IGF-I but not insulin inhibits apoptosis at a low concentration in vascular smooth muscle cells

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Abstract

Apoptosis of vascular smooth muscle cells (VSMCs) is of importance in the development of diabetic angiopathy. Our aim was to evaluate the effect of insulin and IGF-I on apoptosis in VSMCs. Rat aortic VSMCs were used and apoptosis was induced by serum starvation. As apoptotic markers we measured caspase-3 activity, histone-associated DNA fragments by ELISA and nuclear morphology by DAPI (4',6-diamidino-2-phenylindole) staining. Phosphorylation of IGF-I receptors was evaluated by Western blot. Serum starvation had increased caspase-3 activity even after 3 h. The highest activity was found after 3–12 h. IGF-I 10^{-9} M inhibited serum starvation-induced caspase-3 activity with a maximal effect after 12 h. When studied after starvation for 12 h, significant inhibitory effects on caspase-3 were found at IGF-I

concentrations of 10^{-8} – 10^{-7} M ($P < 0.01$) and at an insulin concentration of 10^{-6} M ($P < 0.01$). DNA fragmentation was detected by ELISA after 24 h and chromatin condensation and nuclear fragmentation by DAPI staining after 24 and 48 h respectively. IGF-I dose-dependently reduced apoptosis evaluated by ELISA, reaching a maximal effect at 10^{-9} M. Insulin reduced apoptosis but the effect was weaker and a higher concentration was needed. IGF-I (10^{-8} M) and insulin at a very high concentration (10^{-6} M) phosphorylated IGF-I receptors. Taken together, IGF-I and insulin have anti-apoptotic effects on VSMCs but the effect of insulin is only found at high unphysiological concentration.

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Introduction

Vascular smooth muscle cells (VSMCs) are involved in development of vascular disease by processes such as cell proliferation, migration, apoptosis and production of extracellular matrix (Davies *et al.* 1993, Davies 1996, Bennett & Boyle 1998, Goetze *et al.* 2001). Diabetes mellitus causes a variety of abnormalities in the vascular system, affecting both micro- and macrovasculature (Donnelly *et al.* 2000). It has been hypothesized that the pathogenesis of diabetic vasculopathy involves abnormal regulation of VSMC apoptosis (Hall *et al.* 2000). Diabetes even of short duration accelerates the death of capillary cells in the retina by apoptosis (Podesta *et al.* 2000). In atherosclerotic disease, apoptosis of VSMCs has a critical role in plaque stability (Davies 1996, Okura *et al.* 2001).

Initiation of apoptosis can result from multiple stimuli such as growth factor withdrawal, cytokines, free radicals and toxins (Cohen 1994, Pollman *et al.* 1996). It occurs by rapid, irreversible condensation of cytoplasm, accompanied by an increase in cell density and condensation of the nuclear chromatin (Saini & Walker 1998). Activation of the caspase cascade is responsible for many of

the hallmarks of apoptosis, such as DNA fragmentation, chromatin condensation and apoptotic body formation (Janicke *et al.* 1998). Caspases are cysteine proteases which have an unusual specificity among proteolytic enzymes to perform highly selective proteolysis during the course of cell death (Thornberry *et al.* 1997, Kidd 1998). Caspases can be grouped according to their principal function: group I (caspases 1, 4, 5) are primarily involved in the processing of pro-inflammatory cytokines; group II (caspases 2, 3, 7) act as downstream terminal effector caspases responsible for cleavage of intracellular substrates required for cellular survival, architecture and metabolic function (Chinnaiyan & Dixit 1996, McCarthy & Bennett 2000); group III (caspases 6, 8, 9) are involved in the cleavage and activation of group II caspases.

Activation of the insulin-like growth factor (IGF)-I receptor (IGF-IR) exerts antiapoptotic effects, protecting multiple cell types against a variety of death signals (Peruzzi *et al.* 1999). Suppression of IGF-I signalling induces apoptosis *in vitro* and *in vivo* (Resnicoff *et al.* 1994). IGF-I has an important role in the survival of VSMCs in different ways, including a marked suppression of apoptosis (Bennett *et al.* 1995). Also insulin has been reported

to inhibit apoptosis in VSMCs (Jung *et al.* 2000, Goetze *et al.* 2001). There are few insulin receptors in VSMCs and it has been suggested that the effect of insulin is mediated through IGF-IRs (Lee *et al.* 1988, Arnqvist *et al.* 1995). The aim of this study was to determine the effect of IGF-I and insulin in a time- and dose-dependent manner on apoptosis in VSMCs.

Materials and Methods

Cell culture

Aortic smooth muscle cells were isolated from male Sprague-Dawley rats (~250 g) according to Nilsson *et al.* (1983). Animal procedures were approved by the local ethical committee for animals. Cells were grown in Ham's medium (Invitrogen, Stockholm, Sweden) with 50 µg/ml ascorbic acid, 50 µg/ml gentamicin sulphate, 2 µg/ml Fungizone and 10% new-born calf serum (NCS) (Invitrogen) in F12 medium (pH 7.4) (Invitrogen) at 37 °C and air with 5% CO₂. Trypsin (0.05%) and EDTA (0.02%) solution was used to passage the cells. Cells between passages three and ten were used for the experiments. The cells were characterized as smooth muscle cells according to morphological criteria (Ross 1971) and with a murine monoclonal antibody (clone asm-1, IgG2a; Boehringer Mannheim GmbH, Mannheim, Germany) against a unique epitope of smooth muscle α -actin, as described by Skalli *et al.* (1986). This type of α -actin is specific for smooth muscle cells (Gabbiani *et al.* 1981). Cells grown on glass coverslips were washed twice with PBS and fixed in ice-cold acetone for 5 min. Thereafter the cells were preincubated in 2% goat serum and 1% BSA in PBS for 15 min, and then incubated with fluorescein-linked anti-mouse IgG from sheep (Amersham Pharmacia Biotech) for 30 min. A Nikon Eclipse E800 fluorescence microscope equipped with a VFM EPI-fluorescence attachment (Nikon Instruments Inc., Melville, NY, USA) was used.

Morphological evaluation by DAPI

(4',6-diamidino-2-phenylindole) staining

Cells were cultured in 35 mm Petri dishes and fixed with 4% formaldehyde, washed twice with PBS, stained with DAPI 5×10^{-7} M for 10 min, and mounted with Vectashield with DAPI (ImmunKemi, Järfälla, Sweden). Apoptosis was quantitated by counting a total of 200 nuclei from each Petri dish and calculating the percentage of apoptotic nuclei.

Determination of caspase-3 activity

To assay caspase-3 activity, Ac-DEVD-AMC, a caspase-3 fluorogenic substrate was used (Cat. No. 66081U; BD PharMingen, BD Biosciences, Stockholm, Sweden).

Activated caspase-3 cleaves Poly (ADD ribose) polymerase from its 116 kDa to an 85 kDa residual fragment. When Ac-DEVD-AMC is treated with active caspase-3, 7-amino-4-methylcoumarin (AMC) is released and monitored in a spectrofluorometer at an excitation wavelength of 380 nm and an emission wavelength range of 430–460 nm.

For analysis of caspase-3 activity, the cells were grown in 10 cm Petri dishes. Near-confluent cells were starved in serum-free medium and then harvested after 0, 3, 6, 12, 24 and 48 h. For evaluation of the effects of IGF-I and insulin, near-confluent cells were incubated for 12 h with serum-free medium and different concentrations of IGF-I (10^{-11} – 10^{-7} M) or insulin (10^{-10} – 10^{-6} M). As positive control, cells cultured in 10% NCS were used. Samples were scratched off with a rubber policeman, centrifuged at 700 g for 10 min, washed with PBS washing buffer recommended by the company and centrifuged at 700 g for 5 min. The pellet was stored at –70 °C until measurement. Pellets were lysed with 100 µl cell lysis buffer (recommended by company) for 30 min on ice. Fifteen microlitres of the lysed suspension were used for protein determination (Lowry *et al.* 1951). The remaining lysate was incubated with 1 ml reaction buffer (20 mM HEPES (pH 7.5), 10% glycerol, 2 mM dithiothreitol) and 20 µM Ac-DEVD-AMC for 1 h in a shaking water-bath at 37 °C. Caspase-3 activity was expressed as arbitrary units of AMC fluorescence per microgram of protein.

Detection of apoptosis by ELISA

Apoptotic cell death was evaluated by a photometric enzyme-linked immunoassay for quantitative determination of cytoplasmic histone-associated DNA fragments (Cell Death Detection ELISA Kit; Roche Diagnostic Scandinavian AB, Stockholm, Sweden). The kit was used according to the manufacturer's instruction. Cells were cultured in 24-well plates with 40 000 cell/well. After 72 h near-confluent cells were starved in 1 ml serum-free medium for 24 h and then incubated with serum-free medium with different concentrations of IGF-I (10^{-11} – 10^{-7} M) or insulin (10^{-10} – 10^{-6} M) for the next 24 h. Control cells were kept in serum-free medium. Cells were harvested including medium by scratching of the wells and centrifuged at 700 g for 10 min. The cell pellet was lysed with the incubation buffer of the kit and centrifuged to pellet cell nuclei, containing high molecular weight DNA. The supernatant containing mono- and oligonucleosomes or low molecular weight DNA was used for the rest of the assay. Anti-histone antibody containing coating buffer was added to the 96-well ELISA plates and incubated overnight at 4 °C. The plates were rinsed (three times) with washing buffer and the samples were added and incubated for 90 min. Plates were rinsed (three times) with washing buffer and the peroxidase-conjugated anti-DNA antibody was added. After incubation with peroxidase substrate

(ABTS) from Roche, optical density of the colour reaction was measured by a spectrophotometer at 405 nm.

Western blot analysis

VSMCs were seeded in 150 mm Petri dishes and cultured for 5–10 days to near confluence. After 24 h starvation, IGF-I (10^{-8} M) or insulin (10^{-8} , 10^{-6} M) was added to the Petri dishes, mixed gently, incubated for 10 min and quickly removed. Petri dishes were placed on liquid nitrogen for about 30 s. Two millilitres of lysis buffer (Tris 20 mM (pH 7.5), NaCl 150 mM, EDTA 5 mM, sodium deoxycholate 0.5%, Triton X-100 0.5%, Na_3VO_4 1 mM, aprotinin 1.5 µg/ml, leupeptin 1.5 µg/ml, phenylmethylsulphonylfluoride 1 mM) were added to Petri dishes and cell lysates left on ice for 30 min. Cells were completely scraped off before totally thawed and transferred to pre-chilled tubes. Cells lysates were centrifuged at 15 000 *g* at 4 °C for 10 min. The supernatant was transferred into other tubes. Samples were stored at -70 °C if not used immediately.

Total protein content was measured by the bicinchoninic acid method to adjust the amount of aliquot used for subsequent experiments. To each tube of cell lysate that contained 1 mg total protein, 2 µl corresponding polyclonal antibody (anti-IGF-IR β -subunit antibody C20; Santa Cruz Biotechnology, Santa Cruz, CA, USA) were added and the test tube was kept on ice for 2 h. Fifty microlitres of protein-A Sepharose (Pharmacia, Uppsala, Sweden) were added and gently shaken at 4 °C overnight. The immunoprecipitates were washed three times with ice-cold lysis buffer and diluted in 50 µl sample buffer. Samples were separated on a 7.5% SDS-PAGE and transferred onto a polyvinylidene difluoride membrane. After blocking in 0.2% polyvinyl alcohol and 0.2% Tween in buffer (Tris base 0.05 M, NaCl 0.15 M, pH 7.5), the membrane was immunoblotted using a 1:1000 dilution of the anti-IGF-IR β -subunit antibody for 2 h at room temperature. A monoclonal phosphotyrosine antibody (anti-phosphotyrosine antibody PY20, sc-508; Santa Cruz Biotechnology) was used to detect receptor phosphorylation. The proteins were detected with a horseradish peroxidase-linked secondary antibodies and an ECL Plus system (Amersham Pharmacia Biotech). In order to show different proteins on the same membrane, the membrane was stripped by heating to 60 °C for 30 min in stripping buffer containing 10% SDS, 100 mM 2-mercaptoethanol in 62.5 mM Tris-HCl.

Statistical analysis

Values are given as means $\pm$ S.E.M. from at least four independent experiments. Statistical analysis was performed by Student's *t*-test for paired unpaired comparisons and, when multiple comparisons were made,

by one-way ANOVA in conjunction with Dunnett's post-hoc test. $P < 0.05$ was considered statistically significant.

Results

DAPI staining

As shown in Fig. 1A, VSMCs had intact nuclear morphology in medium with 10% NCS while cells cultured in serum-free medium for 24 h showed chromatin condensation (Fig. 1B). DNA fragmentation and nuclear shrinkage of VSMCs were found after starvation for 48 h (Fig. 1C). The percentage of apoptotic nuclei after 48 h was $6.0 \pm 1.0\%$ in serum and $11.8 \pm 1.5\%$ ($n=6$, $P < 0.02$) in serum-free media.

Caspase-3 activity

Caspase-3 activity was already increased after serum starvation for 3 h in VSMCs (Fig. 2). The highest activity was observed after 3–12 h. IGF-I 10^{-9} M reduced serum starvation-induced caspase-3 activity with a maximal effect after 12 h (Fig. 3). The effect of IGF-I was of borderline significance after 6 h ($P < 0.055$) and significant after 12 h ($P < 0.04$). After starvation for 24 and 48 h no effect of IGF-I was found. When studied after starvation for 12 h, significant inhibitory effects on caspase-3 were found at IGF-I concentrations of 10^{-8} – 10^{-7} M ($P < 0.01$) and at an insulin concentration of 10^{-6} M ($P < 0.01$) (Fig. 4). Borderline significances were obtained for IGF-I 10^{-9} M and insulin 10^{-7} M, $P < 0.056$ and $P < 0.058$ respectively.

Antiapoptotic effect of insulin and IGF-I evaluated by ELISA

ELISA analysis of mono- and oligonucleosomal DNA fragmentation demonstrated a high DNA fragmentation in VSMCs between 24 and 72 h of serum starvation compared with cells incubated with serum (Fig. 5). Cells incubated with IGF-I (10^{-11} – 10^{-7} M) or insulin (10^{-10} – 10^{-6} M) showed different inhibitory effects on apoptosis (Fig. 6). The highest inhibitory effects of IGF-I and insulin were seen at concentrations of 10^{-9} M and 10^{-8} M respectively; $P < 0.003$ compared with control.

Phosphorylation of IGF-IRs

Phosphorylation of IGF-IR β -subunit was found by IGF-I (10^{-8} M) and insulin (10^{-6} M) (Fig. 7A). A very weak band was also found with insulin (10^{-8} M) (Fig. 7A). Figure 7B shows the gel blotted with an antibody against IGF-IR. The experiment was repeated three times with similar results.

Figure 1 Morphological evidence of apoptosis in VSMCs stained with DAPI. Confluent cells were starved with serum-free medium and control cells were grown in 10% NCS. (A) Cells in 10% NCS had intact nuclei. (B) After 24 h in serum-free medium, nuclei showed chromatin condensation. (C) Nuclear shrinkage after serum-free medium for 48 h.

Figure 2 Time-dependent caspase-3 activity in VSMCs. Near-confluent cells were starved in serum-free medium (SF) and harvested after 0, 3, 6, 12, 24 and 48 h. Caspase-3 activity at 0 h was used as control. Values are given as percent of the activity at 0 h. Means $\pm$ S.E.M.; $n=8$.

Figure 3 Time-course for inhibitory effect of IGF-I (10^{-9} M) on caspase-3 activity. Confluent cells were starved in serum-free medium with or without IGF-I. Cells were also cultured with 10% NCS. Caspase-3 activity at 0 h starvation was used as control. Means $\pm$ S.E.M., $n=4$.

Discussion

We found that IGF-I and insulin had inhibitory effects on apoptosis induced by serum starvation in VSMCs. Compared with IGF-I, insulin had a weaker effect that was found at a higher concentration, 10^{-9} M and 10^{-8} M respectively. This suggests that the effect of insulin is elicited through the IGF-IR.

Proteolytic cleavage of cellular proteins evaluated by caspase-3 activity showed highest activity between 3 and 12 h in this study. That activation of caspase-3 occurs early in serum-deprived smooth muscle cells is supported by the observation of Shichiri *et al.* (2000), who found an increase in caspase-3 activity even after 30 min serum deprivation of rat VSMCs. After activation of the caspase protease family, followed by cleavage of nuclear lamins and

Figure 4 Evaluation of inhibitory effect of IGF-I and insulin on caspase-3 activity. Confluent cells were starved in serum-free medium and incubated for 12 h with insulin (10^{-10} – 10^{-6} M) or IGF-I (10^{-11} – 10^{-7} M). Data are given as 100 minus percent of control (starved cells). Means $\pm$ S.E.M.; $n=4$.

Figure 5 Time course for serum starvation-induced apoptosis in VSMCs evaluated by ELISA. Near-confluent cells were starved in serum-free (SF) medium for different times. Cells were also incubated with 10% NCS (serum). The cells were harvested after 0, 24, 48 and 72 h. Data are given as the percent at 0 h. Means $\pm$ S.E.M., $n=4$.

destruction of the cytoskeleton, the cells enter into an irreversible phase. Proteases mediating cell destruction are conserved in virtually all cells (Bennett 1997). Caspase-3 alone appears essential to complete the destructive phase of apoptosis (Avaughan *et al.* 2002). In our study, an inhibitory effect on caspase-3 activity was found after exposure to IGF-I for 3–12 h. An inhibitory effect of IGF-I (1.3×10^{-8} M) has previously also been reported in human VSMCs (Bai *et al.* 1999). Evaluation of the effect of IGF-I (10^{-11} – 10^{-7} M) on caspase-3 activity showed that IGF-I (10^{-9} – 10^{-7} M) inhibited caspase-3 activity. Serum starvation induced the highest apoptosis in VSMCs after 48 h when assessed as histone-associated DNA fragments (mono- and oligonucleosomes) by ELISA. With morphological evaluation by DAPI staining we found chromatin condensation after 24 h and after 48 h nuclear fragmentation in serum-starved VSMCs. Also caspase-3 was markedly inhibited by serum, which thus had a pronounced antiapoptotic effect. Serum contains several growth factors with possible effects on apoptosis, of which IGF-I and platelet-derived growth factor have been identified as potent survival factors (Bennett *et al.* 1995). Our results are consistent with previous reports of apoptosis in rabbit and human VSMCs (Pollman *et al.* 1996, Aoki *et al.* 1997, Gouni-Berthold *et al.* 2001). The effect of IGF-I

Figure 6 The effect of IGF-I and insulin on apoptosis in VSMCs evaluated by ELISA. Near-confluent cells were starved for 24 h and incubated with different concentration of IGF-I (10^{-11} – 10^{-7} M) (A) or insulin (10^{-10} – 10^{-6} M) (B) for the next 24 h. For control, cells remained in serum-free medium. Data shown as 100 minus percent of control. Means $\pm$ S.E.M., $n=4$.

(10^{-11} – 10^{-7} M) on DNA fragmentation showed that IGF-I at a concentration of 10^{-9} M had a maximal antiapoptotic effect on our cells. Jung *et al.* (2000) showed that H_2O_2 -induced apoptosis in rat VSMCs is inhibited by IGF-I at a concentration of 10^{-9} M. Using evaluation by nuclear chromatin morphology after 12 h in rat VSMCs, Bai *et al.* (1999) found that the effect of IGF-I appeared at a concentration of 1.3×10^{-10} M and was maximal at 1.3×10^{-8} M.

With the evaluation of insulin (10^{-10} – 10^{-6} M) on caspase-3 activity, we found that insulin (10^{-6} M) inhibited caspase-3 activity significantly. To the best of our knowledge there are no previous dose-response studies on inhibition of caspase-3 activity by IGF-I or insulin in vascular smooth muscle. Goetze *et al.* (2001) showed that insulin (10^{-6} M) protects VSMCs from apoptosis. Jung *et al.* (2000) found that insulin (10^{-7} M) induces Akt phosphorylation after 30 min to 6 h in VSMCs, and inhibits apoptosis. The available data thus suggest that IGF-I inhibits apoptosis in concentrations 10–100 times lower than insulin (Jung *et al.* 2000).

The insulin receptor and IGF-IR are homologous and insulin and IGF-I can cross-react with each other's receptors with 100- to 1000-fold less potency than to their own receptors (Arnqvist *et al.* 1995). We used concentrations of

Figure 7 Phosphorylation of the IGF-IR β -subunit. Near-confluent cells were starved for 24 h and incubated with IGF-I (10^{-8} M) or insulin (Ins) (10^{-8} , 10^{-6} M) for 10 min. Phosphorylation of IGF-IR was evaluated by Western blot after immunoprecipitation. (A) Shows the SDS-PAGE gel after blotting with a phosphotyrosine antibody and (B) the same gel after blotting with an antibody against the IGF-IR β -subunit. The experiment was repeated three times with similar results.

10^{-11} – 10^{-7} M IGF-I and 10^{-10} – 10^{-6} M insulin to evaluate their effects. The circulating free IGF-I concentration is difficult to assess due to binding proteins, but is probably around 10^{-9} M (Frystyk *et al.* 1994). The normal plasma insulin concentration is in the range 10^{-11} – 10^{-9} M (Olsson *et al.* 1988). Half-maximal displacement of labelled IGF-I from its receptor by unlabelled IGF-I is obtained at 0.3×10^{-9} M (Bornfeldt *et al.* 1991) and the corresponding concentration for displacement of labelled insulin from its receptor by unlabelled insulin is around 10^{-11} M (Kurtzhals *et al.* 2000). We found that IGF-I (10^{-8} M), and at a very high unphysiological concentration also insulin (10^{-6} M) phosphorylated the IGF-IRs. This observation is in agreement with Banskota *et al.* (1989), who found that insulin (1.6×10^{-7} M) and IGF-I (1.3×10^{-8} M) stimulate *c-myc*, and who demonstrated that the effects of insulin and IGF-I on DNA synthesis and proliferation of human smooth muscle cells occur via the IGF-IR. Specific binding of insulin in cultured VSMCs has been found to be very low, and affinity cross-linking studies have not been able to demonstrate specific insulin receptors (Jialal *et al.* 1985, King *et al.* 1985). Insulin stimulates metabolism in

isolated arteries, but is approximately 100 times less potent in arterial smooth muscle compared with skeletal muscle (Arnqvist 1974). Ruiz-Torres *et al.* (1998) showed that under special conditions that also occur with ageing, insulin interacts with receptors in VSMCs, especially IGF-I, stimulating these cells to produce collagen. Thus there are several lines of evidence suggesting that the metabolic effects of insulin on VSMCs observed *in vitro* are elicited through IGF-IRs. Our results suggest that also the inhibitory effects of IGF-I and insulin on apoptosis in VSMCs probably are mediated through IGF-IRs. The effects of insulin are only found at insulin concentrations which are much higher than those that occur *in vivo*, indicating that the effect of insulin can only be obtained *in vitro*. From a clinical point of view, our results suggesting that IGF-I but not insulin inhibits apoptosis in VSMCs is of great interest. In patients with type 1 diabetes, IGF-I levels are low, and largely independent of glycaemic control, probably due to lack of insulin delivery to the liver in the portal vein (Hanaire-Broutin *et al.* 1996, Ekman *et al.* 2000). Low circulating IGF-I could increase apoptosis and thereby promote vascular disease.

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