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Expression and function of receptors for insulin-like growth factor-I and insulin in human coronary artery smooth muscle cells

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Abstract *Aims/hypothesis:* Hyperinsulinaemia and insulin resistance, as well as low IGF-I, have been implicated in the pathogenesis of cardiovascular disease. Little is known about direct effects of IGF-I and insulin on human coronary artery smooth muscle cells (HCASMCs). Our aim was to characterise the expression and function of IGF-I receptor (IGF-IR) and insulin receptor (IR) in HCASMCs. *Materials and methods:* Cultured HCASMCs were used. mRNA expression was measured by quantitative real-time RT-PCR analysis. Receptor proteins, phosphorylation of β-subunits and the presence of hybrid IR/IGF-IR were analysed by immunoprecipitation and western blotting. DNA synthesis and glucose metabolism were assessed using [^{3}H]thymidine incorporation and D-[U-^{14}C]glucose accumulation respectively. *Results:* The mRNA expression of IGF-IR was approximately eight-fold higher than that of IR in HCASMCs. The presence of IGF-IR and IR could be demonstrated by immunoprecipitation and western blot analysis. Phosphorylation of the IGF-IR β-subunit was obtained by IGF-I at 10^{-10}–10^{-8} mol/l and insulin at 10^{-8} mol/l. Insulin and IGF-I at 10^{-10}–10^{-9} mol/l phosphorylated the IR β-subunit. When immunoprecipitated with monoclonal anti-IR α-subunit or IGF-IR α-subunit antibodies, we found bands in slightly different positions, suggesting the presence of hybrid IR/IGF-IR. IGF-I at 10^{-9}–10^{-8} mol/l significantly stimulated [^{3}H]thymidine incorporation and at a concentration of 10^{-9}–10^{-7} mol/l also D-[U-^{14}C]glucose accumulation in HCASMCs. Insulin at 10^{-9}–10^{-7} mol/l had no effect on DNA synthesis, but increased glucose accumulation at 10^{-7} mol/l. *Conclusions/interpretation:* Our study provides experimental evidence that IGF-IR and possibly hybrid IR/IGF-IR play a role in HCASMCs.

Keywords Human coronary artery smooth muscle cells · Hybrid insulin receptor/IGF-I receptor · IGF-I receptor · Insulin receptor

Abbreviations ECL: enhanced chemiluminescence · FAM: 6-carboxyfluorescein · GAPDH: glyceraldehyde-3-phosphate dehydrogenase · HCASMC: human coronary artery smooth muscle cell · HRP: horseradish peroxidase · IGF-IR: IGF-I receptor · IR: insulin receptor · PY20: anti-phosphotyrosine antibody · TAMRA: 6-carboxytetramethylrhodamine · VIC: fluorescent dye · VSMC: vascular smooth muscle cell

Introduction

Coronary heart disease is a common cause of morbidity and mortality in patients with diabetes [1, 2]. Also, insulin-resistant states without diabetes are associated with cardiovascular disease [3]. Insulin and the related peptide IGF-I have been reported to be associated with the pathogenesis of vascular disease [4, 5]. In atherosclerotic vascular lesions and restenosis after percutaneous transluminal coronary angioplasty, proliferation of vascular smooth muscle cells (VSMCs) takes place [6, 7]. IGF-I has been reported to be a chemoattractant factor and to regulate metabolism, cell proliferation, differentiation and survival in VSMCs of animal origin [8–12]. Although several reports have documented the expression of IGF-I and its receptor (IGF-IR) in VSMCs from bovine [13–15], porcine [16] and rat [17, 18], little is known about the expression and function of insulin receptor (IR) and IGF-IR in human VSMCs [19–21].

Insulin receptor and IGF-IR are homologues, sharing >50% of their amino acid sequence and having 84% homology in the β-subunit tyrosine kinase domains [22, 23]. Investigating the physiological effects of insulin and IGF-I is complicated, because, besides acting on their receptors, each hormone can cross-react, with a lower affinity, with the other's receptor [24], and IGF-I also interacts with specific

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binding proteins [25]. Furthermore, insulin and IGF-I can also bind to hybrid IGF-IR/IR [26, 27], which can form when both receptors are expressed in the same cells.

The first step in activation of IGF-IR and IR is autophosphorylation of the β-subunit, followed by recruitment and phosphorylation of docking proteins involved in the regulation of cell metabolism, proliferation and survival [22, 23]. Knowing how insulin and IGF-I act on human coronary artery smooth muscle cells (HCASMCs) could have a great impact on our understanding of the pathogenesis of cardiovascular disease. Therefore the aim of this study was to characterise IGF-IR and IR in HCASMCs with regard to expression and function.

Materials and methods

Materials

Primers and probes for quantitative real-time RT-PCR were purchased from SGS (Scandinavian Gene Synthesis AB, Köping, Sweden). Mono-^{125}I-(Tyr A14)-human insulin (56 TBq/mmol), ^{125}I-IGF-I (74 TBq/mmol), D-[U-^{14}C]glucose (11.7 GBq/mmol) and [6-^{3}H]thymidine (740 GBq/mmol) were obtained from Amersham Pharmacia Biotechnology (Little Chalfont, Bucks, UK).

Polyclonal anti-IGF-IR β-subunit antibody c-20 and anti-IR β-subunit antibody c-19 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Monoclonal anti-IGF-IR α-subunit 17-69 and anti-IR α-subunit 83-7 antibodies were a kind gift of Professor K. Siddle, University of Cambridge, UK. Horseradish peroxidase-conjugated goat anti-rabbit (goat anti-rabbit-HRP) (Zymed, San Francisco, CA, USA), sheep anti-mouse-HRP (Amersham) and streptavidin-HRP (Amersham) were used as secondary antibodies. An enhanced chemiluminescence (ECL) detection system was obtained from Amersham.

Culture of cells

HCASMCs were obtained from Clonetics (San Diego, CA, USA) and tested negative for von Willebrand Factor VIII and positive for α-smooth muscle actin. Cells were cultured in accordance with the manufacturer's instruction in smooth muscle cell growth medium consisting of 500 ml smooth muscle cell basal medium, 2.5 mg insulin, 1 μg human fibroblast growth factor, 5 ng human recombinant epidermal growth factor, 25 mg gentamicin, 25 μg amphotericin B and 25 ml fetal bovine serum. HCASMCs were incubated at 37°C in 95% air–5% CO_2. All experiments were performed in triplicate with cells in passage five to eight in confluent cultures.

Quantitative real-time RT-PCR

RNA was extracted using the RNeasy Mini Kit (Qiagen, Hilden, Germany). From 1 μg RNA, first-strand cDNA was transcribed using a commercial kit (Invitrogen, Life Technologies, Stockholm, Sweden). The gene expression of IR and IGF-IR, with primer and probe pairs for IGF-I and IR mRNA, was estimated by a quantitative real-time PCR assay using the ABI PRISM 7700 Sequence Detection System (PE Applied Biosystems, Stockholm, Sweden). The oligonucleotides were purchased from SGS. For human IR we used as forward primer 5′-AGGAGCCCAATGGTCTGA-3′, as reverse primer 5′-GAGACGCAGAGATGCAGC-3′ and as probe 5′-(6-carboxyfluorescein [FAM])-ACCATATCGCCG ATAACTCACTTCATACAGT-(6-carboxytetramethylrhodamine [TAMRA])-3′. For human IGF-IR we used as forward primer 5′-CGATGTGTGAGAAGACCACCA-3′, as reverse primer 5′-ACATTTTCTGGCAGCGGTTT-3′ and as probe 5′-(FAM) CAATGAGTACAACTACCGC TGCTGGACCAT-(TAMRA)-3′. The primers and probe for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA were obtained from PE Applied Biosystems with the sequence for forward primer 5′-GAAGGTGGAGG TCGGAGTC-3′, for reverse primer 5′-GAAGATGGTGA TGGGATTTC-3, and for probe 5′-(fluorescent dye [VIC])-CAAGCTTCCCGTTCTCAGCC-(TAMRA)-3′.

The reaction consisted of 25 ng cDNA, 50 nmol/l probe, 300 nmol/l sense and anti-sense primers, 12.5 μl 2×TaqMan Mastermix (PE Applied Biosystems) and water in a total volume of 25 μl. After 2 min at 50°C and 10 min at 95°C, the reaction ran for 40 cycles consisting of a denaturation or melting step at 95°C for 15 s, followed by an annealing/extension step at 60°C for 1 min. The detection of the PCR products was possible through the combination of 5′–3′ nuclease activity of AmpliTaq Gold DNA Polymerase 6-carboxy-*X*-rhodamine by the release of the fluorescent reporters FAM for human mRNA IR and IGF-IR and VIC for GAPDH probe oligonucleotides during the RT-PCR reaction. The fluorescence was measured at each cycle. The data were analysed using Sequence Detector version 1.7 (PE Applied Biosystems).

The relative amount of mRNA was normalised to the housekeeping gene encoding GAPDH. GAPDH reactions of all samples were run in the same 96-well plate in the same run as the reactions of the samples for the gene of interest. GAPDH mastermix (1.25 μl) (PE Applied Biosystems) consisting of primers and probe for GAPDH was added to 25 ng cDNA, 12.5 μl TaqMan Mastermix and water for a total reaction volume of 25 μl. Reactions were performed in duplicate. The relative quantity of IGF-IR and IR mRNA was calculated using the comparative C_T method after initial experiments showed similar quantitative PCR efficiency rates for IGF-IR, IR and GAPDH.

Immunoprecipitation of the IGF-IR and IR β-subunit

To determine the tyrosine phosphorylation state of IGF-IR and IR β-subunit, subconfluent HCASMCs were cultured in 75-cm^2 flasks. They were serum starved for 24 h before the experiments. The cells were then washed in cold F-12-BSA medium (1 mg BSA/ml F-12) and incubated for 30 min on ice with 50 μmol/l Na_3VO_4 solution diluted in

F-12-BSA medium. The cultures were incubated in warm (37°C) F-12-BSA medium (1 mg BSA/ml F-12) at 37°C for 10 min with concentrations of 10^{-10}–10^{-8} mol/l for IGF-I and 10^{-10}–10^{-8} mol/l for insulin. After the incubation, cells were lysed for 30 min on ice with a lysis buffer containing 20 mmol/l Tris (pH 7.5), 150 mmol/l NaCl, 5 mmol/l EDTA, 0.5% sodium deoxycholate, 0.5% Triton X-100, 1 mmol/l Na_3VO_4, 1.5 μg/ml aprotinin, 1.5 μg/ml leupeptin and 1 mmol/l phenylmethylsulphonyl fluoride. Cell lysates were centrifuged at 4°C for 15 min at 20,000 *g*. The supernatant was transferred into new tubes and stored at −70°C. Total protein content was measured by the bicinchoninic acid method (Pierce, Rockford, IL, USA) in order to adjust the amount of protein used for subsequent analysis.

To immunoprecipitate the IGF-IR or IR, we incubated cell lysates that contained 0.5–1 mg total protein with 2.5 μl polyclonal anti-IGF-IR β-subunit antibody c-20 (Santa Cruz Biotechnology) and monoclonal anti-IGF-IR α-subunit 17–69 antibodies or polyclonal anti-IR β-subunit antibody c-19 (Santa Cruz Biotechnology) and monoclonal anti-IR α-subunit 83-7, respectively. Protein-A Sepharose (Pharmacia-Upjohn, Uppsala, Sweden) or Protein G (Amersham) was added and samples were shaken gently at 4°C overnight. The immunoprecipitates were washed three times with ice-cold lysis buffer and diluted in 50 μl 2×Laemmli sample buffer (0.125 mol/l Tris base, 4% SDS, 10% glycerol, 0.02% bromophenol, 4% β-mercaptoethanol, pH 6.8).

Western blot analysis

Immunoprecipitated samples were boiled for 2 min. After centrifugation, proteins in the supernatant were separated on a 7.5% SDS-PAGE gel. The separated proteins were electrotransferred onto a polyvinyl difluoride membrane and blocked overnight with blocking buffer (5% milk powder, 0.1% Tween 20, TBS). The membrane was immunoblotted using 1:1,000 dilution of the anti-phosphotyrosine PY20 antibody (Santa Cruz Biotechnology) for 2 h at room temperature. The proteins were visualised by using a specific secondary HRP-linked anti-mouse monoclonal IgG antibody (Amersham), followed by an ECL detection system detection. Autoradiographs were obtained by exposure to a Hyperfilm ECL (Amersham).

The membranes were stripped by heating at 55°C for 30 min in stripping buffer (10% SDS, 100 mmol/l β-mercaptoethanol, 62.5 mmol/l Tris–HCl). They were again blotted with either polyclonal anti-IGF-IR β-subunit antibody c-20 or polyclonal anti-IR β-subunit antibody c-19 and the proteins were visualised as described above.

^{3}H[Thymidine] incorporation into DNA

DNA synthesis was quantified by measuring [^{3}H]thymidine incorporation into DNA in HCASMCs. The cells were grown in 24-well plates, serum starved for 24 h and then incubated with 37 kBq/ml [^{3}H]thymidine (Amersham) with and without insulin and IGF-I at concentrations of 10^{-10}–10^{-7} mol/l. DNA was precipitated with 5% cold trichloroacetic acid and then solubilised in 0.5 ml 0.1 mol/l KOH. Part of the solution, 0.4 ml, was added to 4 ml scintillation solution, and the radioactivity was measured in a liquid scintillation counter (Rackbeta 1217; LKB Wallac, Turku, Finland). The data are expressed as percentage increase in [^{3}H]thymidine incorporation of control cells.

D-[U-^{14}C]glucose accumulation

Confluent HCASMCs grown in six-well plates were starved in serum-free media for 24 h before experiment. The cells were then incubated at 37°C for 2 h with the addition of 37 kBq/ml D-[U-^{14}C]glucose (Amersham) and in the absence or presence of insulin and IGF-I at concentrations of 10^{-10}–10^{-7} mol/l. In a separate experiment, cells were pretreated for 2 h with IGF-IR antibody, clone α-IR$_3$ at 10^{-8} mol/l followed by insulin or IGF-I at concentrations of 10^{-9}–10^{-7} mol/l for another 2 h. Thereafter cells were rinsed three times with PBS, lysed with 0.5 ml 0.1% SDS and 0.4 ml of the solubilised cells was added to 4 ml scintillation solution, and the radioactivity was measured. The data are expressed as percentage of control cells.

Statistical analysis

Values are given as means±SEM. Statistical comparisons were made with the SPSS program (SPSS, Chicago, IL, USA) by one-way ANOVA. A p value of less than 0.05 was considered statistically significant.

Results

Quantitative real-time RT-PCR

The mRNA expression of IGF-IR and IR was demonstrated in HCASMCs by quantitative real-time RT-PCR in cells starved for 24 h before experiments. The amount of IGF-IR mRNA was approximately eight times more abundant than that of IR mRNA (p<0.001) (Fig. 1).

Immunoprecipitation and western blot for IGF-IR and IR

The effect of IGF-I or insulin on phosphorylation of IGF-IR and IR β-subunit in HCASMCs and also the presence of hybrid IR/IGF-IR were assessed by immunoprecipitation and western blotting (Figs. 2, 3 and 4). In order to show receptor phosphorylation, cells were exposed to either IGF-I or insulin at concentrations indicated for 10 min. After immunoprecipitation of receptors with an anti-IGF-IR antibody and blotting the membrane with the same antibody, we found a band in the vicinity of 97 kDa, a position corresponding to IGF-IR β-subunit (Fig. 2). When developed with PY20 the immunoprecipitated β-subunit was found to be phosphory-

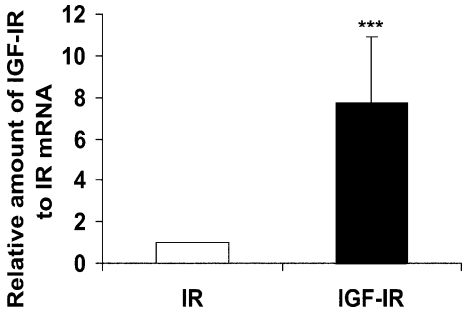

Fig. 1 Expression of mRNA IGF-IR and IR in cultured HCASMCs. IGF-IR and IR mRNA expression were measured by real-time RT-PCR. Results are presented as the mean amount of IGF-IR mRNA relative to the mean amount of IR mRNA±SEM (n=4 for each). ***p<0.001

lated after exposure to IGF-I at 10^{-10}–10^{-8} mol/l and insulin at 10^{-8} mol/l (Fig. 2). Insulin at 10^{-9} mol/l did not consistently phosphorylate the IGF-I β-subunit (Fig. 2) and insulin at 10^{-10} mol/l had no effect on IGF-IR phosphorylation (Fig. 2).

By immunoprecipitation with a polyclonal anti-IR antibody we demonstrated a band with a molecular mass in a slightly lower position than 95 kDa, corresponding to the IR β-subunit, which has a molecular mass of 95 kDa (Fig. 3). Phosphorylation of the β-subunit shown by developing the membrane with PY20 was obtained with IGF-I at 10^{-10}–10^{-9} mol/l and insulin at 10^{-10}–10^{-9} mol/l (Fig. 3).

Immunoprecipitation and western blot for hybrid IR/IGF-IR

Immunoprecipitation of the IR or IGF-IR with monoclonal anti-IR or anti-IGF-IR α-subunit antibody brings down the whole $\alpha_2\beta_2$-heterotetradimeric holoreceptor complex. From the same lysate used for receptor phosphorylation we also immunoprecipitated the receptors with specific monoclonal anti-IR 83-7 (Fig. 4a,b) or anti-IGF-IR 17-69 α-subunit antibodies (Fig. 4c,d) and detected the receptor bands by using polyclonal anti-IR or anti-IGF-IR

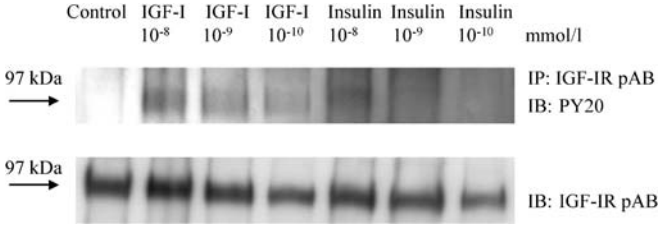

Fig. 2 Tyrosine phosphorylation of IGF-IR β-subunit and protein expression of IGF-IR β-subunit after immunoprecipitation with IGF-IR c-20 antibody in HCASMCs. Immunoprecipitation (*IP*) and western blot analysis of IGF-IR β-subunit and tyrosine phosphorylation of IGF-IR β-subunit after exposure of near-confluent HCASMCs to either IGF-I at 10^{-10}–10^{-8} mol/l or insulin at 10^{-10}–10^{-8} mol/l for 10 min. Immunoblotting (*IB*) with anti-phosphotyrosine antibody PY20 and with anti-IGF-IR β-subunit (pAB) antibody polyclonal, as indicated. Results are representative of three experiments. The *arrow* indicates the molecular mass at 97 kDa

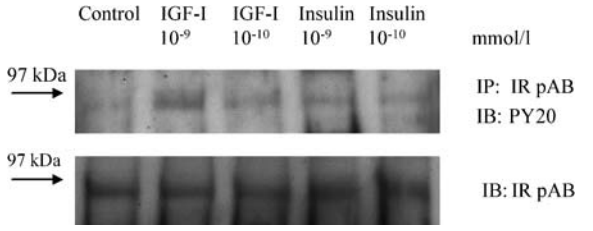

Fig. 3 Tyrosine phosphorylation of IR β-subunit and protein expression of IR β-subunit after immunoprecipitation with IR c-19 antibody in HCASMCs. Immunoprecipitation (*IP*) and western blotting analysis (*IB*) of IR β-subunit and tyrosine phosphorylation of IR β-subunit after exposure of near-confluent HCASMCs to either IGF-I at 10^{-10}–10^{-9} mol/l or insulin at 10^{-10}–10^{-9} mol/l for 10 min. Immunoblotting with anti-phosphotyrosine antibody PY20 and anti-IR β-subunit (pAB) antibody polyclonal, as indicated. The *arrow* indicates the molecular mass at 97 kDa

β-subunit antibodies. When immunoprecipitating the receptors with monoclonal anti-IR α-subunit antibody and then developing the membrane with polyclonal anti-IR β-subunit antibody (Fig. 4a), we observed a band situated at a slightly lower molecular mass position (97 kDa) than when it was developed with the polyclonal anti-IGF-IR β-subunit antibody (Fig. 4b). The opposite result was obtained for the receptors initially immunoprecipitated with the monoclonal anti-IGF-IR α-subunit antibody, when we developed the membrane with the polyclonal anti-IGF-IR β-subunit antibody (Fig. 4c) or thereafter with the polyclonal anti-IR α-subunit antibody (Fig. 4d). In this case, the band was situated at a higher molecular mass position than when it was developed with the polyclonal anti-IR β-subunit antibody.

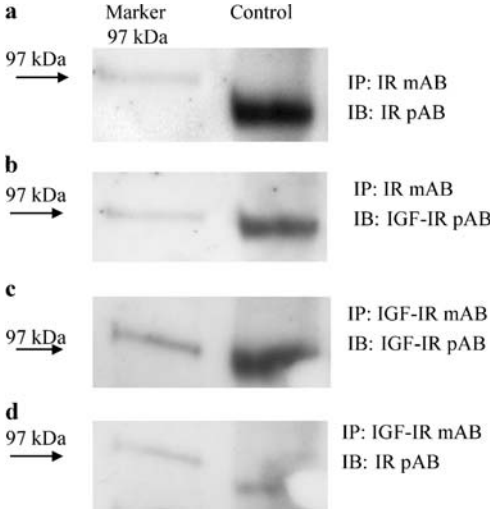

Fig. 4 Hybrid IR/IGF-IR in HCASMCs after immunoprecipitation with monoclonal antibodies (*mABs*). **a** Immunoprecipitation (*IP*) with IR 83-7 mouse monoclonal antibody and immunoblotting (*IB*) with IR polyclonal antibody (*pAB*) c-19. **b** Immunoprecipitation with IR 83-7 mouse monoclonal antibody and immunoblotting with IGF-IR polyclonal antibody c-20. **c** Immunoprecipitation with IGF-IR 17-69 mouse monoclonal antibody and immunoblot with IGF-IR polyclonal antibody c-19. **d** Immunoprecipitation with IGF-IR 17-69 mouse monoclonal antibody and immunoblot with IR polyclonal antibody c-20. Immunoreactive bands are located at different locations on the blot. Results are representative of three experiments

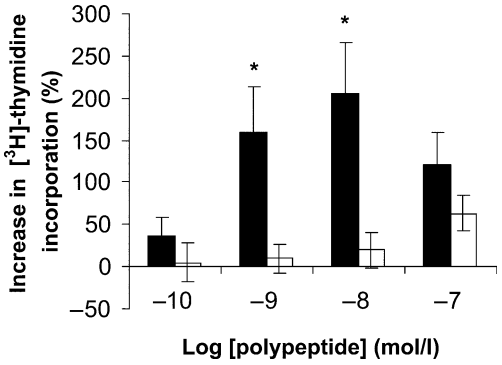

Fig. 5 Effect of insulin (*open bars*) and IGF-I (*black bars*) on [^{3}H] thymidine incorporation in HCASMCs. Confluent cultured cells were incubated at 37°C for 24 h in serum-free medium in the presence of [^{3}H]glucose and polypeptides. Values are means±SEM of measurements from four experiments. *p<0.05

^{3}H[Thymidine] incorporation into DNA

DNA synthesis was determined by [^{3}H]thymidine incorporation in HCASMCs (Fig. 5). The incorporation of [^{3}H] thymidine was stimulated by addition of IGF-I and was significant at concentrations of 10^{-9} mol/l (p=0.017) and 10^{-8} mol/l (p=0.01), but not at a lower concentration of 10^{-10} mol/l or at a higher concentration of 10^{-7} mol/l. Insulin in concentrations of 10^{-10}–10^{-7} mol/l had no significant effect.

D-[U-^{14}C]glucose incorporation

The effect of IGF-I and insulin on glucose metabolism in HCASMCs was studied as D-[U-^{14}C]glucose accumulation into the cells (Fig. 6). IGF-I significantly stimulated glucose accumulation, at concentrations of 10^{-8}–10^{-7} mol/l (p<0.001) and 10^{-9} mol/l (p=0.002), respectively, whereas insulin at 10^{-10}–10^{-8} mol/l had no significant effect, except at the highest concentration of 10^{-7} mol/l (p=0.004).

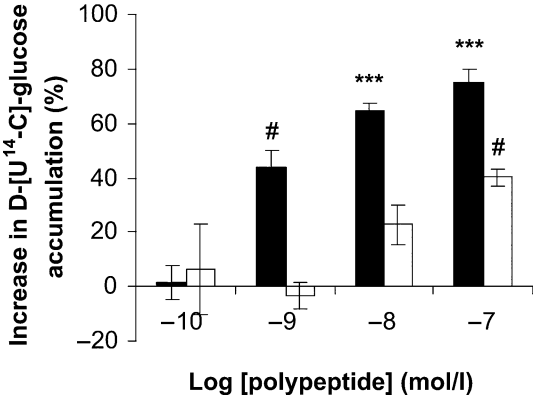

Fig. 6 Effect of insulin (*open bars*) and IGF-I (*black bars*) on D-[U-^{14}C] glucose incorporation in HCASMCs. Confluent cultured cells were incubated at 37°C for 2 h in serum-free medium in the presence of D-[U-^{14}C]glucose and polypeptides. *Bars* are means± SEM of measurements from four experiments. $^{\#}p$=0.05–0.001; ***p<0.001

Pretreatment of HCASMCs with IGF-IR antibody, clone α-IR3 at 10^{-8} mol/l, followed by insulin or IGF-I at a concentration of 10^{-9}–10^{-7} mol/l resulted in inhibition of IGF-I at 10^{-9} mol/l and a tendency to partial inhibition of insulin at 10^{-8}–10^{-7} mol/l (data not shown).

Discussion

Here we show that cultured HCASMCs express IGF-IR and IR as detected by mRNA expression and western blotting. Gene expression indicated that IGF-IR was more abundant than IR. We also found evidence for hybrid IR/IGF-IR. Both IGF-I and insulin were capable of phosphorylating their own receptors at low physiological concentrations of 10^{-10}–10^{-9} mol/l [28, 29], whereas only IGF significantly stimulated glucose metabolism and DNA synthesis at a low concentration in HCASMCs.

IGF-IR mRNA expression in HCASMCs was demonstrated in this study by quantitative real-time RT-PCR. In VSMCs from experimental animals, the presence of IGF-IR has been demonstrated by ligand binding and western blotting [13, 30–33]. Ligand binding of IGF-I has been reported in cultured human newborn aortic smooth muscle cells [20]. The presence of IGF-IR α-subunit was demonstrated in HCASMCs using affinity cross-linking of ^{125}I-IGF-I [21]. By immunoprecipitation and western blot analysis, we showed the IGF-IR β-subunit in HCASMCs. In this investigation, phosphorylation of IGF-IR β-subunit in HCASMCs by low, physiological concentrations of IGF-I was demonstrated. Insulin was only able to activate the β-subunit of IGF-IR at a supraphysiological concentration. Circulating insulin concentrations in vivo are probably too low for insulin to act directly on the IGF-IR in VSMCs [19]. In porcine VSMCs activation of IGF-IR by IGF-I has been shown [30, 31, 34]. The available data thus indicate that both human and animal VSMCs have functioning IGF-IRs activated at physiological IGF-I concentrations.

In VSMCs, specific insulin binding is low and uncertain [19, 35] and affinity cross-linking of ^{125}I-insulin to its receptor has not been shown. In this study, however, we found mRNA expression of IR in HCASMCs, and demonstrated the presence of the β-subunit of the IR as well as phosphorylation of IR β-subunit by a physiological concentration of insulin. This clearly shows the presence of IRs in VSMCs and phosphorylation of the β-subunit by low concentrations of insulin in HCASMCs, suggesting that IRs were activated.

In order to distinguish between IR, IGF-IR and hybrid IR/IGF-IR in HCASMCs, receptor immunoprecipitation was done with anti-IGF-IR or anti-IR α-subunit-specific monoclonal antibodies and then blotting with polyclonal IGF-IR or IR antibody, respectively. The results were similar and showed bands for both insulin and IGF-IRs on the same gels situated in slightly different positions in the vicinity of a molecular mass marker of 97 kDa when developed subsequently by the two receptor antibodies. These observations indicate either the presence of hybrid IR/IGF-IR or that the antibodies are not specific for IR and

IGF-IR. Using the same monoclonal antibodies as we did, several previous studies have reported the presence of hybrid IR/IGF-IR in various types of mammalian tissues and in several cell lines [26, 27, 36–38]. These monoclonal antibodies are specific for IR and IGF-IR respectively, and also bind to hybrid receptors [39], which strongly suggests the presence of hybrid receptors in human coronary artery endothelial cells. Our finding on IGF-IR and IR phosphorylation should therefore be interpreted and judged in light of the presence of hybrid IR/IGF-IRs. Hybrid IR/IGF-IR could have different biological characteristics, depending on whether IR isoform A or B is involved. Hybrid IR-B/IGF-IR has binding characteristics similar to the IGF-IR, while hybrid IR-A/IGF-IR has a high affinity for insulin [40]. Although we did not characterise the IR isoforms, our observation that insulin stimulates glucose accumulation only in very high concentrations could be explained by activation of IGF-IR or the presence of hybrid IR/IGF-IR acting as IGF-IR [39, 41]. The lack of phosphorylation of the IGF-IR by low concentrations of insulin and the phosphorylation of the IR by low concentrations of IGF-I is also consistent with the properties of hybrid IR-B/IGF-IR [40].

In agreement with our findings in this study, it has previously been shown in HCASMCs that IGF-I has a weak mitogenic effect, increasing [^{3}H]thymidine incorporation at a concentration of 10^{-8} mol/l [30]. In human aortic VSMCs, IGF-I at 10^{-9} mol/l also stimulated DNA synthesis [20, 42, 43]. Evidence also exists that IGF-I plays a functional role in the regulation of DNA synthesis, assayed by [^{3}H]thymidine incorporation, in cultured porcine and rat aortic and coronary smooth muscle cells [11, 13, 25, 35, 36, 44]. In our study, glucose accumulation into HCASMCs was also significantly stimulated by IGF-I in low concentration. These results suggest that IGF-I has metabolic and growth effects at low physiological concentrations in human VSMCs.

Similar results, supporting our finding that HCASMCs were insensitive to insulin, have been reported in HCASMCs [21] and in human aortic VSMCs [20, 43, 45]. Our results from pretreatment of HCASMCs with an IGF-IR antibody are in agreement with earlier studies indicating that effects of insulin at high concentrations (10^{-7} mol/l) are probably mediated via IGF-IR rather than IR [20, 43, 45]. Although we demonstrated the presence and activation of the IR in HCASMCs, insulin had no metabolic or growth effects at low concentrations of 10^{-10}–10^{-9} mol/l. A possible explanation for this is that the insulin signal is too attenuated due to the larger amount of IGF-IR. IGF-IR and IR form hybrid IGF-IR/IR in HCASMCs, as discussed above, and results from other tissues suggest that the non-dominating receptor will be incorporated into hybrid IGF-IR/IR to a large extent. In spite of activation of IR β-subunit at an insulin concentration of 10^{-9} mol/l, insulin at 10^{-9}–10^{-8} mol/l, in contrast to the same concentration of IGF-I, had no effect on D-[U-^{14}C]glucose or [^{3}H]thymidine incorporation. Whereas IGF-I can activate all the IGF-IR and the IR αβ-subunits incorporated into hybrid IGF-IR/IR, insulin can only activate its own αββα-holoreceptor.

Speculating on the consequence, if HCASMCs respond to IGF-IR or hybrid IR/IGF-IR, we would point out that there are several conditions in which low circulating IGF-I is associated with different vascular diseases. These conditions include type 1 diabetes mellitus [46, 47], growth hormone deficiency [48, 49] and normal ageing [50]. An increasing prevalence of vascular diseases and low IGF-I levels has been reported in epidemiological studies [4]. It is therefore conceivable that the prevalence of IGF-IR and possibly the existence of hybrid IR/IGF-IR acting as IGF-IR in human endothelial cells play a significant role in the pathogenesis of cardiovascular diseases.

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