**Supplementary Material**

Bifunctional fusion proteins containing the sequence of the bradykinin homologue maximakinin: activities at the rat bradykinin B₂ receptor

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**SUPPLEMENTARY METHODS**

Design of human serum albumin (HSA) fusion proteins that contain the BK sequence at their C-terminus

In an effort to generate high molecular weight agonists of the B₂ receptor (B₂R), many HSA fusion proteins were generated from a commercially available vector coding for the secreted HSA (obtained from Origene, Rockville, MD; catalog number 319937). The first HSA fusion protein that was generated used the same spacer agonist combination as the bright fluorescent B₂R agonist EGFP-MK. Indeed, HSA-MK was generated by fusing the amphibian peptide sequence maximakinin (MK) to the C-terminus of HSA. To do so, the sequence coding for HSA was amplified using the following PCR primers: 5’- GTT TAA ACG GGC CCT ATG AAG TGG GTA ACC TT-3’ (sense) and 5’- TT AGG CAA ATC TAA GCC TAA GGC AGC TTG ACT TGC-3’ (antisense) while the MK sequence was extracted from the previously described EGFP-MK vector (Charest-Morin et al. 2013) using the following PCR primers: 5’- C TTA GGC TTA GAT TTG CCT AAG ATC AAC CGC AAA GG-3’ (sense) and 5’- TTG GTA CCG AGC TCG TTA TCG AAA AGG GGA GAA CCC CG-3’ (antisense). The two PCR products were cloned in the BamHI /XbaI digestion product of pcDNA3.1 using the Gibson assembly technique (Gibson Assembly® Master Mix; New England Biolabs, Ipswich, MA).
Several other HSA fusion proteins were generated with different spacer-agonist combinations. HSA-(NG)₅-BK was derived from the B₁ receptor agonist EGFP-S4-P1 (Charest-Morin et al. 2016), which uses 15 repeats of the Asn-Gly dimer. To generate HSA-(NG)₅-BK, the HSA sequence was amplified using the following PCR primers: 5’-C GTT TAA ACG GGC CCT ATG AAG TGG GTA ACC TTT ATT TC-3’ (sense) and 5’-TTG GTA CCG AGC TCG TTA TCG AAA AGG GGA GAA CCC CGG TGG ACG GCC GTT GCC GTT GCC GTT GCC GTT TAA GCC TAA GGC AGC TTG-3’ (antisense). The presence of a nucleotide spacer (underscored) between the sequence overlapping HSA and the one overlapping the pcDNA3.1 vector allowed for the insertion of the (NG)₅-BK sequence in the resulting fusion protein. This PCR product was cloned into the BamHI/XbaI digestion product of the pcDNA3.1 vector using the Gibson assembly technique. The HSA-MISI (MK with Ile-Ser insert) fusion protein was generated through oligonucleotides-directed mutagenesis using the following primers: 5’-CGC AAA GGA CCA ATC AGC CGT CCA CCG GGG-3’ (sense) and 5’-CCC CGG TGG ACG TCG TTA AAA AGG GGA GAA CCC CGG TGG ACG GCC GTT GCC GTT GCC GTT GCC GTT TAA GCC TAA GGC AGC TTG-3’ (antisense). The presence of a nucleotide spacer (underscored) between the sequence overlapping EGFP and the one overlapping the Asn-Gly spacer allowed for the insertion of the FLAG tag sequence (DYKDDDDK) allowing easier further manipulation of the Asn-Gly spacer. Further attempts were made to generate HSA fusion protein with longer spacer linking the agonist and the HSA domain. The strategy that was applied aimed to insert a high number of repeats of the Asn-Gly dimer between HSA and MK. The desired spacer-agonist combination was obtained by two consecutive rounds of PCR. Firstly, the sequence coding for (NG)₁₅-Lys-des-Arg⁹-BK from the EGFP-S4-P1 vector was amplified using the following PCR primers: 5’GAC GAG CTG TAC AAG GAC TAC AAG GAC GAC GAC GAC AAG AAC GGC AAC GGC AAC GGC-3’ (sense) and 5’-TG GTA CCG AGC TCG TTA AAA AGG GGA GAA CCC-3’ (antisense). The presence of a nucleotide spacer (underscored) between the sequence overlapping the Asn-Gly dimer sequence and the one overlapping the pcDNA3.1 vector allowed for the insertion of the MK sequence in the resulting fusion protein.
following PCR primers: 5’- C GTT TAA ACG GGC CCT ATG AAG TGG GTA ACC TTT ATT TC-3’ (sense) and 5’- CTT GTA GTC TAA GCC TAA GGC AGC AGC TTG-3’ (antisense). The two final PCR products were cloned inside the BamHI/XbaI digestion product of the pcDNA3.1 vector using the Gibson assembly technique. Due to the repetitive nature of the Asn-Gly dimer, HSA fusion protein with different numbers of Asn-Gly repeats were obtained. HSA-(NG)$_{15}$-MK, HSA-(NG)$_{30}$-MK, HSA-(NG)$_{45}$-MK and HSA-(NG)$_{60}$-MK were obtained using this strategy, but only the three latter were thoroughly studied.

The final HSA fusion protein that was generated is the cytosolic form of myc-HSA-MK. This fusion protein consists of the full HSA-MK sequence where the secretion signal was replaced by the myc epitope. Indeed, the first 18 amino acids of HSA required for the correct secretion (MKWVTFISLLFLFSSAYS) were deleted and replaced by the myc epitope preceded by a Met codon (MEQKLISEEDL) using once again the Gibson assembly technique. The following PCR primers were used to amplify the HSA sequence (minus the secretion signal): 5’- C GTT TAA ACG GGC CCT ATG GAG CAG AAA CTC ATC TCA GAA GAG GAT CTC AGG GGT GTG TTT CGT CGA-3’ (sense) and 5’- TTG GTA CCG AGC TCG TTA TCG AAA AGG GGA GAA CC-3’ (antisense). The presence of a nucleotide spacer (underscored) between the sequence overlapping the pcDNA3.1 vector sequence and the one overlapping the HSA sequence allowed for the insertion of the myc epitope sequence at the N-terminus of the resulting fusion protein. The HSA PCR product was cloned in the BamHI/XbaI digestion product of the pcDNA3.1 vector. The sequence of all the vectors described above were validated using automated sequencing.

Production of HSA fusion proteins

The cytosolic protein of myc-HSA-MK was produced exactly in the same way as the APEX2-(NG)$_{15}$-MK fusion protein (see Materials and Methods in main text), without the hemin supplementation. However, since all other HSA fusion proteins were secreted, an alternate procedure was elaborated. Producer HEK 293a cells were transfected with a vector encoding a secreted HSA construction using PEI overnight, and the medium of these cells was replaced the day following the transfection with DMEM containing 10% Cell-Ess® (Essential Pharmaceuticals, Ewing, NJ). Cell-Ess® is serum replacement solution of synthetic origin designed to replace FBS in cell culture. In this case, Cell-Ess® was used instead of FBS because the latter contains large amounts of bovine serum
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albumin which could interfere with the subsequent purification and quantification processes. Producer HEK 293a cells were maintained in Cell-Ess® containing medium for 72 hours after which the conditioned medium (CM) was harvested and centrifuged (10 min, 3200 rpm, 4°C) to remove cellular debris. The resulting CMs were then purified and concentrated using Mimetic Blue® SA HL P6XL 1-ml prepacked columns (ProMetic BioSciences Ltd, Rockville, MD) and an infusion syringe pump. The Mimetic Blue® SA HL P6XL is an immobilized chromatography adsorbent that selectively binds albumin and albumin-related proteins. Briefly, using the infusion syringe pump the Mimetic Blue® column was equilibrated with 20 ml of PBS before being loaded with the HSA fusion protein containing CM (50-100 ml). Then, the column was washed using 30 ml of PBS and the HSA fusion protein was eluted with 60 mM sodium octanoate in PBS, pH 7.4 (Sigma-Aldrich). The column was then cleaned with 15 ml of NaOH 1 M, rinsed with 15 ml of PBS and then loaded with storage buffer (20% ethanol/80% 0.1 M NaCl (v/v)). Each column was used several times. Following this purification procedure, the concentration of HSA fusion proteins in the eluates was determined using an anti-HSA ELISA (Abcam, Cambridge, MA).

SUPPLEMENTARY RESULTS

Production and pharmacology of HSA fusion proteins

Immunoblot experiments were exploited to monitor the expression, molecular masses and homogeneity of fusion proteins (Supplementary Figure 3). The secretable constructions s-HSA (natural sequence), s-HSA-MK, s-HSA-MISI and s-HSA(NG)5-BK were all present in the conditioned media of HEK 293a producer cells in concentrations usually exceeding 25 nM, as judged by the intensity of bands produced by authentic solutions of HSA used for calibration (data not shown). Further, the insert determined molecular weight shifts compatible with their size (see details in Supplementary Figure 3 legend). Three additional constructions included long Asn-Gly (NG) repeats (30 to 60) along with a Flag tag (Supplementary Figure 2). Their apparent molecular weight was overestimated by SDS-PAGE (Supplementary Figure 3), possibly indicating a migration anomaly associated with the particular repetitive sequence (however, validity of all vectors was confirmed by sequencing of the coding DNA). The extracts of fusion proteins containing the BK sequence were also reactive with anti-BK antibodies known to react with the C-terminal sequence of
BK, but not at all with des-Arg\(^9\)-BK, indicating an intact C-terminus (Supplementary Figure 3). The 3 extended constructions were as well detected with the anti-Flag tag antibody (Supplementary Figure 3).

The Mimetic blue resin exploits the capacity of HSA to bind small molecule dyes in specific binding pockets. s-HSA and the various secretable fusion proteins based on s-HSA were both concentrated and purified in the eluate of Mimetic blue column as shown by Supplementary Figures 4 and 5: immunoblots for HSA showed that the flow through fluid contained less of the protein than the CM, but that the early eluate was enriched. Fusion proteins possessing the terminal BK sequence and/or the internal Flag tag reacted with the corresponding antibodies. The concentration of the HSA fusion proteins in eluates was determined using the HSA immunoassay, allowing their pharmacological evaluation at a known molarity (Supplementary Figure 6). All purified constructions in the 50-600 nM concentration range failed to mobilize calcium in HEK 293a cells that stably expressed rat myc-B\(_2\)R; the lots of recipient cells were responsive to either BK or MK (Supplementary Figure 6).

Lysates of producer HEK 293a cells transiently expressing myc-HSA-MK, a sequence devoid of signal peptide, contained an abundant protein heavier than mature HSA and reactive with all three antibodies, anti-HSA, anti-BK and anti-myc-tag, supporting the accumulation of a cytosolic fusion protein intact at both termini (Supplementary Figure 7A). Interestingly, the protein applied to Mimetic blue column flowed through and nothing was later eluted from the resin (Supplementary Figure 7B), suggesting the loss of critical binding pockets in the presumably denatured HSA sequence formed in the cytosol of producing cells. The myc-HSA-MK-containing lysate induced significant calcium mobilization (Supplementary Figure 7C), and this effect was specific because it was abated by co-treatment with the B\(_2\)R antagonist anatibant. The agonist effect of myc-HSA-MK is not likely to derive from a C-terminal fragment since no degradation of the protein was evidenced using immunoblots. These results suggest that the BK or MK sequences are not available for receptor binding in the other non-denatured secreted constructions.
Supplementary Figure 1. Constructions based on the hemoprotein APEX2, endowed with peroxidase activity. Top: sequences (one-letter amino acid codes) of the modules used in fusion proteins. Bottom: schematic representation of the hypothetical docking of BK or of the APEX2-(NG)\textsubscript{15}-MK protein to the B\textsubscript{2}R.
Supplementary Figure 2. Constructions based on human serum albumin (HSA). Top: sequences (one-letter amino acid codes) of the modules used in fusion proteins. Constructions that include the natural HSA signal peptide are secreted by producer cells and conventionally identified by the s- prefix. The Met-myc tag sequence at the N-terminus encodes a protein that is cytosolic in producer cells.

Bottom: schematic representation of the hypothetical docking of BK or of HSA fusion proteins that contain the BK sequence at their C-terminus to the B2R.

**Mature HSA**
= DAHKSE VAHRFKDLGE ENFKALVLIA FAQYLQQCPF EDHVKLVNEV TFAKTCVAD EASAENCDSL HTLFGDKLCT VATLRETYGE MADCACQKEP ERNECFLQHK DDNPNLRVL RPEVDVMCTA FHDNEETFLK KYLYINARRH PFYAPELFL FARKYKAFT ECCQAAKAA CLLPLKLDELR DEGKASSAKQ RLKCASLQKF GERAQKAWAY ARLSQRFPPKA EFAEVSKLVT DLTKVHTTEGC HGLLLECADD RADLAKYICE NQDSISLLK ECCEPKPLEK SHCIAEPEnd EMDALPSLA ADVEKSDVCE KNYAEKADVY LGFMYEVAY RHPDYSTYLL LRLKTYETTL ELECCAAADP HECYKVFDE PKPLVEEPQ FONI KONCQEFDE IGEYGFQONLA LLVRYTKVP QVSTPTLVEV SRNLGKVGSK COCKHEPARKM PCAEDYLSVW LGNLVLHEK TPVSDRTYKCG TESLVLNRP RFSALEVEDET YVPEFNAET EFTHADICTL SEKERQIKQG TALVEVYHK PKATKEOLKA VMDDFAAFVE KCKADKDKET CFAEGKGLV AASQAALGL

**Signal Peptide**
= MKWVTFRSSL FLFSSAYSRG VRFR-

**Met, myc tag**
= MEQKLISEEEL-

**Maximakinin**
= DLPKINRKGP RPPGFSPFR Stop

**Maximakinin with Ile-Ser insert (MISI)**
= DLPKINKGPI RPPGFSPFR Stop

**Flag-**
= NGNQNHGNGG RPPGFSPFR Stop

**BK**
= NGNGNGNGNG RPPGFSPFR Stop

**B2R**
= NGNGNGNGG KR

**HSA-sprec-BK**
= DYKDDDDK(NG)nKR

**s-BK**
= DLPKINRKGP RPPGFSPFR Stop

**n = 30, 45 or 60**
Supplementary Figure 3. Immunoblots of the conditioned media of producer HEK 293a cells transiently expressing the indicated constructions (pcDNA3.1 is the empty form of the exploited expression vector). 10 µl of conditioned medium were migrated in 7% gels. An identical volume of a solution of authentic HSA (66.5 kDa) was also migrated. Blots were reacted with horseradish peroxidase-conjugated anti-HSA antibodies, anti-BK antibodies that recognize the C-terminal sequence of the peptide, the last being revealed by secondary HRP-conjugated antibodies. For each of the fusion protein, the observed band is heavier than that of HSA and consistent with the predicted size of the insert (MK: 2.16 kDa; MISI: 2.36 kDa; (NG)₅-BK: 1.90 kDa). However, SDS-PAGE migration overestimated the predicted molecular weight of s-HSA-(NG)₃₀-MK (76.1 kDa), s-HSA-(NG)₄₅-MK (78.7 kDa) and s-HSA-(NG)₆₀-MK (80.2 kDa) which were also detected by anti-Flag tag antibodies. Representative blots from 3 separate experiments.
Supplementary Figure 4. Purification and concentration of the CM of producer HEK 293a cells transiently expressing the indicated construction (first group; the s-prefix indicate a secreted protein; thus s-HSA is authentic HSA). The proteins were purified from the CMs using the affinity resin Mimetic blue. All constructions containing the MK sequence reacted with the anti-BK antibodies.
Supplementary Figure 5. Purification and concentration of the CM of producer HEK 293a cells transiently expressing the indicated construction (second group). Presentation as in Supplementary Figure 4.
Supplementary Figure 6. Lack of calcium mobilization in HEK 293a cells stably expressing rat myc-B$_2$Rs stimulated with secreted fusion proteins that contain the HSA and MK of BK sequences. Protein concentrations were determined using an HSA immunoassay (the final concentrations are indicated). Lots of recipient cells were responsive to BK and/or MK.
Supplementary Figure 7. Cell lysates of producer cells expressing myc-HSA-MK: characterization and pharmacology. A. The construction is expressed as a single band with a proper molecular weight (the N- and C-terminal extensions in myc-HSA-MK amount to 3.48 kDa) and reacts with both anti-BK and anti-myc antibodies, indicating intact N- and C-terminal sequences. B. Attempted purification of myc-HSA-MK using the mimetic blue resin. The protein did not bind to the resin, suggesting denaturation of the HSA binding pockets. C. myc-HSA-MK has agonist activity in recipient HEK 293a cells that stably expressed rat myc-B₂Rs (calcium mobilization) and the effect is abated by the B₂R antagonist anatibant. Presentation as in Supplementary Figure 6.