Inositol phospho-oligosaccharides from rat fibroblasts and adipocytes stimulate 3-*O*-methylglucose transport

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Inositol phospho-oligosaccharides (IPOs), which are released from liver membranes upon stimulation by insulin, mimic a wide spectrum of insulin effects in different cells, but not the stimulation of glucose transport. We investigated whether other insulin-sensitive tissues release glucose transport-stimulating IPOs and whether this is related to the human insulin receptor isoform-A or -B (HIR-A or HIR-B). Rat1 fibroblasts over-expressing HIR-A or -B (rat1-HIR cells) were labelled with [³H]-glucosamine, [³H]mannose or *myo*-[³H]inositol. IPOs from the cell supernatant were partially purified by an AG1X2 anion-exchange column, and fractions were eluted at different pH values (pH 3, pH 2 and pH 1.3). The label from glucosamine, mannose and *myo*-inositol appeared predominantly in the pH 2 fraction. The biological activity of the fractions was determined by measuring 3-*O*-methylglucose transport and lipogenesis in fat cells. Using the pH 2 fraction from the supernatant of rat1-HIR fibroblasts, insulin increased the release of 3-*O*-methylglucose-transport-stimulating activity (HIR-A: without insulin, 22.4 ± 5.4%; with insulin 54.0 ± 8.4%; HIR-B: without insulin

21.6 ± 7.5%, with insulin, 44.7 ± 10.6%, given as a percentage of equilibrium glucose transport reached after 4 s) and lipogenesis-stimulating activity (HIR-A: without insulin, 1.24 ± 0.17; with insulin, 4.69 ± 0.2; HIR-B: without insulin, 1.34 ± 0.18; with insulin, 4.98 ± 0.31, given as nmol of [³H]glucose converted into lipids/min per 10⁶ cells). Analogous experiments were performed with isolated rat fat cells expressing the physiological level of insulin receptors. Upon insulin stimulation of fat cells in the presence of 2.5 mM mannose, the release of 3-*O*-methylglucose-transport-stimulating activity was detected (for purified supernatant of adipocytes without insulin, 6.9 ± 1.12%; with insulin, 41.0 ± 3.6%) and lipogenesis-stimulating activity (without insulin, 0.93 ± 0.17, with insulin 2.96 ± 0.31 nmol/min per mg). These data suggest (1) that adipocytes and rat1-HIR fibroblasts release IPOs that are able to stimulate glucose transport, (2) that both insulin receptor isoforms (HIR-A and HIR-B) mediate the effect of insulin on IPO release, and (3) that overexpression of insulin receptors increases the basal release of IPOs.

INTRODUCTION

Inositol phospho-oligosaccharides (IPOs) isolated from liver and hepatoma cells have similar actions to those of insulin [1–9]. It has been shown that IPOs that are released from hepatocyte membranes upon insulin stimulation are able to mimic a great number of insulin effects in different cell types [1–9]. There is increasing evidence that several subclasses of insulin-sensitive IPOs exist [10]. It is believed that these compounds are released from cell membranes due to the stimulation of phospholipase C activity [3–5,9]. Since the insulin effect on IPO release was attenuated in cells expressing kinase-negative receptors [9], it can be assumed that this effect is mediated by the insulin receptor kinase. It is interesting to note that the IPOs from liver are not able to mimic the effect of insulin on glucose transport [1,4,6,9]. We have observed previously that putative IPOs isolated from bovine blood cells by anion-exchange chromatography are able to mimic a similar spectrum of insulin effects [11] as the IPOs from liver and hepatoma cells [1,2,4,9], and in addition the stimulation of glucose transport in fat cells [11] has been observed. This finding suggests that glucose-transport-stimulating IPOs do exist, and we speculate that insulin-sensitive tissues other than liver might be able to release certain subclasses of IPOs which are specifically able to stimulate glucose transport. The ability to release these postulated subclasses of IPOs might be a con-

sequence of a tissue-specific composition of plasma membrane glycopospholipids; alternatively, it could be related to the different tissue-specific expression of the insulin receptor isoforms (HIR-A and HIR-B). HIR-A and HIR-B might have different abilities to induce the release of specific subclasses, and the lack of glucose transport-stimulating IPOs in liver might be related to the receptor isoform expression, since it is known that liver expresses predominantly HIR-B [12,13].

In the present study we have addressed the following questions: (1) do fat cells and rat1-HIR fibroblasts (rat1 fibroblasts over-expressing HIR-A or HIR-B) release IPOs upon insulin stimulation, (2) do these IPOs stimulate glucose transport, and (3) are both receptor isotypes able to induce the release of IPOs with the same biological activity?

MATERIALS AND METHODS

Materials

Anti-insulin antibodies were from Miles Laboratories. Dowex AG1X2 was from Bio-Rad. [³H]Glucosamine, [³H]mannose, *myo*-[³H]inositol, [³H]glucose and 3-*O*-methyl-D-[¹⁴C]glucose were from Amersham. Fat cells were prepared from male Sprague–Dawley rats fed *ad libitum* (180–220 g body wt.). 3-*O*-Methylglucose, mannose, porcine insulin, collagenase and lipid markers were from Sigma. Silica gel 60 t.l.c. plates were from

Abbreviations used: IPO, inositol phospho-oligosaccharide; HIR-A and -B, human insulin receptor isoforms A and B; DMEM, Dulbecco's modified Eagle's medium; PI, phosphatidylinositol; PIP₂, phosphatidylinositol 4,5-bisphosphate.

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Merck. Fetal calf serum, Dulbecco's modified Eagle's medium (DMEM)/F12, DMEM, glutamine, PBS and penicillin/streptomycin were from Gibco. Plastic material for cell culture was from Greiner.

Cell culture

Rat1 fibroblasts transfected with HIR-A ($\sim 1.9 \times 10^6$ receptors/cell) or HIR-B ($\sim 3.6 \times 10^6$ receptors/cell) were cultured in DMEM/F12 supplemented with 10% fetal calf serum, 2 mM glutamine and penicillin (100 units/ml)/streptomycin (100 μ g/ml). For isolation of insulin-mimetic substances, the supernatant of one Petri dish (145 cm²) with a confluent cell layer was used. Prior to stimulation, the medium was removed and the cells were washed three times with PBS, and allowed to stand for 30 min in serum-free DMEM/F12 medium and subsequently in 10 ml of DMEM/F12 with or without 100 nM insulin. After a 10 min incubation at 37 °C, the medium was removed from the cell layer and centrifuged to separate remaining cellular particles.

Fat cell preparation

Fat cells were prepared from male Wistar rats according to the method of Rodbell [14]. The epididymal fat pads were digested with collagenase (0.8 mg/ml) in buffer A (125 mM NaCl, 5 mM KCl, 2.5 mM MgCl₂, 1 mM CaCl₂, 1 mM KH₂PO₄, 25 mM Tris/HCl, pH 7.4, and 50 mg of BSA/ml). The cells were filtered through a nylon mesh and washed three times. Adipocytes were incubated with 10 nM insulin for 20 min at 37 °C in a Krebs/Ringer/Hepes buffer. Subsequently the buffer was separated from the cells by centrifugation and further processed as described below.

Isolation of insulin-mimetic IPOs

For isolation of insulin-mimetic IPOs, 10 ml of the supernatant from 200×10^6 cells was chromatographed in 2 ml AG1X2 anion-exchange columns eluted successively with 20 ml of distilled water and three solutions of aqueous HCl adjusted to pH 3, pH 2 and pH 1.3 respectively. The eluates were concentrated, neutralized to pH 7.4 and lyophilized. The lyophilized material was resuspended in 500 μ l of water and tested for its ability to stimulate 3-O-methylglucose transport and lipogenesis.

Separation of AG1X2 fractions by t.l.c.

The presence of oligosaccharides in the medium was tested by staining with AgNO₃/NaOH after t.l.c. The fractions from the AG1X2 column were applied on silica gel 60 thin-layer plates. Portions of 5 μ l of lyophilized and resuspended samples were applied to the plates and the chromatograms were developed in a chamber with chloroform/acetone/methanol/acetic acid/water (40:15:13:12:20, by vol.). The oligosaccharide-containing fractions were detected by AgNO₃ staining with 3% NaOH and exposure to u.v. light (253 nm) by a modification of the method of Miyamoto et al. [15].

Labelling of cells

Rat1-HIR fibroblasts were incubated with [³H]glucosamine (10 μ Ci/ml) in DMEM/F12 containing 1% BSA. The medium

containing the label was removed after 5 h, and the cell layer was washed eight times with DMEM/F12 and allowed to stand for an additional 30 min in DMEM/F12 without fetal calf serum. For labelling with [³H]mannose and *myo*-[³H]inositol (each 5 μ Ci/ml), rat1-HIR cells were incubated for 12 h with the radioactive material, washed and then incubated for 30 min with serum-free DMEM/F12. IPO release was then stimulated with 100 nM insulin at 37 °C for 10 min. The released IPOs were further isolated and purified as described above. For labelling of rat adipocytes, isolated fat cells were incubated in DEM with [³H]glucosamine (5 μ Ci/ml) and 1% BSA for 12 h. The medium was removed and adipocytes were washed three times with DEM and then incubated with 10 nM insulin for 20 min at 37 °C. IPOs released from adipocytes were isolated as described above.

3-O-Methylglucose transport in rat adipocytes

Glucose transport was measured as described [16]. Isolated rat adipocytes (epididymal fat) were incubated for 20 min at 37 °C in a Krebs/Ringer/Hepes buffer with 20 μ l of purified IPOs released from rat1 fibroblasts or rat adipocytes. Samples (100 μ l) of the concentrated fat cell suspension (12×10^6 cells/ml) were drawn together with 200 μ l of 3-O-methylglucose (final concentration 0.5 mM) and with 0.1 μ Ci of 3-O-methyl-D-[¹⁴C]glucose into a mixing pipette (Gilson Medical Electronics, Villier-le-Bel, France). After 4 s the uptake was stopped by diluting the cells in 5 ml of 0.9% NaCl containing phloretin (1 mM). Cells were separated by centrifugation through silicone oil (700 g for 60 s). The cell layer was removed using a pipette and added to scintillation fluid, and the radioactivity was measured. The 3-O-methylglucose uptake measured during 4 s is expressed as a percentage of the 3-O-methylglucose accumulated at equilibrium in fat cells after 30 min of incubation under saturating insulin concentrations.

Lipogenesis in rat fat cells

Lipogenesis was measured by the method of Moody et al. [17] as incorporation of D-[3-³H]glucose into toluene-extractable lipid under conditions where the glucose transport is not rate-limiting [18]. Adipocytes (0.2 ml of cell suspension; 1.5×10^4 cells/ml) were incubated for 60 min at 37 °C in buffer A in polypropylene scintillation vials in the presence of tracer glucose, 0.5 mM unlabelled glucose and 10 μ l of IPOs released from rat1-HIR fibroblasts or rat adipocytes. The radioactivity in the lipid phase was measured in a liquid scintillation counter.

RESULTS

Transfected rat1 fibroblasts

IPOs were isolated from rat1 fibroblasts overexpressing HIR-A or HIR-B. After incubation in the presence or absence of insulin, the supernatants of these cells were purified as described in the Materials and methods section. Fractions were eluted from an AG1X2 column at pH 3, 2 and 1.3. Staining with AgNO₃/NaOH was performed as described previously [15] to localize separated oligosaccharides on t.l.c. Figure 1 shows that material was stained which migrated in t.l.c. between phosphatidylinositol (PI) and phosphatidylinositol 4,5-bisphosphate (PIP₂). Under basal conditions there was less staining of the material from HIR-A compared with HIR-B cells; however, insulin stimulation increased the amount of stained material in both cell types. As a control we also performed protein staining. The material could not be detected by protein staining with ninhydrin on t.l.c. (results not shown).

Figure 1 T.L.C. of compounds eluted at pH 2 from the AG1X2 column

IPO release from rat1-HIR fibroblasts was stimulated (+) or not (–) with 100 nM insulin. The supernatant was applied on an AG1X2 column. The fraction eluted at pH 2 was lyophilized and resuspended, and portions of 5 μ l were applied to a silica gel 60 plate. T.L.C. was performed as described in the Materials and methods section. Lipid markers were phosphatidylinositol (PI) and PIP_2 . The T.L.C.-separated material was silver-stained with AgNO_3 and 3% NaOH.

Table 1 Release of radioactivity from [^3H]glucosamine labelled rat1-HIR fibroblasts

Rat1-HIR fibroblasts were labelled with [^3H]glucosamine (10 $\mu\text{Ci}/\text{ml}$) for 5 h at 37 $^\circ\text{C}$ and stimulated or not with 100 nM insulin for 10 min. The supernatant was applied to an AG1X2 column and fractions were eluted at pH 3, 2 and 1.3. Portions of 200 μl of the eluted fractions were counted for radioactivity in a liquid scintillation counter. The data are means $\pm$ S.E.M. of results obtained from two labelling experiments (triplicate determinations).

Fraction	Release of radioactivity (c.p.m.)			
	HIR-A		HIR-B	
	Basal	+ Insulin	Basal	+ Insulin
pH 3.0	144 $\pm$ 12	192 $\pm$ 12	203 $\pm$ 23	187 $\pm$ 28
pH 2.0	1500 $\pm$ 101	3506 $\pm$ 137	1810 $\pm$ 125	3640 $\pm$ 98
pH 1.3	694 $\pm$ 80	980 $\pm$ 103	1046 $\pm$ 112	1237 $\pm$ 154

To further characterize this material, we labelled rat1 fibroblasts with [^3H]glucosamine, [^3H]mannose and *myo*-[^3H]inositol, which are putative components of IPOs isolated from liver and blood cells [2,4,11,19]. Table 1 shows the distribution of released radioactivity in fractions from the AG1X2 column eluted at pH 3, pH 2 and pH 1.3 after labelling of rat1-HIR cells with [^3H]glucosamine and stimulation or not with insulin. The fraction eluted at pH 2 contained most of the radioactively labelled material (this could also be demonstrated after labelling of rat1-HIR cells with [^3H]mannose or *myo*-[^3H]inositol), suggesting that the putative IPOs appear in this fraction. The release of labelled material from rat1-HIR fibroblasts was increased by 2.3-fold (pH 2.0, HIR-A) and 2.0-fold (pH 2.0, HIR-B) after insulin stimulation. As a control for insulin stimulation we measured the phosphotyrosine content in the β -subunit of the insulin receptor by Western blotting. After insulin stimulation, increases of 2.6-fold and 3.1-fold in the phosphotyrosine content of the HIR-A and HIR-B β -subunits respectively were found.

Figure 2 Effect of AG1X2 fraction from rat1-HIR fibroblasts on 3-O-methylglucose transport in rat adipocytes

IPO release from rat1-HIR fibroblasts was stimulated without (IPO) or with (IS-IPO) 100 nM insulin in serum-free DMEM/F12. The cell supernatant was purified on an AG1X2 anion-exchange column. 3-O-Methylglucose transport was stimulated by 20 μl of the neutralized, lyophilized and resuspended fraction eluted at pH 2 from the AG1X2 column. The hatched bars show the effect of AG1X2 fractions after treatment with anti-insulin antibodies. The basal value (on the left) was determined by addition of Krebs/Ringer/Hepes buffer instead of the AG1X2 pH 2 fraction. The results of four experiments (means $\pm$ S.E.M., * P < 0.05 versus basal; Student's t test) are shown.

To determine the biological activity of this material, we tested the AG1X2 fractions for their ability to stimulate 3-O-methylglucose transport and lipogenesis in isolated rat fat cells. Figure 2 shows the stimulation of 3-O-methylglucose uptake with the pH 2 fraction isolated from the supernatant of rat1 fibroblasts after treatment or not with insulin. The pH 2 fraction increased 3-O-methylglucose uptake via IPOs released upon insulin stimulation (HIR-A, 2.4-fold stimulation; HIR-B, 2.1-fold stimulation). To assess whether the increased effect observed after insulin stimulation was indeed due to increased IPO release and not to contamination with insulin that had been carried through the purification procedure, we treated the AG1X2 fractions with anti-insulin antibodies. None of the 3-O-methylglucose transport-stimulating activity was lost after the addition of anti-insulin antibodies to the fraction eluted at pH 2, demonstrating that the stimulation is not due to a contamination of this fraction with insulin. As a further control, we added to the basal cell supernatant before the purification procedure, the same insulin concentration that was used in the other experiments to stimulate IPO release by the cells. No bioactivity above the basal value could be found under these conditions in the AG1X2 fraction after the purification procedure (results not shown). These results clearly exclude the possibility that the bioactivity of fibroblasts in the pH 2 fraction is due to contamination by insulin.

To determine whether the insulin-stimulated release of IPOs is induced through the overexpressed HIR-A or HIR-B, or through the parental receptor, we compared parental and transfected cells after stimulation with 100 nM insulin. At this insulin concentration we found no difference between all three cell types. However, earlier studies with cells that have been transfected with the insulin receptor [20,21] have shown that differences between parental and transfected cells may only be detectable when submaximal insulin concentrations are used. Therefore we repeated the experiments with 0.1 nM insulin. At this insulin concentration, glucose transport was significantly increased (P < 0.01) in transfected cells compared with non-transfected cells (Table 2). These results suggest that both receptor isotypes

Table 2 Effect of AG1X2 (pH 2) fraction from transfected rat1-HIR fibroblasts and parental cells on 3-*O*-methylglucose transport in rat adipocytes

IPO release from rat1 fibroblasts transfected with HIR-A or HIR-B and from non-transfected cells was stimulated with 0.1 nM insulin in serum-free DMEM/F12. The cell supernatant was purified on an AG1X2 anion-exchange column. 3-*O*-Methylglucose transport in rat adipocytes was stimulated by 20 μ l of lyophilized and resuspended fraction eluted at pH 2 from the AG1X2 column. Results of three experiments (duplicate determinations; means $\pm$ S.E.M.) are shown. The basal value without insulin and IPOs was 3.4% of equilibrium. * $P < 0.01$ versus control (Student's *t* test).

	3- <i>O</i> -Methylglucose transport (% of equilibrium)
Untransfected cells	13.4 $\pm$ 1.8
Rat1-HIR-A cells	22.3 $\pm$ 3.2*
Rat1-HIR-B cells	24.2 $\pm$ 3.3*

Figure 3 Effect of AG1X2 fraction from rat1-HIR fibroblasts on lipogenesis in rat adipocytes

IPO release from rat1-HIR fibroblasts was stimulated without (IPO) or with (IS-IPO) 100 nM insulin in serum-free DMEM/F12. The cell supernatant was purified on an AG1X2 anion-exchange column. Lipogenesis was stimulated by 10 μ l of the lyophilized and resuspended fraction eluted at pH 2 from the AG1X2 column. The hatched bars show the effect of AG1X2 fractions after treatment with anti-insulin antibodies. The basal value (on the left) was determined by addition of Krebs/Ringer/Hepes buffer instead of the AG1X2 pH 2 fraction. The results of five experiments (means $\pm$ S.E.M.) are shown.

are able to transduce the insulin signal leading to the release of IPOs.

Figure 3 shows the effect of the AG1X2 pH 2 fraction from the supernatants of rat1-HIR-A and rat1-HIR-B cells on [³H]glucose incorporation into total lipids of fat cells. The stimulation of lipogenesis was 3.7-fold over basal (the basal value was determined as the effect of IPOs released without insulin stimulation) with fractions from rat1-HIR-A, and 3.8-fold over basal with fractions from rat1-HIR-B. The addition of anti-insulin antibodies did not suppress the effect significantly, demonstrating that no major insulin contamination contributes to stimulation of lipogenesis.

Rat fat cells

To elucidate whether an insulin-dependent release of these insulin-mimetic compounds can only be demonstrated in cells

Figure 4 Effect of AG1X2 fractions from adipocytes on 3-*O*-methylglucose transport in rat adipocytes

IPO release from rat adipocytes was stimulated without (IPO) or with (IS-IPO) 10 nM insulin in serum-free DEM. The cell supernatant was purified on an AG1X2 anion-exchange column. 3-*O*-methylglucose transport was stimulated by 20 μ l of the neutralized, lyophilized and resuspended fraction eluted at pH 1.3. The hatched bars show the effect of AG1X2 fractions after treatment with anti-insulin antibodies. The basal value (left) was determined by addition of Krebs/Ringer/Hepes buffer instead of the AG1X2 fraction. The results of five experiments (means $\pm$ S.E.M.) are shown.

overexpressing the insulin receptor or whether this phenomenon also occurs in rat adipocytes expressing the physiological level of insulin receptors, we stimulated rat adipocytes with insulin and isolated putative IPOs from the incubation buffer, as described for rat1 fibroblasts. After labelling of the adipocytes with [³H]glucosamine and incubation with insulin, no significant insulin-induced release of labelled material was detectable (results not shown). However, the failure to detect insulin-released label in the supernatant might be due to a rapid re-uptake of these compounds by the fat cells, and thus we stimulated IPO release in the presence of 2.5 mM mannose. We have shown previously [11] that mannose is able to block putative IPO uptake in fat cells. In the presence of 2.5 mM mannose, the radioactivity released from [³H]glucosamine-labelled adipocytes after insulin stimulation could be eluted from the AG1X2 column at pH 3.0 (2145 c.p.m./200 μ l), pH 2.0 (1786 c.p.m./200 μ l) and pH 1.3 (3621 c.p.m./200 μ l). In contrast to the results obtained with rat1-HIR fibroblasts, labelled material from the supernatant of rat adipocytes was not clearly separated at the different pH values. Although the insulin-stimulated release of [³H]glucosamine-labelled material occurred predominantly at pH 1.3 (insulin stimulation, 3786 c.p.m./200 μ l; basal, 1856 c.p.m./200 μ l; mean values of three separate experiments), [³H]glucosamine-labelled substances were also eluted at pH 2 and pH 3. We tested the biological activity of the AG1X2 fractions from the supernatant of rat adipocytes in the same way as for rat1-HIR fibroblasts, but in the presence of 2.5 mM mannose. Figure 4 shows the effect on 3-*O*-methylglucose transport of the AG1X2 fractions from supernatant obtained in the presence of mannose from rat adipocytes stimulated or not with insulin. In the absence of insulin, almost no stimulatory activity was found. This is in contrast to the observation with transfected fibroblasts expressing high receptor levels, which released insulin-like activity under basal conditions. In the presence of insulin the stimulation of 3-*O*-methylglucose uptake (5.9-fold stimulation) could be determined with supernatant from rat adipocytes. The effect on 3-*O*-methylglucose transport was obtained predominantly with the fraction eluted at pH 1.3, while in rat1-HIR fibroblasts the pH 2 fraction was active. This effect was not suppressed with anti-

Figure 5 Effect of AG1X2 fractions from adipocytes on lipogenesis in rat adipocytes

IPO release from rat adipocytes was stimulated without (IPO) or with (IS-IPO) 10 nM insulin in serum-free DEM. The cell supernatant was purified on an AG1X2 anion-exchange column. Lipogenesis was stimulated by 10 μ l of the lyophilized and resuspended fraction eluted at pH 3 from the AG1X2 column and measured as [³H]glucose incorporation into total lipids. The hatched bars show the effect of AG1X2 fractions after treatment with anti-insulin antibodies. The basal value (left) was determined by addition of Krebs/Ringer/Hepes buffer instead of the AG1X2 fraction. The results of five experiments (means $\pm$ S.E.M.) are shown.

insulin antibodies. The effect on lipogenesis is shown in Figure 5. Lipogenesis was stimulated significantly by the IPO fraction eluted at pH 3 (3.2-fold stimulation over the basal value obtained after treatment with anti-insulin antibodies). Approx. 50% of the lipogenesis-stimulating activity could be suppressed by anti-insulin antibodies, suggesting that insulin co-purifies at pH 3 and causes part of the effect on lipogenesis.

DISCUSSION

IPO release from rat1 fibroblasts overexpressing the human insulin receptor

The present study shows that rat1 fibroblasts overexpressing HIR-A or HIR-B are able to release, upon stimulation by insulin and also under basal conditions, insulin-like activity which activates 3-*O*-methylglucose uptake and lipogenesis in fat cells. There is some evidence suggesting that the active components of these fractions are IPOs. The arguments for this conclusion are as follows. Throughout the purification protocol the insulin-like activity behaves identically to IPOs isolated by other workers from liver or hepatoma cells [1,2,4,6,7] and by ourselves from bovine blood [11]. The insulin-like activity is not detectable after nitrous acid treatment, which is compatible with the idea that glucosamine is a part of the active compound [11]. It was demonstrated previously that, in addition to glucosamine mannose also appears to be an important component of insulin-mimetic IPOs [11]. In our preparation [³H]glucosamine, [³H]mannose and *myo*-[³H]inositol label all co-purified with the active fractions. Furthermore, the substance in the active fractions could be detected by carbohydrate staining with AgNO₃/NaOH. This, and the fact that the stained material ran on t.l.c. plates between PI and PIP₂, is compatible with the conclusion that the insulin-stimulated compound in the fraction with insulin-like activity is an IPO. The amount of this material in the medium was increased by insulin stimulation, which suggests insulin-dependent release. On the other hand, high basal values of the insulin-mimetic material were seen in the medium

of rat1 fibroblasts. This is in contrast to the observation with rat fat cells. This difference between the two cell types might be a consequence of the overexpression of the insulin receptor in rat1 fibroblasts, suggesting that high receptor levels increase the basal release of these compounds. In general, the data suggest that both receptor isoforms are able to induce this insulin effect. Since both cell types, expressing either HIR-A or HIR-B, were able to release glucose-transport-stimulating IPOs, it appears unlikely that the absence of glucose-transport-stimulating IPOs in liver is related to the fact that liver expresses almost exclusively HIR-B, not HIR-A. It must instead be due to other liver-specific membrane characteristics.

IPO release from rat adipocytes

The release of IPOs was not only observed in cells overexpressing the insulin receptor, but it was also detectable in rat adipocytes which express the physiological level of insulin receptors. However, there were some different features in rat adipocytes compared with rat1-HIR fibroblasts. Rat1-HIR fibroblasts that express high levels of insulin receptors release the putative IPOs in the absence of insulin, but this is not the case for adipocytes. Both cell types release glucosamine-labelled material upon insulin stimulation; however, the glucosamine label of rat1-HIR fibroblasts was found in the pH 2 fraction, whereas the label from adipocytes occurred predominantly in the fraction eluted at pH 1.3, and to only a minor extent in the pH 2 and pH 3 fractions. There is evidence that several subtypes of IPOs with insulin-like activity do exist [10]. Therefore it might be possible that the plasma membranes of rat adipocytes and rat1-HIR fibroblasts release distinct subtypes of IPOs that show different behaviour during purification. Another difference between rat adipocytes and rat1 fibroblasts overexpressing the insulin receptor concerns the role of mannose. Mannose was shown to block a postulated transport system for insulin-mimetic IPOs in fat cells [11]. In our experiments, mannose was required in order to detect IPO release by fat cells, while it was not required for IPO release by rat1-HIR fibroblasts. This might again be explained by the fact that rat1 fibroblasts contain an approx. 1000-fold higher concentration of insulin receptors on the cell surface than do rat adipocytes. Since rat1 fibroblasts overexpressing the insulin receptor release large amounts of IPOs, the presence of mannose might not be necessary to block a putative re-uptake system, as in this cell re-uptake is overcome by production. Alternatively, it seems possible that the suggested re-uptake might not exist or might work differently in fibroblasts.

Possible role of IPOs in the regulation of glucose carrier activity in fat cells

The IPOs from liver mimic many effects of insulin, and the potential role of these IPOs in insulin signalling has been extensively discussed elsewhere [1,9]. In our experiments we tested only one cellular effect which is known to be induced by IPOs from liver, i.e. the stimulation of lipogenesis, which is also inducible by the IPOs released from rat adipocytes or rat1-HIR fibroblasts. It remains to be studied whether, besides different abilities to stimulate glucose transport, other differences exist in the spectrum of insulin effects induced by IPOs from liver and IPOs from fat cells and rat1-HIR fibroblasts. It is interesting to note that liver, a tissue which contains no insulin-stimulated glucose transport system, does not release transport-stimulating IPOs, whereas fat cells and transfected rat1 fibroblasts, which contain an insulin-sensitive glucose transport system, do release transport-stimulating IPOs. The physiological role of glucose-

transport-stimulating IPOs from fat cells and rat1-HIR fibroblasts is unclear. As this phenomenon is only detectable in the presence of mannose in adipocytes, we have to consider whether it is likely to play a role *in vivo* in the intact tissue. It is clear that the insulin-induced translocation of the transporter isoform GLUT 4 plays a central role in glucose transport [22]. However, we have shown earlier [22] that, in addition to translocation, carrier activation is also required in order to obtain the full stimulation of glucose transport. Substances that were able to mimic the effect of insulin on carrier activity included exogenously added phospholipase C or IPOs from bovine blood cells [23]. IPOs from bovine blood were not able to induce carrier translocation [11]. Taking these earlier findings together with the present observations, we propose that IPOs released from fat cells and rat1-HIR fibroblasts might play a role in the effect of insulin on glucose carrier activity. While the insulin-induced carrier translocation must be an intracellular sequence of reactions, it seems possible that the signal that effects carrier activity might be generated at the cell surface. In this context, a cell-to-cell signalling system through IPO release from the cell surface might be involved.

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