germany) with BSA as standard. Homogenates (25 μg) were submitted to slot-blotting (nitrocellulose membrane, Schleicher & Schuell, Dassel, Germany) using a polyclonal antibody against GLUT4 (East-Acres, Southbridge, MA, U.S.A.) and a polyclonal antibody against UCP (Ricquier et al., 1983). Detection of GLUT4 was performed with 0.2 μCi/ml 125I-Protein A (Amersham), followed by autoradiography. Quantification was performed with scanning autoradiographs using a densitometer. Detection of UCP was achieved by alkaline phosphatase-linked anti-sheep IgG (Bio-Rad) reacting with 5-bromo-4-chloro-3-indolyl phosphate and p-NitroBlue Tetrazolium chloride (Bio-Rad). Quantification was performed with scanning blots using a reflectancemeter (Hoeffer Scientific Instruments).

Statistical analysis

Results are expressed as means ± S.E.M. Statistical analysis was performed by Student’s t test for unpaired data.

RESULTS AND DISCUSSION

The present study was undertaken (1) to analyse the time course of the changes in UCP mRNA concentration in BAT of diabetic rats and (2) to assess the relative effects of hypoinsulinaemia and hyperglycaemia on this parameter. The changes in GLUT4 mRNA were followed in parallel as a control of insulin action.

Changes in blood glucose and plasma insulin concentrations

Diabetic rats

After STZ injection, a typical hyperglycaemia and hypoinsulinaemia appeared from 30 h onwards (Table 1).

Phlorizin-treated diabetic rats

In phlorizin-treated diabetic rats, the blood glucose concentration was maintained at a mean value of 7.2 mmol/l from the 18th hour onwards (Table 1). Plasma insulin concentration in phlorizin-treated diabetic rats was not statistically different from the untreated diabetic rats.
### Table 1  Changes in blood glucose and plasma insulin after injection of STZ

Values are means ± S.E.M. The number of rats in each group is indicated in parentheses. Some non-diabetic rats were infused with phlorizin for 3 days (Controls + Phi). Some diabetic rats were infused with either phlorizin (STZ + Phi) from 18 to 72 h or insulin (STZ + Ins) from 18 to 48 h. *Difference statistically significant (P < 0.05) when compared with fed non-diabetic rats. †Difference statistically significant (P < 0.05) when compared with non-treated diabetic rats.

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Blood glucose (mmol/l)</th>
<th>Plasma insulin (µunits/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls (6)</td>
<td>6.1 ± 0.1</td>
<td>25 ± 3</td>
</tr>
<tr>
<td>Controls + Phi (4)</td>
<td>5.5 ± 0.2</td>
<td>18 ± 4</td>
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<tr>
<td>STZ injection</td>
<td></td>
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<tr>
<td>48 h (5)</td>
<td>22.8 ± 2.8*</td>
<td>&lt; 5*</td>
</tr>
<tr>
<td>72 h (6)</td>
<td>25.0 ± 2.3*</td>
<td>&lt; 5*</td>
</tr>
<tr>
<td>STZ + Phi (6)</td>
<td>7.2 ± 0.4†</td>
<td>&lt; 5</td>
</tr>
<tr>
<td>STZ + Ins (6)</td>
<td>6.6 ± 0.4†</td>
<td>45 ± 5†</td>
</tr>
<tr>
<td>Insulin withdrawal</td>
<td></td>
<td></td>
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<tr>
<td>6 h (5)</td>
<td>22.1 ± 1.9</td>
<td>&lt; 5</td>
</tr>
<tr>
<td>24 h (4)</td>
<td>25.2 ± 2.2</td>
<td>&lt; 5</td>
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</table>

### Table 2  Quantification of UCP and GLUT4 mRNA concentrations in BAT of control and diabetic rats

The mRNA was quantified by scanning densitometry and normalized against the 18S rRNA, as described in the Materials and Methods section. Results are expressed as % of control values and are means ± S.E.M. for the number of rats indicated in parentheses. Some diabetic rats were infused with either phlorizin (STZ + Phi) from 18 to 72 h or insulin (STZ + Ins) from 18 to 48 h, and compared with controls or 48 h or 72 h non-treated diabetic rats. Some insulin-infused diabetic rats had their insulin infusion stopped after 48 h. They were studied 24 h after insulin withdrawal and compared with insulin-infused diabetic rats (STZ + Ins). *Difference statistically significant (P < 0.05) when compared with fed non-diabetic rats. †Difference statistically significant (P < 0.05) when compared with non-treated diabetic rats. ‡Difference statistically significant (P < 0.05) when compared with insulin-treated diabetic rats.

<table>
<thead>
<tr>
<th>Conditions</th>
<th>UCP mRNA (%) of control</th>
<th>GLUT4 mRNA (%) of control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls (6)</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>STZ injection</td>
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<tr>
<td>48 h (4)</td>
<td>51 ± 10*</td>
<td>25 ± 9*</td>
</tr>
<tr>
<td>72 h (6)</td>
<td>40 ± 8*</td>
<td>20 ± 5*</td>
</tr>
<tr>
<td>STZ + Phi (6)</td>
<td>10 ± 2†</td>
<td>17 ± 8*</td>
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<tr>
<td>STZ + Ins (6)</td>
<td>110 ± 11†</td>
<td>103 ± 7†</td>
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<tr>
<td>Insulin withdrawal</td>
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<td></td>
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<tr>
<td>6 h (5)</td>
<td>97 ± 9</td>
<td>95 ± 11</td>
</tr>
<tr>
<td>24 h (4)</td>
<td>25 ± 11‡</td>
<td>30 ± 9‡</td>
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Phlorizin-treated non-diabetic rats

After 3 days of phlorizin infusion, blood glucose concentration was 5.5 mmol/l and plasma insulin concentration was 18 µunits/ml (Table 1). These values were not statistically different from those measured in control rats.

Insulin-treated diabetic rats

Blood glucose concentration in diabetic rats was maintained between 18 and 48 h at a mean value of 6.6 mmol/l by using an insulin infusion rate of 0.25 unit/h/kg. The mean plasma insulin concentration reached 45 µunits/ml, a value close to physiological values (Table 1). When insulin infusion was stopped, a rapid decrease in plasma insulin concentration was observed and a marked hyperglycaemia developed after 1 h (Table 1). The rapid disappearance of plasma insulin allowed us to study short-term effects of insulin deprivation and hyperglycaemia on UCP and GLUT4 mRNA and protein concentrations.

### Changes in UCP and GLUT4 mRNA and protein concentrations

Fed non-diabetic rats were used as controls. UCP and GLUT4 mRNA (Table 2) and protein (Table 3) concentrations in control rats were taken as 100%.

Diabetes induced by STZ resulted in a 60% decrease in UCP mRNA concentration after 72 h with a decrease of 80% in GLUT4 mRNA in BAT (Figure 1). UCP and GLUT4 concentrations were decreased by respectively 36% and 44% (Figure 2). Previous studies in rats and mice have shown that
They were insulin-infused diabetic rats had their insulin infusion stopped after 48 h (Ins withdrawal). They were studied 24 h after insulin withdrawal and compared with insulin-infused diabetic rats (STZ + Ins).

Some diabetic rats were infused with insulin (STZ + Ins) from 18 to 48 h. Some insulin-infused diabetic rats had their insulin infusion stopped after 48 h. They were studied 6–24 h after insulin withdrawal and compared with insulin-infused diabetic rats (STZ + Ins).

Some diabetic rats were infused with either phlorizin (STZ + Phl) from 18 to 72 h or insulin (STZ + Ins) from 18 to 48 h, and compared with controls (C) or non-treated diabetic rats (STZ). Some insulin-infused diabetic rats had their insulin infusion stopped after 48 h (Ins withdrawal). They were studied 24 h after insulin withdrawal and compared with insulin-infused diabetic rats (STZ + Ins).

After 10–12 days of diabetes the concentration of UCP and the number of GDP-binding sites was decreased by 35–80% in BAT (Seydoux et al., 1984; Jamal and Saggerson, 1988; Göloën and Trayhurn, 1990a). Our study shows that a large decrease in UCP mRNA and protein concentrations was observed for only 2 days of hypoinsulinaemia and could contribute to the impairment of the BAT function. When a physiological dose of insulin was infused into diabetic rats (maintaining a normal plasma insulin of 45 μunits/ml) (Figures 1 and 2), the decrease in UCP mRNA and protein concentration was prevented. Previous observations have shown that UCP concentration in BAT was normalized by infusion of increasing amounts of insulin in diabetic mice and rats (Géloën and Trayhurn, 1990a,b). The precise time course of UCP mRNA concentration during hypoinsulinaemia in vivo was investigated after insulin treatment of diabetic rats was stopped. UCP and GLUT4 mRNA concentrations were not significantly decreased 6 h after insulin infusion was stopped, but after 24 h a reduction of 75% was observed (Figure 3). UCP and GLUT4 concentrations had not changed 24 h after insulin withdrawal (Figure 2). Recent in vitro experiments have also emphasized the direct role of insulin on the expression of the UCP gene in adipocytes differentiated from interscapular brown preadipocytes of Phodopus sungorus (Klaus et al., 1991a) and in brown fat tumour cells derived from a transgenic mouse (Kozak et al., 1992).

However, it was difficult to investigate the separate roles of hypoinsulinaemia and hyperglycaemia on UCP gene expression in vivo in untreated and insulin-treated diabetic rats. The specific role of hyperglycaemia has been studied by infusing phlorizin into diabetic rats. Surprisingly, the decrease in UCP mRNA and protein concentrations in BAT was more pronounced (respectively –90% and –60%) in diabetic rats that had had their glycaemia normalized by phlorizin treatment than in untreated diabetic rats (respectively –60% and –36%). In contrast, the marked decreases in GLUT4 mRNA and protein concentrations in BAT were similar in hyperglycaemic and normoglycaemic diabetic rats (Figures 1 and 2). This suggests that hyperglycaemia had a specific effect on UCP mRNA and protein concentrations in BAT. It is noteworthy that phlorizin infusion in non-diabetic rats did not change UCP and GLUT4 mRNA concentrations significantly compared with control rats (results not shown). Thus phlorizin per se was not responsible for the dramatic decrease (–90%) in UCP mRNA in BAT of phlorizin-treated diabetic rats. It has been reported that hyperglycaemia can directly increase or potentiate the effect of insulin on the expression of a number of hepatic genes. Liver GLUT2 gene expression is increased by hyperglycaemia in vivo and in vitro (Oka et al., 1990; Asano et al., 1992; Burcelin et al., 1992), and hyperglycaemia, in the presence of insulin, increases the expression of L-pyruvate kinase (Decaux et al., 1991), aldolase B (Munnich et al., 1985) and S14 gene (Goto and Mariash, 1992). The present study shows that BAT UCP is another glucose-dependent gene. The 5′-flanking regions of L-pyruvate kinase and S14 genes have been shown to contain a regulatory sequence of 10 nucleotides responsible for activation in response to elevated glucose metabolism (Thompson and Towle, 1991; Shih and Towle, 1992). Four segments (−1625 to −1615, −1566 to −1556, −866 to −856, −795 to −785) with 80% sequence similarity to the carbohydrate-response element of S14 and L-pyruvate kinase genes have been found in the 5′-flanking region of UCP gene (Bouillaud et al., 1988).

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REFERENCES
Uncoupling protein and GLUT4 and their mRNAs in brown adipose tissue


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