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REVISED MANUSCRIPTGit Johansson *et al***Human microvascular endothelial cells are sensitive to IGF-I but resistant to insulin at the receptor level****G. S. Johansson, S. I. Chisalita and H. J. Arnqvist**

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IGF-I receptor, insulin receptor, hybrid receptor, RT-PCR, western blot, thymidine incorporation

Summary

Human microvascular endothelial cells (HMVEC) are sensitive to IGF-I but insulin resistant and express several times more IGF-I receptors (IGF-IR) than insulin receptors (IR). Our aim was to investigate the mechanism of this insulin resistance in cultured HMVEC by studying receptor activation and signal propagation downstream.

The IGF-IR β -subunit and the IR β -subunit were detected and found to co-precipitate. IRA was the major IR isoform expressed in HMVEC. IGF-I 10^{-9} - 10^{-8} M phosphorylated its cognate receptor β -subunit. IGF-I also phosphorylated the IR β -subunit at 10^{-9} M. Phosphorylation of insulin receptor substrate 1 was obtained by IGF-I 10^{-9} - 10^{-8} M. Akt was phosphorylated by IGF-I at 10^{-8} - 10^{-7} M and by insulin 10^{-7} M. IGF-I at 10^{-8} - 10^{-6} M significantly increased DNA synthesis. We conclude that microvascular endothelial cells are sensitive to IGF-I but resistant to insulin due to a preponderance of IGF-I receptors and sequestration of insulin receptors into insulin/IGF-I hybrid receptors.

1. Introduction

Vascular complications in diabetes largely affect the microcirculation, and the integrity and function of the endothelium is an important factor in the development of these complications (Kim et al., 2006, Pambianco et al., 2006). In diabetes circulating insulin levels as well as the insulin-like growth factor (IGF) system are altered (Bereket et al., 1999, Delafontaine et al., 2004, Hedman et al., 2004). Insulin and IGF-I initiate their biological effects by binding to their cognate cell surface receptors; the IGF-I receptor (IGF-IR) and the insulin receptor (IR), which are highly homologous (Siddle et al., 2001). Both belong to the tyrosine kinase family having a tetrameric structure formed by two $\alpha\beta$ -dimers consisting of an extracellular α -subunit and a transmembrane β -subunit (Siddle et al., 2001). Due to their structural similarity, insulin and IGF-I can cross-react with each other's receptor but with a 100-1000-fold lower affinity than to their own receptor. The IR exists in two isoforms, IRA which lacks exon 11 and IRB with exon 11. These have been shown to have different binding affinities for IGF-I and IGF-II (Frasca et al., 1999). The IR and the IGF-IR can also heterodimerize to form hybrid receptors, consisting of one IR $\alpha\beta$ -dimer and one IGF-IR $\alpha\beta$ -dimer, shown to have a binding affinity similar to the IGF-IR, *i.e.* to bind IGF-I, but not insulin, with high affinity (Soos and Siddle, 1989).

We have previously reported that human microvascular endothelial cells (HMVEC) are sensitive to IGF-I but insensitive to insulin (Chisalita and Arnqvist, 2004). HMVEC express both IR and IGF-IR but IGF-IR are several times more abundant than IR (Chisalita and Arnqvist, 2004). In human macrovascular endothelial cells IR and IGF-IR have been found to form hybrid insulin/IGF-I receptors (Chisalita et al., 2006, Nitert et al., 2005) which may be of importance for insulin sensitivity. The aim of this study was to investigate at what level insulin resistance occurs in HMVEC. We therefore studied the dose-response of IGF-I and insulin with regard to receptor activation, downstream signalling and biological effect. The relative mRNA abundance for the IR isoform A compared to the IR isoform B, and the presence of hybrid receptors were also determined since this may be of importance for insulin sensitivity.

2. Materials and Methods

2.1 Cell culture

Primary human microvascular endothelial cells (HMVEC) isolated from adult dermis were provided by Clonetics™ (San Diego, USA). Cells were cultured in EBM-2 medium supplemented with EGM-2 Single Quots (Clonetics™). All experiments were performed on near-confluent cultures from passages 3-9 made quiescent by incubation for 18–24 h in serum-free EBM-2 medium.

2.2 Quantitative RT-PCR

Quantitative real time RT-PCR analysis was performed in order to compare the relative abundance of mRNA for the two IR isoforms in HMVEC. The procedure was performed as described previously (Chisalita and Arnqvist, 2004). The following primers and probes purchased from SGS (Scandinavian Gene Synthesis AB, Köping, Sweden), were used: IRA forward primer: 5'-GTTTAGGAAGACGTTTGAGGATT-3', IRA reverse primer: 5'-TCACATTCCCAACATCGCCA-3', IRA receptor probe: 5'-(FAM)-CACACGTGGTTTTTCGTCCCCAGGCCAT (TAMRA)-3'; IRB primer: 5'-

ATTACCTGCACAACGTGGTTTTTC -3', IRB reverse primer: 5'-
GGACCTGCGTTTCCGAGA T-3' and IRB probe: 5'- (FAM)-
AACCTCTTCAGGCACTGGTGCCGAG (TAMRA)-3'.

2.3 Receptor phosphorylation and cell lysis

The cells were stimulated for 10 min at 37°C with recombinant human IGF-I (Groppe, Adelaide, Australia) or recombinant human insulin (SIGMA-Aldrich Sweden AB, Stockholm, Sweden) at concentrations 10^{-10} - 10^{-8} M or 10^{-10} - 10^{-7} M. Stimulation with VEGF was used as a positive control for extracellular kinases 1 and 2 (ERK 1/2). In experiments on receptors and insulin receptor substrate 1 (IRS-1) phosphorylation, the cells were preincubated with 50 μ M sodium vanadate on ice for 30 min. After stimulation the cells were solubilized with ice-cold cell lysis buffer [20 mM Tris, 150 mM NaCl, 5 mM EDTA, 0.5% (wt/vol) sodium deoxycholate, and 0.5% (vol/vol) Triton X-100, pH 7.5] supplemented with 1 mM phenylmethylsulfonyl fluoride, 1.5 mg/ml leupeptin, 1.5 mg/ml aprotinin and 1 μ M sodium vanadate for 30 min on ice (all chemicals from Sigma-Aldrich). The lysed cells were scraped and further disrupted by passage through a syringe needle, followed by centrifugation at 12 000 x g for 15 min at 4°C to pellet cell debris. The lysate supernatants were stored at -70°C pending further analysis. The protein content of the lysates was determined using the bicinchoninic acid protein method (Pierce, Rockford, IL).

2.4 Immunoprecipitation

Primary antibodies used for immunoprecipitation (IP) included rabbit polyclonal anti-IR β -subunit (C-19), rabbit polyclonal anti-IGF-IR β -subunit (C-20) and rabbit polyclonal anti-IRS-1 (C-20) from Santa Cruz Biotechnology (Santa Cruz, USA). The lysates were immunoprecipitated as described previously using protein A-Sepharose (Pharmacia-Upjohn, Uppsala, Sweden) to collect the immune complexes (Johansson and Arnqvist, 2006). The receptor antibodies used in this study have previously been shown to be highly specific for the β -subunits of the IR and IGF-IR, respectively (Johansson and Arnqvist, 2006). The previous study was, however, performed on cell lysate from vascular smooth muscle cells of rat origin, so, in order to assure the specificity of the antibodies in human endothelial cell lysate as well, we performed the same reducing experiments prior to IP in HMVEC lysate, confirming earlier results (data not shown).

2.5 SDS-PAGE and western blot analysis

Levels of phosphorylated insulin and IGF-I receptor β -subunit proteins and signalling molecules were analyzed with SDS-PAGE and Western blot analysis using standard methods. Primary antibodies used for immunoblotting studies included monoclonal mouse anti-PY20 antibody and rabbit polyclonal antibodies for IR β -subunit (C-19), IGF-IR β -subunit (C-20), IRS-1 (C-20) and phospho-Akt 1/2/3 (Thr308), all purchased from Santa Cruz Biotechnology. Rabbit polyclonal antibodies for total Akt, phospho-ERK 1/2 (Thr202/Tyr204) and total ERK 1/2 were obtained from Cell Signalling (Boston, USA). The membranes were incubated with a horseradish peroxidase (HRP)-conjugated antibody, either HRP-conjugated sheep anti-mouse antibody (Amersham Biosciences, Uppsala, Sweden) or HRP-conjugated goat anti-rabbit antibody obtained from Zymed (San Francisco, USA). The proteins were then visualized with enhanced chemiluminescence (ECL) detection reagents and exposure to ECL Hyperfilm™, both from Amersham Biosciences. After detection of phosphorylated receptor β -subunit or signalling molecule, the membranes were stripped and

reprobed to measure the total (both phosphorylated and unphosphorylated) amount of the specific protein. All membranes were scanned with a LAS1000 camera (Image-Guage, Fujifilm, Tokyo, Japan) with subsequent densitometric evaluation using Multi Gauge Version 3.0 (Fujifilm, Tokyo, Japan). Data were registered as per cent phosphorylated/total protein of maximum phosphorylated/total protein on each membrane, and the results presented as the mean of 3 - 4 experiments.

2.6 [$6\text{-}^3\text{H}$] thymidine incorporation

DNA synthesis was quantified as [$6\text{-}^3\text{H}$] thymidine (Amersham Biosciences) incorporation into DNA. The cells were grown on 6-well plates until near confluent when they were serum starved for 18–20 h in serum-free EBM-2 medium. They were then stimulated for 18–20 h with hormones at indicated concentrations in fresh serum-free EBM-2 medium, and then incubated for a further 3 h with [$6\text{-}^3\text{H}$] thymidine (1 $\mu\text{Ci/ml}$). The cells were washed free of medium with PBS, and the DNA precipitated with ice-cold 5% (wt/vol) trichloroacetic acid for 15 min at 4°C. The cells were then solubilized in 1.0 ml of 0.1 mol/l potassium hydroxide for 1–2 h at room temperature. 0.7 ml of cell solution was added to 4 ml of UltimaGold scintillation fluid and the radioactivity measured in a liquid scintillation counter (Rackbeta 1217).

2.7 Statistical analysis

Statistical comparisons of means \pm SEM were performed using SPSS 12.0.1 for Windows (Chicago, IL) with one-way ANOVA and post hoc testing with Dunnett t-test (2-sided). A p value of 0.05 was considered statistically significant.

3. Results

3.1 Quantification of IRA and IRB mRNA

In HMVEC, expression of receptor mRNA for the IR isoforms IRA and IRB, was demonstrated by real time RT-PCR. The relative abundance of IRA mRNA to IRB mRNA expression in HMVEC was approximately 7 times higher ($p < 0.01$) (Fig. 1).

3.2 Presence of receptor co-precipitation

The cell lysates were analyzed by IP and SDS-PAGE. After IP with the IGF-IR β -subunit specific antibody we detected a band at the level of the 97.4 kDa molecular marker, which corresponds to the molecular weight of the IGF-IR β -subunit (Fig. 2A). When the membrane was stripped and reprobed with the IR β -subunit specific antibody a band at a clearly lower position was detected, corresponding to the IR β -subunit at 95 kDa (Fig. 2C). After IP with the IR β -subunit specific antibody bands at the same levels were detected with the specific IR (Fig. 2D) and IGF-IR antibodies (Fig. 2B). This implies co-precipitation of the IGF-IR β -subunit and the IR β -subunit.

3.3 Detection of phosphorylated receptors

Phosphorylation of the different receptors was studied by detecting phosphorylated tyrosine residues on the immunoprecipitated receptor β -subunits using PY20 antibody. As

shown in Figure 3A significant phosphorylation of the IGF-IR β -subunit was obtained by IGF-I stimulation at concentrations 10^{-9} - 10^{-8} M compared to baseline. Phosphorylation of the IR β -subunit was obtained by stimulation with IGF-I 10^{-9} - 10^{-8} M compared to baseline (see Fig. 3B).

3.4 Detection of intracellular substrates phosphorylated downstream

To further study the biological effects downstream of the activated receptors we investigated the phosphorylation of IRS-1, ERK 1/2 and Akt (also known as protein kinase B). Phosphorylation of IRS-1 was detected with PY20 and was obtained by IGF-I at concentrations 10^{-9} - 10^{-8} M compared to baseline, as seen in Figure 4A. Akt was significantly phosphorylated compared to baseline at threonine 308 by IGF-I stimulation at concentrations 10^{-8} - 10^{-7} M and insulin 10^{-7} M (Fig. 4B). ERK 1/2 phosphorylation at threonine 202 and tyrosine 204 was not significantly higher than baseline, as shown in Fig. 4C.

3.5 Effects on cell proliferation

The effect of IGF-I and insulin on cell proliferation was studied by [$6\text{-}^3\text{H}$] thymidine incorporation into DNA. [$6\text{-}^3\text{H}$] thymidine incorporation was significantly increased from baseline in cells stimulated with IGF-I 10^{-8} - 10^{-6} M, with maximum levels reached at 10^{-8} M, whereas insulin stimulation did not show any significant increase from baseline, although an increase in effect could be seen at 10^{-8} M (see Fig. 5). Data are expressed as per cent above control (unstimulated) cell radioactivity.

4. Discussion

We studied the effects of insulin and IGF-I on microvascular endothelial cells at the receptor level and further downstream. Stimulation with IGF-I or insulin at both physiological and supraphysiological concentrations revealed that IGF-I at 10^{-9} - 10^{-8} M phosphorylated its cognate receptor as well as the IR, indicating activation at physiologically relevant concentrations (Frystyk et al., 1994). Co-precipitation of the IGF-IR β -subunit and the IR β -subunit, and the phosphorylation of IR β -subunits at even low IGF-I concentration, suggests the presence of hybrid insulin/IGF-I receptors (Chisalita et al., 2006, Johansson and Arnqvist, 2006, Nitert et al., 2005). Receptor activation by IGF-I was followed by downstream signalling and also increased DNA-synthesis. In contrast insulin 10^{-9} - 10^{-8} M did not activate the IR, IRS-1, Akt or ERK 1/2 and had no significant effect on DNA-synthesis. This suggests that HMVEC already exhibits insulin resistance at the receptor level.

It is conceivable that both a reduction in the number of IR and the presence of hybrid insulin/IGF-I receptors contribute to insulin resistance in HMVEC. The specific binding of insulin in HMVEC is several times lower than the specific binding of IGF-I indicating a lower number of homodimeric IR compared to IGF-IR or insulin/IGF-I hybrid receptors (Chisalita and Arnqvist, 2004). For high affinity binding of insulin to IR to occur, binding to both receptor α -subunits is necessary (De Meyts and Whittaker, 2002, Ward et al., 2007) whereas IGF-I needs only one IGF-IR α -subunit to bind with high affinity. This probably explains why insulin/IGF-I hybrid receptors have a low affinity for insulin but bind IGF-I with the same high affinity as IGF-I homodimers. However, the IR $\alpha\beta$ -dimer can be cross-activated through activation of the IGF-IR $\alpha\beta$ -dimer in the hybrid receptor (Ward et al., 2007), as was also shown in this study. The formation of hybrid receptors is regulated by a process of random assembly determined by the molar proportion of receptors, i.e. the less

abundant receptor will, to a large extent, be incorporated into hybrid insulin/IGF-I receptor (Pandini et al., 1999). In osteoblasts, a cell type with both IGF-IR and IR, and containing insulin/IGF-I hybrid receptors, disruption of the IGF-IR was found to enhance insulin signalling and action indicating that the insulin-generated signal was tempered through interactions with the IGF-IR (Fulzele et al., 2007). Correspondingly, sequestration of insulin receptors into insulin/IGF-I hybrid receptors in HMVEC probably makes the cells insulin resistant.

We analyzed the gene expression of insulin receptor isoforms IRA and IRB, and found IRA to be ~7 times more prevalent than IRB, indicating that IRA in HMVEC, as in coronary artery endothelial cells (Chisalita et al., 2006), is the dominating isoform. A difference in binding characteristics depending on the incorporated IR half dimer, IRA or IRB, has been reported (Pandini et al., 2002), but recent studies suggest that both types of hybrid receptors have the same affinity for IGF-I and insulin (Benyoucef et al., 2007, Slaaby et al., 2006). The lack of insulin effect on DNA-synthesis in HMVEC is in agreement with our previous observation (Chisalita and Arnqvist, 2004) together with several other reports on microvascular cells (Artwohl et al., 2007, Giannini et al., 2001) suggesting that human microvascular endothelial cells are insulin resistant. Because of the cross-reactivity of insulin and IGF-I at high concentrations it is very important to use physiological or near physiological concentrations of the peptides when studying insulin and IGF-I effects in vitro on cells that express both IR types and IGF-IR, and also to make sure of which receptor is activated. Several studies demonstrating insulin effects on vascular cells have used insulin concentrations which could well cross-react with the IGF-I receptor (Salt et al., 2003, Zeng and Quon, 1996).

In this and our previous report (Chisalita and Arnqvist, 2004) low concentrations of IGF-I were found to stimulate DNA-synthesis in HMVEC. Other studies on microvascular endothelial cells suggest that HMVEC as well as glomerular (Giannini et al., 1999) and retinal microvascular endothelial cells (Giannini et al., 2001) are very sensitive to IGF-I indicating a physiological function of IGF-I.

Our results were obtained on cultured endothelial cells and it is possible that the phenotype may differ between cultured cells and cells in intact vascular tissue. However, in human umbilical vein endothelial cells the ratio of IGF-IR to IR was similar in cultured and freshly isolated cells (Nitert et al., 2005). The function of IGF-I in intact vascular tissue has been studied in vivo, where the infusion of IGF-I in the human forearm model has been shown to increase blood flow (Pendergrass et al., 1998). Furthermore, in patients with diabetes the IGF system is dysregulated with low circulating IGF-I levels and changes in the IGF-binding proteins (Hedman et al., 2004) and it is possible that these changes affect the function of microvascular endothelial cells (Tooke et al., 1996).

In conclusion, due to a preponderance of IGF-I receptors and sequestration of insulin receptors into insulin/IGF-I hybrid receptors, microvascular endothelial cells are sensitive to IGF-I but are insulin resistant.

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Figure legends**Figure 1**

Gene expression of insulin receptor A (IRA) and insulin receptor B (IRB) isoforms in cultured dermal microvascular cells (HMVEC). IRA and IRB mRNA expression was measured by real time RT-PCR. Grey filled bar represents IRA mRNA and unfilled bar represents IRB mRNA. Data is expressed as amount IRA mRNA relative to IRB mRNA, where IRB was set to 1. Bars are means \pm SE of four experiments. ** = $p < 0.01$.

Figure 2

Co-precipitation of IR β -subunits and IGF-IR β -subunits in HMVEC. IGF-I receptor (IGF-IR) β -subunit (97kDa) and insulin receptor (IR) β -subunit (95kDa) was detected by Western blot in the same IP using specific IR β -subunit or IGF-IR β -subunit antibodies, implying co-precipitation. The figure shows blots after immunoprecipitation (IP) against either IGF-IR β -subunit (left column, 2A & 2C) or IR β -subunit (right column, 2B & 2D), and immunoblot against the IGF-IR β -subunit (upper blots, 2A & 2B) and the IR β -subunit (lower blots, 2C & 2D). Similar results were obtained in three independent experiments.

Figure 3

Phosphorylation of IR and IGF-IR in HMVEC. Effects of IGF-I and insulin on tyrosine phosphorylation of IGF-IR β -subunit (Fig 3A) or IR β -subunit (Fig 3B) in HMVEC were analyzed by immunoprecipitation with an IGF-IR or IR β -subunit specific antibody and immunoblotting with an anti-phosphotyrosine antibody (PY20). Significant phosphorylation of the IGF-IR β -subunit compared to baseline was found at IGF-I stimulations at 10^{-9} - 10^{-8} M, Fig 3A. Phosphorylation of the IR β -subunit compared to baseline was found at IGF-I stimulations at 10^{-9} - 10^{-8} M, Fig 3B. Dark grey filled bars represent IGF-I stimulation and light grey filled bars represent insulin stimulation. Data are means from three independent experiments. Data were quantified by densitometry and are expressed as phosphorylated/total protein, fractional of maximal response. * = $p < 0.05$, ** = $p < 0.01$.

Figure 4

Activation of insulin receptor substrate 1 (IRS-1), Akt (PKB) and ERK 1/2 in HMVEC. Effects of IGF-I and insulin on tyrosine phosphorylation of IRS-1 was analyzed by immunoprecipitation of IRS-1 with an IRS-1 antibody and immunoblotting with an anti-phosphotyrosine antibody (PY20). Significant phosphorylation of IRS-1 compared to baseline was found at IGF-I stimulations at 10^{-9} - 10^{-8} M, see Fig. 4A. Akt phosphorylation was analyzed using a specific antibody detecting Akt 1/2/3 phosphorylated at threonine 308. Significant phosphorylation of Akt compared to baseline was found at IGF-I stimulations at 10^{-8} - 10^{-7} M, insulin 10^{-7} M, see Fig. 4B. No significant phosphorylation compared to baseline was observed on ERK 1/2 phosphorylation at threonine 202 and tyrosine 204 (Fig. 4C). Grey filled bars represent IGF-I stimulation and unfilled bars represent insulin stimulation. Data are means from four and eight independent experiments respectively. Data were quantified by

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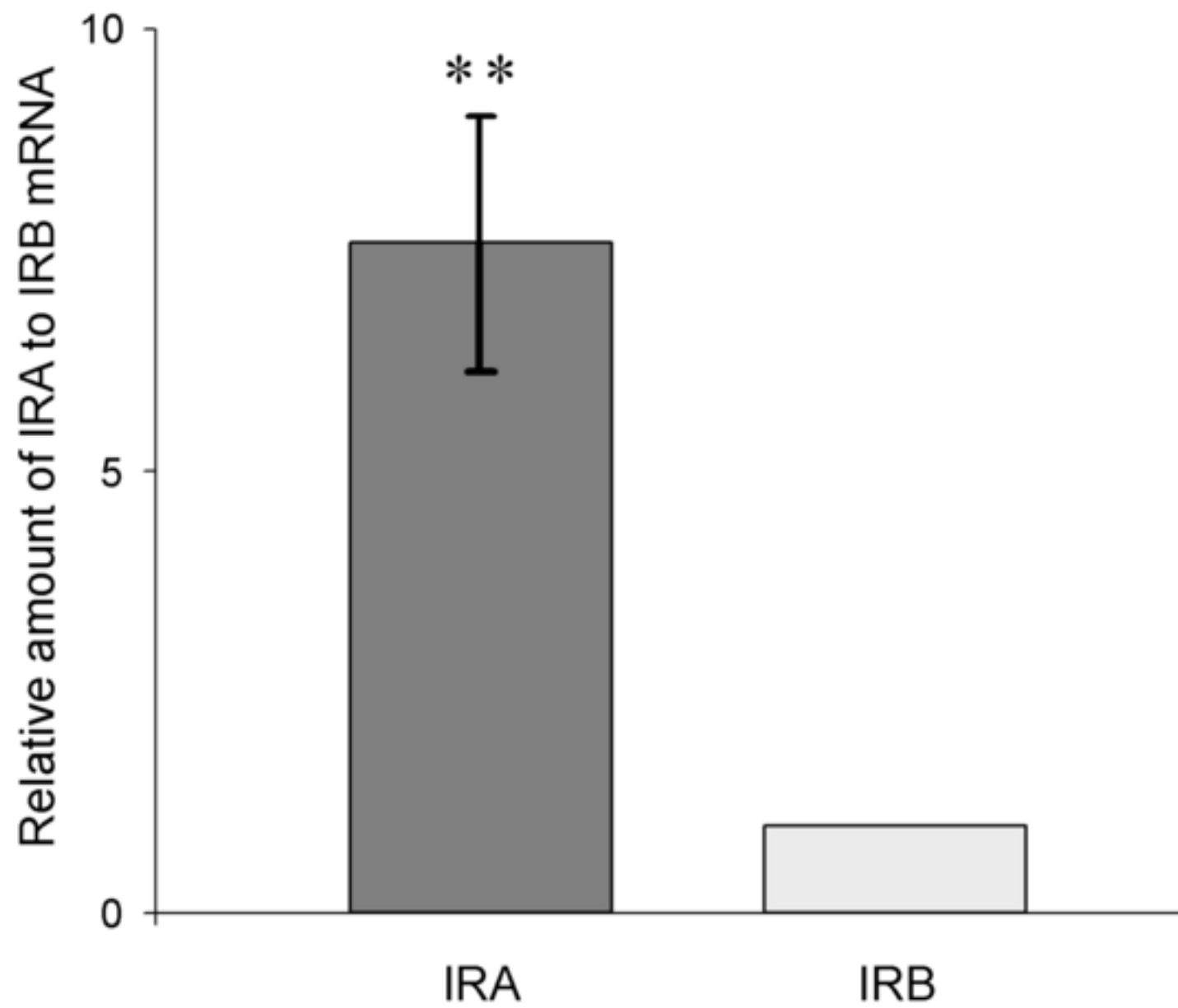
Human microvascular endothelial cells are sensitive to IGF-I
but resistant to insulin at the receptor level

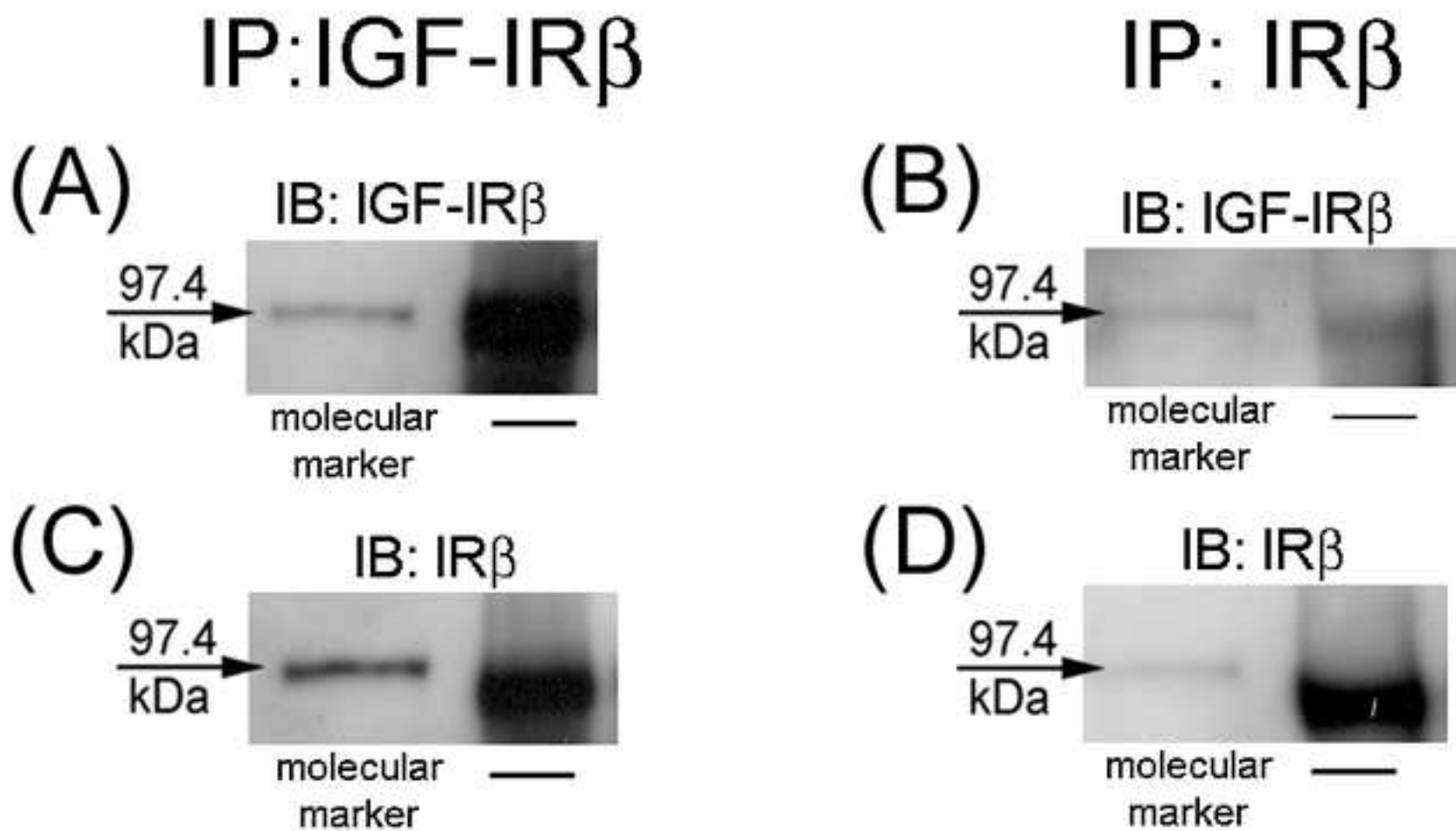
REVISED MANUSCRIPTGit Johansson *et al*

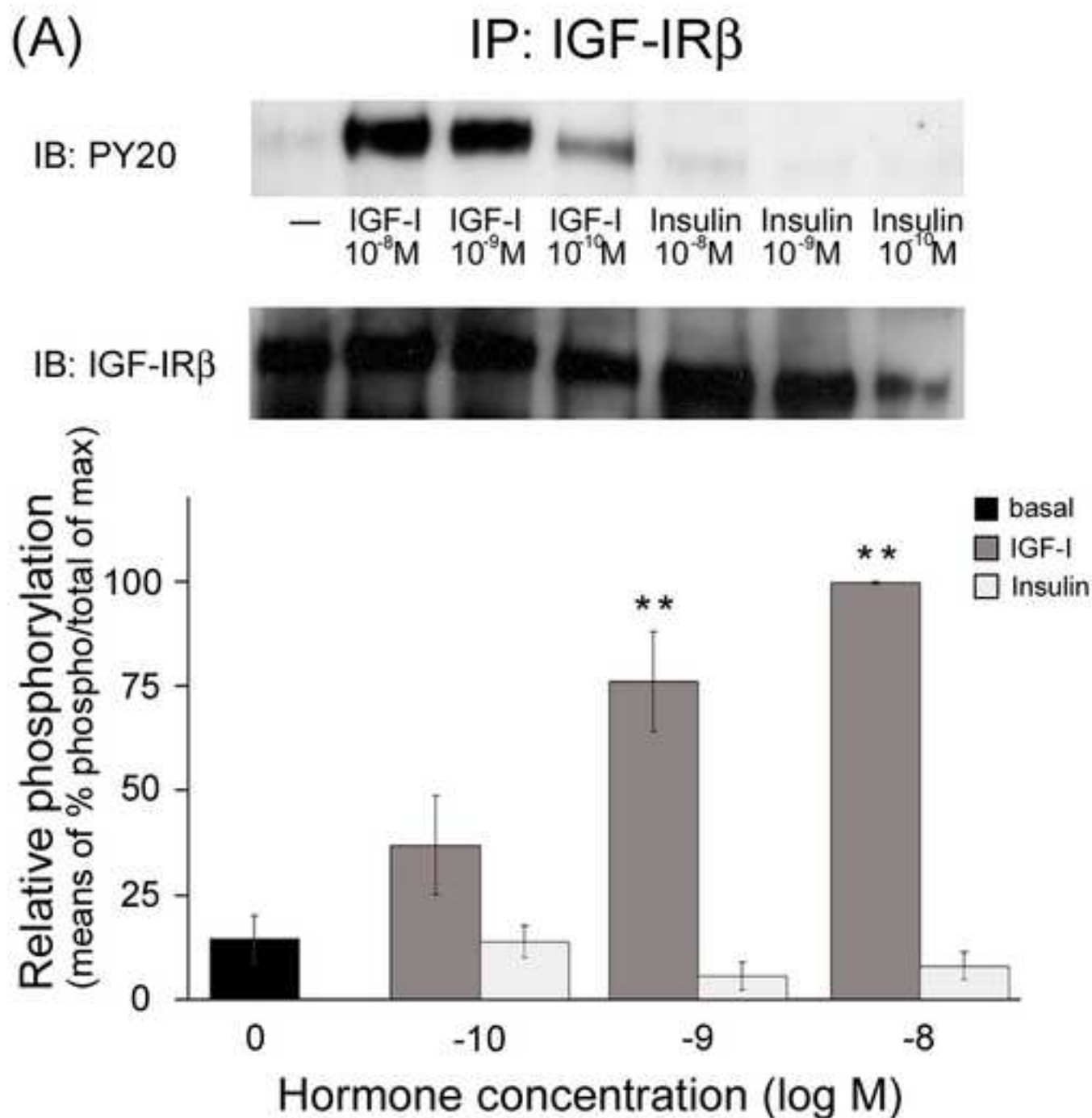
densitometry and are expressed as means of per cent phosphorylated/total protein of maximal response. $\ast = p < 0.05$, $\ast\ast = p < 0.01$.

Figure 5

Effects of IGF-I and insulin on [^3H] thymidine incorporation into DNA in HMVEC. Grey filled bars represent IGF-I stimulation and unfilled bars represent insulin stimulation. Data are expressed as thymidine incorporation above baseline, and are compared to baseline. Bars are mean \pm SE of 10 experiments. $\ast = p < 0.05$.







(B)

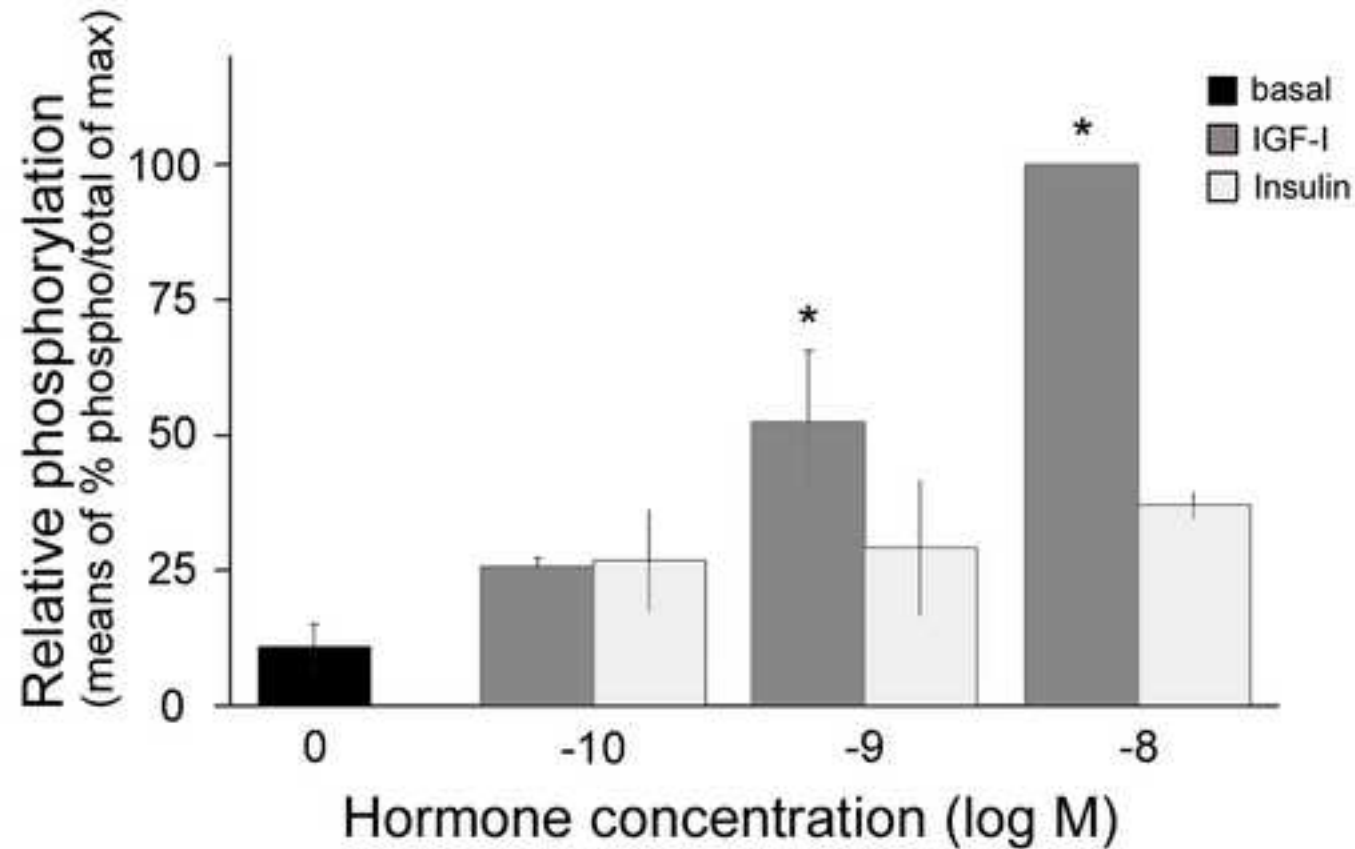
IP: IR β

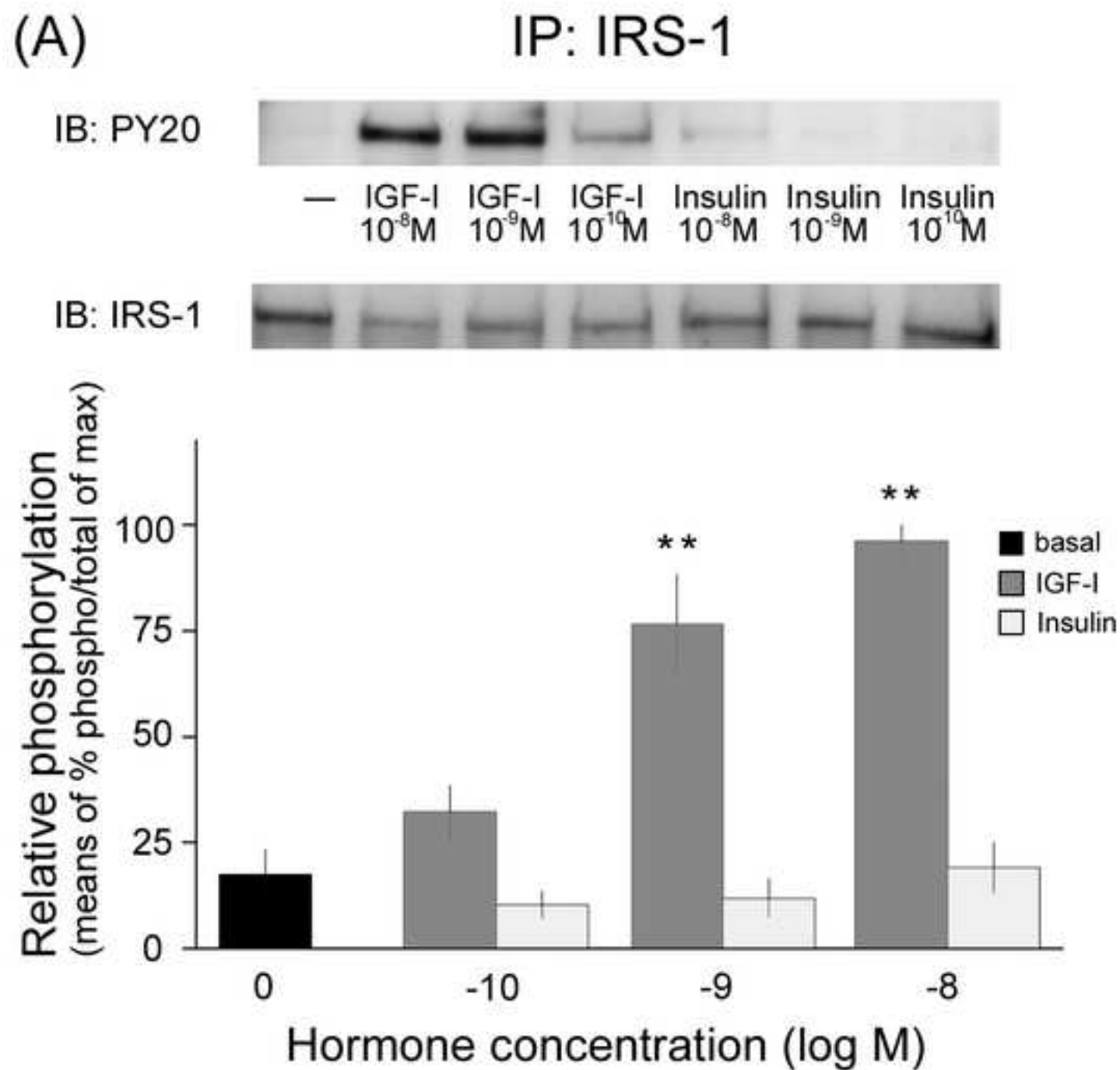
IB: PY20

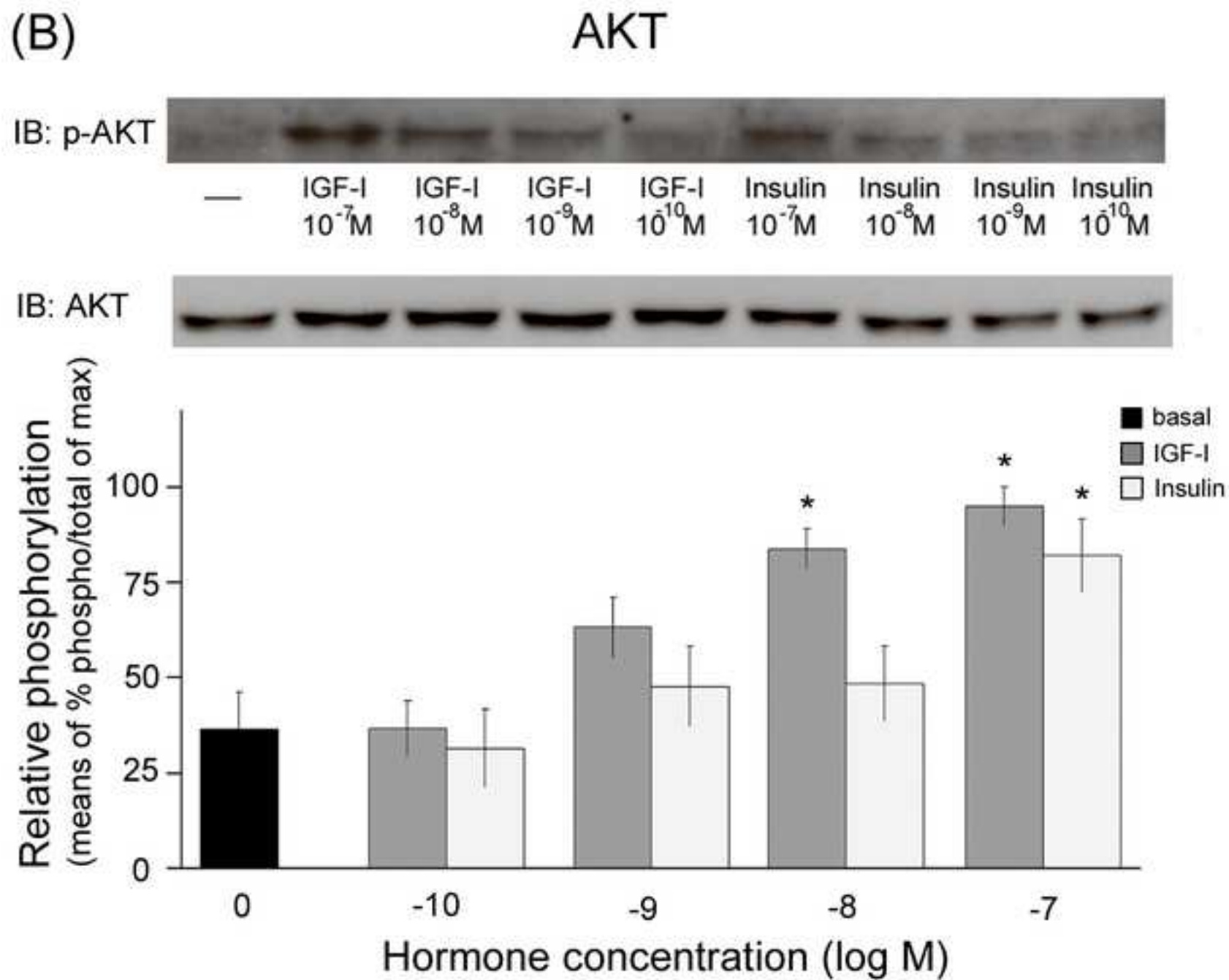


— IGF-I 10⁻⁸M IGF-I 10⁻⁹M IGF-I 10⁻¹⁰M Insulin 10⁻⁸M Insulin 10⁻⁹M Insulin 10⁻¹⁰M

IB: IR β







(C)

ERK 1/2

IB: p-ERK 1/2



— IGF-I 10⁻⁷ M IGF-I 10⁻⁸ M IGF-I 10⁻⁹ M IGF-I 10⁻¹⁰ M Insulin 10⁻⁷ M Insulin 10⁻⁸ M Insulin 10⁻⁹ M Insulin 10⁻¹⁰ M

IB: ERK 1/2

