Involvement of Extracellular Signal-regulated Kinase 5 in Kinin B₁ Receptor Up-regulation in Isolated Human Umbilical Vein

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Running Title: ERK5 in Kinin B1 Receptor Up-regulation in HUV

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Number of text pages: 39
Number of tables: 1
Numbers of figures: 7
Numbers of references: 64
Number of words:
Abstract: 250
Introduction: 741
Discussion: 1856

Abbreviations:; HUV, human umbilical vein; DAKD, des-Arg10-Kallidin; ERK5, extracellular signal-regulated kinase 5; MAPK, mitogen-activated protein kinases; JNK, c-Jun N-terminal kinase; ERK1/2, extracellular signal-regulated kinase 1/2; NF-κB, nuclear transcription factor-κB; AP-1, activator protein-1; MEKK, MAPK kinase kinase; MEK, MAPK kinase; CRC, Concentration-response curve; BAEC, bovine
artery endothelial cells; BLMEC, bovine lung microvascular endothelial cells; 5-HT, 5-hydroxytryptamine, serotonin; qRT-PCR quantitative real-time reverse transcription polymerase chain reaction; GAPDH, glyceraldehyde phosphate dehydrogenase; DMSO, dimethyl sulfoxide; E\textsubscript{max}, maximal response; n\textsubscript{H}, slope factor; ANOVA, one-way analysis of variance; VSMC, vascular smooth muscle cells.

**Recommended section assignment:** Inflammation, Immunopharmacology, and Asthma
Abstract

The up-regulated kinin B₁ receptors exert a pivotal role in modulating inflammatory processes. In isolated human umbilical vein (HUV), kinin B₁ receptor is up-regulated as a function of in vitro incubation time and pro-inflammatory stimuli. The aim of this study was to evaluate the involvement of extracellular signal-regulated kinase 5 (ERK5), p38 MAPK, c-Jun N-terminal kinase (JNK) and extracellular signal-regulated kinase 1/2 (ERK1/2) on kinin B₁ receptor up-regulation process in HUV, employing functional and biochemical methods. Real time PCR 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-Kallidine (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 (CRC) to DAKD after 5h-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-h post-isolation followed by a significant reduction in the last 3-h. None of the treatments modified the responses to serotonin (5-HT), an unrelated agonist, suggesting a specific effect on kinin B₁ receptor up-regulation. The present work provides for the first time pharmacological evidence indicating that ERK5 plays a significant role on kinin B₁ receptor up-regulation. 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 for a better understanding of MAPK involvement in inflammatory and immunological 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). While 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 knock-out 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 up-regulation phenomenon. In this sense, the 5′-flanking region of the human kinin B₁ 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 provide evidence that several pro-inflammatory cytokines are involved in kinin
B₁ receptor up-regulation through NF-κB activation (Baldwin, 1996; Ni et al., 1998; Marceau et al., 1998).

Our group demonstrated in HUV, that kinin B₁ receptor-mediated responses are potentiated by pro-inflammatory mediators like lipopolysaccharide, interleukine-1β and tumor necrosis factor-α and inhibited by anti-inflammatory mediators such as dexamethasone and retinoic acid probably by repressing the activity of NF-κB and AP-1 (Sardi et al., 1997; Sardi et al., 2000a). Furthermore, Sardi et al., (1999 and 2002) have demonstrated in this tissue, employing several pharmacological tools, that NF-κB activation plays a key role in the development of kinin B₁ 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 Davies, 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 non-transcriptional changes that result in specific cellular responses including cellular proliferation/differentiation or inflammation (Plotnikov et al., 2011). Several stimuli such as pro-inflammatory 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 (MEKK), 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 recent identified MAPK, is able to induce and regulate several physiological processes including proliferation, angiogenesis, immunological processes and stress responses (Plotnikov et al., 2011). However, there is less available information about this kinase than the other MAPK pathways and the full scope of its functions is not clear. In this sense, while many authors have postulated p38 MAPK, ERK1/2 and JNK as signaling pathways involved in the up-regulation of kinin B1 receptors (Larrivée et al., 1998; Zhang et al., 2004; Medeiros et al., 2004; Phagoo et al., 2005; El Sayah et al., 2006; Brecthter et al., 2008), there is still no evidence involving ERK5 in kinin B1 receptor up-regulation. The aim of this study was to evaluate the involvement of ERK5 and the others MAPK signaling pathways in kinin B1 receptor up-regulation process in our isolated HUV experimental model employing together 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-h. The cords were placed onto dissecting dishes containing Krebs’ solution. Veins were carefully dissected free from Warthon’s jelly using micro-dissecting instruments and cut into rings of approximately 3 mm width.

**Functional Studies.** To performed this 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).

CRCs to DAKD, a kinin B₁ receptor-selective agonist, were constructed after a 15-, 120- or 300-min *in vitro* incubation by cumulative addition, in 0.25 log₁₀ increments in order to determinate a tissue sensitization to DAKD. Only one agonist CRC was performed on a single ring.
With the purpose to evaluate 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-h equilibration period. Some HUV rings were continuously exposed to selective MAPK pathways inhibitors before cumulative addition of DAKD at 5-h. 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 min before and throughout the construction at 5-h of the CRC to the kinin B₁ receptor selective agonist, DAKD.

At the end of each CRC, serotonin (5-HT) 10 μM 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-h 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 (N₂) immediately after isolation until processed (basal conditions) and other were incubated for 120 or 300 min. In other series of experiments, rings were incubated for 5-h 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 and then were frozen in 
N₂ until processed.

RNA isolation, cDNA synthesis and quantitative real-time reverse transcription 
polymerase chain reaction 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-min *in vitro* incubation or as fresh, nonincubated 
tissue. In other series of experiments, HUV were snap-frozen after a 30-min *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 following 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); des-Arg¹⁰-Kallidine 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 
SelleckChem (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 in order to rule out any non-specific action of this solvent on the tonus or contractility of the tissue preparations as well as on the RNA isolation, cDNA synthesis, performance of qRT-PCR or mRNA levels. These experiments showed that 0.1% v/v DMSO fails to modify DAKD induced responses in HUV or GAPDH 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 ± S.E.M. All data are expressed as mean ± S.E.M. From each umbilical cordon 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 5-HT 10 µM. Responses obtained for each cord tested in the same group were averaged and then fitted to a four parameter logistic model expressed as follows: $Y = \alpha - E_{\text{max}} / 1 + (X / \text{EC}_{50})^{n_H + E_{\text{max}}}$; where $Y$ is the response; $X$, the arithmetic dose; $\alpha$, the response when $X=0$; $\text{EC}_{50}$, the agonist concentration that produces 50% of the maximal response; $E_{\text{max}}$, the maximal response and $n_H$, the slope factor (DeLean et al., 1978). Estimates for the parameters mentioned above were determined using Graph Pad Prism Version 4.00 (Graph Pad Software Inc., La Jolla, CA, USA). The $\text{EC}_{50}$ values were transformed into $\text{pEC}_{50}$ ($-\log \text{EC}_{50}$). 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 GAPDH endogenous control using $2^{-\Delta C_t}$ and $2^{-\Delta\Delta C_t}$.

Statistical analysis was performed by means of one-way analysis of variance (ANOVA) 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 IUPHAR 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 min of *in vitro* incubation yielded a pEC₅₀ 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 during 120 min, the response to DAKD 1 µM was significantly higher than after 15 min incubation (Eₘₐₓ: 74.33 ± 2.94%, *n*=9, *p*<0.05; Fig 1A). The incubation during 300 min produced a significant leftward shift of the CRC to DAKD compared to 120 min incubated tissues (pEC₅₀ 120 min: 8.23 ± 0.07, *n*=9, 300 min: 8.94 ± 0.02, *n*=12, *p*<0.001; Fig.1A) but the maximal response was not modified (Eₘₐₓ: 81.33 ± 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-h of *in vitro* incubation. In this respect, continuous exposure to BIX02188 10 µM produced a significant rightward shift of the CRC to DAKD (pEC₅₀: 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) while continuous exposure to a lower dose (1 µM) of this inhibitor failed to modified neither pEC_{50} nor maximal response (Fig. 2A, Table 1). In according with this results, qRT-PCR analysis demonstrated that continues treatment with BIX02188 10 µM produced a reduction of 60.37% in kinin B1 receptor mRNA expression after 5-h incubation when compared with control (P<0.05, Fig. 2B).

To rule out any toxic effect of BIX02188 10 µM, some HUV rings were incubated with the selective inhibitor 15 min before the construction at 5-h of the CRC to the kinin B1 receptor selective agonist. Under these experimental conditions, BIX02188 10 µM failed to modify kinin B1 receptor mediated responses induced by DAKD (Table 1). In addition, continues exposure to BIX02188 10 µM failed to affect the CRC to an unrelated agonist, 5-HT, in HUV rings after a 5-h 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). Taking together, both results 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-h 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 were 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

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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 ($E_{\text{max}}$: 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 continues 5-h treatment with SB203580 10 µM or VX-702 200 nM produced a reduction of 64.39% ($P<0.01$) and 55.92% ($P<0.05$), respectively, in kinin B$_1$ receptor mRNA levels when compared with control (Fig. 3C).

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 min before the construction at 5-h 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-h in vitro incubation were modified by continuous exposure to SB203580 10 µM (Fig. 3D, pEC$_{50}$ control: 8.16 ± 0.04, treated: 8.29 ± 0.03; $E_{\text{max}}$ 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; $E_{\text{max}}$ control: 90.11 ± 2.15, treated: 92.75 ± 2.46, $n$=7). Taking 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-h 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 pEC50 nor maximal response, were 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-h in vitro incubation in about 55.52% (P<0.05, Fig. 4B).

On the other hand, short exposure (15 min before the construction at 5-h of the concentration-response curve) to SP600125 3 µM failed to modify kinin B1 receptor mediated responses induced by DAKD (Table 1). Furthermore, continues exposure to SP600125 3 µM failed to affect the CRC to 5-HT in HUV rings after a 5-h 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). Taking together, these results indicate the lack of toxic effects of the JNK inhibitor on HUV 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 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-h in vitro incubation (Fig. 5A and Table 1). In line with functional results, continued treatment with PD184352 1 µM caused no significant change in B1 receptor mRNA levels after 5-h 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. 6A and 6D). *In vitro* incubation of isolated HUV resulted in a marked and time-dependent phosphorylation of ERK5 reaching the maximal levels between 60 and 120 min and returning to basal values after 180 min. On the other hand, the phosphorylation of p38 MAPK and JNK was detected under basal conditions (0 min) and similar level of phosphorylation was maintained up to 30 min of *in vitro* incubation, in the case of p38 MAPK (Fig. 6B and 6E) and to 60 min reaching the maximal levels at 120 min, in the case of JNK (Fig. 6C and 6F).

**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 min of *in vitro* incubation. The treatment of isolated HUV with BIX02188 10 µM in the first 30 min 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).

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

In relation with JNK, as shown in Fig. 6C, JNK was clearly phosphorylated after 30 min of *in vitro* incubation. The 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 up-regulation in a 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; Féélétou et al., 1995; Gobeil et al., 1996). On the other hand, in isolated HUV, our group have 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, 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, coronary and umbilical artery has been reported (Couture et al., 1981; Zuzack et al., 1996; Drummond and Cocks, 1995; Pelorosso et al., 2007).

To further study the kinin B₁ receptor up-regulation process in isolated HUV, we first considerate necessary to evaluate the kinin B₁ receptor mRNA expression under basal conditions and after 120 and 300 min 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 up-regulation process in isolated HUV.

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

The ERK5 is twice the size of other MAPK (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 heart, skeletal muscle, placenta, lungs and kidneys (Nithianandarajah-Jones et al., 2012) and there are reports of its presence in 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 up-regulation 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-h of incubation. A wide array of inhibitors of kinin B₁ receptor up-regulation have shown to be ineffective when applied to tissues only minutes before the agonist stimulation after 5-h of incubation (Pelorosso et al., 2009; Sardi et al., 1998; Sardi et al., 1999; Sardi et al., 2000a). In agreement with this, further analysis revealed that BIX02188 inhibitory effect is time dependent since a 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, CRC to an unrelated agonist, 5-HT, in HUV rings after a 5-h 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 affect the phosphorylation of other closely related MAPK.

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

The involvement of p38 MAPK in kinin B1 receptor up-regulation 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) demonstrated in rat portal vein the relevance of this kinase in kinin B1 receptor up-regulation process. In human tissues, the involvement of these kinases was observed only in osteoblastic osteosarcoma cell line (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 B1 receptor up-regulation 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-h of incubation were inhibited in a dose-dependent manner by continuous incubation with p38 MAPK and JNK selective inhibitors and this evidence was correlated with a significant reduction in kinin B1 receptor mRNA expression after 5h-in vitro incubation with these inhibitors in HUV. Moreover, the selectivity of p38 MAPK and JNK inhibitors at the concentration used in the present work, was confirm by Western Blot analysis in which the ability to inhibited MAPKAPK-2 and c-Jun phosphorylation, respectively, without affect the phosphorylation of other closely related MAPK was observed.

Furthermore, the 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 min for p38 MAPK and 120 min for JNK, coinciding with the early times of the kinin B1 receptor up-regulation process. These results, similarly with the observed with ERK5, strongly indicate that these p38 MAPK and JNK represent an important signaling pathway associated with kinin B1 receptor up-regulation. 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, similarly to the results obtained with the ERK5 inhibitor.

Regarding ERK1/2 relevance in kinin B1 receptor up-regulation, 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 HUV. In agreement with our results, in vitro studies in rat portal vein (Medeiros et al., 2004), human fetal lung
fibroblast (Phagoo et al., 2005), human lung fibroblast (Haddad et al., 2000),
oстеобластная остеосаркома клеточная линия (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 B₁ receptor up-regulation 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-h of VUH in vitro incubation. The evaluation of the time sequence of the results obtained in our tissue relative to the phosphorylation of the different MAPK, clearly indicates that these enzymes are at maximum functional activity during the first 2-h and a significant and marked reduction in phosphorylation was observed during the last 3-h, consistent with a participation in the early stages of the kinin B₁ receptor up-regulation 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 there are evidence indicating the presence of NF-κB 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 up-regulation by an mRNA transcription induction rather than enhancing the stability of kinin B₁ receptor mRNA.

In our study the lack of increase in the maximum response to DAKD in the sensitization process between 2-h and 5-h, 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, in our experimental model,
isolated HUV, has been suggested the presence of a proportion of spare receptors in the kinin B₁ receptor population after 5-h of *in vitro* incubation (Sardi et al., 1998, 1999).

It has been shown, that kinin B₁ receptors are central to the aetiology 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 up-regulation can provide a novel target for treatment of airway hyperreactivity in asthmatic 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 pain neuropathy (Talbot et al., 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 et al. (2011) demonstrated the participation of kinin B₁ receptor in neurodegenerative processes, suggesting a link between this receptor and the neuroinflammation in Alzheimer's disease. Moreover, da Costa et al. (2013) have shown evidence which 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 up-regulation in a human tissue which may be relevant for a better understanding of MAPK inhibitors effects on the mentioned pathological conditions. Furthermore, this study provides pharmacological and biochemical evidence indicating that ERK5 plays a novel clear and significant role in this process in a human tissue, thus ERK5 may be a new
therapeutic target for the rational development of pharmacotherapeutic tools for inflammatory and immunological diseases as well as painful process.

Acknowledgments

We wish to thank the Instituto Médico de Obstetricia (Ciudad Autónoma de Buenos Aires) and the Servicio de Obstetricia — Hospital General de Agudos 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 and Rothlin

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

Contributed new reagents or analytic tools: Errasti and Armesto

Performed data analysis: Kilstein and Armesto

Wrote or contributed to the writing of the manuscript: Kilstein and Rothlin
References


human lung fibroblasts by tumor necrosis factor-alpha: modulation by dexamethasone. 


**Footnotes**

This research was supported by grants from Universidad de Buenos Aires (UBA, 20020100100611) and Consejo de Investigaciones Científicas y Técnicas (CONICET — PIP2620), Argentina.
Figures Legends

**Fig. 1. A**, Concentration response curves to DAKD at 15 min (■, n=9), 120 min (△, n=9) and 300 min (○, 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 \( \text{pEC}_{50} \) values; #, \( P<0.05 \), significant differences between maximal responses.

**Fig. 2. A**, Concentration-response curves 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 determination made after a 5-h *in vitro* incubation, and the vertical lines show S.E.M. *****, \( P<0.001 \), significant differences between \( \text{pEC}_{50} \) values. **B**, Expression of B1 receptor mRNA at basal conditions or after 120 min and 300 min 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; *, \( P<0.05 \) and ***, \( P<0.001 \), significant differences between means.

**C**, Concentration-response curves 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 determination made after a 5-h *in vitro* incubation, and the vertical lines show S.E.M.
Fig. 3. A, Concentration-response curves to DAKD on control HUV rings (■, n=11) and on tissues continuously exposed to SB203580 1 μM (○, n=5) or 10 μM (△, n=7). B, Concentration-response curves to DAKD on control HUV 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 determination made after a 5-h in vitro incubation, and the vertical lines show S.E.M. **, P<0.01 and ***, P<0.001, significant differences between pEC50 values. C, Expression of kinin B1 receptor mRNA after a 5-h 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 and **, 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-h in vitro incubation, and the vertical lines show S.E.M.

Fig. 4. A, Concentration-response curves to DAKD on control HUV rings (■, n=9) and on tissues continuously exposed to SP600125 1 μM (△, n=7) or 3 μM (○, n=7). Each symbol represents the mean of n determination made after a 5-h 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 5h-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, Concentration-response curves 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 determination made after a 5-h in vitro incubation, and the vertical lines show S.E.M.
**Fig. 5.** A, Concentration-response curves to DAKD on control HUV rings (■, n=13) and on tissues continuously exposed to PD184352 0.1 μM (△, n=7) and 1 μM (○, n=7). Each symbol represents the mean of n determination made after a 5-h *in vitro* incubation, and the vertical lines show S.E.M. B, Expression of B_{1} receptor mRNA after a 5-h *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.

**Fig. 6,** Time course of MAPKs activation after isolation and *in vitro* incubation of HUV. Tissue 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 and ***, P<0.001, significantly different from non-incubated tissue (0 min).

**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 min of *in vitro* incubation in 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.

TABLE 1
Effect of various in vitro treatments on the concentration-response curves to DAKD in 5h-incubated HUV rings.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Period (h)</th>
<th>pEC₅₀</th>
<th>Eₘₐₓ</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Control</td>
<td>Treated</td>
<td>Control</td>
</tr>
<tr>
<td>BIX02188 (1 µM)</td>
<td>0-5</td>
<td>8.88 ± 0.04</td>
<td>8.87 ± 0.05</td>
<td>85.30 ± 1.68</td>
</tr>
<tr>
<td>BIX02188 (10 µM)</td>
<td>0-5</td>
<td>8.88 ± 0.04</td>
<td>8.08 ± 0.05***</td>
<td>85.30 ± 1.68</td>
</tr>
<tr>
<td>BIX02188 (10 µM)</td>
<td>4.75-5</td>
<td>8.81 ± 0.04</td>
<td>8.73 ± 0.08</td>
<td>86.90 ± 1.96</td>
</tr>
<tr>
<td>SB203580 (1 µM)</td>
<td>0-5</td>
<td>8.96 ± 0.05</td>
<td>8.76 ± 0.05</td>
<td>88.23 ± 2.00</td>
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<tr>
<td>SB203580 (10 µM)</td>
<td>0-5</td>
<td>8.96 ± 0.05</td>
<td>8.61 ± 0.07**</td>
<td>88.23 ± 2.00</td>
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<tr>
<td>SB203580 (10 µM)</td>
<td>4.75-5</td>
<td>8.81 ± 0.04</td>
<td>8.76 ± 0.05</td>
<td>86.90 ± 1.96</td>
</tr>
<tr>
<td>VX-702 (20 nM)</td>
<td>0-5</td>
<td>9.00 ± 0.02</td>
<td>8.97 ± 0.08</td>
<td>77.47 ± 1.13</td>
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<tr>
<td>VX-702 (200 nM)</td>
<td>0-5</td>
<td>9.00 ± 0.02</td>
<td>8.47 ± 0.03***</td>
<td>77.47 ± 1.13</td>
</tr>
<tr>
<td>Drug</td>
<td>pEC50 (Range)</td>
<td>E_max (Mean ± S.E.M.)</td>
<td>pEC50 (Mean ± S.E.M.)</td>
<td>5-HT (Mean ± S.E.M.)</td>
</tr>
<tr>
<td>--------------------</td>
<td>---------------</td>
<td>-----------------------</td>
<td>-----------------------</td>
<td>----------------------</td>
</tr>
<tr>
<td>VX-702 (200 nM)</td>
<td>4.75-5</td>
<td>8.81 ± 0.04</td>
<td>8.79 ± 0.09</td>
<td>86.90 ± 1.96</td>
</tr>
<tr>
<td>SP600125 (1 µM)</td>
<td>0-5</td>
<td>8.87 ± 0.03</td>
<td>8.83 ± 0.03</td>
<td>89.74 ± 1.47</td>
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<tr>
<td>SP600125 (3 µM)</td>
<td>0-5</td>
<td>8.87 ± 0.03</td>
<td>8.28 ± 0.04***</td>
<td>89.74 ± 1.47</td>
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<tr>
<td>PD184353 (0.1 µM)</td>
<td>0-5</td>
<td>8.85 ± 0.03</td>
<td>8.94 ± 0.06</td>
<td>80.12 ± 1.44</td>
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<tr>
<td>PD184353 (1 µM)</td>
<td>0-5</td>
<td>8.85 ± 0.03</td>
<td>8.75 ± 0.07</td>
<td>80.12 ± 1.44</td>
</tr>
</tbody>
</table>

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 pEC50 values.
Figure 3

A. Isometric contraction (% 5-HT) vs. Log [DAKD] (M)

B. Isometric contraction (% 5-HT) vs. Log [DAKD] (M)

C. B₁ receptor mRNA expression (relative to GAPDH)

D. Isometric contraction (% 5-HT) vs. Log [5-HT] (M)

5-h incubation

- Control
- SB203580 1 μM
- SB203580 10 μM
- VX-702 20 nM
- VX-702 200 nM
- SB203580 10 μM
Figure 5

A. Isometric contraction (%5-HT) vs. Log [DAKD] (M)

- Control
- PD184352 0.1 μM
- PD184352 1 μM

B. B_{1} receptor mRNA expression (relative to GAPDH)

- Control
- PD184352 1 μM

5-h incubation