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2-furoyl-LIGRLO-amide: A potent and selective Proteinase-Activated Receptor 2 (PAR-2) agonist.

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Abbreviations: Amino acids are abbreviated by their one-letter codes; HEK293, human embryonic kidney cells; KNRK, Kirsten virus-transformed rat kidney cells; PAR_n, Proteinase-Activated Receptor n, as per recommendations by the Nomenclature Committee of the International Union of Basic and Clinical Pharmacologists (Hollenberg and Compton, 2002).

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Abstract

A peptide corresponding to a Proteinase-Activated Receptor 2 (PAR-2) activating peptide with an N-terminal furoyl group modification, 2-furoyl-LIGRLO-NH_2, was assessed for PAR-2-dependent and –independent biological activities. 2-furoyl-LIGRLO-NH_2 was equally effective to, and 10- to 25-times more potent than SLIGRL-NH_2 for increasing intracellular calcium in cultured human and rat PAR-2-expressing cells, respectively. In bioassays of tissue PAR-2 activity, measured as arterial vasodilation and hyperpolarization, 2-furoyl-LIGRLO-NH_2 was 10- to 300-times more potent than SLIGRL-NH_2. Unlike trans-cinnamoyl-LIGRLO-NH_2, 2-furoyl-LIGRLO-NH_2 did not cause a prominent non-PAR-2 mediated contraction of murine femoral arteries. In conclusion, 2-furoyl-LIGRLO-NH_2 represents the most potent and selective activator of PAR-2 in biological systems described to date.

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Proteinase-Activated Receptor 2 (PAR_2) is associated with the pathophysiology of many diseases typically involving inflammatory responses. It is a receptor for several trypsin-like serine proteinases that proteolytically cleave its N-terminus to elicit a G-protein-coupled activation signal involving in part, increases in intracellular calcium (Hollenberg and Compton, 2002). Its expression on the endothelium of blood vessels suggests that PAR_2 may have a significant role in the modulation of vascular reactivity during tissue injury or inflammation (McGuire and Triggle, 2003;Vergnolle et al., 2001). Peptide agonists that have been designed to mimic the proteolytic activation of PAR_2 in isolated cell systems have proved of considerable utility to delineate the effects of activating PAR_2 in more complex biological systems *in vitro* and *in vivo* (Macfarlane et al., 2001;Hollenberg and Compton, 2002). The peptide agonists for PAR_2 have been designed on the basis of the so-called tethered ligand sequences of the murine and human receptors, SLIGRL and SLIGKV, that are unmasked by tryptic proteolysis of the receptor N-terminal domain, so as to bind to and activate the receptor (Vu et al., 1991;Hollenberg and Compton, 2002).

Recently, a new compound from among a series of PAR_2 activating peptides, 2-furoyl-LIGKV-OH, was found to mimic the actions of the PAR_2-activating peptide, SLIGRL-NH_2, but with an extended duration of action *in vivo* in a mouse model of chronic arthritis (Ferrell et al., 2003). In this mouse model of arthritis, the intra-articular injection of SLIGRL-NH_2 caused knee joint swelling that reached a maximum at 4 hr and then declined, whereas 2-furoyl-LIGKV-OH caused swelling that was not only equivalent to that of SLIGRL-NH_2 at 4 hr, but reached a maximum at 24 hr that was almost double the maximal swelling caused by SLIGRL-NH_2, and then persisted for at least an additional 48 hr thereafter (Ferrell et al., 2003). The N-terminal furoyl-modification of the human PAR_2-activating peptide sequence was in keeping with prior studies using N-terminal acylation to yield PAR antagonists (Bernatowicz et al., 1996) and to protect PAR peptide-based agonists from endogenous aminopeptidases that are expected to decrease their bioavailability (Coller et al., 1993;Vergnolle et al., 1998;Maryanoff et al., 2001). Among the list of selective PAR_2 activating peptides was the compound trans-cinnamoyl-LIGRLO-NH_2, which is a potent and selective PAR_2 agonist, like SLIGRL-NH_2, and which possesses a carboxy-terminal

ornithine, that when acylated with ^{3}H-propionate, permits its use as a radioligand receptor binding probe (Al Ani et al., 1999). However, our continued experience with the use of the trans-cinnamoyl PAR_2-activating peptide for studies done *in vitro* and *in vivo* revealed that it also can stimulate receptors other than PAR_2 in the mouse vasculature (McGuire et al., 2002a). Thus, despite its selective and potent action on PAR_2 in cultured cells, this acylated agonist appears not to be the best probe for PAR_2 functions in complex biological systems *in vitro* or *in vivo*, where it can have other actions (McGuire et al., 2002a). It is interesting to note that the N-acylated PAR_2 peptide is a full agonist, whereas the N-trans-cinnamoyl derivatives of PAR_1 and PAR_4 activating peptides are antagonists for their respective receptors (Bernatowicz et al., 1996;Hollenberg and Saifeddine, 2001).

Given the limited pharmacological profile described for 2-furoyl-LIGKV-OH (Ferrell et al., 2003), we sought to evaluate in more depth, the action of the comparable peptide, 2-furoyl-LIGRLO-NH_2 to determine whether it might be a more useful compound than trans-cinnamoyl-LIGRLO-NH_2 to assess the potential effects of activating PAR_2 in complex biological systems. We designed the new PAR_2-activating peptide to be more potent than 2-furoyl-LIGKV-OH by taking advantage of the increase in peptide potency we have previously observed upon substituting an arginine for lysine at position 5 and upon C-terminal amidation (Al Ani et al., 1999;Hollenberg et al., 1996).

Methods

Materials

All peptides were synthesized as carboxy amides (> 95% purity, assessed by HPLC and mass spectrometry) by Dr. Denis McMaster and Tyler Vanderputten (Peptide Core Facility at the University of Calgary, Faculty of Medicine, Calgary AB Canada). Unless indicated otherwise all remaining chemicals were purchased from Aldrich-Sigma Co. (St. Louis, MO. USA). Peptides were dissolved in phosphate buffered saline (pH 7.4) containing 25 mM HEPES.

Animals

Sprague-Dawley male rats (350 g) and C57BL/6 male mice (8-10 weeks) were supplied by Charles River (PQ). Procedures that involved animals were approved by the Animal Resources Committee at the University of Calgary and were in accordance with the guidelines of the Canadian Council on the Care of Animals in Research.

Global intracellular calcium measurements

The details of the experimental protocol for measuring changes in global intracellular calcium in cells suspended in solution have been described previously (Al Ani et al., 1999;Compton et al., 2000;Kawabata et al., 1999). The experiments described herein employed either human or rat PAR_2-expressing KNRK cells (Al Ani et al., 1999;Compton et al., 2000) or HEK293 cells, which constitutively co-express human PARs 1 and 2 (Kawabata et al., 1999). Cells harvested without the use of trypsin in an isotonic EDTA-containing dissociation medium were incubated in a solution of α-MEM that contained 10% (v/v) fetal calf serum and 0.25 mM sulphinpyrazone, 22 μM Fluo-3 acetoxymethyl ester (Molecular Probes Inc., Eugene, OR) for 25 min at room temperature. Cells were then re-suspended in a buffered solution (pH 7.4) that contained the following (in mM) NaCl 150, KCl 3, $CaCl_2$ 1.5, glucose 10, HEPES 20 and sulphinpyrazone 0.25. Light emission at 530 nm using 480 nm excitation wavelength was monitored on a AMINCO Bowman Series 2 Luminescence Spectrometer (Spectronic Unicam, Rochester, NY). Cell suspensions (2 ml) in 4 ml cuvettes were mixed continuously with a magnetic stirrer and maintained at 24 °C. The response that resulted from the addition of a test agonist was standardized relative to the peak

fluorescence elicited by the addition of calcium ionophore, (2 μM A23187). In the cross-desensitization experiments, a sufficient time was allowed between the sequential addition of agonists (5 to 10 min) to enable a complete refilling of the intracellular calcium stores (Kawabata et al., 1999).

Rat platelet activation assay

The platelet aggregation assay that was utilized to test for PAR_4 activation of isolated washed rat platelets, was as described (Hollenberg and Saifeddine, 2001).

Isometric tension and membrane potential measurements.

The details of experiments for measuring the isometric tension of isolated rat aorta, murine femoral and mesenteric arteries, the latter whilst recording membrane potential, were as described previously (Compton et al., 2002;McGuire et al., 2002b;McGuire et al., 2002a;McGuire et al., 2003). In brief, segments of arteries were isolated from animals that had been killed by cervical dislocation. Tissues were continuously maintained in a standard physiological salt solution buffer containing (in mM) NaCl 114, KCl 4.7, KH_2PO_4 0.8, $MgCl_2$ 1.2, D-glucose 11, $NaHCO_3$ 25, $CaCl_2$ 2.5, that was bubbled with 95%/5% O_2/CO_2 gas mixture to maintain the buffer at pH 7.4. Rings of rat aorta were suspended vertically by two metal hooks, the upper hook connected to a isometric force transducer and the lower hook connected to an unmovable support, in 5 ml cuvettes containing the standard physiological solution. Two gold-plated tungsten wires (0.02 mm diameter) suspended murine arteries horizontally, one connected to a force transducer and the second to a micro-positioner, in a Mulvany-style myograph chambers (Danish Myograph Technologies (DK)). For electrophysiological studies, a motorized micropositioner was used to place a sharp glass microelectrode that was filled with 3M KCl (resistance 50-100 MΩ) close enough to impale cells from the adventitial side of an artery that was suspended in a single myograph chamber. Drugs were added directly to the chambers that contained the vessels. Isometric tension was recorded from rat aorta and murine femoral arteries with a standard paper chart recorder and a personal computer using MyoDaq/MyoData 2.1 (Danish Myograph Technologies (DK)), respectively. Isometric tension and membrane potentials of murine mesenteric arteries were recorded simultaneously from the same artery with a personal computer using Axotape 2.0 software.

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Data Analysis

pD_2 is equal to the negative logarithm, base 10, of the effective concentration of peptide that elicits 50% of the maximal observed response (E_{max}). Unless stated otherwise, data represent the mean ± s.e. mean (error bars on graphs). *n*, equals the number of separate experiments. Statistical comparison of concentration-response parameters (pD_2 and E_{max}) between two experimental groups was made using Student's t-test for paired data. Comparison of contractile responses by femoral arteries was made by one-way ANOVA after logarithmic transformation of the data (logarithm, base 10, of % of 120 mM KCl-induced contraction) and was followed by Newman Keuls post-hoc test. $*p<0.05$ was considered statistically significant.

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Results

Selective and potent activation of PAR_2 by 2-furoyl-LIGRLO-NH_2

To determine whether 2-furoyl-LIGRLO-NH_2 activates PAR_2, KNRK cells that over-express the native rat PAR_2 or that were transfected with an empty vector were treated with this peptide and the resulting global intracellular calcium changes were monitored (Fig. 1). In the cells over-expressing rat PAR_2, 2-furoyl-LIGRLO-NH_2 (0.01 to 10 μM) caused concentration-dependent increases in intracellular calcium (Fig. 2A) and was about 25-times more potent than the receptor-selective PAR_2-activating peptide, SLIGRL-NH_2. The pD_2 values for 2-furoyl-LIGRLO-NH_2 (*n*=4) and SLIGRL-NH_2 (*n*=3) were 7.0 ± 0.1 and 5.6 ± 0.1, respectively. The maximal response to 2-furoyl-LIGRLO-NH_2 (E_{max} 88 ± 3% of 2 μM A23187-induced response) was not different than that elicited by SLIGRL (E_{max} 89% ± 2% of 2 μM A23187-induced response; **p*>0.05 Student's paired t-test). In the empty pcDNA3 vector-transfected KNRK cells that do possess mRNA for PAR_2, 2-furoyl-LIGRLO-NH_2 (10 μM) raised intracellular calcium only to a small extent (*n*=4, E_{max} 14±4%). Likewise, SLIGRL-NH_2 at 100 μM produced a minimal response (*n*=4, E_{max} 16±3%).

A homo-specific receptor cross-desensitization protocol (Kawabata et al., 1999) was used to determine unequivocally that the 2-furoyl-LIGRLO-NH_2-induced calcium response was mediated through activation of PAR_2. Rat PAR_2-overexpressing KNRK cells were first treated with trypsin (10 U ml^{-1}; 20 nM) for 5 to 10 min prior to the addition of 2-furoyl-LIGRLO-NH_2. Under these conditions of PAR_2 desensitization by prior trypsin-dependent PAR_2 activation, a calcium signal generated by the subsequent addition of 2-furoyl-LIGRLO-NH_2 was completely absent (Fig. 1A). Similarly, desensitization of KNRK cell PAR_2 by prior exposure of the cells to SLIGRL-NH_2 completely eliminated a subsequent response to 2-furoyl-LIGRL-NH_2 and vice versa (Fig. 1B, 1C). The control responses to the agonists *without* prior desensitization are shown on the right in each tracing. These experiments demonstrated a homologous desensitization of rat PAR_2 by all agonists. Cross-desensitization data that mirrored exactly the results

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shown for rat PAR_2-expressing KNRK cells in Fig. 1 were obtained for KNRK cells expressing human instead of rat PAR_2.

Since HEK cells constitutively express both human PARs 1 and 2 (Kawabata et al., 1999), it was possible to evaluate the potential action of 2-furoyl-LIGRLO-NH_2 on both receptors concurrently in this cell line. As shown in Fig. 3, a calcium signal was generated in the HEK cells, upon activation by the selective PAR-activating peptides, TFLLR-NH_2 (PAR_1) and SLIGRL-NH_2 (PAR_2). Like SLIGRL-NH_2, (Kawabata et al., 1999), 2-furoyl-LIGRLO-NH_2 caused concentration-dependent increases in the intracellular calcium concentration of HEK293 cells (Fig. 2B). The pD_2 value for this action of 2-furoyl-LIGRLO-NH_2 (n=4) was 5.4 ± 0.1. The E_{max} value for 2-furoyl-LIGRLO-NH_2 was 92 ± 3% of the maximum calcium response generated by 2 μM A21387. A reverse-sequence peptide synthesized as a control that should not be able to interact with PAR_2, 2-furoyl-OLRGIL-NH_2 (≤200 μM), did not cause a calcium signal in either PAR_2-expressing KNRK or HEK293 cells.

To determine whether 2-furoyl-LIGRLO-NH_2 might also activate or antagonize PAR_1, the HEK293 cells were exposed to the receptor-selective PAR_1 agonist, TFLLR-NH_2, after prior exposure of the cells to desensitizing concentrations of 2-furoyl-LIGRLO-NH_2 (Fig. 3A). A prior desensitization of the HEK293 cells by 2-furoyl-LIGRLO-NH_2 did not significantly diminish the subsequent signal generated by TFLLR-NH_2 in the continued presence of 2-furoyl-LIGRLO-NH_2 (Fig. 3A). Thus, the 2-furoyl peptide neither activated nor antagonized PAR_1. Moreover, after the prior desensitization of PAR_1 by thrombin-dependent (10 U ml^{-1}; 100 nM) activation of PAR_1 (Fig. 3B) or by the PAR_1-selective agonist, TFLLR-NH_2, (100 μM) (Fig. 3C), 2-furoyl-LIGRLO-NH_2 elicited changes in intracellular calcium in HEK293 cells that were at least 70% (n=3) of the control value. Under these desensitizing conditions, the calcium responses to the PAR_1-selective agonists were completely absent (Fig. 3B and 3C). In keeping with the results obtained with the KNRK cells expressing human PAR_2, prior desensitization of the HEK cells with the PAR_2-selective agonist, SLIGRL-NH_2 abrogated the cell response to 2-furoyl-LIGRLO-NH_2 and vice-

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versa (Figs. 3D and 3E). In the desensitization protocols illustrated in Fig. 3, the calcium signals generated by the several agonists without prior desensitization are shown on the right of each tracing.

To determine whether 2-furoyl-LIGRLO-NH_2 might activate or inhibit PAR_4, the actions of this peptide in a PAR_4-dependent rat platelet aggregation assay (Hollenberg and Saifeddine, 2001) were assessed. 2-furoyl-LIGRLO-NH_2 (200 μM) did not cause rat platelet aggregation (n=3); nor did it antagonize platelet aggregation triggered by the PAR_4 activating peptide, AYPGKF-NH_2 (15 μM).

Vascular reactivity of rat aorta preparations

Initially, to assess the vascular activity of 2-furoyl-LIGRLO-NH_2, rat aorta rings with intact endothelium were contracted with phenylephrine (1 μM) and then treated with 2-furoyl-LIGRLO-NH_2 to determine its relaxation activity, as previously documented for SLIGRL-NH_2 (Hollenberg et al., 1996). 2-furoyl-LIGRLO-NH_2 (0.01 to 10 μM) caused a concentration-dependent relaxation of the rat aorta preparation (Fig. 4A). As illustrated by Fig. 4a, 2-furoyl-LIGRLO-NH_2 was about 10-times more potent than SLIGRL-NH_2 (*p<0.05, compared to pD_2 value for SLIGRL-NH_2, Student's t-test for paired data). The pD_2 value and E_{max} values of 2-furoyl-LIGRLO-NH_2 (n=3) for relaxation of rat aorta were 6.5 ± 0.1 and 68 ± 4% relaxation. The pD_2 and E_{max} values of SLIGRL-NH_2 (n=3) for relaxation of rat aorta were 5.6 ± 0.1 and 63 ± 5% relaxation, respectively. The reverse-sequence peptide, 2-furoyl-OLRGIL-NH_2 (10 μM) did not relax the phenylephrine-contracted rat aorta.

Vascular reactivity of murine femoral artery preparations

To assess the relative PAR_2-dependent and PAR_2-independent biological activities of 2-furoyl-LIGRLO-NH_2, the effects of this peptide on vascular reactivity were measured in murine femoral arteries with intact endothelium and these responses were compared to those triggered by other PAR_2-activating peptides under the same conditions. The PAR_2-dependent activity of 2-furoyl-LIGRLO-NH_2 in murine femoral arteries was compared to SLIGRL-NH_2, as measured by the relaxation of these cirazoline (0.1 μM)-contracted arteries. 2-furoyl-LIGRLO-NH_2 (0.01 μM to 10 μM) caused a concentration-dependent relaxation of murine femoral arteries (Fig. 4B). The pD_2 and E_{max} values for 2-furoyl-LIGRLO-NH_2 were

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(n=4) 7.9 ± 0.3 and 86 ± 6% relaxation, respectively. The pD_2 and E_{max} values for SLIGRL-NH_2 were (n=4) 5.5 ± 0.1 and 74 ± 7% relaxation, respectively (Fig. 4B). The reverse-sequence peptide, 2-furoyl-OLRGIL-NH_2 (0.1-10 μM; n=4) did not relax cirazoline-contracted murine femoral arteries.

The PAR_2-independent activity of 2-furoyl-LIGRLO-NH_2 in murine femoral arteries with intact endothelium was compared to that of trans-cinnamoyl-LIGRLO-NH_2, as measured by the contraction of these arteries from baseline tension (Fig. 5) (McGuire et al., 2002a). At a concentration of 50 μM i.e. 3000-times the pD_2 value for its relaxation activity, 2-furoyl-LIGRLO-NH_2 caused less than 15% of the contraction elicited by an equal concentration of trans-cinnamoyl-LIGRLO-NH_2. The contraction of arteries treated a second time with 2-furoyl-LIGRLO-NH_2 after a prior exposure to 50 μM 2-furoyl-LIGRLO-NH_2 followed by a tissue wash (homologous desensitization protocol), was not statistically different than the first exposure. However, the contractile responses of tissues treated with 50 μM 2-furoyl-LIGRLO-NH_2 after prior exposure to 50 μM trans-cinnamoyl-LIGRLO-NH_2 and washout (heterologous desensitization protocol) were abolished. The contractile responses of arteries treated with 50 μM trans-cinnamoyl-NH_2 after prior exposure to 50 μM 2-furoyl-LIGRLO-NH_2 and washout (heterologous desensitization protocol) were unaffected. The reverse-sequence peptide, 2-furoyl-OLRGIL-NH_2 (50 μM; n=4) did not contract murine femoral arteries at baseline tension.

Electrophysiological responses of murine small caliber mesenteric arteries to 2-furoyl-LIGRLO-NH_2

To assess the electrophysiological activity of 2-furoyl-LIGRLO-NH_2, the membrane potential of murine mesenteric arteries with intact endothelium were measured either at baseline tension or during contraction with cirazoline, and then during treatment with 2-furoyl-LIGRLO-NH_2 (0.01, 0.1, 1 μM). 2-furoyl-LIGRLO-NH_2 caused a concentration-dependent hyperpolarization of murine mesenteric arteries at baseline tension (Fig. 6A). When murine mesenteric arteries were contracted by cirazoline, 2-furoyl-LIGRLO-NH_2 (0.01, 0.1, 1 μM) caused simultaneous hyperpolarization and relaxation of these arteries (Fig. 6B, 6C). The reverse-sequence peptide, 2-furoyl-OLRGIL-NH_2 (0.01-1 μM; n=3) caused neither hyperpolarization nor relaxation of murine mesenteric arteries.

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Discussion

Receptor selectivity and increased potency of 2-furoyl-LIGRLO-NH_2 for PAR_2

The main finding of our study was that the N-acylated peptide, 2-furoyl-LIGRLO-NH_2, is a potent and selective activator of PAR_2 that, like the isoserine derivative of the PAR-activating peptide SFLLRN (Coller et al., 1993), would be expected to be resistant to aminopeptidases. Our new data considerably extend the limited pharmacological information published previously about the receptor selectivity of a comparable peptide, 2-furoyl-LIGKV-OH, used for studies in a murine arthritis model *in vivo* (Ferrell et al., 2003). Our newly designed PAR_2 activating peptide agonist, 2-furoyl-LIGRLO-NH_2, prompted by that previous study (Ferrell et al., 2003), was almost an order of magnitude more potent than SLIGRL-NH_2 for the activation of rat and human PAR_2 as measured by intracellular calcium signalling in cultured PAR_2-expressing cells and in causing an endothelium-dependent relaxation of rat aorta rings. In this regard, our novel N-acylated PAR_2 agonist, which would provide for the development of a PAR_2 receptor binding probe (Al Ani et al., 1999), was also more potent than trans-cinnamoyl-LIGRLO-NH_2, which is equipotent with SLIGRL-NH_2 in such assays (Al Ani et al., 1999;Vergnolle et al., 1998). 2-furoyl-LIGRLO-NH_2 was specific for the activation of PAR_2 as compared to the activation of PARs 1 and 4, which were unaffected. The homologous receptor cross-desensitization experiments using the calcium signaling assay with either PAR_2-expressing KNRK cells or HEK293 cells that constitutively express both PARs 1 and 2 confirmed the receptor selectivity of 2-furoyl-LIGRLO-NH_2, as did its lack of activity in the PAR_4-triggered rat platelet aggregation assay. Interestingly, this N-acylated peptide was not an antagonist for PAR_1 as is the trans-cinnamoyl derivative of PAR_1-related peptide sequences (Bernatowicz et al., 1996). The much greater pD_2 value of 2-furoyl-LIGRLO-NH_2 for relaxation of the rat aorta preparation, compared to the pD_2 value of SLIGRL-NH_2, was comparable to the greater relative potency of 2-furoyl-LIGRLO-NH_2 relative to SLIGRL-NH_2 in the calcium signaling assay using PAR_2-expressing KNRK cells. Thus, the increased potency of the 2-furoyl derivative would appear not to depend on the PAR_2-bearing target cell or tissue on which it acts.

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We hypothesize that the addition of the more sterically compact furoyl group to the amino terminus of the PAR_2-activating peptide sequence (LIGRLO-NH_2), compared with the bulky N-terminal trans-cinnamoyl goup, provides an even more conformationally favourable motif for interacting with the receptor than does the serine of the native tethered ligand activating peptide, SLIGRL-NH_2 under the same conditions. Based on structure-activity data obtained by us and by others for PAR_2-activating peptides (Hollenberg et al., 1996;Al Ani et al., 1999;Maryanoff et al., 2001), it was expected that our derivative with arginine in the penultimate C-terminal position and a C-terminal amide would be more potent than the previously described peptide with lysine at that position and without a C-terminal amide (Ferrell et al., 2003). It is also possible, given our previous structure-activity data for PAR_2-activating peptides (Hollenberg et al., 1996;Al Ani et al., 1999), that the additional ornithine residue at the C-terminus would confer increased potency of our peptide over the non-carboxy-amidated 2-furoyl derivative previously described (2-furoyl-LIGKV-OH) (Ferrell et al., 2003). This assumption would have to be confirmed experimentally in future work that would be facilitated by the synthesis of radiolabeled ligands, and the future discovery of a PAR_2 antagonist. Notwithstanding, our new derivative is two- to three-times more potent in the HEK293 cell calcium signalling assay (pD_2 5.5) than the potency reported for the action of 2-furoyl-LIGKV-OH on human PAR_2-expressing NCTC 2544 cells (pD_2 5.08) (Ferrell et al., 2003). A resistance to aminopeptidase activity in the assays done on cells *in vitro* cannot explain the comparatively high potency of the 2-furoyl derivative, since our previous work has shown that amino-peptidase inhibitors like amastatin do not influence the potency of non-acylated peptide agonists in the assay (Kawabata et al., 1999). The likely resistance of the 2-furoyl derivative to amino-peptidase action makes it an attractive agonist to employ for studies done *in vivo*, as demonstrated by the use of the agonist, 2-furoyl-LIGKV-OH in studies of murine arthritis (Ferrell et al., 2003).

Relative PAR_2 versus non-PAR_2-activities of 2-furoyl-LIGRLO-NH_2 in the murine vasculature

It has been demonstrated conclusively that PAR_2 activation is responsible for SLIGRL-NH_2–induced relaxation of contracted murine femoral arteries (McGuire et al., 2002a), as is the SLIGRL-NH_2 -induced blood pressure lowering activity of SLIGRL-NH_2 in mice measured *in vivo* (Damiano et al., 1999),

because both responses are absent in PAR_2-deficient mice. Remarkably, 2-furoyl-LIGRLO-NH_2 was up to 300-times more potent than SLIGRL-NH_2 in the murine isolated blood vessel assays, wherein the responses are PAR_2-dependent. In terms of its relaxant action, the peptide trans-cinnamoyl-LIGRLO-NH_2 has been observed to be equipotent with SLIGRL-NH_2 (McGuire et al., 2002a;Al Ani et al., 1999;Vergnolle et al., 1998). In contrast with SLIGRL-NH_2, trans-cinnamoyl-LIGRLO-NH_2 causes a PAR_2-independent contraction of murine femoral arteries from baseline tension at concentrations that are about 50-times greater than its pD_2 value for relaxation (McGuire et al., 2002a). In comparison with the trans-cinnamoyl derivative, 2-furoyl-LIGRLO-NH_2 caused only a minor contraction of the murine femoral arteries (about 15% of the trans-cinnamoyl-LIGRLO-NH_2-induced response) at a concentration that was 3000-times greater than the pD_2 value for its PAR_2-mediated response (relaxation). Therefore, its combined increased receptor potency and its very low predicted non-PAR_2-dependent activity enhanced the useful range of concentrations for 2-furoyl-LIGRLO-NH_2 for activating PAR_2, at least as assessed in mouse-based assays. This property of our new agonist is important because of the number of transgenic mouse models that have been developed to predict human diseases in which it will be of considerable interest to determine the consequence of PAR_2 activation via the use of PAR_2-activating peptides. Such studies would preclude the use of PAR_2 agonists that do not have the potency and selectivity of our newly designed PAR_2 agonist.

2-furoyl-LIGLRO-NH_2 had the same actions qualitatively as SLIGRL-NH_2 in the murine small caliber mesenteric arteries and had the same increase in relative potency (>100-times) compared to SLIGRL-NH_2 (pD_2 value of 6 in murine mesenteric arteries (McGuire et al., 2002b) as had been found in the murine femoral arteries. Interestingly, the potency of 2-furoyl-LIGRLO-NH_2 relative to SLIGRL-NH_2 in the murine bioassays was even greater than the potency found for the 2-furoyl derivative relative to SLIGRL-NH_2 in the rat and human PAR_2-mediated calcium signalling and rat aorta relaxation assays. The reason for these differences between assays may possibly relate to subtle species differences between the interactions of the 2-furoyl derivative with the distinct mouse, rat and human receptor sequences.

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Alternatively, differences in PAR_2 coupling to signal transduction and calcium regulation in native murine endothelial cells versus the coupling to calcium signaling in PAR_2-expressing cell lines may account for differences in the relative potencies of 2-furoyl-LIGRLO-NH_2 to SLIGRL-NH_2 in the different assay preparations. The premise that the differences in relative potencies are due to differences in the sequences of PAR_2 between species is supported by the apparent difference in potency of 2-furoyl-LIGRLO-NH_2 relative to SLIGRL-NH_2 for calcium signalling in the rat compared to human receptor expressed in cultured cells. Furthermore, the PAR_2-dependent activation of mouse endothelial calcium-activated K^+ channels that elicit the hyperpolarization response in mesenteric arteries (McGuire et al., 2002b;McGuire and Triggle, 2003) and endothelial NO synthase activation in femoral arteries (McGuire et al., 2002b) would also be expected to be more indicative of subcellularly localized changes in calcium than are the gross global changes to intracellular calcium arising from PAR_2 activation in transformed cells.

Conclusion

The peptide 2-furoyl-LIGRLO-NH_2 greatly surpasses the potency and receptor selectivity *in vitro* of previously described PAR_2 activating peptides. Given the markedly increased activity of this peptide for PAR_2-dependent activities and the enhanced selectivity for activating PAR_2, compared with the triggering of PAR_2-independent effects in more complex biological assays, this compound should prove to be very useful for studies done in intact animals, aimed at elucidating the potential pathophysiological responses due to PAR_2, when activated *in vivo* by locally generated proteinases.

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Footnotes

[1] Canadian Institutes of Health Research Group on Regulation of Vascular Contractility, Smooth Muscle Research Group.

[2] Mucosal Inflammation Research Group.

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Legends for Figures

Fig. 1. Calcium signalling and ligand cross-desensitization in rat PAR_2-expressing KNRK cells

Rat PAR_2-expressing KNRK cell suspensions containing intracellular fluo-3 were exposed twice to PAR_2-desensitizing concentrations of either (A) trypsin (Trp, 10 Uml^{-1}; 20 nM) or (B) SLIGRL-NH_2 (100 μM), followed by a test concentration of 2-furoyl-LIGRLO-NH_2 (1μM; 2-f-LIGRLO-NH_2). Alternatively, cells were first exposed to desensitizing concentrations of (C) 2-furoyl-LIGRLO-NH_2 (50 μM), followed 10 min later by a test concentration of the selective PAR_2-activating peptide, SLIGRL-NH_2 (25 μM). The calcium signals (fluorescence at 530 nm (E_{530}: upward deflection) generated by the test concentrations of either 2-furoyl-LIGRLO-NH_2 (1 μM) or SLIGRL-NH_2 (25 μM) *without* prior desensitization are shown to the right of each tracing (A, B, C). The scales for time (min) and calcium signal (upward deflection in cm) are shown by the inset. On average, intracellular calcium concentrations rose from a baseline value of about 30 nM to a maximum value of about 340 nM.

Fig. 2. Concentration-effect curves for calcium signalling by 2-furoyl-LIGRLO-NH_2 in PAR_2-expressing cells.

Suspensions of either (A) rat PAR_2-expressing KNRK or (B) HEK293 cells were treated with 2-furoyl-LIGRLO-NH_2 (n=4) at different concentrations and the relative global changes in intracellular calcium were measured by fluorescence of fluo-3. Values were standardized as a percentage relative to a maximal intracellular calcium-dependent fluorescence signal caused by the addition of calcium ionophore (2 μM A23187).

Fig. 3. Calcium signalling and ligand cross-desensitization in human HEK cells co-expressing PARs 1 and 2.

Cross-desensitization protocols like those outlined in the legend to Fig. 1 were done with fluo-3-containing human HEK cells that co-express both PAR_1 and PAR_2. The sequences of the desensitization protocols were: A) desensitization with 2-furoyl-LIGRLO-NH_2 (100 μM; 2-f-LIGRLO-NH_2), followed by a test concentration of the PAR_1-selective agonist, TFLLR-NH_2 (5 μM); B) desensitization with thrombin

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(100 nM) followed by a test concentration of 2-furoyl-LIGRLO-NH_2 (2.5 μM); C) desensitization with TFLLR-NH_2 (100 μM) followed by a test concentration of 2-furoyl-LIGRLO-NH_2; D) desensitization with the PAR_2-selective agonist, SLIGRL-NH_2 (100 μM) followed by a test concentration of 2-furoyl-LIGRLO-NH_2 (2.5 μM); E) desensitization with 2-furoyl-LIGRLO-NH_2 (100 μM) followed by a test concentration of SLIGRL-NH_2 (25 μM). The calcium signals (E_{530}, upward deflection) generated by the various agonists without prior desensitization are shown on the right of each tracing A to E. The scales for time (min) and calcium signal (upward deflection, cm) are shown by the inset. On average, intracellular calcium concentrations rose from a baseline value of about 30 nM to a maximum value of about 340 nM.

Fig. 4. Concentration-response curves for relaxation of rat aorta and murine femoral arteries by 2-furoyl-LIGRLO-NH_2.

Rings of (A) rat aorta (n=4) and (B) murine femoral arteries (n=4) were contracted by sub-maximal concentrations of phenylephrine and cirazoline, respectively, and then tissues were exposed to increasing concentrations of either 2-furoyl-LIGRLO-NH_2 or SLIGRL-NH_2. 100% relaxation represents the complete reversal of the initial contractions by alpha-adrenergic-agonists.

Fig. 5. A comparison of the PAR_2-independent contraction of murine femoral arteries by trans-cinnamoyl-LIGRLO-NH_2 to 2-furoyl-LIGRLO-NH_2.

Rings of murine femoral arteries with intact endothelium at baseline tension conditions were exposed to 50 μM of either trans-cinnamoyl-LIGRLO-NH_2 or 2-furoyl-LIGRLO-NH_2 and then the contractile reposes were measured. Contractions are expressed as a percentage relative to a maximal contraction caused by 120 mM KCl. For the homologous desensitization protocol, rings of femoral arteries were exposed to 50 μM 2-furoyl-LIGRLO-NH_2 after the washout of the prior addition of 50 μM 2-furoyl-LIGRLO-NH_2. For heterologous desensitization protocols, 50 μM of either 2-furoyl-LIGRLO-NH_2 or trans-cinnamoyl-LIGRLO-NH_2 were added after the washout of the prior addition of 50 μM of the other agonist. One-way ANOVA $F(5,14)=28.89$, $p<0.0001$. Newman Keuls post-hoc test for multiple comparisons: ** $p<0.01$ compared to trans-cinnamoyl-LIGRLO-NH_2. [a] $p<0.01$ compared to 2-furoyl-LIGRLO-NH_2. NS, $p>0.05$,

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2-furoyl-LIGRLO-NH_2 homologous desensitization protocol compared to 2-furoyl-LIGRLO-NH_2, and trans-cinnamoyl-LIGRLO-NH_2 after heterologous desensitization protocol compared to trans-cinnamoyl-LIGRLO-NH_2. *n*=3 for each group. Error bars represent the standard deviation. The average contraction of femoral arteries (*n*=30) by 120 mM KCl was 5 ± 0.4 mN.

Fig. 6. Vascular smooth muscle cell hyperpolarization and relaxation by 2-furoyl-LIGRLO-NH_2. Data summarize the change in membrane potentials of vascular smooth muscle cells in murine small caliber mesenteric arteries (A) at baseline tension (-57 ± 2 mV steady state membrane potential) or (B) whilst contracted by cirazoline (–46 ± 1 mV steady state membrane potential) after the exposure to different concentrations of 2-furoyl-LIGRLO-NH_2. The lower bar graph (C) summarizes the relaxation that was measured coincident with the hyperpolarization shown in panel b and is expressed as a percentage of the reversal of cirazoline-induced tone. During some experiments, the intracellular electrodes pulled out of the cells prior to obtaining stable membrane recordings during relaxation responses of contracted arteries to 2-furoyl-LIGRLO-NH_2, and thus, the n values for B and C are different.

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Fig. 1

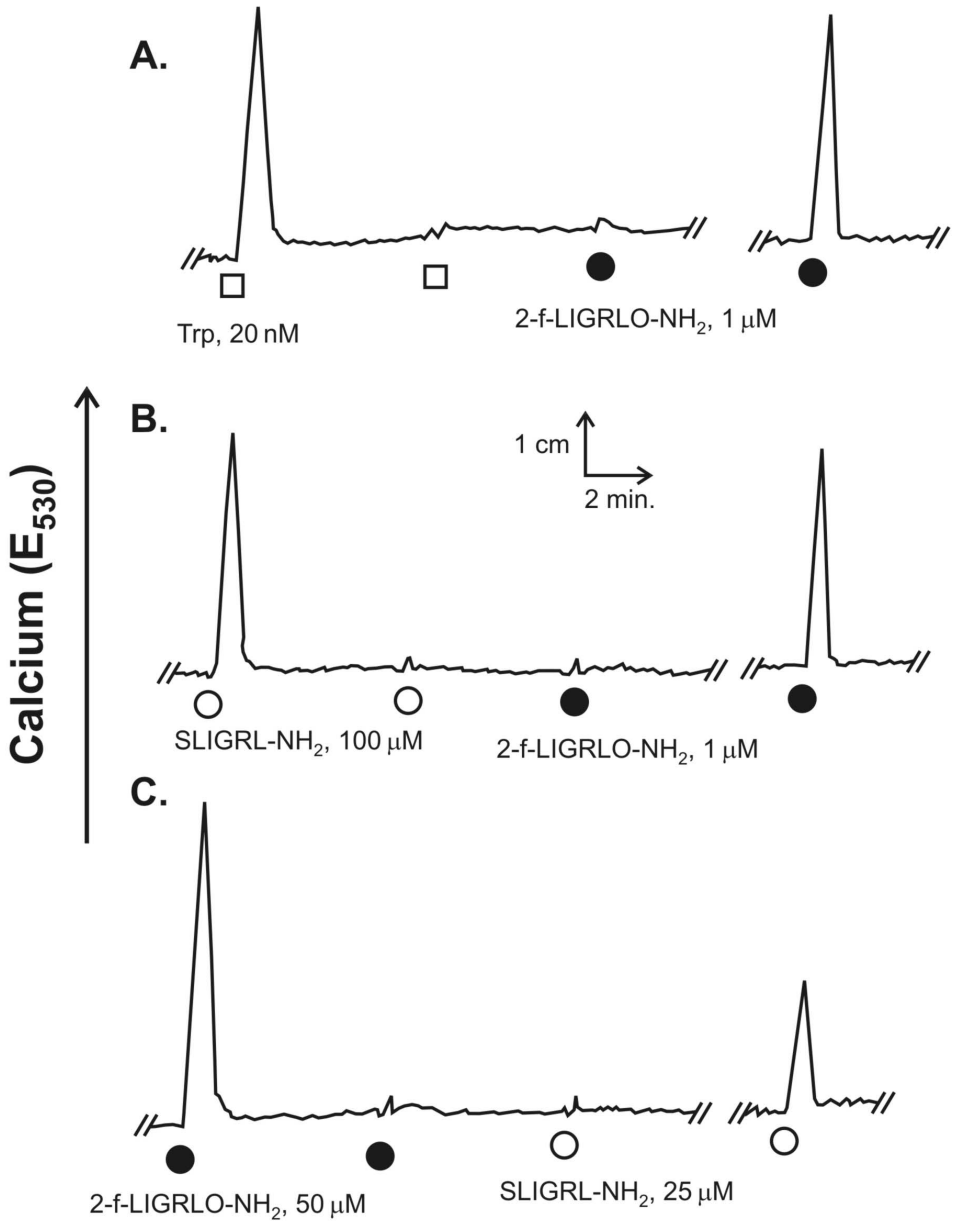

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Fig. 2

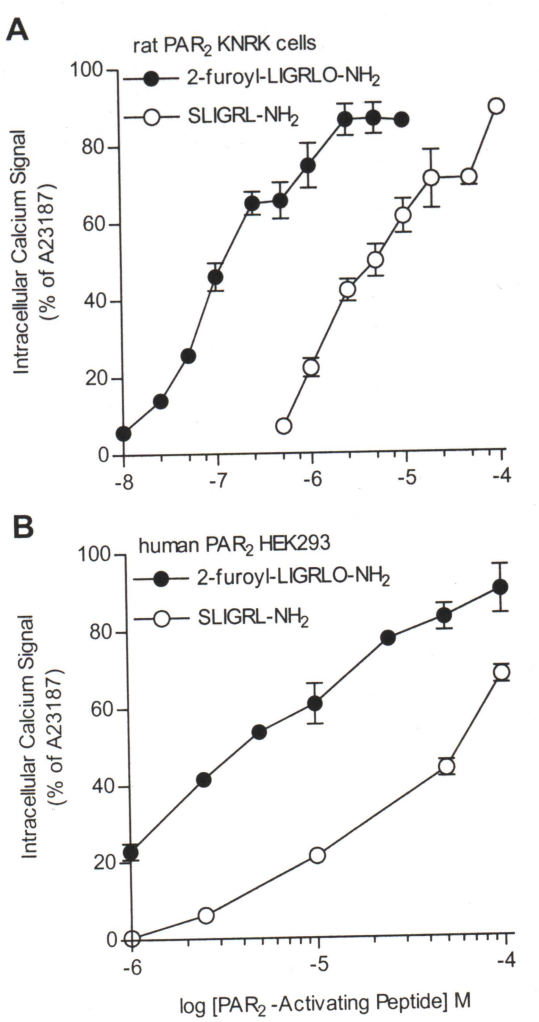

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Fig. 3

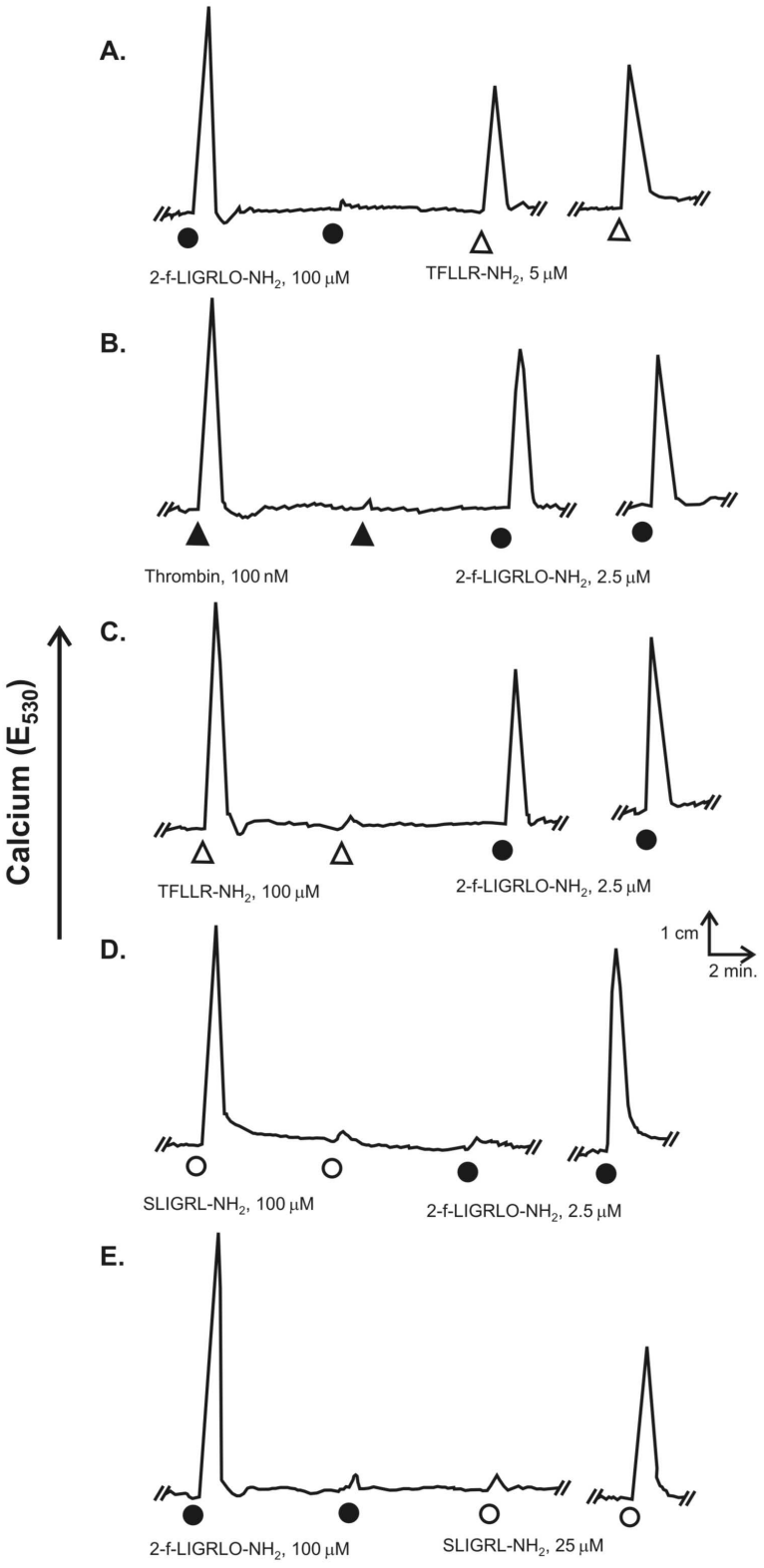

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Fig. 4

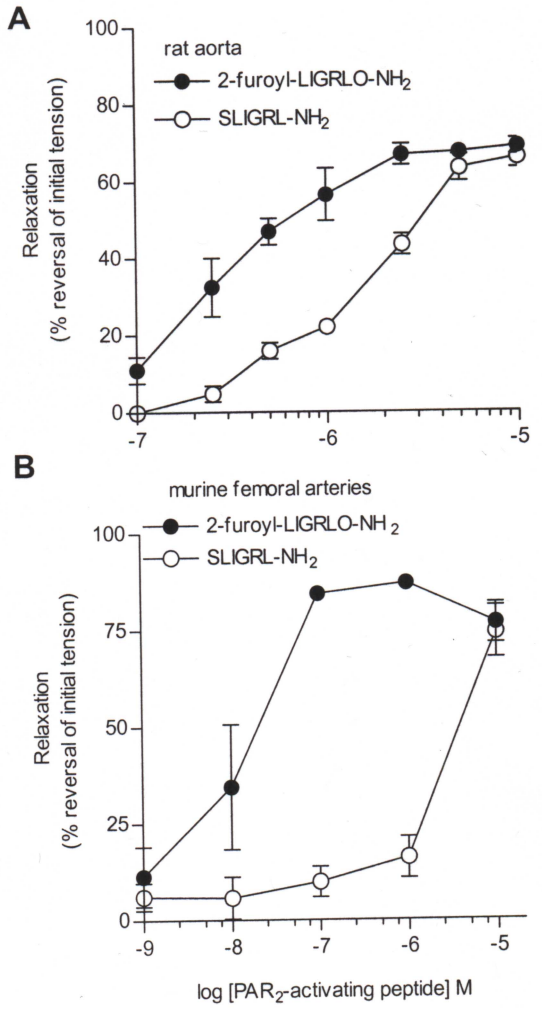

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Fig. 5

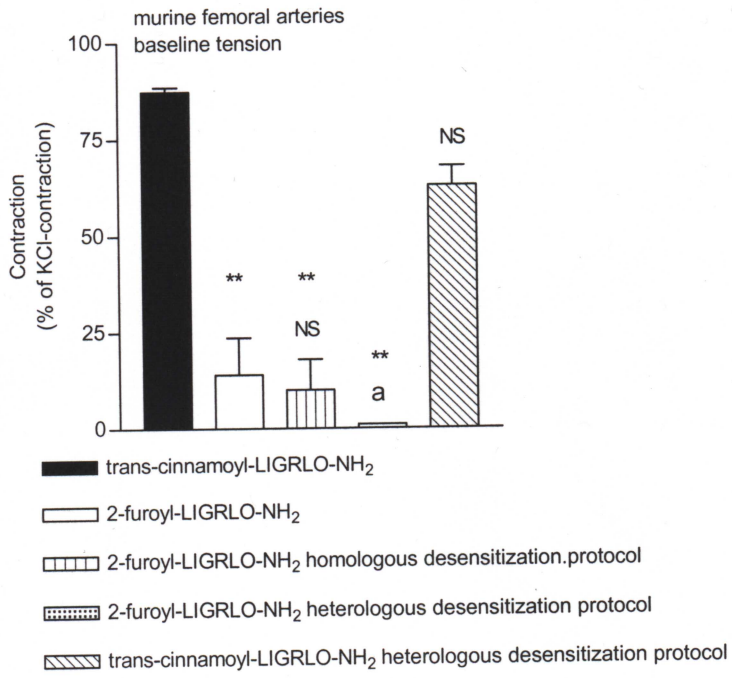

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Fig. 6