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Title page

Title: Neutral endopeptidase up-regulation in isolated human umbilical artery: involvement in desensitization of bradykinin-induced vasoconstrictor effects.

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Running title page

Running title: NEP up-regulation and desensitization of bradykinin effects.

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Abstract

Previous reports show that bradykinin B_2 receptors (BKB_2) mediate contractile responses induced by bradykinin (BK) in human umbilical artery (HUA). However, though it has been reported that BK-induced responses can desensitize in several inflammatory models, the effects of prolonged *in vitro* incubation on BK-induced vasoconstriction in HUA have not been studied. In isolated HUA rings, BK-induced responses after a 5-h *in vitro* incubation showed a marked desensitization compared to responses at 2-h. Inhibition of either angiotensin converting enzyme (ACE) or neutral endopeptidase (NEP), both BK-inactivating enzymes, failed to modify responses to BK at 2-h. After 5-h, ACE inhibition produced only a slight potentiation of BK-induced responses. In contrast, BK-induced vasoconstriction at 5-h was markedly potentiated by NEP inhibition. Moreover, NEP activity, measured by hydrolysis of its synthetic substrate (Z-Ala-Ala-Leu-p-Nitroanilide), showed a 2.4-fold increase in 5-h incubated vs. 2-h-incubated tissues which was completely reversed by cycloheximide (CHX) treatment. Furthermore, CHX significantly potentiated BK-induced responses suggesting that NEP-mediated kininase activity increase at 5-h depends on *de novo* protein synthesis. What's more, under NEP inhibition, CHX treatment failed to produce an additional potentiation of BK-induced vasoconstriction. Still, NEP up-regulation was confirmed by Western blot showing a 2.1-fold increase in immunoreactive-NEP in 5-h-incubated vs. 2-h-incubated HUA. In summary, the present study provides strong pharmacological evidence that NEP is up-regulated and plays a key role in desensitization of BK-induced

vasoconstriction following prolonged *in vitro* incubation in HUA. Our results provide new insights into the possible mechanisms involved in BK-induced responses desensitization during sustained inflammatory conditions.

Introduction

Functional (Regoli *et al.*, 1977) and molecular studies (McEachern *et al.*, 1991; Menke *et al.*, 1994) have unveiled the existence of two bradykinin (BK) receptor subtypes in mammalian tissues, BKB_2 and BKB_1. Whereas BKB_2 receptor expression is constitutive and mediates most of the *in vivo* effects of kinins (Bathon and Proud, 1991), BKB_1 receptor is not present in any significant amount in normal tissues and its expression is often inducible rather than constitutive (Regoli *et al.*, 1977; Sardi *et al.*, 1997). *De novo* synthesis of BKB_1 receptor can be induced during tissue isolation trauma and incubation or under certain pathophysiological conditions conveying tissue injury or inflammation (Leeb-Lundberg *et al.*, 2005). This molecular phenomenon results in a marked sensitization of responses elicited by BKB_1 receptor agonists (Marceau *et al.*, 1998). In contrast, it has been shown that BKB_2 receptor mediated responses can be desensitized, through mechanisms not yet fully elucidated, following prolonged exposure to pro-inflammatory stimuli (Cruwys *et al.*, 1994; Campos *et al.*, 1996; El Sayah *et al.*, 2006). Though it has been shown that BK-induced contractile responses in human umbilical artery (HUA) are mediated by BKB_2 receptor subtype activation (Feletou *et al.*, 1995; Abbas *et al.*, 1998), possible changes in BK-mediated effects after prolonged *in vitro* incubation have not been yet analyzed in this tissue.

Whereas receptor expression is a major determinant of kinins´ actions, agonist production and inactivation rates have been shown to play an important role in BKB_1 and BKB_2 receptor mediated effects (Marceau *et al.*, 1998). Vascular

inactivation of BK can be mediated by several enzymes. Among them angiotensin converting enzyme (ACE) has been classically described as the main inactivator of BK in vascular tissues (Erdos, 1990). In addition, Gafford et al. (1983) have shown that neutral endopeptidase (NEP) is able to hydrolyze BK, yielding inactive fragments. Interestingly, we have recently shown that ACE and NEP are present in HUA smooth muscle after prolonged *in vitro* incubation (Pelorosso *et al.*, 2005).

Taking into account the above mentioned evidence, our objectives were: 1) To evaluate changes in HUA contractile sensitivity to BK following prolonged *in vitro* incubation and 2) To elucidate the mechanisms involved and the role played, if any, by ACE and NEP in these phenomena.

Methods

Tissue preparation.

Human umbilical cords were obtained from normal full term deliveries and excised midway between the child and the placenta. Immediately, cords were placed in modified Krebs´ solution at 4ºC (of the following mM composition: NaCl 119, KCl 4.7, $NaHCO_3$ 25, KH_2PO_4 1.2, $CaCl_2$ 2.5, $MgSO_4$ 1.0, EDTA 0.004, D-glucose 11). The use of umbilical samples was approved by the ethics committee of the School of Pharmacy and Biochemistry of the University of Buenos Aires and written informed consent was obtained from each parturient.

Usually within 3 h after delivery, the samples were placed onto dissecting dishes containing Krebs´ solution and arteries (internal diameter approximately 1 mm) were carefully dissected free from Wharton's jelly using micro-dissecting instruments and cut into rings of approximately 3 mm width.

Functional studies.

Immediately after dissection, rings were suspended in 5 ml organ baths and stretched with an optimal resting tension of 2 to 4 g (Tufan *et al.*, 2003). Changes in tension were measured with Grass isometric force transducers (FT-03C, Grass Instruments Co., Quincy, MA, U.S.A.) and displayed on Grass polygraphs (model 7D). During incubation, Krebs´ solution was maintained at 37 ºC and pH 7.4 by constant bubbling with 95 % O_2: 5 % CO_2. Bath solution was replaced every 15 min with fresh bubbled buffer. Concentration response curves (CRCs) to BKB_2 receptor subtype agonists were constructed after a 2-h or 5-h *in vitro* incubation

by cumulative addition in 0.25 log_{10} increments. In other experiments, HUA rings were incubated for 2, 3 or 5 h and then sequentially challenged with 1 μM des-Arg^{10}-kallidin, 0.1 μM BK and 10 μM serotonin (5-HT). Whenever necessary, effective inhibitory doses of peptidase inhibitors were employed: 1 μM captopril (ACE inhibitor, IC_{50} 38 nM; Miyamoto *et al.*, 2002), 10 μM phosphoramidon (NEP inhibitor, IC_{50} 10 nM; Loffler, 2000), 10 μM thiorphan (NEP inhibitor, IC_{50} 1.4 nM; Miyamoto *et al.*, 2002), and 10 μM amastatin (aminopeptidase M [APM] inhibitor, IC_{50} 50 nM; Proud *et al.*, 1987) were applied 30 min before addition of BKB_2 and BKB_1 receptor agonists. The concentrations of amastatin, captopril and phosphoramidon used in this study were previously shown to produce the inhibition of des-Arg^{10}-kallidin inactivation as assessed by functional contractility studies in HUA (Pelorosso *et al.*, 2005). Phosphoramidon is also able to inhibit ACE (IC_{50} 78 μM; Kukkola *et al.*, 1995) and endothelin converting enzyme (ECE, IC_{50} 2.5 μM; Hoang and Turner, 1997). However, we have previously shown that 10 μM phosphoramidon is unable to inhibit ACE kininase activity in functional contractility studies carried out in HUA (Pelorosso *et al.*, 2005). Although partial inhibition of ECE could be expected at phosphoramidon concentrations used in the present work, Hoang and Turner (1997) have shown that up to 100 μM phosphoramidon is necessary to completely inhibit human ECE. On the other hand, ECE is highly resistant to inhibition by thiorphan (IC_{50} higher than 200 μM; Hoang and Turner, 1997). Nevertheless, it is important to note that thiorphan is also able to inhibit ACE (IC_{50} 295 nM; Miyamoto *et al.*, 2002). Therefore, all experiments involving thiorphan were carried out in presence of 1 μM captopril in

order to avoid possible ACE interference. In addition, whenever necessary 10 μM cycloheximide (protein synthesis inhibitor) was added to the bath during the entire incubation. None of the inhibitors tested produced any significant change in HUA rings´ basal tone when applied.

At the end of each CRC, 10 μM 5-HT was applied to determine tissue maximal contractile response (Altura *et al.*, 1972). All experiments were performed in parallel with rings from the same umbilical cord. Only one agonist CRC was performed in each ring.

NEP assay.

HUA rings were snap frozen after a 2-h or 5-h *in vitro* incubation or as fresh, non-incubated tissue. In the day of the experiment, tissues were ground to powder and then resuspended in ice cold lysis buffer (50 mM TRIS-HCl, pH 7.4, 1 % Triton X-100, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 mM PMSF and 1 mM benzamidine hydrochloride). Whole cell extracts were prepared by centrifugation at 3000 x g for 15 min at 4 ºC. Supernatants were collected and assayed for protein content.

Whole cell extracts (150 μg of protein) from fresh or incubated tissues were incubated with 500 μM Z-Ala-Ala-Leu-p-nitroanilide (ZAAL-pNA) in 100 μl of 50 mM HEPES (pH 7.4) for 1 h at 37ºC. Reaction was stopped by addition of 10 μM phosphoramidon. The reaction mixture was then added with 5 mU of leucine-aminopeptidase and further incubated for 30 min at 37ºC. Reaction mixtures where taken to ice and then measured for absorbance at 405 nm. NEP activity

was determined by the absorbance of the liberated p-nitroaniline and from the decrease in digestion rate caused by 10 μM phosphoramidon. Specific activity was calculated upon construction of a standard curve with known Leu-p-nitroanilide concentrations.

Western blot.

HUA rings were snap frozen after a 2-h or a 5-h *in vitro* incubation or as fresh, non incubated tissue. In the day of the experiment, tissues were ground to powder and then resuspended in ice cold lysis buffer (50 mM TRIS-HCl, pH 7.4, 150 mM NaCl, 1 % Triton X-100, 0.1 % SDS, 1 mM EDTA, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 mM PMSF and 1 mM benzamidine hydrochloride). Whole cell extracts were prepared by centrifugation at 3000 x g for 15 min at 4 ºC. Supernatants were collected and assayed for protein content.

Whole cell extracts (70 μg of proteins) were separated by electrophoresis on 8 % SDS polyacrylamide gels and resolved proteins were electrotransferred onto PVDF membranes. Membranes were blocked in Tris-buffered saline (TBS) containing 0.5% Tween 20 and 5% non-fat dried milk and then incubated overnight with either anti-NEP or anti-APM rabbit polyclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.). Membranes were washed three times in TBS containing 0.5% Tween 20 prior to incubation with alkaline phosphatase-conjugated goat anti-rabbit IgG for 1 h. Immunoreactive bands were detected by enhanced chemiluminescence, scanned and quantified using Quantity One software (Bio-Rad Laboratories, CA, U.S.A.). Equal gel loading

was verified by membrane's Ponceau Red staining. Changes in immunoreactive NEP levels were calculated based on a standard curve constructed with protein extracts from rat kidney. Negative controls were carried out in the absence of primary antibody.

Expression of results and statistical analysis.

All data are expressed as mean ± SEM. The number of experiments *n* represents the number of rings from different cords tested. Contractile responses are expressed as percentage of tissue maximum response elicited by 10 μM 5-HT. The estimates of EC_{50} values (i.e., the agonist concentration that produces 50% of the maximal response), the maximal response (E_{max}) and the slope factor (n_H) were obtained using ALLFIT (DeLean *et al.*, 1978). Briefly, responses obtained for each agonist concentration in each ring tested in the same group were averaged and then fitted to a four parameter logistic model expressed as (1):

$$(1) \qquad Y = \frac{a - E_{max}}{1 + (X/\ EC_{50})^{nH}} + E_{max}$$

where Y is the response; X, the arithmetic dose and a, the response when X = 0. EC_{50} were transformed into pEC_{50} (-log EC_{50}). Statistical analysis was performed by means of unpaired Student´s t-test or one-way analysis of variance (ANOVA) followed by Tukey's post-test, when appropriate. *P* values lower than 0.05 were taken to indicate significant differences.

Reagents.

The following compounds were used for functional studies: 5-hydroxytryptamine creatine sulphate complex from RBI (Natick, MA, U.S.A.); des-Arg^{10}-kallidin, BK, N-methyl-D-Phe^{7}-BK from Bachem Bioscience Inc. (King of Prussia, PA, USA); amastatin hydrochloride ((2S,3R)-3-Amino-2-hydroxy-5-methylhexanoyl-Val-Val-Asp hydrochloride), captopril (N-[(S)-3-Mercapto-2-methylpropionyl]-L-proline), cycloheximide (3-[2-(3,5-dimethyl-2-oxocyclohexyl)-2-hydroxyethyl]glutarimide) and thiorphan ((±)-N-(3-Mercapto-2-benzylpropionyl)glycine) from SIGMA Chemical Co. (St. Louis, MO, U.S.A.); phosphoramidon (N-alpha-L-rhamnopyranosyloxy(hydroxyphosphinyl)-L-Leucyl-L-Tryptophan) and Z-Ala-Ala-Leu-p-nitroanilide (Benzyloxycarbonyl-L-Alanyl-L-Alanyl-L-Leucine p-nitroanilide) from Peptides International Inc. (Louisville, KY, USA).

Preparation of all stock solutions and their subsequent dilutions were performed in glass bidistilled water. Stock solutions were stored in frozen aliquots and thawed and diluted daily.

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Results

Effects of *in vitro* incubation time on BK induced contractile responses in HUA.

Contractile responses induced by BK obtained after a 2-h *in vitro* incubation yielded a pEC_{50} of 8.38 ± 0.05 and a maximal response of 82.9 ± 7.9 % (n = 8, figure 1). When tissues were incubated during 5 h, pEC_{50} was significantly lower (7.98 ± 0.06, n=8, $P<0.05$) but maximal response was not modified (E_{max} 69.5 ± 9.3 %, n=8, figure 1).

Effects of ACE inhibition on BK induced contractile responses after 2 or 5 h *in vitro* incubation.

Contractile responses induced by BK after a 2-h *in vitro* incubation in HUA rings (pEC_{50} 8.38 ± 0.05, E_{max} 82.9 ± 7.9 %, n=8, figure 2A) failed to be modified by exposure to 1µM captopril (pEC_{50} 8.30 ± 0.05, E_{max} 86.31 ± 2.5 %, figure 2A). However a small but significant potentiation of BK induced responses was observed in rings treated with 1µM captopril after a 5-h incubation (control: pEC_{50} 7.98 ± 0.06, E_{max} 69.5 ± 9.3 %, n=8; captopril: pEC_{50} 8.26 ± 0.06, $P<0.05$, E_{max} 75.9 ± 7.9 %, n=8, figure 2B).

Effects of NEP inhibition on BK induced contractile responses after a 2 or 5-h *in vitro* incubation.

NEP inhibition did not modify BK induced contractile responses in rings incubated for 2 h (control: pEC_{50} 8.38 ± 0.05, E_{max} 82.9 ± 7.9 %, n=8; phosphoramidon: pEC_{50} 8.48 ± 0.07, E_{max} 78.7 ± 10.4 %, n=8, figure 3A).

However, contractile responses induced by BK after a 5-h *in vitro* incubation in HUA rings (pEC_{50} 7.98 ± 0.06, E_{max} 69.5 ± 9.3 %, n=8) were significantly potentiated by treatment with 10 μM phosphoramidon (pEC_{50} 8.61 ± 0.10, $P<0.05$, E_{max} 89.1 ± 5.9 %, n=8, figure 3B).

Lack of effects of phosphoramidon on responses elicited by N-methyl-D-Phe^7-BK after a 5-h *in vitro* incubation.

Contractile responses induced by N-methyl-D-Phe^7-BK, a BKB_2 receptor agonist analogue (Reissmann *et al.*, 1996), after a 5-h *in vitro* incubation in HUA in presence of 1 μM captopril (pEC_{50} 6.23 ± 0.06, E_{max} 92.0 ± 3.5 %, n=6) failed to be modified by further addition of 10 μM phosphoramidon (pEC_{50} 6.28 ± 0.09, E_{max} 85.7 ± 3.5 %, n=6, figure 4A). As a positive control, we evaluated the effects of the addition of 10 μM phosphoramidon on BK-elicited responses obtained in presence of 1 μM captopril after a 5-h *in vitro* incubation (captopril: pEC_{50} 8.26 ± 0.06, E_{max} 75.9 ± 7.9 %, n=8; phosphoramidon plus captopril: pEC_{50} 8.60 ± 0.05, $P<0.05$, E_{max} 91.8 ± 2.5 %, n=8, figure 4B).

Further functional evidence of changes in NEP kininase activity as a function of *in vitro* incubation time in HUA.

Contractile responses induced by BK after a 2-h *in vitro* incubation in HUA rings in presence of 1 μM captopril (pEC_{50} 8.38 ± 0.09, E_{max} 91.4 ± 4.4 %, n=5) failed to be modified by additional exposure to 10 μM thiorphan (pEC_{50} 8.54 ± 0.05, E_{max} 93.4 ± 3.3 %, n=5, figure 5A). However, when CRCs to BK were carried out

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after a 5-h *in vitro* incubation in presence of 1 μM captopril, further addition of 10 μM thiorphan produced a significant potentiation of contractile responses induced by the agonist (captopril: pEC_{50} 7.89 ± 0.10, E_{max} 78.6 ± 12.1 %, n=5; captopril plus thiorphan: pEC_{50} 8.40 ± 0.09, $P<0.05$, E_{max} 94.0 ± 1.9 %, n=5, figure 5B).

Role of *de novo* protein synthesis in changes in NEP kininase activity as a function of *in vitro* incubation time in HUA.

Contractile responses induced by BK after a 5-h *in vitro* incubation in HUA rings in presence of 1 μM captopril (pEC_{50} 7.77 ± 0.05, E_{max} 83.6 ± 3.6 %, n=6) were significantly potentiated by treatment with 10 μM cycloheximide (pEC_{50} 8.10 ± 0.04, $P<0.05$, E_{max} 89.3 ± 2.4 %, n=6, figure 6A). However, when rings were treated with 1 μM captopril plus 10 μM phosphoramidon, continuous exposure to 10 μM cycloheximide failed to modify BK induced contractile responses at 5 h (captopril plus phosphoramidon: pEC_{50} 8.48 ± 0.03, E_{max} 94.8 ± 2.2 %, n=6; captopril plus phosphoramidon plus cycloheximide: pEC_{50} 8.42 ± 0.03, E_{max} 93.9 ± 2.2 %, n=6, figure 6B). In addition, neither potency nor maximal responses to BK were modified when 10 μM cycloheximide was applied 15 min previous to the construction of the CRC (data not shown).

Role of *in vitro* incubation time and *de novo protein* synthesis in changes in NEP enzymatic activity in HUA whole cell extracts.

NEP activity was measured on HUA whole cell extract preparations from fresh tissues and from rings incubated during 2 and 5 h (figure 7). Activity in fresh

tissues was 0.20 ± 0.05 nmol/mg/min (n=6). In addition, activity at 2 h was 0.29 ± 0.07 nmol/mg/min (n=3), which was not higher than in fresh tissues. However, activity measured after a 5-h incubation period was higher than those observed in fresh and 2 h-incubated tissues (0.70 ± 0.08 nmol/mg/min, n=7, *P*<0.05). Treatment with 10 μM cycloheximide during 5 h produced a decrease in NEP activity when compared to 5 h-incubated tissues (0.27 ± 0.07 nmol/mg/min, n=2, *P*<0.05, figure 7).

Role of *in vitro* incubation time in changes in immunoreactive NEP content in HUA whole cell extracts.

NEP content was measured by densitometric analysis of Western blots using rabbit polyclonal anti-NEP antibody on HUA whole cell extract preparations from fresh tissues and from rings incubated during 2 h and 5 h.

A single band of approximately 100 kDa was detected in extracts obtained from fresh, 2 h and 5 h-incubated HUA (figure 8A). NEP content after a 2-h incubation was 1.71 ± 0.40 (n=3, figure 8B) fold higher than in fresh HUA. Moreover, NEP content after a 5-h incubation was higher than that observed at 2 h (3.62 ± 0.26, n=6, *P*<0.05, figure 8B).

Role of *in vitro* incubation time in changes in contractile responses mediated by BKB_1 receptor subtype in HUA.

Maximal contractile responses in HUA evoked by single exposure to 1 μM des-Arg^{10}-kallidin in presence of 10 μM amastatin, 10 μM phosphoramidon and 1 μM

captopril were determined at different incubation times. Responses obtained after a 2-h *in vitro* incubation were 37.9 ± 11.8 % (n=9). Responses at 3 h were higher although not significantly different (64.4 ± 12.1 %, n=9). However, when HUA rings were challenged with 1 μM des-Arg^{10}-kallidin after a 5-h *in vitro* incubation maximal responses were significantly higher than those obtained at 2 h (81.0 ± 11.4 %, P<0.05, n=9). In contrast, maximal contractile responses evoked by single exposure to 0.1 μM BK remained unchanged through the incubation times tested (2 h: 90.2 ± 3.0 %, n=9; 3 h: 94.1 ± 2.0 %, n=9; 5 h: 95.6 ± 1.1, n=9, figure 9).

Discussion

BKB_2 receptors are constitutively expressed in a variety of tissues and mediate most of *in vivo* kinins´ effects (Bathon and Proud, 1991). However, in several *in vivo* models, BKB_1 receptors up-regulate upon challenge with different pro-inflammatory stimuli, thus enabling responses induced by BKB_1 receptor agonists (Leeb-Lundberg *et al.*, 2005). In the present work we have demonstrated for the first time that, in HUA, contractile responses induced by the selective BKB_1 receptor agonist, des-Arg^{10}-kallidin, are sensitized after a 5-h *in vitro* incubation period. A similar time-dependent profile of BKB_1 receptor induction has been described in other vascular smooth muscle preparations including, among others, rabbit aorta (Audet *et al.*, 1994), human umbilical vein (Sardi *et al.*, 1997), rat portal vein (Medeiros *et al.*, 2004) and rabbit mesenteric artery (Deblois and Marceau, 1987). Induction of BKB_1 receptor after tissue injury can be mimicked by exposure to lipopolysaccharide (LPS) and several inflammatory mediators (IL-1β, TNF-α; Marceau *et al.*, 1998). In agreement with this, Angers e*t al.* (2000) have provided evidence that BKB_1 receptor mRNA levels in primary cultures of smooth muscle cells obtained from HUA are sharply up-regulated by IL-1β. Moreover, the inflammatory nature of the spontaneous BKB_1 receptor up-regulation process after isolation injury and *in vitro* incubation has been further suggested by its inhibition upon tissue exposure to IL-1 receptor antagonist (Marceau, 1995), anti-inflammatory cytokines (Sardi *et al.*, 2002) and nuclear factor-κB inhibitors (Sardi *et al.*, 1999).

Yet, our report also shows that the selective BKB_2 receptor agonist, BK, displays a significant decrease in potency for contractile effects as a function of *in vitro* incubation time in isolated HUA. Similar findings have been reported in other models in which sensitization of responses mediated by BKB_1 receptor is associated with a clear reduction of BKB_2 receptor mediated effects (Cruwys *et al.*, 1994; Campos *et al.*, 1996; El Sayah *et al.*, 2006). For instance, Campos *et al.* (1996) have shown that lipopolysaccharide (LPS) causes an increase in BKB_1 receptor mediated rat paw edema, accompanied by a patent drop in BKB_2 receptor mediated edematogenic response. Recently, El Sayah *et al.* (2006) have reported that BK-induced contractile responses in isolated LPS-treated pig iris sphincter muscle are markedly reduced in a time dependent process. Still, mechanisms underlying the functional desensitization of BKB_2 receptor mediated responses are currently unknown. True BKB_2 receptor down-regulation is only observed in some forms of inflammation (acute renal transplant rejection; Naidoo *et al.*, 1996). Nevertheless, sustained, agonist-mediated stimulation does not lead to detectable degradation of rabbit BKB_2 receptor (Bachvarov *et al.*, 2001). Moreover, clinical samples of chronic inflammatory tissues (psoriatic skin, nasal tissue samples from subjects with allergic rhinitis) show the up-regulation of BKB_1 receptor mRNA without the down-regulation of BKB_2 receptor mRNA (Schremmer-Danninger *et al.*, 1999; Christiansen *et al.*, 2002). In addition, whereas a patent up-regulation of BKB_1 receptor is observed in aortas obtained from LPS-treated pigs, neither autoradiographic visualization nor displacement studies using BKB_2 receptor ligands yield differences in BKB_2 receptor population

characteristics between control and LPS-treated animals (Schremmer-Danninger *et al.*, 1998). Interestingly, our group has recently demonstrated a relevant role for ACE and NEP in the functional inactivation of kinins following prolonged *in vitro* incubation of HUA (Pelorosso *et al.*, 2005). This fact, in addition to poor evidence in favor of the possible down-regulation of BKB_2 receptor, led us to analyze the potential role of these enzymes in BK desensitization phenomenon observed in isolated HUA.

In the present report we've shown that, while BK-induced responses after a 2-h *in vitro* incubation failed to be modified by captopril treatment, a small but significant potentiation was observed by exposure to the ACE inhibitor after a 5-h incubation. There are several studies employing recombinant BKB_2 receptor models that suggest a possible interaction of ACE inhibitors with BKB_2 receptors leading to the potentiation of BK-induced responses independently of inhibiting ACE activity (Tom *et al.*, 2003). However, strong pharmacological evidence suggests that potentiation of vasoactive effects of BK by ACE inhibitors in physiological models is due to inhibition of kinin metabolism (Dendorfer *et al.*, 2001; Tom *et al.*, 2002). Therefore, our results are compatible with a possible increase in ACE kininase activity after a 5 h-incubation period in isolated HUA.

Furthermore, though BK-elicited responses obtained after 2 h *in vitro* incubation were not modified by phosphoramidon treatment, contractile responses induced by BK in HUA at 5 h were markedly potentiated by NEP inhibition. These results suggest that NEP ability to perform functional inactivation of BK in biophase is significantly increased after a 5-h *in vitro* incubation in isolated HUA. Moreover,

BK potency at 5 h in presence of phosphoramidon (pEC_{50} 8.61) was not significantly different from that observed at 2 h with (pEC_{50} 8.48) or without (pEC_{50} 8.38) phosphoramidon treatment. These results, in addition, suggest that the possible occurrence of BKB_2 receptor down-regulation in our model is unlikely.

Given the magnitude of NEP inhibition effects in comparison to those observed under ACE blockade we decided to pursue a more profound characterization of NEP activity in HUA. Therefore, all subsequent CRCs were carried out in presence of 1 μM captopril to rule out any involvement of ACE in further observations.

In the following series of experiments, we tested the effects of phosphoramidon treatment on responses elicited by the BKB_2 analogue, N-methyl-D-Phe^7-BK (Reissmann *et al.*, 1996) at 5 h. This compound behaved as a full, although less potent agonist, in comparison to BK. These observations are consistent with those reported for this analogue in different BKB_2 receptor models (Dendorfer *et al.*, 1999; Dendorfer *et al.*, 2001). In addition, 10 μM phosphoramidon failed to modify N-methyl-D-Phe^7-BK-induced contractile responses in HUA. This result suggests that preservation of the original Pro^7-Phe^8 bond structure of BK, the main cleavage site of NEP, is necessary for phosphoramidon potentiating effects. Previous reports indicate the possibility that NEP inhibitors may potentiate BK-induced responses by other mechanisms beside enzyme inhibition (Deddish *et al.*, 2002). However, our results suggest that potentiation of BK-induced

responses by NEP inhibition at 5 h in HUA is due to impairment of kinin biotransformation in biophase.

To confirm that potentiation of BK-induced contractile responses at 5 h in HUA is mediated by NEP inhibition, a chemically unrelated NEP inhibitor, thiorphan, was tested (Roques *et al.*, 1995). Although thiorphan failed to modify BK-induced responses in HUA rings after a 2-h *in vitro* incubation, it significantly potentiated responses elicited by BK at 5 h. Moreover, potentiation of BK-elicited responses produced by thiorphan was quantitatively equivalent to that observed with phosphoramidon. These results provide further functional evidence of changes in NEP kininase activity as a function of *in vitro* incubation time in HUA.

In order to evaluate the possible correlation between *de novo* protein synthesis and NEP kininase activity increase in isolated HUA incubated for 5 h, responses to BK were evaluated in rings continuously treated with cycloheximide, a protein synthesis inhibitor. The significative potentiation of BK-induced responses after a 5-h incubation with cycloheximide suggests that NEP might be up-regulated during prolonged *in vitro* incubation in HUA. Furthermore, the lack of effect of cycloheximide on BK-induced responses in presence of phosphoramidon further substantiates our hypothesis that cycloheximide effects depend on NEP synthesis inhibition.

The increase in NEP activity as a function of *in vitro* incubation time in HUA was further evaluated by means of its biochemical analysis in HUA whole cell extracts. The sharp increase in NEP activity after a 5-h incubation is consistent with the enzyme's up-regulation in HUA. Furthermore, the abolishment of this

increase observed with cycloheximide treatment constitutes strong biochemical evidence suggesting that NEP is newly synthesized in HUA during prolonged *in vitro* incubation. In addition, the significant increment in NEP immunoreactive protein content observed in HUA whole cell extracts after a 5-h incubation further suggests that NEP is up-regulated in this tissue.

Previous reports have provided evidence that NEP up-regulation might constitute a common response to tissue injury. For instance, Walther *et al*. (2002) showed that NEP activity in 6-day infarcted left ventricles from rats was 100 % higher than in control (sham-operated) group. Further evidence in support of this hypothesis has been provided by Olerud *et al*. (1999). This group has shown that while in normal skin NEP is strikingly localized to keratinocytes of the epidermal basal layer, incisional wounds produce an up-regulation of NEP already evident 6 h after wounding in these cells.

During sustained inflammatory insult, kinin-mediated responses adapt from a BKB_2 receptor type in the acute phase to a BKB_1 receptor type in the chronic phase (Dray and Perkins, 1993). This adaptation is explained in part by BKB_1 receptor induction from essentially a null level, which is triggered by several pro-inflammatory stimuli (Leeb-Lundberg *et al.*, 2005). However, additional synchronous processes may take part in the shift leading to the prevalence of BKB_1 over BKB_2 receptor mediated responses during continuous inflammatory stimuli. For instance, Schremmer-Danninger *et al*. (1998) have shown that LPS treatment is able to produce an increase of up to 200 % in carboxipeptidase M activity in pig aortic samples. Carboxipeptidase M cleaves carboxyterminal

arginine in BK and kallidin yielding the BKB_1 selective agonists des-Arg^9-BK and des-Arg^{10}-kallidin, respectively. In contrast, preincubating rabbit aortic rings for 6 h, a procedure that sharply up-regulates BKB_1 receptor expression, produces a negligible increase in activity of APM, a major inactivating enzyme of des-Arg^{10}-kallidin, the endogenous BKB_1 receptor agonist (Fortin *et al.*, 2005). This result coincides with that found in our model where APM level as determined by Western blot remains unchanged in fresh vs. 5 h incubated HUA tissue (data not shown). Thus, the above mentioned evidence, in addition to that described in the present study, suggests that kinin-mediated responses may shift from a BKB_2 type to a BKB_1 during sustained inflammatory insult not only by changes in relative receptor densities but also by changes in kininases´ expression leading to increased BKB_1 agonists production and, at the same time, higher BKB_2 agonists inactivation rates.

In summary, our work clearly demonstrates that prolonged *in vitro* incubation of HUA rings leads to NEP up-regulation, evidenced by the increase in immunoreactive NEP content and its correlation with increased enzymatic activity. Furthermore, data presented here provide strong pharmacological evidence that NEP up-regulation produces an increase in BK biological inactivation resulting in a marked reduction of kinin-induced contractile responses in HUA rings. Taking into account that NEP constitutes a minor functional inactivator of des-Arg^{10}-kallidin (Pelorosso *et al.*, 2005) our results suggest that NEP up-regulation might play a role in the shift of kinin-mediated responses from

a BKB_2 type in the acute phase to a BKB_1 type during sustained inflammatory processes.

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Footnotes

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Legends for figures

Figure 1. Responses elicited by BK after a 2 (■) or 5 (●) h *in vitro* incubation; ✶ represents significant differences between pEC_{50} (P<0.05).

Figure 2. A. Responses elicited by BK after a 2 h *in vitro* incubation in presence (●) or absence (■) of 1 μM captopril. **B.** Responses elicited by BK after a 5 h *in vitro* incubation in presence (●) or absence (■) of 1 μM captopril; ✶ represents significant differences between pEC_{50} (P<0.05). Data of BK effects in absence of peptidase inhibitors correspond to that shown in Figure 1.

Figure 3. A. Responses elicited by BK after a 2 h *in vitro* incubation in presence (●) or absence (■) of 10 μM phosphoramidon. **B.** Responses elicited by BK after a 5 h *in vitro* incubation in presence (●) or absence (■) of 10 μM phosphoramidon; ✶ represents significant differences between pEC_{50} (P<0.05). Data of BK effects in absence of peptidase inhibitors correspond to that shown in Figure 1.

Figure 4. A. Responses elicited by N-methyl-D-Phe^7-BK after a 5 h *in vitro* incubation in presence of 1 μM captopril (■) or 1 μM captopril and 10 μM phosphoramidon (●). **B.** Responses elicited by BK after a 5 h *in vitro* incubation in presence of 1 μM captopril (■) or 1 μM captopril and 10 μM phosphoramidon (●); ✶ represents significant differences between pEC_{50} (P<0.05).

Figure 5. A. Responses elicited by BK after a 2 h *in vitro* incubation in presence of 1 μM captopril (■) or 1 μM captopril and 10 μM thiorphan (●). **B.** Responses elicited by BK after a 5 h *in vitro* incubation in presence of 1 μM captopril (■) or 1

μM captopril and 10 μM thiorphan (●); ✶ represents significant differences between pEC_{50} (P<0.05).

Figure 6. A. Responses elicited by BK after a 5 h *in vitro* incubation in presence of 1 μM captopril with (●) or without (■) continuous exposure to 10 μM cycloheximide. **B.** Responses elicited by BK after a 5 h *in vitro* incubation in presence of 1 μM captopril and 10 μM phosphoramidon with (●) or without (■) continuous exposure to 10 μM cycloheximide; ✶ represents significant differences between pEC_{50} (P<0.05).

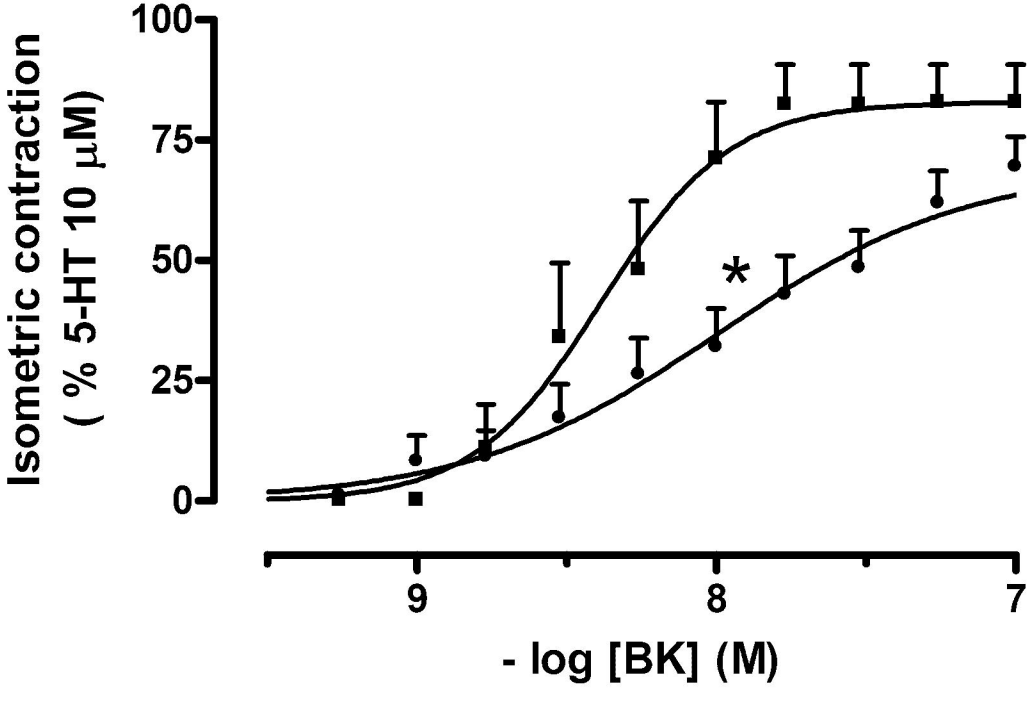
Figure 7. NEP enzymatic activity measured in whole cell extracts from fresh HUA (open bar), tissues incubated for 2 h (hatched bar) tissues incubated for 5 h (filled bar) and tissues incubated for 5 h with 10 μM cycloheximide (CHX, reverse hatched bar); ✶ represents significant differences between means (P<0.05).

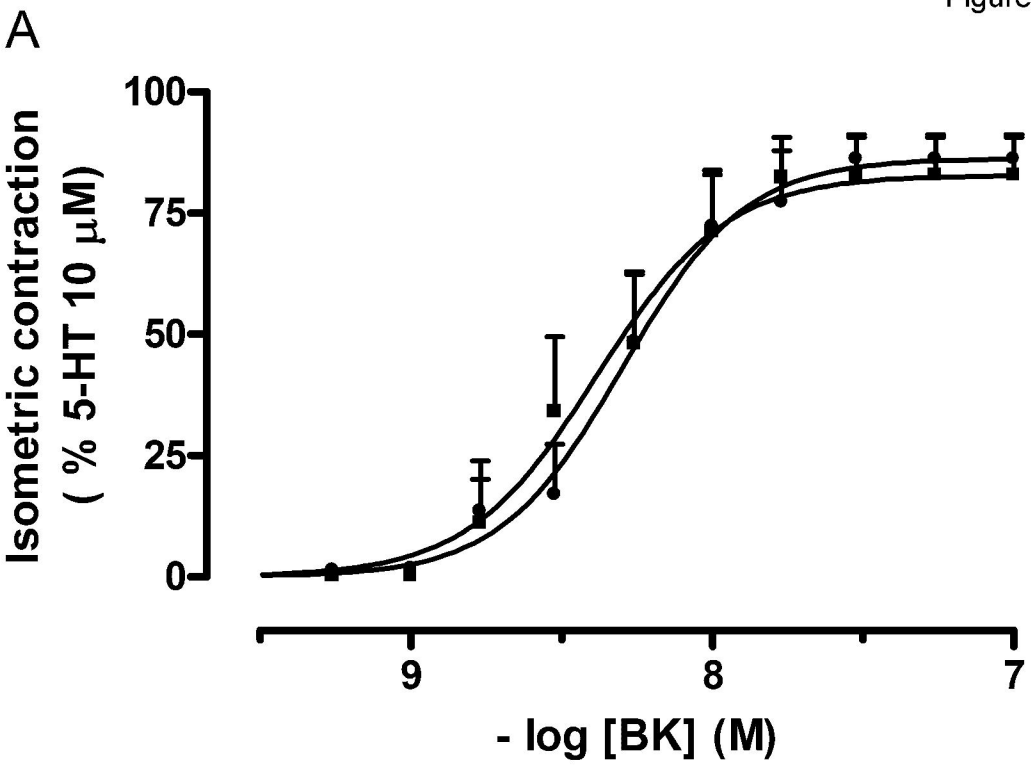
Figure 8. A. Representative blot showing a 100 kDa band corresponding to immunoreactive NEP protein in whole cell extracts from fresh HUA, HUA incubated for 2 h, HUA incubated for 5 h and fresh rat kidney (positive control). **B.** Densitometric analysis of NEP protein content in membrane extracts obtained from fresh HUA (open bar, n=6), HUA incubated for 2 h (hatched bar, n=3), and HUA incubated for 5 h (filled bar, n=6). Results are expressed as fold increase of NEP protein content in comparison to fresh tissues; ✶ represents significant differences between means (P<0.05).

Figure 9. Maximal responses elicited by single exposure to 1 μM des-Arg^{10}-kallidin (open bars) or 0.1 μM BK (filled bars) in HUA rings incubated for 2, 3 and

5 h. All rings were exposed to 10μM amastatin, 1 μM captopril and 10 μM phosphoramidon; ✶ represents significant differences between E_{max}.

Figure 1




A
100
75
50
25
0
Isometric contraction
(% 5-HT 10 μM)
9
8
7
- log [BK] (M)

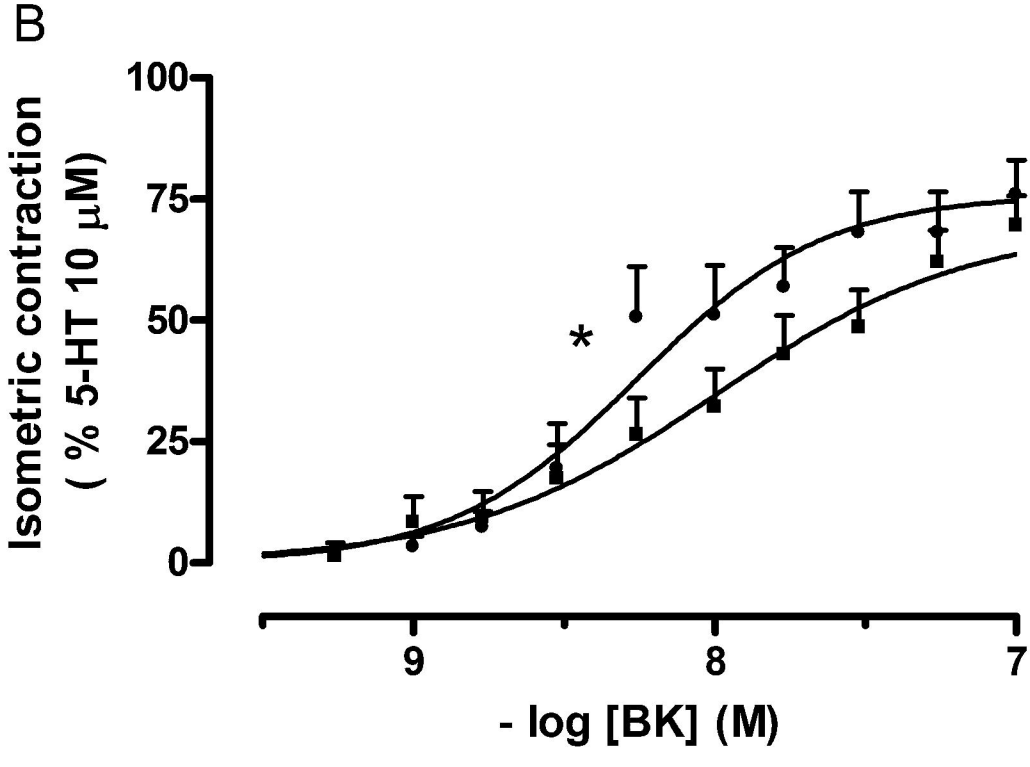
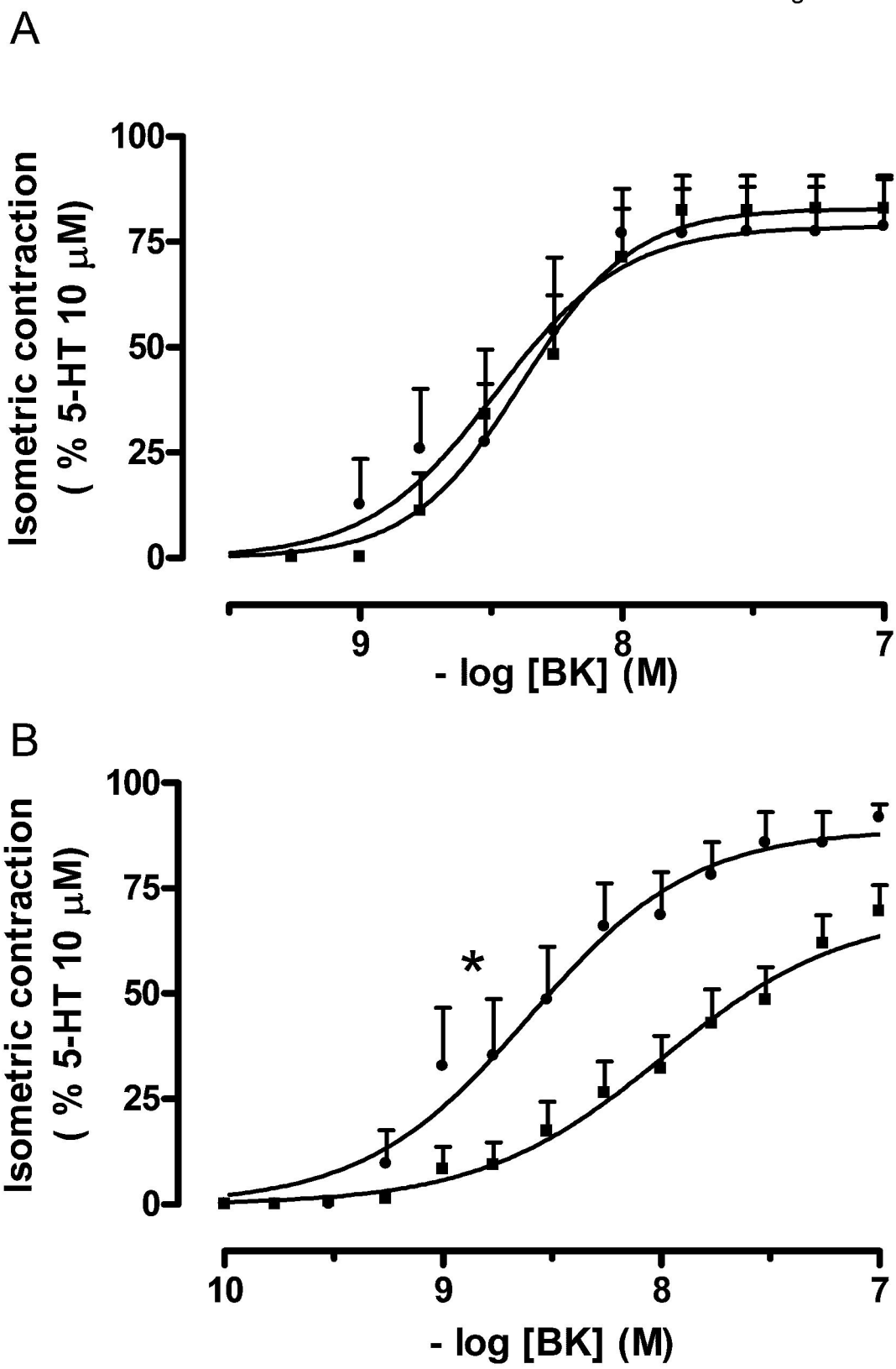
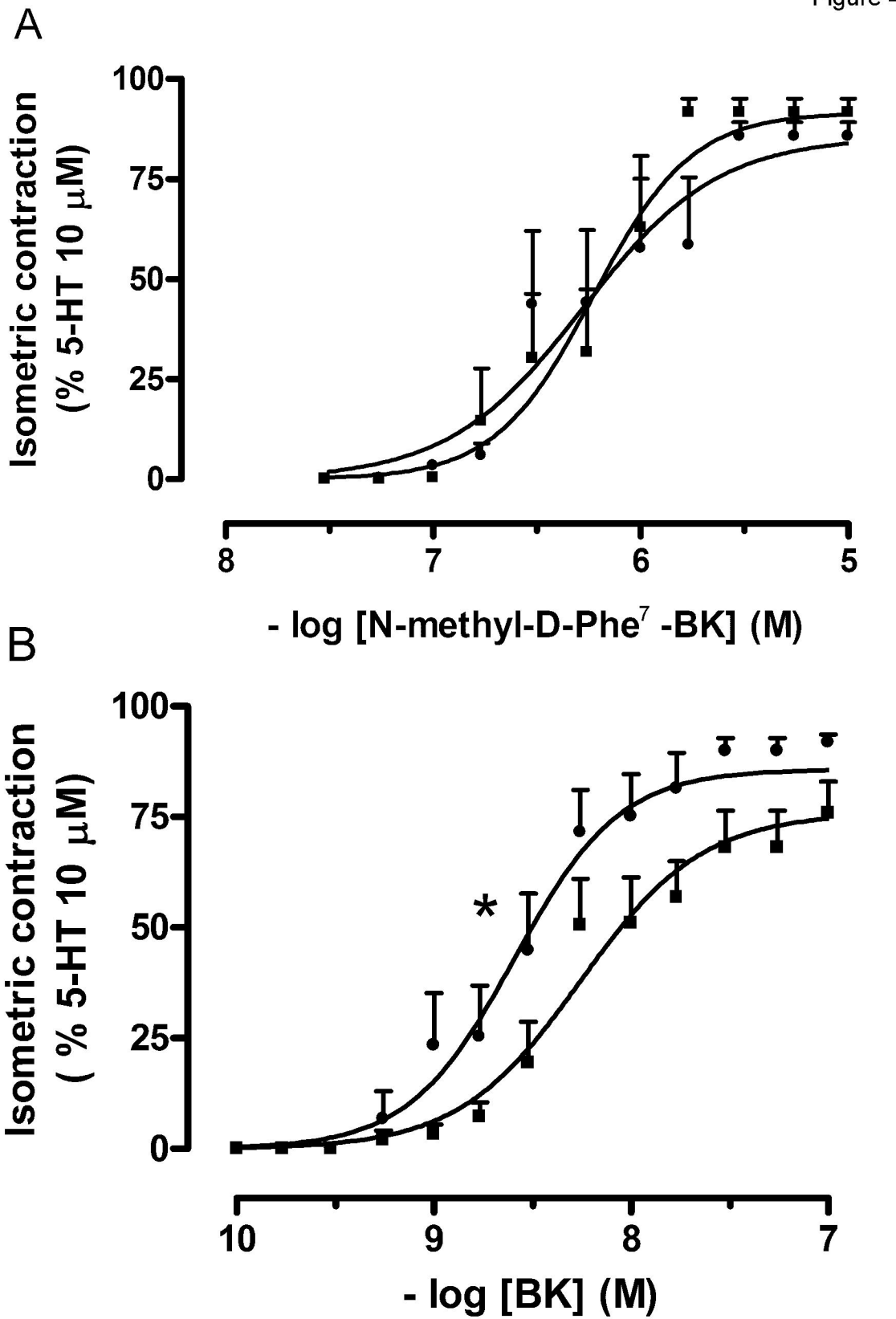
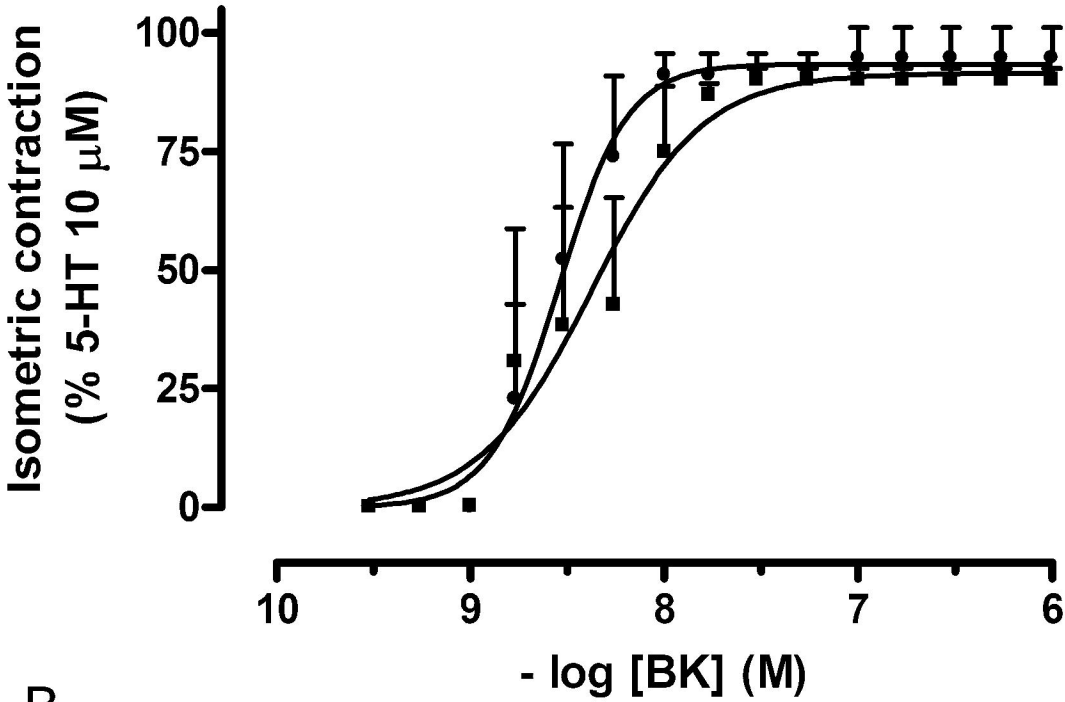

B
100
75
50
25
0
Isometric contraction
(% 5-HT 10 μM)
*
9
8
7
- log [BK] (M)

Figure 3

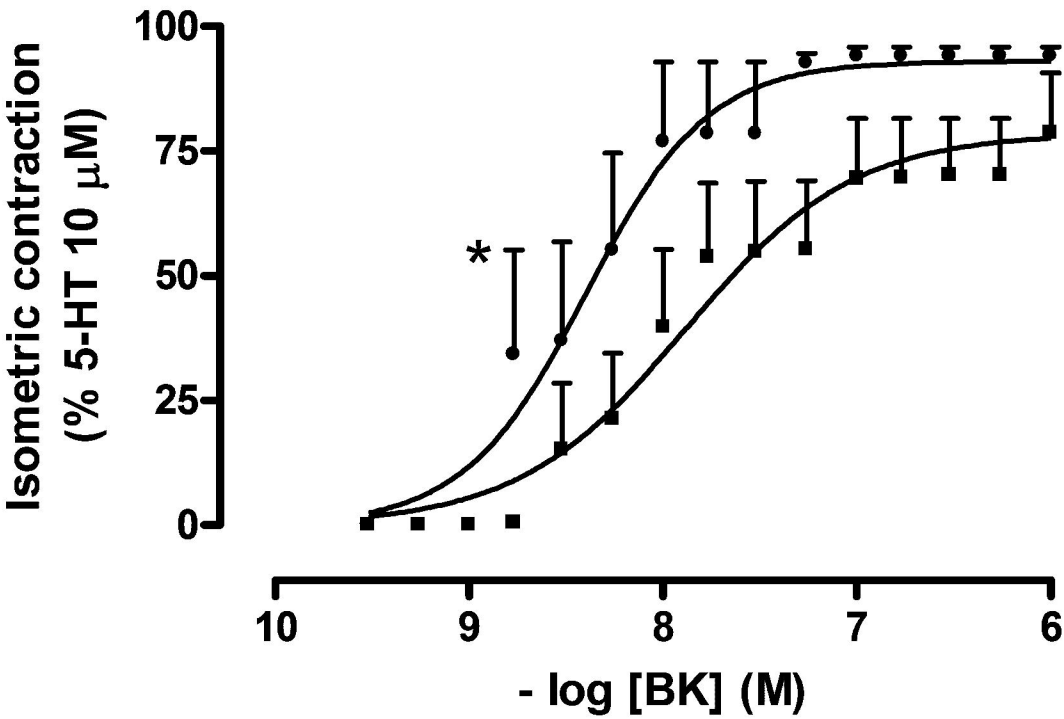

A
Isometric contraction
(% 5-HT 10 μM)
100
75
50
25
0
9
8
7
- log [BK] (M)
B
Isometric contraction
(% 5-HT 10 μM)
100
75
50
25
0
*
10
9
8
7
- log [BK] (M)


A
Isometric contraction
(% 5-HT 10 μM)
100
75
50
25
0
8
7
6
5
- log [N-methyl-D-Phe7 -BK] (M)
B
Isometric contraction
(% 5-HT 10 μM)
100
75
50
25
0
*
10
9
8
7
- log [BK] (M)

A


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

B


Isometric contraction
(% 5-HT 10 μM)
100
75
50
25
0
*
10
9
8
7
6
- log [BK] (M)

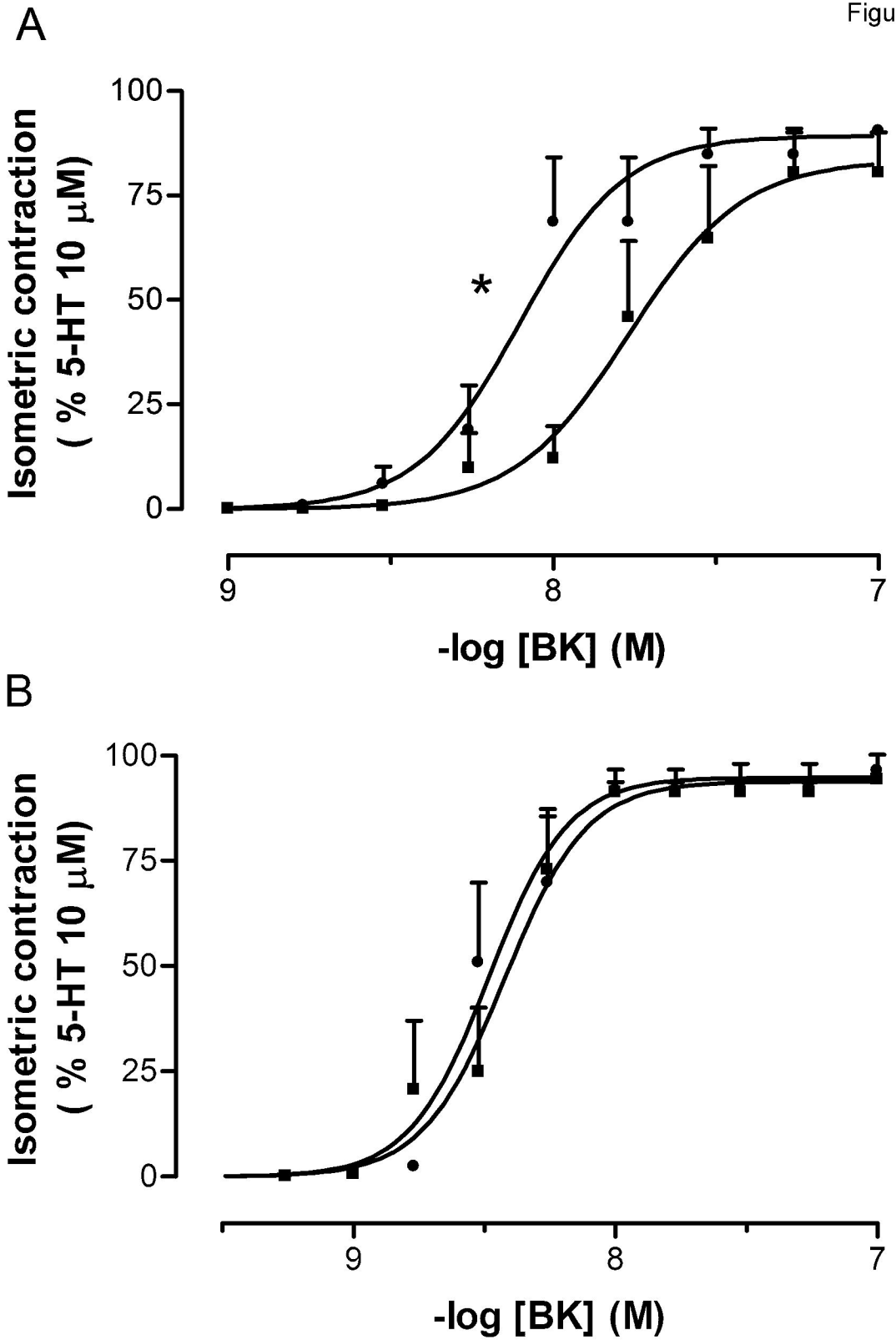
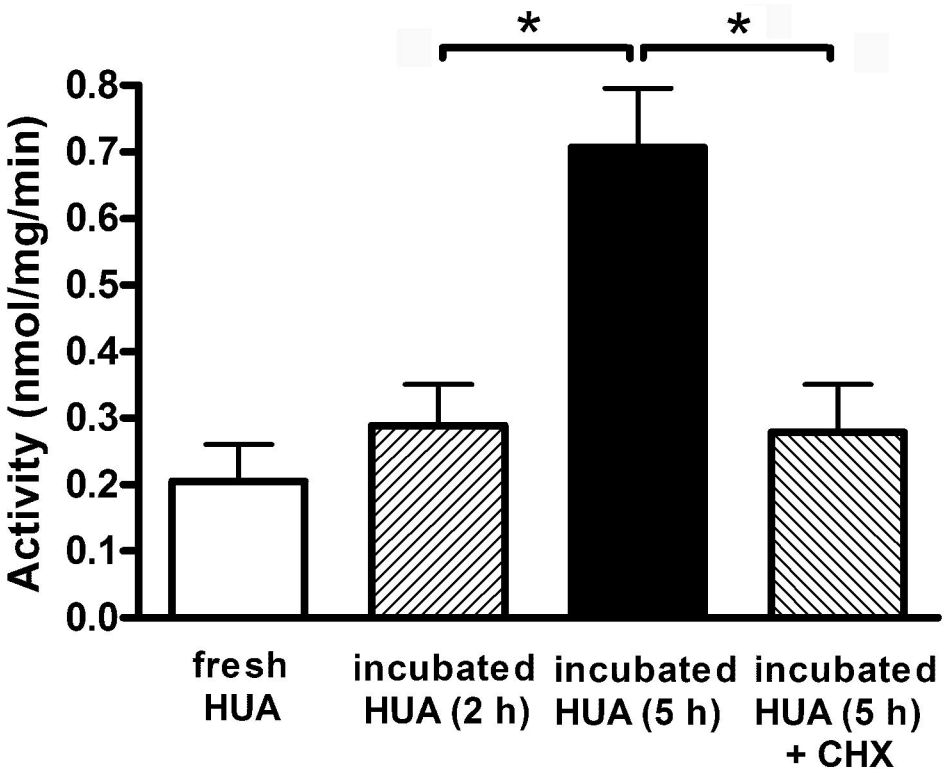
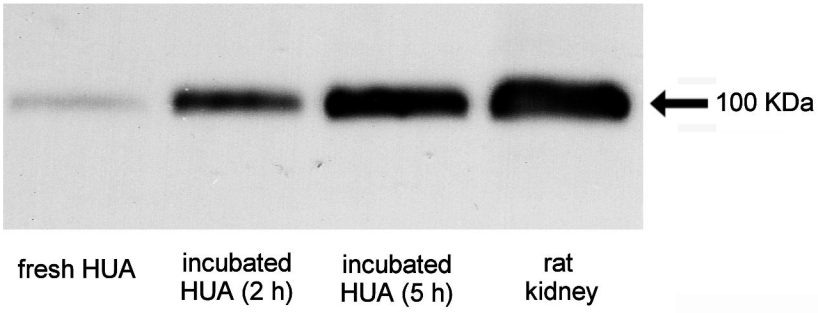

A
Isometric contraction
(% 5-HT 10 μM)
100
75
50
25
0
*
9
8
7
-log [BK] (M)
B
Isometric contraction
(% 5-HT 10 μM)
100
75
50
25
0
9
8
7
-log [BK] (M)

Figure 7


*
*
Activity (nmol/mg/min)
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
fresh HUA
incubated HUA (2 h)
incubated HUA (5 h)
incubated HUA (5 h) + CHX

A


100 KDa
fresh HUA
incubated HUA (2 h)
incubated HUA (5 h)
rat kidney

B

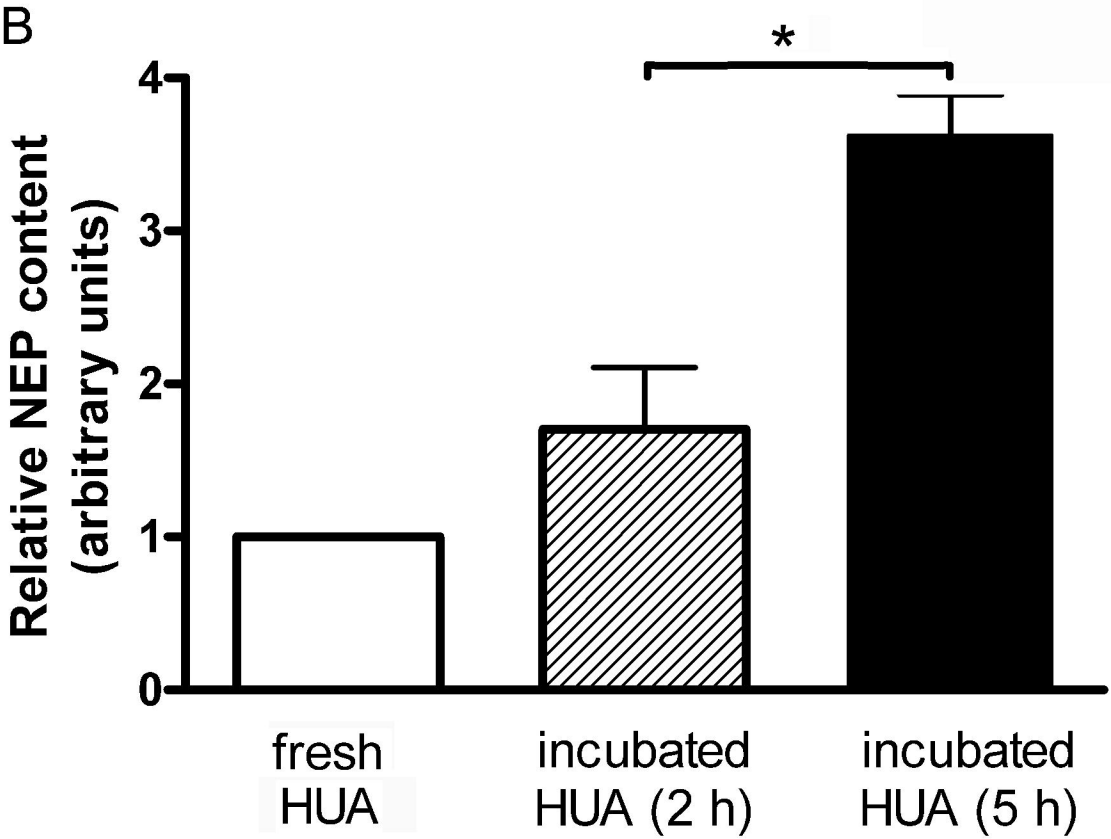
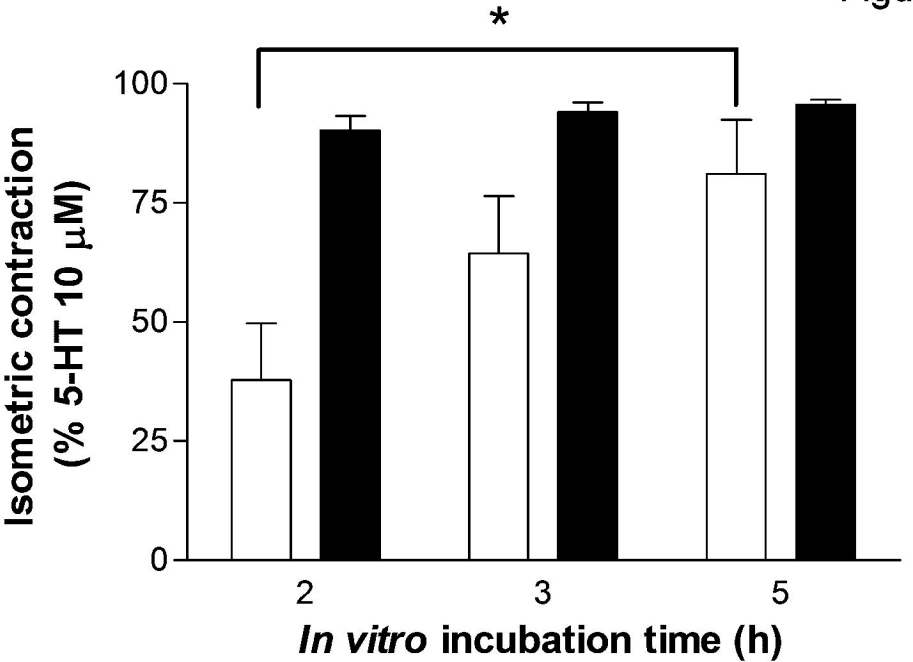

*
Relative NEP content (arbitrary units)
0
1
2
3
4
fresh HUA
incubated HUA (2 h)
incubated HUA (5 h)

Figure 9


*
100
75
50
25
0
Isometric contraction
(% 5-HT 10 μM)
2
3
5
In vitro incubation time (h)