

Role of reactive oxygen species in bradykinin-induced proliferation of vascular smooth muscle cells

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ABSTRACT

In addition to the induction of cell proliferation and migration, bradykinin (BK) can increase c-fos mRNA expression, activate ERK 1/2 and generate reactive oxygen species (ROS) in vascular smooth muscle cells (VSMC). It is not known, however, whether BK can induce cellular proliferation and extracellular matrix production via redox-sensitive signaling pathways. We investigated the role(s) of ROS in proliferation, migration and collagen synthesis induced by BK in VSMC derived from Sprague Dawley rat aorta. BK (10 nM) increased VSMC proliferation by 30 % (n=5); this proliferation was inhibited by the antioxidants N-acetylcysteine (20 mM) and α -lipoic acid (LA, 250 mM). In addition, BK induced an increase in cell migration and in collagen levels that were blocked by LA. ROS production induced by BK (n=10) was significantly inhibited by bisindolylmaleimide (4 μ M) and by PD98059 (40 μ M). These results suggest that: 1) ROS participate in the mechanism(s) used by bradykinin to induce cellular proliferation; 2) bradykinin induces ROS generation through a pathway that involves the kinases PKC and MEK; and 3) ROS participate in the pathways mediating cell migration and the production of collagen as a response to treatment with bradykinin. To our knowledge, this is the first report describing mechanisms to explain the participation of ROS in the cellular proliferation and extracellular matrix pathway regulated by BK.

Key terms: bradykinin, antioxidants, migration, signal transduction, reactive oxygen species

INTRODUCTION

Vascular smooth muscle cells (VSMC) in mature animals are highly specialized cells that have as one of their major functions the regulation of vascular tone via contraction (Owens, 1995). VSMC proliferation is normally quiescent in the adult, and mitogenic activity is rarely exhibited when cells contract during the hemodynamic regulation state. However, in disease where significant vascular injury occurs, such as diabetes and hypertension, VSMC growth is stimulated when the physiologic and regulatory balances between inhibitory and stimulatory signals are disrupted. These alterations in the signals for cell growth and matrix production can ultimately lead to

atherosclerosis and its subsequent progression. Elucidation of the early cellular events accompanying the deregulation of VSMC growth and differentiation during the initiation and subsequent course of atherogenesis is of great importance in understanding the etiology of this disorder (Ross, 1993). Dysfunction of and/or damage to the endothelial cells lining the vasculature accompanied by reductions in the production of nitric oxide are also postulated to have a major role in increased VSMC proliferation (Bauer et al., 2001).

Several important cellular signaling mediators have been implicated in the dedifferentiation of the VSMC and its change from a contractile to a proliferative

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phenotype. They include the vasoactive peptides (angiotensin II, endothelin-1, and bradykinin) and polypeptide growth factors (EGF, PDGF, IGF-1, FGF-2 and TGF- β) (Schwartz and Liaw, 1993). Recent evidence also suggests that many of these mediators can induce the production of reactive oxygen species (ROS). ROS production is significantly increased in injured vasculature and to a variety of experimental and clinical conditions, such as ischemia-reperfusion (Bertuglia and Giusti, 2003), thrombosis, diabetes (Giugliano et al., 1996), myocardial infarction (Ide et al., 2001) and angioplasty (Roller et al., 2001). In addition, in several types of models, experimental observations suggest that ROS are important in inducing cell proliferation, DNA synthesis and proto-oncogene mRNA expression (Baas and Berk, 1995). Reactive oxygen intermediates, such as superoxide anion ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and the hydroxyl radical ($HO\cdot$), are increasingly considered as a new family of second messenger molecules (Finkel, 1999).

The vasoactive peptide bradykinin (BK) is an important regulator of the normal physiologic function of the vasculature. Increasingly, however, pathophysiologic actions attributable to BK including phenotypical and structural changes of VSMC are also being recognized. It has been reported that BK induces VSMC proliferation (Yau et al., 2001), and we have previously observed that BK induces c-fos mRNA expression via pathways dependent on the generation of ROS and on ERK 1/2 activation, a step in the signaling pathway considered prerequisites for cellular proliferation (Velarde et al., 1999). In addition, BK induced ERK 1/2 activation and c-fos mRNA expression are inhibited by antioxidants (Greene et al., 2000).

Previous reports in studies involving HaCaT keratinocytes (Coutant et al., 1997) and lymphocytes (McFadden and Vickers, 1989) also indicate that BK induces migration in a dose-dependent manner, suggesting an important role for BK in this event. BK also induces VSMC to synthesize extracellular matrix proteins. We have observed that BK increases TGF- β mRNA and the mRNA for

the $\alpha 2$ -subunit of collagen (Douillet et al., 2000). Treatment of cells with BK also activates intracellular signaling mediators, such as NF- κ B (Xie et al., 2000) and AP-1 (El-Dahr et al., 1996, 1998). However, the upstream or downstream signaling intermediates attributable to BK that activate NF- κ B are currently not known. ERK 1/2 activation in response to BK stimulation occurs via a signaling cascade involving the B2 receptor, PKC, MEK and ultimately ERK 1/2 (Velarde et al., 1999). Although this pathway is mediated by ROS (Greene et al., 2000), the specific sequence of events resulting in the production of ROS and downstream effects affected by the pathway have not been previously identified. The purpose of the current set of studies is to investigate the specific role(s) of ROS in proliferation, migration and collagen synthesis induced by BK treatment in VSMC.

METHODS

VSMC culture

Aortic vascular smooth muscle cells obtained from 75-150 g male Sprague-Dawley rats (*Rattus norvegicus*) were prepared by a modification of the Majack method (Majack and Clowes, 1984). A two-cm segment of aorta, cleaned of fat and adventitia, was incubated in 1 mg/ml collagenase for 3 hours at room temperature. The aorta was then cut into small sections and fixed to a culture flask for explantation in Minimal Essential Medium (MEM) (Gibco-BRL, Gaithersburg, MD, USA) supplemented with 10% fetal Calf Serum (FCS), 100 units/ml of penicillin and 100 μ g/ml of streptomycin, and incubated at 37°C in a humidified atmosphere of 95% air and 5% CO_2 . Cells were identified as vascular smooth muscle cells by their characteristic morphology and contractile properties, by their positive staining with an antibody against α -actin, and their negative staining with an antibody against Von-Willebrand's factor. Cells were used between passages 2 and 6 in all experiments. Quiescence was induced by transferring the 60-70% confluent cell cultures to MEM devoid of

serum and growth factors for 48 hours prior to the treatments with various chemicals.

[³H]-Thymidine incorporation and DNA synthesis

Rate of DNA synthesis in cultured rat VSMC was estimated by the rate of incorporation of [³H]-thymidine into cellular DNA. Quiescent VSMC cultured in 6-well cell culture plates were treated with different agonists in serum-free medium for 24 hours and then pulsed labeled with 1μCi/ml of [³H]-thymidine (DuPont NEN) for another 16 hours. Cells were then washed 3 times with PBS, precipitated with perchloric acid, washed with PBS and solubilized with 0.5 ml of SDS 0.1 % /NaOH 0.1N. Radioactivity was determined using a liquid scintillation counter (Watson et al., 2001).

Cell migration assay

VSMC migration was assessed using a modified Boyden chamber (Corning, Inc.). VSMC suspended in MEM were added to the upper chamber, and tested samples were added to the bottom chamber. After 4 hours of incubation at 37°C, cells in the filter were fixed, the upper surface was scraped and the filter was stained with hematoxylin. The average number of cells from 4 randomly-chosen, high-power (400x) fields on the lower surface of the filter were counted (Miao et al., 2000).

Collagen determination

Quiescent VSMC in 6-well plates were treated with BK (10 nM) for 48 hours in the presence of ascorbic acid (50 μM). Twenty-four hours before the end of the incubation period, ³H-Proline was added to each well. Following the subsequent incubation, the medium was collected and separated into two fractions. One fraction was treated with collagenase (20 U) for 90 min at 37°C. Both fractions were concentrated using a speed-vac, and proteins were subsequently resolved on a 5 % polyacrylamide gel. Following electrophoresis, the gels were fixed in methanol/acetic acid for 60 min and incubated in increasing concentrations of

acetic acid. Finally, the gels were incubated in a 20 % 2,5-diphenyloxazole (PPO) solution in acetic acid for 2 hours, dried and exposed to an X-ray film for 48/72 hours. Bands were scanned, and densitometric analysis was performed using the NIH-Image program (Ichiki et al., 1997).

Determination of NF-κB activation

VSMC were harvested following treatment with BK for 30 min in the presence or absence of antioxidants. Nuclear extracts were prepared as described by Dignam et al. (1983). Briefly, cells were resuspended in 400 μl of a hypotonic buffer (10 mM HEPES pH 7.9; 1.5 mM MgCl₂; 10 mM KCl; 0.5 mM DTT; 0.5 mM PMSF; 0.5% Nonidet P-40) at 4°C. Extracts were then centrifuged at 15,000 g for 1 min (at 4°C), and the supernatants collected. The nuclear pellets were resuspended in hypertonic buffer (10 mM HEPES pH 7.9; 1.5 mM MgCl₂; 420 mM NaCl; 0.5 mM DTT; 0.2 mM EDTA; 0.5 mM PMSF; 25 % glycerol), centrifuged at 15,000 g for 10 min (at 4°C), and the supernatants collected.

Double-stranded oligonucleotides were radio-labeled with γ³²P-ATP and T4 kinase. Binding assays were performed with 5μg of protein extract, 1.5 μg of poly[d(I-C)], 5 μl of buffer [50 mM HEPES (pH 7.8), 5mM spermidine, 15 mM MgCl₂, 36 % glycerol, 3 mg/ml BSA, 0.3% Nonidet P-40, 15 mM DTT], and 40,000 to 70,000 cpm of ³²P-labeled oligo-nucleotide, with water added for a final volume of 25 μl. Reactions were incubated for 15 min on ice before addition of ³²P-labeled oligonucleotide, then for an additional 15 min at room temperature. For competition with unlabeled oligonucleotide, a 100-fold molar excess of oligonucleotide relative to the radio-labeled probe was added to the binding assay. Samples were electrophoresed on 4 % non-denaturing polyacrylamide gels, dried and autoradiographed (El-Dahr et al., 1996).

Measurement of intracellular ROS generation

VSMC were plated into 96-well plates and cultured as described above. When cells

reached confluency, they were loaded with 10 μM of the H_2O_2 -sensitive fluorescent probe 2',7'-dichlorofluorescein diacetate (DCF-DA; Molecular Probes) for 2 hours at 37°C. Once inside the cell, non-fluorescent DCF-DA is deacetylated enzymatically by cellular esterases to 2',7'-dichlorofluorescein, which remains trapped within the cell. DCF fluoresces in the presence of intracellular peroxides. After loading, VSMC were washed with Earle's buffer and pre-incubated with the antagonists or inhibitors for the appropriate times, followed by treatment with BK or H_2O_2 as positive control. Readings were taken in a fluorometer using an excitation wavelength of 485 and an emission wavelength of 530, immediately after the stimulation and every 5 minutes for the following 30 min (Royall and Ischiropoulos, 1993).

Statistical analysis

Data are expressed as mean \pm SEM and were analyzed by the non-parametric methods of Kruskal-Wallis for multiple comparisons and the Mann-Whitney U test for unpaired two sample analyses. Differences were considered significant at $p < 0.05$.

RESULTS

Participation of ROS in bradykinin-induced proliferation

It has previously been reported that BK induces VSMC proliferation (Yang et al., 2003). To determine whether cell proliferation was mediated by the generation of ROS, VSMC were pre-incubated with the antioxidants α -lipoic acid (LA, 250 μM) for 2 hours or N-acetylcysteine (NAC, 15 mM) for 45 minutes followed by a 24-hour incubation with BK (10 nM). As shown in figure 1, BK induced a significant increase in cell proliferation (133 \pm 10 %) (n=8, $p < 0.05$). Pre-incubation with either antioxidant alone decreased basal proliferation (48 \pm 5 % for LA and 61 \pm 20 % for NAC) (n=4, $p < 0.05$). Proliferation induced by BK in the presence of antioxidants was significantly inhibited compared to BK alone and was not significantly different from controls incubated with the antioxidants alone (60 \pm 5 % for BK+LA and 54 \pm 13 % for BK+NAC) (n=4), suggesting that ROS are involved in bradykinin's proliferative effect.

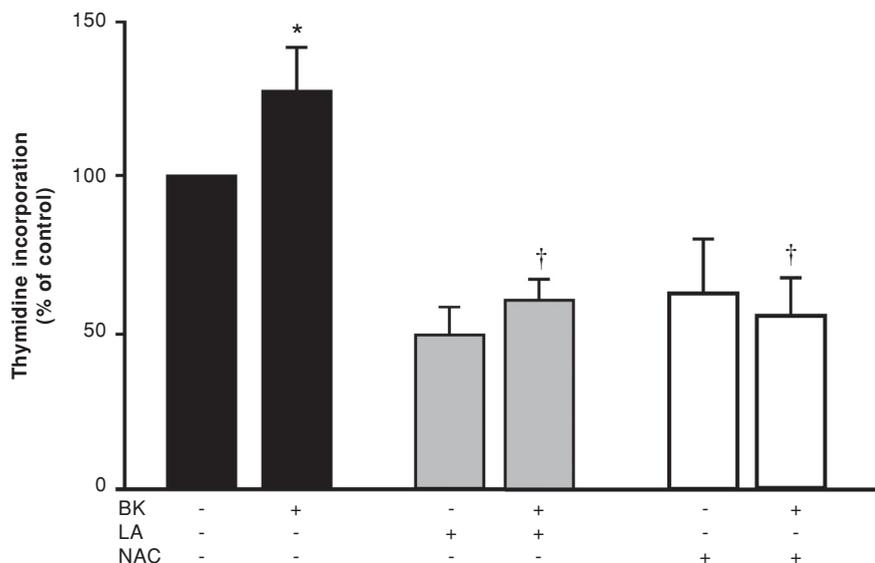


Figure 1. Basal and bradykinin-induced proliferation are inhibited by antioxidants. VSMC were pre-incubated with antioxidants Lipoic acid (LA, 250 μM) for 2 hours or with N-Acetyl-Cysteine (NAC, 15 mM) for 45 min followed by a 24-hour incubation with BK 10nM. Bars represent average \pm standard error for 4 experiments. * = $p < 0.05$ compared to control. † = $p < 0.05$ compared to BK.

Participation of ROS on the activation of NF- κ B by bradykinin

To determine whether ROS was mediating BK-induced NF- κ B activation in VSMC, we stimulated the cells for 30 min with increasing concentrations of BK (10^{-9} to 10^{-7} M) and observed a dose-dependent increase in NF- κ B signaling in the nucleus (Fig 2A). To determine whether NF- κ B activation is mediated by ROS production, cells were pre-incubated with LA for 2 hours or NAC for 45 min followed by treatment with BK (10^{-8} M) for 30 min. Incubation with LA alone induced an increase in NF- κ B activity, and the activation of NF- κ B induced by BK was not modified by LA (data not shown). Incubation with NAC alone did not modify the activation of NF- κ B when compared to control, but significantly blocked BK-induced NF- κ B activation (Fig 2B).

ROS production induced by bradykinin is dependent on the activation of PKC and MEK

To identify the potential position in VSMC BK signaling pathway(s) where ROS are generated, we used a pharmacological approximation using inhibitors of two kinases (PKC and MEK) known to be activated in response to BK. BK induces a significant increase in ROS production after 10 min ($159 \pm 19\%$, $n=5$, $p<0.05$). This increase is significantly inhibited by pre-incubation with bisindolylmaleimide (Bim, $4\mu\text{M}$), a broad-spectrum inhibitor of PKC, and by the MEK inhibitor PD98059 ($40\mu\text{M}$) (Fig. 3). Incubation of the cells with Bim or PD98059 alone did not modify ROS production.

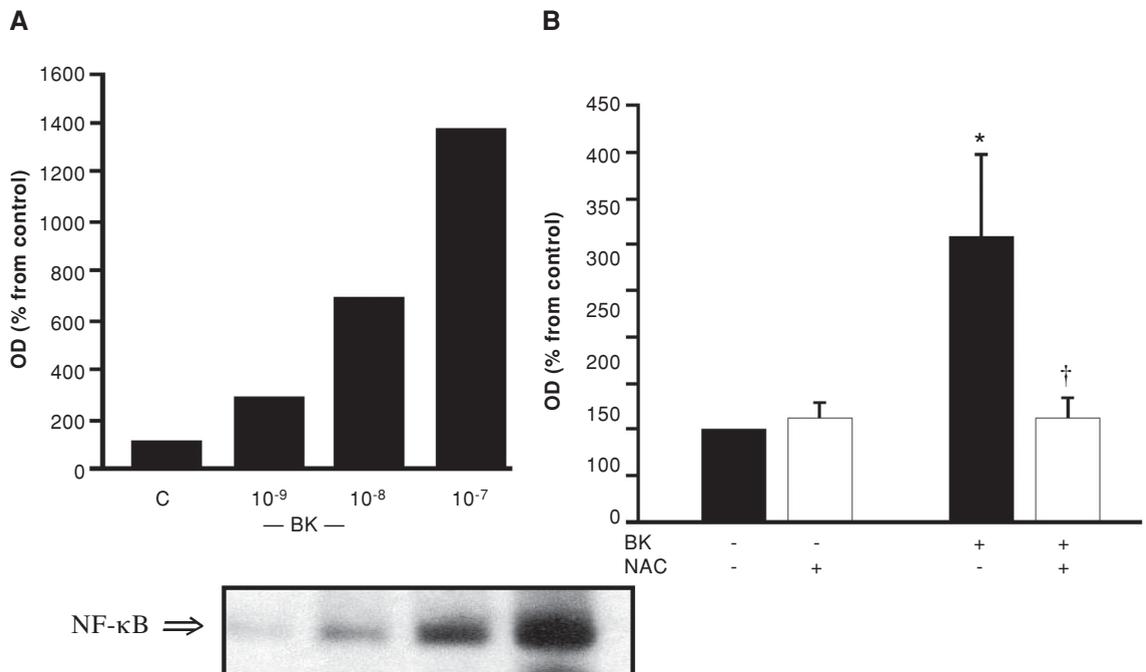


Figure 2. Effect of NAC on the activation of NF- κ B induced by bradykinin. VSMC were stimulated with BK for 30 min. A) dose response in NF- κ B activation. B) Pre-incubation with NAC (20 mM) for 45 min. Bars represent average \pm standard error for 4 experiments. * = $p<0.05$ compared to control. † = $p<0.05$ compared to BK.

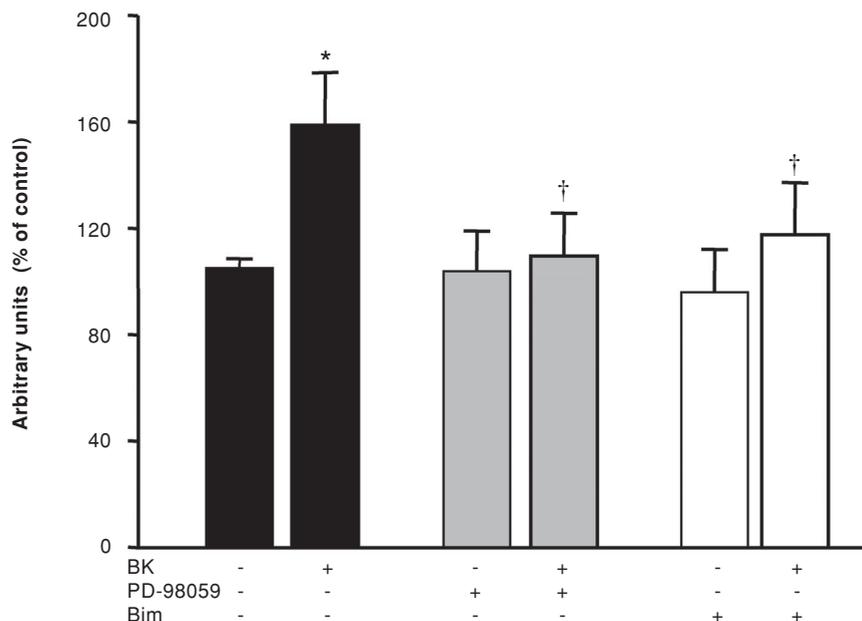


Figure 3. ROS production induced by bradykinin is dependent on PKC and MEK activities. VSMC were loaded with DCF for 1 hour and pre-incubated with PD98059 (40 μ M) or bisindolylmaleimide (Bim, 4 μ M) for 30 minutes followed by the stimulation with BK for 10 min. Bars represent average \pm standard error for 8 experiments. * = $p < 0.05$ compared to its own control in the absence of BK.

Migration induced by bradykinin is mediated by ROS

To determine whether BK was able to induce cell migration in VSMC, cells were incubated for 2 and 4 hours with BK. PDGF-BB was used as a positive control (Arita et al., 2002). At two hours, BK did not induce cell migration, whereas PDGF induced a marked increase in cell migration. However after 4 hours, BK induced a significant increase in cell migration when compared to control cells. The increase in migration was similar to that observed for PDGF (Fig 4A). To determine if the increase in migration was mediated by changes in ROS, cells were pre-incubated with the antioxidant LA for 2h and stimulated with BK for 4 hours. Pre-incubation with LA did not change migration pattern in control cells but blocked migration induced by BK ($p < 0.05$) and also reduced the cellular migration induced by PDGF (Fig 4B).

Collagen production induced by bradykinin is mediated by ROS

To determine the effects of BK on the production of extracellular matrix, we measured the production of collagen and also determined whether BK-induced alterations in collagen are affected by the production of ROS. For this purpose, VSMC were incubated with BK for 48 hours in the presence of ^3H -Proline and ascorbic acid to induce collagen secretion. Secreted collagen was identified among the proteins that incorporated proline, based on a higher level of ^3H -Proline incorporation, molecular weight and sensitivity to collagenase digestion (Ichiki et al., 1997). As expected, BK induced an increase in the quantity of ^3H -Proline incorporated into collagen protein secreted from VSMC. Collagen secretion was not affected by pre-incubation with LA alone. However, BK-induced secretion of collagen was blocked when cells were treated with BK in the presence of LA (Fig 5). These results suggest that ROS participate in the signaling pathway that results in collagen synthesis and secretion.

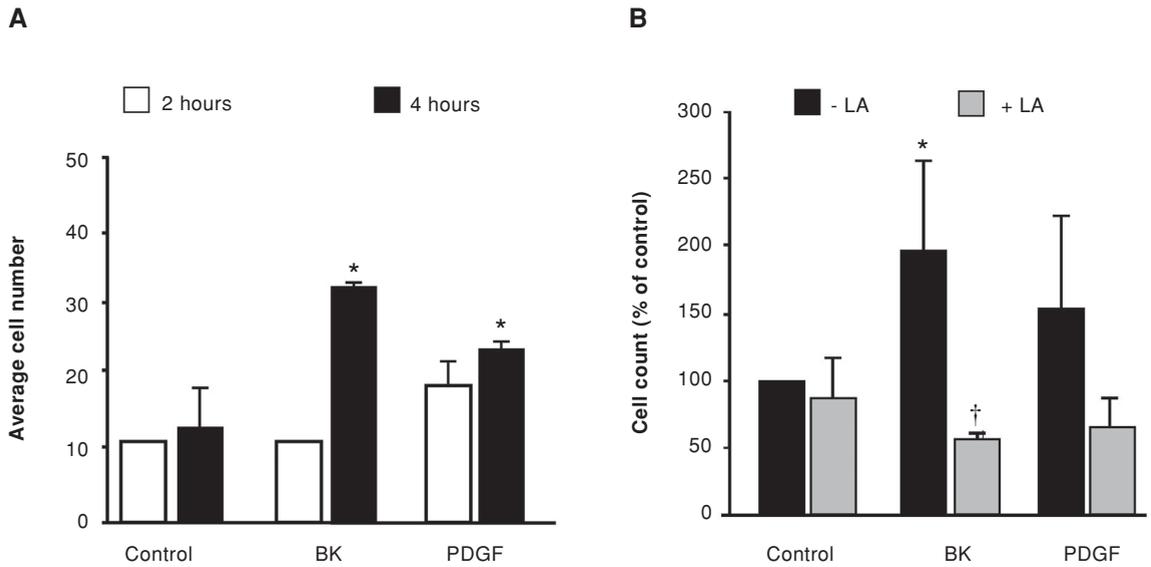


Figure 4. Lipoic acid inhibits cell migration induced by BK. A) VSMC were incubated with BK (10nM) or PDGF-BB (1 nM) for 2 or 4 hours. B) VSMC were preincubated with lipoic acid (250 μ M) for 2 hours, followed by the incubation with BK(10nM) or PDGF-BB (1 nM) for 4h. Bars represent average \pm standard error for 4 experiments. * = $p < 0.05$ compared to control. † = $p < 0.05$ compared to BK.

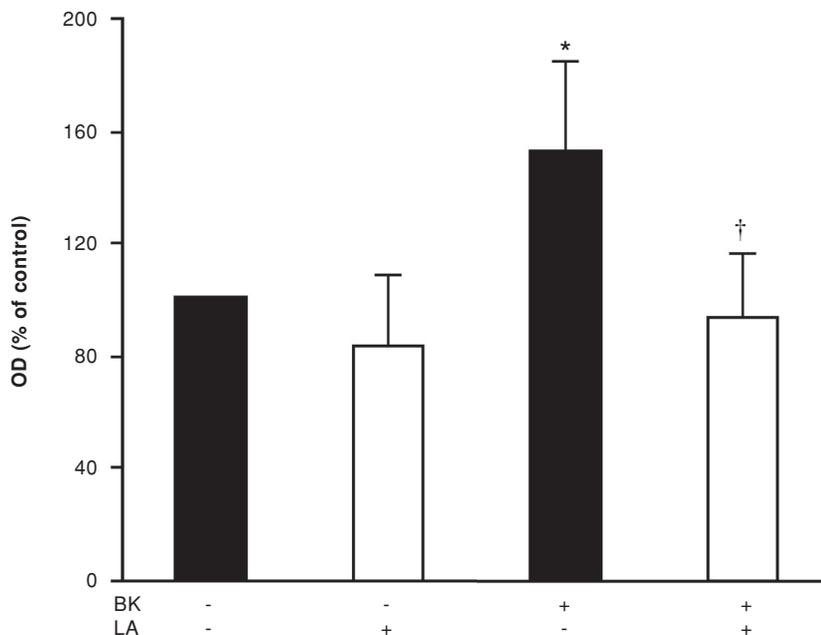


Figure 5. Bradykinin-induced collagen production is inhibited by lipoic acid. Cells were preincubated with lipoic acid (250 μ M) for 2 hours followed by the incubation with BK for 48 hours. Proline was added to the medium 24 hours before the end of the incubation. Bars represent average \pm standard error for 4 experiments. * = $p < 0.05$ compared to control. † = $p < 0.05$ compared to BK.

DISCUSSION

In the present work we have observed that: 1) ROS participate in the mechanism(s) used by bradykinin to induce cellular proliferation; 2) bradykinin induces ROS generation through a pathway that involves PKC and MEK; and 3) ROS participate in the pathways mediating cell migration and the production of collagen as a response to treatment with bradykinin.

Bradykinin is a potent polypeptide hormone released by the activation of the intrinsic pathway of coagulation. Once released, BK activates B₂ receptors expressed on the surface of endothelial cells, which leads to the activation of ERK 1/2 (Leiva-Salcedo et al., 2002) and also to the liberation of NO and prostaglandin I₂ to the space between the endothelium and the smooth muscle cell layer (Ruschitzka et al., 1999), producing an endothelium-dependent relaxation. However, in several pathologic situations including diabetes and hypertension and following procedures like angioplasty, which are associated with vascular injury, the endothelium may become dysfunctional. In the presence of a dysfunctional and/or absent endothelium, circulating vasoactive peptides can interact directly with the VSMC layer. We, and others, have observed that BK can induce proliferation of VSMC in vitro (Yau et al., 2001), and we have postulated that BK can be considered among the factors involved in the phenotypic dedifferentiation of vascular cells and the development of atherosclerosis. In addition, many of the pathophysiological disease processes affecting the vasculature are known to be associated with increases in oxidative stress. In this investigation, we have postulated that several of the effects on VSMC that are induced by BK, such as proliferation, migration and synthesis of matrix proteins, are mediated by the generation of ROS.

The induction of proliferation by bradykinin has been a controversial topic. Several groups have observed an increase in proliferation in different cell types (Higashida et al., 1996; El-Dahr et al., 1998), while others have observed the opposite effect (Alric et al., 1999; Dixon et

al., 1997). Our results in VSMC have shown a modest but significant effect of BK on the induction of proliferation, similar to what has been observed for angiotensin II (Makita et al., 1995), another vasoactive peptide postulated to induce proliferation in VSMC. This is in contrast to the effects observed by others in VSMC from mesenteric artery (Dixon et al., 1997). The differing results between the two studies could be due to differences in the origin of the smooth muscle cells (ours came from the aorta, while theirs come from the mesenteric artery) and the treatment given to the cells (FBS was absent in our cells; they stimulated theirs in the presence of fetal serum). This modest but significant effect of BK on cell proliferation is consistent with the notion that BK and angiotensin II are not competence factors as are PDGF or FGF, but rather progression factors.

Pre-incubation with the antioxidants LA and NAC without any other stimulus produces a decrease in cell proliferation when compared to control, suggesting that ROS partly mediate the basal proliferation rate of VSMC in primary culture. This effect could be explained considering that the preparation of primary cultures is possibly a stress stimulus that could induce mild changes in the phenotype of VSMC. Despite the presence of a basal proliferation rate, our results indicate that BK induces a significant additional increase in proliferation that was inhibitable by antioxidants. These results suggest the participation of ROS in the signaling pathway leading to cellular proliferation induced by BK. ROS are known to be involved in both proliferative and anti-proliferative events depending on the concentration generated, site of production, whether they are secreted or remain within the cell, the cellular type that is generating them, and the presence of an anti-oxidant defense system. In macrophages, the production of ROS is abundant and not only stays within the cell but is secreted to the extracellular compartment as well; these conditions favor cell death (Forman and Torres, 2001). On the other hand, in vascular cells the production of ROS is

limited to the intracellular compartment and in smaller concentrations, favoring a proliferative state (Li and Shah, 2003). Potential sources of ROS in VSMC have been suggested, and a major source recently identified in these cells is the enzyme NAD(P)H oxidase. It is possible that this enzyme complex is responsible for generating the ROS production that we observed in our current studies. In a previous set of studies where we used diphenylene-iodonium (DPI), a potent NAD(P)H oxidase inhibitor, we blocked ROS production and ERK 1/2 activation induced by BK (Greene et al., 2000).

Extensive evidence indicates that ROS regulate gene expression by modulating a large number of transcription factors, including the nuclear transcription factor kappa B (NF- κ B). It has been observed that H₂O₂ is capable of activating NF- κ B in some cell types. In addition, in human aortic smooth muscle cells IL-1 β induces the activation of NF- κ B via the production of ROS (Hoare et al., 1999). Several authors have proposed that BK can induce NF- κ B activation (Xie et al., 2000) but most of these studies have been done in cell lines. Based on this evidence, we studied the effect of BK on NF- κ B activation in VSMC. As was expected, BK induced an increase in NF- κ B that was concentration-dependent, and in agreement with the evidence in literature, we proposed that ROS could be mediating this effect. Unexpectedly, LA did not reduce BK-induced increase in nuclear NF- κ B. To confirm this result, we used another antioxidant (DPI) with similar results (data not shown). To our surprise, the pre-incubation with a third antioxidant, NAC, inhibited BK-induced NF- κ B activation. At present, we have no clear explanation for these results but propose two potential explanations. First, in tissues LA can be converted into its reduced form, dihydrolipoic acid, and as such, can act as a pro-oxidant (Packer et al., 1995). On the other hand, it has been postulated that NAC acts through a pathway independent of scavenging ROS (Zafarullah et al., 2003). NAC raises intracellular GSH levels and thereby provides GSH peroxidase with the

co-substrate to eliminate ROS (Zhang et al., 1991).

In order to determine some key intracellular signaling intermediates that require activation for the generation of ROS, we used a pharmacologic approach of two important kinases (PKC and MEK). The PKC inhibitor Bim inhibited BK stimulation of ROS, suggesting that this enzyme is required for BK-induced generation of ROS. When MEK was inhibited by PD98059, we also observed an inhibition of the response induced by BK. We did not expect this result, since we previously have observed that antioxidants blocked p42/44 MAPK activation, suggesting that ROS were generated before the activation of MEK. One way to explain our results is to consider the existence of two separate but not inconsistent scenarios for how ROS are involved in ERK signaling. First, it is possible that PKC activation is important for the generation of ROS via NADPH oxidase. An additional mechanism to explain how PD-98059 might reduce ROS production is the existence of a second instance where ROS are produced, which is dependent on ERK activation (Seshiah et al., 2002). Future experiments in which the time frame of the experimental protocol is manipulated will be required to address this issue.

Migration of medial smooth muscle cells into the intima is an important process in various vascular proliferative diseases. Although the mechanisms responsible for this migration are not fully understood, several factors produced in response to vascular injury have been implicated in this process. BK has long been considered as an inflammatory factor, which has an important effect on neutrophil migration (Paegelow et al., 2002). In addition, BK can induce the migration of keratinocytes (Coutant et al., 1997) but not of endothelial cells from post-capillary venules in culture (Morbidelli et al., 1998). We have observed that BK can induce VSMC migration in a similar magnitude to the one observed for PDGF, a classical chemotactic agent for medial VSMC (Liu et al, 1989). There is abundant evidence suggesting the participation of ROS on cell migration. In

fact, H₂O₂ induces cell migration in bovine aortic endothelial cells (Yasuda et al., 1999). In addition, arachidonic acid (AA) induces migration through a pathway that involves the production of ROS (Nishio and Watanabe, 1997). Our results indicate that ROS mediate the migration induced by BK, although we have not yet determined whether this effect is mediated by AA production. However, it has been shown that BK is capable of mobilizing AA in VSMC (Zhang et al., 1991).

In addition to proliferation and migration, the remodeling of the extracellular matrix is an important feature of atherosclerosis and other diseases. In myocardial infarction, it has been observed that angiotensin converting enzyme (ACE) inhibitors reduce hypertrophy and fibrosis. This antifibrotic effect is in part mediated by an increase in the concentration of BK (Gallagher et al., 1998). However, this inhibition seems to be mediated by the generation of NO and prostacyclin from the endothelium (Farhy et al., 1993). In addition, ACE inhibitors have not been effective in all studies (the Mercator Study Group, 1992); this evidence suggests that the beneficial effect of BK on the myocardium depends on a functional endothelial layer. In previous experiments, we observed an increase in collagen mRNA in response to BK in VSMC (Douillet et al., 2000). Our current results show that the protein for collagen is also increased and that this effect is likely to be mediated via a pathway involving ROS. This observation has also been reported in a set of studies suggesting that angiotensin II uses a similar pathway to generate increased collagen production in cardiomyocytes (Sorescu and Griendling, 2002). Although we have used ascorbic acid in the incubation medium as a cofactor to increase collagen secretion (Davidson et al., 1997), its effect as an antioxidant is not relevant because of the low concentrations used (De Tullio and Arrigoni, 2004).

In summary, our results suggest that, under conditions in which BK is present at high concentrations, such as inflammation and diabetes, and in the absence of a functional endothelial surface layer, BK

participates in the induction of cell proliferation, migration and increase in extracellular matrix production through a mechanism that involves the generation of ROS. To our knowledge, this is the first report implicating ROS in the proliferative and migratory pathways of bradykinin.

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