Glucose transporter expression and glucose utilization in skeletal muscle and brown adipose tissue during starvation and re-feeding

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Starvation (48 h) decreased the concentration of mRNA of the insulin-responsive glucose transporter isoform (GLUT 4) in interscapular brown adipose tissue (IBAT) (56%) and tibialis anterior (10%). Despite dramatic [7-fold (tibialis anterior) and 40-fold (IBAT)] increases in glucose utilization after 2 and 4 h of chow re-feeding, no significant changes in GLUT 4 mRNA concentration were observed in these tissues over this re-feeding period. The results exclude changes in GLUT 4 mRNA concentration in mediating the responses of glucose transport in these tissues to acute re-feeding after prolonged starvation.

INTRODUCTION

The cellular uptake of glucose is facilitated by a family of glucose transporter isoforms, the expression of which is tissue-specific (Bell *et al.*, 1990). Two glucose transporter isoforms have been identified in adipose tissue and muscle. The GLUT 1 isoform accounts for basal (constitutive) uptake, whereas the insulin-regulated glucose transporter isoform (GLUT 4) is responsible for insulin-stimulated glucose uptake. GLUT 4 is the major isoform expressed in these tissues (Koranyi *et al.*, 1990; Kahn *et al.*, 1991). Both glucose utilization (Issad *et al.*, 1987; Holness *et al.*, 1991) and GLUT 4 mRNA (Kahn *et al.*, 1989) have been reported to decrease in adipose tissue during fasting, but, paradoxically, the expression of GLUT 4 mRNA in skeletal muscle does not appear to change in parallel with the changes in glucose utilization observed *in vivo*: Northern blotting has revealed a 3-fold increase of GLUT 4 mRNA in mixed hindlimb muscle from rats subjected to prolonged (48–96 h) starvation (Charron & Kahn, 1990). The interpretation of the latter results, obtained with RNA from a hindlimb preparation containing muscles of different fibre composition, may be complicated by the considerable heterogeneity in the responses of glucose utilization by individual skeletal muscles to starvation and re-feeding (Issad *et al.*, 1987; Sugden *et al.*, 1990; Holness & Sugden, 1991). Glucose utilization by oxidative working muscles reflects not only the insulin status, but also their energy requirements and the availability of alternative energy substrates to glucose: their rates of glucose utilization are high in the fed state, decline dramatically with starvation [in which state lipid fuels are preferentially utilized (see Holness & Sugden, 1990)] and are rapidly restored to fed values by re-feeding. In contrast, rates of glucose utilization by non-working muscles are low in the fed state, only slightly decreased by 48 h starvation, but substantially increased to values greatly exceeding the fed value within 2–4 h of re-feeding (Sugden *et al.*, 1990). The augmentation of glucose uptake and phosphorylation by non-working muscles observed after re-feeding is thought to be a direct consequence of increased utilization of glucose 6-phosphate for glycogen synthesis (reviewed by Felber *et al.*, 1988; Sugden & Holness, 1990). Thus the maintenance of a relatively high glycogen concentration (which inhibits glycogen synthase) limits the extent to which glucose

utilization can be stimulated by an increase in insulin (Thiebaud *et al.*, 1982; Holness & Sugden, 1991).

The present study was undertaken to evaluate the regulatory importance of changes in GLUT 4 mRNA abundance in directing the concerted changes in glucose uptake and phosphorylation induced *in vivo* by prolonged (48 h) starvation and acute (2–4 h) chow re-feeding in skeletal muscle and adipose tissue. We have selected for study a non-working skeletal muscle (tibialis anterior), since its glucose utilization rate correlates with changes in insulin-stimulated glucose disposal as glycogen (Holness & Sugden, 1991). Changes in GLUT 4 mRNA were also studied in interscapular brown adipose tissue (IBAT). IBAT exhibits rates of insulin-stimulated glucose disposal which are considerably higher than those of white adipose tissue in the fed state (Cooney & Newsholme, 1982; Ferré *et al.*, 1986) and, whereas glucose utilization by white adipose tissue is only modestly decreased by starvation (Issad *et al.*, 1987), glucose utilization by IBAT is quite substantially decreased (Holness *et al.*, 1991). In addition, re-feeding after prolonged starvation results in rates of glucose utilization by IBAT which greatly exceed (on a wet-weight basis) those of any other tissue of the body (Holness *et al.*, 1991).

MATERIALS AND METHODS

Materials

Sources of materials for measurements of glucose utilization are given in Holness *et al.* (1988) and Holness & Sugden (1990). Hybond N nylon membranes and [α - 32 P]dATP were obtained from Amersham International (U.K.). Terminal deoxynucleotidyltransferase was obtained from Boehringer Mannheim (U.K.). Oligonucleotides were custom-synthesized by Oswel DNA Services, Edinburgh, U.K. All other chemicals were of molecular-biology grade.

Experimental procedures

Rats. Female albino Wistar rats (200–250 g) on a 12 h-light/12 h-dark cycle were either fed *ad libitum* on a standard rodent diet (supplied by Special Diets Services, Witham, Essex, U.K.; 52% digestible carbohydrate, 15% protein, 3% lipid and 30% non-digestible residue, all by weight), starved for 48 h (food

Abbreviations used: GLUT 4, the insulin-responsive glucose transporter isoform; IBAT, interscapular brown adipose tissue; GUI, glucose utilization index.

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removal at the end of the dark phase of the light/dark cycle), or starved for 48 h and then re-fed on standard rodent diet *ad libitum* for 2 or 4 h. Experiments with rats with continuous access to food *ad libitum* (termed fed *ad libitum*) were completed before 11:00 h, i.e. within 2.5 h of the end of the dark (feeding) period.

Measurement of glucose utilization index (GUI). GUI values were estimated by using 2-deoxy[^3H]glucose as described by Ferré *et al.* (1985). The study of changes in GUI values in tibialis anterior and IBAT *in vivo* was conducted using conscious unrestrained rats, each fitted with an indwelling cannula (see Issad *et al.*, 1987) at least 5 days before the experiment. Details of the experimental procedures involved in the measurements of GUI values and blood glucose concentrations are given in Sugden *et al.* (1990). GUI values were calculated as described by Issad *et al.* (1987) by dividing the radioactivity (d.p.m.) of tissue 2-deoxy[^3H]glucose 6-phosphate by the calculated integral of blood 2-deoxy[^3H]glucose/[glucose]. Mean coefficients of variance of blood glucose concentrations over each 1 h period of sampling were < 15% in each instance (see Sugden *et al.*, 1990). As in Issad *et al.* (1987), values were not corrected for the discrimination factor (lumped constant) for deoxyglucose in glucose metabolic pathways (Ferré *et al.*, 1986). Times of starvation and re-feeding given in relation to measurement of values of GUI refer to the time of injection of 2-deoxy[^3H]glucose, since most uptake of radiolabel occurs during the first 20 min after injection (Sokoloff *et al.*, 1977; James *et al.*, 1985).

Northern-blot analysis. Tissues were freeze-clamped in liquid N_2 and stored at -70°C for subsequent RNA extraction. Total cellular RNA was extracted from approx. 1 g of pooled tissue by the acid guanidinium thiocyanate/phenol/chloroform method (Chomczynski & Sacchi, 1987). Typically tissues from four animals were pooled. RNA concentration was determined from its A_{260} . RNA (40 μg) was denatured and size-separated by electrophoresis on a Mops/formaldehyde/1% agarose denaturing gel (Maniatis *et al.*, 1982). RNA was transferred to a nylon membrane and fixed by baking for 2 h at 80°C . The oligonucleotide probe for GLUT 4 [a 43-base oligonucleotide corresponding to bases 1564–1606 in the sequence given by Birnbaum (1989) and containing about 50% G+C bases] was labelled with [α - ^{32}P]dATP by using terminal deoxynucleotidyl-transferase (Roychoudhury & Wu, 1980). Hybridization was carried out for 16 h at 55°C in a solution consisting of $5\times\text{SSC}$ ($1\times\text{SSC} = 0.15\text{ M-NaCl}/0.015\text{ M-sodium citrate}$, pH 7.0), $20\times\text{Denhardt's solution}$ ($1\times\text{Denhardt's solution} = 0.01\%$ Ficoll/0.1% BSA/0.01% polyvinylpyrrolidone), 100 μg of denatured sonicated herring sperm DNA, 20 mM-sodium phosphate, pH 6.8, 10% (w/v) dextran sulphate, 2 ng of labelled oligonucleotide/ml and 7% (v/v) SDS. Membranes were washed for $3\times 20\text{ min}$ at 55°C in $3\times\text{SSC}/0.01\text{ M-sodium phosphate}$ (pH 6.8)/ $10\times\text{Denhardt's}/5\%$ SDS, and then twice at 22°C for 3 min with $1\times\text{SSC}/1\%$ SDS. Finally, two stringent washes, each of 30 min at 60°C with $1\times\text{SSC}/1\%$ SDS, were always used.

Signal detection and quantification. Washed membranes were exposed to Kodak XAR-5 film for 24 h at -70°C and autoradiographs developed. Hybridized bands were sized by comparison with the positions of 18 S and 28 S rRNA.

Signal intensities were determined by using a direct-capture two-dimensional surface counter-image system (Autograph; Oxford Positron Systems, Oxford, U.K.). This necessitated the conversion of counts for individual blots into percentages to allow the data from replicate studies to be used in analysis. We determined empirically that the counter was linear between 0 and 22000 c.p.m., which was within the range of radioactivity hybridizing to the filters. Blots were normalized for RNA loading by re-probing with oligo(dT). The Autograph analysis system

was used only to provide data on the relative intensities of the signal in individual blots, owing to differences between blots resulting from probe-labelling, efficiency of hybridization and washing etc.

Statistics

Statistical significance of differences in GUI was assessed by Student's unpaired *t* test. GUI values are given as means $\pm$ S.E.M. for five to eight rats. Each blot was repeated at least three times to ensure that the patterns were reproducible.

RESULTS AND DISCUSSION

Oligonucleotide probe specificity

The specificity of the 43-base oligonucleotide probe used to examine GLUT 4 mRNA expression in the present experiments was tested by hybridization to total RNA from specific tissues established to differ in their glucose-transporter-isoform mRNA expression (see Bell *et al.*, 1990; Kahn & Flier, 1990). Results are shown in Fig. 1. The probe for GLUT 4 hybridized with RNA from skeletal muscle and heart (which contain predominantly GLUT 4), but not with RNA from liver or pancreatic islets of Langerhans (which predominantly express GLUT 2) or brain (GLUT 1 and GLUT 3). This confirms the specificity of the oligonucleotide probe for GLUT 4 mRNA predicted from its base sequence (see the Materials and methods section). A second transcript not corresponding to the GLUT 4 transcript was occasionally, but not consistently, detected in the approximate region of 28 S rRNA.

Effects of starvation and re-feeding on GLUT 4 mRNA concentrations and rates of glucose utilization in tibialis anterior and IBAT

Figs. 2 and 3 show the relative abundance of GLUT 4 mRNA in tibialis anterior and IBAT from fed, 48 h-fasted and 48 h-fasted/2 h- or 4 h-re-fed rats. The major transcript was detected at the position predicted for the GLUT 4 transcript (Birnbaum, 1989). Relative levels of GLUT 4 mRNA in tibialis anterior and IBAT of fed, 48 h-starved, and 48 h-starved/2 h- or 4 h-re-fed rats (estimated by Autograph; see the Materials and methods

Fig. 1. Northern-blot analysis of total RNA hybridized with the GLUT 4 oligonucleotide probe

Total RNA (40 μg) obtained from tissues from fed rats was electrophoresed after denaturation and hybridized with an oligonucleotide probe to GLUT 4 as described in the Materials and methods section. Tissues were: lane *a*, heart; lane *b*, liver; lane *c*, mixed hindlimb muscle; lane *d*, pancreas; lane *e*, brain; lane *f*, pancreatic islets of Langerhans. The numbers to the side of the Figure indicate the positions of 28 S and 18 S rRNAs.

Fig. 2. Northern-blot analysis of total RNA from tibialis anterior muscles of rats in different nutritional conditions hybridized with the GLUT 4 oligonucleotide probe

Total RNA (40 μ g) obtained from eight pooled tibialis anterior muscles from four rats was extracted and subjected to Northern-blot analysis as described in the Materials and methods section. Muscles were obtained from: lane *a*, rats fed *ad libitum*; lane *b*, 48 h-starved rats; lane *c*, 48 h-starved/2 h-chow-re-fed rats; lane *d*, 48 h-starved/4 h-chow-re-fed rats.

Fig. 3. Northern-blot analysis of total RNA from IBAT of rats in different nutritional conditions hybridized with the GLUT 4 oligonucleotide probe

Total RNA (40 μ g) obtained from IBAT pooled from four rats was extracted and subjected to Northern-blot analysis as described in the Materials and methods section. IBAT was obtained from: lane *a*, rats fed *ad libitum*; lane *b*, 48 h-starved rats; lane *c*, 48 h-starved/2 h-chow-re-fed rats; lane *d*, 48 h-starved/4 h-chow-re-fed rats.

section) are compared with changes in tissue glucose utilization in Table 1.

Tibialis anterior. Northern blots loaded with equivalent amounts of RNA per lane demonstrated only minor (less than 10%) changes in GLUT 4 mRNA abundance in this muscle after 48 h starvation or during re-feeding for 2 or 4 h (Fig. 2). The modest (38.5%) decline in glucose utilization observed in tibialis anterior after 48 h starvation is consistent with the failure to observe a major effect on GLUT 4 gene expression (Table 1). Despite significant increases in insulin concentrations after re-

feeding (Holness & Sugden, 1991), there is no quantitative correlation between the significant (7-fold) increase in glucose utilization observed *in vivo* at both 2 and 4 h after re-feeding and the modest (< 10%) increase in GLUT 4 mRNA expression in tibialis anterior (Table 1).

IBAT. There were clear effects of starvation and re-feeding on GLUT 4 mRNA levels in IBAT (Fig. 3). GLUT 4 mRNA abundance was decreased ($P < 0.001$) after starvation for 48 h. The magnitude of the decline in the rate of glucose utilization elicited by starvation (72%; $P < 0.05$) is greater than that of

Table 1. GUIs and relative abundance of GLUT 4 mRNA in tibialis anterior and IBAT of fed, 48 h-starved and 48 h-starved/re-fed rats

GUIs were measured by using 2-deoxy[3 H]glucose. Further details of methodology are given in the Materials and methods section and the legends of Figs. 2 and 3. Statistical significance of the effects of re-feeding are shown: * $P < 0.001$.

	GUI				GLUT 4 mRNA relative abundance (% of fed)	
	(ng of glucose/min per mg)		(% of fed)		Tibialis anterior	IBAT
	Tibialis anterior	IBAT	Tibialis anterior	IBAT		
Fed	5.6 $\pm$ 0.8	23.0 $\pm$ 6.8	100	100	100	100
48 h-starved	3.5 $\pm$ 1.5	6.5 $\pm$ 1.2	61.5	28.1	90.0 $\pm$ 2.5	44.5 $\pm$ 2.4
48 h-starved/2 h-re-fed	24.3 $\pm$ 2.1*	260.4 $\pm$ 38.7*	432.6	1132.7	108.0 $\pm$ 8.3	46.8 $\pm$ 7.6
48 h-starved/4 h-re-fed	24.3 $\pm$ 2.7*	196.0 $\pm$ 29.4*	432.3	852.5	109.7 $\pm$ 2.6	71.8 $\pm$ 16.1

GLUT 4 mRNA (56%; Table 1). Therefore, changes in GLUT 4 mRNA expression may contribute to, but are not entirely responsible for, the suppression of brown-fat glucose observed after 48 h starvation. The decrease in GLUT 4 mRNA abundance was not reversed after re-feeding for 2 h (Fig. 3), despite dramatic (40-fold) increases in glucose utilization to over 10 times the fed value (Table 1). The starvation-induced decrease in GLUT 4 mRNA abundance was reversed partially by re-feeding for 4 h (to 72% of the fed value), whereas glucose utilization was not further increased (Table 1). GLUT 4 mRNA levels in IBAT were still below those in controls fed *ad libitum* after 8 h of re-feeding (results not shown).

Glucose utilization by white adipose tissue

It is of interest to compare the effects of starvation and re-feeding on GLUT 4 mRNA abundance and glucose utilization rates in brown adipose tissue and in white adipose tissue. GLUT 4 mRNA expression in epididymal white adipose tissue decreases by 90% after starvation for 48 h (Sivitz *et al.*, 1989). GUI values (ng of glucose/min per mg) measured in white adipose tissue *in vivo* (the present study) were not significantly decreased after 48 h starvation [fed, 2.2 ± 0.3 (8); 48 h-starved, 1.8 ± 0.4 (3); $P > 0.05$]. The half-time of recovery of GLUT 4 mRNA in white adipose tissue is 3 h; by 8 h of re-feeding it is about 2-fold greater than in the fed state (Sivitz *et al.*, 1989). Glucose utilization (the present study) increased by 2.9-fold within 2 h of re-feeding [to 5.3 ± 0.4 (5) ng/min per mg; $P < 0.001$ versus starvation]. Thus, as in brown fat, there is a poor relationship between GLUT 4 mRNA abundance and rates of glucose utilization *in vivo* in white fat.

General discussion

Dramatic variations in glucose transport and disposal by the insulin-sensitive tissues of the body occur during nutritional transitions. The present study was undertaken to assess the regulatory importance of changes in the relative abundance of mRNA coding for GLUT 4 in IBAT and skeletal muscle for the marked changes in glucose utilization evoked in these insulin-sensitive tissues by starvation and re-feeding. The study was prompted in part by the finding (Sivitz *et al.*, 1989) of dramatic changes in GLUT 4 mRNA abundance in white adipose tissue in response to changes in the nutritional status. Although we obtained a qualitatively similar pattern of changes in IBAT GLUT 4 mRNA abundance in response to starvation and re-feeding to that observed by Sivitz *et al.* (1989) in white fat, they were far less marked. More importantly, despite a dramatic increase in rates of IBAT glucose utilization after re-feeding, there was no evidence for an overshoot in GLUT 4 mRNA abundance over the fed value in this tissue.

Northern blotting of mRNA from a preparation of mixed gastrocnemius and soleus muscles revealed that starvation for periods in excess of 48 h was associated with a 3-fold induction of GLUT 4 mRNA (Charron & Kahn, 1990). Re-feeding for 24–48 h restored the mRNA level towards the fed value (Charron & Kahn, 1990). The relative amount of GLUT 4 glucose transporter protein paralleled the changes in mRNA abundance (Charron & Kahn, 1990). The pattern of response of tibialis anterior (predominantly fast fibres; Ariano *et al.*, 1973) differed markedly from this. GLUT 4 mRNA abundance was unchanged by 48 h starvation. Furthermore, when we examined the acute (2–4 h) response to re-feeding in detail, we obtained no evidence that changes in GLUT 4 mRNA abundance were instrumental in or necessary for the increased rates of glucose utilization. Clearly, there is no requirement for increased expression of GLUT 4

mRNA to facilitate glucose uptake in this non-working muscle, where the major fate of glucose during hyperinsulinaemia is storage as glycogen. Starvation is associated with a decrease in translational efficiency (reviewed by Sugden & Fuller, 1991); the possibility is therefore not excluded that acute re-feeding is associated with increased translational efficiency and GLUT 4 protein synthesis. Alternatively, additional regulatory factor(s) influencing rates of glucose utilization by tibialis anterior and other non-working muscles during the starved-to-fed transition may be effects of insulin to promote recruitment and activation of transporters (see below) and/or stimulate glucose utilization at a post-transport site (e.g. glycogen synthase).

Insulin can stimulate glucose uptake by favouring the translocation of glucose transporters from an intracellular pool to the plasma membrane (Cushman & Wardzala, 1980; Wardzala & Jeanrenaud, 1981; Greco-Perotto *et al.*, 1987a,b; Zaninetti *et al.*, 1988), and by increasing the intrinsic activity of the transporters once they have been translocated to the plasma membrane (revealed by changes in the Hill coefficient and the dissociation constant; for review see Cusin *et al.*, 1990). The present study would appear to exclude changes in GLUT 4 transcription as an additional factor in mediating the acute and dramatic response of glucose transport in IBAT and non-working skeletal muscle to physiological hyperinsulinaemia induced by re-feeding.

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