

Peptide fragments of bradykinin show unexpected biological activity not mediated by B1 or B2 receptors

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Abstract

Background and purpose: Bradykinin [BK-(1-9)] is an endogenous nonapeptide involved in multiple physiological and pathological processes. A long-held belief is that peptide fragments of BK-(1-9) are biologically inactive. Here, we have tested the biological activities of BK-(1-9) and two major peptide fragments in human and animal systems. **Experimental Approach:** Levels of BK peptides in male Wistar rat plasma were quantified by mass spectrometric methods. Nitric oxide was quantified in human, mouse and rat cells, and loaded with DAF-FM. We used aortic rings from adult male Wistar rats to test vascular reactivity. Changes in blood pressure and heart rate were measured in conscious adult male Wistar rats. **Key results:** Plasma levels of BK-(1-7) and BK-(1-5) in rats were increased following infusion of BK-(1-9). All tested peptides induced NO production in all cell types tested. However, unlike BK-(1-9), NO production elicited by BK-(1-7) or BK-(1-5) was not inhibited by B₁ or B₂ receptor antagonists. BK-(1-7) or BK-(1-5) also induced concentration-dependent vasorelaxation of aortic rings, without involving B₁ or B₂ receptors. In vivo, either intravenous or intra-arterial administration of BK-(1-7) or BK-(1-5) induced similar hypotension response. **Conclusions and implications:** BK-(1-7) and BK-(1-5) are endogenous peptides present in plasma. They are formed, at least partially, through the BK-(1-9) proteolysis. BK-related peptide fragments show biological activity, not mediated by B₁ or B₂ receptors. These BK-fragments could constitute new, active components of the kallikrein-kinin system.

1. INTRODUCTION

Bradykinin [BK-(1-9)] is a nonapeptide of the kallikrein-kinin system (KKS) and was first characterized over 70 years ago (Rocha *et al.*, 1949), as a potent hypotensive agent in vivo, and to induce a slow contraction of guinea-pig uterus (hence *brady* kinin). This biological activity was generated by incubating ox plasma with snake venom (from *Bothrops jararaca*) or trypsin. A decade later, the nonapeptide BK-(1-9) was purified by (Elliott *et al.*, 1960) and shown to induce hypotension, slow contraction of smooth muscle and pro-inflammatory effects. Since then, our knowledge of the biochemistry, physiology, and pharmacology of the KKS and BK-(1-9) has greatly increased. In the present context the most important features include (i) the inactivation of BK-(1-9) by plasma kininases I and II (Yang *et al.*, 1970), (ii) the rapid and extensive inactivation of BK-(1-9) in the pulmonary circulation (Ferreira *et al.*, 1967), later associated with the angiotensin-converting enzyme (ACE) (Bakhle, 1968; Ferreira *et al.*, 1970); ii) elucidation of two proteoforms of kininogens (Habal *et al.*, 1976); iii) identification of des-Arg⁹-BK [BK-(1-8)] as a biologically active component of KKS (Regoli *et al.*, 1977); iv) cDNA cloning of the constitutive B₂ receptor, a selective target for BK-(1-9) (McEachern *et al.*, 1991); v) cDNA cloning of B₁ receptor, a selective target for BK-

(1-8) (Menke *et al.* , 1994) and (vi) FDA approval of the selective B₂ receptor antagonist icatibant (Cicardi *et al.* , 2010) as a treatment for hereditary angiodema.

The bioactivities of the peptide fragments of BK-(1-9) have been mostly overlooked for the past 40 years. In the late 1960s, BK-(1-9) was shown to have a biological half-life of about 17 seconds in blood, being extensively metabolized in a single pass on the pulmonary circulation (clearance of about 80%) (Ferreira *et al.* , 1967; Ryan *et al.* , 1968). Given this short half-life, several studies (Suzuki *et al.* , 1969; Regoli *et al.* , 1977; Park *et al.* , 1978; Redman *et al.* , 1979; Roch-Arveiller *et al.* , 1983) aimed at characterizing the potential activity of the fragments of BK-(1-9) in different species and using different protocols. However, the overall conclusion was that the fragments derived from BK-(1-9) were devoid of biological activity, except for the BK-(1-8) (des-Arg⁹-BK).

Over the last 20 years, several new biological activities for BK-(1-9) have been uncovered (Regoli *et al.* , 2015; Regoli *et al.* , 2016), particularly its ability to generate NO, which have considerably increased the range of effects that could be mediated by the nonapeptide. However, the fragments of BK-(1-9) have not been assessed for these new, potential, activities. One problem inherent in such re-assessment of the fragments is the choice of the peptides to study. BK-(1-9) is a substrate for carboxypeptidases (e.g., ACE and ACE2), aminopeptidases (e.g., aminopeptidase P) and endopeptidases (e.g., neutral endopeptidase, now neprilysin), thus generating many different cleavage products (Campbell, 2013; Verano-Braga *et al.* , 2020). A general conclusion of BK-(1-9) metabolism is that its major proteolytic fragment is the BK-(1-8), which is acknowledged as the only biologically active BK peptide known to date, together with BK-(1-7) and BK-(1-5) that are regarded as biologically inert fragments (Kopylov *et al.* , 2016; Semis *et al.* , 2019).

Thus, we present here a re-evaluation of the biological activities exhibited by BK-(1-7) and BK-(1-5), compared with those of the parent nonapeptide BK-(1-9), in a range of *in vitro*, *ex vivo* and *in vivo* systems, using human and rodent tissues. Our results show that, contrary to previous results, these BK-(1-9) fragments are indeed biologically active, stimulating NO production *in vitro*, and inducing vasorelaxation *ex vivo*. Such actions were not mediated by either of the kinin B₁ or B₂ receptors. We also report *in vivo* actions of the BK-(1-9) peptide fragments in cardiovascular parameters (arterial blood pressure and heart rate), and under inflammatory conditions.

2. METHODS

2.1. Synthesis, purification and chemical characterization of BK-(1-9), BK-(1-5) and B₁ receptor antagonist

BK-(1-9) [RPPGFSPFR], BK-(1-5) [RPPGF] and the B₁ receptor selective antagonist Lys-(des-Arg⁹-Leu⁸)-BK-(1-9) [KRPPGFSPPL] were synthesized by solid-phase peptide synthesis employing the Fmoc strategy (Chan *et al.* , 2000), using NovaSyn-TGA resins (Merck, Kenilworth, NJ, USA). The final unprotected peptides were purified by HPLC using a DiscoveryBIO® Wide Pore C18 semi-preparative column. Purified products were analysed by MALDI TOF/TOF Autoflex III Smartbeam mass spectrometer (Bruker Daltonics, Hamburg, Germany) for synthesis quality control.

2.2. Animals

All animal care and experimental procedures were in accordance with the current edition of the NIH *Guide for the Care and Use of Laboratory Animals* and were approved by the local Animal Ethical Committee (CEUA-UFGM; protocol 213/2016). Adult male Wistar rats (8-12 weeks old; weighting 200-250 g), neonatal male Wistar rats (3-5 days old) and adult male C57Bl/6 mice (8-12 weeks old; weighting 20-24 g) were obtained from the CEBIO-UFGM. B₁ and B₂ receptor knockout (B1RKO and B2RKO, respectively) male mice were obtained from the animal facility of the Department of Biochemistry and Immunology at UFGM. All animals were housed in standard cages at the animal facility of the Department of Physiology and Biophysics (UFGM), with controlled temperature (22 ± 2° C) and under a 12/12 h light-dark cycle. Standard chow and filtered tap water were available *ad libitum* . Animal studies were done in accordance with the ARRIVE guidelines (Kilkenny *et al.* , 2010), and with the recommendations made by the editorial board of the *British*

Journal of Pharmacology (Lilley et al., 2020). Animals were divided randomly in the experimental groups and the investigator involved in the data analyses was blinded to the experimental conditions.

2.3. In vivo quantification of BK-(1-9) fragments by multiple reaction monitoring (MRM) mass spectrometry analysis

Adult male Wistar rats were anesthetized with *i.p.* administration of urethane (1300 mg kg⁻¹). A polyethylene catheter was inserted into the inferior vena cava through the femoral vein for *i.v.* administration (0.1 mL) to add the standards, BK-(1-9) (7µg kg⁻¹ or ~2nmol per animal) and heavy-isotope labelled [Pro³(¹³C₅; ¹⁵N)]-BK-(1-9) (7µg kg⁻¹ or ~2nmol per animal) diluted in sterile saline (0.9% NaCl). Blood (~ 3 mL) was collected directly from the left ventricle following 15 seconds or 5 minutes of the BK-(1-9) or [Pro³(¹³C₅; ¹⁵N)]-BK-(1-9) *i.v.* administration. Samples from control animals (no peptide administration) were used to provide as zero-time (time = 0 s) values. This experimental group was important to define the circulating concentration of any endogenous BK peptide fragments evaluated in this experiment (i.e., BK-(1-7) and BK-(1-5)). The blood was collected in a tube containing the protease inhibitors: 1 mmol L⁻¹ p-hydroxymercuribenzoate, 30 mmol L⁻¹ 1,10-phenanthroline, 1 mmol L⁻¹ phenylmethylsulfonyl fluoride (PMSF), 1 mmol L⁻¹ pepstatin A, and 7.5% EDTA – 140 µL mL⁻¹ of blood. Plasma samples were collected by centrifugation (2000 x *g* at 4°C for 20 minutes) and stored at -80degC until further use. The peptides were extracted from plasma using the HF Bond Elut C18 (Variant, Palo Alto, CA, USA). The C18 resin was activated by 2 sequential washes with 10 mL of 99% acetonitrile (ACN) / 0.1% trifluoroacetic acid (TFA), followed by 10 mL of 0.1% TFA. Then, the resin was washed with 3 mL of 0.1% TFA / 0.1% BSA, 10 mL of 10% acetonitrile / 0.1% TFA, and 3 mL of 0.1% TFA. The sample (1.2 mL) was loaded and washed with 20 mL of 0.1% TFA and then 3 mL of 20% acetonitrile / 0.1% TFA. The peptides were eluted with 3 mL of 99% ACN / 0.1% TFA into low-binding polypropylene tubes (Eppendorf, Hamburg, Germany) and dried down using a SpeedVac (Eppendorf, Hamburg, Germany). Each sample was resuspended in 60 µL 0.1% formic acid (concentration factor of the sample = 20-fold) and then loaded (5 µL) onto the ACQUITY I-Class UPLC system (Waters, Milford, MA, USA) coupled to electrospray ionization (ESI) tandem mass spectrometry (LC-ESI-MS/MS Xevo TQ-S, Waters, Milford, MA, USA). The chromatographic separation was done in a C18 column (ACQUITY UPLC BEH C18 Column, 130Å, 1.7 µm, 2.1 mm X 100 mm, Waters, Milford, MA, USA) and each chromatography lasted for 5.5 min. Solvent A was made of 0.1% formic acid in H₂O and solvent B of 0.1% formic acid in ACN. The chromatographic gradient was as follows (expressed as % of solvent B): i) 3-40% in 3.5 min, ii) 40-99% in 0.01 min, iii) 99% for 0.99 min, iv) 99-3% in 0.01 min, and v) 3% for 0.99 min. Regarding the MS analysis, the main parameters were as follows: i) capillary = 3.5 kV; ii) cone = 20V; iii) temperature of the desolvation gas (hydrogen) = 550°C. The collision energy (CE) (argon gas) was tuned for each target peptide spanning from 10 to 20 CE. The following transitions were monitored by the MS in the MRM mode. BK-(1-9) [RPPGFSPFR]: 354.4 419.3 and 354.4 408.2. BK-(1-7) [RPPGFSP]: 379.42 555.3 and 379.42 527.4. BK-(1-5) [RPPGF]: 278.1 408.4 and 278.1 166.1. For the heavy-isotope labelled peptides, we used the following transitions. [Pro³(¹³C₅; ¹⁵N)]-BK-(1-9) [RPP(¹³C₅; ¹⁵N)GFSPFR]: 356.2 419.3. [Pro³(¹³C₅; ¹⁵N)]-BK-(1-7) [RPP(¹³C₅; ¹⁵N)GFSP]: 382.2 561.3. [Pro³(¹³C₅; ¹⁵N)]-BK-(1-5) [RPP(¹³C₅; ¹⁵N)GF]: 290.2 414.2. The calibration curve was obtained using a stock solution containing a mixture of synthetic BK-(1-9), BK-(1-7) and BK-(1-5). The applied calibration curve model ($y = ax + b$) proved accurate over the concentration range 10 to 1000 pg mL⁻¹ ($r^2 = 0.968$). The limit of quantitation (LOQ) was below 10 pg per sample. The inter- and intra-variability of this method was < 20%.

2.5 Nitric oxide measurements in cell culture

Human glioblastoma cell line U-87 MG was cultured in DMEM-F12 cell media, supplemented by 10% fetal bovine serum (FBS) (Cultilab, Campinas, Brazil) and 1% of antibiotic/antimycotic solution (Thermo-Fisher, Waltham, MA, USA) at 37°C. Rat neonatal cardiomyocytes were isolated, as previously reported (Guatimosim *et al.*, 2008). Rat neonatal cardiomyocytes were maintained with DMEM-F12 cell media supplemented by 10% FBS and 1% of antibiotic/antimycotic solution at 37°C until usage. Mice ventricular adult cardiomyocytes were isolated by collagenase type-2 (Worthington, Columbus, OH, USA), as previously described (Rocha-Resende *et al.*, 2012) and kept in Tyrode media (composition: 140 mM NaCl; 4 mM KCl;

1 mM MgCl₂; 1.8 mM CaCl₂; 10 mM Glucose; 5 mM HEPES; pH = 7.4) until used.

For NO quantification, cells were starved with Hank's Balanced Salt Solution (HBSS) for 1 hour and then loaded with DAF-FM diacetate (Thermo-Fisher, Waltham, MA, USA) at a final concentration of 5 μM for 30 minutes. Cells were rinsed with HBSS and stimulated for 15 minutes with BK-(1-9), BK-(1-7) or BK-(1-5), at the final concentration of 100 nM, all diluted with sterile saline. Kinin receptors involvement in NO production by the tested peptides was assessed by selective antagonism of B₁ and B₂ receptors with Lys-(des-Arg⁹-Leu⁸-BK-(1-9) and HOE-140 (Tocris, Bristol, UK), respectively, at a final concentration of 100 nM, in sterile saline, prior to stimulation by BK-(1-9) or its fragments. A concentration-response curve was generated by stimulating male neonatal rat cardiac myocytes with BK-(1-9) and its fragments using concentrations of 1, 10 and 100 nM, in sterile saline, for 15 minutes. After stimulation under these experimental conditions, cells were fixed with a 4% paraformaldehyde solution for 15 minutes and then mounted. Fluorescence emitted from cells was captured by either a confocal microscope Zeiss LSM 5 LIVE (Zeiss, Oberkochen, Germany) or a Nikon Eclipse Ti (Nikon, Melville, NY, USA), with λ_{Ex} = 495nm and λ_{Ex} = 505nm. Fluorescence emitted by cells was quantified by ImageJ (Schneider *et al.*, 2012).

2.6 Vascular reactivity

Vascular reactivity of BK-(1-9) and its fragments were assessed as previously described (Rocha-Resende *et al.*, 2017). Briefly, 8-12 weeks old male Wistar rats were killed by decapitation. The thoracic aorta was quickly dissected, connective and adipose tissues were removed and then the vessel was cut into 3-5mm wide rings. The rings were carefully placed between two stainless-steel stirrups and coupled to an isometric force transducer. The preparations were kept at 37°C, bathed in Krebs-Henseleit salt solution (118 mM NaCl; 4.7 mM KCl; 1.17 mM KH₂PO₄; 1.12 mM MgSO₄·7H₂O; 11.2 mM glucose; 25 mM NaHCO₃; 2.5 mM CaCl₂·2H₂O; pH = 7.3), lightly bubbled with 95% O₂ / 5% CO₂. Endothelial cells of some aortic rings were removed by gently rubbing the rings with a thin wire, to evaluate the contribution of endothelial cells to the vasorelaxation. Aortic rings were stabilized for at least 60 minutes under a passive tension of 1.6 g. During the stabilization period, Krebs-Henseleit solution was replaced every 15 minutes and passive tension was corrected to 1.6 g if needed. Endothelium integrity was assessed by pre-contracting the rings with phenylephrine (100 nM), dissolved in saline. After stabilization of contraction, acetylcholine (ACh, 1 μM) was added to induce endothelium-dependent vasorelaxation. Endothelium was considered intact when vasorelaxation mediated by this concentration of ACh was greater than 80%. For experiments using rings without functional endothelium [E (-)], only preparations with no response to ACh were used.

Vasorelaxation mediated by BK-(1-9), BK-(1-7) and BK-(1-5) was assessed in endothelium-intact rings by constructing a concentration-response curve for each peptide, after pre-contraction of rings with phenylephrine (100 nM). Peptide concentrations tested ranged from 1 pM (10⁻¹² M) to 1μM (10⁻⁶ M). A vehicle (saline) curve was also generated by adding the same volume of saline to the rings, as used with the peptides, as a negative control. Endothelium dependency to those effects was assessed in denuded rings (E (-)). The contribution of NO and prostanoids to the vasorelaxation response was assessed by blocking nitric oxide synthases (NOS) and cyclooxygenases (COX) with N_ω-nitro-L-arginine methyl ester (L-NAME) and indomethacin, respectively. Both inhibitors were used at a final concentration of 1 μM. L-NAME was dissolved in saline and indomethacin was dissolved in DMSO, with further dilutions in saline. The involvement of kinin receptors in vasorelaxation induced by BK-(1-9), BK-(1-7) or BK-(1-5), was assessed by selectively blocking B₁ or B₂ receptors with Lys-(des-Arg⁹-Leu⁸)-BK-(1-9), or HOE-140, respectively (final concentration 100 nM). Inhibitors and antagonists were incubated with preparations for 20 minutes before contraction by phenylephrine.

2.7 Blood pressure measurements in conscious rats

The day before blood pressure measurements, 8-12 weeks old male Wistar rats were anesthetized by ketamine/xylazine (60 mg kg⁻¹ / 9 mg kg⁻¹, respectively, *i.p.*). A polyethylene cannula was inserted into the abdominal aorta through the femoral artery for blood pressure measurements and another cannula was inserted into the inferior cava vein through the femoral vein or in the aorta through the left common carotid

artery for intravenous (*i.v.*) or intraarterial (*i.a.*) administration, respectively, of BK-(1-9) and its fragments. The cannulas, filled with saline and closed by metallic pins, were exteriorized in the interscapular region of the animals' backs. Rats were individually housed and left to recover for 24 hours. On the following day, rats were coupled to a pressure transducer and then allowed to stabilize until cardiovascular parameters were stable. The cardiovascular parameters were recorded using the MP 150 Biopac System (Goleta, CA, USA). First, a dose-response relationship was obtained to assess whether BK-(1-9) and its fragments were able to elicit biological responses *in vivo*. In order to do so, BK-(1-9), BK-(1-7) or BK-(1-5) was administered *i.v.* as a bolus (0.1 mL), corresponding to a dose of 2.5 nmol, 5 nmol or 10 nmol per animal, in sterile saline. Sterile saline was injected at the same volume, as vehicle control. To further explore the *in vivo* effect mediated by BK-(1-9) and its related fragments, a bolus (0.1 mL) of each peptide was administered, corresponding to a dose of 10 nmol per animal, in the arterial or venous circulation of adult male rats to assess whether passage through the pulmonary vascular beds would alter the response to these peptides. We also decided to test whether *in vivo* inhibition of ACE would affect the responses to BK-(1-9), BK-(1-7) and BK-(1-5). After *i.v.* administration of captopril (5mg kg⁻¹, in sterile saline) and stabilization of cardiovascular parameters, animals were given BK-(1-9) or its related peptides (0.1 mL, 10 nmol per animal, in sterile saline).

2.8 *In vivo* plasma extravasation experiment

Plasma extravasation was performed in 8-12 weeks old C57Bl/6 mice as previously reported (Wick *et al.*, 2018). Briefly, 10 nmol in 10 μ L of BK-(1-9), BK-(1-7) or BK-(1-5) was injected intradermally in the hind paw and sterile saline was injected in the same volume (10 μ L) on the contralateral paw as control. Immediately afterwards, Evans' blue (Merck) solution at 2% was injected intraorbitally for measurement of plasma extravasation. After 30 minutes of peptide administration, mice were killed by cervical dislocation and footpads were dissected. The footpads were dried at 60°C for 24 hours and the tissue was weighted. Evans' blue was extracted by formamide for 72 hours under constant stirring and then the absorbance of this final solution was measured by a spectrophotometer at $\lambda = 620$ nm. Results were expressed as μ g of Evans' Blue per mg of dried tissue.

2.9 *In vivo* nociceptive assay

Potential nociceptive reflexes mediated by BK-(1-9), BK-(1-7) and BK-(1-5) were evaluated as previously described (Amaral *et al.*, 2016). 8-12 weeks old C57Bl/6 mice were submitted to 10 μ L intradermal hind paw injection of BK-(1-9), BK-(1-7) or BK-(1-5) at a dose of 10 nmol. Saline was injected on the contralateral paw as control in the same volume (10 μ L). Animals were placed individually in acrylic cages with wired floor and submitted to an adaptation period of 20 minutes. Evaluation of mechanical nociceptive reflex was assessed with a pressure-meter (Insight Instruments) by applying a crescent pressure in the hind paw until the mice evoked a dorsiflexion withdrawn reflex. The pressure by which this event occurred, expressed in grams (g) was registered.

2.10 Relative gene expression by quantitative polymerase chain reaction (qPCR)

Total RNA from U-87 MG cells, male rat neonatal cardiomyocytes, male murine adult ventricular cardiomyocytes and male rat thoracic aorta was extracted with TRIzol reagent (Thermo-Fisher, Waltham, MA, USA) and cDNA was synthesized using First Strand cDNA Synthesis (Thermo-Fisher, Waltham, MA, USA) kit following the manufacturer's instructions. qPCR was performed in a StepOnePlus Real Time PCR system (Applied Biosystem, Beverly, MA, USA) using Maxima SYBR PCR 2x (Thermo-Fisher, Waltham, MA) and primers (Table 1) (Síntese Biotecnológica, Belo Horizonte, Brazil) at the final concentration of 600 nM. The cDNA was diluted in RNase free water 1:100. Thermal cycling protocol was as follows: i) denaturation at 95°C for 5 minutes followed by; ii) 45 cycles of denaturation at 95°C for 10 seconds; iii) annealing/extension at 60°C for 1 minute. Relative expression of the selected genes was performed by $2^{-\Delta\Delta\tau}$ method (Livak *et al.*, 2001) using the housekeeper gene S26 as normalizer.

2.11 Data and statistical analyses

Experimental design and statistics were as recommended by the *British Journal of Pharmacology* (Curtis

et al., 2018). All experiments were done using 5 or 8 independent replicates ($n = 5$ or $n = 8$). Results are expressed as means \pm SEM. All statistical analyses were performed with GraphPad Prism v.7.0 and $P < 0.05$ was considered to show statistical significance between group means. Confocal microscopy ($n = 5$), plasma extravasation ($n = 8$) and nociception experiments ($n = 8$) were analysed by one-way ANOVA. MS-based plasma peptide quantitation ($n = 5$), vascular reactivity experiments and mean arterial pressure (MAP, $n = 5$) and heart rate (HR, $n = 5$) measurements were analysed by two-way ANOVA. Relative mRNA quantitation (qPCR, $n = 5$) data were analysed by unpaired t test (parametric test due to normal distribution).

2.12 Materials

Bradykinin fragment BK-(1-7) [RPPGFSP] was purchased from GenOne Biotechnologies (Rio de Janeiro, Brazil). The heavy-isotope labelled BK-(1-9) [H-Arg-Pro- $^{13}\text{C}_5$, ^{15}N]Pro-Gly-Phe-Ser-Pro-Phe-Arg-OH; or simply RPP(*)GFSPFR] was purchased from Bachem (Torrance, CA, USA). Unless otherwise stated, all reagents were purchased by Sigma (Saint Louis, MO, USA).

3. RESULTS

3.1 Target mass spectrometry to quantify the BK-(1-9) fragments in plasma from rats

BK-(1-9) (RPPGFSPFR) is metabolized in the plasma producing a variety of fragments, which potentially include BK-(1-7) (RPPGFSP) and BK-(1-5) (RPPGF). However, one should note that the detection and quantitation of a peptide in the plasma with the same molecular weight (572.66 Da) and amino acid sequence (RPPGF) of BK-(1-5), for example, does not imply that it is indeed the BK-(1-5) as such fragment could be produced from the hydrolysis of other plasma protein / peptide than BK-(1-9). Therefore, we decided to evaluate whether an acute infusion of BK-(1-9) would alter the concentrations for the potential BK-(1-9) fragments *in vivo*. We did this to ensure that the putative BK-(1-9) fragments were produced from BK-(1-9) metabolization. As shown in Figure 1A, the plasma levels of BK-(1-7) and BK-(1-5) increased after 15 seconds of BK-(1-9) infusion. The highest plasma concentration for BK-(1-7) was observed after 5 minutes of BK-(1-9) infusion while for BK-(1-5), the peak was at 15 seconds of the administration. This result suggests that indeed BK-(1-7) and BK-(1-5) are peptides endogenously derived from BK-(1-9). To confirm this observation, we gave an *i.v.* infusion of the heavy-isotope labelled [Pro 3 ($^{13}\text{C}_5$, ^{15}N)]-BK-(1-9) in anesthetized male Wistar rats, using the same experimental conditions as above. The results (Figure 1B) showed a similar response profile, in which a rapid increase of the heavy-isotope labelled [Pro 3 ($^{13}\text{C}_5$, ^{15}N)]-BK-(1-5) was detected after 15 seconds of [Pro 3 ($^{13}\text{C}_5$, ^{15}N)]-BK-(1-9) administration while the highest concentration of the heavy-isotope labelled [Pro 3 ($^{13}\text{C}_5$, ^{15}N)]-BK-(1-7) was observed following 5 min of [Pro 3 ($^{13}\text{C}_5$, ^{15}N)]-BK-(1-9) administration (Figure 1B).

3.2 Endogenous BK-(1-9) fragments induce NO production *in vitro*

To test whether BK-(1-9) fragments are biologically active, we tested these BK fragments for their ability to induce NO production in different cell types. We used cells from different species (i.e., human, rat and mouse) and known to express the kinin B $_1$ and B $_2$ receptors. As expected, incubation with BK-(1-9) (100 nM) increased NO production in immortalized human glioblastoma cells (U-87 MG), neonatal male rat cardiomyocytes and male adult mouse ventricular myocytes. BK-(1-9) fragments, BK-(1-7) and BK-(1-5), at the same concentration, also induced NO production in these cell types (Figure 2).

We then generated a concentration-response curve for NO production mediated by BK-(1-9), BK-(1-7) and BK-(1-5) in neonatal male rat cardiomyocytes. At all concentrations tested (1, 10, and 100 nM), BK-(1-9) was able to elicit NO production (Figure 3A). The heptapeptide fragment BK-(1-7) induced NO production in neonatal male rat cardiomyocytes but only at the two higher concentrations (10 and 100 nM) in a dose-dependent manner (Figure 3B). The pentapeptide metabolite BK-(1-5) (Figure 3C) was able to induce NO production at all concentration tested, including 1nM, but no significant difference was observed between 10 and 100 nM. These results suggest that BK-(1-7) and BK-(1-5) are not only metabolites of BK-(1-9) *in vivo* but, *in vitro*, they were able to induce NO production in human and rodent cells.

3.3 The in vitro NO production induced by BK-(1-7) or BK-(1-5) is not mediated by activation of the kinin receptors

To evaluate whether BK-(1-7) or BK-(1-5) were agonists at the kinin receptors in vitro, we pre-incubated the cells with a selective antagonist for B₁ receptors, Lys-des-Arg⁹-BK-(1-9) or a selective antagonist for B₂ receptors, HOE-140. In this manuscript we will also refer to them as B1RB or B2RB, respectively, standing for B₁ or B₂ receptor blockage. In human glioblastoma cell line (U-87 MG), NO production induced by BK-(1-9) was only abolished by the selective B₂ receptor antagonist (Figure 4A). However, the NO production induced by BK-(1-7) (Figure 4B) or BK-(1-5) (Figure 4C) were not affected by neither B₁ nor B₂ receptor antagonists. We observed the same response profile in neonatal rat cardiomyocytes, where the response to BK-(1-9) (Figure 4D) was abolished by the selective antagonist of B₂ receptors. No alteration of NO production induced by BK-(1-7) (Figure 4E) or BK-(1-5) (Figure 4F) was observed when the cells were incubated with antagonists of B₁ or B₂ receptors. These results suggest that the two fragments of BK-(1-9) does not act upon the activation of B₁ and/or B₂ receptors to induce NO production in vitro.

Male mice with knockout for either B₁ (B1RKO) or B₂ receptors (B2RKO) were used to further study our initial results. We found that BK-(1-9) induced NO production only in ventricular cardiomyocytes isolated from B1RKO mice (Figure 4G). On the other hand, BK-(1-7) (Figure 4H) and BK-(1-5) (Figure 4I) induced NO production in ventricular cardiomyocytes isolated from either B1RKO or B2RKO mice.

We employed qPCR to confirm that the cells used in this study expressed the mRNA for B₁ and B₂ receptors. As shown in Figure 4J, the mRNA for both receptors was highly expressed in U-87 MG cells, although the expression level of B₁ receptor mRNA was at least twice higher than the B₂ receptor mRNA. The mRNA for B₂ receptors, but not that for B₁ receptors, was detected in neonatal male rat cardiomyocytes (Figure 4K) and in male adult mouse ventricular myocytes (Figure 4L). Taken together, these results indicate that BK-(1-7) and BK-(1-5) do not act via B₁ or B₂ receptors to stimulate NO production in vitro.

3.4 Effects of the BK-(1-9) fragments on vascular tissue ex vivo

BK-(1-9) is known to induce vasodilation due to NO and prostacyclin production in endothelial cells (Marceau *et al.*, 2020). Given the fact that BK-(1-7) and BK-(1-5) were able to induce NO production in vitro, it was highly likely that these fragments could also promote vasodilation. In aortic rings from male rats, BK-(1-9) induced a concentration-dependent vasorelaxation at lower concentrations and vasoconstriction at higher concentrations (Figure 5A). BK-(1-7) (Figure 5B) and BK-(1-5) (Figure 5C) induced a concentration-dependent vasorelaxation only. The maximum vasorelaxation (E_{max}), expressed as a percentage in relation to the vasoconstriction induced by phenylephrine, was $15 \pm 2\%$ for BK-(1-9), $19 \pm 2\%$ for BK-(1-7) and $18 \pm 2\%$ for BK-(1-5).

Furthermore, the vascular effect of BK-(1-9) was completely blocked when the endothelial cells were mechanically removed (Figure 5D) or when NOS was pharmacologically blocked by L-NAME (Figure 5G), whereas BK-(1-9)-induced vasorelaxation was only partly blocked by pharmacological inhibition of cyclooxygenases by indomethacin (Figure 5J). BK-(1-7)-induced vasodilation was partly dependent on endothelial integrity (Figure 5E) and prostanoid production (Figure 5K), while NO production was essential for the BK-(1-7)-induced vasorelaxation (Figure 5H). BK-(1-5) vasoactivity relied on functional endothelium (Figure 5F), and production of both NO (Figure 5I) and prostanoids (Figure 5L). These results suggest that these three peptides, BK-(1-9), BK-(1-7) and BK-(1-5), induced vasorelaxation by different mechanisms.

3.5 The vascular effects of BK-(1-9) fragments are not mediated by the activation of the kinin receptors

To assess the relevance of kinin receptors in the vasorelaxation of aortic ring preparations induced by BK-(1-9) fragments, we used pharmacological antagonists of either B₁ and B₂ receptors, respectively Lys-(des-Arg⁹-Leu⁸)-BK-(1-9) (100 nM) and HOE-140 (100 nM). The vasorelaxant effect induced by BK-(1-9) was abolished by antagonism of either B₂ receptors (Figure 6A) or B₁ receptors (Figure 6D). Interestingly, vasorelaxation induced by BK-(1-7) (Figures 6B and E) and BK-(1-5) (Figures 6C and F) were not altered in the presence

of either antagonist of the kinin receptors.

The expression of mRNA for the kinin receptors was also assessed in rat thoracic aorta by qPCR. The mRNA for both B₁ and B₂ receptors were detected in the rat thoracic aorta, but the expression of B₂ receptor mRNA was significantly higher than that of B₁ receptor mRNA (Figure 6G). Taken together, the results indicate that the vasorelaxation of rat aortic rings induced by BK-(1-7) and BK-(1-5) was independent of the activation of B₁ receptor and/or B₂ receptors.

3.6 In vivo effects of the BK-(1-9) fragments

Vasodilation is often translated in vivo to hypotension as a result of reduced peripheral vascular resistance. Thus, we aimed at assessing whether BK-(1-7) and BK-(1-5) were also able to reduce the arterial blood pressure. When administered intravenously (*i.v.*) in conscious male rats, BK-(1-9) induced a transient dose-dependent hypotension followed by a transient increase in heart rate (tachycardia) due to baroreflex activation. BK-(1-7) and BK-(1-5) induced a significant hypotension followed by tachycardia but, unlike BK-(1-9), this effect was dose-independent (Figures 7A and B, traces in Supplementary Figure S5). The dose-independent relationship observed for BK-(1-9) fragments could be due to extensive hydrolysis of these peptides in the circulation, mainly in the pulmonary vascular bed. To further explore this, we investigated whether the administration of BK-(1-9) and its fragments on a different vascular bed, thereby bypassing the pulmonary circulation upon their administration, could change the observed responses. Intra-arterial (*i.a.*) administration of the BK-(1-9) led to an increased hypotension when compared to *i.v.* administration (Figures 7C and D, traces on Supplementary Figure S6), since BK-(1-9) is subjected to extensive hydrolysis in the pulmonary circulation (Ferreira *et al.*, 1967; Ryan *et al.*, 1968; Ryan *et al.*, 1994) when it is given *i.v.* On the other hand, we could not detect any significant difference when BK-(1-7) or BK-(1-5) were *i.a.* or *i.v.* administered (Figures 7C and D), suggesting that the BK-(1-9) fragments are somehow more resistant to proteolysis in the pulmonary circulation. As ACE is the main peptidase involved in BK-(1-9) cleavage in vivo and this enzyme is particularly active in the pulmonary circulation (Orfanos *et al.*, 1999), we tested the effects of ACE inhibition in vivo with captopril (ACEi) on the cardiovascular effects led by BK-(1-9) fragments. As expected, treatment with captopril potentiated the hypotensive response to BK-(1-9) (Figures 7E and F, traces on Supplementary Figure S7). On the other hand, the cardiovascular effects of the BK-(1-9) fragments were not affected by ACEi (Figures 7E and F, traces on the Supplementary Figure S7). Taken together, our results suggest that the BK-(1-9) fragments induce a discrete hypotension in vivo, although significant when compared with saline administration, which were not affected by the activity of ACE.

Apart from its cardiovascular effects, BK-(1-9) is a potent pro-inflammatory agent (Caçla *et al.*, 2012). Thus, we evaluated whether the BK-(1-9) fragments could induce nociceptive reflexes and vascular permeability, which are known BK-(1-9) responses. We evaluated nociceptive reflexes in adult male mice following intradermal hind paw injection of BK peptides. Although BK-(1-7) and BK-(1-5) evoked nociceptive responses, the response to BK-(1-9) was significantly greater (Figure 8A). Increased microvascular permeability was assessed by extravasation of Evans' Blue dye, following administration of the BK peptides into the footpad of mice. As expected, BK-(1-9) increased vascular permeability in the footpad, but equivalent molar doses of BK-(1-7) or BK-(1-5) had no significant effect (Figure 8B).

4. DISCUSSION

One of the most significant features of the KKS is that its major agonist component, the nonapeptide BK-(1-9) is rapidly and extensively inactivated in vitro or in vivo, in the presence of biological tissues. Such rapid loss of biological activity is shared by many other mediators and neurotransmitters, including NO, prostaglandins, angiotensin II and acetylcholine, and provides an immediate target for pharmacological intervention. Indeed, the isolation of BK-(1-9) (Elliott *et al.*, 1960) was soon followed by the discovery and characterization of the bradykinin potentiating factor (BPF) as a group of peptides that blocked inactivation of the nonapeptide and potentiated its activities (Ferreira, 1965; Ferreira *et al.*, 1970).

Our re-assessment of the biological activities of two peptide fragments of BK has clearly shown that, in contrast to earlier results (Suzuki *et al.*, 1969; Park *et al.*, 1978; Redman *et al.*, 1979; Roch-Arveiller *et al.*

, 1983), BK-(1-7) and BK-(1-5) do exhibit a range of biological activities, some of which are similar to those of BK-(1-9) and others which are significantly different from those of the parent nonapeptide. Although, as did BK-(1-9), the two peptide fragments increased NO production in vitro, exhibited vasorelaxant effects in vivo and induced hypotension in vivo, these biological activities were, unlike those of BK-(1-9), resistant to antagonists of B₁ or B₂ receptors. Moreover, whereas BK-(1-9) showed, as expected, increased nociception and increased microvascular permeability, the two peptide fragments were clearly less potent nociceptive agents and did not affect microvascular permeability.

4.1. Which peptide fragments of BK-(1-9) are relevant in vivo?

Because BK-(1-9) is a substrate for a variety of peptidases, aminopeptidases, carboxypeptidases and endopeptidases, an equal variety of peptide fragments could be generated in vivo. This situation immediately raises the question of which fragments are, in fact, the most likely endogenous peptides formed from BK-(1-9) in vivo. In several earlier studies, BK-(1-8), BK-(1-7) and BK-(1-5) were identified as the major peptide fragments of BK-(1-9) (Murphey *et al.*, 2000; Marshall *et al.*, 2002; Ahmad *et al.*, 2006; Ramirez-Molina *et al.*, 2006; Kopylov *et al.*, 2016; Semis *et al.*, 2019). In the present work, by monitoring the stable isotope-labelled [Pro³(¹³C₅,¹⁵N)]-BK-(1-9), we were able to detect in vivo production of BK-(1-7) and BK-(1-5), after an infusion of BK-(1-9), thus confirming that these fragments are endogenously produced BK-(1-9) metabolites. Taking all the data together, we could demonstrate that BK-(1-7) and BK-(1-5) were endogenously formed stable metabolites of BK-(1-9) in plasma. As such, it is relevant in the context of the overall response to BK-(1-9) in vivo, to assess the biological activities of these two peptide fragments.

4.2. Earlier work on biological activities of BK-(1-9) fragments

The first evidence for important biological activity of a BK-(1-9) metabolite was presented by (Regoli *et al.*, 1977) who found activity in the octapeptide fragment, BK-(1-8) or des-Arg⁹-BK, and who postulated the existence of two kinin receptors, which was later confirmed by the cloning of the B₂ receptor (McEachern *et al.*, 1991) and the B₁ receptor (Menke *et al.*, 1994). Whereas BK-(1-9) is an agonist at both B₁ and B₂ receptors, its metabolite BK-(1-8) is a selective agonist of B₁ receptors. In our work, the activity of either BK-(1-7) or BK-(1-5) on stimulating NO production and inducing vasorelaxation was not affected by either B₁ or B₂ receptor antagonists. Almost 15 years ago, BK-(1-5) and BK-(1-9) were shown to inhibit α -thrombin-induced platelet aggregation and secretion (Hasan *et al.*, 1996) but BK-(1-5) appeared less potent than BK-(1-9) and both peptides were active at higher concentrations than those we have used (0.1 – 1 mM). No further experiments to identify the kinin receptors involved were reported. Later work with BK-(1-5) showed this peptide to increase the survival of rats in a sepsis model and to antagonize the effects of LPS on the contractile response of aortic rings (Morinelli *et al.*, 2001). The latter effect was observed at 1 nM, a concentration similar to those we have used, and these authors discredited the involvement of either B₁ or B₂ receptors although no data with either receptor antagonist was provided. Moreover, these effects of BK-(1-5) were not endothelium-dependent, excluding a possible mediation by NO. In neither of these earlier reports was the heptapeptide BK-(1-7) studied.

4.3. Effects on NO production in cells

We found significant stimulation by BK-(1-7) and BK-(1-5) of NO production in vitro, using neonatal rat and adult mouse cardiomyocytes, as well as human glioblastoma cells. We chose to study cardiomyocytes because the parent peptide BK-(1-9) is known to induce NO production in cardiac myocytes (Oldenburg *et al.*, 2004) and may contribute to cardiac pre-conditioning (Schoemaker *et al.*, 2000; Heusch *et al.*, 2015). We decided to use an immortalized glioblastoma-like cell line derived from humans (U-87 MG) as this cell line expresses mRNA for both B₁ and B₂ receptors (Uhlen *et al.*, 2017), which most likely translates to a high density of these receptors in the cell membrane. Although the concentrations of the peptides used were not physiological, they were comparable to those used in other similar pharmacological studies, for example, in reports of Ang-(1-7) (Gomes *et al.*, 2010), alamandine (Jesus *et al.*, 2018) and BK-(1-9) actions in vitro (Oldenburg *et al.*, 2004). We acknowledge that a concentration-response curve was needed to confirm that BK-(1-9) fragments action on inducing NO production is not a non-selective event and we

were able to show that in rat neonatal cardiomyocytes that BK-(1-9) and BK-(1-5) displayed activity at the lowest concentration used (1 nM), whereas BK-(1-7) was active at a higher concentration (10 nM). Taken together, these in vitro results suggest that the two fragments of BK-(1-9) tested were biologically active in stimulating NO production and that these activities were most likely driven by receptor activation and not by a non-specific interaction, given the nanomolar activity of BK-(1-7) and BK-(1-5). Further studies are necessary to identify which target(s) these molecules act upon.

4.4. Vascular effects ex vivo and in vivo

Blood pressure is controlled by complex mechanisms that modulate cardiac output and peripheral vascular resistance (see (Guyenet, 2006)). The characteristic effect of BK-(1-9) in vivo is a marked hypotension, which was first observed by (Rocha *et al.* , 1949) and later attributed to vasodilation of systemic vessels, leading to a consequent reduction of peripheral vascular resistance (Leeb-Lundberg *et al.* , 2005).

In our ex vivo experiments with aortic rings, the two fragments were as effective as the parent nonapeptide, although the contribution of the endothelium, NO and vasodilator prostanoids to this relaxation differed between the three peptides, implying differences in the mechanisms of vasorelaxation. Another important difference was that the B₁ or B₂ receptor antagonists were ineffective in blocking the vasorelaxation induced by the fragments, as observed for the stimulation of NO production in cultured cells.

When BK-(1-9) was administered in conscious Wistar rats, we observed the expected transient dose-dependent hypotensive response, while the two fragments induced a transient but dose-independent hypotensive response. The acute hypotensive response observed for BK-(1-7) and BK-(1-5), although less prominent than that for BK-(1-9), was similar to that observed for alamandine (Santos *et al.* , 2019), which is a known cardiovascular modulator of the renin-angiotensin system. However, the acute hypotensive effect mediated by BK-(1-7) and BK-(1-5) was not altered by either ACE inhibition or by bypassing the pulmonary circulation (as per *i.a.* administration), implying a resistance of the two fragment peptides to ACE. This result may seem counter-intuitive as ACE is the main enzyme responsible for the metabolism, in vivo, of BK-(1-9) and some of its fragments (Kopylov *et al.* , 2016), but it is also known that BK-(1-9) is a substrate for several other peptidases and it is highly likely in our in vivo model, that both BK-(1-7) and BK-(1-5) are metabolized by peptidases other than ACE. It is important that inhibitors of bradykininases other than ACE are tested to evaluate precisely which enzymes contribute significantly to the cleavage of these metabolites to even smaller products.

4.5. Effects on inflammation

When BK-(1-9) or its fragments were administered to conscious rats, we observed augmented locomotion, a sign of nociception. Nociception is associated with inflammation and the KKS is known to play a major role in this pathophysiological process (Leeb-Lundberg *et al.* , 2005; Marceau *et al.* , 2020). To assess whether BK-(1-7) and BK-(1-5) played any part in inflammatory events similar to BK-(1-9), we evaluated their activities in two known inflammatory effects of the nonapeptide, nociception (Cayla *et al.* , 2012) and increased microvascular permeability (Kempe *et al.* , 2020). We observed that BK-(1-7) and BK-(1-5) increased nociceptive reflexes in C57Bl/6 mice, but the two fragments were significantly less effective than BK-(1-9). On the other hand, we did not observe increased microvascular permeability mediated by the two BK-(1-9) fragments. Our data suggest that BK-(1-7) and BK-(1-5) would be less potent pro-inflammatory agents than BK-(1-9) and that, in the context of inflammation, cleavage of the nonapeptide to BK-(1-7) and BK-(1-5) would present as a reduction in pro-inflammatory activity. Also, the lack of effects on microvascular permeability could imply that hypotension induced by the fragments in vivo would not be accompanied by oedema, as it is for the parent BK-(1-9). Overall, our data suggest that these peptide fragments may have important outcomes beyond the cardiovascular system, and further and more detailed experiments are needed to evaluate their potential roles in nociception and inflammation. On this regard, since the KSS seems to play an important role in the current SARS-CoV-2 pandemic and its associated disease, COVID-19 (van de Veerdonk *et al.* , 2020; Verano-Braga *et al.* , 2020), and some COVID-19 symptoms like thrombosis, lung inflammation and pulmonary edema may be a direct consequence of the so-called “bradykinin storm”

(Garvin *et al.* , 2020), the relevance of BK-(1-7) and BK-(1-5) in COVID-19 should also be studied.

5. CONCLUSION

Our study shows that two peptide fragments of BK-(1-9) – BK-(1-7) and BK-(1-5) – have significant biological activity in human, rat and mouse cell lines. The cardiovascular response triggered by these fragments seems to be comparable with that of BK-(1-9). However, these peptides exhibit significantly less pro-inflammatory responses than those elicited by BK-(1-9). The observed biological activity for BK-(1-7) and BK-(1-5) is mediated by receptors other than the canonical kinin B₁ and B₂ receptors, implying new pharmacological target(s) yet to be characterized for this peptide mediator system. We suggest that the KKS, like the RAS, is now showing signs of much greater complexity than previously described, in terms of active components and possible targets. Such changes would allow the KKS to fine-tune physiological and pathological processes, including those of the cardiovascular system, inflammation and nociception.

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STATEMENT OF CONTRIBUTION

I.M.S.S.: Experimental design, peptide synthesis, confocal cell imaging, vascular reactivity, manuscript preparation and revision; V.P.T., S.R.A.S.J.: Isolation of cardiac myocytes; A.K.S.: qPCR analysis; V.L.S.O., data acquisition from inflammatory and nociceptive in vivo experiments; I.D.R., C.A.P.: In vivo arterial blood pressure experiments; M.M.A: Data acquisition on confocal cell imaging. L.P.B.C.: Peptide synthesis. F.A.A., J.M.R., G.B.M., M.A.P.F., S.G. and R.A.S.: Experimental design, results discussion and revision of the manuscript. T.V.B.: Experimental design, results discussion, revision and manuscript submission.

CONFLICT OF INTEREST

None declared.

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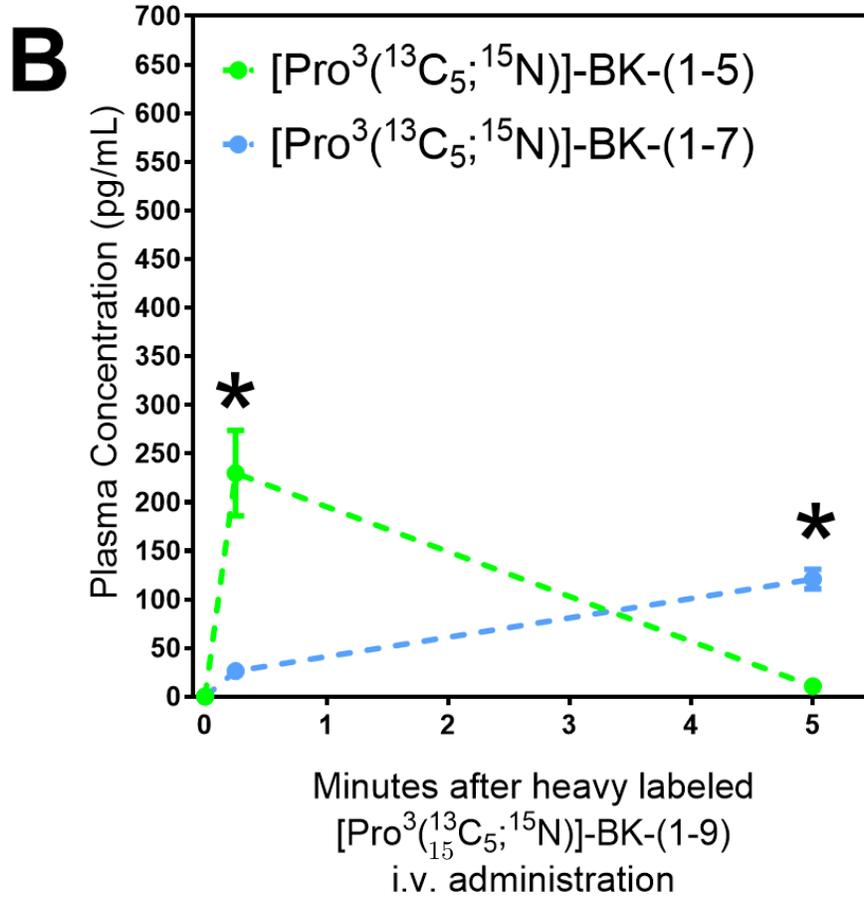
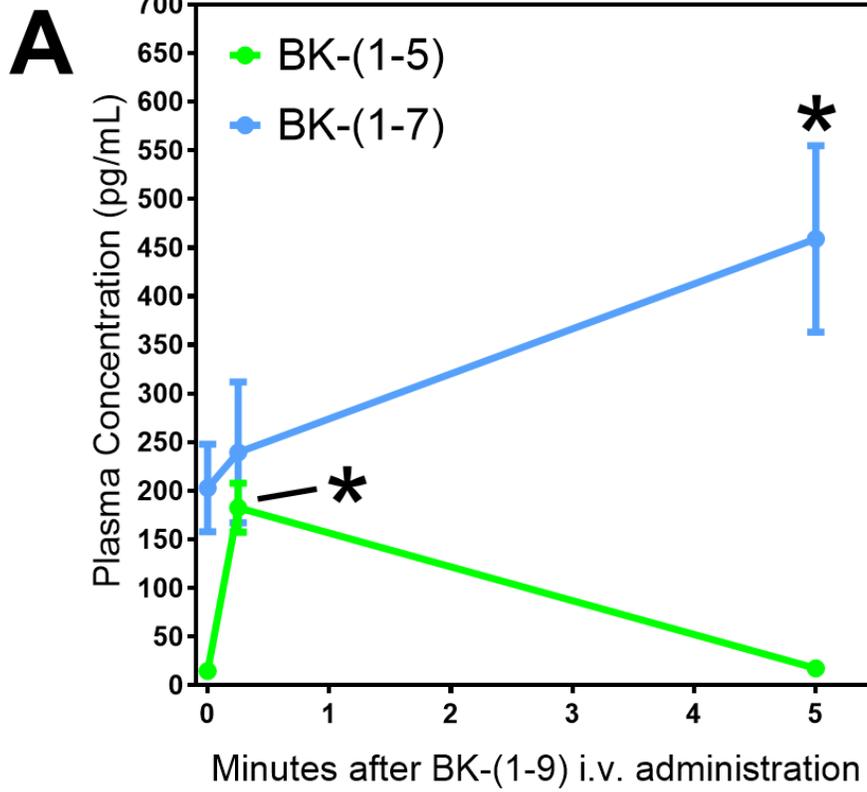
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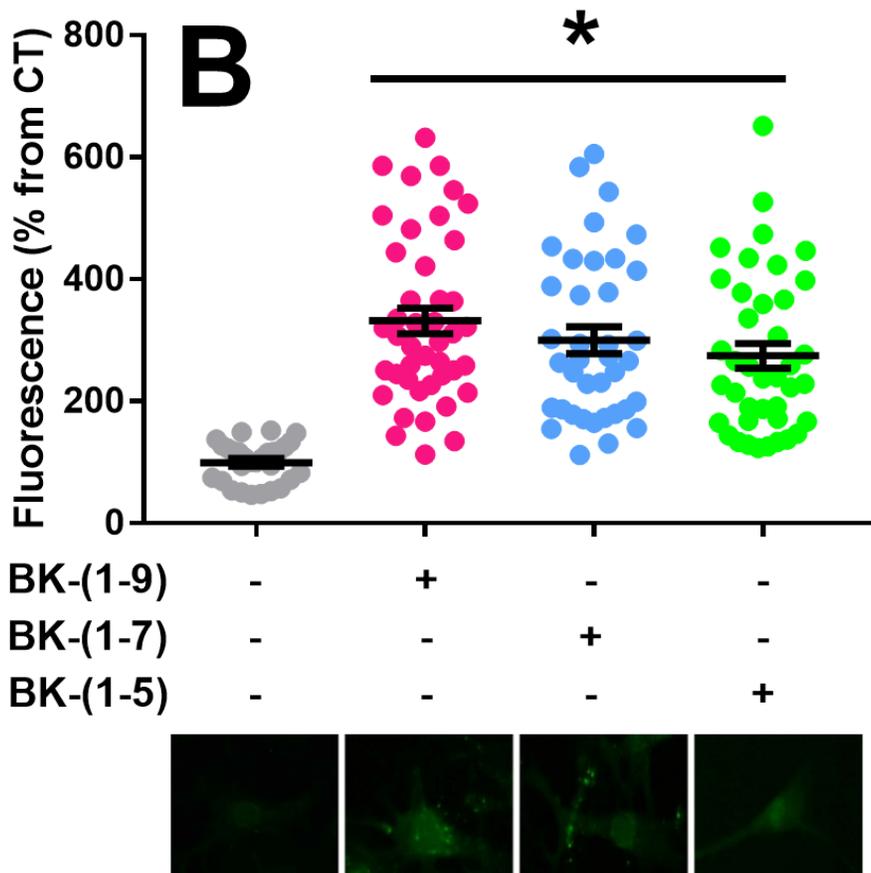
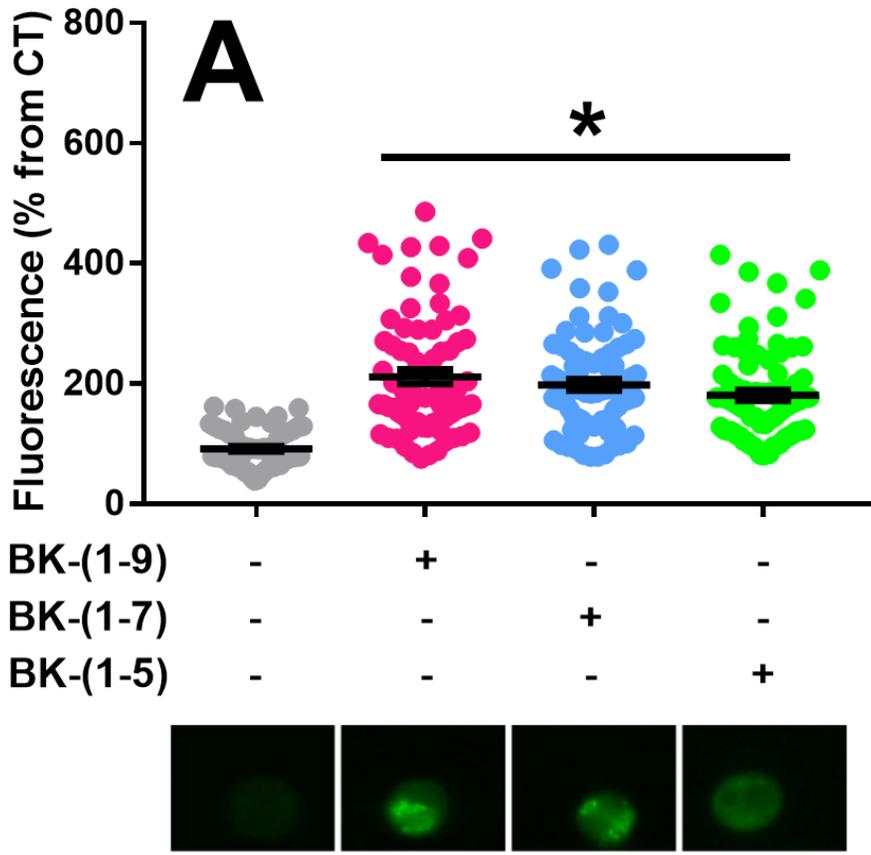
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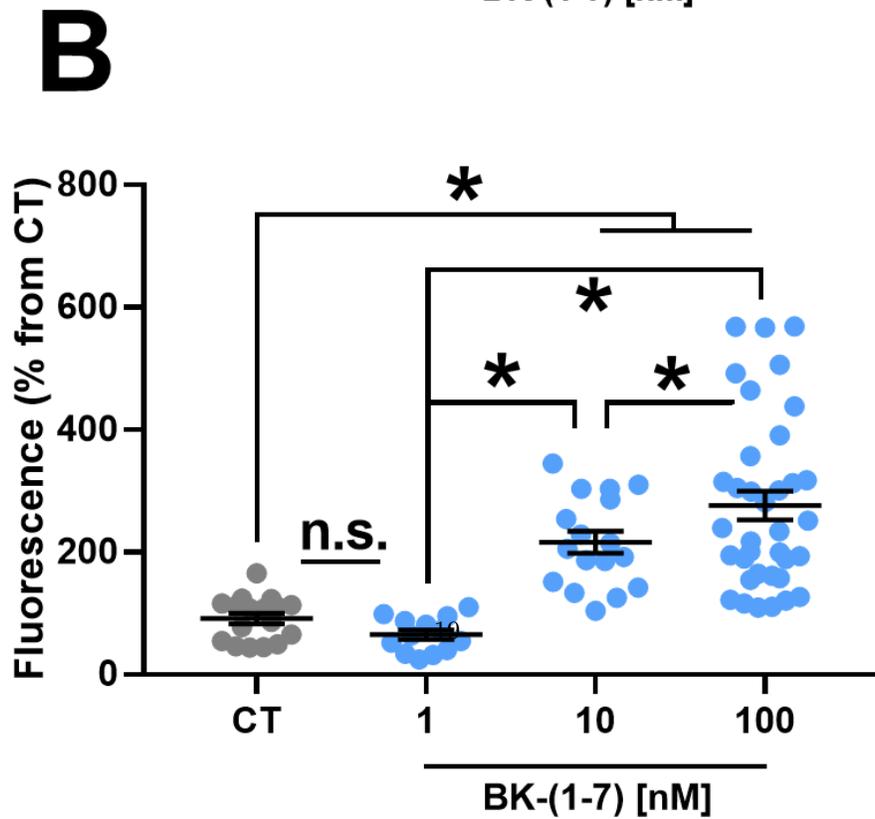
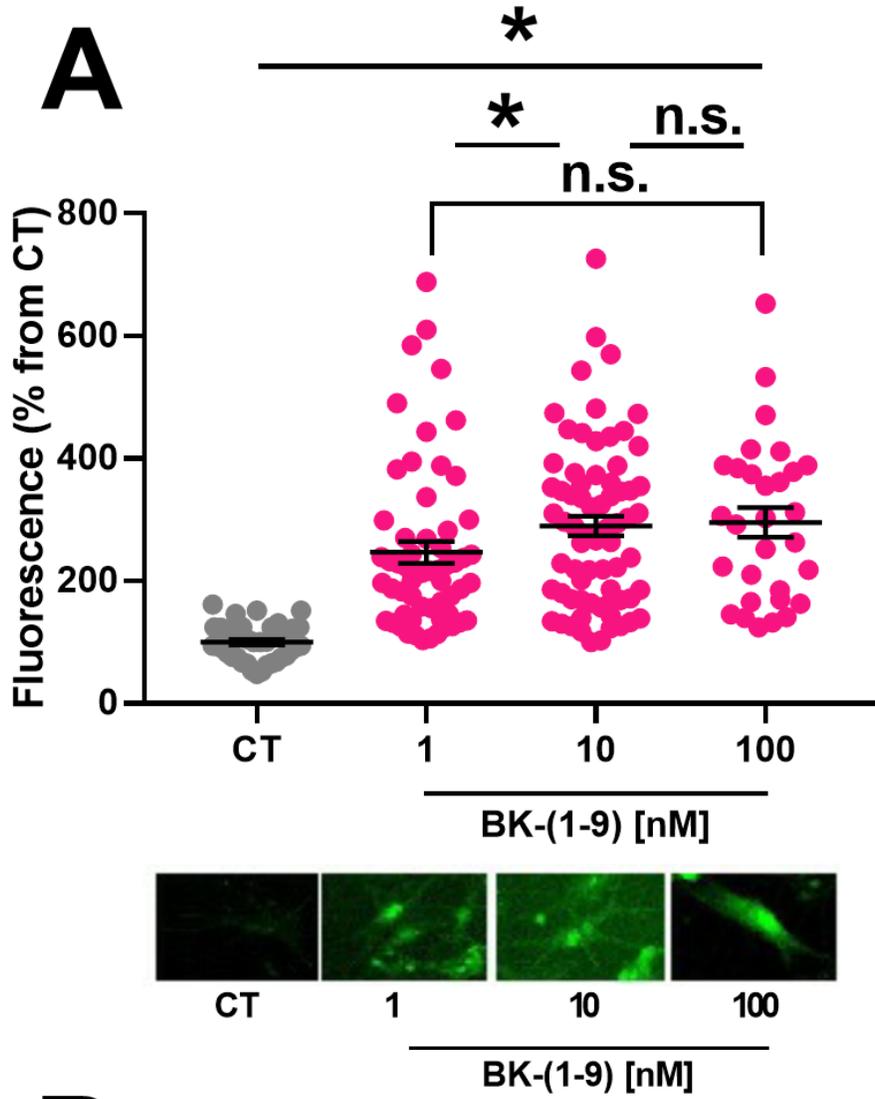
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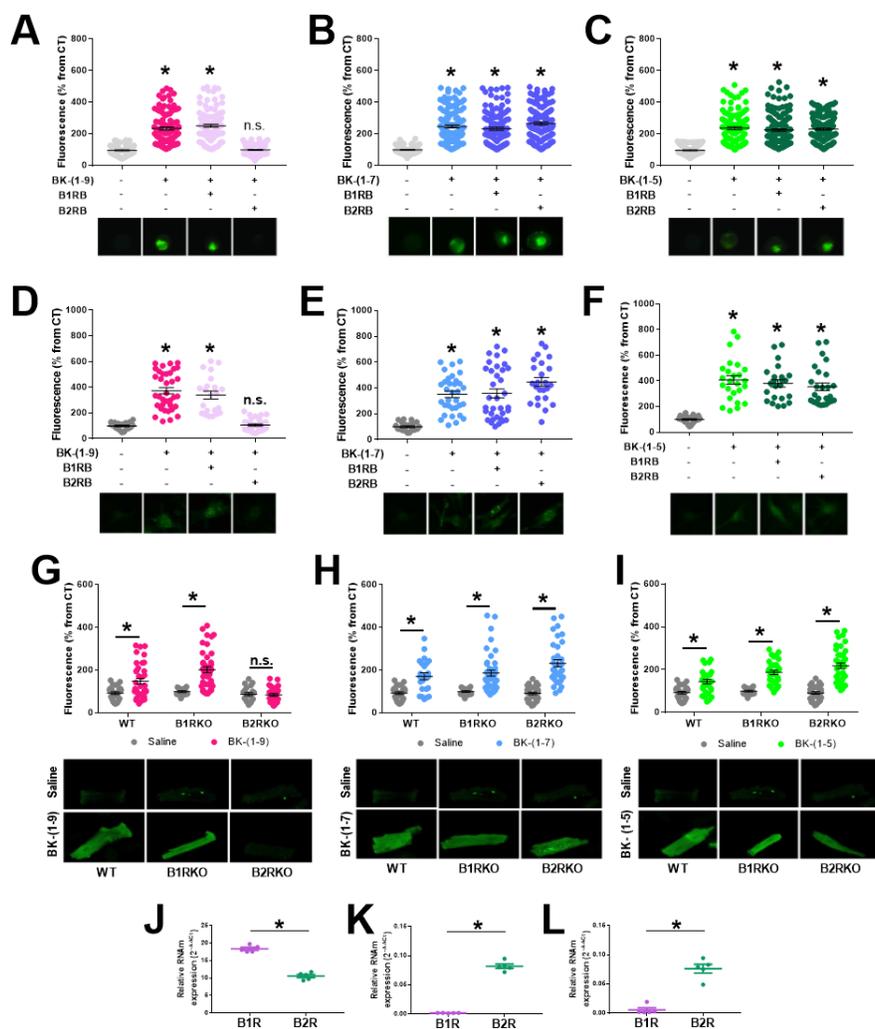
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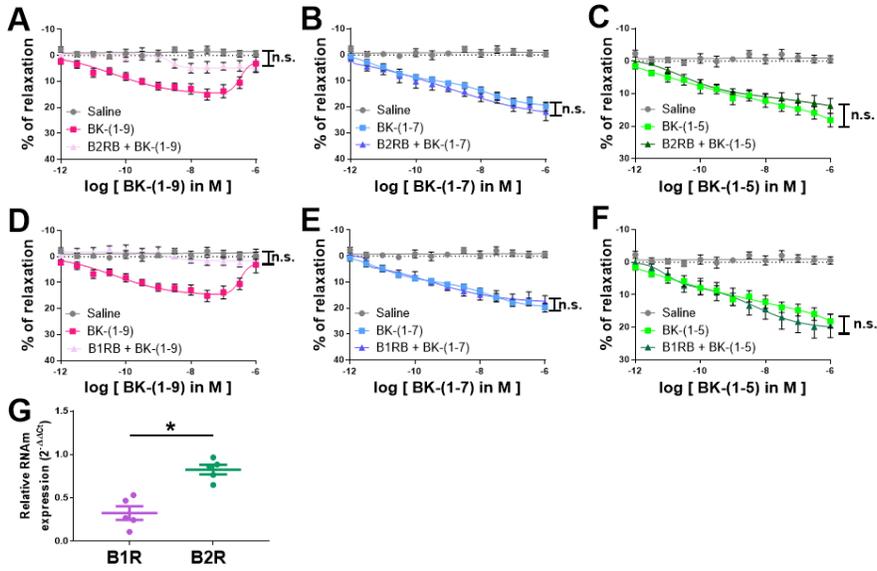
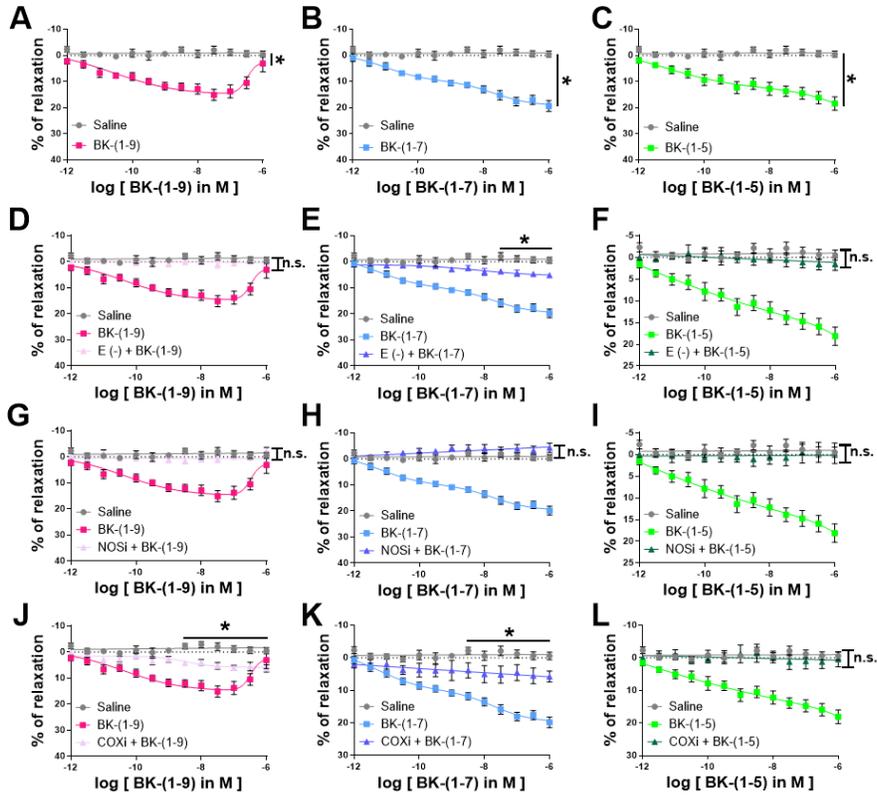


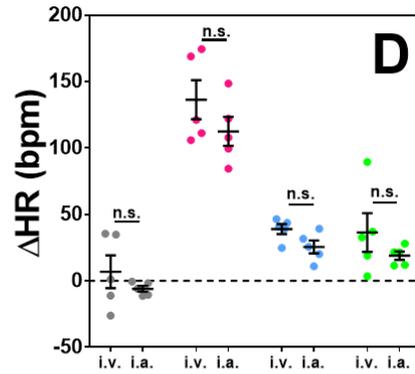
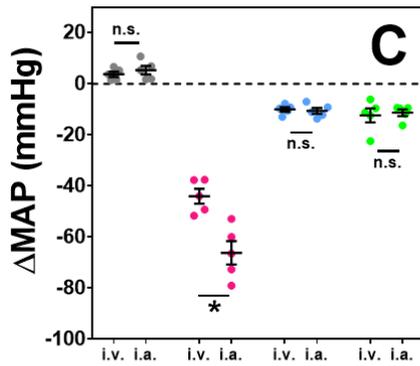
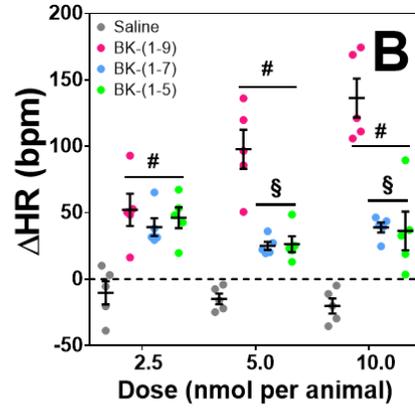
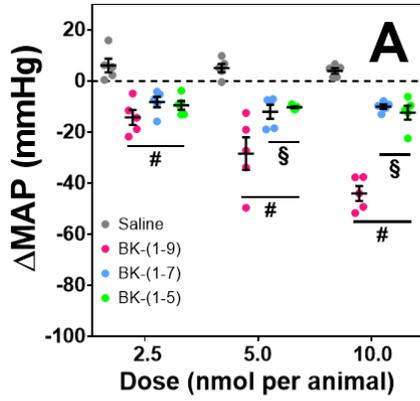


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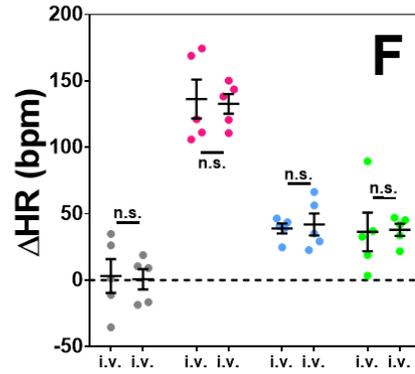
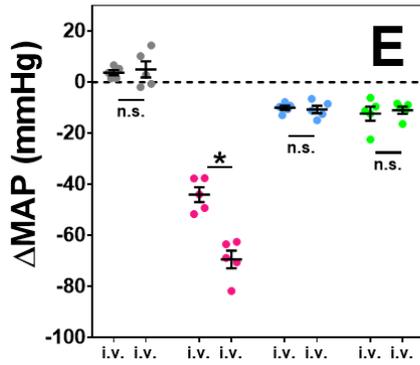






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BK-(1-9)	-	-	+	+	-	-	-	-
BK-(1-7)	-	-	-	-	+	+	-	-
BK-(1-5)	-	-	-	-	-	-	+	+



BK-(1-9)	-	-	+	+	-	-	-	-
BK-(1-7)	-	-	-	-	+	+	-	-
BK-(1-5)	-	-	-	-	-	-	+	+
ACEi	-	+	-	+	-	+	-	+

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BK-(1-5)	-	-	-	-	-	-	+	+
ACEi	-	+	-	+	-	+	-	+

