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Differential effects of IGF-I, IGF-II and insulin in human preadipocytes and adipocytes – Role of insulin and IGF-I receptors

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ABSTRACT

We compared insulin and IGF effects in adipocytes expressing IR (insulin receptors), and preadipocytes expressing IR and IGF-IR (IGF-I receptors).

Treatment of adipocytes with insulin, IGF-II or IGF-I resulted in phosphorylation of IR. Order of potency was insulin > IGF-II > IGF-I. In preadipocytes IR, IGF-IR and insulin/IGF-I hybrid receptors (HR) were detected. Treatment of preadipocytes with IGF-I and IGF-II 10^{-8} M resulted in activation of IGF-IR and IR whereas insulin was more potent in activating IR, with no effect on IGF-IR.

In adipocytes glucose transport was 100-fold more sensitive to insulin than to IGFs and the maximal effect was higher with insulin. In preadipocytes glucose accumulation and DNA synthesis was equally sensitive to insulin and IGFs but the maximal effect was higher with IGF-I. In conclusion, insulin and IGF-I activate their cognate receptors and IGF-I also HR. IGF-II activates IR, IGF-IR and HR. Insulin and IGF-I are partial agonists to each other's receptors.

1. Introduction

Insulin, insulin-like growth factor-I (IGF-I) and insulin-like growth factor-II (IGF-II) are all part of the insulin-like growth factor family and involved in growth, metabolism and survival of the cell (Navarro et al., 1999; Saltiel and Kahn, 2001; Siddle et al., 2001; Chao and D'Amore, 2008). IGF-II is the predominant IGF in humans with circulating levels 5 to 10-fold higher than IGF-I (Frystyk, 2004; Hedman et al., 2004). In contrast to circulating IGF-I, which peaks during puberty, IGF-II is constantly expressed throughout life (Juul et al., 1995).

The IR and the IGF-IR are both members of the tyrosine kinase family of receptors and share an overall homology of more than 50% (Ullrich et al., 1986). As a result of dissimilarities in structure there are differences in tyrosine phosphorylation of the receptors (Lammers et al., 1989). IGF-IR primarily mediates mitogenic effects, while IR primarily mediates metabolic effects (Blakesley et al., 1996; Kim and Accili, 2002). Differences in signalling between the two receptors can also be due to differences in distribution of the IR and IGF-IR in different cell types, receptor activation, rate of internalization and intracellular signal transduction (Blakesley et al., 1996; Kim and Accili, 2002; Werner et al., 2008). The αβ-heterodimers from IR and IGF-IR, respectively, can combine to form insulin/IGF-I hybrid receptors (Moxham et al., 1989). Insulin, IGF-I and IGF-II all bind to IGF-IR and IR but with different affinities, as reviewed by Werner et al. (2008). The IR has a high affinity for insulin, but can also bind IGF-II with 10–50 fold lower affinity and IGF-I with 100–500 fold lower affinity. IGF-II binds to the IGF-IR with 10-fold lower affinity compared with IGF-I. Insulin/IGF-I hybrid receptors function mainly as IGF-IR with much higher affinity for IGF-I and IGF-II than for insulin (Soos et al., 1993; Slaaby et al., 2006). This would imply that the effect of the ligand varies with different receptor distributions.

Our aim was to compare the effects of insulin, IGF and IGF-II and study the impact of differences in distribution of IGF-IR and IR on their action in adipocytes, which express almost exclusively IR, and human preadipocytes, which express both of IR and IGF-IR (Bäck and Arnqvist, 2009).

2. Materials and methods

2.1. Cell culture

Cell culture flasks and plates were purchased from Corning (Schiphol-Rijk, The Netherlands) and all other cell culture materials were purchased from Invitrogen (Paisley, UK) or as indicated in the text. Human preadipocytes and adipocytes were isolated from subcutaneous adipose tissue from elective abdominal surgery. None of the patients had known diabetes. The average BMI was 26.7 kg/m^2, range 21.1–31.2 kg/m^2, except for one patient who was extremely obese, BMI 49.1 kg/m^2. All patients had given their informed consent, the procedures were approved by the local ethics committee at Linköping University and were performed in accordance with the Declaration of Helsinki.

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2.2. Isolation of human adipocytes and preadipocytes

Fat pads were isolated and cut with scissors before incubation with 300 u/ml collagenase type 1 (Sigma–Aldrich Sweden AB, Stockholm, Sweden) in a Krebs–Ringer solution (0.12 M NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$ and 2.5 mM $CaCl_2$) with addition of 20 mM HEPES, pH 7.40 (Sigma), 2 mM glucose, 200 nM adenosine and 1% (w/v) fatty acid-free bovine serum albumin (BSA). Digested tissue was filtered through gauze to remove connective tissue and cells were washed three times with the Krebs–Ringer solution. Fat cells were then isolated by flotation, a procedure which has been shown to give pure adipocytes (Rodbell, 1964). Isolated adipocytes were incubated in 37 °C over night to reverse insulin resistance induced by surgical stress, as previously described in Danielsson et al. (2005).

Preadipocytes were isolated from the Krebs–Ringer solution after the adipocytes were removed by floatation essentially as described in Halvorsen et al. (2001). Briefly, the cell suspension was centrifuged at 500 × *g* for 5 min and the pellet washed once with preadipocyte culture medium (equal amounts of DMEM and Ham's F-12, 10% (v/v) newborn calf serum (NCS), 100 iu/ml penicillin, 100 μg/ml streptomycin, 2 μg/ml Fungizone®) and then cultured in 75 cm^2 culture flasks with preadipocyte culture medium. Medium was changed 2–3 times a week and cells were passaged using trypsin–EDTA when nearly confluence. Cells were not used for more than 5 passages.

2.3. Cell incubation

Preadipocytes were serum-deprived over night in DMEM containing 100 iu/ml penicillin, 100 μg/ml streptomycin, 2 μg/ml Fungizone® and 0.1% (w/v) BSA. Insulin, IGF-I or IGF-II at indicated concentrations were added to the flasks and they were incubated at 37 °C for 10 min. The medium was removed and lysis buffer (pH 7.5) containing 20 mM TrizmaBase, 150 mM NaCl, 5 mM EDTA, 0.5% (w/v) sodiumdeoxycholate and 0.5% (v/v) Triton X-100 with addition of 1 mM phenylmethylsulphonyl fluoride (PMSF), 1.5 μg/ml leupeptin, 1 mM Na_3VO_4 and 1.5 μg/ml aprotinin was added to the flasks. The cells were lysed on ice for 30 min and then harvested by scraping. The lysate was centrifuged at 12,000 × *g* for 15 min at 4 °C and the supernatant was stored at −70 °C until use.

The adipocytes were preincubated for 15 min with 100 nM phenylisopropyl adenosine and 0.5 u/ml adenosine deaminase and then with indicated concentrations of insulin, IGF-I or IGF-II for a further 10 min. The media were removed and the cells resuspended in the lysis buffer. The cells were homogenized using a potter-elvehjem grinder and the lysate was stored frozen until analyzed.

2.4. Immunoprecipitation, SDS-PAGE and immunoblotting

Lysates were immunoprecipitated (IP) with either rabbit polyclonal anti-IR β antibody (c-19) or rabbit polyclonal anti-IGF-IR β antibody (c-20) (Santa Cruz Biotechnology, Heidelberg, Germany), diluted 1:400, and incubated at 4 °C for 2 h. 20 mg/ml Protein A Sepharose (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) in 0.1% (w/v) BSA in the lysis buffer was then added and the lysates were incubated overnight at 4 °C with gentle rocking. The next day lysates were centrifuged at 6500 × *g* for 5 min, supernatants were removed and pellets were washed three times with the lysis buffer. Pellets were then dissolved in SDS-PAGE sample buffer (6.25 mM TrizmaBase, 1% (w/v) SDS, 10% (v/v) glycerol, 0.001% (v/v) bromophenol) with addition of 2% (v/v) β-mercaptoethanol and boiled for 3 min.

Immunoprecipitated proteins were separated in 7.5% acrylamide gels and proteins electrophoretically blotted to polyvinylidene difluoride (PVDF) membranes. After blocking in 3% (w/v) bovine serum albumin, membranes were incubated with antiphosphotyrosine (PY20) or, after stripping membranes in 62 mM Tris–HCl, 2% (w/v) sodium dodecylsulfate, 100 mM β-mercaptoethanol for 30 min at 56 °C, with anti-IGF-IR or anti-IR antibodies. Bound antibodies were detected using horseradish peroxidase-conjugated secondary antibodies and the ECL-plus kit of GE Healthcare. For densitometric evaluation autoradiographs were scanned with a LAS1000 camera (Fujifilm, Tokyo, Japan) and then analyzed using the Multi Gauge software, version 3.0 (Fujifilm). Protein phosphorylation was normalized to total receptor protein and data was expressed as % of maximum effect or as % of unstimulated control.

2.5. Determination of thymidine incorporation in preadipocytes

Cultures of preadipocytes were grown in 6-well plates until near confluence. The cells were incubated with insulin and IGF-I at indicated concentrations in serum free DMEM overnight and then with 1 μCi/ml [6-^{3}H]-thymidine for 3 h at 37 °C, 5% CO_2. Cells were washed and DNA was then precipitated in ice cold 5% (w/v) trichloroacetic acid for 15 min at 4 °C. After treatment with 0.1 M potassium hydroxide for 1–2 h at room temperature 800 μl of the lysate was added to 4 ml of UltimaGold™ scintillation fluid (Chemical Instruments AB (CiAB), Sollentuna, Sweden) and the radioactivity was measured. Data were expressed as percent of radioactivity in untreated control cells.

2.6. Determination of glucose accumulation in preadipocytes

Glucose accumulation was determined as described previously (Bäck and Arnqvist, 2009). Briefly, the cells were grown in 6-well plates, serum-deprived overnight and then incubated for 3 h in serum free DMEM (glucose concentration 1000 mg/l) with addition of 0.2 μCi/ml D-[U-^{14}C]-glucose and insulin or IGF-I at indicated concentrations. The cells were lysed in 0.1% sodium dodecylsulfate and the radioactivity was measured. Data were expressed as percent of radioactivity in unstimulated control cells. EC_{50} values, maximum values and confidence intervals were calculated with GraphPad Prism 4.00 (GraphPad Software Inc., San Diego, CA, USA).

2.7. Determination of glucose transport in adipocytes

Glucose uptake was determined as uptake of 2-deoxy-D-[1-^{3}H]glucose (Amersham Biotech, Uppsala, Sweden) after transfer of cells to medium without glucose (Karlsson et al., 2004). Adipocytes at 10% (v/v) were incubated at 37 °C with the indicated concentration of insulin, IGF-I or IGF-II for 15 min, when 2-deoxy-D-[1-^{3}H]glucose was added to a final concentration of 50 μM (10 μCi/mL) and the cells were incubated for a further 30 min. Incubations were terminated by separating the cells from the medium during 5-s centrifugation through dinonylphtalate oil. The cell cake was dissolved in sodium dodecyl sulfate (SDS) and the amount of radioactivity in the cells determined by scintillation counting. Radioactivity in the medium trapped between cells was corrected for by subtracting radioactivity in incubations that were immediately terminated by addition of cytochalasin B and spinning through the oil. EC_{50} values, maximum values and confidence intervals were calculated with GraphPad Prism 4.00 (GraphPad Software Inc., San Diego, CA, USA).

2.8. Statistical analysis

Statistical comparisons were made with SPSS 17.0 (SPSS Inc. Headquarters, Chicago, IL, USA) by ANOVA. For post hoc analysis Dunnet's test was used. When indicated a nonparametric test, related-samples Wilcoxon signed ranks test, was used. GraphPad Prism 4.00 (GraphPad Software Inc., San Diego, CA, USA) was used for comparison of dose response curves when indicated, and for drawing bar graphs. A p value <0.05 was considered statistically significant. Data is presented as mean ± SE.

3. Results

3.1. Preadipocytes

3.1.1. Receptor phosphorylation

In preadipocytes we detected IGF-IR after immunoprecipitation with IR antibody and then immunoblotting with an IGF-IR antibody, and vice versa, indicating the presence of insulin/IGF-I hybrid receptors.

Preadipocytes were stimulated with either IGF-II, IGF-I or insulin at 10^{-9}–10^{-8} M (Fig. 1), then the receptor proteins were immunoprecipitated either with an antibody against the IGF-IR β-subunit or with an antibody against the IR β-subunit and analyzed by SDS-PAGE and immunoblotting. After immunoprecipitation with the antibody against IGF-IRβ we could detect phosphorylation by IGF-II 10^{-8} M, IGF-I 10^{-8} M, inconsistently also by IGF-I 10^{-9} M, but not by insulin. Immunoprecipitation with the antibody against IRβ revealed phosphorylation by IGF-II 10^{-8} M, insulin 10^{-9}–10^{-8} M and IGF-I 10^{-8} M.

3.1.2. Thymidine incorporation

To quantify DNA synthesis in preadipocytes the incorporation of [6-^{3}H]-thymidine was examined after the cells were stimulated with insulin, IGF-I or IGF-II at 10^{-11}–10^{-7} M (Fig. 2A). IGF-I 10^{-8} M, IGF-II 10^{-8} M or IGF-I 10^{-7} M significantly stimulated DNA synthesis. The effect of IGF-I tended to be higher than that of insulin.

To further examine the higher maximal effects of IGF-I compared to insulin a new set of experiments were performed to see if the effect of IGF-I was additional to the effect of insulin. Cells were treated with insulin 10^{-8}–10^{-5} M alone or with IGF-I 10^{-8} or 10^{-7} M added (Fig. 2B). As seen in Fig. 2A a maximal effect of IGF-I alone was obtained at a concentration of 10^{-8} M and did not increase further at 10^{-7} M. With insulin alone a maximal effect was obtained at 10^{-6} M and then levelled off.

The addition of IGF-I at the concentration of either 10^{-8} or 10^{-7} both resulted in an additional effect compared to insulin alone (Fig. 2B). To compare the maximal effects the peak of each curve