Human aortic smooth muscle cells are insulin resistant at the receptor level but sensitive to IGF1 and IGF2

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Abstract

Whether insulin, at physiological concentrations, has direct effects on vascular smooth muscle cells (VSMCs) remains controversial. Our aim was to characterize the mechanism for insulin resistance in VSMCs. For comparison, the effects of IGF1 and IGF2 were also studied. Cultured human aortic smooth muscle cells (HASMC) were used. Receptor mRNA was analyzed by quantitative reverse transcription PCR and receptor protein by ELISA and western blot. Biological effects were studied by thymidine incorporation and glucose accumulation. In HASMC, both mRNA and protein expression of IGF1 receptors (IGF1R) were fivefold higher compared to insulin receptor (IR). IR isoform A mRNA was 13-fold more expressed than IR isoform B. IR and IGF1R co-precipitated, indicating the presence of hybrid IR/IGF1R. Phosphorylation of the IGF1R β -subunit was obtained by IGF1 10^{-9} – 10^{-8} mol/l and IGF2 10^{-8} mol/l. IR β -subunit was phosphorylated by IGF1 10^{-8} mol/l but not by insulin. IGF1 stimulated IR substrate-1 and AKT at 10^{-8} mol/l and extracellular signal-regulated kinases 1 and 2 at 10^{-9} – 10^{-8} mol/l respectively. IGF1 and 2 at a concentration of 10^{-8} – 10^{-7} mol/l significantly stimulated ^3H -thymidine incorporation, whereas insulin did not. ^{14}C -Glucose accumulation was stimulated by IGF1 or IGF2 10^{-8} – 10^{-7} mol/l, and also by insulin 10^{-7} mol/l. Our results suggest that IGF1R and hybrid IR/IGF1R are activated by physiological concentrations of IGF1 and 2 in HASMC and this propagates downstream signaling and biological effects, while insulin has no effect on its receptor or downstream signaling probably due to a preponderance of IGF1R and incorporation of IR into hybrid IR/IGF1R.

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Introduction

Insulin resistance, diabetes, and circulating insulin-like growth factor 1 (IGF1), have all been implicated in the pathogenesis of cardiovascular disease, but the mechanisms behind these associations are still not clear (De Meyts 1994, Conti *et al.* 2001, 2002, Juul *et al.* 2002, Lakka *et al.* 2002, Nathan *et al.* 2005). In the development of atherosclerosis vascular smooth muscle cells (VSMCs) proliferate, migrate, and produce extracellular matrix (Doran *et al.* 2008). Whether insulin, at physiological concentrations, has direct effects on VSMCs remains controversial. *In vitro*, the effects of insulin on muscular arteries or cultured VSMCs are small and only occur at high supraphysiological concentrations (Arnqvist 1974, Arnqvist *et al.* 1979, King *et al.* 1983, 1985). Insulin is closely related to IGF1 and 2 (Rinderknecht & Humbel 1978*a,b*). High concentrations of insulin can elicit effects on VSMCs by cross reacting with IGF1 receptors (Banskota *et al.* 1989, Avena *et al.* 1999). Insulin receptors (IRs) and IGF1Rs are homologs having 84% homology in the β -subunit tyrosine kinase domains (Ullrich *et al.* 1986). In tissues co-expressing IGF1R and IR, hybrid IGF1R/IR are formed (Moxham *et al.* 1989, Soos & Siddle 1989,

Baillyes *et al.* 1997, Federici *et al.* 1997, Sakai & Clemmons 2003). They consist of an IR $\alpha\beta$ -monomer and an IGF1R $\alpha\beta$ -monomer and have binding characteristics similar to the IGF1R (Baillyes *et al.* 1997, Federici *et al.* 1997, Pandini *et al.* 2002). The human IR exists in two isoforms, IRA and IRB, of which IRA has a high affinity for IGF2 (Mosthaf *et al.* 1990, Yamaguchi *et al.* 1991, Frasca *et al.* 1999, Denley *et al.* 2004). To initiate the biological effects of insulin, IGF1 and IGF2, the first step is binding and activation of membrane-bound receptors by phosphorylation, followed by recruitment and phosphorylation of docking proteins including insulin receptor substrate-1 (IRS1), extracellular signal-regulated kinases 1 and 2 (ERK 1/2), and AKT involved in the regulation of cell metabolism, proliferation, and survival (Dupont & LeRoith 2001, Saltiel & Kahn 2001).

We wanted to study whether insulin resistance of human VSMCs is due to low availability of IRs or impaired downstream signaling. Due to interactions of insulin, IGF1 and IGF2 at the receptor level and common downstream signaling (Kim & Accili 2002), studying their action at physiological concentrations is of great importance for revealing mechanisms of insulin resistance.

Here we investigated effects of insulin, IGF1 and IGF2 on receptors, downstream signaling, and biological effects in human aortic smooth muscle cells (HASMCs).

Materials and methods

Primers and probes for quantitative real-time reverse transcription PCR (RT-PCR) were purchased from Scandinavian Gene Synthesis AB (SGS, Köping, Sweden). Primary antibodies used for immunoprecipitation (IP) and immunoblotting studies included monoclonal mouse anti-tyrosine PY20 antibody and polyclonal rabbit antibodies for IR β -subunit (C-19), IGF1R β -subunit (C-20), IRS-1 (C-20), phospho-AKT 1/2/3 (Thr208), all purchased from Santa Cruz Biotechnology, Inc (Santa Cruz, CA, USA). Polyclonal rabbit antibodies for total AKT, phospho-ERK 1/2 (Thr202/Tyr204) and total ERK 1/2 were bought from Cell Signaling (Boston, MA, USA). Goat anti-rabbit-HRP (Zymed, San Francisco, CA, USA), sheep anti-mouse-HRP (Amersham Pharmacia Biotechnology), and streptavidine-HRP (Amersham Pharmacia Biotechnology) were used as secondary antibodies. Enhanced chemiluminescence (ECL) detection system was obtained from Amersham Pharmacia Biotechnology. D-[U¹⁴C]-glucose (317 mCi nmol/l) and [6-³H]-thymidine (20 Ci nmol/l) were obtained from Amersham Pharmacia Biotechnology.

Culture of cells

HASMC from three different batches were obtained from Gibco Invitrogen. They tested negative for von Willebrand Factor VIII and positive for α -smooth muscle actin. The cells were cultured in accordance with the manufacturer's instructions in smooth muscle cell growth medium, which consisted of medium 231

(smooth muscle cell basal medium), together with smooth muscle growth supplement and amphotericin 1.6 mg/ml, penicillin 100 IU/ml, and streptomycin 20 IU/ml. HASMC were cultured at 37 °C in 95 v/v % air with 5 v/v % CO₂. Cell culture material was bought from Corning (Schiphol-Rijk, The Netherlands). All experiments were performed in triplicate or more, on near confluent cell cultures in passage 4–8.

Quantitative real-time RT-PCR

RNA was extracted using the RNeasy Mini Kit (Qiagen GmbH). From 1 μ g RNA first strand cDNA was transcribed using a commercial kit (Invitrogen AB, Life Technologies). The expression of *IR* and *IGF1R* was estimated by real-time quantitative RT-PCR (QRT-PCR) assay using the ABI PRISM 7500 Sequence Detection System (PE Applied Biosystems, Stockholm, Sweden). The oligonucleotides were purchased from SGS, see used primers and probes listed in Table 1. The real-time RT-PCR reaction was run in a 7500 Prism Sequence Detector System (PE Applied Biosystems). Twelve microlitres TaqMan Universal PCR Master Mix (PE Applied Biosystems), 3 μ l cDNA of reverse transcribed RNA, 300 nmol/l primers, 25 nmol/l probe for *IR*, and 50 nmol/l probe for *IGF1R* respectively were added in a final volume of 25 μ l. 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 allowed through the combination of 5'-3' nuclease activity of AmpliTaq Gold DNA Polymerase ROX by the release of a fluorescent reporter FAM for *IR* and *IGF1R* and VIC for *GAPDH* probes oligonucleotides during the RT-PCR reaction. The fluorescence was measured at each cycle. The relative amount of transcripts,

Table 1 Sequences for the primers and probes used in the quantitative real-time reverse transcription (RT)-PCR

	Primer/probe	Sequence
Gene		
<i>IGF1R</i>	Forward primer	5'-CGA TGT GTG AGA AGA CCA CCA-3'
	Reverse primer	5'-ACA TTT TCT GGC AGC GGT TT-3'
	Probe	5'-(FAM) CAA TGA GTA CAA CTA CCG CTG CTG GAC CA (TAMRA)-3'
<i>IR</i>	Forward primer	5'-AGG AGC CCA ATG GTC TGA-3'
	Reverse primer	5'-GAG ACG CA GAGA TGC AGC-3'
	Probe	5'-(FAM) ACC ATA TCG CCG ATA ACT CAC TTC ATA CAG (TAMRA)-3'
<i>IRA</i>	Forward primer	5'-GTT TAG GAA GAC GTT TGA GGA TT-3'
	Reverse primer	5'-TCA CAT TCC CAA CAT CGC CA-3'
	Probe	5'-(FAM) ACA ACG TGG TTT TCG TCC CCA GGC CAT (TAMRA)-3'
<i>IRB</i>	Forward primer	5'-ATT ACC TGC ACA ACG TGG TTT TC-3'
	Reverse primer	5'-GGA CCT GCG TTT CCG AGA T-3'
	Probe	5'-(FAM) AAC CTC TTC AGG CAC TGG TGC CGA G (TAMRA)-3'
<i>GAPDH</i>	Forward primer	5'-GAA GGT GGA GGT CGG AGT C-3'
	Reverse primer	5'-GAA GAT GGT GAT GGG ATT TC-3'
	Probe	5'-(VIC) CAA GCT TCC CGT TCT CAG CC (TAMRA)-3'

measured during the exponential phase of reaction, was determined by the comparative C_t methods (Bulletin 2, PE Applied Biosystems).

IGF1R ELISA

The amount of IGF1R protein was measured using ELISA. Cell lysate was prepared from near confluent cell cultures. The cells were washed twice with cold PBS and then collected in cold PBS. The cell–PBS suspension was centrifuged at 700 g 4 °C for 5 min. The resulting pellet was lysed in lysis buffer (pH 7.5) containing 20 mmol/l Trizma base, 150 mmol/l NaCl, 5 mmol/l EDTA, 0.5% sodium deoxycholate, and 0.5% Triton X-100 with an addition of 1 mmol/l phenylmethylsulfonyl fluoride (PMSF), 1.5 μ g/ml leupeptin, 1 mmol/l Na_3VO_4 , and 1.5 μ g/ml aprotinin prior to use. The cells were lysed on ice for 30 min and then centrifuged at 4 °C, 12 000 g for 10 min. Supernatant was stored at –70 °C until further use. The plate used in the assay was 96-well Maxisorp plates (NUNC, Roskilde, Denmark) which was coated over night at 4 °C with monoclonal anti-human IGF1R antibodies (MAB 391; R&D Systems, Abingdon, UK) diluted as 2 mg/l in coating buffer (15 mmol/l Na_2CO_3 , 35 mmol/l $NaHCO_3$, pH 9.6). On day 2 the wells were washed three times with 0.05 v/v % Tween 20 in PBS (PBS–T) to remove unbound fractions of antibody before blocking with 5 w/v % BSA and 0.02 w/v % NaN_3 in PBS for 1 h at RT, followed by another wash with PBS–T. Recombinant human IGF1R (R&D Systems) was used as standard. Samples and standards, all diluted in 0.1 w/v % human serum albumin (HSA) in PBS, were incubated on a shaker for 2 h at RT. After washing away the unbound fraction with PBS–T, the secondary antibody was added and incubated on a shaker at RT for 2 h. The biotinylated anti-human IGF1R antibody (BAF 391; R&D Systems) was diluted as 0.5 mg/l in 0.1 w/v % HSA in PBS. After washing HRP conjugated streptavidine diluted 1:500 in 0.1 v/v % PBS–T was added to the plate, and incubated on a shaker for 20 min at RT. The plate was then washed in PBS and substrate for the HRP was incubated with the substrate for 30 min (0.4 mg/l tetramethylbenzidine dihydrochloride dissolved in phosphate-citrate buffer (35 mmol/l citric acid, 67 mmol/l Na_2HPO_4 ; pH 5.0) with 0.006 v/v % H_2O_2). The reaction was then stopped by the addition of 1 mol/l H_2SO_4 and the optical densities were measured at 450 nm.

IR ELISA

The amount of IR protein was measured using an IR Immunoassay Kit (BioSource Europe S.A., Nivelles, Belgium) according to the manufacturer's instructions. Cell lysate was prepared as described above.

Phosphorylation and cell lysis

HASMC were cultured in 75 cm^2 flasks until near confluent and serum starved for 24 h before experiment. The cultures were incubated with concentrations of 10^{-10} – 10^{-8} mol/l for IGF1, IGF2 or insulin in warm F-12-BSA medium for 10 min at 37 °C. After incubation, the 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 w/v % sodium deoxycholate, 0.5 w/v % Triton X-100, 1 mmol/l Na_3VO_4 , 1.5 μ g/ml Aprotinin, 1.5 μ g/ml Leupeptin, and 1 mmol/l PMSF). Cell lysates were centrifuged for 15 min at 12 000 g at 4 °C. The resulting supernatant, the whole cell lysate, 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.

SDS-PAGE and western blot analysis

To study IGF1R and IR β -subunits and also IRS1, cell lysates containing 0.5–1 mg total protein were immunoprecipitated with 0.4–0.6 μ g antibody per mg total protein content. Antibodies used for IP were polyclonal anti-IGF1R β -subunit antibody C-20, polyclonal anti-IR β -subunit antibody C-19 or IRS1 antibody C-20 respectively. All of the above were from Santa Cruz Biotechnology, Inc. Protein-A Sepharose (Pharmacia–Upjohn) was added and samples were shaken gently overnight at 4 °C. The immunoprecipitates were washed and centrifuged three times with ice-cold lysis buffer and diluted in 50 μ l 2 $\times$ Laemmli sample buffer (0.0125 mol/l Trizma base, 2 w/v % SDS, 20 w/v % glycerol, 0.002 w/v % bromophenol, 2 w/v % β -mercaptoethanol, pH 6.8). Whole cell lysate samples were diluted in Laemmli sample buffer to a resulting total protein amount of ~20 μ g per well.

Immunoprecipitated samples or whole cell lysate samples were boiled for 3 min. After centrifugation, proteins in the supernatant were separated on a 7.5 w/v % SDS-PAGE gel. The separated proteins were electrotransferred onto a polyvinylidene difluoride membrane and blocked in blocking buffer. The membrane was then immunoblotted over night at 4 °C with phospho-specific primary antibodies and visualized using secondary HRP-linked antibodies (Amersham, Life Science AB), followed by ECL detection. Autoradiographs were obtained by exposure to a Hyperfilm ECL (Amersham, Life Science AB) and subsequently scanned with a LAS1000 camera (Image-Gauge, Fujifilm, Tokyo, Japan). Densitometric evaluation was performed using Multi Gauge Version 3.0 (FujiFilm), where data was registered as % phosphorylated/total protein of maximum phosphorylated/total protein on each membrane.

Figure 1 Expression of IR and IGF1R mRNA and proteins in HASMC. The relative amount of *IGF1R* to *IR* mRNA expression was measured by real-time RT-PCR, where *IR* was set to 1. The IGF1R and IR protein levels were analyzed by ELISA and are expressed as amount receptor protein relative to amount of total protein, IR was set to 1. The *IGF1R* to *IR* mRNA ratio (5.3 ± 1.3) and the IGF1R to IR protein ratio (5.5 ± 1.2) was found to be almost identical. ***Denotes $P < 0.001$ (mean $\pm$ S.E.M., $n = 4$).

The membranes were stripped by incubation in stripping buffer (62.5 mmol/l Trizma HCl, 2 w/v % SDS, 100 mmol/l β -mercaptoethanol) at 55 °C for 30 min and then reblotted with antibodies detecting the total amount of respective proteins.

[³H]-thymidine incorporation into DNA

DNA-synthesis was quantified by measuring ³H-thymidine incorporation into DNA in HASMC. The cells were grown in 6-well plates until near confluency, serum starved for 24 h before experiment, and then for another 24 h with and without insulin, IGF1 or IGF2 at concentrations of 10^{-10} – 10^{-7} mol/l, followed by incubation with 1 μ Ci/ml ³H-thymidine (Amersham Pharmacia Biotechnology) for 3 h. DNA was precipitated with cold 5 w/v % trichloroacetic acid and then solubilized 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 were expressed as the percentage increased in ³H-thymidine incorporation above basal.

D-[U¹⁴-C]-glucose accumulation

Near confluent HASMC grown in 6-well plates were starved in serum free media for 24 h before experiments. The cells were then incubated at 37 °C for 2 h with the addition of 1 μ Ci/ml D-[U¹⁴-C]-glucose (Amersham Pharmacia Biotechnology) and in absence or presence of insulin, IGF1 or IGF2 at concentrations of 10^{-10} – 10^{-7} mol/l. Thereafter, cells

were rinsed three times with PBS, lysed with 0.5 ml 0.1 w/v % SDS, and 0.4 ml of the solubilized cells was added to 4 ml scintillation solution, and the radioactivity was measured. The data were expressed as percent above basal.

Statistical analysis

Values are given as mean $\pm$ S.E.M. Statistical comparisons were made with SPSS program (SPSS Inc. Headquarters, Chicago, IL, USA) by one-way ANOVA. As *post-hoc* test we used Dunnett's *t*-test (two-sided). A *P* value < 0.05 was considered statistically significant.

Results

Expression of IR and IGF1R

Gene expression of *IR* and *IGF1R* was demonstrated in HASMC by quantitative real-time RT-PCR in cells starved for 24 h before experiments. *IGF1R* mRNA was approximately five times more abundant than *IR* mRNA ($P < 0.001$; Fig. 1). IR and IGF1R were also measured as receptor proteins using ELISA and are expressed as receptor protein per mg of total protein. The amount of IGF1R protein was fivefold higher than IR protein, 8.4 ± 1.2 and 1.5 ± 0.1 ng/mg total protein ($P < 0.001$) respectively (Fig. 1). IR isoform A mRNA was 13-fold more abundant than IR isoform B ($P < 0.001$; Fig. 2).

Figure 2 Expression of IR isoforms A and B in HASMC. The relative amount of *IRA* to *IRB* was analyzed by real-time RT-PCR. IR isoform A was 13-fold (12.9 ± 3.2) more abundant than IR isoform B ($P < 0.001$), IRB was set to 1. ***Denotes $P < 0.001$ (mean $\pm$ S.E.M., $n = 4$).

Receptor β -subunit detection and activation

After IP of receptors with anti-IR β -subunit (Fig. 3A and B) or anti-IGF1R β -subunit (Fig. 3C and D) specific antibodies and blotting the membrane with the same antibody, we found bands at a position slightly lower than the 97 kDa molecular weight marker. The band detected with the IR-specific antibody (Fig. 3A and D) had a position slightly lower than the band detected by the IGF1R antibody (Fig. 3B and C). When the membrane with receptors immunoprecipitated and blotted with polyclonal, anti-IR was reblotted with IGF1R antibody a slightly higher band corresponding to the IGF1R β -subunit was found (Fig. 3B). The opposite result was found when the membrane immunoprecipitated and blotted with polyclonal anti-IGF1R was reblotted with IR antibody (Fig. 3D). The co-precipitation of the IR and IGF1R β -subunits after IP against either of the two receptors β -subunits indicates the presence of insulin/IGF1 hybrid receptors.

To study receptor phosphorylation the cells were exposed to either insulin, IGF1 or IGF2 at concentrations of 10^{-10} – 10^{-8} mol/l for 10 min. Insulin did not activate its cognate receptor or the IGF1 receptor (Fig. 4A and B). IGF1 was found to activate its own receptor at 10^{-9} – 10^{-8} mol/l and even the IR at 10^{-8} mol/l. IGF2 at 10^{-8} mol/l activated the IGF1R and tended to activate the IR.

Intracellular signaling

Insulin 10^{-10} – 10^{-8} mol/l did not activate IRS1, AKT or ERK 1/2 (Figs 5 and 6). IGF1 10^{-8} mol/l significantly phosphorylated IRS1 and AKT and at 10^{-9} – 10^{-8} mol/l phosphorylated ERK 1/2. IGF2 10^{-8} mol/l tended to activate IRS-1. ERK 1/2 was not affected by 10^{-10} – 10^{-8} mol/l IGF2.

Figure 3 Detection of IR and IGF1R β -subunits in HASMC. IR and IGF1R β -subunit protein and evidence for the presence of hybrid IR/IGF1R were demonstrated by immunoprecipitation and western blot. Insulin receptor (IR) β -subunit (95 kDa) and IGF1R (IGF1R) β -subunit (97 kDa) were detected by western blot on the same membrane implying co-precipitation. Figures show blots after immunoprecipitation (IP) against either IR (left column, A and B), or IGF1R (right column, C and D) and immunoblot against the IR β -subunit (A and D) and the IGF1R β -subunit (B and C). Similar results were obtained in four independent experiments.

Figure 4 (A) Activation of IR β -subunit in HASMC. Effect of insulin (dark gray bars), IGF1 (black bars), and IGF2 (gray bars) on tyrosine phosphorylation of IR β -subunit were analyzed by immunoprecipitation of IR by an anti-IR β -subunit antibody and immunoblotting with an anti-phosphotyrosine antibody (PY20) after exposure of near confluent HASMC to insulin, IGF1, and IGF2 at the concentrations of 10^{-10} – 10^{-8} mol/l for 10 min. Data are means from four independent experiments. Data were quantified by densitometry and are expressed as phosphorylated/total protein, fractional of maximal response. * $P < 0.05$. (B) Activation of IGF1R β -subunit in HASMC. Effects of insulin (dark gray bars), IGF1 (black bars), and IGF2 (gray bars) on tyrosine phosphorylation of IGF1R β -subunit were analyzed by immunoprecipitation of IGF1R by an anti IGF1R β -subunit antibody and immunoblotting with an anti-phosphotyrosine antibody (PY20) after exposure of near confluent HASMC to insulin, IGF1, and IGF2 at the concentrations of 10^{-10} – 10^{-8} mol/l for 10 min. Data are means from four independent experiments. Data were quantified by densitometry and are expressed as phosphorylated/total protein, fractional of maximal response. * $P < 0.05$.

Biological effects of IGF2, IGF1, and insulin

The incorporation of [3 H]-thymidine into DNA was stimulated by 10^{-8} – 10^{-7} mol/l of IGF1 ($P = 0.02$ and $P = 0.006$) and IGF2 ($P = 0.001$ and $P \leq 0.001$), whereas

Figure 5 (A) Activation of insulin receptor substrate-1 (IRS1). HASMC were exposed for 10 min to insulin (dark gray bars), IGF1 (black bars), and IGF2 (gray bars) at concentrations of 10^{-10} – 10^{-8} mol/l. Tyrosine phosphorylation of IRS1 was analyzed by immunoprecipitation of IRS1 with an IRS1 antibody and immunoblotting with an anti-phosphotyrosine antibody (PY20). Data are means $\pm$ S.E.M., $n=4$. Data were quantified by densitometry and are expressed as means of percent phosphorylated/total protein, fractional of maximal response. * $P<0.05$. (B) Activation of ERK 1/2. HASMC were exposed for 10 min to insulin (dark gray bars), IGF1 (black bars), and IGF2 (gray bars) at concentrations of 10^{-10} – 10^{-8} mol/l. ERK 1/2 phosphorylation was analyzed using a specific antibody detecting phospho-ERK 1/2 phosphorylated at Thr 202 and Tyr 204. Data are means $\pm$ S.E.M., $n=8$. Data were quantified by densitometry and are expressed as means of percent phosphorylated/total protein, fractional of maximal response. * $P<0.05$.

insulin had no significant effect (Fig. 7A). Glucose accumulation was significantly stimulated by concentrations of 10^{-8} – 10^{-7} mol/l IGF1 ($P=0.03$ and $P=0.001$) and IGF2 ($P<0.001$, $P<0.001$), and also by insulin at the highest concentration of 10^{-7} mol/l ($P=0.001$; Fig. 7B).

Discussion

The effects of insulin, IGF1, and IGF2 on HASMCs were studied at the receptor level and further downstream. Insulin has a high affinity for its own receptor (Ward *et al.* 2007) and physiological effects of insulin are obtained at concentrations of $\leq 10^{-9}$ mol/l in insulin sensitive tissues. In HASMC we could not demonstrate any activation of the IR by insulin; instead it was activated by IGF1 and also tended to be activated by IGF2. This observation can be explained by sequestration of IRs into hybrid IR/IGF1R (Arnqvist 2008). In cells expressing both IGF1R and IR, hybrid IR/IGF1Rs are stoichiometrically formed depending on the relative abundance of IGF1R to IR (Pandini *et al.* 1999). Since IGF1R was several fold more expressed than IR in HASMC, most of the IRs will be incorporated into hybrid IR/IGF1Rs which have a low affinity for insulin, but bind IGF1 and IGF2 with the same affinity as IGF1 receptors (Soos *et al.* 1993, Siddle *et al.* 2001). According to the current model of insulin and IGF-binding to their own receptors, insulin needs to bind to both IR α -subunits in the IR dimer to obtain high affinity binding, whereas for high affinity binding of IGF1 only one IGF1R α -subunit is needed (Ward *et al.* 2007, De Meyts 2008). In the hybrid IR/IGF1R the activated IGF1R β -subunit can transphosphorylate the IR β -subunit (Frattali & Pessin 1993) as shown in this study.

IGF1Rs were expressed and activated at physiological IGF1 concentrations in HASMC, as previously shown in human coronary artery smooth muscle cells (Chisalita & Arnqvist 2005). When membranes are developed with the anti-phosphotyrosine antibody PY20 it will detect

Figure 6 Activation of AKT (PKB). HASMC were exposed for 10 min to insulin, IGF1, and IGF2 at concentrations 10^{-10} – 10^{-8} mol/l. AKT phosphorylation was analyzed using a specific antibody detecting phospho-AKT 1/2/3 phosphorylated at threonine 308. Similar results were obtained in four independent experiments. * $P<0.05$.

Figure 7 (A) Effect of insulin, IGF1, and IGF2 on $[^3\text{H}]$ -thymidine incorporation into DNA in HASMC. Confluent cells, serum starved 24 h before experiment, were incubated at 37°C for 2 h in serum free medium in the presence of $[^3\text{H}]$ -thymidine and polypeptides at concentrations 10^{-10} – 10^{-7} mol/l. Data are expressed as percent thymidine incorporation above basal and are measurements from 10 independent experiments. Bars are mean $\pm$ S.E.M. of 10 experiments. (B) Effect of insulin, IGF1 and IGF2 on D- $[U^{14}\text{C}]$ -glucose accumulation in HASMC. Confluent cells, serum starved 24 h before experiment, were incubated at 37°C for 24 h in serum-free medium in the presence of D- $[U^{14}\text{C}]$ -glucose and polypeptides at the concentrations of 10^{-10} – 10^{-7} mol/l. Data are expressed as percent glucose accumulation above basal and are measurements from 10 independent experiments. Bars are mean $\pm$ S.E.M. of 10 experiments. * $P<0.05$, ** $P<0.01$, *** $P<0.001$.

tyrosine phosphorylated IGF1R β -subunits and also co-precipitated IR β -subunits which are tyrosine phosphorylated. There is little difference in the molecular weight between IR and IGF1R β -subunits and it was not possible to clearly separate them into two different bands as we succeeded to do in rat aortic smooth muscle cells (Johansson & Arnqvist 2006). In HASMC the IRs that co-precipitated with the IGF1Rs were not phosphorylated by low concentrations of insulin which showed a lack of insulin effect at 10^{-10} – 10^{-9} mol/l. This is in conformity with the view mentioned above, that hybrid IR/IGF1R have low affinity for insulin (Soos *et al.* 1993, Siddle *et al.* 2001).

IGF2 10^{-8} mol/l activated the IGF1R β -subunit and tended to activate IR in HASMC. This result is in agreement with the observation that the affinity of IGF2 for the IGF1R is about 10-fold lower than IGF1 itself (Jones & Clemmons 1995). IGF2 at the 10^{-8} mol/l also tended to activate the IR. The affinity of IGF2 for IRA is only 10-fold lower than insulin itself, which is why IGF2 is able to activate the IR (Frasca *et al.* 1999). We found IRA to be the predominant IR isoform

expressed in HASMC. However, since we found no significant activation of IR by insulin itself, probably due to incorporation of IR into hybrid IR/IGF1R, as discussed above, the effect of IGF2 on IR was probably due to activation of IR/IGF1R hybrid receptors. A free IGF2 concentration of 10^{-8} mol/l can probably be obtained *in vivo* (Frystyk *et al.* 1994) and our results therefore suggest that IGF2 may be of importance for human VSMC function (Fig. 7).

Receptor tyrosine kinases of the insulin-like growth factor family promote growth and mediate metabolic signals (Kim & Accili 2002). The IR and IGF1R activated receptor tyrosine kinases phosphorylate IRS proteins linked to the activation of the two main signaling pathways. The phosphatidylinositol 3-kinase pathway (PI3K)–AKT/protein kinase pathway is responsible for most of the metabolic actions, while the Ras-mitogen-activated protein kinase pathway regulates expression of some genes and cooperates with the PI3K pathway to control cell growth and differentiation (Taniguchi *et al.* 2006). In this study we found that the activation of IGF1R was followed by activation of IRS1, AKT, and ERK 1/2 and also by biological effects. We previously showed the activation of IR by insulin 10^{-10} – 10^{-9} mol/l in coronary artery smooth muscle cells indicating that IR can be activated by insulin in human VSMCs (Chisalita & Arnqvist 2005) but this was not accompanied by biological effects. In the current study, there was no activation of the IR by physiological concentrations of insulin in HASMC as mentioned above and insulin 10^{-10} – 10^{-9} mol/l did not activate IRS1,

Figure 8 Proposed model for insulin resistance in HASMC. The insulin resistance of vascular smooth muscle is located at the receptor level. Due to the low number of IRs there are not enough receptors activated by insulin to generate a downstream signal. However, the IR β -subunit is activated by IGF1 when incorporated into hybrid IR/IGF1R. Both IGF1 and IGF2 activate the IGF1R and the hybrid IR/IGF1R and this elicits downstream signaling and biological effects.

ERK 1/2, or AKT and had no biological effects on glucose accumulation or DNA-synthesis. This suggests that the available insulin holoreceptors in the aortic VSMCs are too few to generate a signal that is propagated. The insulin resistance of vascular smooth muscle therefore seems to be located at the receptor level as we recently described in human microvascular endothelial cells (Johansson *et al.* 2008). In accordance with our proposed mechanism for insulin resistance in human VSMCs (Fig. 8), disruption of IGF1R in breast cancer cells, osteoblast, and rat VSMC is followed by an increased fraction of insulin holoreceptors and reduced insulin resistance (Fulzele *et al.* 2007, Zhang *et al.* 2007, Engberding *et al.* 2009).

Insulin was found to stimulate glucose accumulation in HASMC at a concentration of 10^{-7} mol/l, but not at lower concentrations. At such a high concentration that is far above what can be reached *in vivo* (Olsson *et al.* 1988) insulin is known to cross react with IGF1 receptors (Banskota *et al.* 1989, Avena *et al.* 1999) and the effect of insulin on glucose accumulation is probably elicited by an activation of IGF1R or hybrid IR/IGF1R. It is therefore crucial to use physiological concentrations ($\leq 10^{-9}$ mol/l) of insulin and IGF1 in studies *in vitro* to be able to extrapolate the results to *in vivo* conditions.

Our results clearly demonstrate that HASMC are sensitive to IGF1 and IGF2, but insulin resistant at the receptor level due to the low fraction of available IRs and impairment in downstream signaling pathway (Fig. 8). Furthermore, here, we show that IGF1R are considerably more abundant than IR, and reveal the presence of hybrid IR/IGF1R that probably modulates the insulin resistance of HASMC.

Declaration of interest

The authors declare that there is no duality of interest associated with this manuscript.

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