Insulin receptor-deficient cells as a new tool for dissecting complex interplay in insulin and insulin-like growth factors

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Abstract Cell systems derived from knockout mice for the insulin receptor (IR) or the IGF-1 receptor (IGF-1R) represent unique tools for dissecting complex interplay in the actions of insulin and insulin-like growth factors through their cognate versus non-cognate receptor. In this study, we used a fibroblast cell line derived from IR-deficient mice to investigate metabolic and mitogenic effects of IGF-1 and insulin. IGF-1 was able to stimulate glucose uptake, glucose incorporation into glycogen and thymidine incorporation in such cells. Phosphatidylinositol 3-kinase and mitogen-activated protein kinase, two enzymes of major metabolic-mitogenic signaling pathways, were activated upon stimulating these cells with IGF-1. All these effects were also achieved when IR-deficient cells were stimulated with insulin. Thus, IGF-1R can represent an alternative receptor through which insulin might exert some of its effects.

Key words: Insulin receptor; Insulin-like growth factor 1 receptor; Insulin action; Signal transduction

1. Introduction

Insulin and insulin-like growth factors (IGF-1 and IGF-2) lead to a variety of biological effects mediated by the insulin receptor (IR) and the type 1 receptor for IGFs (IGF-1R), respectively, which have very similar heterodimeric $\alpha 2\beta 2$ structure and belong to the family of receptor tyrosine kinases [1]. The analysis of the metabolic and mitogenic effects mediated by IR and IGF-1R in vivo is complicated for a number of reasons. The two receptors are present on the surface of most of the cells in the body although their relative proportions vary in different tissues. The two receptors activate common intracellular signaling pathways, i.e. the phosphatidylinositol 3-kinase (PI 3-kinase) and the mitogen-activated protein kinase (MAP kinase) pathways, by the same mechanisms [2,3]. These receptors can bind the heterologous ligands, albeit with lower affinities [4]. Finally, some biological effects might be achieved through IR/IGF-1R hybrid receptors [5].

The analysis of various knockout mice carrying null mutations in genes encoding these ligands or their receptors [6–11] has shed some light on the issues of specificity and redundancy in the insulin and IGF system in vivo. It is interesting to briefly present certain striking features of the phenotype of some of these mutants in which homologous ligand/receptor interactions were abolished and heterologous interactions could be forced to take place.

IGF-1R deficiency resulted in marked intrauterine growth retardation and several abnormalities of differentiation in various tissues leading to immediate lethality at birth [8]. Interestingly, mutant mice lacking both IGF-1R and the IGF-2/mannose-6-phosphate receptor (IGF-2R) which functions in clearing IGF-2 from the circulation were viable [12]. In this situation, efficient interaction of IGF-2 with IR could take place due to elevated IGF-2 levels. Therefore, IGF-2 action through IR could completely compensate for IGF-1R deficiency. The ability of IGF-2 to lead to biological effects via IR was further demonstrated using IGF-1R-deficient fibroblasts [13].

On the other hand, IR-deficient pups were nearly normal at birth but developed severe diabetes mellitus with ketoacidosis leading to death within 1 week [11]. Interestingly, insulin-deficient pups developed the same metabolic disorders but more rapidly and died sooner [9]. Since IR-deficient mice became hyperinsulinemic, one could wonder whether insulin, at high concentrations, might exert some of its effects through IGF-1R. This issue was further addressed here by examining the ability of IGF-1 and insulin to lead to metabolic and mitogenic effects and to activate PI 3-kinase and MAP kinase through IGF-1R using a fibroblast cell line derived from IR-deficient mice.

2. Materials and methods

2.1. Cell cultures

Primary cultures of fibroblasts from IR-deficient newborn pups were obtained as described [14]. Briefly, heart and kidneys were minced and incubated with trypsin for 30 min at 37°C. After centrifugation, the cells were plated into culture dishes in Dulbecco's modified Eagle's medium (DMEM; Gibco-BRL)/10% fetal bovine serum. After a few passages of cells maintained at subconfluent levels, primary cultures underwent crisis and a cell line was recovered. It was verified that these cells carried a homozygous null mutation for IR by genotype analysis of cellular DNA [11]. Cells were grown to confluence in 24-well plates for metabolic and mitogenic assays and in 6-well plates for the PI 3-kinase and MAP kinase assays.

2.2. 2-Deoxyglucose uptake

The uptake of 2-deoxyglucose was measured essentially as follows. Briefly, cells were serum-starved for 18 h in DMEM with low glucose/0.1% bovine serum albumin (BSA) and then incubated for 2 h at 37°C in DMEM without glucose. The cells were washed with phosphate-buffered saline (PBS), preincubated for 40 min in 1 ml of HEPES buffer (50 mM HEPES pH 7.4, 140 mM NaCl, 1.85 mM $CaCl_2$, 1.3

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Abbreviations: IGF, insulin-like growth factor; IGF-1R, type 1 IGF-1 receptor; IR, insulin receptor; MAP kinase, mitogen-activated protein kinase; PI 3-kinase, phosphatidylinositol 3-kinase

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mM $MgSO_4$, 4.8 mM KCl)/0.1% BSA and stimulated with human recombinant IGF-1 (Gro Pep) or insulin (Sigma) for 10 min at 37°C prior to incubation with 0.1 mM 2-deoxyglucose (Sigma) and 1 μCi 2-deoxy-D-[2,6-^{3}H]glucose (25–55 Ci/mmol; Amersham) for 10 min at 37°C. Cells were washed with cold PBS, solubilized in 250 μl 1 N NaOH and the solution was neutralized with 250 μl 1 N HCl before counting the radioactivity.

2.3. Glucose incorporation into glycogen

Glucose incorporation into glycogen was performed as described [15]. Cells were serum-starved as for the 2-deoxyglucose uptake assay and then incubated in DMEM containing 2.5 mM glucose, 0.1% BSA and 25 mM HEPES pH 7.4 for 3 h at 37°C. The cells were washed with PBS, stimulated with IGF-1 or insulin for 30 min at 37°C and incubated in the presence of 1 μCi D-[U-^{14}C]glucose (250 mCi/mmol; Amersham) for 90 min. Cells were then rinsed with PBS, solubilized with 250 μl 30% KOH for 30 min at 37°C and samples were boiled for 30 min with carrier glycogen at 1 mg/ml. Glycogen was precipitated with 95% ethanol at 4°C during 1 h, recovered on Whatman glass filters that were washed with ice-cold 70% ethanol and their radioactivity was counted.

2.4. Thymidine incorporation into DNA

Confluent cells were grown to quiescence in DMEM/0.5% BSA for 72 h. The ligands (IGF-1 or insulin) were then added to the wells for 16 h prior to a 2-h pulse with 1 μCi or [methyl-^{3}H]thymidine (25 Ci/mmol; Amersham). The cells were washed twice respectively in cold PBS, 5% trichloroacetic acid (TCA) and 95% ethanol and then dissolved in 250 μl 1 N NaOH. The solution was neutralized with 250 μl 1 N HCl and the radioactivity was counted.

2.5. Assay of PI 3-kinase activity

PI 3-kinase activity was measured essentially as described [16]. Briefly, cells were serum-starved for 18 h in DMEM/0.2% BSA, stimulated with IGF-1 or insulin for 10 min at 37°C, washed with PBS and solubilized in lysis buffer (50 mM HEPES pH 7.5, 150 mM NaCl, 1 mM EDTA pH 8, 10% glycerol, 1% Triton X-100, 20 mM sodium pyrophosphate, 2 mM orthovanadate pH 10, 100 mM NaF, 2 mM phenylmethylsulfonyl, 20 μg/ml aprotinin, 20 μg/ml leupeptin and 0.7 μg/ml pepstatin). The lysates were immunoprecipitated using 5 μg anti-phosphotyrosine antibodies (PY20; Transduction Laboratories) and protein A-Sepharose (Pharmacia) for 3 h at 4°C. The immunoprecipitates were washed three times with lysis buffer, three times with the reaction buffer (20 mM HEPES pH 7.5, 0.4 mM EDTA and 0.4 mM sodium pyrophosphate) and pellets were resuspended in 30 μl of the reaction buffer. L-α-Phosphatidylinositol (Sigma) was added (10 μl of a sonicated solution at 10 mg/ml in 5 mM HEPES pH 7.5) and the reaction was initiated by addition of 10 μl of a mixture containing 1 μCi [γ-^{32}P]ATP (4500 Ci/mmol; ICN), 50 mM $MgCl_2$, 0.25 mM HEPES pH 7.5, 0.24 mM EDTA and 0.24 mM sodium pyrophosphate. After 15 min incubation at room temperature with shaking, the reaction was stopped by adding 15 μl 4 M HCl and 130 μl $CHCl_3$/methanol (1:1). The samples were centrifuged and the lower organic phases containing phospholipids were subjected to thin layer chromatography (TLC; Merck) performed in $CHCl_3/CH_3OH/NH_4OH/H_2O$ (45:35:7:3) and the TLC plates were exposed to X-ray films for 24 h.

2.6. MAP kinase assay

Cells were serum-staved, stimulated with IGF-1 or insulin for various times and solubilized as for the PI 3-kinase assays. The lysates were immunoprecipitated using antibodies against extracellular regulated kinase (ERK)-1 and ERK-2 (1 μg each; Santa Cruz) and protein A-Sepharose for 3 h at 4°C. The immunoprecipitates were washed three times with lysis buffer, three times with the reaction buffer (20 mM HEPES, 150 mM NaCl, 0.1% Triton X-100, 10% glycerol) and pellets were resuspended in 40 μl of the reaction buffer. 10 μl of the reaction mixture containing 50 mM $MgCl_2$, 1 mg/ml myelin basic protein (MBP; Sigma), 0.25 mM ATP and 25 mM dithiothreitol was added and the reaction was initiated by addition of 0.5 μl [γ-^{32}P]ATP (4500 Ci/mmol; ICN). After 30 min incubation at room temperature with shaking, the reaction was stopped by spotting the whole samples on Whatman P81 filters that were washed once in 10% TCA, three times in 5% TCA, three times in 1% TCA containing 240 mM sodium pyrophosphate and rinsed with ethanol. The filters were dried and the radioactivity was counted.

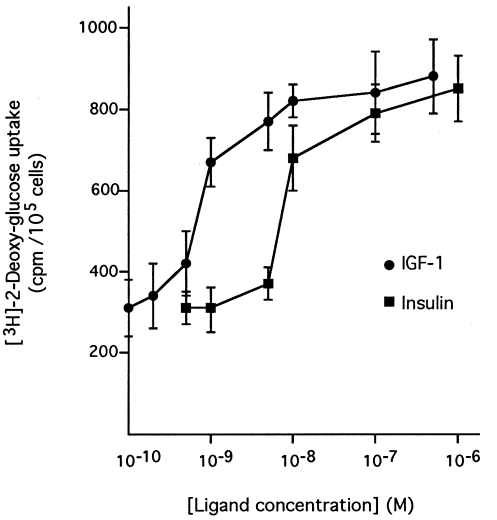

Fig. 1. Stimulation of glucose transport in IR-deficient fibroblasts by IGF-I or insulin. Serum-starved cells were incubated in DMEM without glucose, stimulated in HEPES buffer by increasing concentrations of IGF-I or insulin, followed by incubation with [^{3}H]-2-deoxyglucose. The cells were then washed, solubilized and the radioactivity was counted. A blank value obtained with unstimulated cells was subtracted. Results are presented as the means ± S.E.M. of 10 separate experiments.

3. Results and discussion

The role that IGFs play in cellular growth and differentiation is very well documented. Although there are a number of reports indicating that IGF-1 can lead to metabolic effects and that IGF-1R possesses certain metabolic potential [17–23], the lethal phenotype of IR-deficient mice indicates that IGF-1R cannot entirely substitute for the absence of IR [10,11]. Mutant mice lacking IR or IR-deficient cellular systems that can be derived from these mutants represent unique tools to reinvestigate the metabolic potential of IGF-1R in the absence of IR. In the present study, some metabolic and mitogenic

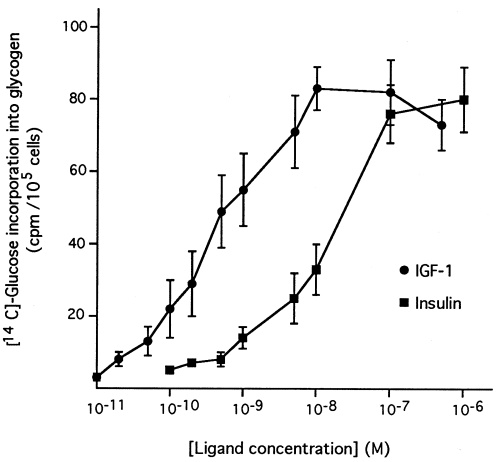

Fig. 2. Glucose incorporation into glycogen in IR-deficient fibroblasts stimulated by IGF-I or insulin. Serum-starved cells were stimulated by increasing concentrations of IGF-I or insulin and then incubated with [^{14}C]glucose. The cells were washed, solubilized and boiled. Glycogen was precipitated, recovered by filtration on Whatman GFB filters and the radioactivity was counted. A blank value obtained with unstimulated cells was subtracted. The data are the means ± S.E.M. of 10 separate experiments.

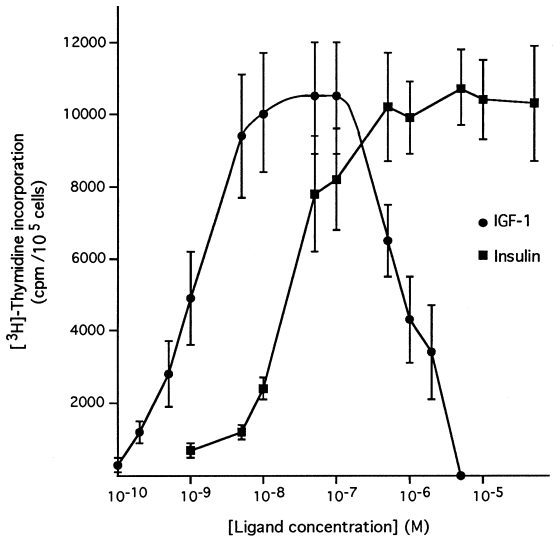

Fig. 3. Incorporation of [^{3}H]thymidine in IR-deficient fibroblasts stimulated by IGF-I or insulin. Cells maintained in serum-free medium were stimulated by increasing concentrations of IGF-I or insulin prior to incubation with [^{3}H]thymidine. The cells were washed, fixed with TCA, dissolved and the radioactivity was counted. A blank value obtained with unstimulated cells was subtracted. Each value is the mean ± S.E.M. of 10 experiments.

actions of IGF-1 as well as activation of PI 3-kinase and MAP kinase were examined using an IR-deficient fibroblast cell line. In parallel, the ability of insulin to lead to these effects mediated by IGF-1R was investigated in such cells.

3.1. Metabolic effects

Firstly, glucose uptake was measured using [^{3}H]-2-deoxyglucose upon stimulation of IR-deficient cells with IGF-1 or insulin. As shown in Fig. 1, IGF-1 was able to stimulate glucose uptake in these cells. The dose-response curve for this metabolic action of IGF-1 was a sigmoid. Stimulation of glucose uptake was also obtained when these cells were stimulated with insulin (Fig. 1). As for IGF-1, the dose-response curve with insulin was also a sigmoid that was however shifted to the right, reflecting the lower affinity of insulin for the heterologous receptor. Ten-fold higher concentrations of insulin were required to reach effects comparable to those obtained with IGF-1. The plateau values were obtained for IGF-1 at $\sim 10^{-8}$ and for insulin at $\sim 10^{-7}$ M.

Secondly, glucose incorporation into glycogen was measured using [^{14}C]glucose upon stimulation of IR-deficient cells with IGF-1 or insulin. As shown in Fig. 2, IGF-1 was able to stimulate glucose incorporation into glycogen in these cells and again the dose-response curve for this metabolic action of IGF-1 was a sigmoid. The data presented in Fig. 2 also show the ability of insulin to stimulate glucose incorporation into glycogen in these cells. Again, the dose-response curve with insulin was shifted rightward.

3.2. Mitogenic effects

The mitogenic effect is classically examined by measuring [^{3}H]thymidine incorporation into cellular DNA upon stimulating the cells with growth factors. As expected, IGF-1 was able to stimulate [^{3}H]thymidine incorporation in IR-deficient cells (Fig. 3). Interestingly, a bell-shaped dose-response curve was obtained for this mitogenic action of IGF-1. The data presented in Fig. 3 show that insulin can stimulate [^{3}H]thymidine incorporation in these cells as well. As for the metabolic effects, higher insulin concentrations are required to reach effects comparable to those obtained with IGF-1. In contrast with IGF-1, the dose-response curve for the mitogenic action of insulin in this system appeared to be a sigmoid in the range of concentrations tested.

3.3. Signaling pathways activated

The activation of PI 3-kinase and MAP kinase, involved in major metabolic and mitogenic signaling pathways activated by IGF-1R, was examined upon stimulation of IR-deficient cells with IGF-1 or insulin.

The PI 3-kinase activity was determined by analyzing phosphorylation of phosphatidylinositols in the presence of [γ-^{32}P]ATP. As shown in Fig. 4, PI 3-kinase activity was increased in cells stimulated with IGF-1 as compared with the basal activity in unstimulated cells. Insulin was also able to stimulate PI 3-kinase activity in these cells. Comparable activation of PI 3-kinase was obtained with 10 nM IGF-1 and 100 nM insulin. Activation of PI 3-kinase was also observed when cells were stimulated with high concentrations of IGF-1 (1 μM) or insulin (50 μM).

The MAP kinase activity was analyzed by measuring phosphorylation of MBP in the presence of [γ-^{32}P]ATP. The results of the kinetics of MAP kinase activation upon stimulation of cells with IGF-1 at 10 nM and insulin at 100 nM are presented in Fig. 5. Clearly, both ligands were able to stimulate MAP kinase although this activation appeared to be more sustained with IGF-1 as compared with insulin. Similar results were obtained in two other experiments. Activation of MAP kinase was also observed when cells were stimulated with high concentrations of IGF-1 or insulin (not shown).

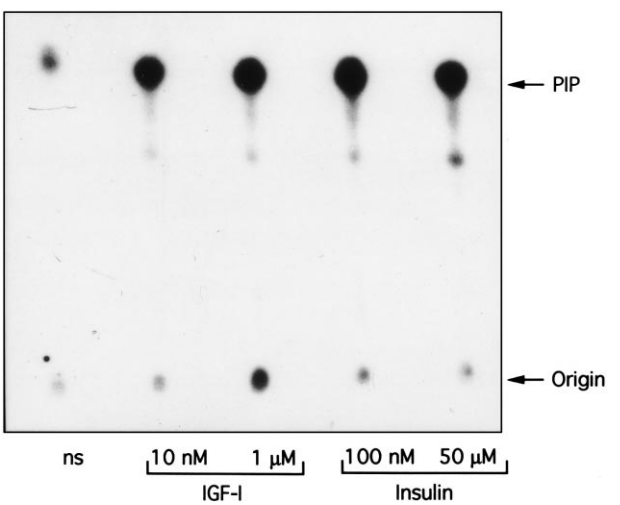

Fig. 4. PI 3-kinase activation by IGF-I or insulin in IR-deficient fibroblasts. Cells were serum-starved prior to incubation with IGF-I or insulin at the indicated concentrations. The cells were solubilized and the lysates were immunoprecipitated with anti-phosphotyrosine antibodies. The PI 3-kinase activity in the immunoprecipitates was determined by phosphorylation of phosphatidylinositols in the presence of [γ-^{32}P]ATP. Labeled phospholipids were separated by chromatography on TLC plates that were exposed to film and the autoradiogram is presented. PIP: phosphatidylinositol phosphate. ns: unstimulated control.

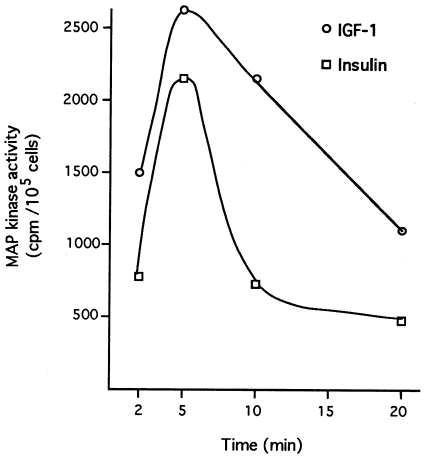

Fig. 5. MAP kinase activation in IR-deficient fibroblasts by IGF-I or insulin. The cells were placed in serum-free medium and then stimulated by IGF-I (10 nM) or insulin (100 nM) for the indicated times. The cells were solubilized and the lysates were immunoprecipitated with anti-ERK1/anti-ERK2 antibodies. MAP kinase activity in the immunoprecipitates was determined by phosphorylation of MBP in the presence of [γ-^{32}P]ATP. The samples were spotted onto Whatman P81 filters, washed and the radioactivity was counted. A blank value obtained with unstimulated cells was subtracted from each time point.

3.4. Limited metabolic potential of IGF-1R?

We have shown in this work that IGF-1 could stimulate glucose uptake and its incorporation into glycogen in IR-deficient fibroblasts, which clearly indicates that IGF-1R possesses a metabolic potential. These effects could also be obtained upon stimulation of these cells by insulin. It is therefore conceivable that in IR-deficient mice which become hyperinsulinemic, insulin, at high concentrations, might exert some of its effects through IGF-1R. This might partly explain the little longer survival of these mutants as compared with mutant mice lacking insulin [9–11]. Attempts to prolong the survival of IR-deficient pups by injecting IGF-1 were unsuccessful [24]. It was, however, reported that IGF-1 injections could lower hyperglycemia but did not reduce serum triglyceride levels. This suggest that IGF-1R might be unable to stimulate lipogenesis in adipose cells and to prevent liver steatosis. The derivation of cell lines from adipose tissue, muscle and liver will make it possible to further assess whether and to what extent IGF-1R can represent an alternative receptor for insulin in these cells.

3.5. Mitogenic actions of IGFs and insulin through IGF-1R or IR

The data presented in this work clearly show that both IGF-1 and insulin were able to stimulate thymidine incorporation in IR-deficient fibroblasts. In this context, it is interesting to recall the results of some recent studies concerning the ability of insulin or IGF-1/IGF-2 to lead to mitogenic effects exclusively through IR in cell lines lacking IGF-1R. One such cell is a fibroblast cell line derived from IGF-1R-deficient mice [14]. It was reported that these cells, if overexpressing IR, were able to proliferate in serum-free medium supplemented only with insulin or IGF-2 whereas IGF-1 failed to promote the growth of these cells [13]. Surprisingly, IGF-2 was even more potent than insulin in promoting growth of these cells. Another cell line lacking IGF-1R is the LB cell line derived from a murine T-cell lymphoma that was insulin-dependent for growth [25]. Insulin stimulated the incorporation of [^{3}H]thymidine in these cells [25,26]. Interestingly, the dose-response curve for this mitogenic effect of insulin through IR was bell-shaped [27], like we observed in this work for the mitogenic effect of IGF-1 acting through IGF-1R. In the LB cell line, negligible mitogenic effects were observed with IGF-1 and none with IGF-2 [25]. In IR-deficient fibroblasts, insulin was clearly able to lead to mitogenic effects but the dose-response curve was sigmoidal. The biological relevance of the bell-shaped dose-response curves for the mitogenic effects of insulin acting through IR or IGF-1 acting through IGF-1R still escapes our understanding and the underlying molecular basis remains to be further explored. It can, however, be recalled that a bell-shaped curve was previously reported for the action of growth hormone on lipogenesis in adipocytes [28]. It was demonstrated that growth hormone binding to its receptor in solution resulted in the formation of a dimer [29,30]. Receptor dimerization was critical for this biological effect and the bell-shaped curve in this case was explained by possible self-antagonism at high ligand concentrations [28]. Since there appear to be some striking differences in the mitogenic action of insulin, IGF-1 and IGF-2 through their homologous or heterologous receptors, this must revive interest in further exploration for possible differences in the nature of the various ligand/receptor interactions. Cell lines lacking IR or IGF-1R represent unique tools for such studies.

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