Cross-talk between angiotensin II and IGF-1-induced connexin 43 expression in human saphenous vein smooth muscle cells

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Abstract

Vascular restenosis following coronary artery bypass graft can cause major clinical complications due to intimal hyperplasia in venous conduits. However, the precise underlying mechanisms of intimal hyperplasia are still unclear. We have recently reported that increased expression of connexin43 (Cx43) is involved in the proliferation of vascular smooth muscle cells (SMCs) in human saphenous vein (SV). In this study, we investigated the signalling transduction pathway involved in Cx43 expression and SV SMC proliferation. Angiotensin-II (AT-II, 100 ng/ml) increased AT-II receptor 1 (AT-1R) protein expression and insulin-like growth factor-1 (IGF-1) (100 ng/ml) up-regulated IGF-1 receptor (IGF-1R) protein expression in SV SMCs. Interestingly, AT-1R expression was also increased by IGF-1 treatment, and IGF-1R expression was increased by AT-II treatment, which was blocked by siRNA-IGF-1R and siRNA-AT-1R, respectively. Furthermore, the effect of AT-II and IGF-1 signal cross-talk inducing up-regulation of their reciprocal receptors was blocked by siRNA against extra-cellular signal-regulated kinases 1/2 (Erk 1/2) in SMCs of SV. Moreover, AT-II and IGF-1-induced Cx43 expression via phosphorylation of Erk 1/2 and activation of transcription factor activator protein 1 (AP-1) through their reciprocal receptors in SV SMCs. These data demonstrate a cross-talk between IGF-1R and AT-1R in AT-II and IGF-1-induced Cx43 expression in SV SMCs involving Erk 1/2 and downstream activation of the AP-1 transcription factor.

Keywords: angiotensin II • connexins • intimal hyperplasia • restenosis • vascular smooth muscle cells • vein graft disease

Introduction

Atherosclerotic occlusion of blood vessels is a major cause of morbidity and mortality worldwide. Current treatment consists of percutaneous transluminal coronary angioplasty, stenting and coronary artery bypass graft [1]. Bypass grafting can be done using either internal mammary artery or saphenous vein (SV). However, stenosis and occlusion of SV conduits commonly limits long-term results following coronary bypass grafting (CABG) surgery [2]. The precise underlying mechanisms of intimal hyperplasia are likely to involve growth factors which promote hyperplasia, inflammation and mitogenesis, leading to progression of the cell cycle and proliferation of smooth muscle cells (SMCs) [3, 4].

Insulin-like growth factor-1 (IGF-1) is involved in protein synthesis, cell migration and mitogenesis. Increased expression of IGF-1 has also been shown in plaque and restenotic areas [5]. Activation of IGF-1 receptors (IGF-1R) triggers several signal pathways, including phosphatidylinositol 3 kinase – Akt- and mitogen-activated protein kinase (MAPK) [6, 7]. Angiotensin II (AT-II), a potent growth factor, has been shown to stimulate proliferation of vascular smooth muscle cells (VSMCs), induce the migration of VSMCs, and induce the accumulation and deposition of collagen – all factors contributing to the pathogenesis of atherosclerosis [8, 9]. There is increasing evidence that connexins are involved in the development of intimal hyperplasia and restenosis involving mouse and human atherosclerotic lesions [10]. Recently, our data further showed that connexin43 (Cx43) expression can be influenced by AT-II and IGF-1 through Erk and p38 pathways, and may contribute to the pathogenesis of vein graft disease.
following CABG [11]. However, we do not know whether there is cross-talk between AT-II and IGF-1 signalling pathways involved in Cx43 expression in SMCs of human SV. In this study, we examined the effect of AT-II, and IGF-1, and cross-talk between their signalling pathways on Cx43 expression in SMCs of human SV.

Methods

Specimen collection, processing and culture of smooth muscle cell

The SV specimens (mean patient age 69.5 ± 5.8 yrs) left over from the bypass surgical procedure were used for the study. Specimens were collected fresh in ice-cold University of Wisconsin solution, which is used to maintain cellular and tissue integrity in the organs for transplantation. The human SV conduits were immediately brought to the laboratory, where all of the procedures were carried out under sterile conditions. SMCs from the SV conduits were isolated by a method previously reported from our laboratory [12, 13]. Briefly, after gentle removal of the endothelial cells and adventitia, the specimen was minced and digested with digestion media [containing elastase, collagenase, M199SF medium, 1 mg/l glucose, 4-(2-hydroxyethyl)-1-piperazine ethane sulfonic acid (HEPES) and fetal bovine serum (FBS)]. The isolated cells were removed by centrifugation at 900 × g for 10 min. at 4°C, suspended in M199SF, incubated at 37°C in a humidified 5% CO2 atmosphere for 10–14 days, then passaged. The sub-cultured VSMCs were used between passages 3 and 5. The confluent cells showed the characteristic hill-and-valley pattern associated with spindle-shaped VSMCs. The purity of isolated VSMCs was examined with positive immunostaining to smooth muscle α-actin and caldesmon. The purity of the isolated cells was >99%, as established in our laboratory and reported previously [12]. Prior to stimulation experiments, cells were serum starved for 24 hrs in DMEM. The experimental protocol for this study was approved by the Institutional Review Board of Creighton University.

Flow cytometry

For IGF-1R and AT-1R expression profiling, 1 × 10^6 cells were washed with phosphate-buffered saline plus 0.2% bovine serum albumin (Sigma-Aldrich, St. Louis, MO, USA). Cells were washed and incubated with AT-1R and IGF-1R monoclonal antibody for 30 min., and then with fluorescein isothiocyanate (FITC)-conjugated goat antimouse secondary antibody (BD, Franklin Lakes, NJ, USA) for 30 min. Cells were washed and fixed in 1% paraformaldehyde. Samples were analysed using the BD FACScan flow cytometer.

Small interfering RNA

All siRNAs were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Transfection of VSMCs was performed using the nucleofection device and solutions (Amxara Biosystems, Amaxa, Inc., Allendale, NJ, USA). Nucleofection device is an apparatus which delivers unique electrical stimuli to the target cells. The resulting transfection efficiency was more than 70% in vascular SMCs in our previous study [13].

Western blot analysis

SMCs were collected and lysed in lysis buffer (60 mmol/l Tris HCl pH 7.4, 1% NP-40, 0.15% sodium deoxycholate, 150 mmol/l NaCl, 1 mmol/l ethylenediaminetetraacetic acid, 1 mmol/l phenylmethylsulfonyl fluoride, 5 mmol/l sodium orthovanadate, 5 mmol/l NaF, 20 μg/ml leupeptin and 5 μg/ml aprotinin), and the protein concentration of the lysate was determined by BioRad protein assay (BioRad, Hercules, CA, USA). Proteins were resolved by SDS-PAGE and transferred to an Immobilon P membrane. Non-specific proteins were blocked by incubation in blocking buffer (5% milk powder/Tris-buffered saline, pH 7.8/0.1% Tween 20) and the membranes were incubated overnight at 4°C with blocking buffer containing antibodies to AT-1R, IGF-1R, Erk and Cx43 (Cell Signaling Technology, Danvers, MA, USA). Membranes were washed and incubated with horseradish peroxidase (HRP)-conjugated secondary antibody and detected using an ECL kit (Pierce, Rockford, IL, USA). As a loading control, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was probed and visualized in all blots.

Activation of transcription factor AP-1

SMCs were transfected with 5 μg of pAP-1-Luc (Panomics, Redwood City, CA, USA) designed to monitor the AP-1 signal transduction pathway with or without dominant-negative AP-1 (A-Fos) (kindly provided by Dr. Charles Vinson) and 0.2 μg of Renilla luciferase reporter control vector (Promega, Madison, WI, USA) to normalize transfection efficiencies. Twenty-four hours after transfection, the cells were stimulated with AT-II and IGF-1 for 3 hrs, following which the cells were harvested and lysates were prepared. Luciferase activity was measured using a luminometer and a Dual Luciferase Reporter Assay System (Promega) as per the manufacturer’s protocol. All values were normalized to Renilla luciferase activity.

Statistical analysis

Data are presented as mean ± S.E. Statistical analysis was performed using Student’s t-test or ANOVA when appropriate to analyse statistically significant differences between groups. For this study P < 0.05 was considered significant.

Results

AT-II and IGF-1 signals cross-talk to up-regulate their reciprocal receptors

In our previous study, we showed that AT-II and IGF-1 increased protein expression of Cx43 in human SV SMCs in a dose- and time-dependent manner via AT-1R and IGF-1R, which may result in the increase in gap junction intercellular communication in VSMCs [11]. We further observed the effect of AT-II and IGF-1 on the Cx43 expression in SV SMCs in a cross-talk manner. Both IGF-1R and AT-1R were strongly expressed in either IGF-1 or AT-II stimulation. The presence of AT-II and IGF-1 together further increased the IGF-1R and AT-1R expression (Fig. 1).
We investigated the possible cross-talk signal between IGF-1 and AT-II that is involved in up-regulation of their reciprocal receptors. Initially, SMCs were transfected with siRNA against AT-1R or IGF-1R. The expression of AT-1R and IGF-1R was determined by Western blot analysis. A marked reduction of AT-1R and IGF-1R protein expression was present 48 and 72 hrs following siRNA treatment (lanes 3 and 4 of Fig. 2A and B). Western blot was done to evaluate AT-1R and IGF-1R protein expression normalized with GAPDH in SV SMCs. IGF-1 increased the AT-1R expression (lane 3 of Fig. 2C), and AT-II up-regulated the expression of IGF-1R (lane 2 of Fig. 2D). This was inhibited by siRNA for IGF-1R (lane 5 of Fig. 2C) and siRNA for AT-1R (lane 4 of Fig. 2D). These data suggest cross-talk signalling between AT-II and IGF-1 in SV SMCs.

AT-II and IGF-1 signals cross-talk to up-regulate their reciprocal receptors via phosphorylation of Erk 1/2 in SMCs of saphenous vein

We further investigated the potential signalling pathways in AT-II and IGF-1-induced expression of their reciprocal receptors in hSV SMCs. Both AT-II and IGF-1 markedly induced the phosphorylation of extracellular signal-regulated kinases 1/2 (Erk 1/2) (lanes 2 and 3 of Fig. 3A). After transfection with siRNA for Erk, the protein expression of Erk was significantly inhibited at 48 and 72 hrs (lanes 3 and 4 of Fig. 3B). The SMCs of SV were incubated with AT-II or IGF-1 for 24 hrs in the absence or presence of ERK siRNA. IGF-1-induced AT-1R protein expression was also blocked by...
inhibitors of Erk 1/2 (Fig. 3C). Meanwhile, AT-II-induced IGF-1R protein expression was blocked by the siRNA for Erk 1/2 (Fig. 3D). These findings demonstrate that AT-II and IGF-1 signals cross-talk to up-regulate their reciprocal receptors via activation of Erk 1/2 in SMCs of SV.

AT-II and IGF-1-induced Cx43 expression via their reciprocal receptors and Erk 1/2 in VSMCs of saphenous vein

We investigated signalling pathways in AT-II and IGF-1-induced Cx43 expression in VSMCs isolated from SV. The VSMCs of SV were incubated with AT-II and IGF-1 for 24 hrs in the absence or presence of inhibitors of AT-1R and IGF-1R. AT-II and IGF-1-induced Cx43 expression in SV SMCs (lanes 4 and 6 of Fig. 4A). However, AT-II-induced Cx43 protein expression was partially blocked by siRNA against IGF-1R (lane 5 of Fig. 4A). Meanwhile, IGF-1-induced Cx43 protein expression was also partially blocked by siRNA for AT-1 receptor (lane 7 of Fig. 4A). These findings suggest that AT-1R and IGF-1R cross-talk in their signalling pathways to up-regulate Cx43 in AT-II- and IGF-1-stimulated SMCs.

We further examined the potential signalling pathways in AT-II and IGF-1-induced Cx43 expression in hSV SMCs. The SMCs of SV were incubated with AT-II or IGF-1 for 24 hrs in the absence or presence of Erk siRNA. Both of AT-II and IGF-1-induced Cx43 protein expression was blocked by siRNA against ERK 1/2, (lanes 4 and 6 of Fig. 4B). These findings demonstrate that ERK 1/2 mediates the expression of Cx43 induced by AT-II and IGF-1 in hSV SMCs.

Involvement of the activation of transcription factor AP-1 in the AT-II and IGF-1-induced Cx43 expression via their reciprocal receptors

The activator protein 1 (AP-1) and its promoter site are likely involved in the cytokine regulation of Cx43. We examined whether AT-II and IGF-1 induce activation of the transcription factor AP-1 through the IGF-1R, AT-1R and ERK1/2 pathways. VSMCs were co-transfected with pAP-1-Luc and 0.2 μg of Renilla luciferase reporter control vector in the absence or presence of siRNA against AT-1R, IGF-1R and Erk 1/2. We found that AT-II and IGF-1-induced activation of AP-1 were blocked by siRNA against IGF-1R, AT-1R and Erk ½ (lanes 6–8, 10–12 of Fig. 5A). These data suggest that AT-II and IGF-1 induce the activation of transcription factor AP-1 through their reciprocal receptors and Erk 1/2 signalling pathways in SV SMCs.

In order to confirm the involvement and activation of transcription factor AP-1 in AT-II and IGF-1-induced Cx43 expression, SMCs were co-transfected with pAP-1-Luc and A-Fos. In our previous data, we have confirmed that the A-Fos significantly blocked AT-II-induced activation of transcription factor AP-1 [11]. In this study, we further found that A-Fos markedly inhibited AT-II and IGF-1-induced Cx43 protein (lanes 4 and 6 of Fig. 5B). These results demonstrate that AT-II and IGF-1 induce the Cx43 expression via activation of transcription factor AP-1 through their reciprocal receptors in SV SMCs.
Discussion

Restenosis, as a result of intimal hyperplasia, is a severe limitation of various interventional procedures, including coronary artery bypass graft, in which SV and internal mammary artery (IMA) are commonly used as bypass conduits [14]. SMCs, under the influence of mitogens, proliferate and migrate towards the lumen, resulting in intimal hyperplasia and restenosis of the vessel, thus influencing the long-term patency of the graft [15]. We recently reported that increased Cx43 expression following stimulation of VSMCs with AT-II and IGF-1 contributed to more proliferation in the SMCs of SV than in the internal mammary artery [11, 13]. In the present research, we further studied the effect of AT-II and IGF-1 on the Cx43 expression via up-regulation of their reciprocal receptors and downstream activation of MAPKs and AP-1 in SMCs of human SV.

First, AT-II and IGF-1 contributed to more proliferation in the SMCs of SV than in the internal mammary artery [11, 13]. In the present research, we further studied the effect of AT-II and IGF-1 on the Cx43 expression via up-regulation of their reciprocal receptors and downstream activation of MAPKs and AP-1 in SMCs of human SV. First, AT-II and IGF-1 contributed to more proliferation in the SMCs of SV. Second, AT-II and IGF-1 induced the expression of AT-1R and IGF-1R via cross-talk signalling between AT-II and IGF-1 in SV SMCs. Secondly, AT-II and IGF-1 induced the activation of MAPKs, subsequently activating the transcription factor AP-1 in SV SMCs. Finally, AT-1R, IGF-1R, Erk 1/2 and AP-1 were involved in AT-II and IGF-1-induced Cx43 expression. These data demonstrate a cross-talk between IGF-1R and AT-1R in AT-II and IGF-1-induced Cx43 expression in SV SMCs involving phosphorylation of Erk 1/2 and downstream activation of AP-1 signalling pathway.

Fig. 3 Effect of siRNA for Erk1/2 on AT-II and IGF-1-induced expression of their reciprocal receptor in SMCs of SV. (A) VSMCs were stimulated with AT-II (10^{-7} mol/l) or IGF-1 (100 ng/ml) for 10 min. The cells were lysed and p-ERK was measured. (B) SMCs were transfected with siRNA against Erk 1/2 and non-silencing siRNA, and total protein expression of Erk 1/2 was determined by Western blot analysis after 48 and 72 hrs. (C) After transfection with Erk 1/2-siRNA, SMCs were treated with IGF-1 (100 ng/ml) for 24 hrs. (D) After transfection with Erk 1/2-siRNA, SMCs were treated with AT-II (10^{-7} mol/l) for 24 hrs. Proteins were quantified, and each well was loaded with 20 µg of protein. Data are mean ± S.E. of triplicate observations. * P < 0.01 compared with control group. # P < 0.01 compared with AT-II group or IGF-1 group.

Initiation and progression of the restenotic vessel involve complex patterns of interaction between the cells of the arterial wall, in which cytokines, chemokines and growth factors are known to play a critical role [16]. Two strong mitogens and chemotactants for SMCs are IGF-1 and AT-II, which elicit their effect via IGF-1R and AT-1R, respectively [16, 17]. Both IGF-1 and AT-II are abundantly present in restenotic human atherectomy specimens. We and others have reported the potent proliferative effect of IGF-1 and AT-II on SVSMCs [18, 19]. In this study, we found that both IGF-1 and AT-II up-regulated their reciprocal receptors. AT-1R activation plays a pivotal role in the pathogenesis of hypertension, atherosclerosis and heart failure. Stimulation of AT-1R by AT-II leads to increasing sympathetic activity, vasoconstriction, reabsorption of water and sodium, cellular growth and apoptosis, and to the accelerated progression of atherosclerosis [20, 21]. Inhibition of AT-1R activation by angiotensin converting enzyme inhibition or AT-1R antagonism resulting in blood pressure reduction in hypertensive patients decreases proliferation of SMCs after cardiac interventional surgery with stent and angioplasty, and attenuates the onset and progression of atherosclerosis [22]. Overexpression of AT-1R, for example by either oestrogen deficiency or hypercholesterolemia, enhances vasoconstriction, cell growth and production of reactive oxygen species [20]. In our study, IGF-1 causes AT-1R up-regulation via activation of p42/44 MAPK. Studies also showed that other growth factors, such as platelet derived growth factor and basic fibroblast growth factor, could affect the AT-1R expression through phosphorylation of tyrosine [23]. IGF-1R is a critical determinant of VSMC growth response [24]. Thus, a functional IGF-1R autocrine loop is required for the mitogenic effects of vari-
ous growth factors, such as platelet derived growth factor, epidermal growth factor and AT-II. Furthermore, a study has shown that AT-II, platelet derived growth factor and epidermal growth factor increased IGF-1 density on vascular cells. Inhibition of the effect by IGF-1R with oligonucleotides also inhibits the AT-II-induced cellular growth [25]. AT-II-induced up-regulation of IGF-1 mRNA and protein was mediated by the activation of a tyrosine kinase [26]. In our study, we showed that AT-II caused IGF-1R up-regulation through activation of p42/44 MAPK. However, their mechanisms of action display remarkable diversity with respect to AT-1R regulation. The further clarification of these intracellular signalling processes will help to understand the obviously distinct roles of growth factors in the setting of cardiovascular disease.

In our study, we found that Cx43 expression can be influenced by AT-II and IGF-1 through ERK 1/2 pathways, and may contribute to the pathogenesis of vein graft disease following coronary artery bypass grafting [11]. Many of the signalling events relevant to cell proliferation and differentiation are mediated through activation of transcription factors via MAPKs [27]. In our previous study, we have showed that activation of the ERK, p38 and JNK signalling pathways may participate in the regulation of cardiac gap junctions [11]. Gap junctions consist of aggregates of transmembrane hemichannels (or connexons) that link the cytoplasmic compartments of neighbouring cells, allowing a direct exchange of ions and second messengers [28]. Cx43 has been reported to be expressed in the vascular wall SMCs of the media, with Cx43 being predominant [29, 30]. Gap junctions have been described in cardiovascular disease [31]. Here, our results further confirm that AT-1R and IGF-1R cross-talk in their signalling pathways to up-regulate Cx43 via Erk 1/2 signal pathway in AT-II- and IGF-1-stimulated SMCs. However, the precise role of MAPKs in the regulation

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of Cx43 expression and activity in VSMCs is uncertain. The regulation of IGF-1 and AT-II-induced Cx43 expression and their signalling pathway in SV SMCs may provide an explanation or elucidate the cause for the selective failure of hSV bypass grafts due to restenosis following CABG.

The AP-1 is a transcription factor which is a heterodimeric protein composed of proteins belonging to the c-Fos, c-Jun, activating transcription factor (ATF) and Jun dimerization protein (JDP) families [32]. It regulates gene expression in response to a variety of stimuli, including cytokines, growth factors and stress. AP-1 in turn controls a number of cellular processes including differentiation, proliferation and apoptosis [33]. The AP-1 and its promoter site are likely involved in the cytokine regulation of Cx43 [34]. Activation of ERK1/2 induces overexpression of c-fos mRNA and enhanced AP-1 DNA-binding activity [35]. We have confirmed that AT-II mediates its effect through the ERK1/2, P38 and JNK pathways via the activation of transcription factor AP-1. These results further confirmed that AT-1R and IGF-1R cross-talk in their signalling pathways to up-regulate Cx43 via Erk 1/2 and downstream activation of AP-1 signal pathway in AT-II- and IGF-1-stimulated SMCs. These findings may explain, at least in part, the proliferative vasculopathy observed in vein graft disease.

In summary, our results support the notion that crosstalk between signalling of AT-II and IGF-1 leads to greater proliferation of SV SMCs in Erk 1/2-dependent activation of AP-1 involving an up-regulation of Cx43 expression. The data in this study advance our understanding of the underlying pathways in the pathogenesis of intimal hyperplasia and restenosis, and their progression in coronary artery bypass graft.

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Conflict of interest

The authors confirm that there are no conflicts of interest.

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