Changes in insulin-receptor mRNA levels in skeletal muscle and brown adipose tissue of weanling rats during fasting and refeeding

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Tissue-specific alterations in insulin sensitivity occur in response to fasting and refeeding, as part of the integrated adaptive mechanisms employed to adjust to major changes in nutritional status. In the present study the effects of fasting and refeeding on insulin-receptor, actin and myosin mRNA levels in skeletal muscle, and insulin-receptor and uncoupling-protein mRNA in brown adipose tissue of rats have been examined. Insulin-receptor mRNA levels increased markedly in both skeletal muscle and brown adipose tissue after a 40 h fast, the increase being greater in brown fat (8-fold) than in muscle (2-fold). On refeeding for 4 h, the insulin-receptor mRNA level in both tissues declined rapidly to control levels. An increase in insulin-receptor mRNA level was also observed in brown adipose tissue after a 16 h fast, although not in skeletal muscle. In contrast to the insulin-receptor mRNA, the level of the mRNA for the mitochondrial uncoupling protein declined markedly in brown adipose tissue during a 40 h fast. These results indicate that insulin-receptor mRNA levels are modulated in response to the alterations in nutritional status that occur during fasting and refeeding; this may reflect a nutritional influence on transcription of the receptor-protein gene.

Insulin-receptor mRNA: Skeletal muscle: Brown adipose tissue

Major hormonal changes occur in response to alterations in nutritional status, particularly with respect to insulin, which plays a central role in influencing tissue responses to nutrient supply. For example, differential changes in the sensitivity of tissues to insulin occur during fasting and refeeding. Thus, skeletal muscle increases its responsiveness to insulin in the fasted animal (Brady *et al.* 1981), while a decrease in basal and insulin-stimulated glucose transport occurs in white adipose tissue (Kahn *et al.* 1988) and an inhibition of glucose output from the liver (Penicaud *et al.* 1985).

The receptors for insulin and growth factors such as insulin-like growth factor (IGF-1), epidermal growth factor (EGF) and platelet-derived growth factors (PDGF) are membrane proteins. The effects of such hormones and growth factors are mediated by first binding to the receptor, which then initiates a cascade of events that induces responses appropriate to the particular agent. The observed changes in insulin sensitivity may be mediated through alterations in the insulin receptor itself, or in the subsequent signal pathway. Several observations indicate post-receptor changes associated with altered insulin sensitivity (Crettaz & Jeanrenaud, 1980), for example in acanthosis nigricans (Grigorescu *et al.* 1984), in lactating rats (Burnol *et al.* 1990) and in streptozotocin-induced diabetes (Kadowaki *et al.* 1984). However, alterations in receptor number have also been associated with some conditions of insulin resistance, such as the resistance that occurs in genetically-obese Zucker rats (Crettaz *et al.* 1980). Although muscle glucose utilization is reduced during

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fasting (Issad *et al.* 1987), the number of insulin receptors present in muscle and the liver increases in fasted animals (Balage *et al.* 1989), while the tyrosine kinase activity of the receptors is normal in muscle but decreased in liver (Balage *et al.* 1989; Contreras *et al.* 1990). Changes in the number or turnover of receptors may be important in the control of tissue sensitivity to hormones and growth factors, but at present little is known of the mechanisms that are involved, particularly at the level of gene transcription.

Differential tissue responses in insulin sensitivity in the fasted animal are reflected by changes in the number of glucose transporters (Kahn *et al.* 1988), and this in turn reflects changes at the mRNA level. The level of the GLUT 4-transporter mRNA decreases in white adipocytes with fasting and increases on refeeding (Charron & Kahn, 1990; Gould & Bell, 1990), with parallel effects on insulin-stimulated glucose transport. In contrast, increased expression of glucose transporters occurs in muscle with fasting, and this returns to control levels on refeeding (Charron & Kahn, 1990).

Skeletal muscle represents about 400 g/kg total body mass, is insulin responsive, and plays a major role in glucose metabolism. Although only 10–20 g/kg body-weight, brown adipose tissue (BAT) is highly sensitive to insulin and its thermogenic function has been shown to be compromised in the presence of insulin resistance (Mercer & Trayhurn, 1984). In the present study we have investigated the effects of fasting and refeeding on the level of the insulin receptor mRNA in these two insulin-sensitive tissues, as part of an examination of the influence of nutritional status on the expression of receptor-protein genes.

METHODS

Animals and experimental regimen

Male, hooded Lister rats (Rowett strain), aged 28 d, were group housed in wire-floored cages. Four experimental groups were defined: control, fasted 16 h, fasted 40 h, and fasted 40 h–refed 4 h. Each of the test groups contained six rats, with a total of twelve control animals. The control group was fed *ab lib.* with a commercial diet (Biosure diet; Special Diet Services, Cambridge), containing (g/kg): carbohydrate 570, starch 450, protein 180, digestable energy 133, fibre 36, fat 24. Food was withdrawn from the fasted groups for the appropriate times, and returned to the refed group for a 4 h period. The body-weight of the rats in each group was determined at the beginning of the experiment and at the time of sampling (Table 1).

Rats were killed by decapitation and blood samples were collected from severed blood vessels. Plasma was obtained by centrifugation at 3000 ***g*** for 10 min at 4°, and stored at −20° for subsequent analysis of insulin and glucose concentrations. Skeletal muscle (gastrocnemius) and interscapular BAT were rapidly removed from each rat and immediately frozen in liquid nitrogen. The tissue samples were then transferred to −70° for storage, before the extraction of RNA.

Plasma insulin was measured by radioimmunoassay using an anti-serum raised in guinea-pigs to porcine insulin (ICN Biochemicals), with ^{125}I-labelled insulin as a tracer and rat insulin (Novo Biolaboratories, UK) as a standard. Plasma glucose levels were determined by a glucose oxidase assay (Trinder, 1969).

RNA extraction, purification and electrophoresis

RNA was extracted as described by Chomczynski & Sacchi (1987). Briefly, tissues were homogenized using an Ultra-turrax T25 in a solution containing 4 M-guanidinium isothiocyanate, 25 mM-sodium citrate (pH 7·0), sarkosyl (5 g/l) and 0·1 M-2-mercaptoethanol. Sodium acetate (2 M), pH 4·0, and chloroform–isoamyl alcohol (49:1 v/v) were added sequentially to 100 and 200 ml/l respectively of the volume of the homogenized

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solution. After thorough mixing, the samples were stored on ice for 15 min and then centrifuged at 12000 *g* for 20 min at 4°. The upper aqueous layer containing the RNA was removed and the RNA precipitated with propan-2-ol. After a second propan-2-ol precipitation and an ethanol wash, the pellet was resuspended in sodium dodecyl sulphate (5 g/l; SDS). The concentration of the RNA was determined spectrophotometrically by absorbance at 260 nm. Absorbance at 280 nm was also determined and the 260:280 nm ratio calculated; this was routinely 1·9–2·1 indicating a high level of purity of the RNA sample. Approximately 2 μg RNA/mg muscle was obtained.

The RNA samples were resolved by electrophoresis in agarose (12 g/l) containing formaldehyde (Sambrook *et al.* 1989). Each sample was run in duplicate using 20 μg total RNA per lane. The RNA was transferred to a nylon membrane (Genescreen) by capillary blotting overnight and immobilized by exposure to u.v. light.

Northern hybridizations

A cDNA probe for the human insulin receptor was kindly donated by Dr L. Ellis (Howard Hughes Medical Institute, University of Texas, Dallas, USA). This probe was a 1·5 kb *Eco*RI/*Pst*I fragment corresponding to the region 1101 to 2602 base pairs, which codes for a region in the extracellular domain (Ebina *et al* 1985). Additional probes included α-actin (originally from Dr K. Schwartz, Unité de Recherches sur le Metabolism du Coeur, U127 INSERM, Paris, France) and adult fast myosin heavy chain (MHC 62), and these were kindly donated by Professor G. Goldspink (Royal Veterinary College, London). The actin probe was a 1·7 kb *Pst*I fragment of a genomic clone subcloned into pBR322. The myosin probe was a 0·6 kb *Pst*I fragment from pBR322. Additionally, a 1·4 kb probe for 18S rDNA (Erickson *et al.* 1981) was kindly donated by Dr R. Fulton (Beatson Institute, Glasgow). A 27-mer synthetic oligonucleotide probe was used to measure uncoupling protein mRNA (MacFarlane & Trayhurn, 1990).

Hybridizations with insulin receptor, actin, myosin and 18S probes were carried out using the following standard procedures. Briefly, nylon membranes were pre-hybridized for 16 h at 42° in a buffer containing 0·05 M-Tris-hydrochloric acid (pH 7·5), 1·0 M-sodium chloride and (g/l): formamide 500, dextran sulphate 100, polyvinylpyrrolidone (PVP) 2, bovine serum albumen (BSA) 2, ficoll 2, and denatured salmon sperm (> 100 μg/ml). The appropriate probe (25–50 ng) was labelled with [α-^{32}P]dCTP by random priming using the Multiprime DNA labelling system (Amersham International Plc, Amersham, Bucks.). The labelling efficiency was determined on a sample of the labelled probe and was found to be a minimum of 10^6 counts/min (cpm) per ng DNA. The hybridization mix was prepared using the appropriate denatured probe in the previously specified hybridization buffer (excluding dextran sulphate) and added to the pre-hybridization mix. Hybridizations were carried out for 16 h at 42°.

Non-specific hybridization to the filters was removed by the following washing procedures: SSC (300 mM-NaCl–30 mM-sodium citrate, pH 7·0) for 5 min at room temperature; SSC (75 mM-NaCl–7·5 mM-sodium citrate, pH 7·0), containing 10 g SDS/l for 60 min at 65°; SSC (15 mM-NaCl–0·15 mM-sodium citrate, pH 7·0) for 30 min at room temperature. Each washing procedure was carried out in duplicate and the membranes were then placed in autoradiography cassettes with hyperfilm-MP (Amersham International Plc). For the insulin-receptor mRNA the membranes were laid down with intensifying screens for 7 d, while for the actin and myosin mRNA and the 18S rRNA the membranes were exposed for 4–16 h, without intensifying screens. All exposures were carried out at −70°.

Pre-hybridization of membranes with the oligonucleotide probe for uncoupling protein was carried out at 37° for 2 h in SSC (900 mM-NaCl–90 mM-sodium citrate, pH 7·0)

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Table 1. *Tissue weights, and plasma insulin and glucose levels in control, fasted and fasted–refed rats**

(Values are the means with their standard errors for six animals in each group)

Treatment group	Body-wt (g)				Tissue wt (mg)				Plasma insulin (ng/ml)		Plasma glucose (mg/ml)	
	Initial		Final		Skeletal muscle		BAT					
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Control	70·7	3·4	78·4	4·4	300	16	107	23	0·41	0·36	1·27	0·19
Fasted, 16 h	71·1	2·4	65·1	2·7	276	22	98	10	< 0·02		0·87	0·04
Fasted, 40 h	71·2	2·1	59·2	2·3	242	11	69	12	< 0·02		0·68	0·19
Fasted 40 h–refed 4 h	71·0	2·1	64·1	1·8	236	12	62	12	0·75	0·28	1·59	0·15

* Animals were fasted for 16 h or 40 h, and one group was refed for 4 h following a 40 h fast; for details, see p. 584.

containing 0·05 M-Tris-HCl (pH 7·5), 1·0 M-NaCl and (g/l): SDS 10, PVP 2, BSA 2, ficoll 2, sodium pyrophosphate 1, and denatured salmon sperm (> 100 μg/ml). The probe (76 ng) was labelled with [$\gamma-^{32}$P]ATP using T4 polynucleotide kinase (Amersham International Plc). The labelled probe was separated from unincorporated nucleotide triphosphates (dNTP) by ethanol precipitation (Sambrook *et al.* 1989); a labelling efficiency of approximately 10^6 cpm/ng DNA was obtained. The probe was added to the pre-hybridization buffer and hybridization was allowed to proceed for 4 h at 42°. Non-specific hybridization was removed by washing the membranes in SSC (300 mM-NaCl–30 mM sodium citrate, pH 7·0) with 1 g SDS/l, for 30 min at 42°. This wash was then repeated and followed by one 30 min wash in SSC (150 mM-NaCl–30 mM sodium citrate, pH 7·0) with 1 g SDS/l at 42°. The membranes were then placed in autoradiography cassettes with hyperfilm-MP and laid down overnight at −70°, with intensifying screens.

Quantitative analysis of mRNA levels

The intensity of the signal on the autoradiographic films was quantified using a QUIPS image-processing work-station operating with visual colour service (VCS) image processing software. The signals for the respective mRNA were calculated relative to the level of 18S rRNA in each preparation, which was used as an internal standard for each RNA preparation to compensate for any variation in the loading of RNA onto the gel. Both bands for the insulin-receptor mRNA were quantified and the sum of these values used in the calculations. The optical density ratio of the group was then calculated relative to the control samples within each autoradiograph. Control samples were represented as 100%. Quantification was carried out on duplicate samples from three animals in each test group. Analysis of significance was carried out using the Student's *t* test on the difference in the mean values from 100% control values.

RESULTS

Tissue weights, plasma insulin and glucose levels

The rats in the 16 h and 40 h fasted groups decreased in weight by 17 and 24% respectively relative to the control groups at the same sampling point. In the refed group the rats refed after the 40 h fast consumed 26 g in the time period. The skeletal muscle and BAT

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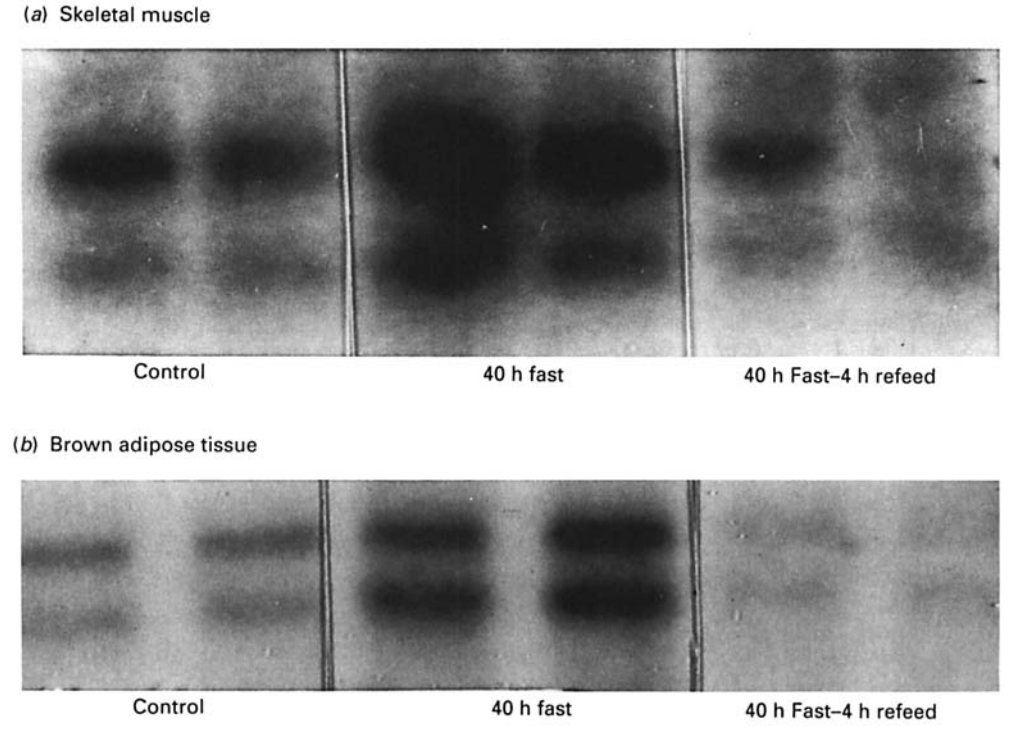

Fig. 1. Effect of fasting and refeeding on insulin-receptor mRNA levels in (*a*) skeletal muscle and (*b*) brown adipose tissue. Rats were fasted for 40 h and refed for 4 h. Results show representative Northern hybridizations of total RNA (20 μg) incubated with a 1·5 kb cDNA probe for the insulin receptor (for details, see pp. 585–586).

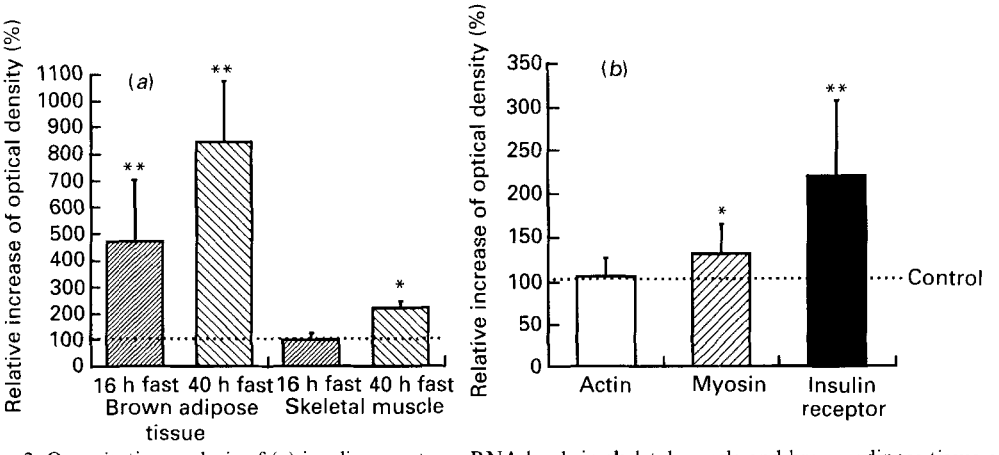

Fig. 2. Quantitative analysis of (*a*) insulin-receptor mRNA levels in skeletal muscle and brown adipose tissue of 16 h and 40 h fasted rats and (*b*) insulin-receptor, actin and myosin mRNA in skeletal muscle of 40 h fasted rats. Values are means with their standard errors represented by vertical bars. Mean values for fasted rats were significantly different from those of controls where controls are represented as 100% (Student's *t* test): * $P < 0{\cdot}05$, ** $P < 0{\cdot}01$. For details of procedures, see p. 586.

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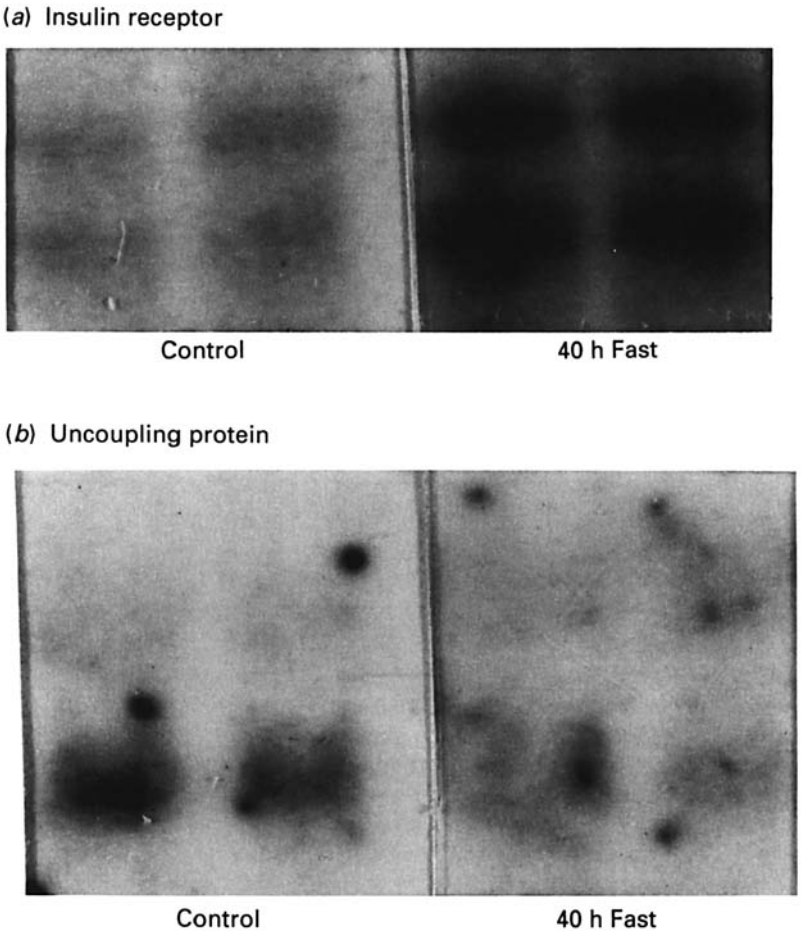

Fig. 3. Effect of a 40 h fast on insulin-receptor and uncoupling-protein mRNA levels in brown adipose tissue of rats. Results show Northern hybridization of total RNA (20 μg) from brown adipose tissue after probing for (*a*) insulin-receptor mRNA and (*b*) uncoupling-protein mRNA. For details, see pp. 585–586.

weights were recorded at the end of each test period (Table 1). A decrease in weight was observed in both tissues of the fasted and the fasted–refed groups, relative to control animals.

The plasma insulin levels declined during the 16 h and 40 h fasting periods, to below the level of detection of the radioimmunoassay (Table 1). The insulin levels were, however, raised after refeeding. Plasma glucose levels were similarly decreased in the fasted animals and increased above control levels in the refed group.

Effects of fasting and refeeding on insulin-receptor mRNA levels

The level of the mRNA for the insulin receptor in BAT and skeletal muscle was determined by Northern hybridization. Each membrane contained RNA from both the control and the test groups for direct reference, so that any variability associated with the hybridization conditions would be overcome. Two bands corresponding to 8·5 and 7·2 kb were evident in both tissues (Fig. 1). Similar bands were also present in white adipose tissue (values not shown). This is consistent with previous observations on rat brain, kidney, liver and muscle

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(Goldstein *et al.* 1987). In human cell lines smaller mRNA (approximately 1·8–3·3 kb) have also been identified (Ullrich *et al.* 1985), but these bands have not been observed in rat tissues and were not found in the present work.

After a 40 h fast the level of the insulin-receptor mRNA was significantly increased in both BAT and skeletal muscle when compared with control animals (Figs 1 and 2(*a*)). The level of the mRNA declined subsequently on refeeding. Relative to control values given as 100%, the insulin-receptor mRNA levels in BAT and skeletal muscle were 844 and 220% respectively. A smaller increase (470%) was evident in BAT after a 16 h fast, but this was still significantly increased above control levels. After the shorter fasting period no change was observed in the level of the insulin-receptor mRNA in skeletal muscle (Fig. 2(*a*)).

Specificity of changes in insulin-receptor mRNA levels

The level of the mRNA for actin and myosin in skeletal muscle was examined by reprobing the membrane in order to determine if the observed increases in the insulin-receptor mRNA on fasting were specific, or a reflection of a generalized change. One band was found each for the actin and myosin mRNA, corresponding to 1·7 kb and 6·9 kb respectively. The actin mRNA level was similar in the fasted and control groups (per μg total RNA), although the level of the myosin mRNA was slightly higher in the fasted animals. The insulin-receptor mRNA level was 2-fold higher in the fasted group than in the controls (Fig. 2(*b*)).

The level of the mRNA for uncoupling protein was determined, to provide a comparison for the insulin-receptor mRNA in BAT. Two bands corresponding to approximately 1·9 and 1·6 kb were detected, as noted previously for rats (Ricquier *et al.* 1986). In the 40 h fasted animals the major band, at 1·6 kb, decreased substantially relative to controls (Fig. 3).

DISCUSSION

Northern hybridization with a cDNA probe for the human insulin-receptor gene indicates that there are two major mRNA species for the insulin receptor in both rat skeletal muscle and BAT. In the case of muscle this is consistent with earlier observations (Goldstein *et al.* 1987), but identification of insulin-receptor mRNA has not previously been reported in BAT. The different lengths of the mRNA have been shown to be due to variable lengths of 3′ untranslated sequences (Goldstein & Kahn, 1989).

The level of the mRNA for the insulin receptor in skeletal muscle and BAT was found to increase markedly in response to fasting; this confirmed our earlier preliminary observation on a separate group of animals (Knott *et al.* 1990). Furthermore, quantification of the autoradiograms showed that the magnitude of the increase in the level of insulin-receptor mRNA in skeletal muscle was greater than 2-fold. This is comparable with the changes in the amount of insulin-receptor protein that has previously been observed in response to similar fasting conditions (Balage *et al.* 1989). The results suggest, therefore, that receptor number may be controlled at a pretranslational level, i.e. involving either gene transcription or the stability of the mRNA.

An increase in the amount of insulin-receptor mRNA was also observed in BAT, although the changes were evident in this tissue after only a 16 h fast, while a 40 h fast was required for changes to be detected in skeletal muscle. The magnitude of the response was also greater after a 40 h fast in BAT (8-fold) than in skeletal muscle (2-fold). Thus, with respect to insulin-receptor mRNA levels, BAT, is more sensitive than muscle to the imposed fasting conditions.

In the absence of information relating to the effects of insulin-receptor protein content in BAT in response to fasting, it is not possible to directly relate the changes in the mRNA levels to a potential physiological response to fasting. However, the qualitatively similar

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response in muscle and BAT, together with the known increase in insulin-receptor number in muscle tissue, suggests that the mRNA changes in BAT are likely to be reflected in an increase in insulin-receptor protein.

The increases observed in the level of the insulin-receptor mRNA do not appear to be due to a general increase in mRNA per unit total RNA. The level of the mRNA for the major muscle protein, actin, did not change in the fasted animals. There was, however, a slight rise in the level of the myosin mRNA, but this increase was not as large as that observed with the insulin-receptor mRNA. This serves to highlight the significance of the increases that were observed in the insulin-receptor mRNA on fasting, and particularly so in the light of the overall decline in total muscle weight that occurs in the fasted animal.

The specificity of the increase in insulin-receptor mRNA on fasting is also evident in BAT, there being a marked decline in the level of uncoupling-protein mRNA in conjunction with the enrichment of the insulin-receptor mRNA. The marked decline in uncoupling-protein mRNA correlated with the reduction in the amount of uncoupling protein itself which has been documented in fasted animals (Trayhurn & Jennings, 1988), and is consistent with a previous report by other workers (Champigny & Ricquier, 1990). It is emphasized that uncoupling protein is a unique marker for BAT and that the level of the protein determines the capacity of the tissue for thermogenesis.

Skeletal muscle plays an important role in the responsiveness of the whole animal to insulin, with respect to glucose utilization. The enhanced insulin sensitivity of skeletal muscle during fasting occurs concomitantly with the development of relative insulin resistance in other tissues, particularly the liver and white adipose tissue. Collectively, these tissue-specific alterations in insulin sensitivity in the fasted animal would be expected to facilitate the preferential channelling of glucose to skeletal muscle. In addition, although BAT represents a relatively small proportion of total body-weight (10–20 g/kg), it is highly sensitive to insulin (Ferré *et al.* 1986). The importance of insulin sensitivity to the functional activity of BAT is underlined by the fact that the thermogenic responsiveness of the tissue is compromised in the presence of insulin resistance (Mercer & Trayhurn, 1984). In general, glucose utilization and the insulin sensitivity of BAT are modulated in accordance with the physiological requirements for thermogenesis (Ferré *et al.* 1986). The present results would suggest that an increase in the level of the insulin-receptor mRNA in both skeletal muscle and BAT may be part of a co-ordinated response to enhance insulin sensitivity. In the case of BAT this appears somewhat paradoxical, given the loss of uncoupling protein and fall in thermogenic capacity that occur in the fasted animal (Trayhurn & Jennings, 1988).

The changes in plasma insulin and glucose levels that are induced by fasting and refeeding are well established (Penicaud *et al.* 1985). Insulin-dependent regulation of insulin-receptor concentration has been demonstrated (Gavin *et al.* 1974) and it is possible that the hypoinsulinaemia of the fasted animal is a prerequisite for the increase in the number of insulin receptors. However, after a 16 h fast the rats were hypoinsulinaemic, and while BAT exhibited an increase in the level of the mRNA for the insulin receptor, no such response was evident in skeletal muscle. As tissue-specific differences in response occur alongside alterations in circulating insulin concentration, insulin may not be the only factor in the control of the level of insulin-receptor mRNA. Indeed, it has been demonstrated in vitro in human cell lines that insulin-receptor mRNA levels are reduced by depletion of glucose levels (Briata *et al.* 1990; Levy *et al.* 1991), but at present it is not possible to relate this observation to the situation in vivo described here.

In summary, the present results indicate that insulin-receptor mRNA levels in both skeletal muscle and BAT can be altered by gross changes in food intake. Nutritional status may, therefore, regulate hormone-receptor number and tissue sensitivity to the hormone, at least in part through control at the level of gene expression. A change in the level of

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mRNA is a general indication of pretranslational regulation, but it does not distinguish between alterations in mRNA stability or alterations in gene transcription (Nielson & Shapiro, 1990). Since insulin sensitivity and receptor number are modified in fasting (Brady *et al.* 1981, Balage *et al.* 1989), it appears probable that the observed changes in insulin-receptor mRNA levels are reflected in the production of an active receptor.

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