Elevated protein tyrosine phosphatase activity and increased membrane viscosity are associated with impaired activation of the insulin receptor kinase in old rats

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Insulin resistance is very common in the elderly, and may be associated with glucose intolerance or frank diabetes. In previous studies we demonstrated that insulin resistance in old Wistar rats is associated with decreased autophosphorylation and activation of the hepatic insulin receptor kinase (IRK) *in vivo*. We now show that this defect can be reproduced *in vitro*, where the extent of insulin-induced activation of IRK in liver membranes of old rats was decreased by ~ 50% compared with young controls. The defect could be largely abolished after solubilization of the membranes with Triton X-100. We also show that: (*a*) the viscosity of membranes from the old rats was significantly ($P < 0.001$, $n = 4$) higher (by 15%) compared with young controls; (*b*) incubation of plasma membranes from old animals with lecithin liposomes, which lowered their cholesterol levels, partially abolished the defect in IRK activation; and (*c*) Triton extracts of liver membranes prepared from old rats did not interfere with the activation of IRK derived from young controls. Additionally, non-membrane components did contribute to the development of this defect. We observed a significant (~ 30%) ($P < 0.001$, $n = 18$) elevation of cytosolic protein tyrosine phosphatase (PTP) activity directed against the β subunit of the insulin receptor in livers of old rats. No such elevation of PTP activity could be demonstrated with synthetic substrates. Our findings are consistent with a model in which increased membrane viscosity as well as enhancement of a cytosolic PTP activity both markedly inhibit the activation *in vivo* of the hepatic IRK in old animals.

INTRODUCTION

The insulin receptor (IR) is a transmembrane glycoprotein consisting of two α and two β subunits linked by disulphide bonds (reviewed in [1,2]). Insulin binding to the α subunits leads to autophosphorylation of a tyrosine kinase which forms part of the receptor β subunit. A variety of genetic and acquired syndromes of insulin resistance are associated with defects in the structure, function and action of the IR [3–7]. In several syndromes of extreme insulin resistance, as well as in a few cases of non-insulin-dependent diabetes mellitus (NIDDM), the defects in IR function are associated with mutations in the IR gene [3–6]. More common syndromes of acquired insulin resistance, such as NIDDM, obesity and aging, have not yet been linked to such mutations, however.

In a previous study [7] we demonstrated that insulin resistance in old Wistar rats is associated with impaired (50%) autophosphorylation and activation of the hepatic insulin receptor kinase (IRK) *in vivo*. Since there was no defect in insulin-dependent kinase activity of receptors purified from non-treated old rats [7], we concluded that the intrinsic hepatic kinase activity in the old animals is normal, and the impairment of the activation *in vivo* is due to elements distal to the receptor itself. IRK activity is known to be regulated by several elements [1] including serine/threonine kinases [8–11], protein tyrosine phosphatases (PTPs) [12–14], specific protein inhibitors [15,16] and specific lipids [17–20]. In the present study we attempted to determine which elements contribute to the impaired activation of the hepatic IRK in old rats. For this purpose we used a model system *in vitro*, in which the contribution of lipid membrane components as well as other cellular constituents could be evaluated.

MATERIALS AND METHODS

Materials

[γ-^{32}P]ATP and mono[^{125}I]iodo-labelled (at tyrosine-14 of the A chain) human insulin were from Amersham International, Amersham, Bucks., U.K. ATP, CTP and poly(Glu,Tyr)(4:1) were from Sigma. Pig insulin was from Elanco, Indianapolis, IN, U.S.A. Wheat-germ agglutinin (WGA) coupled to agarose was from Bio-Makor, Rehovot, Israel. Rabbit polyclonal antibodies generated against a synthetic peptide corresponding to amino acids 1305–1324 of the human IR (ST-50) were generously supplied by P. Gorden and S. I. Taylor, N.I.H., Bethesda, MD, U.S.A.

Animals

Female Wistar rats were fed *ad libitum* and fasted for 24 h before use. Two groups of animals were studied; young rats (2–3 months old, 169 ± 17 g body wt.; $n = 27$) and old rats (24–27 months old, 221 ± 17 g body wt.; $n = 16$).

Insulin administration to animals

Animals were anaesthetized with Nembutal (60 mg/kg body wt.), and insulin was injected in PBS via the portal vein. Control

Abbreviations used: IR, insulin receptor; IRK, insulin receptor kinase; IRS-1, insulin receptor substrate 1; PMSF, phenylmethanesulphonyl fluoride; PTP, protein tyrosine phosphatase; NIDDM, non-insulin-dependent diabetes mellitus; WGA, wheat-germ agglutinin; DPH, 1,6-diphenyl-1,3,5-hexatriene.

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rats were injected with PBS alone. After injection, livers were excised and frozen in liquid N_2 within 30 s.

Preparation of crude liver membranes

A portion (0.8 g) of liver (frozen in liquid N_2) was homogenized with 50 mM Hepes/1 mM EDTA/1 mM EGTA/0.25 M sucrose containing 0.2 mM phenylmethanesulphonyl fluoride (PMSF), 5 μg/ml leupeptin, 10 μg/ml trypsin inhibitor and 10 μg/ml aprotinin, at pH 7.4. The homogenate was centrifuged at 450 *g* for 15 min (4 °C), and the supernatant was further centrifuged at 100000 *g* for 1 h. The pellet was resuspended in KRP buffer (118 mM NaCl, 5 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 10 mM Na_2HPO_4, 0.2 mM PMSF, 5 μg/ml leupeptin, 10 μg/ml trypsin inhibitor and 10 μg/ml aprotinin, pH 7.4), and centrifuged for 30 min at 100000 *g*. The final pellet was resuspended in KRP buffer supplemented with 10% glycerol and stored at −70 °C.

Generation of lectin-purified IR

To generate lectin-purified IRs, Triton-soluble particulate fractions were subjected to affinity chromatography over columns of WGA coupled to agarose as previously described [7]. Glycoproteins, including the IR, were eluted from the columns with buffer A (50 mM Hepes, pH 7.6, 150 mM NaCl, 0.1% Triton X-100, 0.5 M *N*-acetyl-D-glucosamine and 10% glycerol) [7]. These fractions were referred to as 'WGA eluates'.

Determination of IR number by ^{125}I-insulin binding assay

The assay was performed on WGA-purified IR preparations as previously described [21]. When indicated, ^{125}I-insulin binding to IR in intact membranes was also assayed as for WGA-purified IR preparations. IR concentration was calculated from the ratio of bound/free (B/F) insulin, as previously described.

Assay of IRK activity

IRK activity was determined essentially as described previously [22]. Samples (50 μl) of eluates from WGA columns (in buffer A), normalized for receptor number, were incubated (30 min, 22 °C) with 10 μl portions of 0.1 μM insulin in buffer B (50 mM Hepes, 0.01% BSA, 0.1% Triton X-100, pH 7.6) or buffer B alone. Phosphorylation was initiated with 40 μl of a 'reaction mix' containing 125 μM [γ-^{32}P]ATP, 2.5 mM CTP, 100 mM magnesium acetate, 0.1% Triton X-100 and 5 mg/ml poly(Glu,Tyr) (4:1). Reactions were allowed to proceed for 10 min at 22 °C and were terminated by applying 80 μl samples on to Whatman 3 MM filter papers that were extensively washed in 10% trichloroacetic acid/10 mM sodium pyrophosphate, rinsed with ethanol, dried, and counted for radioactivity by liquid scintillation in a Betamatic counter. One unit of kinase activity was defined as the amount of enzyme required to incorporate 1 pmol of ^{32}P into poly(Glu,Tyr) (4:1) during 10 min.

Autophosphorylation of IR

Autophosphorylation was carried out essentially as for phosphorylation of exogenous substrates, except that poly(Glu,Tyr) (4:1) was omitted from the reaction mixture and magnesium acetate was replaced with manganese acetate to yield a final concentration of 4 mM. The reaction was terminated by adding 2.5-fold-concentrated Laemmli buffer [23] containing 5 mM ATP, pH 6.8. After heating for 5 min at 95 °C, the samples were resolved by SDS/PAGE (7.5% gels), dried and radiographed. In some experiments, as indicated, the autophosphorylation assay was performed in the presence of non-radioactive ATP (1 mM).

Autophosphorylation of IR in liver membranes

The reaction was carried out as the autophosphorylation of the IR, except that non-radioactive ATP (1 mM) was used. The reaction was terminated by adding 'stopping solution', composed of 0.25 M EDTA, 2 mM vanadate, 80 mM β-glycerophosphate, 10 mM sodium pyrophosphate, 100 mM NaF, 10 μg/ml aprotinin, 10 μg/ml trypsin inhibitor, 5 μg/ml leupeptin, 1 mM PMSF, 1% Triton X-100 and 50 mM Hepes, pH 7.6. The insoluble material was precipitated by centrifugation at 12000 *g* for 30 min, and the IR present in the supernatant were immunoprecipitated by using antibodies directed against the C-terminus of the IR (ST-50) antibodies). Laemmli sample buffer [23] was then added to the immunocomplex, and the proteins were subjected to SDS/PAGE and immunoblotting with anti-phosphotyrosine antibodies.

Western blotting with anti-phosphotyrosine antibodies

Affinity-purified anti-phosphotyrosine antibodies were generated as previously described [24]. The proteins were electrophoretically transferred to nitrocellulose, incubated with anti-phosphotyrosine antibodies and with ^{125}I-labelled goat anti-rabbit antibodies as previously described [24].

Partial purification of IRK inhibitor from rat liver cytosol

Livers of young (3 months) or old (24–27 months) Wistar rats were frozen in liquid N_2 and ground to a powder. Then 0.5 g of powder was homogenized in a Heidolph homogenizer in 3 vol. of 0.25 M sucrose/4 mM EDTA/0.14 M 2-mercaptoethanol, containing 0.2 mM PMSF, 5 μg/ml leupeptin, 10 μg/ml trypsin inhibitor, 10 μg/ml aprotinin and 10 mM Mes, pH 6.5. The suspension was centrifuged at 100000 *g* for 1 h at 4 °C, and the supernatant was collected and applied to a DEAE-cellulose column which had been pre-equilibrated with buffer C (5 mM Mes, 1 mM EDTA, 9 mM NaCl and 15 mM 2-mercaptoethanol, pH 6.5). After incubation for 1 h the effluent was collected and the column was washed with 5 column vol. of buffer C. Proteins bound to the column were eluted with 1.0 M NaCl in buffer C.

Assay of IRK inhibitor activity

Lectin-purified IR (50–200 μg/ml, in buffer A) were incubated with 0.1 μM insulin for 30 min at 22 °C, and then for an additional 10 min with 50 μl of inhibitory fractions (effluents of the DEAE-cellulose column in buffer C). Assay of IRK activity was subsequently performed by adding 25 μl of a 'reaction mixture' to yield the following final concentrations: 50 μM [γ-^{32}P]ATP, 1 mM CTP, 40 mM magnesium acetate, 2 mg/ml poly(Glu,Tyr) (4:1) and 0.1% Triton X-100. The reaction proceeded for 10 min and was terminated as described under 'Assay of IRK activity'.

Extraction of PTPs from rat liver

Livers frozen in liquid N_2 were homogenized in buffer D (3 ml/g), composed of 0.25 M sucrose, 5 mM EDTA, 0.5 mM EGTA, 1 mM dithiothreitol, 2 mM benzamidine, 0.2 mM PMSF,

5 μg/ml leupeptin, 10 μg/ml trypsin inhibitor, 10 μg/ml aprotinin and 50 mM Hepes, pH 7.4. The homogenate was centrifuged at 500 *g* for 15 min at 4 °C, and the supernatant was further centrifuged for 60 min at 100000 *g* (at 4 °C). The resulting supernatant was kept and is referred to as the cytosolic fraction. The pellet, referred to as the particulate (membrane) fraction, was solubilized in 1% Triton X-100 in the same buffer. After centrifugation for 60 min at 100000 *g* (4 °C), the supernatant was collected and is referred to as the 'membrane extract'.

Assay of PTP activity with ^{32}P-labelled poly(Glu,Tyr)(4:1) as substrate

The assay was based on measurements of the decrease in amount of ^{32}P-labelled poly(Glu,Tyr)(4:1), and was carried out as previously described [25]. The reaction was terminated by applying samples on to Whatman filter papers that were extensively washed in 10% trichloroacetic acid, dried, and counted for radioactivity by liquid scintillation. The percentage decrease in trichloroacetic acid-precipitable ^{32}P-poly(Glu,Tyr)(4:1) was taken as a measure of PTP activity.

Immunoprecipitation of the IR by anti-receptor antibodies

A 7.5 μl portion of ST-50 antibodies were incubated with a 30 μl suspension of Protein A–Sepharose (50%, v/v) and 100 μl of 0.1 M Tris/HCl buffer, pH 8.5, for 2 h at 4 °C. The precipitate was collected by centrifugation for 3 min at 12000 *g* at 4 °C. After three washes with Tris/HCl buffer, pH 8.5, the complex was resuspended with 100 μl of lectin-purified IR preparation (in buffer A), and was shaken for 18 h at 4 °C. After precipitation (as described above), the immune complexes were washed three times in HTN buffer (150 mM NaCl, 0.1% Triton X-100 and 50 mM Hepes, pH 7.6) and subjected to autophosphorylation as follows.

Autophosphorylation of immunoprecipitated IRs

The aforementioned precipitated receptors were suspended in 20 μl of HTN buffer containing 10 μg/ml aprotinin, and incubated with 10 μl of 0.1 μM insulin for 30 min at 22 °C. Phosphorylation was initiated by addition of 20 μl of a reaction mixture to yield the following final concentrations: 5 μM [γ-^{32}P]ATP (1 Ci/mmol), 20 mM $MnCl_2$, 0.1% Triton X-100 and 50 mM Hepes, pH 7.6. The reaction was allowed to proceed for 10 min at 30 °C, after which the immune complexes were extensively washed with HTN buffer containing 2 mM EDTA. The immunoprecipitated and phosphorylated receptors were subsequently used for assay of receptor-specific PTP activity.

Assay of PTP activity towards the IR

^{32}P-labelled IRs, prepared as described above, were co-incubated with 10 μl of dithiothreitol (1 mM final concn.) and 80 μl of the following liver fractions: cytosol (0.8 mg/ml), membrane extract (0.8 mg/ml) or WGA-eluted proteins (0.2 mg/ml) for 30 min at 30 °C. The reaction was terminated with 25 μl of Laemmli buffer [23] and heating at 95 °C for 5 min. The samples were then subjected to SDS/PAGE and the gel was dried and autoradiographed.

Preparation of lecithin liposomes

Liposomes were prepared from egg lecithin (Lipid Products, Nutfield, Surrey, U.K.) by sonication in PBS and stored at 4 °C as described previously [26]. The lecithin liposomes (10 mg/ml) were used during the first 3 days after preparation.

Estimation of membrane fluidity

1,6-Diphenyl-1,3,5-hexatriene (DPH) was used as a fluorescent probe for estimation of plasma-membrane fluidity [27]. DPH was dissolved in tetrahydrofuran at a concentration of 2.0 mM, just before labelling, and was further diluted 1:1000 in KRP buffer containing 100 μg of liver membranes. Fluorescence polarization was measured after a 30 min incubation at 37 °C with a fluorescence-depolarization instrument, as described previously [28]. Fluorescence polarization and intensity were obtained by measurements of $I_\parallel$ and $I_\perp$, where $I_\parallel$ and $I_\perp$ are the fluorescence intensities polarized parallel and perpendicular, respectively, to the direction of the polarized excitation beam. These values are related to the degree of fluorescence polarization, P, by the equation [29]: $P = (I_\parallel/I_\perp - 1)/(I_\parallel/I_\perp + 1)$. The microviscosity, η, is related to P by the equation: $\eta = 2P/(0.46 - P)$, as described in [29].

Protein determination

Protein concentration was determined by a modified Lowry method [30] with bovine serum albumin as standard.

Statistical analysis

In many experiments, the results are expressed as means ± S.D. The level of significance of the differences between the mean values was determined by unpaired (one-tail) t test.

RESULTS

Defect in autophosphorylation *in vivo* of the IRK in livers from old rats

We have previously shown [7] that old (24–27 months) Wistar rats exhibit impaired activation of the IRK *in vivo*. Insulin injection induces tyrosine phosphorylation of the receptor β subunit, but the extent of phosphorylation in old animals is only 50% of that of the young controls. This impaired autophosphorylation *in vivo* could not be attributed to a defect in insulin binding in the old animals [7], nor could it be accounted for by differences in receptor concentration, since all samples contained equal amounts of receptors, as judged both by ^{125}I-insulin binding studies and by immunoblotting with an antibody (ST-50) directed against the C-terminal end of the receptor β subunit. Additional experiments were therefore carried out to determine the elements which contribute to the impaired activation of the hepatic IRK in old rats. To this end, several approaches were taken.

Effects of an endogenous cytosolic inhibitor on IRK activity

Previous studies [15,16] revealed the presence of a protein inhibitor of the IRK whose concentration is elevated in fibroblasts from patients with insulin resistance and NIDDM [16]. To determine whether the decreased activation *in vivo* of IRK in old rat livers could result from an elevation of such an inhibitory activity, cytosolic liver extracts were prepared, applied to DEAE-cellulose columns, and the effluent was collected. Proteins bound to the column were eluted with 1 M NaCl. An IRK-inhibitory activity could be detected in both the DEAE-cellulose effluent and eluate fractions, but the activity in the effluent was 4-fold higher (results not shown). Incubation of the DEAE-cellulose effluent with lectin-purified receptors inhibited IRK activity in a dose-dependent manner (Figure 1a) with 50% inhibition being

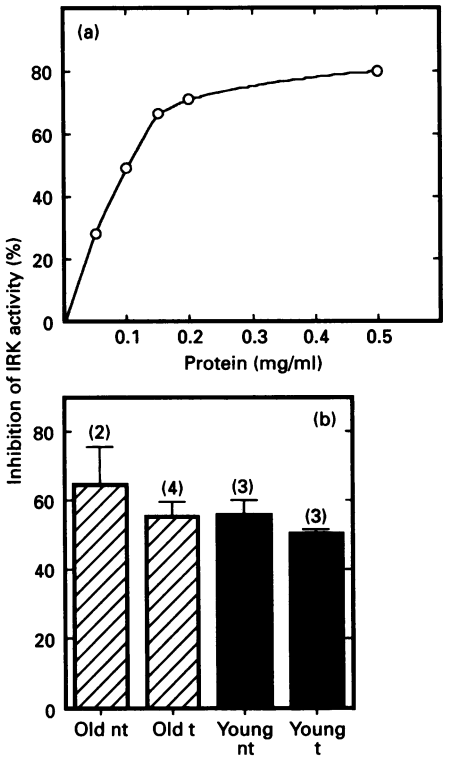

Figure 1 Effect of cytosolic liver extracts on IRK activity

(**a**) Lectin-purified IR preparations were incubated (30 min, 23 °C) in the presence of 0.1 μM insulin and then for a further 15 min in the presence of increasing concentrations of a DEAE-cellulose effluent fraction (in buffer C). Phosphorylation reactions were initiated by addition of a reaction mixture containing poly(Glu,Tyr) (4:1) as described in the Materials and methods section. 100% IRK activity was defined as activity in the presence of buffer C only. (**b**) Liver cytosolic extracts derived from young or old rats that were injected with either PBS (Young nt, Old nt) or insulin (Young t, Old t) were applied to DEAE-cellulose columns, and the effluents were collected as described in the Materials and methods section. Samples (0.1 mg of protein/ml) were assayed for their inhibitory effect on IRK activity. Results are presented as percentage inhibition (mean ± S.D.). Numbers of rats are indicated in parentheses.

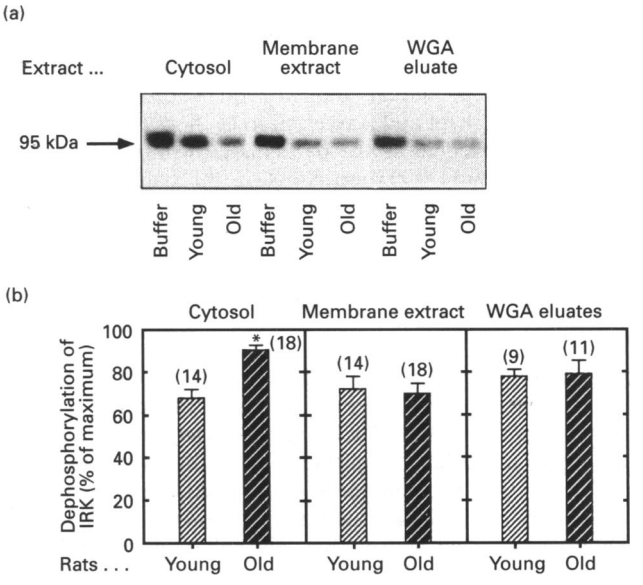

Figure 2 Dephosphorylation of autophosphorylated IR by liver extracts derived from young and old rats

IRs of young non-injected rats were immunoprecipitated and then autophosphorylated as described in the Materials and methods section. The phosphorylated receptors were extensively washed and resuspended in 10 μl of 10 mM dithiothreitol with 90 μl of buffer alone or with one of the following liver extracts derived from young or old rats: cytosol (0.8 mg/ml), 'membranes extracts' (0.8 mg/ml) or 'WGA eluate' (0.2 mg/ml). Dephosphorylation was carried out as described in the Materials and methods section. Samples were analysed by SDS/PAGE and autoradiographed. The results of a representative experiment are shown in (**a**). The intensity of the 95 kDa band corresponding to the IR β subunit was quantified by densitometry. The mean values (± S.E.M.) of dephosphorylation of several experiments are shown in (**b**). Numbers of animals assayed are indicated in parentheses. $^*P < 0.0001$ according to Student's unpaired t test.

obtained with 0.1 mg/ml of the inhibitory fraction. However, we found no significant difference in the inhibitory activity derived from livers of either young or old rats (Figure 1b), and both inhibited the IRK activity by 55–65% at 0.1 mg/ml. Insulin injection into the portal vein had no effect on this inhibitory activity (Figure 1b). We therefore conclude that the impaired activation of the hepatic IRK in old rat liver most likely does not involve a significant elevation of cytosolic inhibitory activity.

PTP activity in young and old rat liver

(i) PTP activity towards the phosphorylated β subunit of the IR

Elevated PTP activity could be one of the underlying causes for the deceased autophosphorylation *in vivo* of the IRK in old rats. To address this possibility, livers from either young or old rats were extracted, and cytosolic, particulate and lectin-purified (glycoprotein-enriched) fractions were prepared. ^{32}P-labelled receptors were used as substrates for these PTPs. PTP activity toward the ^{32}P-labelled IR β subunit (IR-PTP) was detected in all fractions (Figure 2a); however, there were no significant differences in this activity in membranes or lectin-purified liver preparations between young and old rats. By contrast, the cytosolic fractions of old rat liver possessed significantly ($P < 0.001$) higher ($\sim 30\%$) IR-PTP activity than did those derived from young controls (Figure 2b). This IR-PTP activity was linear for at least 30 min, indicating that the differences from young and old rats were not underestimated because of rate-limiting amounts of ^{32}P-labelled IR. No differences in IR-PTP activity were found between insulin- or PBS-injected young or old animals (results not shown), suggesting that, if present, insulin-induced alterations in IR-PTP activity were too small to be detected in our system.

(ii) PTP activity towards ^{32}P-labelled poly(Glu,Tyr)(4:1)

To determine whether the higher dephosphorylation rate of IR in old rats was due to increased activity of a specific PTP (i.e. IR-PTP), or whether it reflected a general age-related increase in total hepatic PTP activity, we used ^{32}P-labelled poly(Glu,Tyr) (4:1) as a common PTP substrate. With this substrate, we found no difference between cytosolic PTP activity in young and old rats (results not shown). Similarly, no difference was found in PTP activity obtained from control or insulin-injected rats (results not shown). It appears therefore that aging is accompanied by a selective increase in specific PTPs, such as those directed towards the IR. Furthermore, our results suggest that elevation of IR-PTP activity may contribute to the decreased activation *in vivo* of the IRK in old rat liver.

Effect of membrane viscosity on IRK activation in old rats

Aging is accompanied by changes in plasma membrane composition, elevation of membrane cholesterol content, and an

Table 1 Microviscosity of liver membranes derived from young or old rats

Liver membranes were prepared as described in the Materials and methods section from 4 old and 3 young rats. The apparent mean microviscosity ($\eta \pm$S.E.M.) of these membrane preparations was determined by using the fluorescent probe DPH and measuring its fluorescence depolarization as described in the Materials and methods section. The significance of the difference in the results obtained from the two groups of rats was analysed by Student's unpaired *t* test.

Animals	Mean *P*	Mean η	t test
Young	0.207 ± 0.0016	0.823 ± 0.012	$P = 0.001$
Old	0.224 ± 0.0019	0.946 ± 0.016	

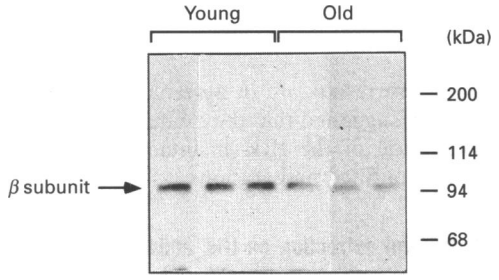

Figure 3 Activation of IRK in liver membranes of PBS-injected young and old rats

Liver membranes were prepared from PBS-injected young or old rats (3 animals each). A 600 μg portion of membranes was incubated with 0.1 μM insulin for 1 h. Autophosphorylation for 1 min, followed by immunoprecipitation and SDS/PAGE, was carried out as described in the Materials and methods section. An autoradiogram of the gel immunoblotted with anti-phosphotyrosine antibodies is shown. Densitometric analysis of the 95 kDa bands revealed that autophosphorylation of IRK in the old rats decreased, on average, by 66% compared with young controls. The kDa values represent migration of protein standards.

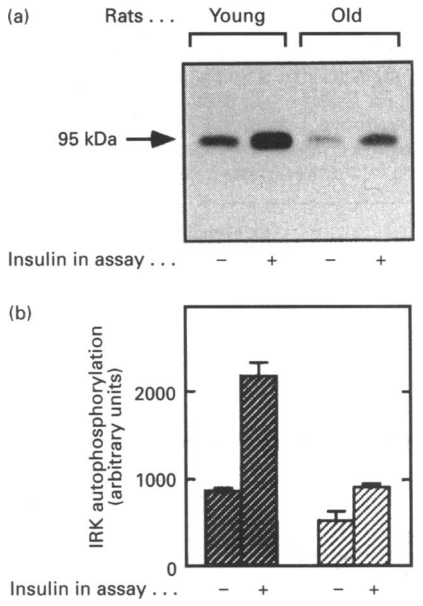

Figure 4 Effects of insulin treatment *in vitro* on IRK activity in membranes from insulin-injected rats

Liver membranes were prepared from insulin-injected young and old rats. After incubation with either 0.1 μM insulin or buffer for 1 h at 23 °C, the membranes were subjected to autophosphorylation and were further processed as described in the legend to Figure 3. An autoradiogram of a representative experiment (one of three) is presented in (**a**). The intensity of the phosphorylated IR β subunits was quantified by densitometry and is presented in (**b**). The results are expressed as means ($\pm$S.E.M.) of 3 animals in each treatment.

increase in membrane viscosity [31,32]. Consistent with these findings, we detected a significant 15% increase in the viscosity of membranes derived from livers of old animals (Table 1). Such an increase could alter the capacity of IR to undergo aggregation, a necessary step in the activation process of the IRK [33–35]. We were therefore interested in determining how the decreased membrane fluidity could affect the activation of the IRK.

No autophosphorylation of IRK could be detected in the absence of insulin in membranes prepared from young livers (results not shown). When membranes were preincubated with insulin *in vitro*, half-maximal and maximal autophosphorylation of the IR β subunit were observed at 200 μM and 1 mM ATP respectively (results not shown). This autophosphorylation was decreased by 50–75% in membranes from old rats (Figure 3), suggesting that the elements which contribute to the impaired activation of the IRK in intact livers [7] are present and also exert their effects in membranes of old animals.

The activation of IRK in membranes from insulin-injected rats was also compared. Autophosphorylation of IRK could be detected in membranes from both young and old rats, even in the absence of insulin added *in vitro* (Figure 4a). This was in contrast with the results obtained with membranes from PBS-injected livers, where no autophosphorylation *in vitro* could be detected in the absence of insulin. Autophosphorylation (in the absence or presence of insulin added *in vitro*) in the membranes of the old animals was significantly decreased (Figure 4b), indicating that, as long as the receptors of the old rats were embedded in the membrane, the impaired activation of IRK which occurred *in vivo* could not be reversed, even by additional incubation *in vitro* with insulin.

Effects of membrane solubilization on IRK activation

To assess further the contribution of membrane lipids to the impaired activation of IRK in old rats, we studied how membrane solubilization affected this activation process. Membranes derived from PBS-injected young and old rats were incubated with insulin in the presence of increasing concentrations of Triton X-100, and autophosphorylation of IRK was monitored. Low concentrations of Triton X-100 (up to 0.1%) had little effect on the activation of IRK; however, higher Triton X-100 concentrations potentiated the insulin-dependent autophosphorylation of IRK, in both young and old rats (Table 2), and the defect in activation of the latter was markedly decreased. Moreover, Triton extracts of membranes prepared from livers of old rats did not interfere with the activation of IRK isolated from livers of young animals (results not shown). Consistent with previous findings [36], these results suggest that membrane lipids pose constraints on the extent of activation of the IRK even in young normal rats, and may have a major role in the impaired activation of the IRK observed in old animals.

Reversal of the impaired activation of IRK in old rats liver by lectin purification

To support further the notion that there is no intrinsic defect in the IRK of old rats, lectin-purified receptors derived from membranes of PBS-injected livers were assayed for tyrosine

Table 2 Effect of Triton solubilization on IRK activation

Liver membranes were prepared from PBS-injected young or old rats. Membranes were incubated (1 h, 23 °C) with 0.1 μM insulin in the absence or in the presence of 1% Triton X-100. Autophosphorylation of IRK was assayed in these preparations in the presence of 1 mM ATP as in Figure 3. After immunoprecipitation with ST-50 antibodies, the IRs were subjected to SDS/PAGE and immunoblotted with anti-phosphotyrosine antibodies. The results of densitometric analysis of 5 young and 8 old rat preparations are presented as means ± S.E.M.

Animals	Triton X-100	IRK autophosphorylation (arbitrary units)
Young	−	100 ± 3
Old	−	18 ± 7
Young	+	134 ± 10
Old	+	90 ± 12

Table 3 Effect of lecithin liposomes on IRK activation in liver membranes

Liver membranes of PBS-injected young or old rats were incubated for 18 h at 4 °C with lecithin liposomes (1:1, v/v) or with buffer alone. At the end of incubation, the membranes were centrifuged at 12000 ***g***, and the supernatant which contained the liposomes was discarded. The membranes were resuspended in KRP buffer, and the activation of the IRK in those membranes was assayed after incubation with insulin as described in the legend to Figure 3. The results of densitometric analysis of the phosphorylated IR β-subunits from three separate experiments are presented as the means ± S.E.M.

Animals	Lecithin liposomes	IRK autophosphorylation (arbitrary units)
Young	−	100 ± 2
Old	−	60 ± 7
Young	+	153 ± 30
Old	+	117 ± 17

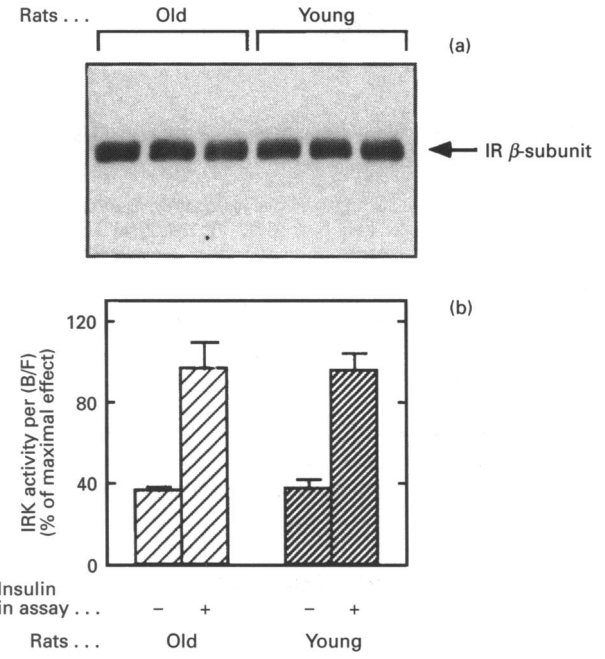

Figure 5 Reversal of the impaired activation of IRK in old rat liver by lectin purification

Liver membranes of PBS-injected young and old rats were solubilized with 1% Triton X-100 in buffer A, and IRs were further purified over lectin columns as described in the Materials and methods section. Equal amounts of IR (based on their ^{125}I-insulin-binding activity) were then incubated with 0.1 μM insulin for 30 min (at 23 °C), and autophosphorylation (in the presence of 1 mM unlabelled ATP) was carried out as described in the Materials and methods section. The proteins were then subjected to SDS/PAGE and immunoblotted with anti-phosphotyrosine antibodies (**a**). After incubation in either the absence or the presence of 0.1 μM insulin for 30 min (at 23 °C), IRK activity in the lectin-purified preparations was also assayed with poly(Glu,Tyr) (4:1) as the substrate. The results of this experiment are presented in (**b**) as means ± S.E.M. of 3 different rats from each group.

kinase activity after incubation *in vitro* with insulin. Although impaired activation of IRK was evident when insulin was added to intact membranes of PBS-injected old rats (Figure 3 and Table 2), no defect in kinase activity was observed when insulin was added to lectin-purified receptor preparations derived from these membranes. This was evident both by assaying autophosphorylation of the IR β subunit (Figure 5a) and by measuring phosphorylation of the exogenous substrate poly(Glu,Tyr) (4:1) (Figure 5b). These results are in agreement with our previous studies [7], which suggested that the elements responsible for the impaired activation of the IRK in intact membranes are not present in lectin-purified preparations.

Effect of cholesterol extraction on IRK activity in intact membranes

Cholesterol is one of the major elements which affects membrane viscosity and whose content in liver membranes, as well as in other tissues, increases during aging [37]. Incubation of membranes with lecithin liposomes is an effective method of decreasing their cholesterol content [26]. Lecithin liposomes were therefore used to study the effect of cholesterol extraction on IRK activation in liver membranes from young and old rats. After cholesterol extraction, an elevated insulin-stimulated autophosphorylation of IRK was observed in membranes from both young and old animals (Table 3), but the difference between the mean levels of autophosphorylation in young and old animals was preserved. However, although the IRK activity in non-treated membranes from old rats was significantly decreased as compared with young controls, only slight and non-significant differences were observed after cholesterol extraction.

DISCUSSION

An impaired activation of the IRK is one of the underlying mechanisms which contributes to the insulin-resistant state that develops with aging [38–41]. In the present work, we provide evidence that this defect in IRK activity is related to a multitude of elements, each of which contributes to this phenomenon. From the present and previous studies [7], it is obvious that the intrinsic activity of the receptor is not affected during aging, but other membrane and non-membrane elements interfere with its activation.

(i) Altered IR-PTP activity in old rats

An age-related increase in activity of a specific inhibitor of IRK [15,16] was ruled out, although we could demonstrate the presence of such an IRK-inhibitory activity in liver cytosol (Figure 1). On the other hand, we could demonstrate in liver of old rats a significant (30–100%) elevation in cytosolic PTP activity directed against the IR β subunit (IR-PTP) (Figure 2). Two lines of evidence support the notion that a specific increase in IR-PTP takes place. First, when the same cytosolic fractions

were assayed with a common substrate for PTPs, a tyrosine-phosphorylated poly(Glu,Tyr) (4:1), there was no difference in activity between cytosolic PTPs from young or old rats. Second, we have previously shown that, in liver extracts of old rats, insulin-dependent tyrosine phosphorylation of the IR β-subunit and of its cellular substrate IRS-1 is decreased, but there is no age-related change in the phosphotyrosine content of other proteins that are constitutively tyrosine-phosphorylated in the liver (e.g. pp 110 and pp 125) [7]. These findings suggest that there is no general elevation in cytosolic PTP activity in the liver of old rats, but rather a selective increase in activity of certain PTPs, such as IR-PTP. Since this cytosolic IR-PTP activity is not affected by insulin injection in either young or old rats, this activity is probably not regulated to a large extent by insulin. Elevated PTP activity in the liver cytosol also occurs in two rat models of insulin-deficient diabetes: genetically diabetic BB rats and streptozotocin-induced diabetic rats [42]. Similarly to our findings, insulin treatment did not affect the PTP activity in the liver cytosol of these animals [42].

It should be noted, however, that not all insulin-resistant states involve increased PTP activity. Insulin resistance induced by fasting is associated with inhibition of an IR-PTP activity [43]; decreased activity is also found in *ob/ob* mice [44], and in hepatic particulate IR-PTP from streptozotocin-induced diabetic rats [14]. Thus, although an insulin-resistant state is a common feature of all the animal models described above, the molecular basis underlying these syndromes may vary and may differently affect IR-PTP activity.

Hepatic IR-PTP activity is present in the cytosol, membrane and glycoprotein-enriched fractions (Figure 2), but the increased activity in old animals was confined to the cytosol fraction. This suggests that the IR-PTP activity in the various subcellular fractions is differently affected by aging, and could be attributed, for example, to the presence of either the same IR-PTP at different states of post-translational modifications, or to the presence of different IR-PTP isoforms that are independently regulated. Such a hypothesis is supported by the observation that particulate PTP activity is increased in adipocytes of streptozotocin-induced diabetic rats, whereas cytosolic PTP activity is decreased [45]. Similarly, a PTP activity is decreased in the liver cytosol, but not in the particulate fraction, of *db/db* diabetic mice [44].

The existence of several forms of PTP has been shown both biochemically and by molecular cloning [12,13,46,47]. PTPs belong to diverse families of intracellular and transmembrane enzymes which may take part in signal transduction and cell-cycle regulation. It is therefore reasonable to assume that the discrepancies in the observations obtained from studies of PTP activity in various insulin-resistant states could be due to altered regulation of different members of the PTP family.

Our observations are consistent with a model where a receptor–insulin complex serves as a substrate for a cytosolic IR-PTP, whose activity is elevated in the old animals. This results in enhanced dephosphorylation of the autophosphorylated IR and, as a consequence, the isolated receptors from the insulin-injected old rats are less phosphorylated and less active as compared with young controls.

(ii) Decreased membrane fluidity and its effects on IRK activation in old rats

Changes in the lipid composition of cell membranes, which decrease their fluidity, occur during the normal physiological process of aging [31,32,48–51]. Consistent with these findings, we observed an elevated viscosity in the liver membranes of old rats (Table 1). This elevation, although moderate, is physiologically significant, since it may modulate the activity of various membrane enzymes [49,50,52]. As shown in the present study, the factors responsible for the impaired activation *in vivo* of the hepatic IRK in old animals are preserved in the membrane preparations of these livers (Figure 3). This impaired activation occurs in spite of the fact that the number and affinity of IRs were similar in liver membrane preparations from young and old animals. Furthermore, incubation of membranes from the old animals with pharmacological doses of insulin *in vitro* (Figure 4) did not correct the defect, suggesting that lower availability or enhanced degradation of insulin could not account for the defect *in vivo*. Studies of the insulin-stimulated IRK activity after solubilization of the membranes with Triton X-100 (Table 2) provide further indication that membrane constituents constrain IRK activity. Once the receptors are solubilized, they are readily activated by insulin (Table 2), and the defect in the activation of IRK in membranes from old rats is largely abolished. Thus the impaired activation of IRK in these intact membranes may be due to age-related alterations in membrane lipid composition which exert a greater inhibitory effect on IRK activation. This notion is supported by the fact that soluble membrane extracts of old rats exert no inhibitory effect on the IRK activity of young controls. Hence, the impaired IRK activation in liver membranes of old rats is not the result of alterations in some non-lipid constituent (i.e. enzymes or inhibitors) which affect the IRK activity.

The major change in membrane composition during aging is an increase in the cholesterol/phospholipid ratio. Many membrane-associated activities are affected by this change, which decreases membrane fluidity [37,49,50,52–54]. Our results provide evidence that changes in membrane fluidity may have an inhibitory effect on the activation process of the IRK. Two possible mechanisms could account for the inhibition. A decrease in membrane fluidity could impede the capacity of IRK to undergo aggregation, a necessary step in its activation process [33–35]. Alternatively, the catalytic activity of the IRK could be directly modulated by the altered phospholipid composition in its vicinity.

Incubation of membranes with lecithin liposomes was previously shown to be an effective tool to lower their cholesterol level [26,27]. Indeed, after incubation of liver membranes (of either young or old rats) with lecithin liposomes, an elevation of the insulin-stimulated activity of the IRK was observed (Table 3). Furthermore, after cholesterol extraction, no significant differences in IRK activity could be detected between membrane preparations derived from young or old animals (Table 3). This therefore suggests that decreasing the cholesterol content of plasma membranes increases the activation of the IRK in response to insulin, and may thereby improve the responsiveness of intact cells to insulin. Conversely, the elevated cholesterol levels in membranes of old rats seem to contribute to the impaired activation *in vivo* of the IRK in these old animals.

Cholesterol has also been shown to affect directly the activity of several enzymes, in addition to its physical effects on bulk membrane properties [55,56]. Similarly, the altered levels of saturation of fatty acids which occur during aging were shown to generate specific effects on membrane enzyme activities, in addition to their effect on membrane fluidity [57]. Thus it is tempting to speculate that specific membrane constituents which are generated during aging may also have a direct inhibitory effect on IRK activity. Indeed, evidence suggesting that phospholipids, sphingosine and cholesterol may function as a modulators of IRK activity has been previously reported [17–20]. Taken together with our findings, these observations suggest that a

specific membrane milieu, such as that existing in membranes of young animals, is required for optimal hormone-responsiveness of the IRK, and imply that the age-related changes in the physicochemical properties of the membranes might affect the molecular conformation of the IRK, as well as its function, both through changes in bulk lipid fluidity and through specific lipid–protein interactions.

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