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Circulating insulin-like growth factors may contribute substantially to insulin receptor isoform A and insulin receptor isoform B signalling

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ABSTRACT

Background: Only a fraction of circulating insulin-like activity is due to insulin itself. The aim of this study was to determine total serum insulin-like activity mediated via the insulin receptor isoform A (IR-A) and isoform B (IR-B) by using kinase receptor activation (KIRA) assays specific for the IR-A and IR-B.
Methods: The IR-A and IR-B KIRA assays use human embryonic kidney cells which have been transfected with the human IR-A or IR-B gene and quantify serum-mediated phosphorylation of the IR.
Results: Both IR KIRA assays were sensitive (detection limit 32 pmol/L) and precise (intra- and inter assay CV: <12% and <15%). The EC_{50}s of insulin, IGF-I and IGF-II were 1.4, 11.2 and 6.7 nmol/L for the IR-A KIRA assay, and 1.3, 31.0 and 15.7 nmol/L for the IR-B KIRA assay.
Results: The operational range of both assays allowed for determination of total insulin-like activity in human serum. Analysis of serum samples showed that there was a significant positive correlation between serum insulin-like and immunoreactive insulin concentrations (IR-A: $r = 0.56$, $p = 0.01$, IR-B: $r = 0.68$, $p = 0.001$). Importantly, addition of IGF-I or IGF-II antibodies to human serum samples could substantially decrease the endpoint signal in both KIRA assays.
Conclusions: We showed that serum IGF-I and IGF-II may substantially contribute to IR signalling. Since IR isoform specific KIRA assays also take into account the contribution of IGFs present in serum on IR signalling, they may help to gain more insight into the roles of IGF mediated IR-A and IR-B activation in health and disease.

1. Introduction

Early studies have shown that insulin-like mediated effects of serum on target tissues are much greater *in vitro* and *in vivo* than what would be expected on the basis of the measured immunoreactive insulin concentrations (Froesch et al., 1985).

Insulin elicits its various biological responses by binding to the insulin receptor (IR), which is then followed by activation of its intrinsic tyrosine kinase (Okada et al., 1998). In the human body, due to alternative splicing of exon 11 of the IR gene, two IR transcripts are generated, resulting in IR isoform A (IR-A) (lacking exon 11) and in IR isoform B (IR-B) (full length) (Belfiore et al., 2009). The relative abundance of mRNAs encoding the IR-A and IR-B isoforms is regulated in a tissue-specific manner (Moller et al., 1989) and also differs by stage of cell development and differentiation. IR-A is the predominant isoform in fetal tissues and cancer cells, while the IR-B is the classical receptor for insulin with metabolic effects in muscle, liver and adipose tissues. (Moller et al., 1989; Mosthaf et al., 1990). Although studies have suggested that differences between IR-A and IR-B in terms of receptor activation and signalling may result in different functions of each IR isoform (Belfiore et al., 2009), it appears that most cells have both IR isoforms and that the ratio of the two seems to be very important (Belfiore et al., 2009).

IGF-I and IGF-II primarily activate the IGF-I receptor (IGF-IR), but they can also activate the IRs (Boucher et al., 2010). According to the literature, the IR-B binds insulin with high affinity but the IGFs poorly, while the IR-A binds insulin and IGF-II with high affinity and IGF-I with low affinity (Denley et al., 2004). In this respect, it is important to underline that although the IGFs have a lower

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affinity for the IR than insulin, *in vivo* circulating total concentrations of IGFs are much higher than of insulin (picomolar vs. nanomolar range) and that IGF bioactivity (1–2% of the total IGF concentration) is modulated by the presence of IGF binding proteins (IGFBPs) and IGFBP proteases (Blakesley et al., 1996). Nevertheless, insulin immunoassays measure only immunoreactive insulin and by doing so they ignore at least the potential insulin-like effects of the IGFs in blood. Moreover, insulin immunoassays do not assess potential biological effects of circulating insulin-like factors on the IR-A and the IR-B.

The aim of this study was to determine the potential biologic actions of serum on the IR-A and the IR-B and to assess the relative contribution of circulating IGFs in this respect. For this purpose we used cell-based kinase receptor activation (KIRA) assays, one specific for the human IR-A and one specific for the human IR-B. Since there are no specific antibodies for the two isoforms available, specificity was determined not by isoform-specific antibodies but by transfecting HEK cells with either the IR-A or the IR-B.

The principle of these two assays is based on quantification of phosphorylated tyrosine residues within the IR after *in vitro* stimulation with serum. The same principle has been used for IGF-I KIRA assay specific for the IGF-IR (Brugts et al., 2008; Chen et al., 2003).

2. Materials and methods

2.1. Peptides

MAI1, a monoclonal antibody directed against the extracellular domain of both human IRs, was used as capture antibody (Novozymes-Gropep (Aidelade, Australia)). Europium-labelled PY20 (Eu-PY20); a monoclonal anti-phosphotyrosine antibody was used as detection antibody (PerkinElmer life sciences (Groningen, The Netherlands)). Human insulin (Actrapid®) was obtained from Novo Nordisk (Bagsvaerd, Denmark), human recombinant IGF-I from Invitrogen (Breda, The Netherlands) and human recombinant IGF-II was obtained from Sigma-Aldrich (Zwijndrecht, The Netherlands). An IGF-I neutralizing antibody was obtained from R&D Systems Europe Ltd. (Abingdon, UK). Monoclonal IGF-II antibody IgG1 m610 was developed in our laboratories (Feng et al., 2006).

2.2. Cell line and media

The human embryonic kidney (HEK) cell-line Flip-in™-293 from Invitrogen was transfected with plasmids (pNTK-2) containing a cDNA insert of the human IR-A (pNTK2-IR-A) or IR-B (pNTK2-IR-B) using Fugene® transfection reagens according to manufacturer's protocol. The plasmids were kindly provided by Axel Ullrich (Martinsried, Germany). After 48 h, cells were trypsinized and cultured in Dulbecco's Modified Eagles Medium (DMEM: gluc+, L-Glutamin +, Pyr+) from Invitrogen supplemented with 10% fetal bovine serum (FBS) from Invitrogen and geneticin 1000 μg/mL from Invitrogen. Separate colonies were isolated, expanded and tested for IR expression defined by quantitative RT-PCR as relative copy number of mRNA (see below) and by immunocytochemistry (see below). The transfected cells were cultured in 75 cm^3 culture flasks from Corning (Amsterdam, The Netherlands) using DMEM containing 10% FBS, 100 U/mL penicillin, 100 μg/L streptomycin from Invitrogen and 500 μg/mL geneticin.

2.3. Quantitative RT-PC

Total RNA was isolated from 10^6 cells, using a commercially available kit (High pure RNA isolation kit) from Roche (Almere, The Netherlands), according to the recommendation by the manufacturer. Complementary DNA was synthesized using 500 ng of total RNA in a Super Reverse Transcriptase (RT) buffer from HT Biotechnology Ltd. (Cambridge, UK), together with 40 nmol of each deoxynucleotide triphosphate, 15 ng oligo-dT primer, 20 U RNAse inhibitor, and 4 U AMV Super RT also from HT Biotechnology, in a final volume of 40 μl. This mixture was incubated for 1 h at 40 °C and thereafter diluted 5 times in bidest. A quantitative PCR was performed using the TaqMan Gold nuclease assay from Roche, according to the manufacturer's protocol. The primer and probe sequences (Sigma Aldrich (Zwijndrecht, The Nederlands)) were: IR-A forward, 5′-CGTTTGAGGATTACCTGCACAA-3′; IR-A reverse, 5′-GCCAAGGGACCTGCGTTT-3′; and IR-A probe, 5′-FAM-TGGTTTTCGTCCCCAGGCCATC-TAMRA-3′. IR-B forward, 5′-CCCAGAAAAACCTCTTCAGGC-3′; IR-B reverse, 5′-GGACCTGCGTTTCCGAGA-3′; and IR-B probe, 5′-FAM-CTGGTGCCGAGGACCCTAGGCC-TAMRA-3′. IGF-II Receptor forward, 5′-ACCGACCCCTCCACGC-3′; IGF-II Receptor reverse, 5′-CCTCCAAGGCCACCTTCAG-3′; and IGF-II Receptor probe, 5′-FAM-AGCAGTACGACCTCTCCAGTCTGGCAAA-TAMRA-3′. Samples were normalized against the expression of the housekeeping gene hypoxanthine-phospho-ribosyl-transferase (HPRT). Dilution curves were constructed for calculating the PCR efficiency for every primer set (Rasmussen, 2001). PCR efficiencies were: HPRT = 1.98, IR-A = 1.89, IR-B = 1.92, IGF-II Receptor = 1.87. The primer and probe sequences for IGF-I, IGF-II and for the IGF-IR have been previously described (van Koetsveld et al., 2006; Vitale et al., 2009). The relative expression of genes were calculated using the comparative threshold method, 2-ΔCt (Schmittgen and Livak, 2008), after efficiency correction (Pfaffl, 2001) of target and reference transcripts.

2.4. Total human IR sandwich ELISA

HEK IR-A and HEK IR-B cells were plated into a 6 well culture plate (Corning, NY, USA), 300,000 cells/well in 2 mL of culture medium at 37 °C and 5% CO_2. After 72 h medium was removed and replaced with 2 mL DMEM containing 0.1% HSA, 100 U/mL penicillin and 100 μg/L streptomycin and 500 μg/mL geneticin. The next day, a total IR sandwich ELISA was performed according to manufactures protocol (Human total Insulin R, R&D Systems Europe Ltd., Abingdon, UK). Quantification of protein concentration was performed by using Nanodrop ND-1000 (Thermo Scientific). For the HEK IR-A 200 μg of lysate was used to perform the ELISA, compared to 100 μg for the HEK IR-B.

2.5. Immunocytochemistry

HEK IR-A and HEK IR-B cells were cultured on chamber slides from Invitrogen (Breda, The Netherlands) for 2 days (20.000 cells/chamber) in DMEM containing 10% FBS, 100 U/mL penicillin, 100 μg/L streptomycin and 500 μg/mL geneticin. Prior to immunostaining, cells were fixed with 4% paraformaldehyde and 0.2% picric acid in phosphate buffer, pH 6.9 for 40 min at room temperature. After washing with Tris/HCl/Tween 0.5%, fixation of cells was finalized by incubating them with 50% methanol (3 min at room temperature) and 100% methanol (3 min at room temperature). Fixed cells were washed again with Tris/HCl/Tween 0.5% followed by washing with PBS. Subsequently they were incubated with H_2O_2 (30%) (15 min at room temperature) to quench endogenous peroxidase and were washed with Tris/HCl/Tween 0.5% thereafter. Fixed cells were then incubated with the following primary antibodies for 1 h at room temperature: anti-IR (Enzo Life Sciences, Antwerp, Belgium; mouse monoclonal, 1:25) and anti-IGF-IR (Novus Biologicals, Cambridge, United Kingdom; mouse monoclonal, 1:500). After incubation, cells were washed and two drops of HRP-Rabbit/Mouse from Dako (Heverlee, Belgium) were added to chamber slides and incubated for 30 min. Bound antibodies were visualized with freshly prepared 100 μl of DAB from Dako