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Evidence for Prostacyclin and cAMP Upregulation by Bradykinin and Insulin-Like Growth Factor 1 in Vascular Smooth Muscle Cells

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Abstract

Although bradykinin (BK) and insulin like growth factor-1 (IGF-1) have been shown to modulate the functional and structural integrity of the arterial wall, the cellular mechanisms through which this regulation occurs is still undefined. The present study examined the role of second messenger molecules generated by BK and IGF-1 that could ultimately result in proliferative or anti-proliferative signals in vascular smooth muscle cells (VSMC).

Activation of BK or IGF-1 receptors stimulated the synthesis and release of prostacyclin (PGI₂) leading to increased production of cAMP in VSMC. Inhibition of p42/p44^{mapk} or src kinases prevented the increase in PGI₂ and cAMP observed in response to BK or IGF-1, indicating a role for these kinases in the regulation of cPLA₂ activity in the VSMC. Inhibition of PKC failed to alter production of PGI₂ in response to BK, but further increased both p42/p44^{mapk} activation and the synthesis of PGI₂ produced in response to IGF-1. In addition, both BK and IGF-1 significantly induced the expression of c-fos mRNA levels in VSMC, and this effect of BK was accentuated in the presence a cPLA₂ inhibitor. Finally, inhibition of cPLA₂ activity and/or cyclooxygenase activity enhanced the expression of collagen I mRNA levels in response to BK and IGF-1 stimulation.

These findings indicate that the effect of BK or IGF-1 to stimulate VSMC growth is an integrated response to the activation of multiple signaling pathways. Thus, the excessive cell growth that occurs in certain forms of vascular disease could reflect dysfunction in one or more of these pathways.

INTRODUCTION

Vascular smooth muscle cell (VSMC) proliferation and deposition of extracellular matrix are characteristic of progressive atherosclerotic lesions (1). Vascular injury leading to endothelial dysfunction is often a contributing factor (2, 3). Multiple growth factors and hormones have the potential to stimulate growth of VSMCs (4, 5) and may play a role in the

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evolution of atherosclerotic vascular disease. Alternatively, locally-generated signaling molecules such as nitric oxide and PGI₂ act to antagonize cell growth and matrix deposition (6–9). The balance of signaling by such opposing influences will determine the proliferative state of the VSMC under different physiological and pathophysiological conditions.

Mitogen activated protein kinases (MAPKs) represent a family of serine-threonine kinases that are rapidly activated in response to growth factor stimulation. In mammalian cells these include the extracellular regulated kinases 1 and 2 (ERK1 and ERK2 or p44^{MAPK} and p42^{MAPK}), the c-Jun NH₂-terminal kinase or JNK, and p38^{MAPK} (10). These kinases integrate multiple signal inputs and activated MAPKs are capable of phosphorylating a variety of diverse targets including effector kinases and transcription factors involved in the regulation of genes associated with cellular proliferation and hypertrophy (11). MAPK activation has been linked to neointimal proliferation in response to arterial injury (12). On the other hand, nitric oxide and PGI₂ are generally released by the vascular endothelium to stimulate production of cGMP and cAMP (6, 7, 13) by VSMCs and inhibit ornithine decarboxylase activity (8), actions that serve to attenuate injury-or growth factor-induced cellular proliferation.

All components of the kallikrein/kinin system have been localized within the vascular wall. Kallikrein is expressed in isolated arteries and veins and by VSMCs (14, 15). Kininogen, the substrate for kinin generation by kallikrein activity, kininase and B₂ kinin receptors are also present in the VSMC (16). The physiological action of kinins is to relax the arterial blood vessel through synthesis and release of nitric oxide from the vascular endothelium (17). However, in vascular injury where endothelial integrity is lost, kinins can act to constrict the VSMC and promote cellular proliferation (18). Similarly, VSMCs express and secrete IGF-1 (19). IGF-1 is a weak mitogen for VSMCs (20) and enhances the effects of other growth factors (21, 22). Moreover, IGF-1 and IGF-1 receptor mRNA are increased in injury-induced proliferation of VSMCs (23) and overexpression of IGF-1 leads to hyperplasia of smooth muscle cells in mouse aorta (24). Thus, locally generated kinins and IGF-1 may act in an autocrine fashion to influence vascular function.

In the present study, we tested the hypothesis that BK and IGF-1 activate both proliferative and anti-proliferative pathways in VSMC and that the mitogenic effect of these molecules is a complex integrated response. The effects of BK and IGF-1 on the activation of early response signaling pathways in primary cultures of rat aortic smooth muscle cells. Each molecule stimulated MAPK activity in VSMCs through similar, though not identical, second messenger mechanisms. In addition, both BK and IGF-1 acted on the VSMC to increase PGI₂ production, leading to elevated cAMP levels and consequent attenuation of BK-induced c-fos expression as well as BK and IGF-1-induced collagen I expression. The results indicate that the proliferative response of VSMCs to BK or IGF-1 stimulation reflects the integration of multiple signaling processes.

METHODS

Cell Culture

Rat aortic VSMC from male Sprague-Dawley rats (Charles-River, Laboratories, Wilmington, MA) were prepared by a modification of the method of Majack et al (25). A 2-cm segment of artery cleaned of fat and adventitia was incubated in 1 mg/ml collagenase for 3h at room temperature. The artery was then cut into small sections and fixed to a culture flask for explantation in minimal essential media (MEM) containing 10% fetal calf serum (FCS), 1% non-essential amino acids, 100 mU/ml penicillin and 100 µg/ml Streptomycin. Cells were incubated at 37°C in a humidified atmosphere of 95% air –5% CO₂. Medium was changed every 3–4 days and cells were passaged every 6–8 days by harvesting with trypsin-EDTA. VSMC were identified by the following criteria. They stained positive for intracellular cytoskeletal fibrils of actin and smooth muscle cell specific myosin and negative for factor VIII antigens. VSMC isolated by this procedure were homogeneous and were used in all studies between passages 2–6.

MAPK Assay

Quiescent VSMC stimulated with BK (10⁻⁸ M) and/or IGF-1 (10⁻⁸ M) for 5 min. were suspended in 250 µl of lysis buffer (20 mM Tris, 130 mM NaCl, 10% glycerol, 10 mM Chaps, 1 mM PMSF, 2 mM Na Vanadate, 100 mU/ml aprotinin, 0.15 mg/ml benzamidine, pH 8.0), sonicated for 10 sec and centrifuged at 13,000g for 10 min. 25–30 µg of cytosolic fraction was analyzed by SDS-polyacrylamide gel electrophoresis and the separated proteins were transferred to PVDF membranes and immunoblotted with anti-phospho-MAPK polyclonal antibody that detects p42 and p44 MAPK only when activated by phosphorylation at Thr202 and Tyr204 (1:1000 dilution, Cell Signaling, Beverly, MA). Immunoreactive bands were visualized using the ECL reagent (Amersham Corp.) according to the procedure described by the supplier.

Determination of cAMP

Serum starved VSMC sub-cultured into 24-well culture dishes were stimulated with BK (10⁻⁸ M) and/or IGF-1 (10⁻⁸ M) for 10 min at 37°C. After the stimulation period the media was removed and the cells were incubated at 4°C for 10 min in 0.1 N HCl to extract intracellular cAMP. The acid extract was then analyzed for cAMP by radioimmunoassay, sensitivity of 10 pg (26).

Determination of Prostacyclin

VSMC cultured in 6-well plate dishes were serum deprived for 24 h followed by stimulation with either BK (10⁻⁸ M) or IGF-1 (10⁻⁸ M) for 10 min at 37°C. The release of 6-keto-PGF_{1α} the stable metabolite of PGI₂ into the media was measured by RIA (sensitivity 25pg) as performed previously (27). Aliquots of 50 µl and 100 µl were added to the RIA tube and then antibody and [³H] 6-keto-PGF_{1α} were added (final volume 6-keto-PGF_{1α}, 450 µl). After incubation for 24 h, the free [³H] 6-keto-PGF_{1α} was separated from the bound [³H] 6-keto-PGF_{1α} using 1ml of charcoal-dextran solution and the bound [³H] 6-keto-PGF_{1α} was counted in liquid scintillation counter.

RNA extraction and Northern Blotting and Real-Time PCR

Quiescent VSMC grown in 15-cm plates were stimulated with BK (10^{-8} M) for 30 min. Total RNA from the cells was extracted by the Tri reagent Method. RNA yield was determined spectrophotometrically (Ultraspec III, Pharmacia) by absorbance at 260 nm.

Total RNA (20 μ g) obtained as described above was denatured at 65°C for 15 min, and ran on a 1.5 % agarose gel. The gel was stained with ethidium bromide to determine the position in each lane of the 28S and 18S ribosomal RNA and to demonstrate, that similar amounts of intact RNA was used for each sample. Total RNA was transferred from the gel to Nytran membrane filters by a Possiblot Pressure Transducer (Stratagene, La Jolla, Ca) and hybridized at 60°C for 18–24 h with a c-fos cDNA probe labeled with 32 P by random priming. The hybridized membranes were washed and exposed to film. Autoradiographs (Kodak XAR-5 film, Eastern Kodak, Rochester, New York) of the membranes were obtained scanned and the intensities of the bands were quantified by a microdensitometer.

Total RNA (2 μ g) was converted to cDNA using MLV Reverse Transcriptase (Promega, Madison, WI) according to the manufacturer's protocol at 37°C for 1 hr. To determine the validity of primers and appropriate T_m for Real Time PCR, the primers were first amplified in a PCR reaction to ensure that only one band is amplified. The following primers were designed so that all of the PCR products are within 75–150 bp (Integrated DNA Technologies Inc). Collagen I: 5'-CAC ACA TCC TGT GTC TCT TCC CAT-3'; 5'-GAT CAA GCA TAC CTC GGG TTT CCA-3'. s-actin:5'-ACT GCC GCA TCC TCT TCC TC-3';5'-CCG CTC GTT GCC AAT AGT GA-3'.

For each target gene, a standard curve was established. This was achieved by performing a series of 3-fold dilutions of the gene of interest. Negative control was made using the same volume of Rnase-free water instead of sample. The master mix was prepared as follows: 2 $\times$ SYBR Green Supermix (cat.No. 170-8880, BIO-RAD) 12.5 μ l, forward and reverse primer 0.25 μ l respectively and ddH₂O 12 μ l. For each well, 22 μ l of master mix was loaded first, followed by 3 μ l of sample, and mixed well to get total reaction volume of 25 μ l. For plate setup, SYBR-490 was chosen as fluorophore. The plate was covered with a sheet of optical sealing film. PCR conditions were 95°C for 3 min, followed by 40 cycles of 95°C for 10 sec, 58°C for 1 min (for β -actin and Collagen I), then 95°C for 1 min, 55°C for 1 min and 100 cycles of 55°C for 10 sec. All of the reactions were done in duplicate. The correlation coefficient is between 0.99–1; PCR efficiency is between 80–120%. The mRNA levels were expressed relative to β -actin. Realtime PCR using iCycleTM iQ optical system software (version 3.0a).

Protein Determination

The concentration of protein was determined by the method of Lowry (28) with bovine serum albumin as the standard.

Statistical analysis

Data are expressed as mean $\pm$ SE and were analyzed by analysis of variance ANOVA, and by Student's t-test for two-tailed unpaired analysis. Differences were considered significant if $P < 0.05$.

RESULTS

Regulation of MAPK activation and prostacyclin (PGI₂) synthesis by BK and IGF-1 in VSMC

To examine the effects of either BK or IGF-1 on MAPK activation and PGI₂ synthesis in VSMC, cells were serum-starved for 24 hours and then stimulated with BK or IGF-1. MAPK activation was measured as an increased phosphorylation of ERK1 (p44^{mapk}) and ERK2 (p42^{mapk}). Treatment of quiescent VSMCs for 10 min with BK or IGF-1 produced approximately a two-fold increase in phosphorylation of both p44^{mapk} and p42^{mapk} compared to that observed in unstimulated control cells (Figure 1, $P < 0.05$, $n = 8$ experiments). To examine effects on PGI₂ synthesis, release into the incubation medium of the stable metabolite of PGI₂, 6-keto-PGF_{1 α} , was measured by RIA. In figure 2, serum-deprived VSMC cultured in 6-well plates were treated with various concentrations of BK (10^{-10} – 10^{-6} M) for 10 min. BK stimulated the release of 6-keto-PGF_{1 α} into the culture media in a concentration-dependent manner with a peak response at 10^{-8} M ($P < 0.05$, $n = 8$ experiments). Similar results were obtained with IGF-1.

To evaluate the time response through which BK or IGF-1 maximally stimulates the release of 6-keto-PGF_{1 α} , VSMC were treated with BK (10^{-8} M) and/or IGF-1 (10^{-8} M) for various times (0–30 min) as shown in figure 3 ($n = 6$ experiments). The results demonstrate that both BK and IGF-1 increased the synthesis of 6-keto-PGF_{1 α} in VSMC within 5 min of addition with a peak effect achieved by 10 min. Basal release of 6-keto-PGF_{1 α} did not change throughout the experimental study period. The composite of these findings demonstrate that both BK and IGF-1 promote MAPK activation and stimulate the synthesis of PGI₂ in VSMCs.

Role of the MAPK pathway in the regulation of 6-keto-PGF_{1 α} and cAMP synthesis by BK and IGF-1

To define the signaling mechanism through which BK and IGF-1 stimulates 6-keto-PGF_{1 α} synthesis, the following experiments were carried out to assess the role of the MAPK pathway on this process. It is well established that the ERK family of MAPKs (p42^{mapk} and p44^{mapk}) is rapidly activated in response to growth stimuli and appears to integrate multiple intracellular signals transmitted by various second messengers. To explore the role of MAPK activation on PGI₂ and cAMP production by BK and IGF-1 in VSMC, cells were pretreated for 30 min with PD 98059 (20 μ M, NEN-Biolabs, Beverly, MA), a specific cell permeable inhibitor of the MAPK activator MEK followed by BK (10^{-8} M) or IGF-1 (10^{-8} M) stimulation for 10 min. BK or IGF-1 produced a 2–3 fold increase in ERK phosphorylation compared to unstimulated cells (BK or IGF-1 vs. control, $P < 0.05$, Figure 4A, $n = 8$ experiments). The MEK inhibitor PD98059 decreased basal ERK activity and reduced the level of ERK phosphorylation observed in response to either BK or IGF-1 (BK vs. BK+PD; IGF-1 vs. IGF-1+PD, $P < 0.05$, Figure 4A).

With regard to 6-keto-PGF_{1α} production, both BK and/or IGF-1 resulted in a 5–8 fold increase in the release of 6-keto-PGF_{1α} compared to unstimulated control VSMC (BK, IGF-1 vs. control, $P < 0.05$, Figure 4B, $n = 6$ experiments). Inhibition of ERK phosphorylation by the MEK inhibitor resulted in a significant decrease in the synthesis of 6-keto-PGF_{1α} in response to either BK or IGF-1 (BK vs. BK+PD; IGF-1 vs. IGF-1+PD, $P < 0.05$, Figure 4B). This suggests that treatment with the MEK inhibitor reduced ERK activity to a level below that necessary to promote PGI₂ formation. However, it is also possible that not all of the effects of BK or IGF-1 on prostacyclin production are mediated by MAPK alone. It should be noted that the MEK inhibitor had no significant effect on the basal release of 6-keto-PGF_{1α} by VSMC.

Since activation of PGI₂ receptors by PGI₂ results in adenylate cyclase activation, which in turn leads to an increase in cAMP synthesis, we examined the effects of PD 98059 on cAMP production in response to either BK or IGF-1. Figure 4C demonstrates that cAMP production is increased 2-fold in VSMC treated with BK and/or IGF-1 compared to untreated cells (BK, IGF-1 vs. control, $P < 0.05$, $n = 7$ experiments). This increase in cAMP production by BK or IGF-1 was completely inhibited by the MEK inhibitor PD 98059 (BK vs. BK+PD; IGF-1 vs. IGF-1+PD, $P < 0.05$, Figure 4C). Inhibition of MEK activity had no significant effect on basal cAMP production but completely inhibited the increase in cAMP levels by BK or IGF-1 (BK vs. BK+PD; IGF-1 vs. IGF-1+PD, $P < 0.05$, Figure 4C). This is consistent with the effect of PD 98059 to significantly blunt BK and IGF-1 stimulation of PGI₂ production (Figure 4B).

In the next series of experiments the role of Src family of tyrosine kinases on the regulation of ERK activity, 6-keto-PGF_{1α} and cAMP synthesis was explored. Quiescent VSMC were treated with BK (10^{-8} M) or IGF-1 (10^{-8} M) for 10 min. Both BK and IGF-1 resulted in a marked increase in phosphorylation of p44^{mapk} and p42^{mapk} in VSMC compared to unstimulated control cells (Figure 5A, $P < 0.05$, $n = 8$ experiments). To demonstrate a role for Src kinases in ERK activation by BK and IGF-1, VSMC were pretreated for 40 min with a specific cell permeable inhibitor of Src family tyrosine kinases PP1 (10 μM, Biomol Research Laboratories Inc., Plymouth, PA) followed by stimulation with BK (10^{-8} M) or IGF-1 (10^{-8} M) for 10 min. Treatment of cells with PP1 lowered basal MAPK activity. However in the presence of PP1 inhibitor, the increase in MAPK phosphorylation achieved in response to BK and IGF-1 was partially reduced (BK vs. BK+PP1; IGF-1 vs. IGF-1+PP1, $P < 0.05$, figure 5A).

Figure 5B, shows the effects of BK and IGF-1 on 6-keto-PGF_{1α} production in the presence and absence of Src kinase inhibitor PP1. Both BK and IGF-1 resulted in a significant increase in the synthesis of 6-keto-PGF_{1α} compared to control cells (BK and IGF-1 vs. Control, $P < 0.05$, $n = 6$ experiments). This increase in synthesis of 6-keto-PGF_{1α} elicited by BK and IGF-1 was significantly decreased by pretreatment of cells with the Src kinase inhibitor PP1 (BK vs. BK+PP1; IGF-1 vs. IGF-1+PP1, $P < 0.05$, Figure 5B), but was higher than cells treated with PP1 alone. Since complete inhibition of PGI₂ synthesis in response to BK and IGF-1 stimulation in the presence of PP1 inhibitor was not achieved, it would suggest that other mediators besides src kinases are utilized by BK and IGF-1 to promote synthesis of PGI₂.

To determine whether the decrease in 6-keto-PGF_{1α} stimulation following blockade of src kinase would translate into decreased production of cAMP, experiments were conducted to measure the production of cAMP in VSMC treated with either BK (10⁻⁸ M) or IGF-1 (10⁻⁸ M) for 10 min in the presence and absence of the Src kinase inhibitor PP1. The results presented in figure 5C show that BK and IGF-1 produced a 2-fold increase in cAMP production compared to controls (BK, IGF-1 vs. Control, P<0.05, n=6 experiments). However, this increase in cAMP induced by BK and/or IGF-1 was eliminated when VSMC were pretreated with PP1 (BK vs. BK+PP1; IGF-1 vs. IGF-1+PP1, P<0.05, Figure 5C). Inhibition of Src kinases had no significant effect on basal cAMP levels. These studies demonstrate for the first time the requirement for Src kinase activity to stimulate synthesis of PGI₂ and cAMP in response to BK and IGF-1 in VSMC. Taken together, the results demonstrate that BK and IGF-1 share common signaling mechanisms to increase the synthesis of PGI₂ and cAMP in the VSMC and provide strong support for a role for ERK activation in regulating this process.

Role of PKC in BK and/or IGF-1-induced MAPK phosphorylation and 6-keto-PGF_{1α}

In VSMC activation of phospholipase C by BK leads to production of two second messengers, inositol phosphates and diacylglycerol that induce the release of intracellular calcium and PKC activation (29). We have previously shown that BK leads to PKC activation in VSMC and that PKC is upstream of the MAPK pathway (30). Therefore, in the present study we sought to determine whether inhibition of PKC activity would alter BK-induced production of PGI₂ in VSMC. VSMC were pretreated with a PKC inhibitor, bisindolylmaleimide (GFX 109203, 2 μM) for 30 min, followed with BK (10⁻⁸ M) stimulation for 10 min. Treatment of VSMC with BK produced a significant increase in 6-keto-PGF_{1α} production compared to unstimulated control cells (25.46±7.16 vs. 4.53±2.02 pg/μg protein, BK vs. Control, respectively, P<0.002, n=6). Pretreatment with the PKC inhibitor GFX did not significantly alter the production of 6-keto-PGF_{1α} in response to BK stimulation (19.22±5.09 vs. 25.46±7.16 pg/μg protein, BK+GFX vs. BK, respectively, P=0.24, n=6). Another study was carried out to assess the effects of down-regulation of PKC by 24 h of pretreatment with phorbol ester [5μM phorbol 12-myristate 13-acetate (PMA)] on 6-keto-PGF_{1α} production in response to BK-stimulation. Once again, BK produced a significant increase in 6-keto-PGF_{1α} production compared to control cells (6.78±0.46 vs. 0.22±0.05, BK vs. Control, respectively, P<0.01). However, down-regulation of PKC activity by PMA did not significantly alter the response of BK to stimulate 6-keto-PGF_{1α} synthesis (7.46±1.97 vs. 6.78±0.46, BK+PMA vs. BK, respectively, P=0.42, n=6). Overall, these studies demonstrate that BK stimulates 6-keto-PGF_{1α} synthesis via a PKC-independent pathway.

To address whether PKC is essential for IGF-1 to regulate MAPK activity and hence PGI₂ synthesis, VSMC were treated with IGF-1 (10⁻⁸ M) for 10 min in the presence of a PKC inhibitor, GFX (2 μM). The results shown in Figure 6A demonstrate once again that IGF-1 stimulation results in ERK activation in VSMC (IGF-1 vs. Control, P<0.05, n=5 experiments). Treatment with the PKC inhibitor GFX did not significantly alter basal MAPK phosphorylation, but further increased ERK phosphorylation in response to IGF-1 (IGF-1 vs. IGF-1+GFX, P<0.05, n=5 experiments).

We next examined the effects of PKC inhibition on the production of 6-keto-PGF_{1α} by IGF-1. The results presented in Figure 6B show that IGF-1 produced a significant increase in 6-keto-PGF_{1α} production compared to unstimulated VSMC (IGF-1 vs. Control, $P<0.05$). The PKC inhibitor did not alter the basal production of 6-keto-PGF_{1α}. However, the production of 6-keto-PGF_{1α} was further increased by IGF-1 when PKC activity was inhibited by GFX (IGF-1 vs. IGF-1+GFX, $P<0.05$, $n=8$ experiments, Figure 6B).

Effect of cPLA₂ inhibitor on BK-induced and IGF-1 induced c-fos mRNA expression

Activation of MAPK pathway by either BK or IGF-1 provides a link in the signal transduction pathway from the cytosol to the nucleus. The nuclear targets for MAPK include the activation of transcription factors such as c-fos and the non-nuclear targets include the activation of cPLA₂ (31, 32). Once activated, cPLA₂ rapidly catalyzes the release of arachidonic acid from the sn-2 position of phospholipids in the plasma membrane leading to synthesis of prostaglandins. We sought to determine whether inhibition of cPLA₂ would alter c-fos mRNA expression in response to either BK or IGF-1. VSMC were pretreated with AACOCF₃ (20 μM), a cPLA₂ inhibitor, followed by BK (10⁻⁸ M) or IGF-1 (10⁻⁸ M) stimulation for 30 min. The findings demonstrate that BK and IGF-1 treatment produced a 7.5-fold and 6-fold increase in c-fos mRNA/GAPDH mRNA levels compared to untreated cells (0.92 ± 0.17 [$n=7$] or 0.75 ± 0.34 [$n=4$] vs. 0.12 ± 0.05 [$n=8$] c-fos mRNA/GAPDH mRNA; BK or IGF-1 vs. Control, $P<0.001$, respectively, Figure 7). However, in the presence of the cPLA₂ inhibitor, AACOCF₃, BK produced a 12-fold increase in the induction of c-fos mRNA levels above control, and a 1.5-fold increase above BK alone (1.42 ± 0.26 [$n=4$] vs. 0.12 ± 0.05 [$n=8$] or 0.92 ± 0.17 [$n=7$] c-fos mRNA/GAPDH mRNA; BK +AACOCF₃ vs. control or BK, $P<0.01$, respectively). With respect to IGF-1, a 7.3-fold increase in c-fos mRNA induction was observed above control, and a 1.2-fold increase was observed above IGF-1 alone (0.88 ± 0.18 [$n=5$] vs. 0.12 ± 0.05 [$n=8$] or 0.75 ± 0.34 [$n=4$] c-fos mRNA/GAPDH mRNA; IGF-1+AACOCF₃ vs. control, $P<0.01$, respectively). Treatment of VSMC with AACOCF₃ alone produced a 5.5-fold increase in c-fos mRNA levels compared to control cells (0.66 ± 0.17 vs. 0.12 ± 0.05 , AACOCF₃ vs. control, $p<0.01$). GAPDH mRNA level, measured in the same samples was not altered by either BK or IGF-1 treatment.

Effect of cPLA₂ and cyclooxygenase inhibitors on BK-induced and/or IGF-1 induced collagen I mRNA expression

To further explore the link between cPLA₂, BK and IGF-1 on aspects of cellular function, studies were designed to assess whether inhibition of cPLA₂ will modulate collagen I mRNA expression in response to either BK or IGF-1 stimulation. VSMC were pretreated with AACOCF₃ (20 μM), a cPLA₂ inhibitor, followed by BK (10⁻⁸ M) or IGF-1 (10⁻⁸ M) stimulation for 6h. Collagen I mRNA levels were measured by real-time PCR and expressed relative to β-actin mRNA levels. However, when VSMC were pretreated with AACOCF₃, AACOCF₃ alone caused a significant increase in collagen I mRNA levels compared to control, as has been previously shown using other inhibitors of PLA₂ activity (13). AACOCF₃ pretreatment resulted in a 303 ± 72 % increase in collagen I mRNA/β-actin mRNA above unstimulated control cells ($p<0.003$, $n=5$). This finding confounded our ability to decipher the role of cPLA₂ in modulating the expression of collagen I in response to BK or IGF-1 stimulation.

Consequently, in a subsequent study we sought to determine whether blockade of PGI₂ generation by inhibition of cyclooxygenase activity, will alter the expression of collagen I mRNA in response to either BK or IGF-1. VSMC were pretreated with indomethacin (20 μ M), a cyclooxygenase inhibitor, followed by BK (10⁻⁸ M) or IGF-1 (10⁻⁸ M) stimulation for 6h. Collagen I mRNA levels were measured by real-time PCR and expressed relative to β -actin mRNA levels. Neither, BK or IGF-1 increased collagen I mRNA levels significantly when used alone. However, both significantly stimulated collagen I expression in cells in which PGI₂ generation was inhibited by pretreatment with indomethacin (figure 8). Indomethacin alone did not significantly influence collagen I mRNA levels.

DISCUSSION

Although BK and IGF-1 have been shown to stimulate proliferation of a number of cells including VSMC (7, 20, 33, 34) and mesangial cells (35), the cellular mechanisms through which this regulation occurs is still undefined. BK mediates its effects via activation of the B₂-kinin receptor, which is a G-protein-coupled receptor (36) that lacks an intrinsic tyrosine kinase, whereas IGF-1 mediates its effects via activation of a receptor which is a tyrosine kinase receptor (37). The results of the present study demonstrate that activation of BK or IGF-1 receptors in VSMCs generate second messenger signals that can either promote or inhibit growth of the VSMC. Both BK and IGF-1 promoted activation of MAPK, which stimulates the expression of nuclear transcription factors implicated in VSMC growth (11), and each molecule also elicited the synthesis and release of PGI₂ which elevates cAMP to inhibit proliferation of the VSMC (7, 9).

The mechanisms involved in MAPK activation are complex and vary with the receptor stimulated and cell type. On binding to the B₂ kinin receptor in VSMCs, BK activates phospholipase C via the heterotrimeric GTP binding protein, Gq. Activation of phospholipase C stimulates the production of inositol 1, 4, 5 triphosphate to elevate cytosolic free Ca²⁺ and generates diacylglycerol, an activator of PKC (29). These second messengers then initiate a complex network of intracellular signaling leading to activation of the extracellular regulated MAPKs, p42^{mapk} and p44^{mapk} (30). In comparison, the IGF-1 receptor is a tyrosine kinase receptor and interaction of the receptor with IGF-1 activates the intracellular kinase domain. This initiates a series of phosphorylation events wherein Shc associates with the adaptor protein Grb2 which promotes the interaction of the guanine nucleotide-releasing protein Sos with Ras (37, 38). Ras activates Raf-1 which phosphorylates MAPK kinase (MEK) leading to the activation of MAPK (39). In the present study pretreatment of VSMCs with PD 98059, an inhibitor of MEK, decreased BK- and IGF-1-induced phosphorylation of p42^{mapk} and p44^{mapk}, indicating a similarity of downstream signaling by the two mitogens. Further, we previously demonstrated a role for the Src family of cytoplasmic tyrosine kinases in BK stimulation of MAPK activity. To determine if Src kinases might similarly contribute to IGF-activation of p42^{mapk} and p44^{mapk} in the VSMC, cells were treated with IGF-1 or BK in the presence of the Src kinase inhibitor, PP1. Inhibition of Src kinases dampened MAPK phosphorylation in response to IGF-1 stimulation. This result suggests that while BK and IGF-1 act through distinctly different types of cellular receptors, the signaling pathways initiated by each molecule

become common very early in the cascade of events required for stimulation of MAPK activity in the VSMC.

It is recognized that eicosanoids can modulate the actions of BK and IGF-1 on renal function and vascular tone. However, the cellular mechanisms through which BK and IGF-1 may stimulate eicosanoid production in VSMCs have not been fully addressed. Our data show that both BK and IGF-1 act on the VSMC to produce a concentration- and time-dependent increase in the synthesis and release of PGI₂, which is the major cyclooxygenase product synthesized by arterial smooth muscle cells. Increased PGI₂ synthesis, in turn, elevated VSMC cAMP levels. A similar action of BK to stimulate PGE₂ formation in VSMCs was recently demonstrated in cells cultured from human pulmonary artery (40). PGE₂ and the PGI₂ analogue, iloprost, also promoted cAMP formation in these cells. Interestingly, prolonged exposure of pulmonary cells to high concentrations of BK led to decreased PGI₂-stimulated cAMP production. These findings suggest that excessive and continuous stimulation of prostaglandin formation by VSMCs may desensitize the mechanisms for cAMP regulation.

The cellular mechanisms through which BK and IGF-1 may stimulate eicosanoid production in VSMCs have not been fully addressed. In the present study, stimulation of PGI₂ synthesis and cAMP elevation was preceded by a significant increase in p42^{mapk} and p44^{mapk} phosphorylation, and, consequently, experiments were performed to determine if the MAPK signaling pathway might have a role in BK or IGF-1-induced eicosanoid production. Pretreatment of cells with PP1 to inhibit Src kinases, which we have previously shown to be upstream of MAPK, dampened p42^{mapk} and p44^{mapk} phosphorylation observed in response to BK or IGF-1 and decreased BK-or IGF-1-induced PGI₂ production to a level below that required to increase cAMP in the VSMC. Moreover, blockade of MAPK phosphorylation by the MEK inhibitor, PD 98058, abolished the increase in PGI₂ and cAMP produced by either BK or IGF-1. Taken together, these findings indicate that members of the Src kinase family and p42^{mapk} and p44^{mapk} may have a key role in the regulation of eicosanoid synthesis by BK and IGF-1 in VSMCs.

An interesting difference between BK and IGF-1 actions was observed in VSMCs that were pretreated with GFX, an inhibitor of PKC activation. Inhibition of PKC did not significantly alter BK stimulation of PGI₂ synthesis, whereas, inhibition of PKC enhanced IGF-1–stimulated p42^{mapk} and p44^{mapk} phosphorylation and also increased IGF-1 stimulation of PGI₂ production. This observation provides further support for a direct link between MAPK activation and PGI₂ synthesis in VSMCs and suggests that PKC may be a negative modulator of these responses when initiated through stimulation of the IGF-1 receptor.

Activation of MAPK by BK or IGF-1 provides a link in the transduction of signals from the cytosol to nucleus. The nuclear targets for MAPK include transcription factors such as c-fos which is commonly expressed in response to mitogenic stimuli. Eicosanoids such as PGI₂, by stimulating the production of cAMP, have opposing effects and the potential to antagonize mitogen-induced cell proliferation. In the present study, treatment of VSMCs with BK or IGF-1 increased p42^{mapk} and p44^{mapk} phosphorylation and produced a 6-to7-fold induction of c-fos mRNA expression. These effects were accompanied by a

concomitant increase in the synthesis and release of PGI₂. When cells were pretreated with AACOCF₃, an inhibitor of PLA₂, to eliminate the production of PGI₂, the level of induction of c-fos mRNA was enhanced significantly for BK. A similar trend was observed for IGF-1 but the effect was small and not statistically significant. Such findings suggest that stimulation of PGI₂ synthesis in the VSMC serves to counter the proliferative actions of BK and, perhaps, IGF-1 as well. Consistent with this idea is the finding that inhibition of PGI₂ synthesis with the cyclooxygenase inhibitor, indomethacin, enhanced the ability of both BK and IGF-1 to stimulate collagen I expression in the VSMC. This result provides additional support for the concept that PGI₂ plays a significant role in modulating the proliferative effects of BK and IGF-1 in the arterial vasculature.

In summary, BK and IGF-1 generate second messengers that stimulate early mitogenic and anti-mitogenic signals in VSMCs. While each molecule activates a different type of cellular receptor, similar pathways are utilized for p42^{mapk} and p44^{mapk} activation and for stimulation of PGI₂ synthesis. Activation of Src-family of tyrosine kinases appears to be an early event in the signaling cascade wherein the mechanisms of BK and IGF-1 actions become common. BK or IGF-1 induced phosphorylation of p42^{mapk} and p44^{mapk} increases expression of the transcription factor, c-fos, and is also key, to the activation of cPLA₂ and increased production of PGI₂ by the VSMC. Once formed, PGI₂ elevates intracellular cAMP which then serves to modulate the influence of BK and IGF-1 on c-fos expression, as well as the expression of collagen I. These findings indicate that the effect of BK or IGF-1 to stimulate proliferation of VSMCs is an integrated response to the activation of multiple signaling pathways. The excessive cell growth that occurs in certain forms of vascular disease could reflect dysfunction in one or more of these pathways.

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Figure 1. Activation of MAPK by BK and/or IGF-1

VSMC were stimulated with either BK (10⁻⁸ M) and/or IGF-1 (10⁻⁸ M) for 10 min. Cell proteins were separated with SDS-PAGE. MAPK phosphorylation (p42^{mapk} and p44^{mapk}) were measured by immunoblot using anti-phosphotyrosine-MAPK antibodies (p-MAPK) and total MAPK was measured in the same immunoblot by stripping the membrane and re-immunoblotting with anti-total MAPK antibodies (t-MAPK). Data are expressed as mean $\pm$ SE and the bar graphs are representative of 6 separate experiments. * P<0.05 vs. control.

Figure 2. Concentration-dependent effects of BK on PGI₂ (6-keto-PGF_{1α}) production in VSMC Serum starved VSMC were stimulated with BK (10⁻¹⁰–10⁻⁶ M) for 10 min., and the release of 6-keto-PGF_{1α} into the media was measured by RIA. BK produced a concentration dependent increase in 6-keto-PGF_{1α} with a peak response at 10⁻⁸M. The figure is representative of 8 separate experiments. *P<0.05 vs. basal.

Figure 3. Time-response effects of BK and IGF-1 on PGI₂ (6-keto-PGF_{1α}) release in VSMC
 VSMC were stimulated with either BK (10⁻⁸M) or IGF-1 (10⁻⁸ M) for various times (0–30 min). Both BK and IGF-1 each produced a significant increase in 6-keto-PGF_{1α} production compared to control values. The peak response was observed 10 min. post stimulation. Figure is representative of 6 separate experiments. *P<0.05 vs. control.

Figure 4. Role of MAPK kinase on MAPK activation (A), the synthesis of PGI₂ (6-keto-PGF_{1 α} , B) and cAMP (C) by BK and IGF-1

VSMC were stimulated with either BK (10⁻⁸M) or IGF-1 (10⁻⁸M) for 10 min in the presence and absence of MEK inhibitor PD98059 (20 μ M). MAPK phosphorylation (p42^{mapk} and p44^{mapk}) were measured by immunoblot using anti-phosphotyrosine-MAPK antibodies (p-MAPK) and total MAPK was measured in the same immunoblot by stripping the membrane and re-immunoblotting with anti-total MAPK antibodies (t-MAPK). Release of 6-keto-PGF_{1 α} and cAMP into the media, were measured by RIA. Data are expressed as mean $\pm$ SE and the bar graphs are representative of 6 separate experiments. * P<0.05 vs. control, †P<0.05 vs. BK, #P<0.05 vs. IGF-1.

Figure 5. Role of Src-kinases on MAPK activation (A), the synthesis of PGI₂ (6-keto-PGF_{1α}, B) and cAMP (C) by BK and IGF-1

VSMC were stimulated with either BK (10⁻⁸M) or IGF-1 (10⁻⁸M) for 10 min in the presence and absence of Src kinase inhibitor PP1 (10 μM). MAPK phosphorylation (p42^{mapk} and p44^{mapk}) were measured by immunoblot using anti-phosphotyrosine-MAPK antibodies (p-MAPK) and total MAPK was measured in the same immunoblot by stripping the membrane and re-immunoblotting with anti-total MAPK antibodies (t-MAPK). Release of 6-keto-PGF_{1α} and cAMP into the media, were measured by RIA. Data are expressed as mean±SE and the bar graphs are representative of 6–8 separate experiments. * P<0.05 vs. control, †P<0.05 vs. BK, #P<0.05 vs. IGF-1.

Figure 6. Role of PKC in IGF-1 induced MAPK phosphorylation and PGI₂ (6-keto-PGF_{1 α}) production

VSMC were stimulated with either BK IGF-1 (10^{-8} M) for 10 min in the presence and absence of PKC inhibitor bisindolylmaleimide (GFX, 2μ M). MAPK phosphorylation (p42^{mapk} and p44^{mapk}) were measured by immunoblot using anti-phosphotyrosine-MAPK antibodies (p-MAPK) and total MAPK was measured in the same immunoblot by stripping the membrane and re-immunoblotting with anti-total MAPK antibodies (t-MAPK). Release of 6-keto-PGF_{1 α} into the media, was measured by RIA. Data are expressed as mean $\pm$ SE and the bar graphs are representative of 5 separate experiments. * $P < 0.05$ vs. control, † $P < 0.05$ vs. IGF-1.

Figure 7. Role of cPLA₂ on IGF-1 and BK-induced c-fos mRNA expression

VSMC were pretreated with AACOCF₃ (20μM), a cPLA₂ inhibitor, followed by BK (10⁻⁸M) or IGF-1 (10⁻⁸M) stimulation for 30 min. c-fos and GAPDH mRNA levels were measured by Northern blot analysis. The bar graph represents the relative intensities of c-fos mRNA levels/GAPDH mRNA levels. Blot shown is representative of at least 5–7 experiments. *P<0.001 vs. control; †P<0.01 vs. BK.

Figure 8. Effect of cyclooxygenase inhibitors on BK-induced and/or IGF-1 induced collagen I mRNA expression

VSMC were pretreated with Indomethacin (Indo 50μM), a cyclooxygenase inhibitor, followed by BK (10^{-8} M) or IGF-1 (10^{-8} M) stimulation for 6h. Collagen I and β-actin mRNA levels were measured by real-time PCR. The bar graph represents the relative intensities of collagen I mRNA levels/ β-actin mRNA levels. Blot shown is representative of 3 experiments. *P<0.04 vs. control; †P<0.02 vs. IGF-1.

Figure 9. Scheme depicting BK receptor and IGF-1 receptor signaling through MAPK-dependent pathways

Activation of src-family of tyrosine kinases result in the activation of MAPK which in turn stimulates the activity of cPLA₂. Once activated, cPLA₂ in turn will activate cyclooxygenases (COX) resulting in the production of inhibitory prostaglandins such as PGI₂. PGI₂ in turn will lead to elevated cAMP levels, and consequent attenuation of the responses of BK and IGF-1 to stimulate matrix proteins.