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Title Page

Involvement of Extracellular Signal-regulated Kinase 5 in Kinin B_1 Receptor Up-regulation in Isolated Human Umbilical Vein

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Running Title Page

Running Title: ERK5 in Kinin B_1 Receptor Up-regulation in HUV

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Abbreviations:; HUV, human umbilical vein; DAKD, des-Arg^{10}-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

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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_{max}, maximal response; n_H ,slope factor; ANOVA, one-way analysis of variance; VSMC, vascular smooth muscle cells.

Recommended section assignment: Inflammation, Immunopharmacology, and Asthma

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Abstract

The up-regulated kinin B_1 receptors exert a pivotal role in modulating inflammatory processes. In isolated human umbilical vein (HUV), kinin B_1 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 B_1 receptor up-regulation process in HUV, employing functional and biochemical methods. Real time PCR 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}-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 B_1 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 B_1 receptor up-regulation. The present work provides for the first time pharmacological evidence indicating that ERK5 plays a significant role on kinin B_1 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.

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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). While 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 knock-out 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 up-regulation 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 provide evidence that several pro-inflammatory cytokines are involved in kinin

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B_1 receptor up-regulation through NF-κB activation (Baldwin, 1996; Ni et al., 1998; Marceau et al., 1998).

Our group demonstrated in HUV, that kinin B_1 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-kB 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 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 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

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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 B_1 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 B_1 receptor up-regulation. The aim of this study was to evaluate the involvement of ERK5 and the others MAPK signaling pathways in kinin B_1 receptor up-regulation process in our isolated HUV experimental model employing together functional and molecular methods.

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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-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 B_1 receptor-selective agonist, were constructed after a 15-, 120- or 300-min *in vitro* incubation by cumulative addition, in 0.25 $\log_{10}$ increments in order to determinate a tissue sensitization to DAKD. Only one agonist CRC was performed on a single ring.

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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 B_1 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 (N_2) 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

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nM, SP600125 3 μM, BIX02188 10 μM and PD184352 1 μM and then were frozen in N_2 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-Arg^{10}-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

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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 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. 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: $Y=\alpha-E_{max}/1+(X/EC_{50})n_H+E_{max}$; where Y is the response; X, the arithmetic dose; α, the response when X=0; EC_{50}, the agonist concentration that produces 50% of the maximal response; E_{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 EC_{50} values were transformed into pEC_{50} ($-\log EC_{50}$). The

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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 $2^{-\Delta Ct}$ and $2^{-\Delta\Delta Ct}$.

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).

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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 min 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 during 120 min, the response to DAKD 1 μM was significantly higher than after 15 min incubation (E_{max}: 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 (pEC_{50} 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 (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-h of *in vitro* incubation. In this respect, continuous exposure to BIX02188 10 μM produced a significant rightward shift of the CRC to DAKD (pEC_{50}: control 8.88

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± 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 B_1 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 B_1 receptor selective agonist. Under these experimental conditions, BIX02188 10 μM failed to modify kinin B_1 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 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-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_{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_{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). 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

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± 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, 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 B_1 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 B_1 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, 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). Taking 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-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 B_1 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

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observed under basal conditions (Fig. 6A and 6D). I*n 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 MAPKAKP-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).

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Discussion

Sensitization to kinin B_1 receptor agonists in isolated HUV is a *bona fide* system to study kinin B_1 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 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 have 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, 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, 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 B_1 receptor up-regulation process in isolated HUV, we first considerate necessary to evaluate the kinin B_1 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

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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 up-regulation process in isolated HUV.

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

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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 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 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 B_1 receptor up-regulation process. Taken as a whole, the functional and molecular results support the hypothesis that ERK5 signaling pathway clearly participates in kinin B_1 receptor up-regulation in isolated HUV.

The involvement of p38 MAPK in kinin B_1 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 B_1 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

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fibroblast (Phagoo et al., 2005) but it is important to mention that, although all these experiments evaluated the kinin B_1 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 B_1 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 B_1 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 B_1 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 B_1 receptor signal transduction, similarly to the results obtained with the ERK5 inhibitor.

Regarding ERK1/2 relevance in kinin B_1 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 B_1 receptor mRNA in HUV. In agreement with our results, *in vitro* studies in rat portal vein (Medeiros et al., 2004), human fetal lung

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fibroblast (Phagoo et al., 2005), human lung fibroblast (Haddad et al., 2000), osteoblastic osteosarcoma cell line (Brechter et al., 2008), and murine tracheae (Zhang et al., 2007) demonstrated that ERK1/2 inhibition did not resulted in a significant reduction of kinin B_1 receptor up-regulation 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-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 B_1 receptor up-regulation 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 there are evidence indicating 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 up-regulation by an mRNA transcription induction rather than enhancing the stability of kinin B_1 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 B_1 spare receptors in the HUV. In accordance with this, in our experimental model,

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isolated HUV, has been suggested the presence of a proportion of spare receptors in the kinin B_1 receptor population after 5-h of *in vitro* incubation (Sardi et al., 1998, 1999).

It has been shown, that kinin B_1 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 B_1 receptor expression in the airway, suggesting that MAPK-dependent kinin B_1 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 B_1 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 B_1 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 B_1 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 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 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

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therapeutic target for the rational development of pharmacotherapeutic tools for inflammatory and immunological diseases as well as painful process.

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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

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Zuzack J, Burkard M, Cuadrado D, Greer R, Selig W, and Whalley E (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.

Footnotes

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Figures Legends

Fig. 1. A, Concentration response curves to DAKD at 15 min (■, *n*=9), 120 min (Δ, *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 pEC_{50} values; #, $P<0.05$, significant differences between maximal responses. **B,** Expression of B_1 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.

Fig. 2. A, Concentration-response curves to DAKD on control HUV rings (■, *n*=11) and on tissues continuously exposed to BIX02188 1 μM (Δ, *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 pEC_{50} values. **B,** Expression of kinin B_1 receptor mRNA after a 5-h *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,** 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.

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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 (Δ, *n*= 7). **B,** Concentration-response curves 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 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 pEC_{50} values. **C**, Expression of kinin B_1 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 (Δ, *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 pEC_{50} values. **B,** Expression of kinin B_1 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.

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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-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

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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.

Treatment	Period (h)	pEC_{50}		E_{max}		*n*	
		Control	Treated	Control	Treated	Control	Treated
BIX02188 (1 μM)	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

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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

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.

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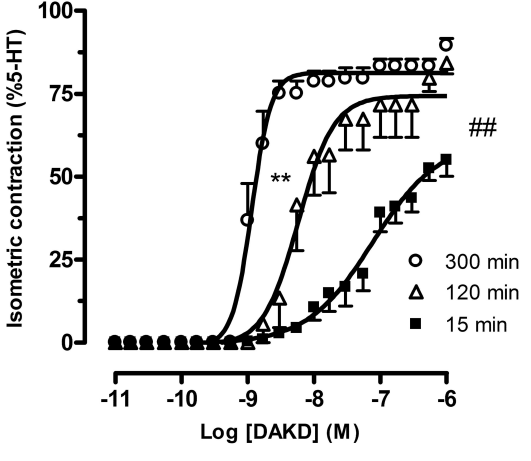
Figure 1

A.


Isometric contraction (%5-HT)
100
75
50
25
0
-11
-10
-9
-8
-7
-6
Log [DAKD] (M)
**
##
300 min
120 min
15 min

B.

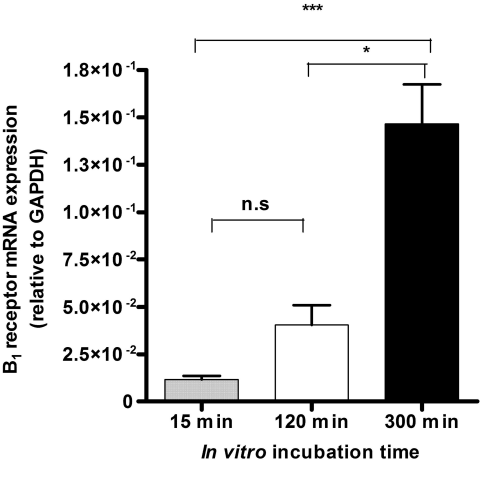
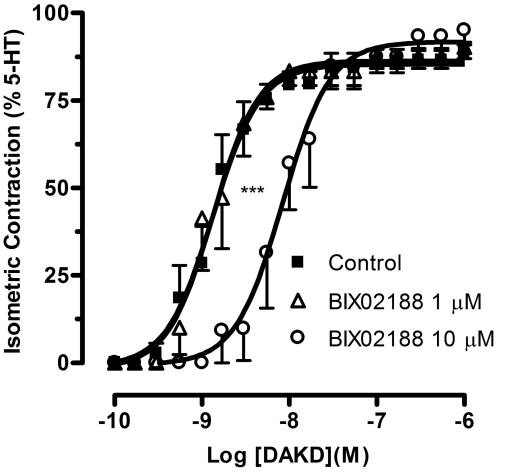
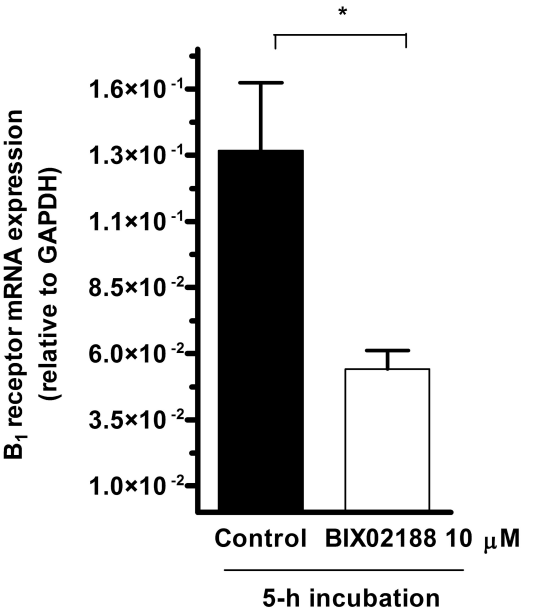

***
*
n.s
B_1 receptor mRNA expression (relative to GAPDH)
1.8×10^{-1}
1.5×10^{-1}
1.3×10^{-1}
1.0×10^{-1}
7.5×10^{-2}
5.0×10^{-2}
2.5×10^{-2}
0
15 min
120 min
300 min
In vitro incubation time

Figure 2

A.


Isometric Contraction (% 5-HT)
100
75
50
25
0
***
Control
BIX02188 1 μM
BIX02188 10 μM
-10
-9
-8
-7
-6
Log [DAKD](M)

B.


*
B1 receptor mRNA expression
(relative to GAPDH)
1.6×10-1
1.3×10-1
1.1×10-1
8.5×10-2
6.0×10-2
3.5×10-2
1.0×10-2
Control
BIX02188 10 μM
5-h incubation

C.

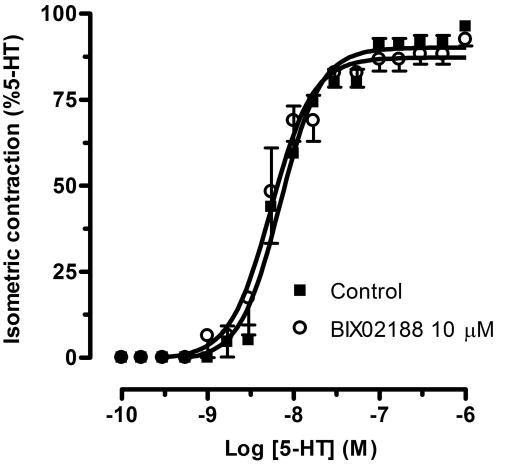

Isometric contraction (%5-HT)
100
75
50
25
0
Control
BIX02188 10 μM
-10
-9
-8
-7
-6
Log [5-HT] (M)

Figure 3

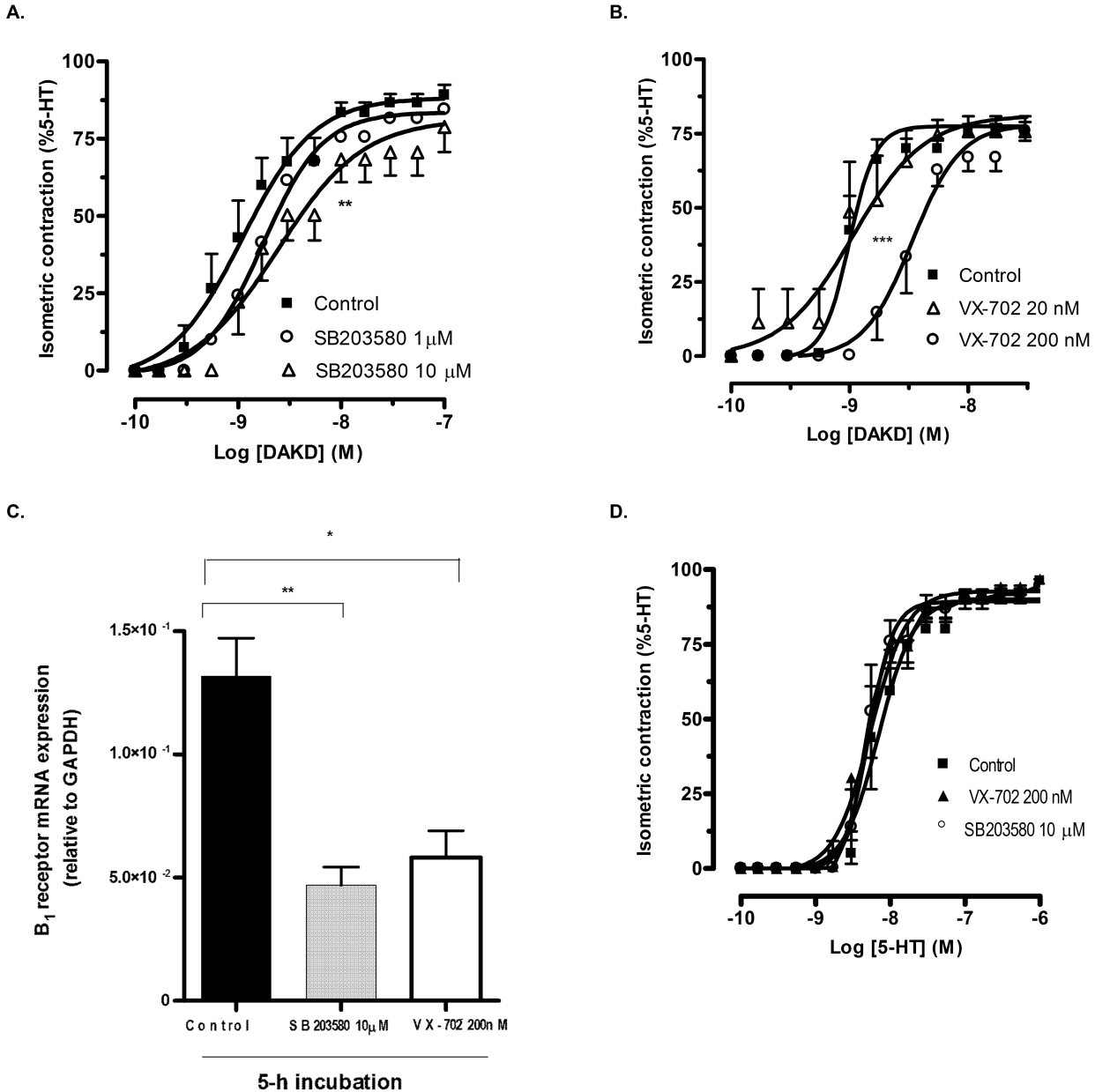
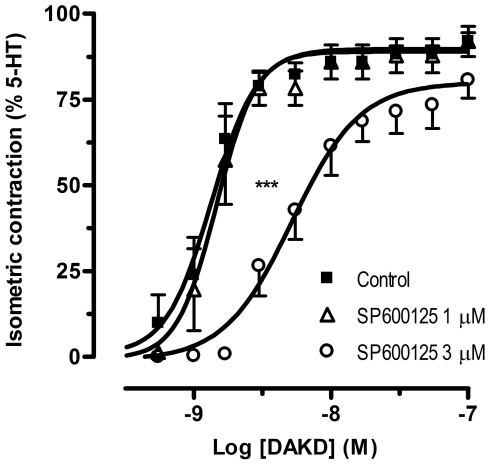
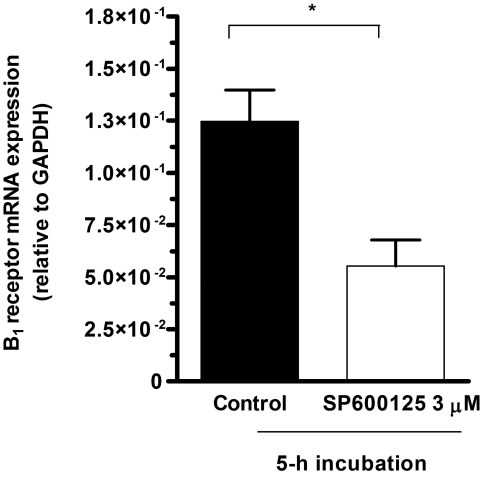

A.
Isometric contraction (%5-HT)
100
75
50
25
0
-10
-9
-8
-7
Log [DAKD] (M)
**
Control
SB203580 1 μM
SB203580 10 μM
B.
Isometric contraction (%5-HT)
100
75
50
25
0
-10
-9
-8
Log [DAKD] (M)
***
Control
VX-702 20 nM
VX-702 200 nM
C.
B1 receptor mRNA expression (relative to GAPDH)
1.5×10 -1
1.0×10 -1
5.0×10 -2
0
*
**
Control
SB 203580 10μM
VX-702 200n M
5-h incubation
D.
Isometric contraction (%5-HT)
100
75
50
25
0
-10
-9
-8
-7
-6
Log [5-HT] (M)
Control
VX-702 200 nM
SB203580 10 μM

Figure 4

A.


Isometric contraction (% 5-HT)
100
75
50
25
0
***
Control
SP600125 1 μM
SP600125 3 μM
-9
-8
-7
Log [DAKD] (M)

B.


*
B1 receptor mRNA expression (relative to GAPDH)
1.8×10-1
1.5×10-1
1.3×10-1
1.0×10-1
7.5×10-2
5.0×10-2
2.5×10-2
0
Control
SP600125 3 μM
5-h incubation

C.

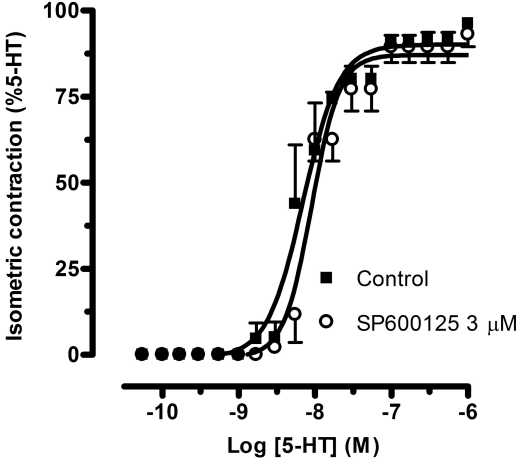

Isometric contraction (%5-HT)
100
75
50
25
0
Control
SP600125 3 μM
-10
-9
-8
-7
-6
Log [5-HT] (M)

Figure 5

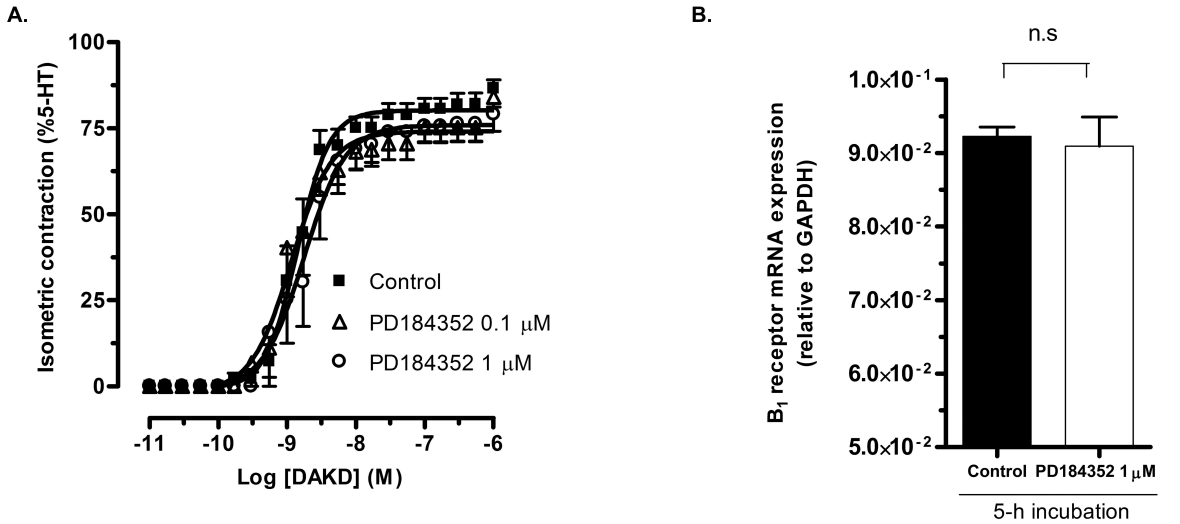

A.
Isometric contraction (%5-HT)
100
75
50
25
0
-11
-10
-9
-8
-7
-6
Log [DAKD] (M)
Control
PD184352 0.1 μM
PD184352 1 μM
B.
n.s
B1 receptor mRNA expression (relative to GAPDH)
1.0×10-1
9.0×10-2
8.0×10-2
7.0×10-2
6.0×10-2
5.0×10-2
Control
PD184352 1 μM
5-h incubation

Figure 6

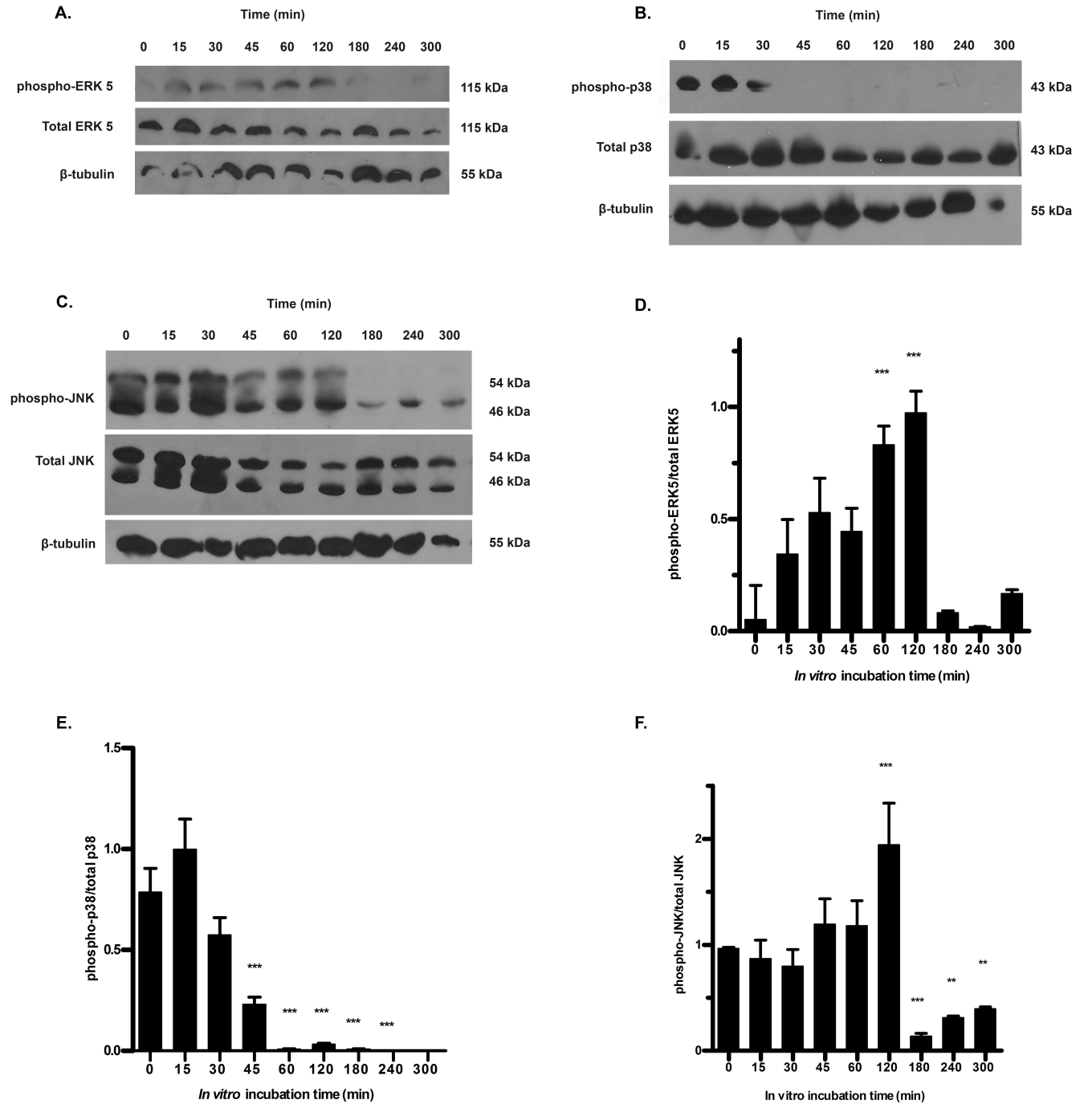
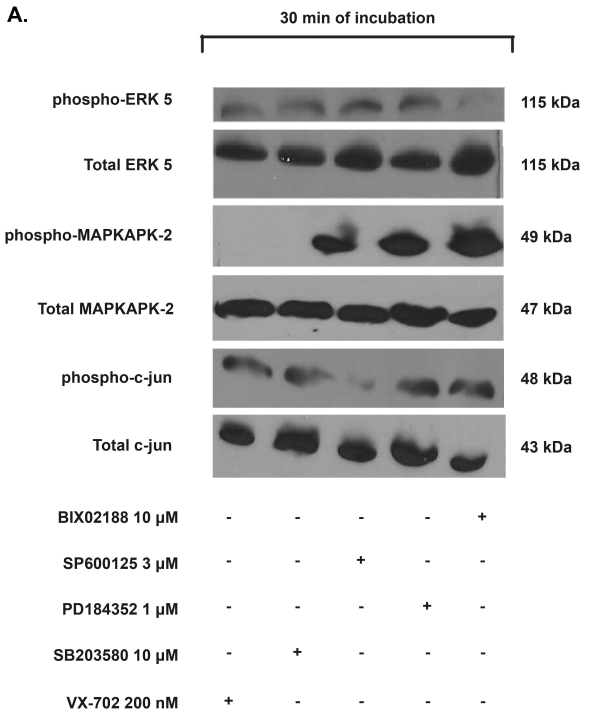
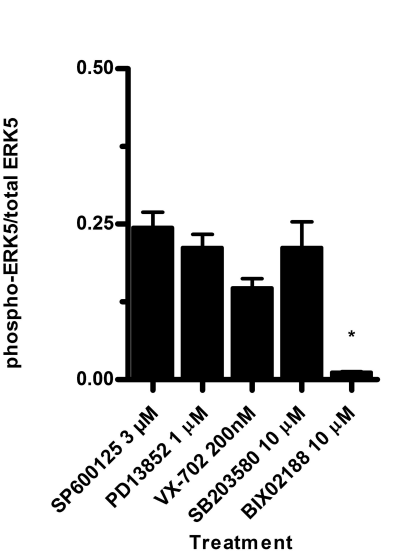
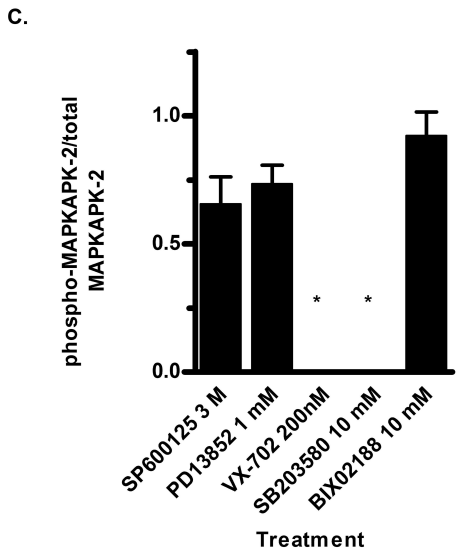
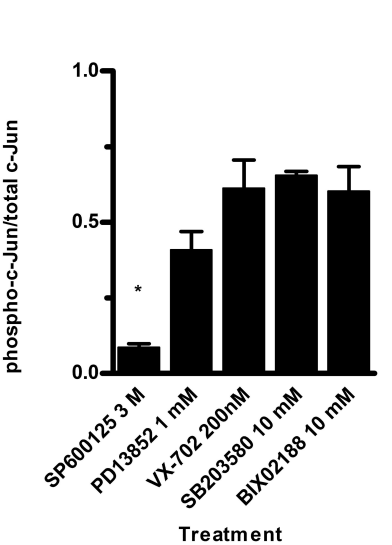

A.
Time (min)
0 15 30 45 60 120 180 240 300
phospho-ERK 5
Total ERK 5
β-tubulin
115 kDa
115 kDa
55 kDa
B.
Time (min)
0 15 30 45 60 120 180 240 300
phospho-p38
Total p38
β-tubulin
43 kDa
43 kDa
55 kDa
C.
Time (min)
0 15 30 45 60 120 180 240 300
phospho-JNK
Total JNK
β-tubulin
54 kDa
46 kDa
54 kDa
46 kDa
55 kDa
D.
phospho-ERK5/total ERK5
0.0
0.5
1.0
***
***
0 15 30 45 60 120 180 240 300
In vitro incubation time (min)
E.
phospho-p38/total p38
0.0
0.5
1.0
1.5
***
***
***
***
0 15 30 45 60 120 180 240 300
In vitro incubation time (min)
F.
phospho-JNK/total JNK
0
1
2
***
***
**
**
0 15 30 45 60 120 180 240 300
In vitro incubation time (min)

Figure 7


A.
30 min of incubation
phospho-ERK 5
115 kDa
Total ERK 5
115 kDa
phospho-MAPKAPK-2
49 kDa
Total MAPKAPK-2
47 kDa
phospho-c-jun
48 kDa
Total c-jun
43 kDa
BIX02188 10 µM - - - - +
SP600125 3 µM - - + - -
PD184352 1 µM - - - + -
SB203580 10 µM - + - - -
VX-702 200 nM + - - - -


B.
phospho-ERK5/total ERK5
0.50
0.25
0.00
*
SP600125 3 µM
PD13852 1 µM
VX-702 200nM
SB203580 10 µM
BIX02188 10 µM
Treatment


C.
phospho-MAPKAPK-2/total MAPKAPK-2
1.0
0.5
0.0
* *
SP600125 3 M
PD13852 1 mM
VX-702 200nM
SB203580 10 mM
BIX02188 10 mM
Treatment


D.
phospho-c-Jun/total c-Jun
1.0
0.5
0.0
*
SP600125 3 M
PD13852 1 mM
VX-702 200nM
SB203580 10 mM
BIX02188 10 mM
Treatment

Supplemental Methods

Involvement of Extracellular Signal-regulated Kinase 5 in Kinin B_1 Receptor Up-regulation in Isolated Human Umbilical Vein. Yael Kilstein, Wanda Nowak, Andrea Emilse Errasti, Antía Andrea Barcia Feás, Arnaldo Raúl Armesto, Facundo Germán Pelorosso and Rodolfo Pedro Rothlin, Journal of Pharmacology and Experimental Therapeutics.

Materials and Methods

Functional Studies Protocols from previous studies from our group (Sardi et al, 1997, 1998, 1999, 2000, 2002; Errasti et al., 2007; Nowak et al., 2007, 2011; Pelorosso et al.,2009) were followed. Briefly, immediately after dissection, rings were suspended in 10-ml organ baths and stretched with an optimal resting tension of 3 to 5 g (Errasti et al., 1999). Changes in tension were measured with Grass isometric force transducers (FT-03C; Grass Instruments, Quincy, MA) and displayed on Grass polygraphs (model 7D). During incubation, the Krebs' solution was maintained at 37°C and pH 7.4 by constant bubbling with 95% O_2/5% CO_2. Bath solution was replaced with fresh warmed Krebs' solution every 15 min and test agents were added after every wash. After 70 min of incubation, each preparation was contracted with KCl 40 mM and then washed with warm Krebs' solution to restore the basal tension. This procedure was designed to test the functional state of the tissue.

The choice of the inhibitors and the concentrations to be used was decided after a search of the literature for investigations describing the kinetic properties of the agents.

To inhibit ERK5 catalytic activity, we used BIX02188, with a reported IC_{50} of 0.8 µM in bovine artery endothelial cells (BAEC) or bovine lung microvascular endothelial cells (BLMEC) (Li et al., 2008). This drug also inhibits the catalytic activity of the upstream MEK5, with an IC_{50} of 4.3

nM without inhibition of the relative closely kinase MEK1/2 (Tatake et al., 2008). The effective concentration for this inhibitor was 10 µM, a 10-fold higher concentration than the respective IC_{50}, which ensure a significant but selective inhibition of the ERK5 catalytic activity without inhibit other closely related MAPK (Li et al., 2008, Tatake et al., 2008, Obara et al., 2009, Badshah et al., 2014).

To inhibit p38 MAPK catalytic activity, we used SB203580 1 and 10 µM, the classic and first reported inhibitor of p38 MAPK by competing binding in the ATP pocket, with an IC_{50} of 0.3 µM in murine lymphocytes CT6 cells (Crawley et al., 1997) and an IC_{50} of 1.59 in rat aorta (Yang et al., 2002). Although this compound has been very useful, SB203580 is also able to inhibit JNK (IC_{50}: 3-10 µM, rat cardiac myocytes, Clerk et al., 1998) among other kinases (Bain et al., 2007). To achieve a selective inhibition, we used a more potent and selective ATP-competitive inhibitor of both p38α and p38β MAPK isoforms, VX-702, that has no appreciable affinity for other related MAPK pathways (Kuliopulos et al; 2004; Ding, 2006; Damjanov et al., 2009; Goldstein et al., 2010). The effective concentration was 200 nM, a 10-fold higher concentration than the IC_{50} reported in human platelets (IC_{50} 4-20 nM, Kuliopulos et al., 2004), which ensure a significant but selective inhibition of p38 MAPK.

In relation to JNK, we used an inhibitor of JNK, SP600125, which competes with ATP to inhibit the enzyme, in concentration of 1 and 3 µM. The reported IC_{50} was 0.04 -0.19 µM (Bennett et al., 2001; Han et al., 2001, Woo et al., 2005; Jing et al., 2008). It is important to mention that this compound presents negligible activity over ERK1/2 or p38 MAPK in a concentration of 3 µM (Lahti et al., 2003).

To selectively inhibit ERK1/2 we used PD184352 0.1 and 1 µM, an ATP non-competitive inhibitor of the activation of ERK1/2, by blocking the upstream enzyme MEK1/2 activity, with a

reporter IC_{50} of 17 nM (Sebolt-Leopold et al., 1999). It has been reported that PD184352 is 100-fold more selective for MEK1/2 than MEK5, and concentrations of it that inhibits MEK1/2 (0.1-1 μM) do not inhibit MEK5-ERK5 although higher concentration (10 μM) could inhibit this kinase (Mody et al, 2001; Bain et al., 2007; Li et al., 2008, Rovida et al., 2008). In this sense, the drug inhibits ERK5 and JNK activation with an IC_{50} of 14.4 μM and 40 μM respectively, but none of these kinases were inhibited at 1 μM in BAEC and BLMEC (Li et al., 2008). All together this evidence suggests that the concentration of PD1843352 employed in the present work was optimal to block ERK1/2 activation specifically without affecting JNK or ERK5.

RNA Isolation, cDNA Synthesis and Quantitative Real-Time Reverse Transcription Polymerase Chain Reaction. In these experiments, tissues were collected as described in previous studies from our group (Errasti et al., 2007). HUV were carefully dissected out of cords free of surrounding tissues, rinsed in ice-cold Krebs solution, cut in small pieces and blotted dry. Some rings were frozen in liquid nitrogen (N_2) immediately after isolation until processed (basal conditions) and other were incubated in 10-ml organ baths (Thermostatic bath model Dubnoff, Vicking, Quincy, MA) with modified Krebs' solution maintained at 37°C and pH 7.4 by constant bubbling with 95% O_2/5% CO_2. Bath solution was replaced every 15 min. After the incubation period, tissues were frozen in N_2 until processed.

RNA isolation, cDNA synthesis and quantitative real-time reverse transcription polymerase chain reaction were performed as described by Linder et al (2010) and Fukushima et al (2014). Briefly, frozen tissues were pulverized to powder with a mortar and pestle under N_2 and total RNA was extracted from approximately 40 mg of each frozen tissue by using RNAeasy mini kit (Quiagen, Hilden, Germany) essentially as described by the supplier. All RNA samples were

subjected to on-column DNase I (Quiagen) treatment to remove trace amounts of genomic DNA. RNA purity and concentration were assessed by using a Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA) and stored at −70 °C for later use.

cDNA synthesis was attained by using iScript cDNA Synthesis kit (Bio-Rad, Hercules, CA) from approximately 0.5 µg of total RNA, according the manufacturer's instructions, by using random hexamer primers.

Quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) was performed to amplify kinin B_1 receptor mRNA. Kapa SYBR Fast qPCR kit Master Mix (2x) Universal (Kapabiosystems, Wilmington, MA) was used for detection of amplified products. qRT-PCR was performed by using the ABI 7500 (Applied Biosystems by Life Technologies, Grand Island, NY). The cycling conditions were as follows: 95°C for 3 min followed by 40 cycles of 95ºC for 15 s and 60ºC for 1 min. Each 20 µl reaction contained approximately 1 µl of cDNA from each sample, 0.4 µl of specific forward and reverse primers (IDT synthesis) for each gene in nuclease-free water, 0.4 µl of Rox Low and 10 µl of Kapa SYBR Fast qPCR kit Master Mix (2x) Universal. An equal amount of template cDNA was used for each sample.

The reaction plate was run in triplicated for every condition. Target gene expression was normalized to housekeeping gene glyceraldehyde phosphate dehydrogenase (GAPDH) in the same sample. Primer specificity was determined by analysis of dissociation (melt) curves (95°C for 1 min, 55°C for 30 s and 95°C for 1 min). A single peak in the rate of change of fluorescence with temperature indicated primer specificity.

The specific primers used in the reaction were: for human kinin B_1 receptor (Christiansen et al., 2002): Sense: 5'-CAACTGAACGTGGCAGAAATCTAC-3' Antisense: 5′-CAAGCCCAAGACAAACACCAG-3'; for human GAPDH (Christiansen et al., 2002): Sense: 5'-

GGGAAGGTGAAGGTCGGAGT-3'; Antisense: 5′-TCCACTTTACCAGAGTTAAAAGCAG-3'. To ensure that subsequent amplification did not derive from contaminant genomic DNA and/or from primer dimer formation, a control without reverse transcriptase was included in parallel for each RNA sample. The amount of mRNA was calculated using the comparative Ct method, which determines the amount of target normalized to an endogenous reference.

Change in expression was calculated as $2^{-\Delta Ct}$ and $2^{-\Delta\Delta Ct}$, where $\Delta Ct = Ct_{target} - Ct_{Gapdh}$ and $\Delta\Delta Ct = (\Delta Ct_{target} - \Delta Ct_{Gapdh})_{treated} - (\Delta Ct_{target} - \Delta Ct_{Gapdh})_{control}$.

Western Blot Analysis. Protocols from previous studies from our group (Errasti et al., 2007; Pelorosso et al., 2007) were followed. In these experiments HUV were carefully dissected out of cords free of surrounding tissues, rinsed in ice-cold Krebs solution, cut in small pieces and blotted dry. Some rings were frozen in liquid nitrogen (N_2) immediately after isolation until processed (basal conditions) and other were incubated in 10-ml organ baths (Thermostatic bath model Dubnoff, Vicking, Quincy, MA) with modified Krebs' solution maintained at 37°C and pH 7.4 by constant bubbling with 95% O_2/5% CO_2. Bath solution was replaced every 15 min. After the incubation period, tissues were frozen in N_2 until processed. On the day of the experiment, HUV (500 mg approximately) were pulverized to powder and then resuspended and homogenized for 2-h in ice-cold rapid immunoprecipitation assay (RIPA) lysis buffer containing protease and phosphatase inhibitors (Complete Mini and PhosSTOP, Roche Diagnostics, Basel, Switzerland). Whole-cell extracts were prepared by centrifugation at 14.000g for 15 min at 4°C to remove cellular debris. The supernatant was recovered and protein concentration was determined by Bradford method (Bradford, 1976) using Bio-Rad Kit (Bio-Rad Laboratories, Hercules, CA, USA).

Proteins (100 μg) were resuspended on loading buffer and boiled at 100 °C for 5 min. Samples were loaded to 10% sodium dodecyl sulphate (SDS)-polyacrylamide electrophoresis gel and ran at 120 V for 1 h. After running, the gel was put into a semi-dry blotting system (Bio-Rad, Hercules, CA, USA), and the proteins were transferred onto a polyvinylidene fluoride (PVDF) membrane (PALL, Port Washington, NY, USA) at 140 mA for 1 h (Towbin et al., 1979). Ponceau Red staining of the membrane verified equal gel loading. Membranes were blocked in Tris-buffered saline (TBS) containing 0.5% Tween 20 (TTBS) and 3% bovine serum albumin (BSA) and then incubated overnight at 4 °C with either anti-phosphorylated-ERK5, anti-phosphorylated-p38 MAPK or anti-phosphorylated-JNK antibodies (#3371, #9211, #9251; Cell Signaling Technology, Beverly, MA, USA) [1:500 to 1:1000 (v/v) dilution in blocking solution]. Membranes were washed four times for 15 min each in TTBS prior to incubation with horseradish peroxidase-conjugated anti-rabbit secondary antibody (#7074; Cell Signaling Technology, Beverly, MA, USA) [1:1000 (v/v) dilution in blocking solution] for 1 h. Excess secondary antibody was removed by washing the membranes were washed three times for 15 min each in TTBS and then given a final 15 min wash with TBS. The bound secondary antibody was detected by enhanced chemiluminescence reagent (ECL Western blotting detection Reagents, Amersham Pharmacia Biotech Inc, Piscataway, NJ, USA) and then exposed to autoradiographic double-emulsion film (Agfa Ortho CP-G plus 100 NIF, Agfa-Gevaert S.A., Argentina). The molecular sizes of the proteins were determined by comparison with standard proteins (Bio-Rad Laboratories, Hercules, CA, USA) in an adjacent lane. The immunoblot was subsequently stripped and reprobed with an antibody to the corresponding nonphosphorylated protein, ERK5, p38 MAPK or JNK (#3372, #9212, #9252; Cell Signaling Technology, Beverly, MA, USA) [1:500 to 1:1000 (v/v) dilution in

blocking solution]. The total amount of protein present in each sample was assessed on the same membrane using anti-β-tubulin (#2128; Cell Signaling Technology, Beverly, MA, USA).

To evaluate the effect of selective inhibitors on MAPK phosphorylation, 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. Membranes were incubated overnight at 4 °C with either anti-phosphorylated-ERK5, anti-phosphorylated- MAPKAPK-2 or anti-phosphorylated-c-Jun (#3371, #3007, #9261; Cell Signaling Technology, Beverly, MA, USA) [1:500 to 1:1000 (v/v) dilution in blocking solution] and subsequently stripped and reprobed with an antibody to the corresponding non-phosphorylated protein, ERK5, MAPKAPK-2 and c-Jun, (#3372, #3042,#9165; Cell Signaling Technology, Beverly, MA, USA) [1:500 to 1:1000 (v/v) dilution in blocking solution] . Negative controls were carried out in the absence of the primary antibody. Immunoreactive bands were detected by enhanced chemiluminescence, scanned, and quantified using GelAnalyzer 2010a software.

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