Originals

Normal insulin receptor tyrosine kinase activity and glucose transporter (GLUT 4) levels in the skeletal muscle of hyperinsulinaemic hypertensive rats

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Summary. The spontaneous hypertensive rat is an animal model characterized by a syndrome of hypertension, insulin resistance and hyperinsulinaemia. To elucidate whether in analogy to other insulin resistant animal models an inactivity of the insulin receptor kinase or an alteration of the glucose transporter (GLUT 4) level in the skeletal muscle might contribute to the pathogenesis of insulin resistance we determined insulin receptor kinase activity and GLUT 4 level in the hindlimbs of spontaneous hypertensive rats and normotensive control rats. Normotensive normoinsulinaemic Lewis and Wistar rats were used as insulin sensitive controls, obese Zucker rats were used as an insulin resistant control with known reduced skeletal muscle insulin receptor kinase activity. Binding of ¹²⁵I-insulin, crosslinking of ¹²⁵I-B²⁶-insulin, autophosphorylation in vitro with ³²P-ATP and phosphorylation of the synthetic substrate Poly (Glu 4: Tyr 1) were performed after partial purification of solubilized receptors on

wheat germ agglutinin columns. GLUT 4 levels were determined by Western blotting of subcellular muscle membranes. Insulin receptors from spontaneous hypertensive rats compared to those from Lewis and Wistar rats showed no difference of the binding characteristics or the in vitro auto- and substrate phosphorylation activity of the receptor, while in the Zucker rats the earlier described insulin receptor kinase defect was clearly evident. Western blots of subcellular muscle membrane fractions with antibodies against GLUT 4 revealed no difference in transporter levels. These data suggest that insulin resistance in spontaneous hypertensive rats is caused neither by an insulin receptor inactivity nor by a decreased number of glucose transporters in the skeletal muscle.

Key words: Spontaneous hypertensive rat, insulin receptor kinase, glucose transporter.

There is increasing evidence that hypertension is associated with insulin resistance and compensatory hyperinsulinaemia [1–8]. The molecular mechanism causing insulin resistance in states of hypertension is not understood. Insulin resistance might be caused by abnormalities of insulin signal transduction at any level of the signal transmitting chain in the target cells of the hormone [9, 10], i.e. at the level of insulin binding, activation of the insulin receptor kinase, post-kinase signal transmitting events or directly at the level of major effector systems such as the glucose transport system or the glycogen synthase. In insulin resistant animal models, which show characteristics of human Type 2 (non-insulin-dependent) or Type 1 (insulin-dependent) diabetes mellitus, such as the Zucker rat [10–12], gold-thio-glucose-mice [13], ob/ob mice [14] or streptozotocin-treated rats [15] an impairment of insulin signal transduction at the level of the insulin receptor kinase in the skeletal muscle has been described. Furthermore, in streptozotocin-induced diabetes defects at the level of the glucose transporter in fat and

skeletal muscle were observed [16, 17]. In human Type 2 diabetes insulin resistance is associated with an inactivity of the insulin receptor kinase of the skeletal muscle [10, 18, 19], the liver and the fat [20, 21]. Furthermore, in fat an abnormality of the glucose transporter system was observed [22], while in the skeletal muscle controversial results were obtained [23–25]. In analogy to these observations it might be speculated that insulin resistance in hypertension could be caused or at least aggravated by an impairment of signal transduction at the level of the insulin receptor kinase or the glucose transporter. An animal model which shows an association of hypertension, insulin resistance and hyperinsulinaemia is the spontaneous hypertensive rat [26]. Recently Reaven et al. [27] have shown that adipocytes from spontaneous hypertensive rats (SHR) display an insulin resistance of the glucose uptake mechanism. In other animal models of insulin resistance [10–17] as well as in human pre-diabetes [28, 29] and Type 2 diabetes [30] it was shown that the predominant role for the pathogenesis of insulin resistance is played by

the skeletal muscle. Therefore the aim of our study was to determine whether the skeletal muscle of the SHR shows an abnormality of insulin signal transduction at the level of the insulin receptor kinase or at the level of the glucose transporter. Insulin receptor kinase activity of the skeletal muscle from SHR as well as GLUT 4 levels were compared with two types of rats with normal insulin sensitivity, normal insulin levels and normal blood pressure i.e. Lewis rats and Wistar rats. The kinase data were also compared with the insulin receptor kinase activity of an animal model with insulin resistance and hyperinsulinaemia, where an inactivity of the insulin receptor kinase has been demonstrated [10], i.e. the Zucker rat.

Materials and methods

Materials

Porcine insulin was purchased from Novo Nordisk (Bagsværd, Denmark), [32 P]ATP, 2900 Ci/mmol, from Du Pont de Nemours (Dreieich, FRG), Triton X-100 from Sigma (Munich, FRG). Aprotinin, benzamidine, vanadate, bacitracin, natriumfluoride, natriumorthophosphate, phenylmethylsulphonyl fluoride (PMSF) and N-acetylglucosamine were obtained from Sigma. Wheat germ agglutinin coupled to agarose was from Miles/ICN (Eschwege, FRG). All reagents for SDS-PAGE were from Bio-Rad (Munich, FRG), and all other reagents were of the best grade commercially available. Insulin (human) was purchased from Hoechst (Frankfurt, FRG). For homogenisation we used an Ultra-Turrax from Janke and Kunkel (Staufen im Breisgau, FRG). For autoradiography X-Omat AR5 diagnostic film was used from Kodak (Rochester, NY, USA). The SHR, Wistar-Kyoto rats and Lewis rats (320–340 g body weight) came from Boehringer (Mannheim, FRG). The polyclonal serum directed against the C-terminal end of GLUT 4 was the kind gift of Dr. T. Plough (Copenhagen, Denmark). The polyclonal serum against GLUT 1 was obtained from Wak-Chemie (Homburg, FRG), nitrocellulose was from Schleicher and Schuell (Dassel, FRG), the chemiluminescence detection system was from Amersham (Braunschweig, FRG).

Insulin receptor kinase

Receptor preparation: muscle receptor was prepared from skeletal muscle as previously described [31]. Skeletal muscle tissue was homogenized in the presence of protease inhibitors (2.5 mmol/l, PMSF); 1000 trypsin inhibiting units/ml, aprotinin; 10 mmol/l benzamidine, 0.01 % bacitracin (g/volume) in a buffer containing 25 mmol/l Hepes (pH 7.4). The homogenates were centrifuged at $11,000 \times g$ for 20 min, the pellet was then solubilized with Triton X-100 at a final concentration of 1 % (by volume). Insoluble material was removed by centrifugation at $50,000 \times g$ for 50 min and the supernatant was applied to columns containing wheat germ agglutinin coupled to agarose. After extensive washing with 25 mmol/l Hepes buffer (pH 7.4) containing 0.05 % Triton X-100, 10 % glycerin, 120 mmol/l NaCl, 2.5 mmol/l KCl, 1 mmol/l $MgCl_2$, 1 mmol/l $CaCl_2$ the bound material was eluted with the same buffer supplemented with 0.3 mol/l N-acetylglucosamine.

Binding to solubilized receptor: solubilized and wheat germ purified receptor was prepared as described above. Aliquots of the wheat germ eluate containing 5 μ g of protein were incubated with [125 I-Tyr^{A14}]-insulin (3×10^{-11} mol/l, 3000 cpm/fmol) and various concentrations of unlabelled insulin for 45 min at 22°C in a medium of 25 mmol/l Tris (pH 7.4), 5 mmol/l $MgSO_4$ and 0.5 g % bovine serum albumin (BSA). Separation of the free and receptor-bound insulin was then performed using dextrane-coated charcoal. Scatchard analysis was performed using a computer program written by G. A. McPherson [32].

Affinity crosslinking of 125 I-B²⁶ insulin to solubilized receptor

Crosslinking experiments were performed as described [33]. A 50 μ l sample (0.5–2 μ g of protein) of wheat germ agglutinin purified receptor eluate was incubated with 0.08 nmol/l [125 I]iodotyrosyl-B²⁶-insulin for 12 h at 4°C. Tubes were chilled on ice and disuccinimidyl substrate (final concentration 25 μ mol/l) was added for 20 min at 4°C. The incubation was terminated by addition of Laemmli buffer containing 100 mmol/l dithiothreitol and Tris/HCl (20 mmol/l, pH 7.4) and subsequent boiling for 25 min at 95°C. The reaction products were analysed by SDS-PAGE (7.5 % gel) and autoradiography [34].

Autophosphorylation of the insulin receptor

For the standard phosphorylation assay approximately 4 μ g of wheat germ purified proteins were pre-incubated at 22°C for 30 min with insulin (10^{-10} – 10^{-6} mol/l) or without insulin. This was followed by an incubation with [32 P]-ATP (10 Ci/75 μ l volume), in 25 mmol/l Hepes buffer (pH 7.4), containing 0.1 % (by volume) Triton X-100, 10 mmol/l $MnCl_2$, 12 mmol/l NaCl, 0.5 mmol/l KCl, 0.25 mmol/l $CaCl_2$, 0.1 mmol/l $MgSO_4$, 0.25 g % BSA, and 1 mmol/l vanadate at 22°C. The incubation was stopped by addition of Laemmli buffer and boiling for 30 min. Subsequently phosphoproteins were separated by PAGE [34]. The phosphoproteins identified by the autoradiography were cut from the gel and counted in a scintillation counter.

Substrate phosphorylation

Substrate phosphorylation activity was analysed by a modification of the method of Braun et al. [35]. Approximately 0.5–1.0 μ g of wheat germ purified proteins were pre-incubated with insulin (10^{-6} – 10^{-10} mol/l) and without insulin for 30 min at 22°C. This was followed by an incubation with [32 P]ATP (50 μ mol/l, 0.005 mCi) in the final volume of 50 μ l containing 25 mmol/l Hepes buffer, containing 0.05 % (by volume) Triton X-100, 12 mmol/l $MgCl_2$, 12 mmol/l $MnCl_2$, 1 mmol/l vanadate, and 1 mmol/l Poly (Glu 4: Tyr 1) for 1 h at 22°C. The phosphorylation was stopped by application of the reaction mixture to Whatman 3MM paper squares washed with 20 mmol/l sodium pyrophosphate. The papers were washed six times in 10 % (by volume) trichloric acid containing 20 mmol/l sodium pyrophosphate and counted with a scintillation mixture in a beta-counter.

Glucose transporter

Treatment of animals and hindlimb preparation: SHR (12–13 weeks old) and control Wistar rats of the same age all with body weights ranging from 320–340 g respectively were injected with insulin (3 mU/l Altinsulin, Hoechst). Subsequently the glucose concentration was checked every 4 min with Glucostix (Bayer, Leverkusen, FRG). The rats were killed 15 min after the injection. The muscles of both hindlimbs were quickly removed, dissected on ice, and deep frozen at -80°C within 3 min of the killing.

Membrane preparation

For the preparation of membrane fractions enriched in plasma membranes or low density membranes (LDM) we used a method as it is also described in the flow sheet (Fig. 1). The essential steps of the purification procedure to obtain fractions enriched with plasma membrane or LDM were as follows: muscles were homogenized with an Ultra-Turrax (Braun, Melsungen, FRG) four times full speed for 10 s. Approximately 4 g tissue was used in each group for the preparation. The homogenate was dissolved in cold (4°C) buffer (0.2 mol/l Tris $\times$ HCl, 10 mmol/l EDTA, 255 mmol/l sucrose, pH 7.4). Containing the following protease and phosphatase inhibitors (0.2 mg/ml bacitracin, 1000 trypsin inhibiting units/ml aprotinin, 1 mmol/l PMSF, 0.2 mg/ml benzamidine, 100 mmol/l sodium flu-

Fig. 1. Schematic presentation of isolation procedure for plasma membranes (PM) and low density membranes (LDM) from the rat hindlimb muscle. TES (2 mmol/l Tris, 100 mmol/l EDTA, 225 mmol/l sucrose)

oride, 10 mmol/l sodium pyrophosphate, 1 mmol/l orthovanadate) and potted in a glass/teflon potter for ten times. After homogenization the tissue was centrifuged at 12,000 g for 20 min. The supernatant consisting of fractions enriched with high density membranes (HDM) and LDM was centrifuged for 15 min at 55,000 g to separate the LDM-enriched membranes from the HDM-enriched membranes. To obtain the crude membrane pellet the supernatant was again centrifuged in a high-speed centrifuge for 60 min at 200,000 g. The pellet was resuspended in buffer, potted again and centrifuged at 7,000 g for 10 min. The resulting plasma membrane-enriched supernatant was centrifuged again for 15 min at 55,000 g to obtain the crude membrane pellet. Both pellets were resuspended in buffer, potted again and then layered on a 38.3 % sucrose gradient. The plasma membrane-enriched fraction was centrifuged in an ultracentrifuge at 80,000 g for 50 min. The LDM-enriched fraction was centrifuged for 65 min at 80,000 g. Both membrane-pellets were collected then at the respective sucrose interphase, diluted four-fold with buffer and dispersed by mechanical disruption through a syringe needle. Finally the plasma membrane-enriched fraction was centrifuged for 40 min at 200,000 g. The LDM-enriched fraction was centrifuged for 60 min at 250,000 g. To determine the purity of the membrane fractions marker enzymes were measured in the cellular homogenate and the membrane fractions. Ouabain sensitive Na^+K^+ -ATPase served as a marker for plasma membrane [36], uridine diphosphate (UDP) galactosyl-transferase as marker for LDM [37]. Protein concentration was determined by Bio-Rad assay.

Table 1. Animal characteristics

	SHR rats	Wistar-Kyoto and Lewis rats
Blood pressure [mm Hg]	215 $\pm$ 5	154 $\pm$ 8
Fasting insulin levels [$\mu\text{U/ml}$]	29.6 $\pm$ 8	19.5 $\pm$ 4
Body weight [g]	335 $\pm$ 3	319 $\pm$ 2

SHR, spontaneous hypertensive rat

Western blotting

Membranes (15 μg protein) were subjected to 10 % SDS-PAGE [34] in the presence of 10 mmol/l dithiothreitol. Proteins were transferred to nitrocellulose by electroblotting (buffer: 192 mmol/l glycine, 25 mmol/l Tris, 10 % methanol, pH = 7.4, 2 h 250 mA). Following transfer the filters were blocked with 5 % non-fat dry milk in Tris buffered saline for 1 h at room temperature and subsequently incubated with the first antibody (GLUT 4 and GLUT 1 dilution in Tris buffered saline with 1 % dry milk) overnight at 4 °C. After incubation with the specific antibody, nitrocellulose was washed several times in Tris buffered saline with 20 mmol/l Tris, 2.5 % non-fat dry milk, 1 % Triton X-100.

Horseradish peroxidase labelled IgG was then bound to the immunocomplexes. Visualization of the immunolabelled bands was carried out by autoradiography after addition of a chemiluminescence reagent.

Scanning densitometry

After autoradiography the density of the labelled 45 kDa bands was measured using a type Elscript 400 scanner from Hirschmann GmbH (Munich, FRG). Measurements were performed with a 0.2 mm window in an extinction range of 2 or 4 by 100 μm steps.

Statistical analysis

The data are expressed as mean values and SD.

Results

Insulin receptor

Table 1 shows the characteristics of SHR, Lewis and Wistar rats. As described earlier blood pressure values of 215 $\pm$ 5 mm Hg were found in SHR, while Lewis and Wistar

Fig. 2. **a** Displacement curves of ^{125}I -insulin to partially purified insulin receptors from skeletal muscle of spontaneous hypertensive rats (Δ) and Lewis (●) and Wistar (□) rats. ^{125}I -insulin-binding was performed as described in the Materials and methods section. Mean values of five preparations are shown. **b** Scatchard plots of ^{125}I -insulin binding to partially purified insulin receptors from skeletal

muscle of spontaneous hypertensive rats (Δ), Lewis (●) and Wistar (□) rats. Mean values of five preparations $\pm$ SEM are shown. B/F, bound/free; Mean $\pm$ SEMs of high affinity binding sites for the three groups: $n = 5$; SHR: $1.08 \times 10^{-10} \pm 0.32 \times 10^{-10}$; Wistar: $1.08 \times 10^{-10} \pm 0.42 \times 10^{-10}$; Lewis: $1.30 \times 10^{-10} \pm 0.47 \times 10^{-10}$

rats had clearly lower levels. Furthermore insulin levels were increased in SHR compared to the normotensive control animals indicating the earlier described insulin resistance of these animals [26, 27]. Receptors were isolated from skeletal muscle of all three groups in parallel experiments. Figure 2a shows the binding values of equal amounts of protein from the wheat germ agglutinin purification step. Insulin displacement curves are shown in Figure 2a. It is evident that identical binding values were found for SHR, Lewis and Wistar rats. In Figure 2b the same data are presented as Scatchard plots. No alteration of receptor affinity or receptor number can be detected. For chemical crosslinking of receptors equal amounts of protein were used. Figure 3 shows the labelling of α -subunits from insulin receptors from all three groups. Scanning densitometry of the labelling of the bands revealed equal amounts of ^{125}I -B 26 insulin crosslinking to receptors from all

three groups as already expected on the basis of the binding data. We now used equal amounts of receptors to compare the insulin effect on ^{32}P incorporation into the β -subunit. Figure 4a shows autoradiograms of the ^{32}P incorporation into the β -subunit of partially purified insulin receptors from SHR, Wistar and Lewis rats. To determine ^{32}P incorporation into the β -subunits the bands were cut out from the gel and the radioactivity was measured in a beta-counter. The mean values $\pm$ SEM of five preparations from each group performed in parallel did not reveal any difference in maximal ^{32}P incorporation and submaximal values. When the data were plotted as shown in Figure 4b as percentage of the maximal insulin effect, it is evident that there is no difference in the dose-response curve of the activation of β -subunit autophosphorylation between the three groups. For comparison isolated receptors were also partially purified in parallel from the skeletal muscle of lean, insulin sensitive and obese hyperinsulinaemic insulin resistant Zucker rats. The data which are used as a further control are shown in Figure 5. As described earlier [11] there is a decrease of the ^{32}P incorporation into the β -subunit of the receptor isolated from the skeletal muscle of the insulin resistant Zucker rat. Figure 6a shows the effect of insulin on substrate phosphorylation. Similarly as seen for autophosphorylation no difference can be detected between SHR, Lewis or Wistar rats. Figure 6b shows for comparison the effect of insulin on substrate phosphorylation by receptors isolated from lean or obese Zucker rats. Clearly a reduction of the kinase activity from the skeletal muscle of the Zucker rats can be seen.

Glucose transporter

To assess the glucose transporter level skeletal muscle was fractionated as described in the Materials and methods section. To determine the purity of the plasma membrane-enriched fraction and the LDM enriched fraction, Na/K-ATPase and UDP-galactosyl-transferase were deter-

Fig. 3. Autoradiogram showing crosslinking of ^{125}I -B 26 -insulin to partially purified insulin receptors from skeletal muscle of spontaneous hypertensive rats (SHR), Lewis and Wistar rats. Crosslinking for equal amounts of the eluate from the wheat germ agglutinin columns was performed as described in the Materials and methods section. A, SHR; B, Wistar-Kyoto rats; C, Lewis rats

Fig. 4a,b. Autoradiogram of $[^{32}\text{P}]$ -incorporation into the β -subunit of partially purified insulin receptor from the skeletal muscle of spontaneous hypertensive rats (SHR), Wistar (WK) and Lewis rats. Insulin receptor was phosphorylated with $[^{32}\text{P}]$ -ATP after *in vitro* insulin stimulation as described under experimental procedures and then applied to SDS-PAGE gels. **a** Three representative autoradiograms showing the dose-response of insulin-stimulated $[^{32}\text{P}]$ -incorporation into the 95 kDa β -subunit of the insulin receptor from SHR (top panel), WK (centre panel) and Lewis (lower panel) muscle. **b** The average dose-response curves of the SHR (Δ) and WK ($\square$) and Lewis ($\bullet$) are plotted as % of maximal $[^{32}\text{P}]$ phosphate incorporation (mean $\pm$ SEM)

Fig. 5. Autophosphorylation of the insulin receptor from the skeletal muscle of the insulin resistant Zucker rat. $[^{32}\text{P}]$ -incorporation into the β -subunit of partially purified insulin receptors from skeletal muscle of lean ($\bullet$) and obese ($\square$) Zucker rats was performed as described in the Materials and methods section. Labelled bands were cut from the gel and counted. Mean values of three experiments are shown.

Fig. 6. a Insulin dose-response curve of Poly(Glu 4: Tyr 1) phosphorylation for spontaneous hypertensive rats (Δ) and control Wistar ($\square$) and Lewis ($\bullet$) rats. The substrate phosphorylation assay was carried out with each muscle sample as described under experimental procedures for 1 h using 1 mmol/l Poly(Glu 4: Tyr 1). The kinase activity was measured in the absence of insulin and at 0.1–1000 nmol/l insulin using equal amounts of partially purified insulin receptor as determined by insulin binding. Mean values $\pm$ SEM of five experiments are shown. **b** Insulin dose-response curve of Poly(Glu 4: Tyr 1) phosphorylation for lean ($\bullet$) and obese ($\square$) Zucker rats. The substrate phosphorylation assay was carried out with each muscle sample as described under experimental procedures for 1 h using 1 mmol/l Poly(Glu 4: Tyr 1). The kinase activity was measured in the absence of insulin and at 0.1–1000 nmol/l insulin using equal amounts of partially purified insulin receptor as determined by insulin binding. The curves represent the mean value of three experiments

Table 2. Protein recovery distribution of marker enzyme specific activities and activity ratios in subcellular membrane fractions from skeletal muscle

Membrane fractions	Plasma membranes	Low density membranes	Homogenate
Ouabain sensitive Na/K-ATPase (nmol Pi · min ⁻¹ · mg of protein ⁻¹)			
	44.3 ± 5.72	14.5 ± 2.54	2.8 ± 0.44
Activity ratio (%)	100	32.3	6.3
UDP-galactosyl-transferase (nmol · min ⁻¹ · mg of protein ⁻¹)			
	2.27 ± 0.19	5.5 ± 0.73	1.3 ± 0.1
Activity ratio (%)	41.3	100	23.6

Results are expressed as the mean ± SEM of each experiment. Activity ratios are calculated by setting the specific activity of the most enriched membrane fraction at 100% and those in other membrane fractions relative to that specific value

mined as marker enzymes (Table 2). Compared to the crude homogenate Na/K-ATPase was enriched approximately 15–18 fold in the plasma membrane fraction. The cross contamination of both fractions is in a similar range to that found in adipocytes [38]. Both fractions were used for PAGE and Western blotting. Figure 7 shows an autoradiogram of an immunoblot with antibody directed against GLUT 4. In membranes from both rats clearly the insulin induced decrease of LDM-labelling and a concomitant increase of plasma membrane labelling is visible. The analogous experiments were performed with antibody directed against GLUT 1. Figure 8 shows an autoradiogram of GLUT 1 labelling. The largest amount of label is found in the LDM fraction. In contrast to the data obtained with GLUT 4 no effect of insulin treatment is visible. The insulin stimulation experiment was repeated once with the same result, i.e. no difference in the effect of insulin between SHR and Wistar-Kyoto rats was detectable. Unstimulated GLUT 4 levels were determined in four experiments. Scanning densitometry revealed no differences between the groups.

Discussion

The data show that no abnormality of the effect of insulin on the insulin receptor kinase from skeletal muscle of SHR is detectable. This discriminates this insulin-resistant animal model from other insulin-resistant animal models such as the Zucker rat [10], the gold-thio-glucose-mouse [13], ob/ob mice [14] and streptozotocin-treated rats [15] as well as human Type 2 diabetes [10, 18, 19]. This suggests that insulin resistance in hypertension is possibly caused by different molecular mechanisms than the insulin resistance in other pathophysiological states. On the other hand the interpretation of our data is limited to this specific animal model of hypertension. It is possible that SHR might not be a representative model for other states of hypertension. Furthermore when SHR are compared to other models of insulin resistance clear differences concerning the degree of insulin resistance are found. The moderate increase of insulin levels suggests that the insulin resistance in SHR is

mild compared to obese Zucker rats which show a clearly more pronounced hyperinsulinaemia [12, 13].

Several mechanisms have been proposed for the effect of insulin on glucose transport in skeletal muscle. While some authors favour a combined model involving carrier translocation and carrier activation as that seen in adipocytes [16] others suggest that only carrier activation is important [39]. In our study we determined two factors, carrier number and carrier translocation. We could not detect differences in carrier numbers. The data concerning GLUT 4 translocation in skeletal muscle clearly confirm the earlier demonstrated insulin-induced translocation from intracellular membranes to the plasma membrane [16]. Similarly, as described by others, an approximately two-fold increase of GLUT 4 labelling is found [17]. There is no evidence for a defect of GLUT 4 translocation in the SHR. An abnormality of intrinsic carrier activity as a possible reason for the insulin resistance clearly cannot be excluded with the present study.

Fig. 7. Western blot analysis of GLUT 4 in subcellular membrane fractions of rat hindlimb muscles under basal conditions and after stimulation with insulin in vivo. Total protein 15 µg from plasma membranes (PM) and low density membranes (LDM) was immunoblotted using anti-GLUT 4 antiserum. Immunolabelled bands were visualized by horseradish peroxidase-labelled anti-rabbit Ig followed by chemiluminescence as described in the Materials and methods section. B, basal; Ins, insulin stimulation in vivo as described. The insulin stimulation experiment was repeated once with the same result **Fig. 8.** Western blot analysis of GLUT 1 in subcellular membrane fractions of rat hindlimb muscles under basal conditions and after stimulation with insulin in vivo. Total protein 15 µg from plasma membranes (PM) and low density membranes (LDM) was immunoblotted using anti-GLUT 1 antiserum. Immunolabelled bands were visualized by horseradish peroxidase-labelled anti-rabbit Ig followed by chemiluminescence as described in the Materials and methods section. B, basal; Ins, insulin stimulation in vivo as described. The insulin stimulation experiment was repeated once with the same result

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