

Involvement of Extracellular Signal-Regulated Kinase 5 in Kinin B₁ Receptor Upregulation in Isolated Human Umbilical Veins^{SI}

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ABSTRACT

The upregulated kinin B₁ receptors exert a pivotal role in modulating inflammatory processes. In isolated human umbilical veins (HUVs), kinin B₁ receptor is upregulated as a function of in vitro incubation time and proinflammatory stimuli. The aim of this study was to evaluate, using functional and biochemical methods, the involvement of extracellular signal-regulated kinase 5 (ERK5), p38 mitogen-activated protein kinase (MAPK), c-Jun N-terminal kinase (JNK), and extracellular signal-regulated kinase 1/2 (ERK1/2) on the kinin B₁ receptor upregulation process in HUV. Real-time polymerase chain reaction analysis revealed for the first time that kinin B₁ receptor mRNA expression closely parallels the functional sensitization to kinin B₁ receptor selective agonist des-Arg¹⁰-kallidin (DAKD) in HUV. Moreover, the selective inhibition of ERK5, p38 MAPK, and JNK, but not ERK1/2, produced a dose-

dependent rightward shift of the concentration-response curves to DAKD after 5-hour incubation and a reduction in kinin B₁ receptor mRNA expression. Biochemical analyses showed that ERK5, p38 MAPK, and JNK phosphorylation is maximal during the first 2 hours postisolation, followed by a significant reduction in the last 3 hours. None of the treatments modified the responses to serotonin, an unrelated agonist, suggesting a specific effect on kinin B₁ receptor upregulation. The present work provides for the first time pharmacologic evidence indicating that ERK5 plays a significant role on kinin B₁ receptor upregulation. Furthermore, we confirm the relevance of p38 MAPK and JNK as well as the lack of effect of ERK1/2 in this process. This study may contribute to a better understanding of MAPK involvement in inflammatory and immunologic diseases.

Introduction

Kinins are small vasoactive peptides generated at the sites of tissue damage during most inflammatory process (Leeb-Lundberg et al., 2005). The actions of kinins are mediated through the stimulation of two subtypes of G-protein-coupled receptors, kinin B₁ and kinin B₂ (Leeb-Lundberg et al., 2005). Whereas kinin B₂ receptors are constitutively expressed in a variety of tissues and mediate most of the in vivo effects of kinins (Leeb-Lundberg et al., 2005), kinin B₁ receptors are not present in any significant amount in normal tissues, and their expression is often inducible rather than constitutive (Regoli et al., 1978; Sardi et al., 1997). Synthesis of kinin B₁ receptors can be induced under certain pathophysiological conditions conveying tissue injury or inflammation or during tissue isolation trauma and incubation (Marceau et al., 1998). Evidence from knockout mice has revealed that kinin B₁ receptor is critically required for a number of important physiological and pathophysiological functions in vivo,

including inflammation and nociception (Pesquero et al., 2000). During sustained inflammatory insult, kinin-mediated responses adapt from a kinin B₂ receptor type in the acute phase to a kinin B₁ receptor type in the chronic phase (Dray and Perkins, 1993).

Many research groups have studied the possible signaling pathways involved in kinin B₁ receptor upregulation phenomenon. In this sense, the 5'-flanking region of the human kinin B₁ receptor gene bears putative nuclear transcription factor- κ B (NF- κ B), as well as activator protein-1 (AP-1) binding motifs, a promoter organization consistent with a highly regulated gene (Bachvarov et al., 1996). This receptor is highly induced under inflammatory conditions, and in vitro and in vivo studies in different tissues provides evidence that several proinflammatory cytokines are involved in kinin B₁ receptor upregulation through NF- κ B activation (Baldwin, 1996; Marceau et al., 1998; Ni et al., 1998).

Our group demonstrated in HUV that kinin B₁ receptor-mediated responses are potentiated by proinflammatory mediators like lipopolysaccharide, interleukin-1 β and tumor necrosis factor- α and are inhibited by anti-inflammatory mediators such as dexamethasone and retinoic acid, probably by repressing the activity of NF- κ B and AP-1 (Sardi et al., 1997, 2000a). Furthermore, Sardi et al., 1999, 2002) have

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ABBREVIATIONS: AP-1, activator protein-1; CRC, concentration-response curve; DAKD, des-Arg¹⁰-kallidin; DMSO, dimethyl sulfoxide; E_{max} , maximal response; ERK1/2, extracellular signal-regulated kinase 1/2; ERK5, extracellular signal-regulated kinase 5; 5-HT, 5-hydroxytryptamine, serotonin; HUV, human umbilical vein; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MEK, MAPK kinase; NF- κ B, nuclear transcription factor- κ B; qRT-PCR, quantitative real-time reverse transcription polymerase chain reaction.

demonstrated in this tissue, using several pharmacologic tools, that NF- κ B activation plays a key role in the development of kinin B₁ receptor-sensitized responses.

Both transcription factors NF- κ B and AP-1 are activated by different members of the mitogen-activated protein kinases (MAPK) family (Whitmarsh and Davis, 1996; Karin et al., 1997; Schulze-Osthoff et al., 1997; Li et al., 2000; Dunn et al., 2002; Sacconi et al., 2002; Tsai et al., 2003; Morimoto et al., 2007) to elicit a range of transcriptional or nontranscriptional changes that result in specific cellular responses, including cellular proliferation/differentiation or inflammation (Plotnikov et al., 2011). Several stimuli (e.g., proinflammatory cytokines; bacterial products; mechanical, osmotic, or oxidative stress) stimulate the MAPK cascade activation (Kyriakis and Avruch, 2001). Mammalian cells have four distinct MAPK cascades, highly conserved and expressed ubiquitously in all eukaryotic cells, extracellular signal-regulated kinase 1 and 2 (ERK1/2), c-Jun N-terminal kinase (JNK), p38 isoforms (α , β , γ , and δ), and ERK5 (Plotnikov et al., 2011). Each group of MAPK is composed of a set of three sequentially acting kinase. The signaling begins with phosphorylation of an apical MAPK kinase kinase, activation of which sequentially phosphorylates and activates the downstream MAPK kinase (MEK). This in turn dual phosphorylates specific threonine and tyrosine residues of a conserved motif present within the kinase domain (Cargnello and Roux, 2011).

The members of p38 MAPK, JNK, and ERK1/2 subgroups are highly homologous and have overlapping, if not redundant, signaling capabilities (Buschbeck and Ullrich, 2005). ERK5, the relatively recently identified MAPK, is able to induce and regulate several physiological processes, including proliferation, angiogenesis, immunologic processes, and stress responses (Plotnikov et al., 2011); however, less information is available about this kinase than about the other MAPK pathways, and the full scope of its functions is not clear. In this sense, whereas many researchers have postulated p38 MAPK, ERK1/2, and JNK as signaling pathways involved in the upregulation of kinin B₁ receptors (Larrivée et al., 1998; Medeiros et al., 2004; Zhang et al., 2004; Phagoo et al., 2005; El Sayah et al., 2006; Brechter et al., 2008), there is still no evidence involving ERK5 in kinin B₁ receptor upregulation. The aim of this study was to evaluate the involvement of ERK5 and the other MAPK signaling pathways in the kinin B₁ receptor upregulation process in our isolated HUV experimental model using together both functional and molecular methods.

Materials and Methods

Tissue Collection and Preparation. Approximately 15- to 35-cm segments were excised from human umbilical cords midway between the placenta and newborn. All cords were collected from healthy normotensive patients after full-term vaginal or cesarean delivery. Approval of a local ethics committee and written informed consents were obtained. Cords were immediately placed in modified Krebs' solution at 4°C (of the following composition: 119 mM NaCl, 4.7 mM KCl, 25 mM NaHCO₃, 1.2 mM KH₂PO₄, 2.5 mM CaCl₂, 1.0 mM MgSO₄, 0.004 mM EDTA, 11 mM D-glucose). The time from delivery until the tissue was set up in the organ bath was approximately 3 hours. The cords were placed onto dissecting dishes containing Krebs' solution. Veins were carefully dissected free from Warthon's jelly using microdissecting instruments and cut into rings of approximately 3 mm wide.

Functional Studies. To perform these experiments, we followed the protocols described in the online Supplemental Material and in our

previous studies (Sardi et al., 1997, 1998, 1999, 2000b, 2002; Errasti et al., 2007; Nowak et al., 2007, 2011; Pelorosso et al., 2009).

Concentration response curves (CRCs) to DAKD, a kinin B₁ receptor-selective agonist, were constructed after a 15-, 120-, or 300-minute *in vitro* incubation by cumulative addition, in 0.25 log₁₀ increments, to determine a tissue sensitization to des-Arg¹⁰-kallidin (DAKD). Only one agonist CRC was performed on a single ring.

With the purpose of evaluating the effect of different MAPK inhibitors on this sensitization process, CRCs were obtained for DAKD in the absence or continuous presence of different MAPK inhibitors after a 5-hour equilibration period. Some HUV rings were continuously exposed to selective MAPK pathways inhibitors before the cumulative addition of DAKD at 5 hours. The choice of the inhibitors and the concentrations to be used is described in the online Supplemental Material.

Some of the tissues were incubated at effective concentrations in the presence of these selective MAPK inhibitors for the last 15 minutes before and throughout the construction at 5 hours of the CRC to the kinin B₁ receptor selective agonist DAKD.

At the end of each CRC, 10 μ M serotonin (5-HT) was added to determine the tissue maximal contractile response (Altura et al., 1972; Sardi et al., 1997). In other series of experiments, CRCs for the unrelated agonist, 5-HT, were constructed on HUV rings after 5 hours of *in vitro* incubation in the presence of effective concentrations of BIX02188, VX-702, SB203580, or SP600125.

All experiments were performed in parallel with rings from the same umbilical cord. Only one CRC to the agonist was performed in each ring. Control trials were performed in the presence of the corresponding concentration of dimethyl sulfoxide (DMSO) 0.1% v/v.

RNA Isolation, cDNA Synthesis, and Quantitative Real-Time Reverse Transcription Polymerase Chain Reaction. In these experiments, tissues were collected and incubated as described in the online Supplemental Material and in previous studies from our group (Errasti et al., 2007). Some rings were frozen in liquid nitrogen immediately after isolation until processed (basal conditions), and others were incubated for 120 or 300 minutes. In other series of experiments, rings were incubated for 5 hours in the same conditions, in the presence or absence of SB203580 10 μ M, VX-702 200 nM, SP600125 3 μ M, BIX02188 10 μ M, and PD184352 1 μ M; then they were frozen in liquid nitrogen until processed.

RNA isolation, cDNA synthesis, and quantitative real-time reverse transcription polymerase chain reaction (RT-PCR) were performed as described in the online Supplemental Material and described by Linder et al. (2010) and Fukushima et al. (2014).

Western Blot. In these experiments, tissues were collected and incubated as described in the online Supplemental Material. Some rings were snap-frozen after a 15-, 30-, 45-, 60-, 120-, 180-, 240-, or a 300-minute *in vitro* incubation or as fresh, nonincubated tissue. In other series of experiments, HUV were snap-frozen after a 30-minute *in vitro* incubation in the presence of either BIX02188 (10 μ M), SB203580 (10 μ M), VX-702 (200 nM), SP600125 (3 μ M), or PD184352 (1 μ M). Western blot was performed after the protocols described in the online Supplemental Material and in our previous studies (Errasti et al., 2007; Pelorosso et al., 2007).

Drugs. The following compounds were used for functional studies as well as RT-PCR assays: 5-hydroxytryptamine creatine sulfate complex from Sigma-Aldrich (St. Louis, MO); DAKD from Bachem California (Torrance, CA); SB203580 4-[4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-1H-imidazol-5-yl]pyridine, VX-702 [1-(5-carbamoyl-6-(2,4-difluorophenyl)pyridin-2-yl)-1-(2,6-difluorophenyl)urea], PD184352 [2-(2-chloro-4-iodophenylamino)-N-(cyclopropylmethoxy)-3,4-difluorobenzamide], BIX01288 [(Z)-3-((3-((dimethylamino)methyl)phenylamino)(phenyl)methylene)-2-oxoindoline-6-carboxamide], and SP600125 (2H-Dibenzo[cd,gl]indazol-6-one) were purchased from Selleck-Chem (Houston, TX). SB203580, VX-702, PD184352, BIX01288, and SP600125 were initially dissolved in DMSO to give stock solution, and subsequent dilutions were prepared in bidistilled water. All stock solutions were stored frozen in aliquots and thawed daily. The rest

of the drugs were dissolved in glass bidistilled water to give stock solution, which were further diluted with glass bidistilled water directly before the experiment. All concentrations of drugs are expressed as a final concentration in the organ bath. The maximal final concentrations of DMSO in the bath solutions were 0.1% v/v. Preliminary experiments were performed in the presence of the corresponding concentrations of DMSO to rule out any nonspecific action of this solvent on the tonus or contractility of the tissue preparations as well as on the RNA isolation, cDNA synthesis, performance of quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR), or mRNA levels. These experiments showed that 0.1% v/v DMSO fails to modify DAKD-induced responses in HUV or glyceraldehyde-3-phosphate dehydrogenase and kinin B₁ receptor mRNA levels from control tissues (data not shown). Nevertheless, all control trials were performed in the presence of the corresponding concentration of DMSO.

Expression of Results and Statistical Analysis. All data are expressed as mean \pm S.E.M. From each umbilical cord, a unique experimental n was obtained to perform the functional and biochemical-molecular studies (CCR, qRT-PCR assays, and Western blot). Responses are expressed as the percentage of tissue maximum response elicited by 10 μ M 5-HT. Responses obtained for each cord tested in the same group were averaged and then fitted to a four-parameter logistic model expressed as follows: $Y = \alpha - E_{\max}/1 + (X/EC_{50})^{n_H} + E_{\max}$, where Y is the response, X is the arithmetic dose, α is the response when $X = 0$, EC_{50} is the agonist concentration that produces 50% of the maximal response, E_{\max} is the maximal response, and n_H is the slope factor (DeLean et al., 1978). Estimates for these parameters were determined using Graph Pad Prism Version 4.00 (Graph Pad Software Inc., La Jolla, CA). The EC_{50} values were transformed into pEC_{50} ($-\log EC_{50}$). The pEC_{50} values between control and treated tissues were compared only when their maximal responses were not significantly different.

Data generated in qRT-PCR were analyzed according to Winer et al. (1999) and Livak and Schmittgen (2001). Calculation of the fold change in kinin B₁ receptor was relative to glyceraldehyde-3-phosphate dehydrogenase endogenous control using $2^{-\Delta\Delta Ct}$ and $2^{-\Delta\Delta Ct}$.

Statistical analysis was performed by means of one-way analysis of variance with Tukey's post hoc test or unpaired Student's t test when appropriate. P values lower than 0.05 were considered to indicate significant differences between means. Terms are as recommended by the International Union of Pharmacology Committee on Receptor Nomenclature and Drug Classification Committee on Receptor Nomenclature and Drug Classification (Neubig et al., 2003).

Results

Effects of In Vitro Incubation Time on DAKD-Induced Contractile Responses and on Kinin B₁ Receptor mRNA Expression in HUV. As shown in Fig. 1A, in vitro incubated HUV rings increased their contractile

response to DAKD as a function of time. Contractile responses induced by DAKD obtained after 15 minutes of in vitro incubation yielded a pEC_{50} of 7.12 ± 0.12 and a response to DAKD 1 μ M of $60.73 \pm 5.70\%$, $n = 9$ (Fig. 1A). When tissues were incubated for 120 minutes, the response to DAKD 1 μ M was significantly higher than after 15-minute incubation (E_{\max} : $74.33 \pm 2.94\%$, $n = 9$, $P < 0.05$; Fig. 1A). Incubation for 300 minutes produced a significant leftward shift of the CRC to DAKD compared with 120-minute incubated tissues (pEC_{50} 120 minutes: 8.23 ± 0.07 , $n = 9$, 300 minutes: 8.94 ± 0.02 , $n = 12$, $P < 0.001$; Fig. 1A), but the maximal response was not modified (E_{\max} : $81.33 \pm 1.21\%$, $n = 12$; Fig. 1A). Interestingly, qRT-PCR analyses demonstrated a time-dependent increase in the expression of kinin B₁ receptor mRNA (Fig. 1B). Taken together, our results indicate a correlation between the increase in the contractile responses induced by the kinin B₁ receptor selective agonist DAKD as a function of incubation time and the increase in kinin B₁ mRNA expression in HUV.

Effects of ERK5 Inhibition on B₁ Receptor-Sensitized Responses and on B₁ Receptor mRNA Expression in HUV. Exposure to the selective MEK5/ERK5 inhibitor BIX02188 demonstrated a dose-dependent inhibition of DAKD-induced responses in HUV after 5 hours of in vitro incubation. In this respect, continuous exposure to 10 μ M BIX02188 produced a significant rightward shift of the CRC to DAKD (pEC_{50} : control 8.88 ± 0.04 , $n = 11$, treated 8.08 ± 0.05 , $n = 6$; $P < 0.001$; Fig. 2A; Table 1) without affecting the maximal response (E_{\max} : control $85.30\% \pm 1.68\%$, treated $91.74\% \pm 2.93\%$; Fig. 2A; Table 1), whereas continuous exposure to a lower dose (1 μ M) of this inhibitor failed to modify either pEC_{50} or maximal response (Fig. 2A; Table 1). In accordance with these results, qRT-PCR analysis demonstrated that continuous treatment with 10 μ M BIX02188 produced a reduction of 60.37% in kinin B₁ receptor mRNA expression after 5-hour incubation compared with control ($P < 0.05$; Fig. 2B).

To rule out any toxic effect of 10 μ M BIX02188, some HUV rings were incubated with the selective inhibitor 15 minutes before the construction at 5 hours of the CRC to the kinin B₁ receptor selective agonist. Under these experimental conditions, 10 μ M BIX02188 failed to modify kinin B₁ receptor-mediated responses induced by DAKD (Table 1). In addition, continued exposure to 10 μ M BIX02188 failed to affect the CRC to an unrelated agonist, 5-HT, in HUV rings after a 5-hour in vitro incubation (Fig. 2C, pEC_{50} control: 8.16 ± 0.04 , treated: 8.26 ± 0.04 ; E_{\max} control: 90.11 ± 2.15 , treated: 87.25 ± 2.05 , $n = 7$). Taken together, both results

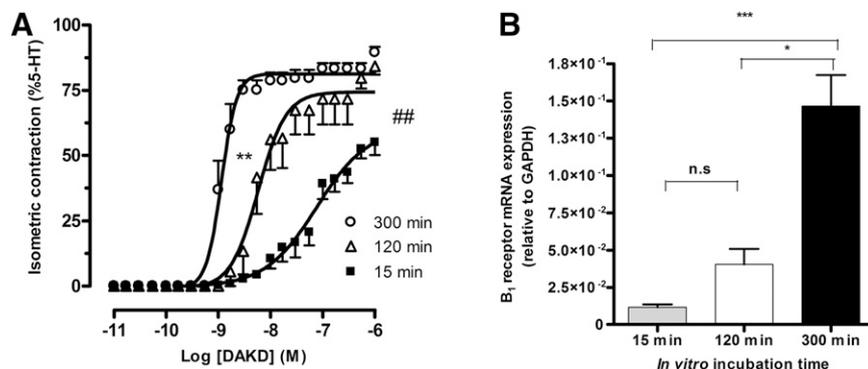


Fig. 1. (A) CRCs to DAKD at 15 minutes (\blacksquare , $n = 9$), 120 minutes (\triangle , $n = 9$), and 300 minutes (\circ , $n = 12$) of incubation in HUV rings. Each symbol represents the mean of n independent determinations and the vertical lines show S.E.M. The responses are expressed as percentage of maximal response to 5-HT (10 μ M) obtained at the end of each experiment. $***P < 0.001$, significant differences between pEC_{50} values; $\#P < 0.05$, significant differences between maximal responses. (B) Expression of B₁ receptor mRNA at basal conditions or after 120 minutes and 300 minutes of incubation in HUV as detected by qRT-PCR. Data are presented as the mean \pm S.E.M. of at least five independent experiments per group. n.s., no significant difference; $*P < 0.05$; $***P < 0.001$, significant differences between means.

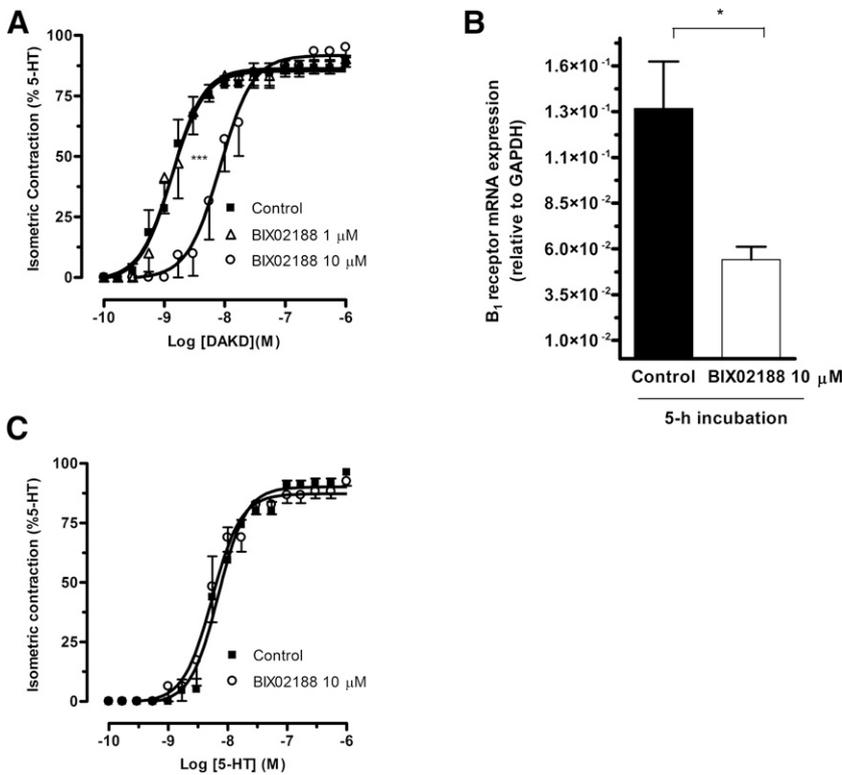


Fig. 2. (A) CRCs to DAKD on control HUV rings (■, $n = 11$) and on tissues continuously exposed to BIX02188 (1 μM , Δ , $n = 7$; or 10 μM , \circ , $n = 6$). Each symbol represents the mean of n determinations made after a 5-hour in vitro incubation, and the vertical lines show S.E.M. *** $P < 0.001$, significant differences between pEC₅₀ values. (B) Expression of kinin B₁ receptor mRNA after a 5-hour in vitro incubation from control HUV rings and tissues continuously treated with BIX02188 (10 μM) as detected by qRT-PCR. Data are presented as the mean \pm S.E.M. of at least five independent experiments per group. * $P < 0.05$, significant differences between treated and control tissues. (C) CRCs to 5-HT on control HUV rings (■, $n = 7$) and on tissues continuously exposed to BIX02188 (10 μM) (\circ , $n = 7$). Each symbol represents the mean of n determinations made after a 5-hour in vitro incubation, and the vertical lines show S.E.M.

indicated the lack of toxic effects of this drug on HUV ring contractility or kinin B₁ receptor signal transduction.

Effects of p38 MAPK Inhibition on Kinin B₁ Receptor-Sensitized Responses and on Kinin B₁ Receptor mRNA Expression in HUV. The selective p38 MAPK inhibitor SB203580 (10 μM) produced a significant rightward shift of the CRC to DAKD after a 5-hour in vitro incubation in HUV rings (pEC₅₀: control 8.96 ± 0.05 , $n = 11$, treated 8.61 ± 0.07 , $n = 7$; $P < 0.01$; Fig. 3A; Table 1) without affecting the maximal response (E_{max} : control $88.23\% \pm 2\%$, treated $81.32\% \pm 2.73\%$; Fig. 3A; Table 1). Neither pEC₅₀ nor maximal response was modified by continuous exposure to a lower dose (1 μM) of this selective inhibitor (Fig. 3A; Table 1).

Another p38 MAPK selective inhibitor, VX-702 (200 nM), produced a significant inhibition of DAKD-induced responses (pEC₅₀: control 9.00 ± 0.02 , $n = 11$, treated: 8.47 ± 0.03 , $n = 7$; $P < 0.001$; Fig. 3B; Table 1) without affecting the maximal response (E_{max} : control $77.47\% \pm 1.13\%$, treated $78.12\% \pm 1.54\%$; Fig. 3B; Table 1). Neither pEC₅₀ nor maximal responses were modified by continuous exposure to a lower dose (20 nM) of this selective inhibitor (Fig. 3B; Table 1). In agreement with functional studies, qRT-PCR analysis demonstrated that continuous 5-hour treatment with 10 μM SB203580 or 200 nM VX-702 produced a reduction of 64.39% ($P < 0.01$) and 55.92% ($P < 0.05$), respectively, in kinin B₁ receptor mRNA levels compared with control (Fig. 3C).

TABLE 1

Effect of various in vitro treatments on the concentration response curves to DAKD in 5-hour incubated HUV rings

Treatment	Period (h)	pEC ₅₀		E_{max}^a		n	
		Control	Treated	Control	Treated	Control	Treated
BIX02188 (1 μM)	h	8.88 ± 0.04	8.87 ± 0.05	85.30 ± 1.68	86.32 ± 1.98	11	7
BIX02188 (10 μM)	0–5	8.88 ± 0.04	$8.08 \pm 0.05^{***}$	85.30 ± 1.68	91.74 ± 2.93	11	6
BIX02188 (10 μM)	4.75–5	8.81 ± 0.04	8.73 ± 0.08	86.90 ± 1.96	80.71 ± 2.80	7	6
SB203580 (1 μM)	0–5	8.96 ± 0.05	8.76 ± 0.05	88.23 ± 2.00	83.52 ± 2.18	11	5
SB203580 (10 μM)	0–5	8.96 ± 0.05	$8.61 \pm 0.07^{**}$	88.23 ± 2.00	81.32 ± 2.73	11	7
SB203580 (10 μM)	4.75–5	8.81 ± 0.04	8.76 ± 0.05	86.90 ± 1.96	80.58 ± 2.07	7	7
VX-702 (20 nM)	0–5	9.00 ± 0.02	8.97 ± 0.08	77.47 ± 1.13	81.15 ± 2.69	11	5
VX-702 (200 nM)	0–5	9.00 ± 0.02	$8.47 \pm 0.03^{***}$	77.47 ± 1.13	78.12 ± 1.54	11	7
VX-702 (200 nM)	4.75–5	8.81 ± 0.04	8.79 ± 0.09	86.90 ± 1.96	87.96 ± 3.08	7	7
SP600125 (1 μM)	0–5	8.87 ± 0.03	8.83 ± 0.03	89.74 ± 1.47	89.01 ± 1.57	9	7
SP600125 (3 μM)	0–5	8.87 ± 0.03	$8.28 \pm 0.04^{***}$	89.74 ± 1.47	79.94 ± 1.91	9	7
SP600125 (3 μM)	4.75–5	8.81 ± 0.04	8.61 ± 0.04	86.90 ± 1.96	77.23 ± 1.81	7	7
PD184353 (0.1 μM)	0–5	8.85 ± 0.03	8.94 ± 0.06	80.12 ± 1.44	74.02 ± 2.13	13	7
PD184353 (1 μM)	0–5	8.85 ± 0.03	8.75 ± 0.07	80.12 ± 1.44	75.89 ± 2.40	13	7

^a E_{max} is expressed as a percentage of maximum responses obtained with 10 μM 5-HT. Values are expressed as mean \pm S.E.M.

** $P < 0.01$, *** $P < 0.001$, significant differences between pEC₅₀ values.

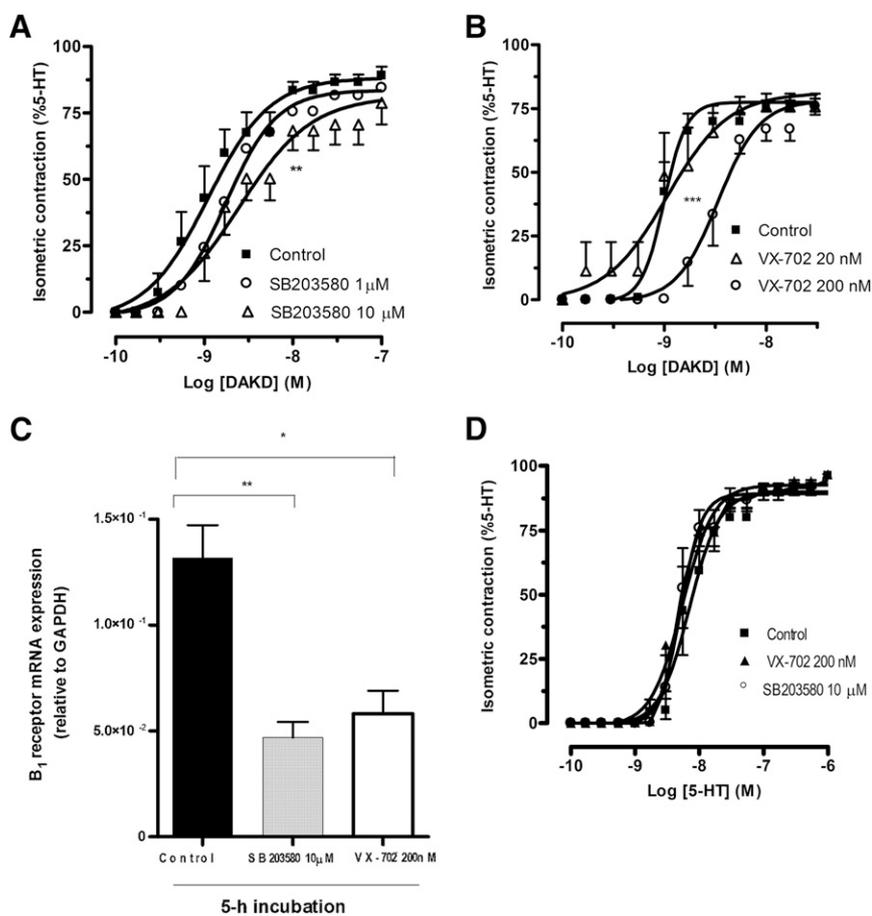


Fig. 3. (A) CRCs to DAKD on control HUVEC rings (■, $n = 11$) and on tissues continuously exposed to SB203580 1 μM (○, $n = 5$) or 10 μM (△, $n = 7$). (B) CRCs to DAKD on control HUVEC rings (■, $n = 11$) and on tissues continuously exposed to VX-702 (20 nM, △, $n = 5$; or 200 nM, ○, $n = 7$). In both figures, each symbol represents the mean of n independent determinations made after a 5-hour in vitro incubation, and the vertical lines show S.E.M. ** $P < 0.01$; *** $P < 0.001$, significant differences between pEC₅₀ values. (C) Expression of kinin B₁ receptor mRNA after a 5-hour in vitro incubation from control HUVEC rings and tissues continuously treated with SB203580 (10 μM) or VX-702 (200 nM), as detected by qRT-PCR. Data are presented as the mean \pm S.E.M. of at least five independent experiments per group. * $P < 0.05$; ** $P < 0.01$, significant differences between treated and control tissues. (D) Concentration-response curves to 5-HT on control HUVEC rings (■, $n = 7$) and on tissues continuously exposed to SB203580 (10 μM , ○, $n = 7$) or VX-702 (200 nM, △, $n = 7$). Each symbol represents the mean of n independent determinations made after a 5-hour in vitro incubation, and the vertical lines show S.E.M.

To rule out any toxic effect of SB203580 (10 μM) or VX-702 (200 nM), some HUVEC rings were incubated with these selective inhibitors 15 minutes before the construction at 5 hours of the CRC to the kinin B₁ receptor-selective agonist. This treatment failed to modify kinin B₁ receptor-mediated responses induced by DAKD (Table 1). In addition, neither pEC₅₀ nor maximal responses of the CRC to an unrelated agonist, 5-HT, in HUVEC rings after a 5-hour in vitro incubation were modified by continuous exposure to 10 μM SB203580 (Fig. 3D, pEC₅₀ control: 8.16 ± 0.04 , treated: 8.29 ± 0.03 ; E_{max} control: 90.11 ± 2.15 , treated: 89.31 ± 2.05 , $n = 7$) or VX-702 200 nM (Fig. 3D; pEC₅₀ control: 8.16 ± 0.04 , treated: 8.26 ± 0.03 ; E_{max} control: 90.11 ± 2.15 , treated: 92.75 ± 2.46 , $n = 7$). Taken together, both results indicated the lack of toxic effects of these drugs on HUVEC ring contractility or kinin B₁ receptor signal transduction.

Effects of JNK Inhibition on Kinin B₁ Receptor-Sensitized Responses and on Kinin B₁ Receptor mRNA Expression in HUVEC. As shown in Fig. 4A, continuous exposure to the selective JNK inhibitor, SP600125 (3 μM), significantly inhibited DAKD-induced responses in HUVEC after 5 hours of in vitro incubation (pEC₅₀: control 8.87 ± 0.03 , $n = 9$, treated 8.28 ± 0.04 , $n = 7$; $P < 0.001$; Fig. 4A; Table 1) without affecting the maximal response (E_{max} : control $89.74\% \pm 1.47\%$, treated: $79.94\% \pm 1.91\%$; Fig. 4A; Table 1). Neither pEC₅₀ nor maximal response was modified by continuous exposure to a lower dose (1 μM) of this selective inhibitor (Fig. 4A; Table 1). In the same sense, qRT-PCR analysis demonstrated that continued treatment with SP600125 3 μM

produced a marked reduction in the increase in B₁ receptor mRNA levels after a 5-hour in vitro incubation in about 55.52% ($P < 0.05$; Fig. 4B).

On the other hand, short exposure (15 minutes before the construction at 5 hours of the concentration-response curve) to SP600125 (3 μM) failed to modify kinin B₁ receptor-mediated responses induced by DAKD (Table 1). Furthermore, continuous exposure to SP600125 (3 μM) failed to affect the CRC to 5-HT in HUVEC rings after a 5-hour in vitro incubation (Fig. 4C, pEC₅₀ control: 8.16 ± 0.04 , treated: 8.05 ± 0.03 ; E_{max} control: 90.11 ± 2.15 , treated: 87.11 ± 1.72 , $n = 7$). Taken together, these results indicate the lack of toxic effects of the JNK inhibitor on HUVEC ring contractility or kinin B₁ receptor signal transduction.

Lack of Effects of ERK1/2 Inhibition on both Kinin B₁ Receptor-Sensitized Responses and on Kinin B₁ Receptor mRNA Expression in HUVEC. The presence of the selective ERK1/2 inhibitor PD184352 (0.1 μM or 1 μM) failed to affect CRC to DAKD in HUVEC rings after a 5-hour in vitro incubation (Fig. 5A; Table 1). In line with functional results, continued treatment with PD184352 (1 μM) caused no significant change in B₁ receptor mRNA levels after 5-hour incubation in HUVEC (Fig. 5B).

Time Course of MAPKs Phosphorylation after In Vitro Incubation of HUVEC. Western blot analyses showed that only very low levels of ERK5 phosphorylation were observed under basal conditions (Fig. 6, A and D). In vitro incubation of isolated HUVECs resulted in a marked and time-dependent phosphorylation of ERK5, reaching maximal levels

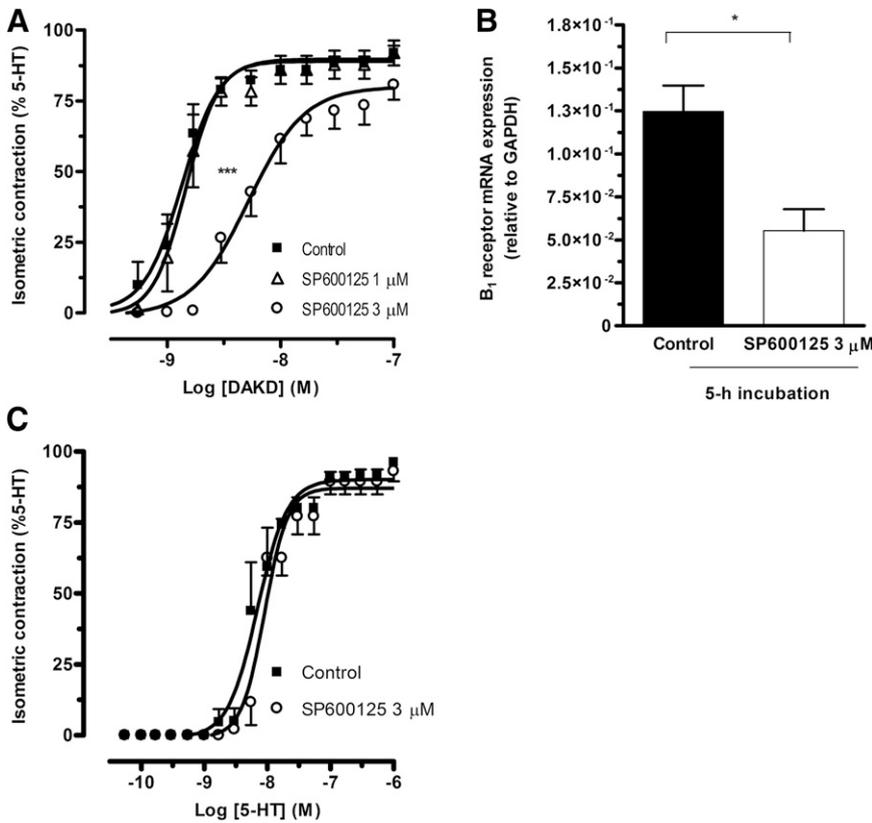


Fig. 4. (A) CRCs to DAKD on control HUV rings (■, $n = 9$) and on tissues continuously exposed to SP600125 (1 μM , Δ , $n = 7$; or 3 μM , \circ , $n = 7$). Each symbol represents the mean of n determinations made after a 5-hour in vitro incubation, and the vertical lines show S.E.M. *** $P < 0.001$, significant differences between pEC_{50} values. (B) Expression of kinin B₁ receptor mRNA after a 5-hour in vitro incubation from control HUV rings and tissues continuously treated with SP600125 (3 μM), as detected by qRT-PCR. Data are presented as the mean \pm S.E.M. of at least five independent experiments per group. * $P < 0.05$, significant differences between treated and control tissues. (C) CRCs to 5-HT on control HUV rings (■, $n = 7$) and on tissues continuously exposed to SP600125 (3 μM , \circ , $n = 7$). Each symbol represents the mean of n determinations made after a 5-hour in vitro incubation, and the vertical lines show S.E.M.

between 60 and 120 minutes and returning to basal values after 180 minutes. On the other hand, the phosphorylation of p38 MAPK and JNK was detected under basal conditions (0 minutes) and a similar level of phosphorylation was maintained for up to 30 minutes of in vitro incubation in the case of p38 MAPK (Fig. 6, B and E) and up to 60 minutes, reaching maximal levels at 120 minutes in the case of JNK (Fig. 6, C and F).

Selective Phosphorylation Inhibition of Different MAPK Pathways in Isolated HUV. The selectivity of the different MAPK inhibitors used in the present work was evaluated by Western blot analysis. As shown in Fig. 6A, in control experiments, ERK5 was clearly phosphorylated after 30 minutes of in vitro incubation. The treatment of isolated

HUV with BIX02188 10 μM in the first 30 minutes of incubation inhibited ERK5 phosphorylation, without a significant effect on the phosphorylation of c-Jun, a downstream target of JNK, or MAPKAPK-2, a downstream target of p38 MAPK (Fig. 7A).

Similar to the results obtained in ERK5 pathway, as shown in Fig. 6B, in control experiments, p38 MAPK was clearly phosphorylated after 30 minutes of in vitro incubation. The treatment of isolated HUV with VX-702 200 nM or SB203580 (10 μM) in the first 30 minutes of incubation inhibited MAPKAPK-2 phosphorylation without affecting ERK5 or c-Jun phosphorylation (Fig. 7A).

As shown in Fig. 6C, JNK was clearly phosphorylated after 30 minutes of in vitro incubation. Treatment of isolated HUV with SP600125 (3 μM) markedly reduced the phosphorylation of c-Jun without affecting ERK5 or MAPKAPK-2 phosphorylation (Fig. 7A).

Discussion

Sensitization to kinin B₁ receptor agonists in isolated HUV is a bona fide system to study kinin B₁ receptor upregulation in human tissue (Sardi et al., 2000b). In the HUV, kinins promote a potent and effective vasoconstrictor response (Altura et al., 1972). It has been demonstrated that this action depends on kinin B₂ receptor stimulus (Marceau et al., 1994; Félétou et al., 1995; Gobeil et al., 1996). On the other hand, in isolated HUV, our group observed a vasoconstricting action of the selective kinin B₁ receptor agonist, des-Arg⁹-bradykinin (Sardi et al., 1997, 1998, 1999, 2000b), as well as an effective vasoconstrictor response of the more potent selective kinin B₁ receptor agonist, DAKD (Nowak et al., 2007). In this model,

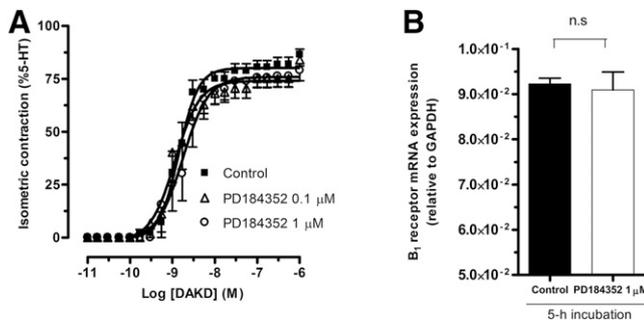


Fig. 5. (A) Concentration-response curves to DAKD on control HUV rings (■, $n=13$) and on tissues continuously exposed to PD184352 0.1 μM (Δ , $n=7$) and 1 μM (\circ , $n = 7$). Each symbol represents the mean of n determination made after a 5-hour in vitro incubation, and the vertical lines show S.E.M. (B) Expression of B₁ receptor mRNA after a 5-hour in vitro incubation from control HUV rings and tissues continuously treated with PD184352 1 μM , as detected by qRT-PCR. Data are presented as the mean \pm S.E.M. of at least five independent experiments per group. n.s., no significant differences from control tissues.

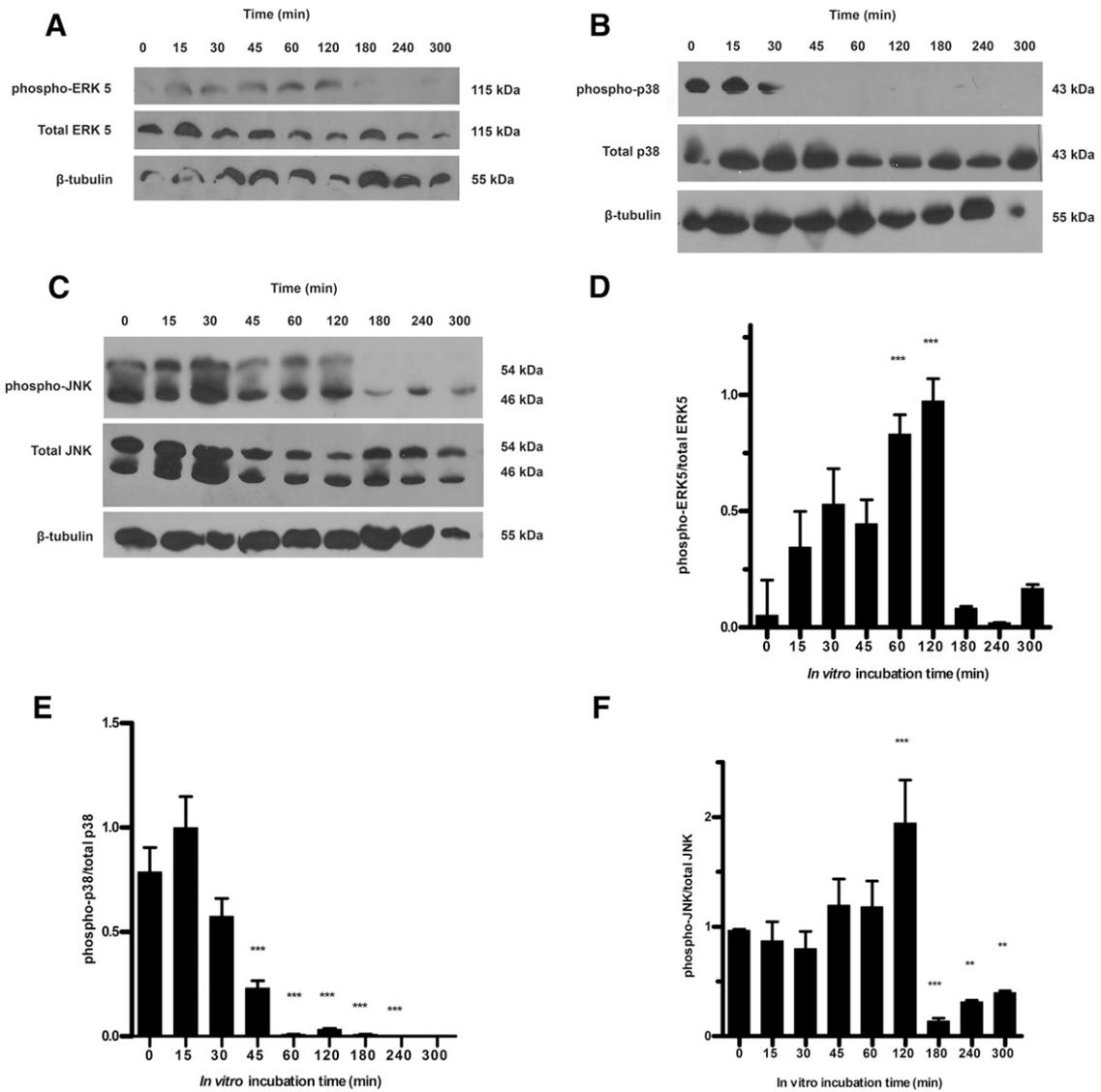


Fig. 6. Time course of MAPK activation after isolation and in vitro incubation of HUV. Tissues were incubated for the indicated times, and tissue lysates were then prepared. Equal volumes of lysate were separated by SDS-PAGE and immunoblotted with the indicated antibodies. Levels of (A) phosphorylated and total ERK5; (B) phosphorylated and total p38 MAPK; and (C) phosphorylated and total JNK were measured by Western blot analysis as described under *Materials and Methods*. β -tubuline was used as an internal control in all the experiments. The histograms represent the densitometric analysis of (D) phosphorylated-ERK5/total ERK5; (E) phosphorylated-p38 MAPK/total p38 MAPK; and (F) phosphorylated-JNK /total JNK determined from immunoblots. The blot shown is representative of four separate experiments. Data represent the mean \pm S.E.M. of four independent experiments. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$, significantly different from nonincubated tissue (0 minutes).

the kinin B₁ receptor-mediated contractile response develops from an initial null level and increases in magnitude as a function of the in vitro incubation time (Sardi et al., 1997). This sensitization process is dependent on the de novo synthesis of receptors (Sardi et al., 1998, 1999). Within human tissues, the in vitro induction of kinin B₁ receptor in colon, ileum, and coronary and umbilical arteries has been reported (Couture et al., 1981; Drummond and Cocks, 1995; Zuzack et al., 1996; Pelorosso et al., 2007).

To study more fully the kinin B₁ receptor upregulation process in isolated HUV, we first considered it necessary to evaluate the kinin B₁ receptor mRNA expression under basal conditions and after 120 and 300 minutes of in vitro incubation to correlate its expression with the functional sensitized responses. The results obtained show that as a function of incubation time the increase in the contractile responses

induced by DAKD was correlated with the increase in kinin B₁ mRNA expression in HUV. The aim of this study was to evaluate the involvement of relevant MAPK signaling pathways (ERK5, p38 MAP, JNK, and ERK1/2) in this kinin B₁ receptor upregulation process in isolated HUVs.

To our knowledge, there is no evidence involving ERK5 in the B₁ receptor up-regulation process. Therefore, we considered it interesting to evaluate the possible participation of the recently identified MAPK, ERK5, in this phenomenon in HUV.

ERK5 is twice the size of other MAPKs (Nishimoto and Nishida, 2006; Wang and Tournier, 2006) and is phosphorylated by MEK5 but not by MEK1 or MEK2 (Hayashi and Lee, 2004). ERK5 is ubiquitously expressed (Buschbeck and Ullrich, 2005) but is particularly abundant in the heart, skeletal muscle, placenta, lungs, and kidneys (Nithianandarajah-Jones et al., 2012, and there are reports of its presence in

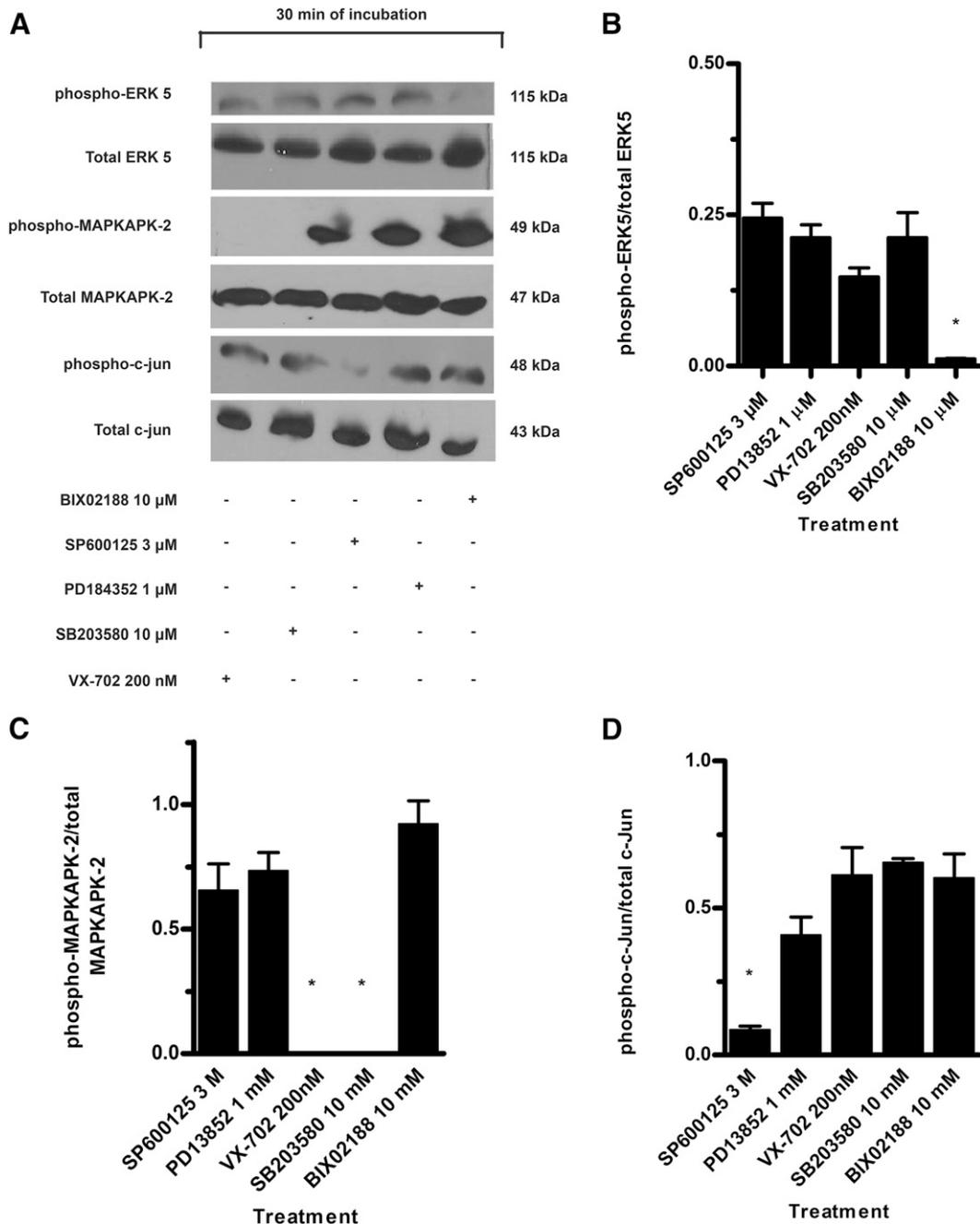


Fig. 7. Selective phosphorylation inhibition of different MAPK pathways in isolated HUV. (A) Representative blots showing the levels of phosphorylated and total ERK5, MAPKAPK-2, and c-Jun in HUV after 30 minutes of in vitro incubation in the presence or absence of SP600125 (3 μ M), PD184352 (1 μ M), VX-702 (200 nM), SB203580 (10 μ M), or BIX02188 (10 μ M). After incubation, tissue lysates were prepared, and equal volumes of lysate were separated by SDS-PAGE and immunoblotted with the indicated antibodies. Levels of phosphorylated and total ERK5, phosphorylated and total MAPKAPK-2, or phosphorylated and total c-Jun were measured by Western blot analysis as described under *Materials and Methods*. The blot shown is representative of three separate experiments. The histograms represent the densitometric analysis of (B) phosphorylated-ERK5/total ERK5; (C) phosphorylated- c-Jun /total c-Jun; and (D) phosphorylated MAPKAPK-2/total MAPKAPK-2 determined from immunoblots. Data represent the mean \pm S.E.M. of three independent experiments. * P < 0.05 significant differences between means.

HUV endothelial cells (Kim et al., 2012) and rat aortic smooth muscle cells (Izawa et al., 2007; Zhao et al., 2011).

For the first time, our results clearly support that the signaling pathway ERK5 is involved in the kinin B₁ receptor upregulation process. In this sense, HUV rings continuously exposed to the selective ERK5 inhibitor, BIX02188, showed a dose-dependent inhibition of vasoconstrictor sensitized-responses elicited by DAKD after 5 hours of incubation. A wide array of inhibitors of kinin B₁ receptor upregulation

have shown to be ineffective when applied to tissues only minutes before the agonist stimulation after 5 hours of incubation (Sardi et al., 1998, 1999, 2000a; Pelorosso et al., 2009). In agreement with this finding, further analysis revealed that BIX02188 inhibitory effect is time dependent since short exposure to this compound failed to modify such responses. On the other hand, these results rule out a direct acute toxic effect of BIX02188 on vascular tone or an acute effect on kinin B₁ receptor signal transduction, in HUV.

Moreover, the CRC to an unrelated agonist, 5-HT, in HUV rings after a 5-hour *in vitro* incubation, were not modified by continuous exposure to BIX02188, thus confirming the lack of toxicity.

Furthermore, we demonstrated that BIX02188 was effective in suppressing not only the increase in DAKD contractile response but also the corresponding expression of kinin B₁ receptor mRNA in HUV. We further confirmed, by Western blot analysis that BIX02188, at the concentration used in the present work, selectively inhibited ERK5 phosphorylation without affecting the phosphorylation of other closely related MAPKs.

Moreover, we have shown, for the first time, in our tissues that the *in vitro* incubation of isolated HUVs resulted in a marked and time-dependent phosphorylation of ERK5, reaching maximal levels at between 60 and 120 minutes and returning to basal values in the last 3 hours of the total 5-hour incubation period, thus demonstrating that this enzyme certainly is at the maximum functional activity in the early stages of the kinin B₁ receptor upregulation process. Taken as a whole, the functional and molecular results support the hypothesis that the ERK5 signaling pathway clearly participates in kinin B₁ receptor upregulation in isolated HUVs.

The involvement of p38 MAPK in kinin B₁ receptor upregulation has been observed in many *in vitro* animal models: isolated rabbit aorta (Larrivé et al., 1998), rat portal vein (Medeiros et al., 2004), pig iris sphincter (El Sayah et al., 2006), chronic inflammatory model in rat trachea (Zhang et al., 2004), or in vascular smooth muscle cells exposed to heat stress (Lagneux et al., 2001), as well as in an *in vivo* inflammatory hyperalgesia model in rats (Ganju et al., 2001). In relation to JNK, Medeiros et al. (2004) have demonstrated in rat portal veins the relevance of this kinase in the kinin B₁ receptor upregulation process. In human tissues, involvement of these kinases was observed only in osteoblastic osteosarcoma cell lines (Brecht et al., 2008) and fetal lung fibroblast (Phagoo et al., 2005), but it is important to mention that although all these experiments evaluated the kinin B₁ receptor upregulation by radioligand binding assays or qRT-PCR, they did not demonstrate a functional correlation of this phenomenon. In the present study, we demonstrated that the CRCs to DAKD after 5 hours of incubation were inhibited in a dose-dependent manner by continuous incubation with p38 MAPK and JNK selective inhibitors, and this evidence correlated with a significant reduction in kinin B₁ receptor mRNA expression after 5-hour *in vitro* incubation with these inhibitors in HUVs. Moreover, the selectivity of p38 MAPK and JNK inhibitors at the concentration used in the present work was confirmed by Western blot analysis in which the ability to inhibit MAPKAPK-2 and c-Jun phosphorylation, respectively, without affecting the phosphorylation of other closely related MAPK was observed.

Furthermore, evaluation of the time sequence of the results obtained in our tissue relative to the phosphorylation of p38 MAPK and JNK, clearly indicates that these enzymes are at maximum functional activity from the beginning of the incubation until about 30 minutes for p38 MAPK and 120 minutes for JNK, coinciding with the early times of the kinin B₁ receptor upregulation process. These results, similar to those observed with ERK5, strongly indicate that these p38 MAPK and JNK represent an important signaling pathway associated with kinin B₁ receptor upregulation. On the other hand, p38 MAPK and JNK selective inhibitors did not

produced a direct toxic effect on HUV contractility or kinin B₁ receptor signal transduction, similar to the results obtained with the ERK5 inhibitor.

Regarding ERK1/2 relevance in kinin B₁ receptor upregulation, we found that a MEK1/2 (upstream ERK1/2) inhibitor, PD184352, failed to inhibit both DAKD-elicited responses and the increase in kinin B₁ receptor mRNA in HUVs. In agreement with our results, *in vitro* studies in rat portal veins (Medeiros et al., 2004), human fetal lung fibroblasts (Phagoo et al., 2005), human lung fibroblasts (Haddad et al., 2000), osteoblastic osteosarcoma cell lines (Brecht et al., 2008), and murine tracheae (Zhang et al., 2007) demonstrated that ERK1/2 inhibition did not result in a significant reduction of kinin B₁ receptor upregulation process.

In the present study, we found that the maximum concentration of kinin B₁ receptor mRNA, as well as the maximum sensitivity to kinin B₁ receptor agonist in functional studies, was achieved after 5 hours of HUV *in vitro* incubation. Evaluation of the time sequence of the results obtained in our tissues relative to the phosphorylation of the different MAPKs, clearly indicates that these enzymes are at maximum functional activity during the first 2 hours, and a significant and marked reduction in phosphorylation was observed during the last 3 hours, consistent with participation in the early stages of the kinin B₁ receptor upregulation process. Considering that it has been well established that kinin B₁ receptor-sensitized responses are abolished by transcription inhibitors (Marceau et al., 1998, Sardi et al., 1998, 1999), supporting the view that the *de novo* synthesis of kinin B₁ receptors mRNA is involved in this process, and that evidence indicates the presence of NF- κ B and AP-1 binding motifs in the 5'-flanking region of the human kinin B₁ receptor gene (Bachvarov et al., 1996), and that both transcription factors are likely to be activated by different MAPK signaling pathways (Morimoto et al., 2007), the evidence suggests that MAPK activation could contribute to kinin B₁ receptor upregulation by an mRNA transcription induction rather than enhancing the stability of kinin B₁ receptor mRNA.

In our study, the lack of an increase in the maximum response to DAKD in the sensitization process between 2 hours and 5 hours, as well as the lack of decline in maximal response using different MAPK inhibitors, may be interpreted by the presence of kinin B₁ spare receptors in the HUV. In accordance with this result, our experimental model, isolated HUVs, has suggested the presence of a proportion of spare receptors in the kinin B₁ receptor population after 5 hours of *in vitro* incubation (Sardi et al., 1998, 1999).

Kinin B₁ receptors are central to the cause of pain and inflammation in various organs. Zhang et al. (2013) demonstrated that blockage of intracellular MAPK signaling prevents kinin B₁ receptor expression in the airway, suggesting that MAPK-dependent kinin B₁ receptor upregulation can provide a novel target for treatment of airway hyper-reactivity in asthma, as well as in other inflammatory airway diseases. Likewise, the inducible kinin B₁ receptor may also represent a target of potential value in the treatment of chronic pain (Calixto et al., 2004), diabetic neuropathy (Talbot and Couture, 2012), and retinal edema in diabetes (Pruneau et al., 2010). The activation of kinin B₁ receptors has also been associated to inflammatory and immunogenic responses in the peripheral and central nervous system. In this sense, Viel and Buck (2011) demonstrated the participation of kinin B₁

receptor in neurodegenerative processes, suggesting a link between this receptor and the neuroinflammation in Alzheimer disease. Moreover, da Costa et al. (2014) have shown evidence that supports the concept that kinin receptors, especially kinin B₁ receptor, are promising targets for cancer therapy, since many tumor cells express aberrantly high levels of these receptors.

In summary, the present work confirms the relevance of p38 MAPK and JNK pathways, as well as the lack of effect of ERK1/2 in kinin B₁ receptor upregulation, in a human tissue, which may be relevant for a better understanding of MAPK inhibitors effects on the mentioned pathologic conditions. Furthermore, this study provides pharmacologic and biochemical evidence indicating that ERK5 plays a novel clear and significant role in this process in a human tissue, and ERK5 thus may be a new therapeutic target for the rational development of pharmacotherapeutic tools for inflammatory and immunologic diseases, as well as painful processes.

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Authorship Contributions

Participated in research design: Kilstein, Nowak, Pelorosso, Rothlin.

Conducted experiments: Kilstein, Barcia Feás, Armesto, Pelorosso, Nowak.

Contributed new reagents or analytic tools: Errasti, Armesto.

Performed data analysis: Kilstein, Armesto.

Wrote or contributed to the writing of the manuscript: Kilstein, Rothlin.

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