Mechanisms by which bradykinin promotes fibrosis in vascular smooth muscle cells: role of TGF-β and MAPK

CHRISTELLE D. DOUILLET,1 VICTORIA VELARDE,1 JULIE T. CHRISTOPHER,1 RONALD K. MAYFIELD,1,3 MARIA E. TROJANOWSKA,1 AND AYAD A. JAFFA1,2

Departments of 1Medicine and 2Cell and Molecular Pharmacology and Experimental Therapeutics, Medical University of South Carolina, and 3Ralph H. Johnson Veteran Affairs Medical Center, Charleston, South Carolina 29425

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Douillet, Christelle D., Victoria Velarde, Julie T. Christopher, Ronald K. Mayfield, Maria E. Trojanowska, and Ayad A. Jaffa. Mechanisms by which bradykinin promotes fibrosis in vascular smooth muscle cells: role of TGF-β and MAPK. Am J Physiol Heart Circ Physiol 279: H2829–H2837, 2000.—Accumulation of extracellular matrix (ECM) is a hallmark feature of vascular disease. We have previously shown that hyperglycemia induces the expression of B2-kinin receptors in vascular smooth muscle cells (VSMC) and that bradykinin (BK) and hyperglycemia synergize to stimulate ECM production. The present study examined the cellular mechanisms through which BK contributes to VSMC fibrosis. VSMC treated with BK (10\(^{-8}\) M) for 24 h significantly increased \(\alpha_2(I)\) collagen mRNA levels. In addition, BK produced a two- to threefold increase in \(\alpha_2(I)\) collagen promoter activity in VSMC transfected with a plasmid containing the \(\alpha_2(I)\) collagen promoter. Furthermore, treatment of VSMC with BK for 24 h produced a two- to threefold increase in the secretion rate of tissue inhibitor of metalloproteinase 1 (TIMP-1). The increase in \(\alpha_2(I)\) collagen mRNA levels and \(\alpha_2(I)\) collagen promoter activity, as well as TIMP-1 secretion, in response to BK were blocked by anti-TGF-β neutralizing antibodies. BK (10\(^{-8}\) M) increased the endogenous production of TGF-β1 mRNA and protein levels. Inhibition of the mitogen-activated protein kinase (MAPK) pathway by PD-98059 inhibited the increase of \(\alpha_2(I)\) collagen promoter activity, TIMP-1 production, and TGF-β1 protein levels observed in response to BK. These findings provide the first evidence that BK induces collagen type I and TIMP-1 production via autocrine activation of TGF-β1 and implicate MAPK pathway as a key player in VSMC fibrosis in response of BK.

Collagen; tissue inhibitor of metalloproteinase 1; transforming growth factor-β; mitogen-activated protein kinase

VASCULAR SMOOTH MUSCLE CELL (VSMC) expansion and the resultant increased extracellular matrix (ECM) deposition play pivotal roles in the progression of atherosclerosis (2). Increases in ECM components such as collagens types I and IV are found in plaque areas of aortas from type I diabetic patients (32). These changes in matrix composition may impair vascular function by changing VSMC from their contractile state to a synthetic state (5). Although the precise mechanisms underlying these changes are undefined, the interaction between arterial smooth muscle cells and the surrounding matrix suggests a dynamic system in which changes in composition and spatial organization of matrix itself may profoundly change the metabolic and proliferative activity of VSMC (34, 42). Collagen formation is the major contributor to the growth of atherosclerotic lesions (2). Of the various types of collagens, type I is the primary component of atherosclerotic plaque and is synthesized by arterial smooth muscle cells in response to growth factors (2). Thus matrix deposition is a characteristic part of the sclerotic process that contributes to vascular function impairment by disrupting normal cell-cell interaction and modifying tissue elasticity.

Among the factors that play a central role in vascular fibrosis is transforming growth factor-β (TGF-β). TGF-β has been shown to stimulate collagen expression in VSMC and also to influence the expression of some matrix metalloproteinases (MMP) and their tissue inhibitors (TIMPs). Since matrix deposition is the result of a balance between synthesis and degradation of ECM components, it is conceivable that the delicate balance between MMP and TIMP, as well as the rate of ECM synthesis, determine the final matrix accumulation.

The localization of components of the kallikrein-kinin system within the vascular wall suggests this system has a role in the regulation of vascular tone, hypertension, and atherogenesis (22, 24). Kallikrein and its mRNA are expressed in isolated arteries and veins and in cultured VSMC (29, 33). Kinogen, the substrate for kinin generation by kallikrein, kininase activity, and B2-kinin receptors are present in VSMC (30). Thus locally generated kinins can act in an autocrine or paracrine fashion to influence vascular function. The physiological actions of bradykinin (BK) are mediated via generation of second messengers such as nitric oxide and eicosanoids (17, 36). BK relaxes VSMC...
through synthesis and release of nitric oxide from the endothelium, but in states of vascular injury in which endothelial integrity is compromised, BK can act directly on VSMC, to increase intracellular calcium and cause contraction (4).

The signaling pathways leading to VSMC fibrosis are just beginning to be studied and seem to be derived from multiple sources. Our recent data demonstrate that BK stimulates early mitogenic signals in VSMC. Through activation of its B2 receptors, BK stimulates activation and nuclear translocation of p42\textsuperscript{mapk} and p44\textsuperscript{mapk} and induces expression of c-fos and c-jun mRNA levels and formation of the AP-1 complex (11, 44). The cellular signals through which BK stimulates p42\textsuperscript{mapk} and p44\textsuperscript{mapk} activation and c-fos mRNA expression in VSMC involves activation of a calcium/calmodulin pathway, src kinase, protein kinase C, and generation of reactive oxygen species (14, 28, 44).

Therefore, the present studies were designed to explore the role of BK in mediating VSMC fibrosis. We found that BK induces \(\alpha_2\) chain of type I (\(\alpha_2(I)\)) collagen mRNA levels, \(\alpha_1(I)\) collagen promoter activity, and TIMP-1 production via autocrine activation of TGF-\(\beta\). In addition, our findings demonstrate that BK signals via the mitogen-activated protein kinase (MAPK) pathway to mediate its effects on \(\alpha_1(I)\) collagen and TIMP-1 production. These findings provide the first evidence for a potential role for BK in VSMC fibrosis.

**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 and Clowes (21). A 2-cm segment of thoracic aorta cleaned of fat and adventitia was incubated in 1 mg/ml collagenase for 3 h at room temperature. The aorta was then cut into small sections and placed into a culture flask for explantation in culture media that were activated by HCl (to measure both active and latent TGF-\(\beta\)) according to the manufacturer instructions, and expressed in picograms per milliliter.

**Transfection of VSMC with the \(\alpha_1(I)\) collagen promoter.** VSMC were transfected by the calcium chloride method with a plasmid containing the \(\alpha_1(I)\) collagen promoter attached to the chloramphenicol acetyl transferase (CAT) gene (~353 COL1A2 CAT); the plasmid was obtained from Promega (Madison, WI), prepared as described by Tamaki et al. (41), purified by double cesium chloride purification, and stored at 4°C. The plasmid (20 \(\mu\)g of plasmid DNA) in a 2 M Ca\(\textsubscript{Cl}^2\) solution was mixed with an equal volume of 2X DNA precipitation buffer (3 Prime →5 Prime Inc., Boulder, CO) and incubated for 30 min before addition to the cells. After 16 h of incubation, the culture medium was changed to 1% FBS in MEM. Two hours later, the cells were stimulated for 24 h as indicated. The cells were then washed in PBS and resuspended in 0.25 M Tris, pH 8.0. The CAT extract was prepared from the cells by four cycles of freezing-thawing and centrifugation at 13,800 g for 15 min. Protein concentrations were determined in each cell extract by Bio-Rad protein assay and resolved on SDS-PAGE. The separated proteins in the gel were transferred to Immobilon-P membrane (Millipore, Bedford, MA) and immunoblotted with goat polyclonal anti-TIMP-1 protein level determination. VSMC were cultured in six-well plates (9.6 cm\(^2\)/well). At 70% confluence, cells were serum starved by the changing of the serum-free media four times within 24 h. Cells were then stimulated for 48 h with 10\(^{-5}\) M BK, in presence or absence of 40 \(\mu\)M of PD-98059, in exactly 1.5 ml. TGF-\(\beta\) protein levels were determined by colorimetric enzyme-linked immunosorbent assay kit (ELISA; R&D Systems, Minneapolis, MN) in the conditioned media that were activated by HCl (to measure both active and latent TGF-\(\beta\)) according to the manufacturer instructions, and expressed in picograms per milliliter.

**Western blotting of TIMP-1.** VSMC in 60-mm dishes were rendered quiescent by growing them in serum-free media for 24 h. Cells were then stimulated as indicated. The conditioned media were collected after 24 h and concentrated by 10.220.33.6 on October 30, 2017 http://ajpheart.physiology.org/ Downloaded from http://alphamorphophysiology.org by 10.220.33.6 on October 30, 2017
subjected to SDS-PAGE. The separated proteins in the gel were transferred to polyvinylidene difluoride membranes, and immunoblotted with rabbit polyclonal phospho-specific MAPK antibodies that specifically recognize tyrosine phosphorylated p42\textsuperscript{mapk} and p44\textsuperscript{mapk} (New England Biolabs, Beverly, MA). The phospho-MAPK antibody was used at 1:6,000 dilution, whereas the control antibody, which recognizes total MAPK, was used at 1:4,000 dilution. The membranes were incubated overnight with the antibody buffer (TBS, 0.05% Tween 20, 1% BSA), washed in TBS-0.05% Tween 20, and exposed to goat anti-rabbit horseradish peroxidase-conjugated IgG (1:5,000) in antibody buffer for 1 h. Immuno-reactive bands were visualized by a chemiluminescent method (Renaissance, New England Biolabs) using Kodak X-LS film.

Statistical analysis. Data are expressed as means ± SE and were analyzed by ANOVA and by using student’s t-test for unpaired analysis. Differences were considered significant if \(P < 0.05\).

RESULTS

Induction of \(\alpha_2(1)\) collagen expression by BK. To investigate whether BK induces ECM formation, we measured \(\alpha_2(1)\) collagen mRNA expression in VSMC treated with BK (10\textsuperscript{-8} M) for 24 h. As shown in Fig. 1A, BK produced a significant increase in \(\alpha_2(1)\) collagen mRNA levels, expressed relative to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. The \(\alpha_2(1)\) collagen mRNA levels were increased to 160% of control VSMC (BK vs. control, \(P < 0.003, n = 3\)).

We next assessed whether BK can induce transcriptional activation of \(\alpha_2(1)\) collagen gene by measuring its effect on the activity of \(\alpha_2(1)\) collagen promoter. VSMC transfected with a plasmid containing the \(\alpha_2(1)\) collagen promoter were stimulated for 24 h with either BK (10\textsuperscript{-8} M) or TGF-\(\beta\)1 (1 ng/ml) as positive control. The CAT activity (used as a reporter for the activity of the collagen promoter) was determined in the cell lysate.

The results (Fig. 1B) demonstrate for the first time that BK can directly increase the activity of the collagen promoter. BK produced a 2.5-fold increase in \(\alpha_2(1)\) collagen promoter activity, whereas TGF-\(\beta\)1 produced a fourfold increase (control vs. BK or TGF-\(\beta\)1, \(P < 0.005, n = 8\) experiments).

Induction of TGF-\(\beta\)1 expression by BK. To define the cellular mechanisms through which BK increased collagen expression, the ability of BK to stimulate the endogenous production of TGF-\(\beta\) was examined. We measured the TGF-\(\beta\)1 mRNA levels in VSMC treated with BK (10\textsuperscript{-8} M) for various times. BK treatment resulted in a significant increase in TGF-\(\beta\)1 mRNA levels/GAPDH mRNA levels above control. This increase in TGF-\(\beta\)1 was observed as early as 4 h, maintained at 6 h, and returned to normal values by 24 h (\(P < 0.02, \)BK vs. 0 h, \(n = 4\) experiments, Fig. 2).

Role of TGF-\(\beta\) in BK-induced \(\alpha_2(1)\) collagen expression. To further define the role of TGF-\(\beta\) in BK signaling, we examined whether the increase in \(\alpha_2(1)\) collagen mRNA levels or promoter activity in response to BK are mediated via autocrine activation of TGF-\(\beta\). VSMC were treated with BK (10\textsuperscript{-8} M) for 24 h in the presence and absence of neutralizing anti-TGF-\(\beta\) antibodies (20 \(\mu\)g/ml, R&D Systems). This concentration of anti-TGF-\(\beta\) antibody has been shown to neutralize the effects of TGF-\(\beta\) on mesangial cell proliferation and matrix formation (39, 45). The results shown in Fig. 3A indicate that the ability of BK to induce \(\alpha_2(1)\) collagen mRNA levels is blocked by the anti-TGF-\(\beta\) neutralizing antibody (\(P < 0.05, \)BK vs. BK + neutralizing antibody, \(n = 3\)). We also examined the effects of TGF-\(\beta\) neutralizing antibody on VSMC transfected with the \(\alpha_2(1)\) collagen promoter and stimulated with BK (10\textsuperscript{-8} M) for 24 h. Although we have observed differences in the magnitude of response to BK between different cell lines, once again, BK produced a significant increase in CAT activity compared with unstimulated control cells (BK vs. control, \(P < 0.01, n = 5, \)Fig. 3B). This increase in CAT activity in
response to BK stimulation was completely blocked in the presence of anti-TGF-β neutralizing antibody. Anti-TGF-β neutralizing antibody had no significant effect on basal CAT activity (Fig. 3B).

**BK increases TIMP-1 secretion via a TGF-β-dependent mechanism.** TIMPs are proteins that regulate the activity of MMPs and play an integral part in the turnover rate of ECM under physiological and pathological conditions (13). Thus it is conceivable that VSMC matrix accumulation is the net result of signals that promote increased TIMP activity. To address this possibility, we examined the effects of BK on the secretion rate of TIMP-1 in VSMC and explored the role of TGF-β in mediating the effects of BK on TIMP-1 production. VSMC were stimulated for 24 h with either BK (10^{-8} M) or TGF-β1 (1 ng/ml), as positive control, in the presence and absence of anti-TGF-β neutralizing antibody (20 μg/ml). The conditioned medium was collected after 24 h, and equal amounts of proteins were resolved on SDS-PAGE and immunoblotted with a polyclonal antibody against rat TIMP-1. Top: intensities of TIMP-1 bands expressed as percentage of control. Bottom: representative blot of 5 separate experiments. *P < 0.05 vs. control. †P < 0.02 vs. BK. ‡P < 0.05 vs. TGF-β.
Fig. 5. Phosphorylation of p42mapk and p44mapk by BK in VSMC. VSMC were stimulated with BK (10^{-8} M) for 5 min in the presence and absence of MEK inhibitor PD-98059 (40 μM). Mitogen-activated protein kinase (MAPK) phosphorylations (p42mapk and p44mapk) 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 immunoblotting with anti-total MAPK antibodies (T-MAPK). Bottom: blots are representative of 6 separate experiments. Top: intensities of both p42mapk and p44mapk bands measured in a densitometer relative to total MAPK and expressed as percent phosphorylation relative to control. *P < 0.01 vs. control. †P < 0.05 vs. BK.

antibody, whereas the antibody alone had no effect on basal TIMP-1 production (Fig. 4).

Activation of p42mapk and p44mapk by BK. MAPKs belong to the group of serine-threonine kinases that are rapidly activated in response to growth factors and appear to integrate multiple intracellular signals transmitted by various second messengers. To understand the signal transduction events mediated by BK, we examined its effects on MAPK activation. Treatment of VSMC with 10^{-8} M BK for 5 min resulted in a rapid increase in tyrosine phosphorylation of p42mapk and p44mapk compared with unstimulated cells (Fig. 5). However, in the presence of the MAPK kinase inhibitor PD-98059 (40 μM), the increase in MAPK phosphorylation in response to BK was reduced. This finding is consistent with our previous observations demonstrating that BK activates MAPK in a concentration and time-dependent manner (28, 44).

Induction of TGF-β1 by BK is mediated via a MAPK-dependent pathway. To determine whether the increase in TGF-β1 mRNA in response to BK translates into an increase in TGF-β1 protein levels, TGF-β1 protein levels were measured in the conditioned media of VSMC stimulated with BK (10^{-8} M). As shown in Fig. 6, BK treatment resulted in a significant increase in TGF-β1 levels, compared with unstimulated control cells (84.5 ± 6.1 vs. 30.8 ± 2.9 pg/ml, BK vs. control, respectively, P < 0.0001, n = 5). To evaluate whether activation of MAPK modulates the BK-induced increase in TGF-β1 protein levels we observed, VSMC were treated with BK (10^{-8} M) in the presence of the MAPK inhibitor, PD-98059. The results shown in Fig. 6 demonstrate that inhibition of the MAPK pathway by PD-98059 completely eliminates the response of BK to stimulate TGF-β1 protein levels (84.5 ± 6.1 vs. 37.7 ± 2.7 pg/ml, BK vs. BK+PD-98059, P < 0.0001, n = 5).

Role of MAPK in BK-induced αs(I) collagen and TIMP-1 expression. To examine whether activation of p42mapk and p44mapk modulates the increase in αs(I) collagen promoter activity in response to BK, VSMC transfected with the αs(I) collagen promoter were pretreated with the MAPK kinase inhibitor PD-98059 (40 μM) for 30 min, followed by BK (10^{-8} M) stimulation for 24 h. In the absence of PD-98059, BK produced a significant increase in CAT activity compared with unstimulated control cells (BK vs. control, P < 0.001, n = 5, Fig. 7A). This increase in CAT activity in response to BK stimulation was reduced significantly in the presence of MAPK kinase inhibitor (P < 0.04, BK vs. BK+PD-98059, Fig. 7A). The MAPK kinase inhibitor had no significant effect on basal CAT activity (Fig. 7A).

To examine whether p42mapk and p44mapk also modulate the increase in TIMP-1 secretion in response to BK, VSMC were pretreated with the MAPK kinase inhibitor PD-98059 (40 μM) for 30 min, followed by BK (10^{-8} M) stimulation for 24 h. The release of TIMP-1 protein levels into the conditioned media was measured by Western blots using specific anti-TIMP-1 antibodies (1:2,000 dilution). BK produced a threefold increase in the secretion rate of TIMP-1 into the media compared with unstimulated cells (BK vs. C, P < 0.05, n = 4) (Fig. 7B). However, inhibition of MAPK activity significantly decreased TIMP-1 secretion in response to BK (BK vs. BK+PD-98059, P < 0.05, n = 4). The

Fig. 6. Induction of TGF-β1 protein secretion by BK in VSMC. Quiescent VSMC were stimulated with BK (10^{-8} M) for 48 h in the presence or absence of MEK inhibitor PD-98059 (40 μM). Conditioned media were collected and activated to determine the total level of TGF-β1 protein, using a colorimetric ELISA kit assay. Values are means ± SE of TGF-β1 protein levels (expressed in pg/ml) and are representative of 5 separate experiments. *P < 0.0001 vs. control. †P < 0.0001 vs. BK.
MAPK kinase inhibitor did not significantly alter basal secretion rate of TIMP-1.

**DISCUSSION**

The role of locally generated vasoactive factors in regulating vascular tone and structure under normal and pathological conditions has been increasingly emphasized. Among these factors, BK and angiotensin II are often considered to have opposite effects on vessel wall with regard to vascular reactivity and arterial compliance. Although the role of angiotensin II has been extensively studied with regard to vascular injury and remodeling, the precise role of BK on VSMC structure and the resultant fibrosis has not been well studied. In the present study we have demonstrated that BK induces the expression and transcriptional activation of $a_2(I)$ collagen gene in VSMC. In addition, we have also shown that BK stimulates the production and secretion rate of TIMP-1. The increase in $a_2(I)$ collagen mRNA levels, $a_2(I)$ collagen promoter activity, and TIMP-1 production in response to BK are mediated via autocrine activation of TGF-$\beta$. Moreover, the increase in $a_2(I)$ collagen promoter activity, TGF-$\beta_1$, and TIMP-1 production by BK involves activation of the MAPK pathway. These findings provide the first evidence that BK stimulates several key signaling pathways that participate in matrix dysregulation in VSMC.

Type I collagen represents up to two-thirds of total collagen, and total collagen constitutes about 60% of total atherosclerotic plaque protein composition (26, 37). Collagen type I is a heterotrimer consisting of two $a_1(I)$ chains and one $a_2(I)$ chain, which are expressed in a coordinated manner. The expression of type I collagen is strictly regulated through development and is tissue specific (31). In the present study, we have shown that BK stimulates the mRNA levels of $a_2(I)$ collagen in VSMC. To begin to understand the mechanism of this collagen regulation by BK, we examined the effects of BK on VSMC transfected with the $a_2(I)$ collagen promoter. Our results demonstrate that BK stimulates the $a_2(I)$ collagen promoter activity, indicating that the increase in $a_2(I)$ collagen mRNA levels in response to BK are mediated via transcriptional regulation. The $a_2(I)$ collagen promoter is regulated by transcription factors such as SP1, SP3, CBF, and AP-1 (7, 41). We have previously shown that BK can induce the expression of protooncogene c-fos and c-jun, and the formation of AP-1 complex transcription factor (15). The increase in c-fos mRNA levels and the formation of AP-1 complex in response to BK were mediated via activation of p42$\text{mapk}$ and p44$\text{mapk}$ pathway (11, 28). In this regard, our data demonstrate that activation of p42$\text{mapk}$ and p44$\text{mapk}$ plays a critical role in mediating the effects of BK on collagen regulation. Thus inhibition of the MAPK pathway significantly reduced the increase in $a_2(I)$ collagen promoter activity in response to BK. Therefore, one can speculate that activation of BK receptors in VSMC in response to BK results in activation of the MAPK pathway, which in turn results in the formation of the transcription factor AP-1, which leads to transcriptional activation of $a_2(I)$ collagen gene.

TGF-$\beta$ is the most potent and consistent activator of collagen synthesis in VSMC (9). It increases the level of steady-state mRNA of $a_2(I)$ and $\alpha_1(III)$ collagen in VSMC (1). In models of atherosclerosis such as rat balloon injury, TGF-$\beta$ was implicated as the factor responsible for the increase in collagen production. Transfection of TGF-$\beta$ gene into pig artery results in enhanced collagen type I production and accumulation (27). In the model of rat balloon injury, the injection of recombinant soluble TGF-$\beta$ type II receptor caused a marked reduction in mRNA levels for collagen type I and III, thus indicating the role of TGF-$\beta$ type II receptor in the fibrotic activity of TGF-$\beta$ (38). Similarly, in VSMC, a dominant-negative mutation of TGF-$\beta$ type II receptor abrogated the enhanced production of collagen type I induced by TGF-$\beta$ (46). However, the response of VSMC to TGF-$\beta$ can be different ac-
According to the cell origin (43). Hence, TGF-β inhibits the cell growth and increases slightly the collagen synthesis in normal VSMC, whereas it stimulates cell growth and markedly induces collagen synthesis in VSMC isolated from atherosclerotic lesions (23). In the present study we have shown that TGF-β plays a crucial role in mediating the effects of BK on matrix dysregulation. Our findings demonstrate that BK stimulates α2(I) collagen mRNA levels and α2(I) collagen promoter activity via autocrine activation of TGF-β. Support for such a notion comes from the findings that addition of anti-TGF-β neutralizing antibody reduces the mRNA levels and promoter activity of α2(I) collagen in VSMC in response to BK. In addition, our findings demonstrate that BK induced the expression of TGF-β1 mRNA levels in VSMC as early as 4 h and also resulted in a marked increase in TGF-β1 protein levels. These findings provide the first evidence that BK stimulates collagen type I production in VSMC via autocrine induction of TGF-β.

Matrix deposition can be viewed as the result of the balance between synthesis and degradation of ECM components. Matrix is degraded by MMPs, a family of neutral zinc proteases, which include the collagenases that degrade type I, III, and IV collagens, the gelatinases, and the stromelysins (10). The matrix proteases activity is blocked by a group of endogenous inhibitory proteins termed TIMP, which include TIMP-1, -2, -3, and -4 (13). Thus it is conceivable that the delicate balance between MMP and TIMP, as well as the rate of ECM synthesis, determines the final fate of matrix accumulation. TIMP-1 is a small glycoprotein synthesized by VSMC, which inhibits all the members of the MMP family and has been shown to participate in various physiological processes that involve tissue remodeling (13). The promoter of TIMP-1 contains multiple response elements in the 5’ region of the gene, including an AP-1-binding site (20). No studies have addressed the effect of BK on TIMP-1 regulation in VSMC. Our study indicates that BK stimulates the production and secretion of TIMP-1 in VSMC and provides an additional pathway through which BK can influence ECM regulation. Since TGF-β has been shown to stimulate the production of TIMP-1, we sought to determine whether BK stimulates TIMP-1 production via autocrine activation of TGF-β. Addition of neutralizing antibody to TGF-β completely eliminated the increase in TIMP-1 production in response to BK, thus implicating TGF-β as a principal stimulator of TIMP-1 by BK in VSMC.

Although the cellular mechanisms through which BK mediates TIMP-1 production in VSMC are not defined, our results suggest a role for the MAPK pathway. Pretreatment of VSMC with the cell-permeable inhibitor of MAPK kinase significantly reduced the increase in TIMP-1 production in response to BK challenge, thus suggesting a role for p42MAPK and p44MAPK in modulating the production of TIMP-1 by BK. Another recent study has shown that the induction of TIMP-1 gene expression in fibroblasts in response to retinoic acid is also mediated via activation of p42MAPK and p44MAPK (3).

The role of MAPK in vascular dysfunction is just beginning to be emphasized. The p42MAPK and p44MAPK members of the MAPK family belong to a group of serine/threonine kinases that are rapidly activated in response to growth factor stimulation. They integrate multiple signals from various second messengers leading to cellular proliferation and differentiation (8, 40). The activated MAPK can translocate to the nucleus, where it is thought to regulate the expression of transcription factors such as c-fos through the phosphorylation of the transcription factor p62TCF and the formation of AP-1 binding site (12). Recently, it has been shown that MAPK activity is transiently activated following vessel wall injury, whereas MAPK phosphorylation is decreased (18, 19). In a model of myocardial infarction, MAPK activity was increased, along with the mRNA levels of TGF-β and collagen types I and III (35). In addition, NIH 3T3 cells transfected with a dominant-negative mutant for MAPK kinase reduced the increase in collagen type I promoter activity in response to TGF-β (25). In the present study we have shown that BK mediates its fibrotic signals in VSMC via activation of the MAPK pathway. Inhibition of MAPK reduced the increase in α2(I) collagen promoter activity, TGF-β1 protein levels, and production of TIMP-1 in response to BK. Other studies also support a role for MAPK in modulating the effects of TGF-β on fibronectin production in VSMC (16).

In summary, our results suggest that BK initiate multiple signals that can result in fibrosis of VSMC. Our findings demonstrate that treatment of VSMC with BK results in an increase in α2(I) collagen mRNA levels and promoter activity and an increase in the production of TIMP-1. The increase in α2(I) collagen promoter activity and TIMP-1 production in response to BK are mediated via autocrine activation of TGF-β and via activation of the MAPK pathway. The significance of these findings to the in vivo actions of BK and the vascular wall changes associated with injury or atherosclerosis is unclear at the present time. Further understanding of the cellular and molecular mechanisms by which BK might modulate vascular fibrosis, a process obligatory to the development of atherosclerosis, could lead to the development of new strategies for intervention and treatment of vascular diseases.

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