1521-0103/357/1/114–124$25.00
THE JOURNAL OF PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS

http://dx.doi.org/10.1124/jpet.115.230169
J Pharmacol Exp Ther 357:114–124, April 2016

Involvement of Extracellular Signal-Regulated Kinase 5 in Kinin B_1 Receptor Upregulation in Isolated Human Umbilical Veins[S]

Yael Kilstein, Wanda Nowak, Andrea Emilse Errasti, Antía Andrea Barcia Feás, Arnaldo Raúl Armesto, Facundo Germán Pelorosso, and Rodolfo Pedro Rothlin

Instituto de Farmacología, Facultad de Medicina, Universidad de Buenos Aires, Ciudad Autónoma de Buenos Aires, Argentina

Received October 21, 2015; accepted January 8, 2016

ABSTRACT

The upregulated kinin B_1 receptors exert a pivotal role in modulating inflammatory processes. In isolated human umbilical veins (HUVs), kinin B_1 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 B_1 receptor upregulation process in HUV. Real-time polymerase chain reaction analysis revealed for the first time that kinin B_1 receptor mRNA expression closely parallels the functional sensitization to kinin B_1 receptor selective agonist des-Arg^{10}-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 B_1 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 B_1 receptor upregulation. The present work provides for the first time pharmacologic evidence indicating that ERK5 plays a significant role on kinin B_1 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 B_1 and kinin B_2 (Leeb-Lundberg et al., 2005). Whereas kinin B_2 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 B_1 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 B_1 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 B_1 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 B_2 receptor type in the acute phase to a kinin B_1 receptor type in the chronic phase (Dray and Perkins, 1993).

Many research groups have studied the possible signaling pathways involved in kinin B_1 receptor upregulation phenomenon. In this sense, the 5′-flanking region of the human kinin B_1 receptor gene bears putative nuclear transcription factor-κB (NF-κB), 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 B_1 receptor upregulation through NF-κB activation (Baldwin, 1996; Marceau et al., 1998; Ni et al., 1998).

Our group demonstrated in HUV that kinin B_1 receptor-mediated responses are potentiated by proinflammatory mediators like lipopolysaccharide, interleukin-1β 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-kB and AP-1 (Sardi et al., 1997, 2000a). Furthermore, Sardi et al., 1999, 2002) have

This research was supported by grants from Universidad de Buenos Aires (UBA, 20020100100611) and Consejo de Investigaciones Científicas y Técnicas (CONICET, PIP2620), Argentina.
dx.doi.org/10.1124/jpet.115.230169.
[S] This article has supplemental material available at jpet.aspetjournals.org.

ABBREVIATIONS: AP-1, activator protein-1; CRC, concentration-response curve; DAKD, des-Arg^{10}-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-κB, nuclear transcription factor-κB; qRT-PCR, quantitative real-time reverse transcription polymerase chain reaction.

demonstrated in this tissue, using several pharmacologic tools, that NF-κB activation plays a key role in the development of kinin B_1 receptor–sensitized responses.

Both transcription factors NF-κB 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; Saccani 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 B_1 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 B_1 receptor upregulation. The aim of this study was to evaluate the involvement of ERK5 and the other MAPK signaling pathways in the kinin B_1 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 $NaHCO_3$, 1.2 mM KH_2PO_4, 2.5 mM $CaCl_2$, 1.0 mM $MgSO_4$, 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-Arg^{10}-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 B_1 receptor selective agonist DAKD.

At the end of each CRC, 10 μM 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 μM, VX-702 200 nM, SP600125 3 μM, BIX02188 10 μM, and PD184352 1 μM; 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 μM), SB203580 (10 μM), VX-702 (200 nM), SP600125 (3 μM), or PD184352 (1 μM). 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,g]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 B_1 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 ± 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 μM 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: $Y = \alpha - E_{\max}/1 + (X/EC_{50})n_{\mathrm{H}} + E_{\max}$, 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 B_1 receptor was relative to glyceraldehyde-3-phosphate dehydrogenase endogenous control using $2^{-\Delta Ct}$ 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 B_1 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 μM of 60.73 ± 5.70%, n = 9 (Fig. 1A). When tissues were incubated for 120 minutes, the response to DAKD 1 μM was significantly higher than after 15-minute incubation ($E_{\max}$: 74.33 ± 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 ± 1.21%, n = 12; Fig. 1A). Interestingly, qRT-PCR analyses demonstrated a time-dependent increase in the expression of kinin B_1 receptor mRNA (Fig. 1B). Taken together, our results indicate a correlation between the increase in the contractile responses induced by the kinin B_1 receptor selective agonist DAKD as a function of incubation time and the increase in kinin B_1 mRNA expression in HUV.

Effects of ERK5 Inhibition on B_1 Receptor-Sensitized Responses and on B_1 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 μM 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% ± 1.68%, treated 91.74% ± 2.93%; Fig. 2A; Table 1), whereas continuous exposure to a lower dose (1 μM) of this inhibitor failed to modified either pEC_{50} or maximal response (Fig. 2A; Table 1). In accordance with these results, qRT-PCR analysis demonstrated that continuous treatment with 10 μM BIX02188 produced a reduction of 60.37% in kinin B_1 receptor mRNA expression after 5-hour incubation compared with control ($P < 0.05$; Fig. 2B).

To rule out any toxic effect of 10 μM BIX02188, some HUV rings were incubated with the selective inhibitor 15 minutes before the construction at 5 hours of the CRC to the kinin B_1 receptor selective agonist. Under these experimental conditions, 10 μM BIX02188 failed to modify kinin B_1 receptor–mediated responses induced by DAKD (Table 1). In addition, continued exposure to 10 μM 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

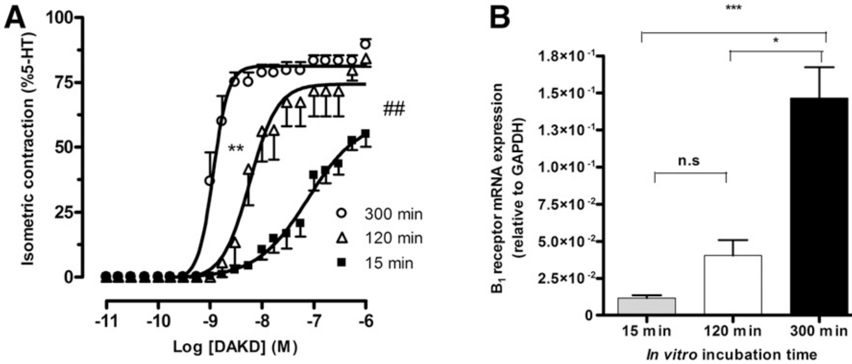

Fig. 1. (A) CRCs to DAKD at 15 minutes (■, n = 9), 120 minutes (Δ, n = 9), and 300 minutes (○, n = 12) of incubation in HUV rings. Each symbol represents the mean of n independent determinations and the vertical lines show S.E.M. The responses are expressed as percentage of maximal response to 5-HT (10 μM) 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 B_1 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 ± 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.

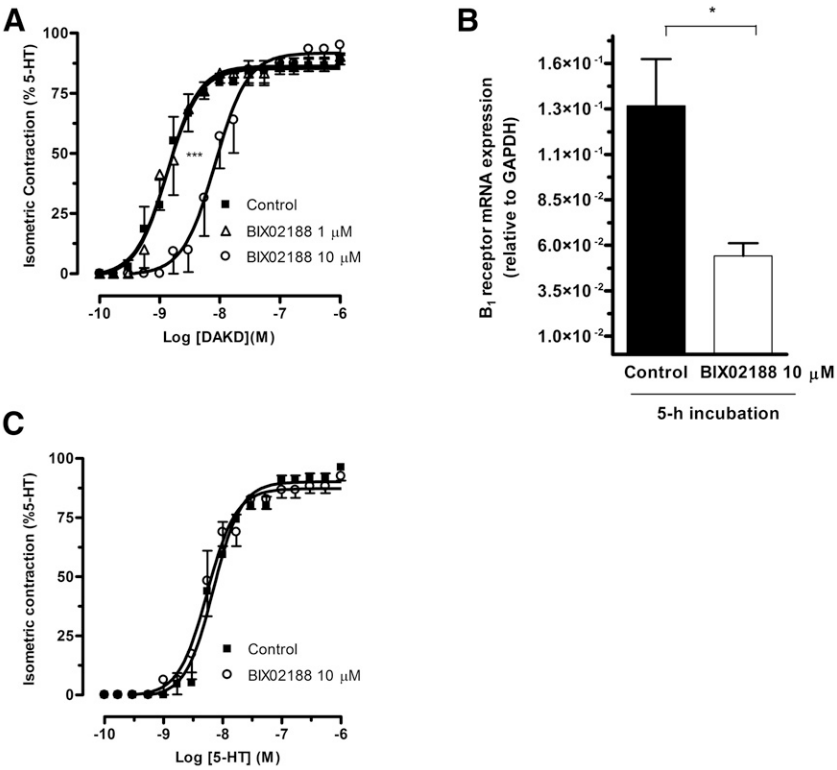

Fig. 2. (A) CRCs to DAKD on control HUV rings (■, n = 11) and on tissues continuously exposed to BIX02188 (1 μM, △, n = 7; or 10 μM, ○, n = 6). Each symbol represents the mean of n determinations made after a 5-hour in vitro incubation, and the vertical lines show S.E.M. *** $P < 0.001$, significant differences between pEC_{50} values. (B) Expression of kinin B_1 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 ± 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) (○, 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 B_1 receptor signal transduction.

Effects of p38 MAPK Inhibition on Kinin B_1 Receptor–Sensitized Responses and on Kinin B_1 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 (pEC_{50}: 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% ± 2%, treated 81.32% ± 2.73%; Fig. 3A; Table 1). Neither pEC_{50} 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 (pEC_{50}: 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% ± 1.13%, treated 78.12% ± 1.54%; Fig. 3B; Table 1). Neither pEC_{50} 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 B_1 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 0–5	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 ± 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 ± 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 ± 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 ± 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 ± S.E.M.
**$P < 0.01$; ***$P < 0.001$, significant differences between pEC_{50} values.

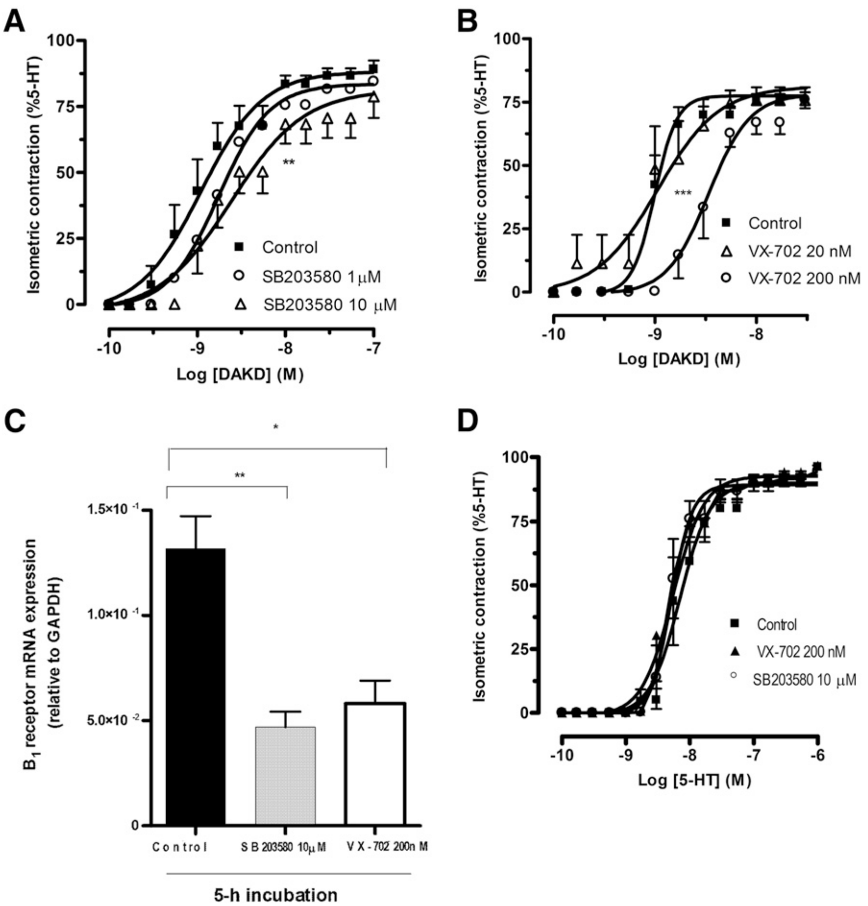

Fig. 3. (A) CRCs to DAKD on control HUV rings (■, n = 11) and on tissues continuously exposed to SB203580 1 μM (○, n = 5) or 10 μM (Δ, n = 7). (B) CRCs to DAKD on control HUV rings (■, n = 11) and on tissues continuously exposed to VX-702 (20 nM, Δ, n = 5; or 200 nM, ○, n = 7). In both figures, each 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 pEC_{50} values. (C) Expression of kinin B_1 receptor mRNA after a 5-hour in vitro incubation from control HUV 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 ± 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 HUV 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 determination 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 HUV rings were incubated with these selective inhibitors 15 minutes before the construction at 5 hours of the CRC to the kinin B_1 receptor-selective agonist. This treatment failed to modify kinin B_1 receptor–mediated responses induced by DAKD (Table 1). In addition, neither pEC_{50} nor maximal responses of the CRC to an unrelated agonist, 5-HT, in HUV rings after a 5-hour in vitro incubation were modified by continuous exposure to 10 μM SB203580 (Fig. 3D, pEC_{50} 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; pEC_{50} 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 HUV ring contractility or kinin B_1 receptor signal transduction.

Effects of JNK Inhibition on Kinin B_1 Receptor–Sensitized Responses and on Kinin B_1 Receptor mRNA Expression in HUV. As shown in Fig. 4A, continuous exposure to the selective JNK inhibitor, SP600125 (3 μM), significantly inhibited DAKD-induced responses in HUV after 5 hours of in vitro incubation (pEC_{50}: 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% ± 1.47%, treated: 79.94% ± 1.91%; Fig. 4A; Table 1). Neither pEC_{50} 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 B_1 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 B_1 receptor–mediated responses induced by DAKD (Table 1). Furthermore, continuous exposure to SP600125 (3 μM) failed to affect the CRC to 5-HT in HUV rings after a 5-hour in vitro incubation (Fig. 4C, pEC_{50} 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 HUV ring contractility or kinin B_1 receptor signal transduction.

Lack of Effects of ERK1/2 Inhibition on both Kinin B_1 Receptor–Sensitized Responses and on Kinin B_1 Receptor mRNA Expression in HUV. The presence of the selective ERK1/2 inhibitor PD184352 (0.1 μM or 1 μM) failed to affect CRC to DAKD in HUV 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 B_1 receptor mRNA levels after 5-hour incubation in HUV (Fig. 5B).

Time Course of MAPKs Phosphorylation after In Vitro Incubation of HUV. 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 HUVs resulted in a marked and time-dependent phosphorylation of ERK5, reaching maximal levels

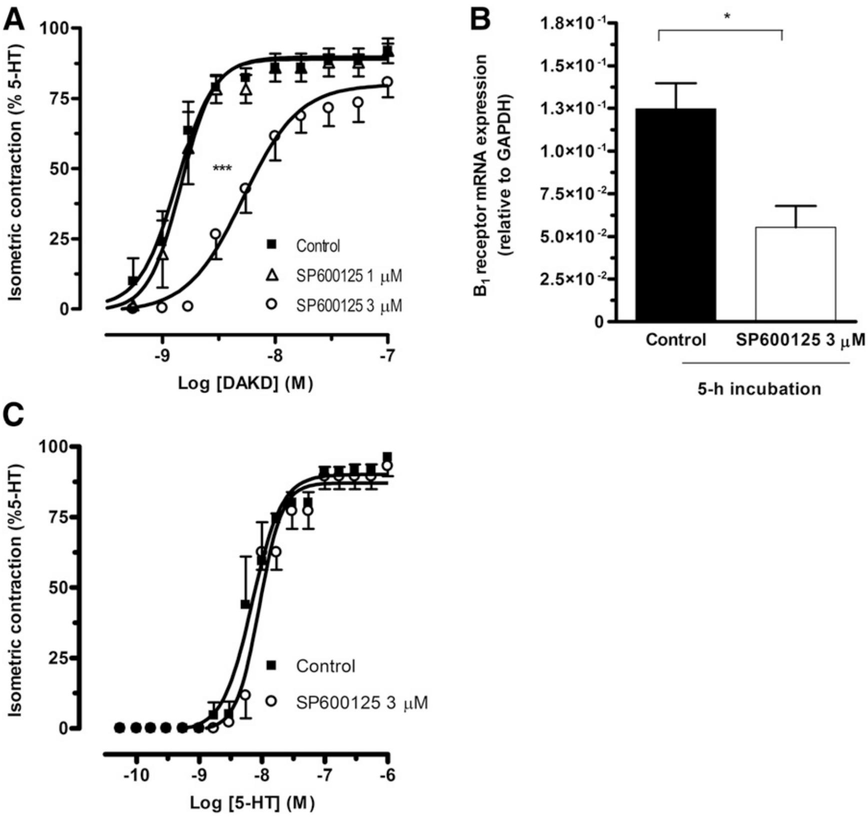

Fig. 4. (A) CRCs to DAKD on control HUV rings (■, n = 9) and on tissues continuously exposed to SP600125 (1 μM, Δ, n = 7; or 3 μM, ○, n = 7). Each symbol represents the mean of n determinations made after a 5-hour in vitro incubation, and the vertical lines show S.E.M. ***$P < 0.001$, significant differences between pEC_{50} values. (B) Expression of kinin B_1 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 ± 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, ○, 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 MAPKAKP-2, a downstream target of p38 MAPK (Fig. 7A).

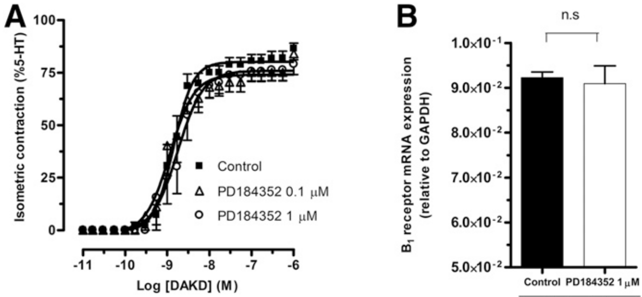

Fig. 5. (A) Concentration-response curves to DAKD on control HUV rings (■, n=13) and on tissues continuously exposed to PD184352 0.1 μM (Δ, n=7) and 1 μM (○, n = 7). Each symbol represents the mean of n determination made after a 5-hour in vitro incubation, and the vertical lines show S.E.M. (B) Expression of B_1 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 ± S.E.M of at least five independent experiments per group. n.s, no significant differences from control tissues.

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 B_1 receptor agonists in isolated HUV is a bona fide system to study kinin B_1 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 B_2 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 B_1 receptor agonist, des-Arg[9]-bradykinin (Sardi et al., 1997, 1998, 1999, 2000b), as well as an effective vasoconstrictor response of the more potent selective kinin B_1 receptor agonist, DAKD (Nowak et al., 2007). In this model,

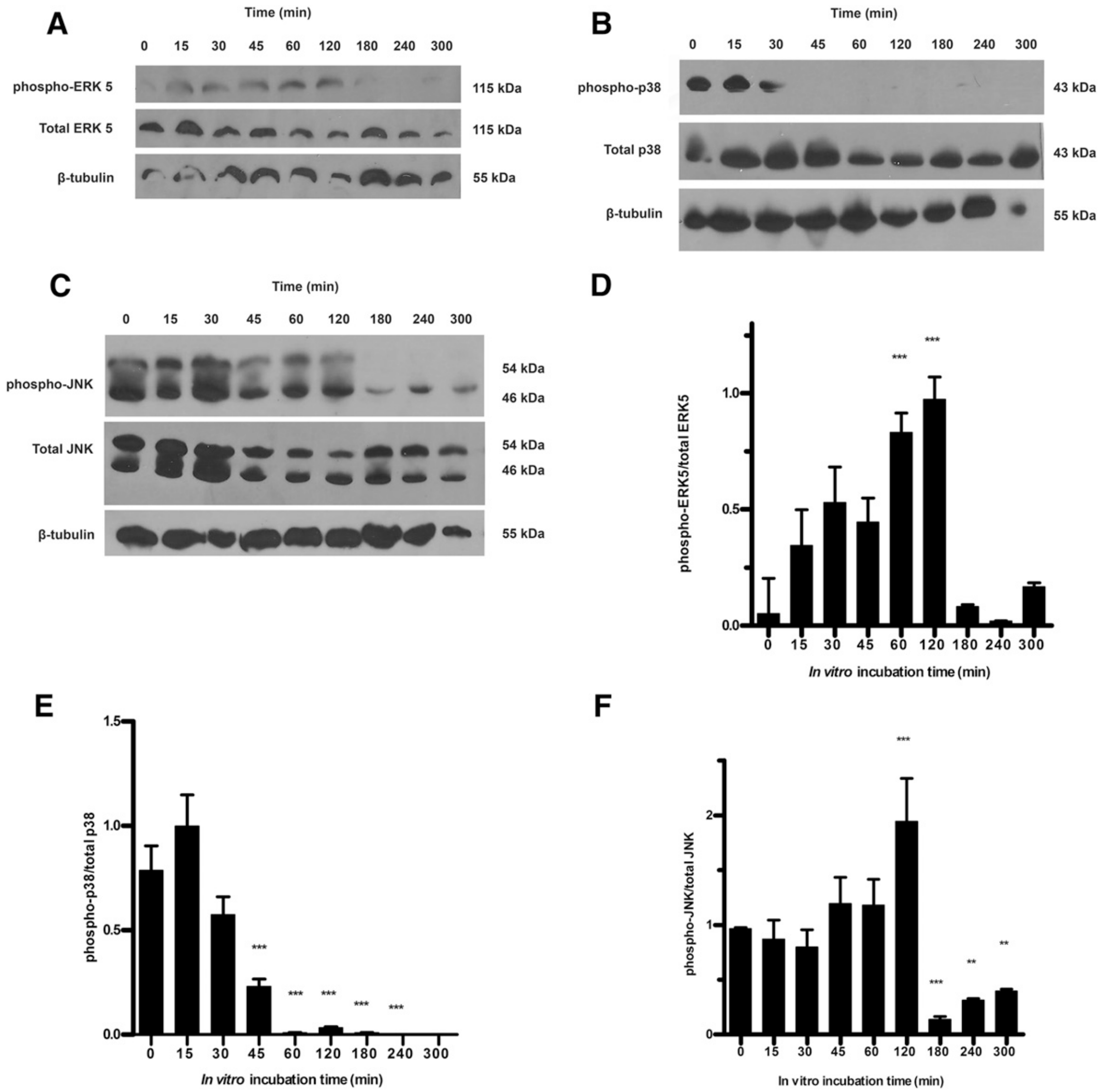

Fig. 6. Time course of MAPK activation after isolation and in vitro incubation of HUV. Tissues were incubated for the indicated times, and tissue lysates were then prepared. Equal volumes of lysate were separated by SDS-PAGE and immunoblotted with the indicated antibodies. Levels of (A) phosphorylated 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 ± 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 B_1 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 B_1 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 B_1 receptor upregulation process in isolated HUV, we first considered it necessary to evaluate the kinin B_1 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 B_1 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 B_1 receptor upregulation process in isolated HUVs.

To our knowledge, there is no evidence involving ERK5 in the B_1 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

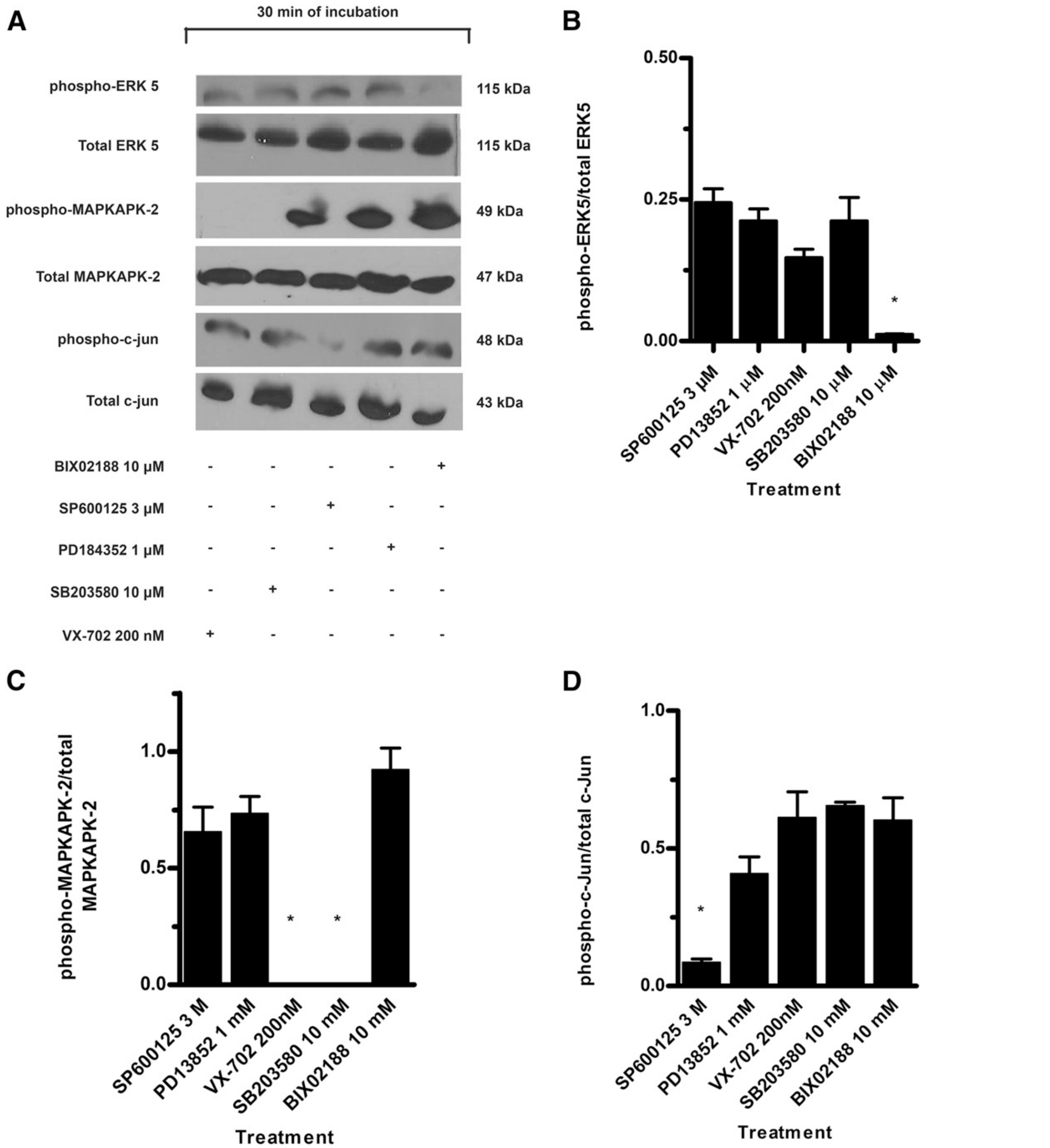

Fig. 7. Selective phosphorylation inhibition of different MAPK pathways in isolated HUV. (A) Representative blots showing the levels of phosphorylated and total ERK5, MAPKAPK-2, and c-Jun in HUV after 30 minutes of in vitro incubation in the presence or absence of SP600125 (3 μM), PD184352 (1 μM), VX-702 (200 nM), SB203580 (10 μM), or BIX02188 (10 μM). 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 ± 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 B_1 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 B_1 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 B_1 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 B_1 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 B_1 receptor upregulation process. Taken as a whole, the functional and molecular results support the hypothesis that the ERK5 signaling pathway clearly participates in kinin B_1 receptor upregulation in isolated HUVs.

The involvement of p38 MAPK in kinin B_1 receptor upregulation has been observed in many in vitro animal models: isolated rabbit aorta (Larrivée 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 B_1 receptor upregulation process. In human tissues, involvement of these kinases was observed only in osteoblastic osteosarcoma cell lines (Brechter 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 B_1 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 B_1 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 B_1 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 B_1 receptor upregulation. On the other hand, p38 MAPK and JNK selective inhibitors did not produced a direct toxic effect on HUV contractility or kinin B_1 receptor signal transduction, similar to the results obtained with the ERK5 inhibitor.

Regarding ERK1/2 relevance in kinin B_1 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 B_1 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 (Brechter et al., 2008), and murine tracheae (Zhang et al., 2007) demonstrated that ERK1/2 inhibition did not result in a significant reduction of kinin B_1 receptor upregulation process.

In the present study, we found that the maximum concentration of kinin B_1 receptor mRNA, as well as the maximum sensitivity to kinin B_1 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 B_1 receptor upregulation process. Considering that it has been well established that kinin B_1 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 B_1 receptors mRNA is involved in this process, and that evidence indicates the presence of NF-κB and AP-1 binding motifs in the 5′-flanking region of the human kinin B_1 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 B_1 receptor upregulation by an mRNA transcription induction rather than enhancing the stability of kinin B_1 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 B_1 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 B_1 receptor population after 5 hours of in vitro incubation (Sardi et al., 1998, 1999).

Kinin B_1 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 B_1 receptor expression in the airway, suggesting that MAPK-dependent kinin B_1 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 B_1 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 B_1 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 B_1

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 B_1 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 B_1 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.

Acknowledgments

The authors thank the Instituto Médico de Obstetricia (Ciudad Autónoma de Buenos Aires) and the Servicio de Obstetricia—Hospital General de Agudos, and Dr. José María Ramos Mejía (Ciudad Autónoma de Buenos Aires) for their efforts in providing umbilical tissues.

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.

References

Altura BM, Malaviya D, Reich CF, and Orkin LR (1972) Effects of vasoactive agents on isolated human umbilical arteries and veins. *Am J Physiol* **222**:345–355.

Bachvarov DR, Hess JF, Menke JG, Larrivée JF, and Marceau F (1996) Structure and genomic organization of the human B1 receptor gene for kinins (BDKRB1). *Genomics* **33**:374–381.

Baldwin AS, Jr (1996) The NF-kappa B and I kappa B proteins: new discoveries and insights. *Annu Rev Immunol* **14**:649–683.

Brechter AB, Persson E, Lundgren I, and Lerner UH (2008) Kinin B1 and B2 receptor expression in osteoblasts and fibroblasts is enhanced by interleukin-1 and tumour necrosis factor-alpha: effects dependent on activation of NF-kappaB and MAP kinases. *Bone* **43**:72–83.

Buschbeck M and Ullrich A (2005) The unique C-terminal tail of the mitogen-activated protein kinase ERK5 regulates its activation and nuclear shuttling. *J Biol Chem* **280**:2659–2667.

Calixto JB, Medeiros R, Fernandes ES, Ferreira J, Cabrini DA, and Campos MM (2004) Kinin B1 receptors: key G-protein-coupled receptors and their role in inflammatory and painful processes. *Br J Pharmacol* **143**:803–818.

Cargnello M and Roux PP (2011) Activation and function of the MAPKs and their substrates, the MAPK-activated protein kinases. *Microbiol Mol Biol Rev* **75**:50–83.

da Costa PL, Sirois P, Tannock IF, and Chammas R (2014) The role of kinin receptors in cancer and therapeutic opportunities. *Cancer Lett* **345**:27–38.

Couture R, Mizrahi J, Regoli D, and Devroede G (1981) Peptides and the human colon: an *in vitro* pharmacological study. *Can J Physiol Pharmacol* **59**:957–964.

DeLean A, Munson PJ, and Rodbard D (1978) Simultaneous analysis of families of sigmoidal curves: application to bioassay, radioligand assay, and physiological dose-response curves. *Am J Physiol* **235**:E97–E102.

Dray A and Perkins M (1993) Bradykinin and inflammatory pain. *Trends Neurosci* **16**:99–104.

Drummond GR and Cocks TM (1995) Endothelium-dependent relaxations mediated by inducible B1 and constitutive B2 kinin receptors in the bovine isolated coronary artery. *Br J Pharmacol* **116**:2473–2481.

Dunn C, Wiltshire C, MacLaren A, and Gillespie DA (2002) Molecular mechanism and biological functions of c-Jun N-terminal kinase signalling via the c-Jun transcription factor. *Cell Signal* **14**:585–593.

El Sayah M, Medeiros R, Fernandes ES, Campos MM, and Calixto JB (2006) Mechanisms underlying lipopolysaccharide-induced kinin B1 receptor up-regulation in the pig iris sphincter in vitro. *Mol Pharmacol* **69**:1701–1708.

Errasti AE, Luciani LI, Cesio CE, Tramontano J, Boveris D, Daray FM, Nowak W, Pelorosso FG, and Rothlin RP (2007) Potentiation of adrenaline vasoconstrictor response by sub-threshold concentrations of U-46619 in human umbilical vein: involvement of smooth muscle prostanoid TP(alpha) receptor isoform. *Eur J Pharmacol* **562**:227–235.

Félétou M, Martin CA, Molimard M, Naline E, Germain M, Thurieau C, Fauchère JL, Canet E, and Advenier C (1995) In vitro effects of HOE 140 in human bronchial and vascular tissue. *Eur J Pharmacol* **274**:57–64.

Fukushima E, Monoi N, Mikoshiba S, Hirayama Y, Serizawa T, Adachi K, Koide M, Ohdera M, Murakoshi M, and Kato H (2014) Protective effects of acetaminophen on ibuprofen-induced gastric mucosal damage in rats with associated suppression of matrix metalloproteinase. *J Pharmacol Exp Ther* **349**:165–173.

Ganju P, Davis A, Patel S, Núñez X, and Fox A (2001) p38 stress-activated protein kinase inhibitor reverses bradykinin B(1) receptor-mediated component of inflammatory hyperalgesia. *Eur J Pharmacol* **421**:191–199.

Gobeil F, Pheng LH, Badini I, Nguyen-Le XK, Pizard A, Rizzi A, Blouin D, and Regoli D (1996) Receptors for kinins in the human isolated umbilical vein. *Br J Pharmacol* **118**:289–294.

Haddad EB, Fox AJ, Rousell J, Burgess G, McIntyre P, Barnes PJ, and Chung KF (2000) Post-transcriptional regulation of bradykinin B_1 and B_2 receptor gene expression in human lung fibroblasts by tumor necrosis factor-alpha: modulation by dexamethasone. *Mol Pharmacol* **57**:1123–1131.

Hayashi M and Lee JD (2004) Role of the BMK1/ERK5 signaling pathway: lessons from knockout mice. *J Mol Med (Berl)* **82**:800–808.

Izawa Y, Yoshizumi M, Ishizawa K, Fujita Y, Kondo S, Kagami S, Kawazoe K, Tsuchiya K, Tomita S, and Tamaki T (2007) Big mitogen-activated protein kinase 1 (BMK1)/extracellular signal regulated kinase 5 (ERK5) is involved in platelet-derived growth factor (PDGF)-induced vascular smooth muscle cell migration. *Hypertens Res* **30**:1107–1117.

Karin M, Liu Zg, and Zandi E (1997) AP-1 function and regulation. *Curr Opin Cell Biol* **9**:240–246.

Kim M, Kim S, Lim JH, Lee C, Choi HC, and Woo CH (2012) Laminar flow activation of ERK5 protein in vascular endothelium leads to atheroprotective effect via NF-E2-related factor 2 (Nrf2) activation. *J Biol Chem* **287**:40722–40731.

Kyriakis JM and Avruch J (2001) Mammalian mitogen-activated protein kinase signal transduction pathways activated by stress and inflammation. *Physiol Rev* **81**:807–869.

Lagneux C, Lebrin F, Demenge P, Godin-Ribuot D, and Ribuot C (2001) MAP-kinase dependent activation of kinin B1 receptor gene transcription after heat stress in rat vascular smooth muscle cells. *Int Immunopharmacol* **1**:533–538.

Larrivée JF, Bachvarov DR, Houle F, Landry J, Huot J, and Marceau F (1998) Role of the mitogen-activated protein kinases in the expression of the kinin B1 receptors induced by tissue injury. *J Immunol* **160**:1419–1426.

Leeb-Lundberg LMF, Marceau F, Müller-Esterl W, Pettibone DJ, and Zuraw BL (2005) International union of pharmacology. XLV. Classification of the kinin receptor family: from molecular mechanisms to pathophysiological consequences. *Pharmacol Rev* **57**:27–77.

Li RC, Ping P, Zhang J, Wead WB, Cao X, Gao J, Zheng Y, Huang S, Han J, and Bolli R (2000) PK-Cepsilon modulates NF-kappaB and AP-1 via mitogen-activated protein kinases in adult rabbit cardiomyocytes. *Am J Physiol Heart Circ Physiol* **279**:1679–1689.

Linder AE, Gaskell GL, Szasz T, Thompson JM, and Watts SW (2010) Serotonin receptors in rat jugular vein: presence and involvement in the contraction. *J Pharmacol Exp Ther* **334**:116–123.

Livak KJ and Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Δ Δ C(T)) Method. *Methods* **25**:402–408.

Marceau F, Hess JF, and Bachvarov DR (1998) The B1 receptors for kinins. *Pharmacol Rev* **50**:357–386.

Marceau F, Levesque L, Drapeau G, Rioux F, Salvino JM, Wolfe HR, Seoane PR, and Sawutz DG (1994) Effects of peptide and nonpeptide antagonists of bradykinin B2 receptors on the venoconstrictor action of bradykinin. *J Pharmacol Exp Ther* **269**:1136–1143.

Medeiros R, Cabrini DA, Ferreira J, Fernandes ES, Mori MA, Pesquero JB, Bader M, Avellar MC, Campos MM, and Calixto JB (2004) Bradykinin B1 receptor expression induced by tissue damage in the rat portal vein: a critical role for mitogen-activated protein kinase and nuclear factor-kappaB signaling pathways. *Circ Res* **94**:1375–1382.

Morimoto H, Kondoh K, Nishimoto S, Terasawa K, and Nishida E (2007) Activation of a C-terminal transcriptional activation domain of ERK5 by autophosphorylation. *J Biol Chem* **282**:35449–35456.

Neubig RR, Spedding M, Kenakin T, and Christopoulos A; International Union of Pharmacology Committee on Receptor Nomenclature and Drug Classification (2003) International Union of Pharmacology Committee on Receptor Nomenclature and Drug Classification. XXXVIII. Update on terms and symbols in quantitative pharmacology. *Pharmacol Rev* **55**:597–606.

Ni A, Chao L, and Chao J (1998) Transcription factor nuclear factor kappaB regulates the inducible expression of the human B1 receptor gene in inflammation. *J Biol Chem* **273**:2784–2791.

Nishimoto S and Nishida E (2006) MAPK signalling: ERK5 versus ERK1/2. *EMBO Rep* **7**:782–786.

Nithianandarajah-Jones GN, Wilm B, Goldring CE, Müller J, and Cross MJ (2012) ERK5: structure, regulation and function. *Cell Signal* **24**:2187–2196.

Nowak W, Errasti AE, Armesto AR, Santín Velazque NL, and Rothlin RP (2011) Endothelial angiotensin-converting enzyme and neutral endopeptidase in isolated human umbilical vein: an effective bradykinin inactivation pathway. *Eur J Pharmacol* **667**:271–277.

Nowak W, Goldschmidt ED, Falcioni AG, Pugliese MI, Errasti AE, Pelorosso FG, Daray FM, Gago JE, and Rothlin RP (2007) Functional evidence of des-Arg10-kallidin enzymatic inactivating pathway in isolated human umbilical vein. *Naunyn Schmiedebergs Arch Pharmacol* **375**:221–229.

Pelorosso FG, Gago JE, Del Rey G, Menéndez SD, Errasti AE, and Rothlin RP (2009) The endocannabinoid anandamide inhibits kinin B_1 receptor sensitization through cannabinoid CB_1 receptor stimulation in human umbilical vein. *Eur J Pharmacol* **602**:176–179.

Pelorosso FG, Halperin AV, Palma AM, Nowak W, Errasti AE, and Rothlin RP (2007) Neutral endopeptidase up-regulation in isolated human umbilical artery: involvement in desensitization of bradykinin-induced vasoconstrictor effects. *J Pharmacol Exp Ther* **320**:713–720.

Pesquero JB, Araujo RC, Heppenstall PA, Stucky CL, Silva JA, Jr, Walther T, Oliveira SM, Pesquero JL, Paiva AC, and Calixto JB, et al. (2000) Hypoalgesia and altered inflammatory responses in mice lacking kinin B1 receptors. *Proc Natl Acad Sci USA* **97**:8140–8145.

Phagoo SB, Reddi K, Silvallana BJ, Leeb-Lundberg LM, and Warburton D (2005) Infection-induced kinin B1 receptors in human pulmonary fibroblasts: role of intact pathogens and p38 mitogen-activated protein kinase-dependent signaling. *J Pharmacol Exp Ther* **313**:1231–1238.

Plotnikov A, Zehorai E, Procaccia S, and Seger R (2011) The MAPK cascades: signaling components, nuclear roles and mechanisms of nuclear translocation. *Biochim Biophys Acta* **1813**:1619–1633.

Pruneau D, Bélichard P, Sahel JA, and Combal JP (2010) Targeting the kallikrein-kinin system as a new therapeutic approach to diabetic retinopathy. *Curr Opin Investig Drugs* **11**:507–514.

Regoli D, Marceau F, and Barabé J (1978) De novo formation of vascular receptors for bradykinin. *Can J Physiol Pharmacol* **56**:674–677.

Saccani S, Pantano S, and Natoli G (2002) p38-Dependent marking of inflammatory genes for increased NF-kappa B recruitment. *Nat Immunol* **3**:69–75.

Sardi SP, Ares VR, Errasti AE, and Rothlin RP (1998) Bradykinin B_1 receptors in human umbilical vein: pharmacological evidence of up-regulation, and induction by interleukin-1 beta. *Eur J Pharmacol* **358**:221–227.

Sardi SP, Daray FM, Errasti AE, Pelorosso FG, Pujol-Lereis VA, Rey-Ares V, Rogines-Velo MP, and Rothlin RP (1999) Further pharmacological characterization of bradykinin B_1 receptor up-regulation in human umbilical vein. *J Pharmacol Exp Ther* **290**:1019–1025.

Sardi SP, Errasti AE, Rey-Ares V, Rogines-Velo MP, and Rothlin RP (2000b) Bradykinin B_1 receptor in isolated human umbilical vein: an experimental model of the *in vitro* up-regulation process. *Acta Pharmacol Sin* **21**:105–110.

Sardi SP, Pérez H, Antúnez P, and Rothlin RP (1997) Bradykinin B_1 receptors in human umbilical vein. *Eur J Pharmacol* **321**:33–38.

Sardi SP, Rey-Ares V, Pujol-Lereis VA, and Rothlin RP (2000a) Retinoids inhibit bradykinin B_1 receptor-sensitized responses in human umbilical vein. *Eur J Pharmacol* **407**:313–316.

Sardi SP, Rey-Ares V, Pujol-Lereis VA, Serrano SA, and Rothlin RP (2002) Further pharmacological evidence of nuclear factor-κ B pathway involvement in bradykinin B_1 receptor-sensitized responses in human umbilical vein. *J Pharmacol Exp Ther* **301**:975–980.

Schulze-Osthoff K, Ferrari D, Riehemann K, and Wesselborg S (1997) Regulation of NF-kappa B activation by MAP kinase cascades. *Immunobiology* **198**:35–49.

Talbot S and Couture R (2012) Emerging role of microglial kinin B1 receptor in diabetic pain neuropathy. *Exp Neurol* **234**:373–381.

Tsai PW, Shiah SG, Lin MT, Wu CW, and Kuo ML (2003) Up-regulation of vascular endothelial growth factor C in breast cancer cells by heregulin-beta 1: a critical role of p38/nuclear factor-kappa B signaling pathway. *J Biol Chem* **278**:5750–5759.

Viel TA and Buck HS (2011) Kallikrein-kinin system mediated inflammation in Alzheimer's disease in vivo. *Curr Alzheimer Res* **8**:59–66.

Wang X and Tournier C (2006) Regulation of cellular functions by the ERK5 signalling pathway. *Cell Signal* **18**:753–760.

Whitmarsh AJ and Davis RJ (1996) Transcription factor AP-1 regulation by mitogen-activated protein kinase signal transduction pathways. *J Mol Med (Berl)* **74**:589–607.

Winer J, Jung CK, Shackel I, and Williams PM (1999) Development and validation of real-time quantitative reverse transcriptase-polymerase chain reaction for monitoring gene expression in cardiac myocytes *in vitro*. *Anal Biochem* **270**:41–49.

Zhang Y, Adner M, and Cardell LO (2004) Up-regulation of bradykinin receptors in a murine in-vitro model of chronic airway inflammation. *Eur J Pharmacol* **489**:117–126.

Zhang Y, Adner M, and Cardell LO (2007) IL-1β-induced transcriptional up-regulation of bradykinin B_1 and B_2 receptors in murine airways. *Am J Respir Cell Mol Biol* **36**:697–705.

Zhang Y, Cardell LO, Edvinsson L, and Xu CB (2013) MAPK/NF-κB-dependent upregulation of kinin receptors mediates airway hyperreactivity: a new perspective for the treatment. *Pharmacol Res* **71**:9–18.

Zhao J, Kyotani Y, Itoh S, Nakayama H, Isosaki M, and Yoshizumi M (2011) Big mitogen-activated protein kinase 1 protects cultured rat aortic smooth muscle cells from oxidative damage. *J Pharmacol Sci* **116**:173–180.

Zuzack JS, Burkard MR, Cuadrado DK, Greer RA, Selig WM, and Whalley ET (1996) Evidence of a bradykinin B1 receptor in human ileum: pharmacological comparison to the rabbit aorta B1 receptor. *J Pharmacol Exp Ther* **277**:1337–1343.

Address correspondence to: Rodolfo Pedro Rothlin, Paraguay 2155, 9° piso, Ciudad Autónoma de Buenos Aires (1121), Argentina. E-mail: farmaco3@fmed.uba.ar