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An in vitro reconstitution system to address the mechanism of the vascular expression of the bradykinin B_1 receptor in response to angiotensin converting enzyme inhibition

Caroline Roy[a], Emilie Marceau[a], Lajos Gera[b], François Marceau[a]

[a]Centre de recherche en Rhumatologie et Immunologie, Centre Hospitalier Universitaire de Québec, Québec QC, Canada G1V 4G2

[b]Department of Biochemistry, University of Colorado Denver, Aurora, CO, 80045, U.S.A.

Corresponding author: Dr. F. Marceau, Centre Hospitalier Universitaire de Québec, Centre de Recherche en Rhumatologie et Immunologie, CHUQ, Pavillon CHUL, T1-49, 2705 Laurier Blvd., Québec (Québec), Canada G1V 4G2. E-mail: francois.marceau@crchul.ulaval.ca

Abbreviations: ACE, angiotensin converting enzyme; Ang, angiotensin; B_1R, B_1 receptor; B_2R, B_2 receptor; B-9972, D-Arg-[trans-4-hydroxyprolyl^3, α-(2-indanyl)glycyl^5, (3as, 7as)-octahydroindol-2-yl-carbonyl^7, α-(2-indanyl)glycyl^8]-bradykinin; BK, bradykinin; CGRP, calcitonin gene related peptide; CM, conditioned medium; hUA-SMC, human umbilical artery smooth muscle cell; HUVEC, human umbilical vein endothelial cell; IFN, interferon; IL, interleukin; IRA, interleukin-1 receptor antagonist; LPS, lipopolysaccharide; NF-κB, nuclear factor-κB; PMA, phorbol 12-mysistate 13-acetate; Sar, sarcosine; SMC, smooth muscle cell; TNF-α, tumor necrosis factor-α.

Abstract

The expression of the bradykinin (BK) B_1 receptor (B_1R), lacking in normal vascular tissues, is induced following innate immune system activation and chronic blockade of angiotensin converting enzyme (ACE). To identify cytokine-dependent or –independent mechanisms for the latter phenomenon, the ACE inhibitor enalaprilat and several peptides potentiated in vivo by ACE blockade were applied either directly to human umbilical artery smooth muscle cells (hUA-SMCs) or to differentiated monoblastoid U937 cells to produce a conditioned medium (CM) that was later transferred to hUA-SMCs. A phagocyte stimulant, lipopolysaccharide, did not upregulate B_1R, measured using [^{3}H]Lys-des-Arg^9-BK binding, or translocate NF-κB to the nuclei if applied directly to the hUA-SMCs. However, the CM of lipopolysaccharide-stimulated U937 cells was active in these respects (effects inhibited by etanercept and correlated to TNF-α presence in the CM). A peptidase-resistant B_1R agonist had no significant direct or indirect acute effect (4 h) on B_1R expression, but repeated hUA-SMC stimulations over 40 h was stimulatory in the absence of NF-κB activation. Other peptides regulated by ACE or enalaprilat did not directly or indirectly stimulate B_1R expression. The reconstitution system supports the rapid cytokine-dependent vascular induction of B_1Rs and a slow "autoregulatory" one potentially relevant for the ACE blockade effect.

Keywords: bradykinin B_1 receptor, lipopolysaccharide, angiotensin converting enzyme, smooth muscle cells, macrophages

1. Introduction

Bradykinin (BK)-related peptides, the kinins, stimulate two related G protein coupled receptors, the widely distributed and preformed B_2 receptor (B_2R) and the B_1 receptor (B_1R), responsive to kinin des-Arg^9 metabolites generated by arginine carboxypeptidases (Moreau et al., 2005b). Vasodilation, increased vascular permeability and smooth muscle cell (SMC) stimulation are mediated by both B_1R and B_2R subtypes, provided that they are expressed. Indeed, the B_1R is generally absent from vascular tissues of healthy animals, but the most documented systems where its expression is induced are related to immunopathology, e.g. following administration of bacterial lipopolysaccharide (LPS) in vivo. B_1R expression then proceeds under the influence of various cytokines, mitogen-activated protein kinases and NF-κB signaling (Larrivée et al., 1998; Marceau et al., 1998; Sabourin et al., 2002; Moreau et al., 2005b). In addition, limited but intriguing evidence has associated B_1R expression to chronic inhibition of angiotensin I converting enzyme (ACE). There is evidence in 3 animal species that B_1R expression is triggered by chronic (≥ 7 days), but not acute (≤ 48 h) ACE blockade in healthy subjects without obvious deleterious effect (Marin-Castano et al., 2002; Moreau et al., 2005a; Bawolak et al., 2008). Notably, in the porcine model, there was no evidence of systemic inflammation (fever, leukocyte count alteration, acute phase protein release) parallel to B_1R expression (Moreau et al., 2005a). The phenomenon may be relevant for both the therapeutic and side effects of ACE inhibitors.

ACE is an ectopeptidase expressed by vascular endothelial and other cells that regulates several humoral systems in vivo and that is an important drug target. ACE inhibitors are nowadays exploited in the therapy of hypertension, congestive heart failure, diabetic nephropathy and other ailments (Hanif et al., 2010). In dissecting which effect of ACE inhibitors may be relevant for

B_1R expression, one must consider their mode of action. Their primary effect may derive from the inhibition of the formation of the vasoconstrictor octapeptide angiotensin (Ang) II from Ang I. As ACE is a prominent BK-destroying enzyme in vascular endothelial cells and the kidney (Moreau et al., 2005b), there is evidence for a vasodilator component of the effect of ACE inhibitors derived from the potentiation of BK action on its widely expressed B_2Rs in acute clinical studies (Gainer et al., 1998; Pretorius et al., 2003; Squire et al., 2000). The endogenous B_1R agonists, des-Arg^9-kinins, also may increase during ACE blockade because they are formed by competing peptidase pathways and also because ACE inactivates them (Décarie et al., 1996). Further, ACE degrades Ac-Ser-Asp-Lys-Pro, an acetylated tetrapeptide derived from thymosin β4 (Fleming, 2006). ACE blockade also promotes the formation of the Ang_{1-7} fragment by diverting the metabolism of Ang I from the formation of Ang II (Santos et al., 2010). Both Ang_{1-7} and Ac- Ser-Asp-Lys-Pro are endowed with anti-inflammatory and anti-fibrotic activities, the former peptide via the G protein coupled receptor mas, and the latter by interacting with unknown molecular/cellular partner (Sharma et al., 2008). Peptides released by afferent nerve terminals, substance P and calcitonin gene related peptide (CGRP), may increase during ACE blockade, either because substance P is an ACE substrate (Fleming, 2006), or because these nerves are activated by endogenous BK (Fox et al., 1996). Finally, the ACE inhibitors, small molecular weight conventional drugs, may elicit an unconventional signaling response mediated by ACE itself. This protein, possessing a small intracellular tail that may be modified by phosphorylation events, may recruit certain signaling molecules (Fleming, 2006).

The general hypothesis tested in this study was that one of the humoral factor influenced by ACE blockade, including endogenous kinins, or an ACE inhibitor itself could induce the expression of B_1Rs in vascular cells via a cytokine-dependent or –independent mechanism. We have exploited

the primary cultures of human umbilical artery smooth muscle cells (hUA-SMC), a well characterized model for B_1R regulation by multiple signaling pathways (Morissette et al., 2006; Moreau et al., 2007; Koumbadinga et al., 2010b), to address the mechanism of ACE inhibitor-induced induction of B_1Rs. Tissue macrophages may be more responsive to innate immune system stimuli than vascular cells and may somehow convert ACE blockade into inflammatory signaling. An ACE inhibitor and several peptides known to be potentiated in vivo by ACE blockade (Ang I, Ang1-7, kinins, Ac-Ser-Asp-Lys-Pro, substance P, CGRP), and Ang II for comparison, were applied either directly to the human vascular cells, or to the U937 macrophage-like cells to produce a conditioned medium (CM) that was later transferred to SMCs. Bacterial LPS was used as a positive control for phagocyte-mediated effects. This in vitro reconstitution system was intended to detect the possible role of the cytokine network in the vascular induction of B_1Rs by ACE inhibition.

2. Materials and Methods

2.1. Drugs

Lys-des-Arg^9-BK (des-Arg^{10}-kallidin) was purchased from Bachem (Torrance, CA, USA) and the ACE inhibitor enalaprilat was obtained in a pure powder form (Kemprotec Ltd., Maltby, Middlesbrough, United Kingdom). The B_1R agonist resistant to peptidases, Sar-[D-Phe^8]des-Arg^9-BK (Drapeau et al., 1991; Audet et al., 1997), was purchased from Phoenix Pharmaceuticals (Burlingame, CA). The design and pharmacological profile of B-9972, a peptide B_2R agonist resistant to several peptidases, are reported elsewhere (Bawolak et al., 2007). Etanercept (Enbrel), a tumor necrosis factor-α (TNF-α) soluble receptor manufactured by Immunex Corp. (Thousand Oaks, CA), was purchased from a hospital pharmacy. Human recombinant interleukin-1β (IL-1β), TNF-α, and IL-1 receptor antagonist (IRA) were purchased from R&D Systems (Minneapolis, MN). The other drugs were obtained from Sigma-Aldrich (St. Louis, MO, USA), including LPS from E. coli (serotype O111:B4, prepared by phenolic extraction).

2.2. Cells

The institutional research ethics board approved the anonymous use of human umbilical cord segments obtained after elective cesarean sections. All culture surfaces were coated with gelatin. Primary cultures of hUA-SMCs were obtained from explants and maintained precisely as described (Moreau et al., 2007). They virtually all expressed the marker α-actin (monoclonal antibody 1A4 from Sigma-Aldrich) but less than 10% expressed smooth muscle myosin heavy chain (Fig. 1; monoclonal antibody SM-M10 from Millipore reactive with both SM1 and SM2 splice variants; Frid et al., 2001). These cells were used at passages 2-5. Under these culture

conditions, the cells proved to be an informative model of B_1R expression and function (Morissette et al., 2006; Moreau et al., 2007; Koumbadinga et al., 2010b). In one control experiment, human umbilical vein endothelial cells (HUVECs), prepared and propagated as described (Koumbadinga et al., 2010b), were utilized. The monoblastoid U-937 cell line, originally obtained from the American Type Culture Collection, was propagated in RPMI-1640 medium supplemented with 10% fetal bovine serum and antibiotics. For the present sets of experiments, these cells were seeded at 1.5 or 3×10^6 par 75-cm^2 flask and were stimulated to differentiate toward adherent macrophage-like cells (Fig. 1, inset) by adding phorbol 12-myristate 13-acetate (PMA; 50 nM) for 48 h; this treatment also determines a mitotic arrest (Minta and Pambrun, 1985). U-937 cells are known to release a mixture of cytokines when stimulated with LPS (Izeboud et al., 1999).

2.3. Cell stimulation

After 48 h of culture in the presence of PMA, the serum-containing culture medium of U-937 cells was replaced by 10 ml of serum-free RPMI-1640 medium containing one of the stimuli to be tested (LPS 1 µg/ml, enalaprilat 1 µM, or a peptide influenced by ACE). After a further 24-h incubation period at 37°C, the culture medium was removed, centrifuged at 3000 g for 20 min to remove any nonadherent cells and subcellular debris and the supernatant was frozen (-20°C) and stored for later use with hUA-SMCs as CM (Fig. 1). The Quantikine immunoassay for human TNF-α (R&D Systems, used as directed) was also applied to the CM. To control the direct effect of stimuli on hUA-SMCs, serum-free RMPI-1640 medium containing the test substances was prepared and applied to the vascular cells in separate experiments.

Confluent hUA-SMCs were serum-starved for the last 48 h of culture to reduce basal expression of the kinin B_1Rs (Moreau et al., 2007), and then the culture medium was removed. Either the conditioned or direct stimulation media were applied for 4 h to confluent hUA-SMCs (12-well plates for the binding assays, 1 ml per well) for a period of 4 h, with incubation at 37°C in a humidified atmosphere containing 5% CO_2. This period is sufficient to record kinin B_1R upregulation by cytokines via the *de novo* synthesis (Moreau et al., 2007). In specific experiments, repeated stimulations with a kinin receptor agonist was applied to cells during the 48-h starvation period.

2.4. Binding assays to detect B_1R and ACE expression in human vascular cells and U937 cells

To evaluate the effect of several cell treatments on B_1R expression, intact hUA-SMCs were incubated with [^{3}H]Lys-des-Arg^9-BK ([^{3}H]des-Arg^{10}-kallidin, PerkinElmer Biosciences, Boston, MA, USA; 64-80 Ci/mmol) in 12-well plates according to published methods (Moreau et al., 2007). PMA-differentiated U937 cells, initially seeded at the density of 10^6 cells per well in 12-well plates, were serum-starved for the last 24-h of incubation, with optional LPS treatment (1 µg/ml) during this period. Either type of cells were exposed to nanomolar concentrations of the B_1R ligand (the saturating 1 nM concentration for hUA-SMCs) and cold competing peptide (1 µM of Lys-des-Arg^9-BK for the determination of non-specific binding) in appropriate wells. The expression of ACE in U937 cells (12-well plates, prepared as above) or, as a positive control, in human umbilical vein endothelial cells maintained in Endothelial Cell Growth Medium (Lonza-Clonetics, Basel, Switzerland) (Koumbadinga et al., 2010b), was established as described using the binding of [^{3}H]enalaprilat (0.25-4 nM) (Morissette et al., 2008). One µM of cold enalaprilat was optionally added to some wells for the determination of specific binding.

2.5. Assay for the nuclear translocation of NF-κB

The translocation of NF-κB p65 subunit from the cytosol to the nucleus was studied in vascular cells using indirect immunofluorescence as described (Moreau et al., 2007; monoclonal antibodies to p65, Transduction Laboratories, 1:100 dilution). Staining was revealed using Alexa fluor 488-conjugated anti-mouse goat IgG (Invitrogen). A 1-h stimulation period is sufficient to record the effect of inflammatory cytokines on the translocation and it has been applied to cells (35-mm petri dishes) stimulated with CMs (2 ml) with optional co-treatments. In specific experiments, repeated stimulations with a kinin receptor agonist was applied to cells during the 48-h starvation period.

2.6. Statistical analysis

Reported numerical values are means ± SE. ANOVA followed by Dunnett's test (comparison with a common control) or Tukey-Kramer multiple comparison test were generally applied to analyze normally distributed data sets involving multiple comparisons (InStat 3.05 computer program, GraphPad Software; San Diego, CA). Non-normal values were analyzed using the Kruskall-Wallis test (nonparametric ANOVA) followed by Dunn's multiple comparison test.

3. Results

3.1. Reconstitution of innate immune system-mediated B_1R expression in hUA-SMCs

The initial experiments on the U937 cell-CM aimed at optimizing the ratio of monoblastoid vs. hUA-SMCs using a relatively low concentration of LPS as the selective leukocyte stimulus (positive control). Two low densities of U937 cells (1.5 or 3×10^6 cells per 75 cm^2 flask containing 10 ml of serum-free RPMI-1640 medium) were compared for the release of factors that would subsequently induce B_1R expression in hUA-SMCs, as judged by the radioligand binding assay (Fig. 2A). Direct stimulation of hUA-SMC with LPS (1 µg/ml) did not upregulate B_1Rs. However, the CMs of unstimulated U937 cells were active in this respect in a density-dependent manner: the binding of the B_1R radioligand was not significantly increased for the lower density of U937, but was significantly stimulated at the higher one (Fig. 2A). LPS stimulation of U937 maintained at either density was highly effective to release factor(s) that increased B_1R density as much as recombinant IL-1β in previously reported experiments (Moreau et al., 2007). The lower density of U937 cells (1.5×10^6 cells per flask) was chosen for the following experiments because it responds well to the positive control stimulus LPS while exerting no overt stimulation of hUA-SMCs when LPS was omitted.

The CM of LPS-treated U937 cells was further combined with cytokine antagonists to gain insight into the identity of the transferred stimulus (Fig. 2B). Etanercept, a soluble receptor for TNF-α, abated the effect of the CM on B_1R expression, alone or with recombinant IRA. The latter agent has no effect when used alone against LPS-CM, suggesting that transferred TNF-α is a major contributor to inflammatory expression of B_1R in SMCs. In hUA-SMCs directly stimulated with LPS, adding cytokine antagonists did not change the low expression of B_1Rs.

Exogenous TNF-α has been previously found to be stimulatory for B_1R expression in vascular cells, including hUA-SMCs (Koumbadinga et al., 2010a). The used concentrations of the cytokines antagonists have been previously validated against TNF-α- or IL-1β-induced B_1R expression in SMCs (Koumbadinga et al., 2009; 2010b).

Direct LPS stimulation of serum-starved hUA-SMCs failed to translocate NF-κB p65 subunit from the cytosol to the nuclei in 60 min; however, the CM of LPS-stimulated U937 cells was active in this respect (Fig. 3). Direct stimulation with IL-1β or TNF-α was a positive control in this assay (Fig. 3). Etanercept abated the effect of the CM of LPS-stimulated U937 and that of TNF-α (Fig. 3).

3.2. Effect of direct or indirect stimulation by an ACE inhibitor or peptides influenced by ACE blockade on B_1R expression in hUA-SMCs

A series of 8 peptides (Ac-Ser-Asp-Lys-Pro, the B_1R agonist Sar-[D-Phe^8]des-Arg^9-BK, the agonist B_2R B-9972, angiotensins, the neuropeptides substance P and CGRP) and the ACE inhibitor enalaprilat were tried at the concentration of 10 μM as direct inducers of B_1R expression (4 h treatment of serum-starved hUA-SMC; Fig. 4); this high concentration is justified by the possible presence of inactivation mechanisms in cells. These agents were not active, although a positive but inconsistent trend was noted for the B_1R agonist (Fig. 4). Exogenous IL-1β, used as a positive control for B_1R induction (Morissette et al., 2006; Moreau et al., 2007), exerted a statistically significant stimulation of B_1R expression at the surface of hUA-SMCs in this series of experiments (fig. 4).

U937 cells (1.5×10^6 cells per 75 cm^2 flask containing 10 ml of serum-free RPMI-1640 medium) were stimulated for 24 h with the same set of peptides and with enalaprilat; the concentrations were limited to 1 μM (Fig. 5). None of the CM was effective to increase the expression of the B_1R in hUA-SMCs when the CM of U937 cells were transferred to the vascular cells for a 4-h incubation period (Fig. 5). Sar-[D-Phe^8]des-Arg^9-BK again exhibited a nonsignificant and inconsistent trend for stimulation. Acute treatment of hUA-SMCs with the B_1R agonist Sar-[D-Phe^8]des-Arg^9-BK or the B_2R agonist B-9972, either direct or via the CM of U937 cells, did not activate NF-κB nuclear translocation (fig. 3).

As only the chronic blockade of ACE is active in animals to induce B_1R expression (see Introduction), a prolonged stimulation of the cultured vascular cells with a kinin may needed to convert the nonsignificant trend for a direct effect of Sar-[D-Phe^8]des-Arg^9-BK into a significant induction of B_1Rs. Prolonged and/or repeated schedules of direct hUA-SMC stimulation with the B_1R agonist Sar-[D-Phe^8]des-Arg^9-BK (1 μM) were attempted to test this idea (fig. 6). Three stimulations over the 48-h serum starvation period produced a significant upregulation of [^{3}H]Lys-des-Arg^9-BK binding sites, while dual stimulation of the standard stimulation 4 h prior to the binding assay did not reach statistical significance (fig. 6). The triple stimulation over 40 h with Sar-[D-Phe^8]des-Arg^9-BK did not activate NF-κB (fig. 3), supporting an alternate, cytokine-independent mechanism.

3.3. Expression of pharmacologic targets in U937 cells

Adherent, differentiated and serum-starved U937 cells did not bind significantly [^{3}H]enalaprilat, whether or not they had been treated with LPS during the last 24-h incubation period (Fig. 7A). A positive control in this assay were cultured HUVECs (Fig. 7A). The binding site parameters of

the radioligand (K_D 0.62 nM, B_{max} 47.7 fmol/well) are similar to those recorded in HUVECs maintained in an alternate culture medium (Morissette et al., 2008). U937 cells also failed to bind [^{3}H]Lys-des-Arg^9-BK (0.125-4 nM, Fig. 7B), suggesting that they do not express any significant endogenous B_1R population.

3.4. Analysis of TNF-α release by stimulated U937 cells

Pharmacologic analyses presented above show the effective antagonism of the effect of the LPS-CM of U937 cells by etanercept, supporting a central role of TNF-α in the reconstitution system. These points were more directly addressed using the analysis of the cytokine concentration in the CMs (fig. 8, immunoassay for human TNF-α). Only LPS was a significant stimulant of TNF-α release in differentiated, serum-starved U937 cells over 24 h. The B_1R agonist Sar-[D-Phe^8]des-Arg^9-BK, the B_2R agonist B-9972 or the ACE inhibitor enalaprilat were ineffective in this respect following 24 h of stimulation. Adding the B_1R agonist (1 μM) to LPS did not induce the production of more TNF-α that the quantity produced under the effect of LPS alone (fig. 8). The results do not support a cytokine-mediated mechanism in the observed kinin-induced B_1R expression.

4. Discussion

4.1. Hypothesis and approach

The aim of the present work was to establish an in vitro model that has the potential to explain an ill-defined physiological response to ACE blockade: the upregulation of vascular B_1R expression. The potential interest of the issue is demonstrated by the fact that B_1Rs may participate to the chronic hypotensive effect of an ACE inhibitor in rats (Marin-Castano et al., 2002). Further, a genetic defect in BK metabolism that favors the formation of the B_1R agonist des-Arg^9-BK, aminopeptidase P deficiency, predicts ACE inhibitor-induced side effects such as angioedema and hypotension during hemodialysis (Duan et al., 2005; Molinaro et al., 2006). Two human cell types, primary SMCs and monoblastoid cells, were exploited to detect any inflammatory cross-talk that would evidence the kinship of B_1R induction by ACE inhibitors with the more familiar effect of LPS. At this occasion, the indirect effect of LPS on vascular cells was better defined.

There is a vast database on U937 histiocytic lymphoma cells, a favored model of monocyte-macrophage. They respond to LPS by the release of a cytokine mixture (TNF-α, IL-1β, IL-6, IL-10) (Izeboud et al., 1999; Tahara et al., 1991), which was confirmed by us for TNF-α. The cells also reportedly possess functional receptors for some of the peptides tested in the CM generation: the NK_1 receptors for substance P (Germonpre et al., 1999), the B_1 and B_2Rs for kinins (Guevera-Lora et al., 2009). However, these receptors are not necessarily coupled to inflammatory cytokine production. There is also evidence that lesional macrophages express B_1Rs, for instance in inflammatory bowel disease (Stadnicki, 2011) and atheromas (Raidoo et al., 1997).

As for the hUA-SMC, the low expression frequency of the differentiation marker smooth muscle myosin heavy chain (fig. 1) suggests that the cells mainly represent the "synthetic" phenotype, as opposed to the contractile one (Cairrão et al., 2009). However, hUA-SMCs were the model for a molecular study about the regulation of B_1R expression (Moreau et al., 2007) that may be usefully connected to the present study (evidence for both cytokine-dependent and –independent expression, see below). Also, synthetic SMCs, are a major physiopathological target of B_1R agonists, based on the expression of these receptors in SMCs located in atheromas (Raidoo et al., 1997) and the anti-proliferative and anti-migratory effect of B_1R agonists in cultured arterial SMCs of animal or human origin (Dixon et al., 2002; Morissette et al., 2006)

4.2. Cytokine-mediated induction of B_1R expression by the stimulation of innate immunity

Innate immune system stimulation by LPS may account for the indirect vascular B_1R induction in rabbits and a non-human primate (deBlois and Horlick, 2001; Marceau et al., 1998). When injected into the rat paw, *Porphyromonas gingivalis* LPS caused the upregulation of B_1Rs that mediated oedema, a response that concerns the microcirculation (Dornelles et al., 2009). In LPS-treated rabbits, contractile functional response to a cognate agonist and measurable B_1R mRNA concentration were observed in the subsequently isolated aorta of these animals (Marceau et al., 1998; Sabourin et al., 2002). B_1Rs also mediated coronary vasodilation and hypotension in this model (Marceau et al., 1998). However, LPS was much less effective than the inflammatory cytokine IL-1β to upregulate $[^3H]$Lys-des-Arg^9-BK binding sites in cultured rabbit aortic SMCs (Bawolak et al., 2008). The present data showed that the direct LPS stimulation does not increase $[^3H]$Lys-des-Arg^9-BK binding sites in hUA-SMCs, whereas the CM of LPS-stimulated U937 cells is highly efficacious in this respect (Fig. 2). A pharmacologic approach showed that the soluble TNF-α receptor etanercept abolished the effect of the CM of LPS-stimulated U937 cells,

supporting a central role of TNF-α. This cytokine is a known and NF-κB-dependent stimulant of B_1R expression in hUA-SMCs, and one that can act in synergy with another cytokine, IFN-γ (Koumbaginga et al., 2010b). The natural IRA did not reduce the effect of the LPS CM, suggesting that IL-1 derived from U937 cells is not abundant enough for an effect on B_1R expression, while exogenous IL-1β has been used as a positive control in our experiments. Dornelles et al. (2009) have shown that TNF-α is a major mediator of B_1R induction when LPS is injected into the rat paw, based on the inhibitory effect of infliximab, a specific neutralizing antibody to the cytokine. Etanercept, a soluble TNF-α receptor, abated the contractile response mediated by a B_1R agonist in the aorta isolated from LPS-treated rabbits (Bawolak et al., 2008). Thus, a model emerges where LPS-activated phagocytes induce B_1R expression via rapidly released TNF-α.

4.3. Cytokine-independent induction of B_1R expression under ACE blockade

Whether an ACE inhibitor or peptide hormones known to be influenced by ACE blockade could reproduce such an indirect effect on vascular expression of inflammatory gene products was the main question examined in present experiments, notably addressing the reason why a 14-day enalapril treatment in rabbits caused a significant contractile response to a B_1R agonist in the subsequently isolated aorta, a vascular SMC system (Bawolak et al., 2008). Although certain macrophages in lesions produce ACE abundantly (in atheromas, sarcoidosis, etc.) (Fleming, 2006; Stanton et al., 2003), U937 did not in previous (Snyder et al., 1985) and present experiments (Fig. 7A, [^{3}H]enalaprilat binding). Acutely stimulating either U937 or hUA-SMCs with peptides that may be affected directly or indirectly by ACE inhibition yielded essentially negative results, except for a trend toward an effect of the peptidase-resistant B_1R agonist Sar-[D-Phe^8]des-Arg^9-BK. In hUA-SMCs, direct treatment for 4 h with the natural agonist Lys-des-Arg^9-

BK had been shown to increase B_1R B_{max} by only the modest factor 1.3 and without a discernible effect on B_1R mRNA concentration or stability (Moreau et al., 2007). We tested whether a much longer stimulation with Sar-[D-Phe^8]des-Arg^9-BK would show a cumulative effect, in line with the slow induction of B_1R by ACE inhibition in vivo (see Introduction). Our simulation of the "chronic" stimulation of hUA-SMCs was limited to 40 h, but was 10 times as long as the 4 h "acute" stimulation generally applied in vitro. The repeated stimulation proved to be effective in a NF-κB-independent manner (fig. 6, 3). It is technically sound to measure B_1R density using a radioligand after cell stimulation with the specific agonist Sar-[D-Phe^8]des-Arg^9-BK because it has a rather low affinity for the human B_1R (Morissette et al., 2006) and is expected to rapidly dissociate from the receptor upon washing. In hUA-SMCs, kinin receptor agonists resistant to peptidases did not activate NF-κB signaling, either directly or indirectly, (acutely or chronically for Sar-[D-Phe^8]des-Arg^9-BK), and in U937 cells these agonists failed to stimulate TNF-α production over 24 h (fig. 8). If the reconstitution model allows accurate predictions, ACE inhibition could induce B_1R expression via an "autoregulatory" stimulation of B_1Rs, which is coupled to protein kinase C. The phorbol ester PMA, that stimulates protein kinase C, is a strong inducer of B_1R expression in hUA-SMCs, but of a different kind from cytokines, as the effect is interpreted as a NF-κB-independent stabilization of B_1R mRNA, as opposed to a transcriptional effect (Moreau et al., 2007). This regulatory signaling is different from the previously defined "autoregulation", i.e., kinin-induced kinin receptor expression (Phagoo et al., 1999). Indeed, B_1R expression in IMR-90 embryonic fibroblasts has been shown to be acutely stimulated by agonists of either the B_1 or B_2Rs, but via the release of autocrine inflammatory cytokines (Phagoo et al., 1999). The protein kinase C-dependent and cytokine-independent pathway for B_1R autoregulation would be compatible with the absence of systemic inflammation in pigs chronically treated with an ACE inhibitor (Moreau et al., 2005a) and with the repressor effect of

a B_1R receptor antagonist on B_1R mRNA expression in tissues of rats treated with such a drug (Marin-Castano et al., 2002). The lack of effect of all other tested peptides potentially affected by ACE inhibition also does not support that a cross-talk between peptides affected by ACE and macrophages has a role in vascular B_1R expression.

4.4. Limitations and conclusion

The reconstitution model has limitations if one wants to survey all possible effects of ACE inhibitors, including direct effects on macrophages. Direct or indirect stimulation of hUA-SMCs with the ACE inhibitor enalaprilat did not influence B_1R abundance, but none of the two exploited cell types expresses ACE (U937 cells, fig. 7A; primary hUA-SMC; Morissette et al., 2008). Despite these and other limitations (e.g., use of SMC with the "secretory" phenotype), the reconstitution system supports separate pathways for the vascular induction of B_1Rs: a rapid phagocyte- and cytokine-dependent one and a slow "autoregulatory" one potentially relevant for ACE blockade. The precise positive feed-back signaling linking B_1R stimulation to its expression remains to be characterized.

Acknowledgements

This work was supported by the grant MOP-93773 from the Canadian Institutes for Health Research (CIHR) and by a Summer Student Award to E.M. from the Faculty of Medicine, Université Laval. We thank Ms. Johanne Bouthillier for technical assistance.

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Figure legends

Fig. 1. Schematic representation of the protocol applied for the generation of conditioned RPMI-1640 medium from PMA-pretreated U937 cells or of medium containing the test substances for the direct stimulation, both of which were used to stimulate the expression of inflammatory genes in human vascular cells. Insets: appearance of adherent U937 cells (phase contrast) and of hUA-SMCs (phase contrast and immunofluorescence for α-actin, positive for virtually all cells, and smooth muscle myosin heavy chain, positive in less than 10% of cells). 25-µm scale bar indicated for each cell type.

Fig. 2. Characterization of the direct and indirect effect of LPS (1 µg/ml) on B_1R expression by hUA-SMCs as measured with the specific binding of a saturating concentration (1 nM) of [^{3}H]Lys-des-Arg^9-BK. The hUA-SMCs were treated for 4 h with the indicated CM from control or LPS-stimulated U937 cells or directly stimulated with LPS. A. Effect of U937 cell density on B_1R expression by hUA-SMCs. Results are expressed as the amount of radioligand specifically bound per cell well. The values were heterogeneous (ANOVA, $P<0.0001$) and further compared to the control direct simulation value (left-most histogram) using Tukey-Kramer multiple comparison test (*$P<0.05$; **$P<0.001$). B. Effect of cytokine antagonists (etanercept, 20 µg/ml, IRA 50 ng/ml, alone or combined in 4-h co-treatments) on direct or indirect (U937-cell conditioned) LPS stimulation of hUA-SMCs. Results are expressed as the percent of the maximal specific binding recorded in each of the 6 experiments, that induced by the LPS-CM. The 4 values corresponding to CM were heterogeneous (Kruskall-Wallis test, $P<0.01$) and further compared to the LPS-CM stimulation using Dunn's multiple comparison test (*$P<0.05$;

**P<0.01). In both panels, values are means ± S.E.M. of the indicated number of determinations (n) each composed of duplicate observations.

Fig. 3. Effect of various agents, applied directly or used in a prestimulation experiment of U937 cells, on the subcellular localization of NF-κB p65 subunit in hUA-SMCs (immunofluorescence, 25-µm scale bar indicated in the top left microphotograph). Nuclear translocation of p65 is an indication of NF-κB activation. Duration of treatments was 60 min, except for one condition, indicated as "repeated", where the hUA-SMCs were stimulated 3 times during the serum-starvation period, at times -40, -24 and -4 h relative to the staining. Some hUA-SMC dishes were co-treated for 60 min with etanercept (20 µg/ml), the other stimulant being indicated with arrows. IL-1β and TNF-α are positive controls. Concentrations of test substances: LPS 1 µg/ml; Sar-[D-Phe^8]des-Arg^9-BK, 1 µM; B-9972 1 µM; IL-1β 5 ng/ml; TNF-α 10 ng/ml.

Fig. 4. Effect of direct stimulation of hUA-SMCs by various agents (4 h, concentration as indicated) on B_1R expression by hUA-SMCs as measured with the specific binding of a saturating concentration (1 nM) of [^{3}H]Lys-des-Arg^9-BK. Number of replicates indicated at the base of each histogram. IL-1β is a positive control. The values were heterogeneous (ANOVA, P=0.05) and further compared to the common control value with Dunnett's test (* P<0.05).

Fig. 5. Effect of stimulation of hUA-SMCs by CM of U937 cells on B_1R expression by hUA-SMCs as measured with the specific binding of a saturating concentration (1 nM) of [^{3}H]Lys-des-Arg^9-BK. The U937 cells (1.5×10^6 / flask) had previously been stimulated by various agents, as

indicated (24 h, 1 μM). Number of replicates indicated at the base of each histogram. The values were not different (ANOVA, P=0.53).

Fig. 6. Effect of the repeated direct stimulation of hUA-SMCs by Sar-[D-Phe^8]des-Arg^9-BK (Sar, 1 μM) as measured with the specific binding of a saturating concentration (1 nM) of [^{3}H]Lys-des-Arg^9-BK. Number of replicates indicated at the base of each histogram. Two primary hUA-SMC lines are represented in the results. IL-1β is a positive control. The values were heterogeneous (ANOVA, P=0.05) and further compared to the common control value with Dunnett's test (* P<0.05; ** P<0.01).

Fig. 7. Expression of pharmacological targets for drugs in PMA-differentiated, adherent, serum starved (-24 h) U937 cells. A. ACE expression measured as specific binding of [^{3}H]enalaprilat (saturation curves). Some cells were treated with LPS (1 μg/ml) during the last 24-h of incubation, as indicated. HUVECs were used as positive controls (n = 2). B. B_1R expression measured as specific binding of [^{3}H]Lys-des-Arg^9-BK (saturation curves, n=2).

Fig. 8. Immunoassay of TNF-α secreted by differentiated, serum-starved U937 cells stimulated for 24 h with LPS, the B_1R agonist Sar-[D-Phe^8]des-Arg^9-BK (Sar), the B_2R agonist B-9972 or enalaprilat (concentrations as indicated). The values were heterogeneous (ANOVA, P<0.001) and further compared to the controls with Dunnett's test (*P<0.01). Values are means ± S.E.M. of the number of determinations indicated close to each bar.

Figure 1

Fig. 1

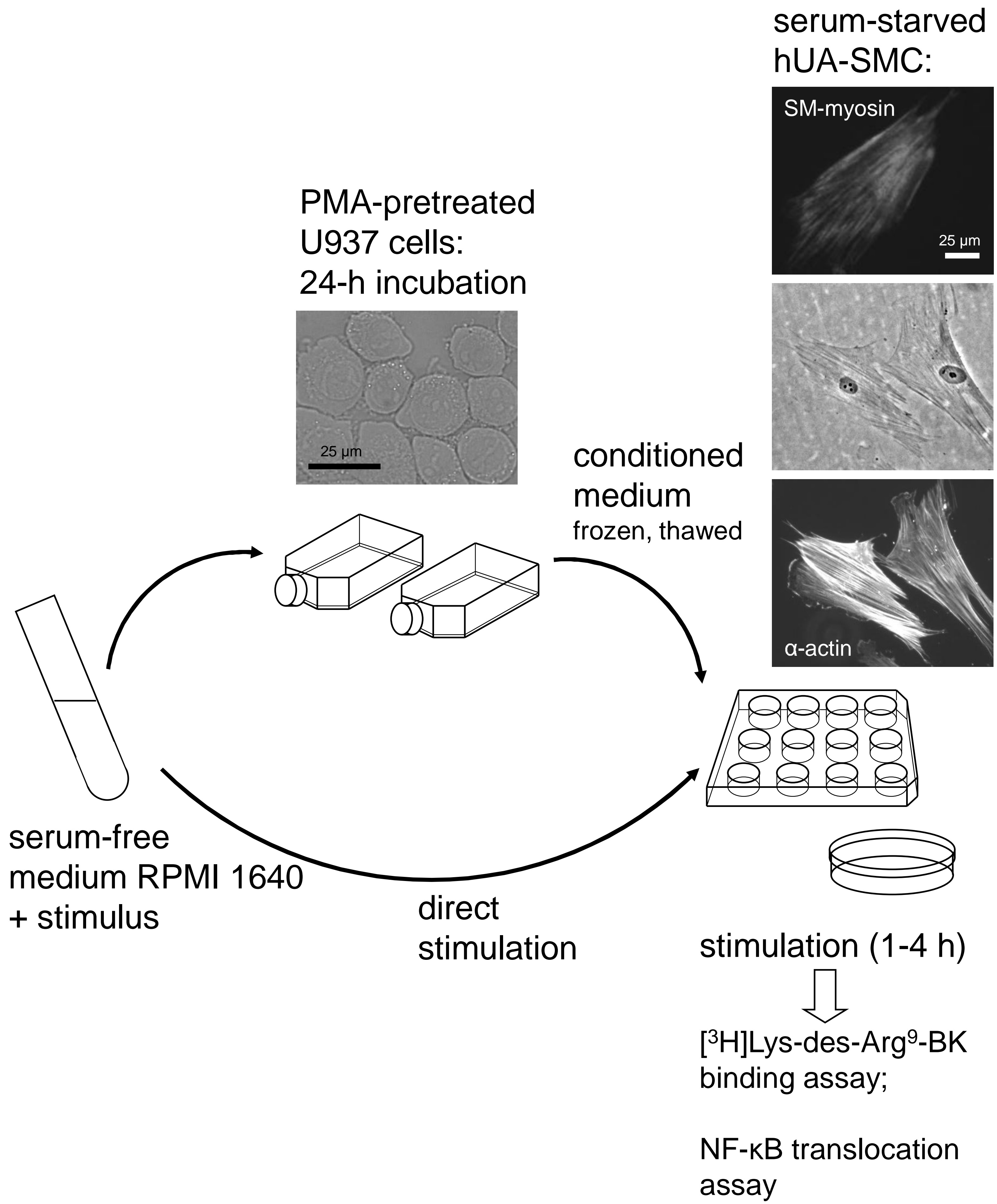
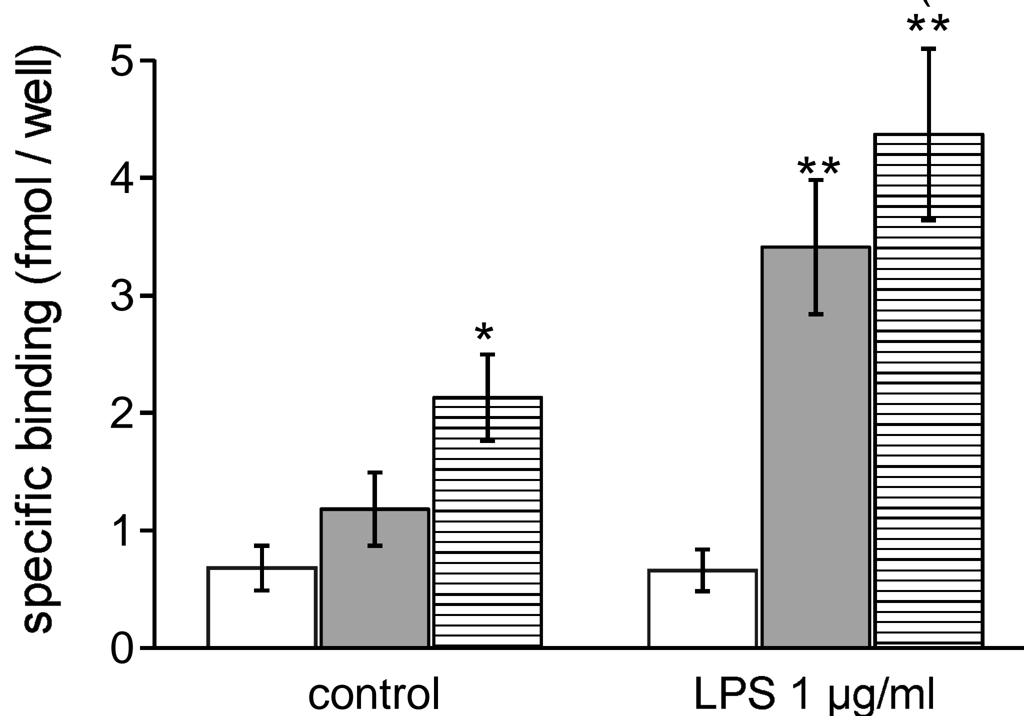
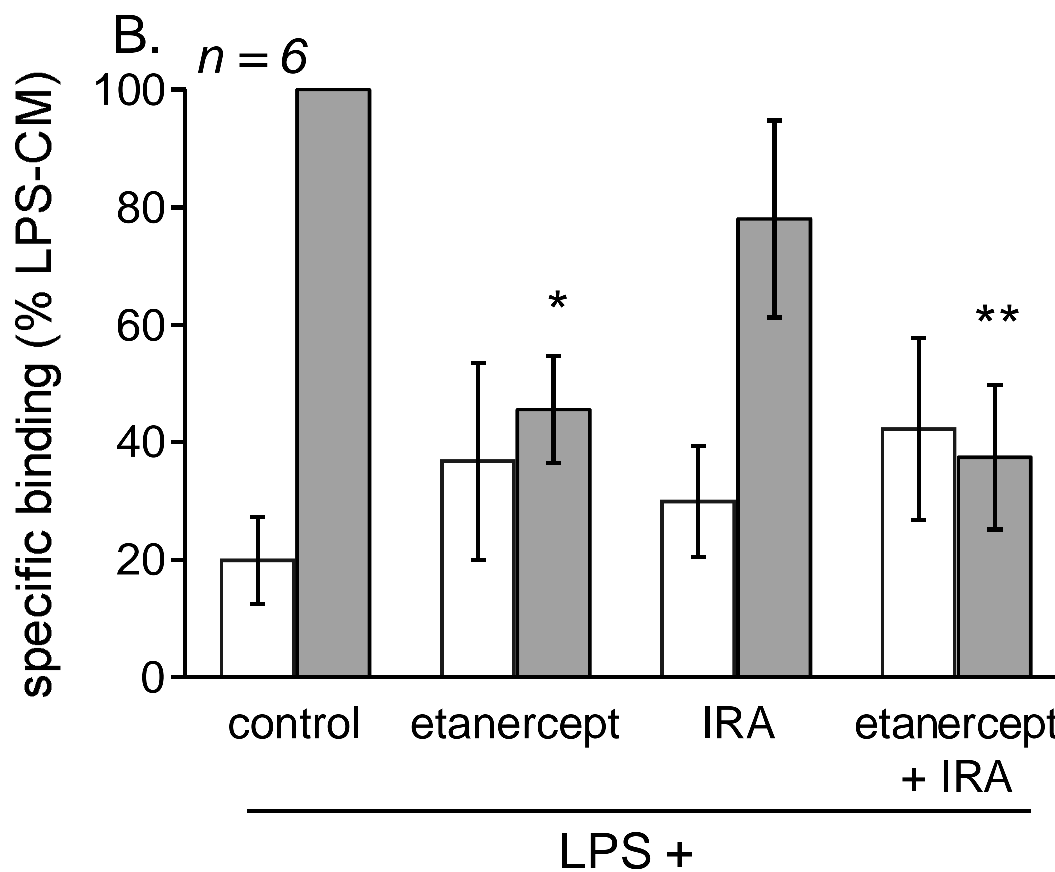


Figure 2

Fig. 2





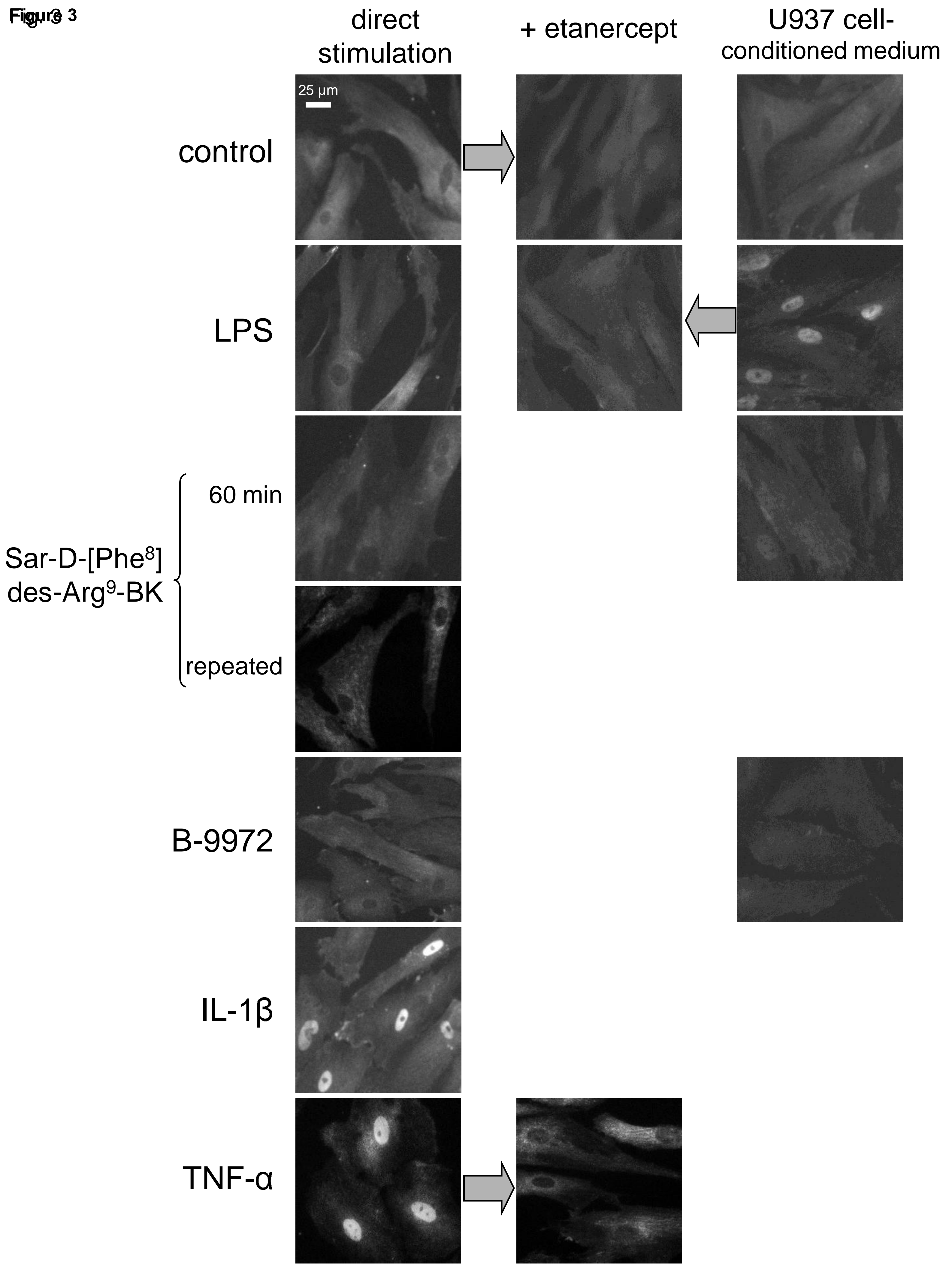
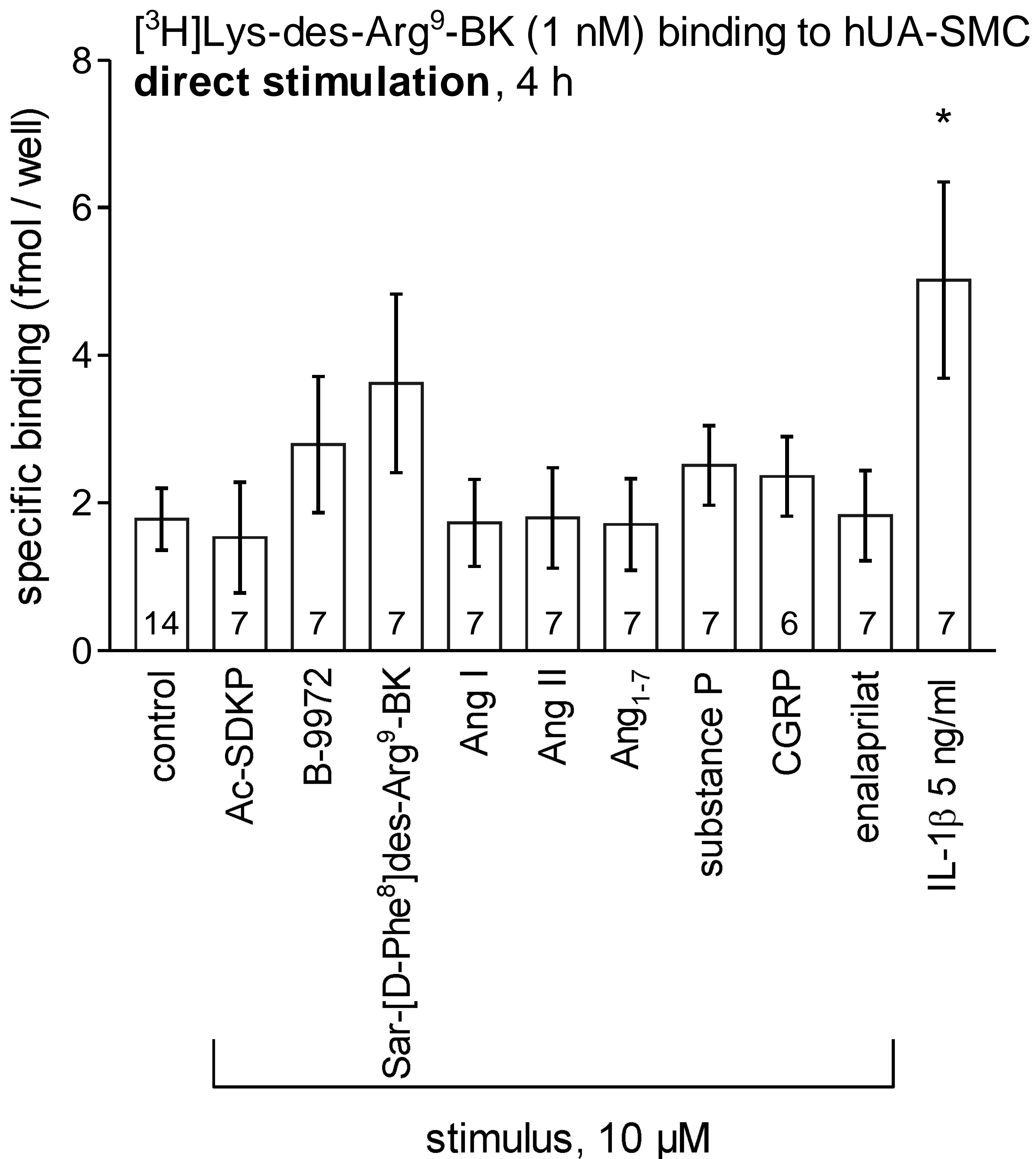
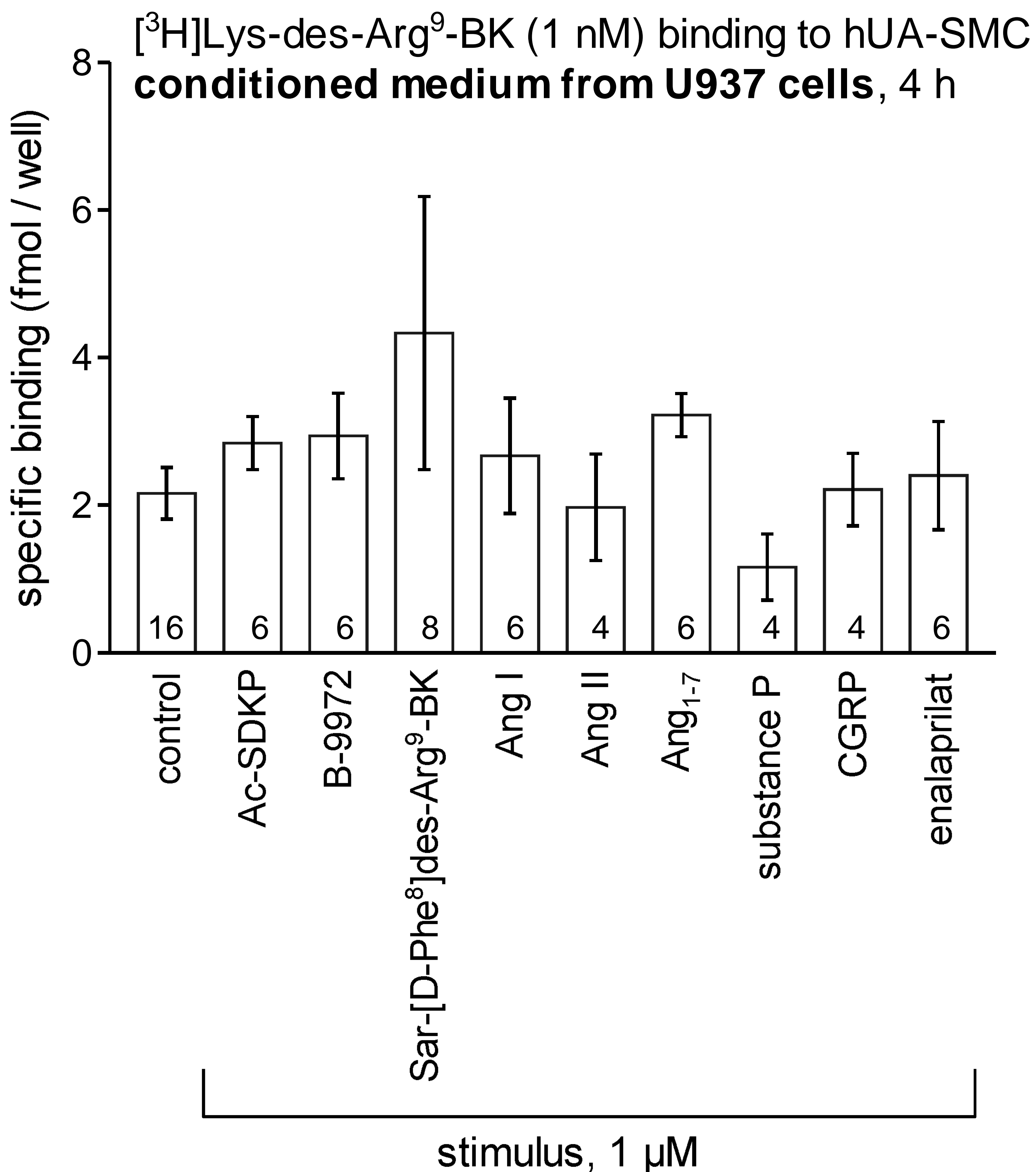
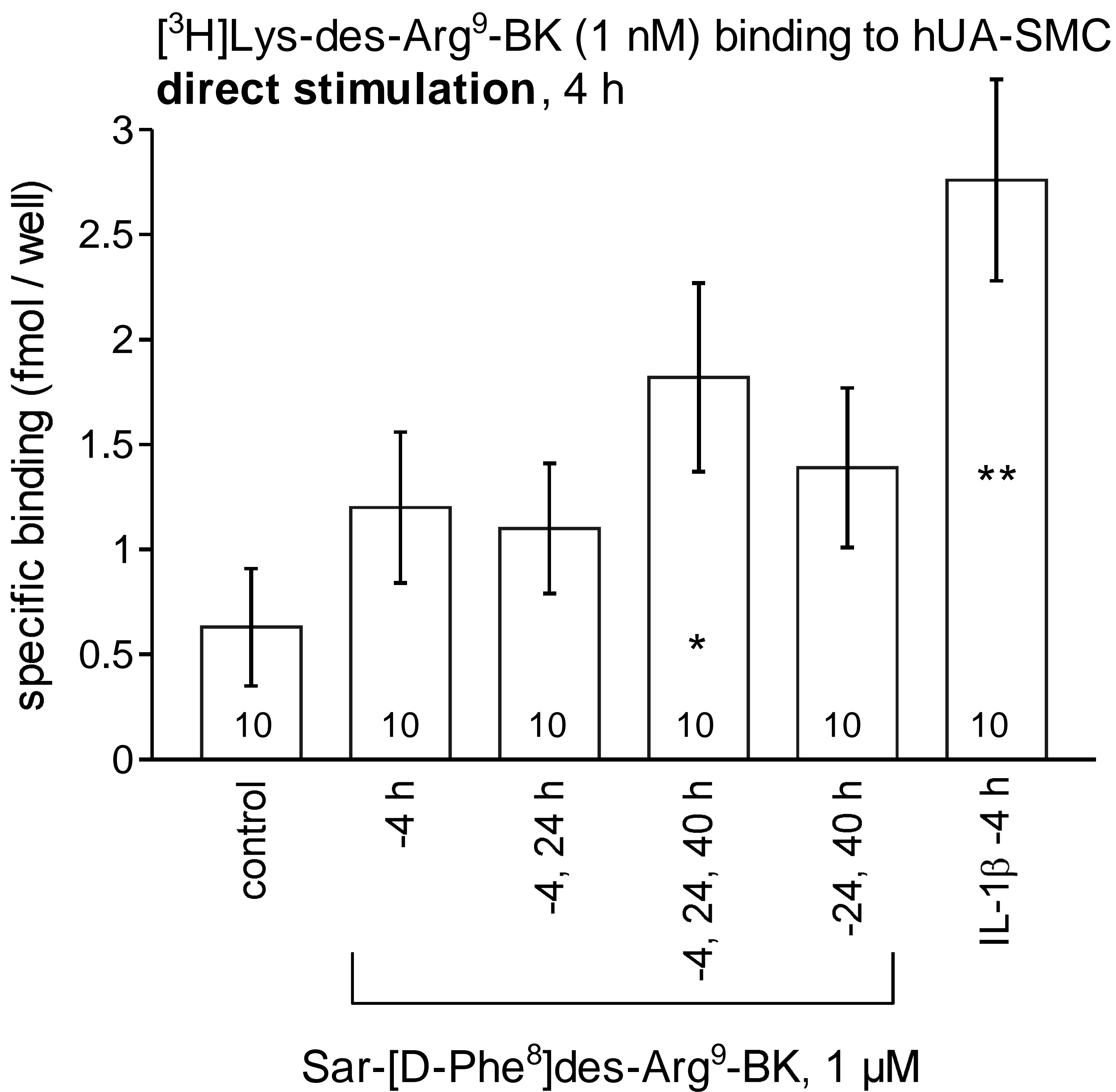


Figure 3
direct stimulation
+ etanercept
U937 cell-conditioned medium
25 µm
control
LPS
60 min
Sar-D-[Phe8] des-Arg9-BK
repeated
B-9972
IL-1β
TNF-α



[3H]Lys-des-Arg9-BK (1 nM) binding to hUA-SMC
direct stimulation, 4 h
specific binding (fmol / well)
0
2
4
6
8
*
14
7
7
7
7
7
7
7
6
7
7
control
Ac-SDKP
B-9972
Sar-[D-Phe8]des-Arg9-BK
Ang I
Ang II
Ang1-7
substance P
CGRP
enalaprilat
IL-1β 5 ng/ml
stimulus, 10 µM


[3H]Lys-des-Arg9-BK (1 nM) binding to hUA-SMC
conditioned medium from U937 cells, 4 h
specific binding (fmol / well)
0
2
4
6
8
16
6
6
8
6
4
6
4
4
6
control
Ac-SDKP
B-9972
Sar-[D-Phe8]des-Arg9-BK
Ang I
Ang II
Ang1-7
substance P
CGRP
enalaprilat
stimulus, 1 µM



[3H]Lys-des-Arg9-BK (1 nM) binding to hUA-SMC
direct stimulation, 4 h
specific binding (fmol / well)
3
2.5
2
1.5
1
0.5
0
*
**
10
10
10
10
10
10
control
-4 h
-4, 24 h
-4, 24, 40 h
-24, 40 h
IL-1β -4 h
Sar-[D-Phe8]des-Arg9-BK, 1 µM

Figure 7

Fig. 7

A. ACE in U937 cells

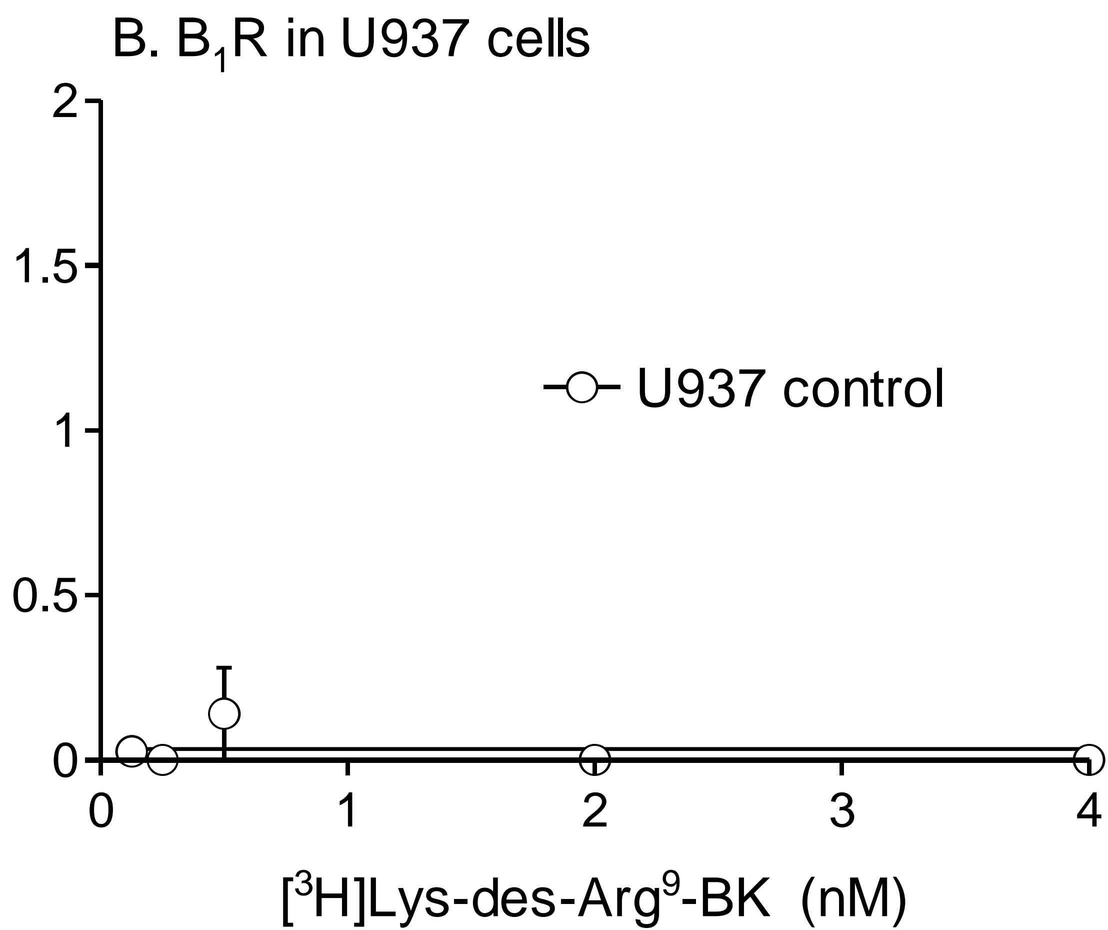
specific binding (fmol / well)

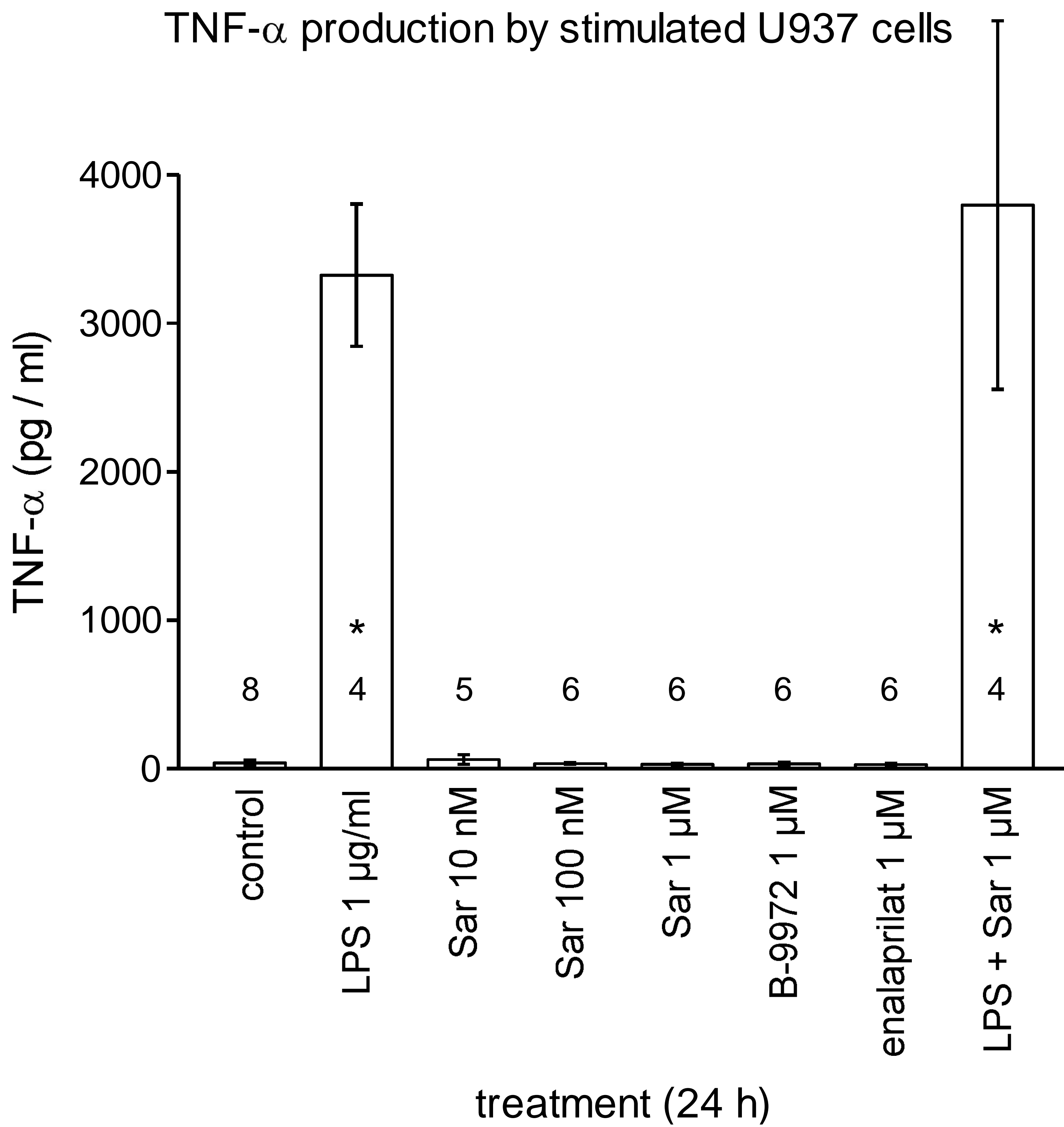
40
30
20
10
0

U937 control
U937 + LPS
HUVECs

0
1
2
3
4

[^{3}H]enalaprilat (nM)




TNF-α production by stimulated U937 cells
TNF-α (pg / ml)
4000
3000
2000
1000
0
*
*
8
4
5
6
6
6
6
4
control
LPS 1 µg/ml
Sar 10 nM
Sar 100 nM
Sar 1 µM
B-9972 1 µM
enalaprilat 1 µM
LPS + Sar 1 µM
treatment (24 h)