A ligand-based approach to investigate the expression and function of angiotensin converting enzyme in intact human umbilical vein endothelial cells

Short title: ACE ligands in endothelial cells

Gérémy Abdull Koumbadinga[1], Marie-Thérèse Bawolak[1], Emilie Marceau[1], Albert Adam[2], Lajos Gera[3], François Marceau[1]

[1]Centre de recherche en rhumatologie et immunologie, Centre Hospitalier Universitaire de Québec, Québec, QC, G1V 4G2, Canada

[2]Faculty of Pharmacy, Université de Montréal, Montréal, QC, H3C 3J7, Canada

[3]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

Abstract

Angiotensin converting enzyme (ACE) is a drug target and an effective bradykinin (BK)-inactivating ectopeptidase. We exploited a recently described [^{3}H]enalaprilat binding assay to quantify the full dynamic range of ACE expression in intact human umbilical vein endothelial cells (HUVECs) stimulated with known or novel modulators of ACE expression. Further, the affinities for ACE of a set of physiological substrates were determined using the same assay. BK has the highest affinity (K_i 225 nM) among known substrates to displace [^{3}H]enalaprilat binding from ACE. Tumor necrosis factor (TNF)-α repressed the expression of ACE in HUVECs while phorbol 12-myristate 13-acetate (PMA) upregulated it in 24 h (~12-fold dynamic range by [^{3}H]enalaprilat binding, corroborated by ACE immunoblotting). Intermediate levels of ACE expression were seen in cells stimulated with both PMA and a cytokine. In contrast, high glucose, insulin or EGF failed to affect ACE expression. The effect of TNF-α was abated by etanercept, the IKK2 inhibitor TPCA-1, or a p38 inhibitor while that of PMA was reduced by inhibitors of PKC isoforms sensitive to phorbol esters and calcium. The short-term PKC- and MEK1-dependent increase of c-Fos expression was best correlated to PMA-induced ACE upregulation. The [^{3}H]enalaprilat binding assay applied to HUVECs supports that ACE is a particularly active kininase and that endothelial ACE expression is dynamically and specifically regulated. This has potential importance in inflammatory diseases and diabetes.

Keywords: bradykinin; angiotensin converting enzyme; endothelial cells; regulation of expression; enalaprilat.

1. Introduction

Bradykinin (BK) is the prototype kinin released from kininogens by kininogenases. It exerts its cardiovascular actions largely via stimulation of B_2 receptors located in endothelial cells [19]. BK is rapidly disposed of in vivo and ACE seems to be the most effective kininase, with high affinity and substrate turnover [4, 7, 8]. ACE inhibitors assume an important place in cardiovascular and renal disease management; they prevent the activation reaction of angiotensin I, but a fraction of their beneficial effects may derive from the potentiation of endogenous BK in humans [12, 26, 33].

ACE expression is constitutive in endothelial cells and some renal epithelial cells and inducible in some other lineages [10]. Endothelial ACE may be regulated by the rate of its synthesis and the cleavage and/or shedding of surface ACE [5, 17, 27]. In human umbilical vein endothelial cells (HUVECs), the transcription of the ACE gene mRNA and ACE activity are known to be stimulated by protein kinase (PK) A and PKC [30, 35, 36] and slightly via NO-controlled PKG [28], and repressed by the inflammatory cytokines IL-1β and TNF-α [25, 29]. It is not known whether these in vitro findings are predictive of enzyme variations of physiopathological importance, although large and opposite variations of ACE activities were recently reported in the heart membranes prepared from rats with type I and II diabetes (decreased and increased activities, respectively [1]).

The present study had 2 major objectives: firstly, we studied the affinities for ACE of a set of physiological or synthetic peptide substrates using the recently described [^{3}H]enalaprilat binding assay [23]. Secondly, the same assay was exploited to quantify the full dynamic range of ACE

expression in intact HUVECs stimulated with previously identified and novel modulators of ACE expression; ancillary experiments were designed to see whether these pathways interact and to further characterize their signaling with reference to PKC isoforms, mitogen-activated protein (MAP) kinases and inflammatory cytokine actions. Results were corroborated using anti-ACE antibodies.

2. Materials and Methods

2.1 Drugs, peptides and reagents

BK, human recombinant insulin, phorbol 12-myristate 13-acetate (PMA), TPCA-1, GF109203x, angiotensins, PD98059 and Ac-SDKP were purchased from Sigma-Aldrich (St. Louis, MO, USA). Additional enzyme inhibitors, Gö6976 and SB203,580, were from Calbiochem (La Jolla, CA, USA). Enalaprilat, an ACE inhibitor, was obtained in a pure powder form (Kemprotec Ltd., Maltby, Middlesbrough, United Kingdom). LY379196 [15] is a generous gift from Eli Lilly (Indianapolis, IN, USA). The cytokines tumor necrosis factor (TNF)-α and interleukin (IL)-1β (human, recombinant) were purchased from R&D Systems (Minneapolis, MN, USA). Etanercept (Enbrel), manufactured by Immunex Corp. (Thousand Oaks, CA, USA), was purchased from a hospital pharmacy. LF 16-0687 (anatibant; 1-[[2,4-dichloro-3-[(2,4-dimethylquinolin-8-yl)oxy]methyl]phenyl]sulfonyl]-N-[3-[[4-(aminoiminomethyl]-phenyl]carbonylamino]propyl]-2(S)-pyrrolidinecarboxamide, mesylate salt), a nonpeptide B_2 receptor antagonist [2], was a gift from Laboratoires Fournier (Daix, France). 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]-BK) is a peptide B_2 receptor agonist with resistance to peptidases [3].

2.2 Cells and radioligand binding assays

The institutional research ethics board approved the anonymous use of human umbilical cord segments obtained after elective caesarean section deliveries. Primary cultures of endothelial cells from the human umbilical vein (HUVECs) were obtained and propagated as described [14]. Briefly, HUVECs were isolated by collagenase digestion of umbilical veins from undamaged sections of fresh cords. The umbilical vein was cannulated, washed with Hank's balanced salt

solution, and perfused for 10 minutes with collagenase (1 mg/ml) in Earle's balancec salt solution at 37°C. After perfusion, the detached cells were collected, the vein was washed with medium 199, and the wash-off was pooled with the perfusate. The cells were washed by centrifugation and plated on gelatin-coated 75-cm^2 culture dishes in medium 199 containing 20% heat-inactivated FBS, ECGS (endothelial cell growth supplement, Sigma-Aldrich, 75 µg/ml), 1% glutamine, 50 U/ml heparin, and antibiotics. Replicated cultures were obtained by trypsinization and were used at passages 2-5. The identity of HUVECs as endothelial cells was confirmed by their polygonal and epithelioid morphology and by detecting their immunoreactivity for von Willebrand factor. Under these conditions, the expression of ACE varied from one cell line to another, but was stable as a function of passage numbers. Presented results are usually representative of several individual donors and a form of normalization has been applied for [^{3}H]enalaprilat binding data (% of control cells).

The density of ACE in these cells treated with cytokines and/or pharmacological agents for 24 h in the complete culture medium was studied using a binding assay involving [^{3}H]enalaprilat and applied precisely as described [23]. Briefly, ([^{3}H]-phenyl)-enalaprilat (20-30 Ci/mmol) was custom-radiolabeled by Dr. Victor Sidorov, successively working at Sibtech, Inc. and AmBios Labs, Inc., Newington, CT, USA from pure enalaprilat (Kemprotec Ltd.). The binding assay was performed using intact, adherent HUVECs (12-well plates) at physiologic ionic strength. The cells were washed twice with the binding medium and filled with 1 ml of prewarmed (37°C) binding medium. The binding medium was Medium 199 supplemented with 0.1% BSA and sodium azide (0.02%, w/v). [^{3}H]Enalaprilat (saturating concentration of 2 nM), with unlabeled enalaprilat (1 µM) for nonspecific binding determination, was the ACE radioligand. Separate protocols dealt with the effect of cell coincubation with a panel of unlabeled peptides. After 60

min of incubation at 37°C, each well was washed three times with 2 ml of ice-cold phosphate-buffered saline (PBS), pH 7.4. One milliliter of 0.1 M NaOH was finally added to dissolve the cells. Radioactivity in the resulting suspension was determined by scintillation counting (5 min per vial).

2.3 Microscopy

To evaluate the localization of ACE in freshly isolated human umbilical blood vessels, immunostaining was applied to paraffin section of the vein and artery using the anti-ACE polyclonal antibodies C28 (dilution 1:200) raised against the C-terminal sequence of human ACE [31] (gift from Prof. P. Corvol, INSERM U36, Paris, France) and applicable to rodent tissue sections [32]. Slides were previously submitted to antigen retrieving (5 min of high-temperature heat denaturation in 0.01 M citrate buffer, pH 6.0) and the staining was revealed by appropriate horseradish peroxidase-coupled secondary antibodies (Sigma) that were allowed to react for 15 min at 25°C with the Immunopure Metal Enhanced DAB substrate (Pierce, Rockford, IL). Endogenous peroxidase was initially inhibited in tissue sections using 3% H_2O_2. Staining for von Willebrand factor (endothelial cell marker; polyclonal from Dako, Mississauga, Canada, dilution 1:200 with antigen retrieving) and α-actin (monoclonal 1A4, Sigma-Aldrich, 1:1000) were also applied to sections to identify vascular endothelial and smooth muscle cells, respectively.

The translocation of NF-κB p65 subunit from the cytosol to the nucleus was studied in HUVECs maintained in their regular culture medium 60-90 min after drug treatments using immunofluorescence as described [22].

2.4 Immunoblots

HUVEC lysates were immunoblotted to detect ACE and signaling molecules after 9% SDS-polyacrylamide gel electrophoresis unless otherwise indicated. The same C28 polyclonal antibodies C28 as used in immunohistochemistry [31] (dilution 1:1000, 7.5% gel) were exploited to detect ACE in variously treated HUVEC. The transcription factor c-Fos (K-25 rabbit polyclonal antibodies, Santa Cruz Biotechnology; 1:100), phospho-ERK1/2 and total ERK 1/2 (respectively monoclonals and polyclonals, Cell Signaling Technology, 1:1000 for each), phospho-p38 (monoclonals, Cell Signaling Technology, 1:1000), phospho-EGR receptor (Tyr^{1068}, monoclonal, Cell Signaling Technology, 1:1000, 7.5% gel), polyclonal anti-phospho-Akt (Ser^{473}, Cell Signaling Technologies, 1:1000), polyclonal anti-AKT (Cell Signaling Technologies, 1:1000) and β-actin (monoclonals, Sigma-Aldrich, 1:50,000) were also detected with appropriate horseradish peroxydase-conjugated secondary antibodies.

For the analysis of ACE and of signaling molecules in total cell lysates, HUVECs (confluent 75-cm^2 flasks) were put in boiling lysis buffer containing 10 mM Tris pH 7.4, 1.0 mM Na_3VO_4, and 1.0% SDS. The lysates were incubated for 5 min at 100°C and then centrifuged at 15,000g for 5 min. Total protein concentrations were then determined using the bicinchoninic acid protein assay (Pierce, Rockford, IL). Twenty-five micrograms of total proteins were run on a 7.5 or 9% SDS-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane. The blots were then incubated 1 h at room temperature in blocking buffer [washing buffer (10 mM Tris pH 7.5, 100 mM NaCl, 0.1% Tween 20) containing 5% skimmed milk]. The primary antibody was added for incubation overnight at 4°C in fresh blocking buffer. The membranes were washed for 30 min in washing buffer at room temperature before adding the appropriate secondary antibody (horseradish peroxidase-conjugated, preadsorbed grade; Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h at room temperature in blocking buffer. The

membranes were washed in washing buffer for another 30 min and then the antibodies were revealed using the Western Blot Chemoluminescence Reagent Plus (NEN Life Science Products), as directed.

2.5 Data analysis

All numerical data were expressed as means ± standard error of the mean (s.e.m.) for *n* values. One-way ANOVA was used to analyze groups of value with appropriate multiple comparison tests (Dunnett's for a comparison to a common control value or Tukey-Kramer multiple comparison test). Binding data were fitted by nonlinear regression to a one-site competition equation to derive IC_{50} values (Prism 4.0, GraphPad Software Inc., San Diego, CA, USA) from which K_i values were calculated using the Cheng and Prusoff [6] method (based on the K_D value of 0.23 nM for [^{3}H]enalaprilat in HUVECs [23]).

3. Results

3.1 Localization of ACE in section of the fresh human umbilical vein

Consistent with the high B_{max} of the ACE ligand [^{3}H]enalaprilat in cultured HUVECs, but very low binding in cultured vascular smooth muscle [23], the anti-ACE antibody labeled the endothelial inner surface of the fresh human umbilical vein (Fig. 1) and artery (data not shown), also positive for von Willebrand factor. However, the underlying smooth muscle layers, positive for α-actin, were negative for ACE (immunohistochemistry). The same anti-ACE antibodies were exploited in immunoblots of HUVEC lysates (see below).

3.2 Binding of BK-related and other potential substrates to ACE

The binding of a saturating concentration (2 nM) of [^{3}H]enalaprilat to intact HUVECs has been previously shown to be displaced by several ACE inhibitor drugs [23]. We extended these investigations to potential ACE substrates related to BK (Fig. 2). BK was the most potent competitor of enalaprilat binding to HUVECs (K_i of 525 nM calculated from an IC_{50} 5.1 μM), closely followed by Lys-BK (K_i 784 nM). The peptides truncated at their C-terminus that are potential B_1 receptor agonists, Lys-des-Arg^9-BK and des-Arg^9-BK, are low affinity competitors (K_i well in excess of 10 μM). The [^{3}H]enalaprilat binding site is distinct from the BK B_2 receptor as it did not recognize the nonpeptide B_2 receptor antagonist LF 16-0687 and had only a small affinity (K_i extrapolated to 18 μM) for B-9972 (D-Arg-[Hyp^3, Igl^5, Oic^7, Igl^8]-bradykinin) (Fig. 2B), a BK analog resistant to hydrolysis by several peptidases. Both LF16-0687 and B-9972 readily compete for [^{3}H]BK binding at the B_2 receptor [2, 3]. Other peptide substrates of ACE of possible or proven physiologic importance include angiotensin I (extrapolated K_i 14.2 μM), but

not angiotensin II, and Ac-SDKP, an acetylated tetrapeptide derived from thymosin β4 [10] (Fig. 2C).

3.3 Modulation of ACE expression as assessed by [^{3}H]enalaprilat binding

HUVECs express ACE in a regulated manner (see Introduction). An original approach, [^{3}H]enalaprilat binding to intact cells, has been exploited to further model modulatory effects of culture conditions. The binding of a saturating concentration (2 nM) of [^{3}H]enalaprilat to intact cells [23] was established in HUVECs maintained in their full culture medium and treated with various agents for 24 h.

We controlled the previously reported effects of a phorbol ester stimulant of PKC and of inflammatory cytokines. We have reproduced both the inhibitory effect of an inflammatory cytokine (TNF-α) and the strong stimulatory effect of PMA on the density of ACE (24 h treatments; Fig. 3A). This representation, in absolute values of fmol/well, establishes for the first time the ~12-fold dynamic range of ACE expression under pharmacological stimulation in these cells. We have verified that TNF-α and IL-1β (4 h) decreased the concentration of ACE mRNA in HUVECs while treatment with PMA increased it (RT-PCR; data not shown), confirming previous findings (see Introduction). Anti-ACE antibodies were exploited in immunoblots of HUVEC lysates (Fig. 3B) and showed that the standard 24-h TNF-α treatment reduced the specific band (~140 kDa), but that PMA increased it considerably, consistent with the results of the [^{3}H]enalaprilat binding assay (Fig. 3A). However, immunoblotting ACE allowed an experiment that was not possible using the binding assay: it was shown that 24-h treatment with a high concentration (1 μM) of the non radioactive ACE inhibitor enalaprilat had no strong effects

on ACE expression (Fig. 3B). One-h treatments with any of the 3 stimuli had no significant effect on ACE abundance.

3.4 Further characterization of signaling pathways that affect ACE expression in HUVECs

The PKC inhibitor GF109203x (5 μM) prevented the effect of PMA on ACE expression, but not that of TNF-α (Fig. 3A). Further, the alternate inflammatory cytokine IL-1β also decreased the density of [^{3}H]enalaprilat binding sites (Fig. 4A) and this effect was not influenced by cell cotreatment with etanercept, a soluble receptor for TNF-α which specifically abolished the inhibitory effect of the latter cytokine. The drug TPCA-1 is a small molecule inhibitor of IKK-2, a kinase that couples inflammatory cytokines to NF-κB signaling [34]. Fig. 4B shows that TPCA-1 at 1 μM significantly reversed the loss of [^{3}H]enalaprilat binding sites induced by TNF-α co-treatment, supporting that the cytokine effect on ACE expression is mediated by NF-κB. Either TNF-α or IL-1β can override the stimulatory effect of PMA on ACE expression (Fig. 4C), leading to a density of binding sites that is intermediate between those of control and PMA-stimulated cells (control values are shown in Fig. 4A for this series of experiments). Many, but not all effects of TNF-α and IL-1 are mediated by the NF-κB signaling system. We exploited a photographic assay for the translocation of the p65 (RelA) subunit of NF-κB (Fig. 5). The assay showed that TNF-α translocated p65 into the HUVEC nucleus in 60 min, an effect that was prevented by TPCA-1 pretreatment (1 μM), but not by the inhibitor of the p38 MAK kinase, SB203,580 (10 μM).

The stimulant effect of PMA on ACE expression was analyzed using staurosporin analogs that show good selectivity within the PKC family and some isoform-selective specificity as a function of the concentration [18] (Fig. 6). The general PKC inhibitor GF109203x (0.5-5 μM) and that of

calcium sensitive isoforms Gö6976 (3 μM) both reduced the effect of PMA on ACE density without affecting the baseline expression of the enzyme in the absence of PMA. LY 379196 is a close analog of GF 109203x that has been developed to inhibit the β isoform of PKC [15]. It had no effect of PMA-induced ACE regulation in a concentration range that is believed to be isoform-specific.

MAP kinases are often signaling intermediates for extracellular stimuli that influence gene expression. MAP kinase and other signaling were probed in lysates of HUVECs submitted to treatments that affect ACE expression and to BK stimulation (Fig. 7A). Acute (1 h) treatment with TNF-α was the only significant stimulator of p38 phosphorylation, taken as an indication of the activation of this MAP kinase pathway, while PMA, enalapril or BK treatments were ineffective in this respect. However, all 4 stimuli increased the phosphorylation state of ERK1/2 after 1 h. Interestingly, only PMA was able to induce c-Fos expression in HUVECs in 1 h, with the heterogenous molecular weight aspect suggestive of the multiple phosphorylation events that are associated with its nuclear translocation [13]. All the observed signaling responses to stimuli faded in cells treated for 24 h (data not shown).

Using pharmacological inhibitors of MAP kinase pathways, we verified the effects of the responsive pathways on ACE expression. Cotreatment with the inhibitor of the α and β isoforms of p38, SB203,580, partially and significantly reversed the inhibitory effect of TNF-α on endothelial ACE (Fig. 7B); however, the drug treatment also slightly but significantly increased the ACE expression in control cells. PD98059, the inhibitor of MEK1, the MAP kinase upstream of ERK1/2, importantly and significantly abated the stimulatory effect of PMA on the density of [^{3}H]enalaprilat binding sites, but also significantly reduced the density of ACE in control or

TNF-α-treated HUVECs (Fig. 7B). We controlled that the acute effect of PMA (30 min) on ERK1/2 phosphorylation was inhibited by cotreatment with PD98059, but also by the PKC inhibitor GF209103x (Fig. 8A), thus positioning the PKC signaling intermediate between the phorbol ester and MEK1. Acute c-Fos induction by PMA (60 min), an event better correlated with ACE upregulation than ERK1/2 phosphorylation (Fig. 7A), was nevertheless suppressed by the MEK1 inhibitor PD98059 and the PKC inhibitor (Fig. 8B). As for the effect of p38 signaling on ACE expression, it was shown that TNF-α-induced p38 phosphorylation was greatly reduced by the IKK2 inhibitor TPCA-1, but much less by the p38 inhibitor SB203,580 (Fig. 8C), positioning NF-κB mediation upstream of p38 signaling. The slight effect of SB203,580 on phospho-p38 may not be specific, as the drug should rather inhibit the kinase activity of p38 on downstream substrates.

3.5 Specificity of stimuli that affect ACE expression

Epidermal growth factor (EGF, 100 nM, 24 h) or high glucose (22.2 mM in the culture medium, 72-h treatment shown, but also applies to 24-h treatment) failed to influence ACE expression (Fig. 9A). EGF exerted an acute effect on cells, EGR receptor phosphorylation in 15 min (Fig. 9A, bottom), while high glucose is reportedly a stimulus for the upregulation of endothelin converting enzyme-1 in HUVECs [16]. Human recombinant insulin (1-5 μM, 24 h) did not appreciably change the density of ACE molecules at the surface of endothelial cells, despite the fact that HUVECs were acutely responsive to insulin (Akt phosphorylation in 30 min, Fig. 9B).

4. Discussion

4.1 Cell type specificity of ACE expression

The expression of the ACE gene product is regulated both in a cell type-specific manner and by physiological factors. Thus, in vascular tissue, ACE is largely restricted to endothelial cells, the smooth muscle cells being negative (immunohistochemistry of umbilical vessel sections, Fig. 1; [^{3}H]enalaprilat binding in cultured vascular cells, [23]). Pathology-induced changes in the vascular composition may alter this picture, as for the ACE-positive macrophages of atheromas [10].

4.2 Peptide binding to ACE

When BK-related peptides are used as competitors of the radiolabelled ACE inhibitor in the binding assay, the known preference for intact kinin C-terminus sequences emerges, with BK and Lys-BK being the substrates of highest affinity (fig. 2A). The des-Arg^9-kinins that are B_1 receptor agonists exhibit only very little capacity to compete for [^{3}H]enalaprilat in the applied assay, although ACE is a significant but slow and nonexclusive degradation pathway for these peptides in various physiological settings [7, 11]. The binding site differs from the BK B_2 receptor by the low or null affinity of ligands that have been designed to resist to inactivation (the agonist B-9972 and the nonpeptide antagonist LF 16-0687, Fig. 2B) and by the measurable affinity of physiological substrates unrelated to kinins, angiotensin I and Ac-SDKP (fig. 2C). However, these peptides exhibit an affinity much lower than that of BK, in line with the fact that ACE is a particularly effective kininase (see Introduction).

4.3 Pharmacological modulators of ACE expression in HUVECs: dynamic range, interactions, specificity and further characterization of signaling

The present study addressed the regulation of endothelial ACE expression using original pharmacological approaches, the [^{3}H]enalaprilat binding assay in intact cells, further corroborated with ACE immunoblots. Relative to the levels recorded in HUVECs maintained in the full culture medium, previously described treatments increased (PMA) or decreased (inflammatory cytokines) the density of ACE [29, 30, 35]. Further, both types of stimuli interacted, as TNF-α or IL-1β abated PMA-induced ACE expression (Fig. 4A). The previously identified pathways have some selectivity, as insulin, high glucose or EGF had no influence on ACE density under similar conditions (Fig. 9). Although acute ERK1/2 phosphorylation was observed in enalaprilat-stimulated HUVECs (Fig. 7A), possibly confirming direct ACE inhibitor-induced signaling via ACE [10, 17], this hypothetical pathway does not importantly feed back on ACE expression, based on ACE immunoblots (Fig. 3B). Functional B_2 receptors are presumably present in HUVECs in this and previous studies [21], notably because BK acutely activates ERK1/2 phosphorylation (Fig. 7A). Thus, it remains to be seen whether autocrine kinins can account for enalaprilat-induced signaling in these cells. Interestingly for the possible effect of diabetic states on ACE expression [1], high glucose failed to influence ACE expression in HUVECs (Fig. 9); the 24-72 h-high glucose treatment applied in the present study is a major modulator of endothelin converting enzyme-1 expression in these cells [16].

The inhibitory effect of TNF-α on ACE expression is mediated by a canonical IKK-2 – NF-κB signaling pathway, as shown by the significant reversal of the inhibition by the IKK-2 inhibitor TPCA-1 (Fig. 4B). We have also observed that TNF-α-induced p38-mediated signaling is downstream of that of NF-κB, being abolished by TPCA-1 (Fig. 8, schematic representation of

signaling, Fig. 10). In fact, the amount of ACE in control cultured HUVECs may be the result of equilibrium between p38- and inflammatory cytokine-mediated downregulation and mitogen- and ERK1/2-mediated upregulation, as based on the effect of MAP kinase inhibitors on control cells. Autocrine IL-1, some of which remains associated to cells, is measurable in control HUVECs and increases in response to mitogens used in the culture medium [20] and may influence this hypothetical equilibrium. However, the addition of exogenous inflammatory cytokines shows the full extent of inflammatory ACE repression. This may be relevant for inflammatory/infectious states, in which the vascular actions of kinins as mediators of inflammation may be potentiated due to loss of this major kininase. In addition, insulin-dependent diabetes models in rodents are associated with high TNF-α expression in tissues and we have previously observed that, in the heart, the activity of ACE decreases in the weeks that follow induction of this pathology with streptozotocin [1]. This alteration, which is a likely result of inflammatory cytokine action in vivo, was fully reversed by insulin treatment. Insulin itself had no effect on ACE expression in HUVECs (Fig. 9B) despite the fact that HUVECs acutely responded to it (Akt phosphorylation).

The previously described PKC-mediated endothelial upregulation of ACE [30, 35] has been reproduced using the [^{3}H]enalaprilat binding assay and ACE immunoblotting. We have extended this line of investigation by showing that a general inhibitor of PMA-sentitive PKC isoforms, GF 109203x, that of a calcium-sensitive isoform (Gö6976), but not that a PKCβ-selective inhibitor (LY 379196), prevented PMA-induced upregulation in HUVECs. The signaling event best correlated with the upregulation of ACE following PMA treatment is the short-term (1 h) increase of c-Fos expression (Fig. 7A), itself controlled at least in part by upstream MEK1-ERK1/2 MAP kinases and the GF109203x-sensitive PKC (Fig. 8A, 8B, 10). It had been previously noted that PMA-induced ACE expression is dependent on the transcription factor

activating protein-1 (AP-1) [9], of which c-Fos is a constituent. A physiopathological example of ACE upregulation by such conditions may be the prediabetic state in Zucker rats where diacylglycerol, the endogenous PKC stimulant, is increased in vascular tissue, as well as the expression of several PKC isoforms [24]. This may explain the high ACE activity and low BK half-life in membranes isolated from young, prediabetic Zucker rats relative to Sprague-Dawley rats [1], and may deprive diabetic individuals from the postulated protective effects of low kinin levels in tissues.

The [^{3}H]enalaprilat binding assay applied to HUVECs supports that BK has the highest affinity for the enzyme among physiological substrates and that the repression of endothelial ACE expression by TNF-α involves the NF-κB and p38 pathways, while PKC-mediated ACE upregulation may recruit MEK1-ERK1/2 and c-Fos signaling. This has potential importance in inflammatory diseases and diabetes.

Acknowledgements

This work was supported by the grant MOP-93773 from the Canadian Institutes of Health Research (CIHR) and by a Studentship Award to M.-T. B. from the Fonds de la Recherche en Santé du Québec. We thank Dr. Marc Pouliot (CHUQ) for facilitating the access to microscopic equipment, Eli Lilly for the gift of LY379196, Prof. Pierre Corvol (Paris, France), for that of the anti-ACE antibodies and Ms. Johanne Bouthillier for technical assistance.

References

[1] Adam A, Leclair P, Montpas N, Koumbadinga GA, Bachelard H, Marceau F. Altered cardiac bradykinin metabolism in experimental diabetes caused by the variations of angiotensin converting enzyme and other peptidases. Neuropeptides 2010;44:69-75.

[2] Bawolak MT, Fortin S, Bouthillier J, Adam A, Gera L, C-Gaudreault R, Marceau F. Effects of inactivation-resistant agonists on the signaling, desensitization and down-regulation of bradykinin B_2 receptors. Br J Pharmacol 2009;158:1375-1386.

[3] Bawolak MT, Gera L, Stewart JM, Marceau F. B-9972 (D-Arg-[Hyp^3, Igl^5, Oic^7, Igl^8]-bradykinin) is an inactivation-resistant agonist of the bradykinin B_2 receptor derived from the peptide antagonist B-9430 (D-Arg-[Hyp^3, Igl^5, D-Igl^7, Oic^8]-bradykinin): pharmacologic profile and effective induction of receptor downregulation. J Pharmacol Exp Ther 2007;323:534–546.

[4] Bunning P, Holmquist B, Riordan JF. Substrate specificity and kinetic characteristics of angiotensin converting enzyme. Biochemistry 1983;22:103-110.

[5] Chattopadhyay S, Santhamma KR, Sengupta S, McCue B, Kinter M, Sen GC, Sen I. Calmodulin binds to the cytoplasmic domain of angiotensin-converting enzyme and regulates its phosphorylation and cleavage secretion. J Biol Chem 2005;280:33847-33855.

[6] Cheng Y, Prusoff WH. Relationship between the inhibition constant (K_i) and the concentration of inhibitor which causes 50 per cent inhibition (I_{50}) of an enzymatic reaction.

Biochem Pharmacol 1973;22:3099-3108.

[7] Cyr M, Lepage Y, Blais C Jr, Gervais N, Cugno M, Rouleau JL, Adam A. Bradykinin and des-Arg^9-bradykinin metabolic pathways and kinetics of activation of human plasma. Am J Physiol Heart Circ Physiol 2001;281:H275-H283.

[8] Erdös EG, Skidgel RA. Metabolism of bradykinin by peptidases in health and disease. In: The Kinin System. Farmer SG ed. Academic Press: San Diego, CA, 1997. pp. 111-141.

[9] Eyries M, Agrapart M, Alonso A, Soubrier F. Phorbol ester induction of angiotensin-concerting enzyme transcription is mediated by Egr-1 and AP-1 in human endothelial cells via ERK1/2 pathway. Circ Res 2002;91:899-906.

[10] Fleming I. Signaling by the angiotensin-converting enzyme. Circ Res 2006;98:887-896.

[11] Fortin JP, Gera L, Bouthillier J, Stewart JM, Adam A, Marceau F. Endogenous aminopeptidase N decreases the potency of peptide agonists and antagonists of the kinin B_1 receptors in the rabbit aorta. J Pharmacol Exp Ther 2005;314:1169-1176.

[12] Gainer JV, Morrow JD, Loveland A, King DJ, Brown NJ. Effect of bradykinin-receptor blockade on the response to angiotensin-converting-enzyme inhibitor in normotensive and hypertensive subjects. N Engl J Med 1998;339:1285–1292.

[13] Glauser DA, Schlegel W. Sequential actions of ERK1/2 on the AP-1 transcription factor allow temporal integration of metabolic signals in pancreatic β cells. FASEB J 2007;21:3240-3249.

[14] Huot J, Houle F, Marceau F, Landry J. Oxidative stress-induced reorganization mediated by the p38 mitogen-activated protein kinase/heat shock protein 27 pathway in vascular endothelial cells. Circ Res 1997;80:383-392.

[15] Jirousek MR, Gillig JR, Gonzalez CM, Heath WF, McDonald JH 3rd, Neel DA, Rito CJ, Singh U, Stramm LE, Melikian-Badalian A, Baevsky M, Ballas LM, Hall SE, Winneroski LL, Faul MM. (S)-13-[(dimethylamino)methyl]-10,11,14,15-tetrahydro-4,9:16, 21-dimetheno-1H, 13H-dibenzo[e,k]pyrrolo[3,4-h][1,4,13]oxadiazacyclohexadecene-1,3(2H)-dione (LY333531) and related analogues: isozyme selective inhibitors of protein kinase Cβ. J Med Chem 1996;39:2664-2671.

[16] Khamaisi M, Dahan R, Hamed S, Abassi Z, Heyman SN, Raz I. Role of protein kinase C in the expression of endothelin converting enzyme-1. Endocrinology 2009;150:1440-1449.

[17] Kohlstedt K, Kellner R, Busse R, Fleming I. Signaling via the angiotensin-converting enzyme results in the phosphorylation of the nonmuscle myosin heavy chain IIA. Mol Pharmacol 2006;69:19-26.

[18] Konopatskaya O, Poole AW. Protein kinase Cα: disease regulator and therapeutic target. Trends Pharmacol Sci 2010;31:8-14.

[19] Leeb-Lundberg LM, Marceau F, Müller-Esterl W, Pettibone DJ, Zuraw BL. International Union of Pharmacology. XLV. Classification of the kinin receptor family: from molecular mechanisms to pathophysiological consequences. Pharmacol Rev 2005;57:27-77.

[20] Marceau F, Grassi J, Frobert Y, Bergeron C, Poubelle PE. Effects of experimental conditions on the production of interleukin-1α and -1β by human endothelial cells cultured in vitro. Int J Immunopharmacol 1992;14:525-534.

[21] Marceau F, Tremblay B, Couture R, Regoli D. Prostacyclin release induced by neurokinins in cultured human endothelial cells. Can J Physiol Pharmacol 1989;67:159 162.

[22] Moreau ME, Bawolak MT, Morissette G, Adam A, Marceau F. Role of nuclear factor-κB and protein kinase C signaling in the expression of the kinin B_1 receptor in human vascular smooth muscle cells. Mol Pharmacol 2007;71:949-956.

[23] Morissette G, Couture JP, Désormeaux A, Adam A, Marceau F. Lack of direct interaction between enalaprilat and the kinin B_1 receptors. Peptides 2008;29:606-612.

[24] Naruse K, Rask-Madsen C, Takahara N, Ha SW, Suzuma K, Way KJ, Jacobs JR, Clermont AC, Ueki K, Ohshiro Y, Zhang J, Goldfine AB, King GL. Activation of vascular protein kinase

C-β inhibits Akt-dependent endothelial nitric oxide synthase function in obesity-associated insulin resistance. Diabetes 2006;55:691-698.

[25] Papapetropoulos A, Antonov A, Virmani R, Kolodgie FD, Munn DH, Marczin N, Ryan JW, Gerrity RG, Catravas JD. Monocyte- and cytokine-induced downregulation of angiotensin-converting enzyme in cultured human and porcine endothelial cells. Circ Res 1996;79:512-523.

[26] Pretorius M, Rosenbaum D, Vaughan DE, Brown NJ. Angiotensin converting enzyme inhibition increases human vascular-type plasminogen activator release through endogenous bradykinin. Circulation 2003;107:579–585.

[27] Ramchandran R, Kasturi S, Douglas JG, Sen I. Metalloprotease-mediated cleavage secretion of pulmonary ACE by vascular endothelial and kidney epithelial cells. Am J Physiol Heart Circ Physiol 1996;271:H744-H751.

[28] Saijonmaa O, Fyhrquist F. Upregulation of angiotensin converting enzyme by atrial natriuretic peptide and cyclic GMP in human endothelial cells. Cardiovasc Res 1998;40:206-210.

[29] Saijonmaa O, Nyman T, Fyhrquist F. Downregulation of angiotensin-converting enzyme by tumor necrosis factor-α and interleukin-1β in cultured human endothelial cells. J Vasc Res 2001;38:370-378.

[30] Saijonmaa O, Nyman T, Kosonen R, Fyhrquist F. Upregulation of angiotensin-converting enzyme by vascular endothelial growth factor. Am J Physiol Heart Circ Physiol 2001;280:H885-H891.

[31] Sibony M, Gasc JM, Soubrier F, Alhenc-Gelas F, Corvol P. Gene expression and tissue localization of the two isoforms of angiotensin I converting enzyme. Hypertension 1993;21:827-835.

[32] Sibony M, Segretain D, Gasc JM. Angiotensin-converting enzyme in murine testis: step-specific expression of the germinal isoform during spermiogenesis. Biol Reprod 1994;50:1015-1026.

[33] Squire IB, O'Kane KP, Anderson N, Reid JL. Bradykinin B_2 receptor antagonism attenuates blood pressure response to acute angiotensin-converting enzyme inhibition in normal men. Hypertension 2000;36:132–136.

[34] Strnad J, Burke JR. IκB kinase inhibitors for treating autoimmune and inflammatory disorders: potential and challenges. Trends Pharmacol Sci 2007;28:142-148.

[35] Villard E, Alonso A, Agrapart M, Challah M, Soubrier F. Induction of angiotensin I-converting enzyme transcription by a protein kinase C-dependent mechanism in human endothelial cells. J Biol Chem 1998;273:25191-25197.

[36] Yang J, Matsukawa N, Rakugi H, Imai M, Kida I, Nagai M, Ohta J, Fukuo K, Nabeshima Y, Ogihara T. Upregulation of cAMP is a new functional signal pathway of Klotho in endothelial cells. Biochem Biophys Res Commun 2003;301:424-429.

Figure legends

Fig. 1. Immunohistochemistry for ACE and cell markers (monoclonal anti-α-actin for the smooth muscle, polyclonal anti-von Willebrand factor [vWF] for the endothelium) in paraffin sections of the human umbilical vein (100 ×). The anti-ACE C28 polyclonal antibodies labeled only the endothelial cells. Control slides show the staining with secondary antibodies only.

Fig. 2. Competition of 2 nM [^{3}H]enalaprilat binding, expressed in % of control, to HUVECs by potential ACE substrates. A. Natural kinin sequences. B. Synthetic ligands of the B_2 receptor. C. Angiotensins and the acetylated tetrapeptide derived from thymosin β4.

Fig. 3. Comparison of 2 approaches to assess ACE expression in HUVECs. A. Specific binding of [^{3}H]enalaprilat (2 nM; expressed in absolute values, fmol/well) to endogenous ACE expressed by adherent, intact HUVECs. A. Cells were treated by drugs or drug combinations as indicated for the last 24 h of culture. Values are means ± s.e.m. of the number of determinations indicated by n, each one being composed of duplicate observations. The values were heterogeneous ($P<0.001$, ANOVA). Tukey-Kramer multiple comparison test vs. control: * $P<0.05$; ** $P<0.001$; effect of GF 109203x vs. values with the same stimulus, † $P<0.001$. B. Immunoblots of HUVEC lysates to document ACE abundance. β-actin was run in the same extracts to document equal loading of tracks.

Fig. 4. Effect of various treatments related to inflammatory cytokines on the specific binding of [^{3}H]enalaprilat (2 nM; expressed in % of control) to HUVECs (all treatments and co-treatments applied for 24 h before the binding assay). A. Effect of the soluble TNF-α receptor etanercept on

cytokine-modulated ACE expression. B. Effect of TPCA-1 on ACE expression in HUVECs co-treated or not with TNF-α (10 ng/ml). C. Dominant inhibitory effect of cytokines in HUVECs treated with PMA. In A-C, ANOVA indicated that values were heterogeneous ($P<0.001$ in A and C, $P<0.01$ for TNF-α-related values only in B). Tukey-Kramer test vs. controls: * $P<0.05$; ** $P<0.001$.

Fig. 5. Photographic assay for the nuclear translocation of NF-κB p65 subunit in HUVECs stimulated with TNF-α for 60 min, with optional treatment with TPCA-1 or SB203,580 applied 30 min before the stimulant. Original magnification 100×.

Fig. 6. Effect of various PKC inhibitors on PMA-induced upregulation of ACE (24 h joint treatments; ACE measured as the specific binding of 2 nM [^{3}H]enalaprilat to HUVECs and expressed in % of control). At the tested concentrations, PKC inhibitors alone had no significant effect on ACE density. However, 2 of the PKC inhibitors had an inhibitory effect on PMA-induced upregulation of ACE (ANOVA, $P<0.001$ for GF109203x, $P<0.05$ for Gö6976). In each situation, Dunnett's test was used to compare the effect of the PKC inhibitor to the appropriate control (* $P<0.05$; ** $P<0.01$).

Fig. 7. MAP kinase signaling and ACE expression in HUVECs. A. Immunoblots of HUVEC lysates to document ACE abundance and cell signaling as a function of acute (1 h) stimulation with the indicated agents. Typical results of duplicate determinations. β-actin or total ERK1/2 was run in the same extracts to document equal loading of tracks. B. Effect of MAP kinase inhibitors on the specific binding of [^{3}H]enalaprilat (2 nM; expressed in % of control) to

HUVECs. The drugs were given alone or with one of the modulators TNF-α (10 ng/ml) or PMA (1 μM) for 24 h. Groups of values corresponding to each or no modulator were heterogeneous (ANOVA, $P<0.001$). Dunnett's test for comparison with DMSO vehicle of MAP kinase inhibitors: * $P<0.05$; ** $P<0.01$.

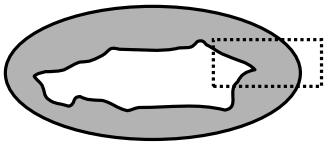
Fig. 8. A and B. Effect of GF109203x and PD98059 on acute signaling induced by PMA in HUVECs (A. ERK1/2 phosphorylation assay, B. c-Fos induction). C. Effect of TPCA-1 and SB203,580 on acute signaling induced by TNF-α in HUVECs. Drug concentration and treatment durations as indicated relative to cell lysis.

Fig. 9. Influence of various treatments (generally applied for 24 h) on ACE expression in HUVECs. (A) Lack of effect of EGF or high glusose (72 h in the latter case) on the specific binding of [^{3}H]enalaprilat to endogenous ACE expressed by adherent, intact HUVECs. Acute EGF receptor autophosphorylation was recorded in these cells (bottom, 15 min treatment, 100 ng/ml, duplicate determinations). (B) Lack of effect of human recombinant insulin on ACE expression (top) despite phosphorylation of Akt following insulin treatment (bottom: sample immunoblots; middle: average densitometric intensity of phospho-Akt in replicated experiments; * $P<0.01$, paired t test with the control).

Fig. 10. Schematic representation of signaling events involved in the regulation of ACE expression in HUVECs.

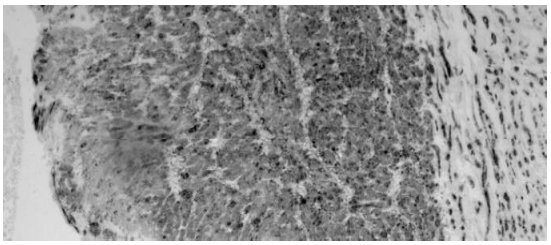
Figure 1

Fig. 1

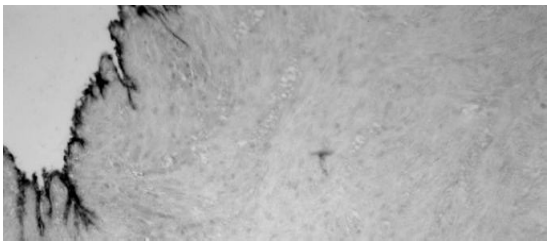


umbilical vein

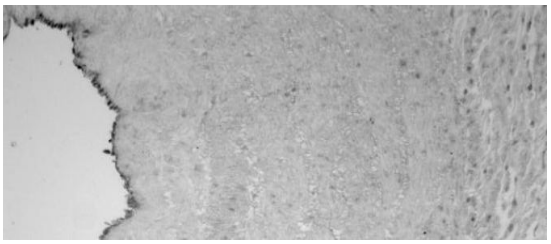
α-actin monoclonal



vWF polyclonal

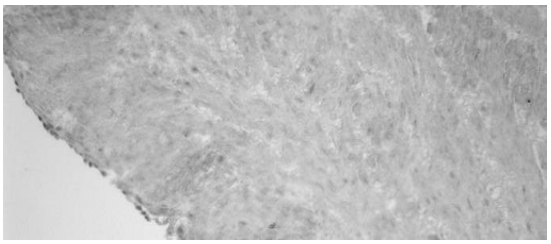


ACE (C28) polyclonal



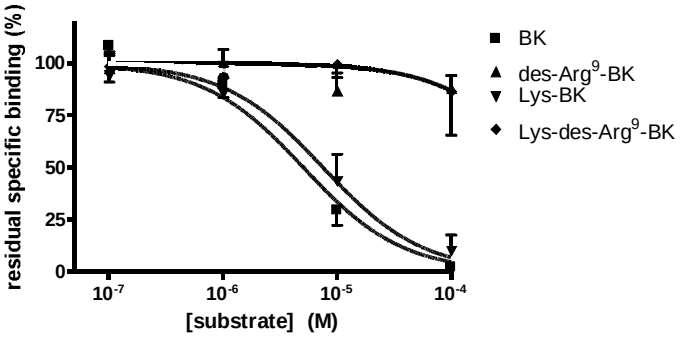
No 1st Ab 2nd anti-mouse

No 1st Ab 2nd anti-rabbit



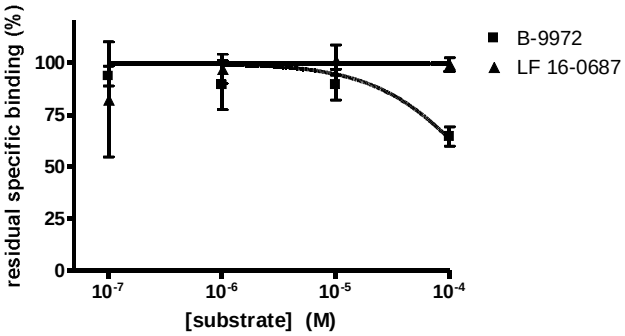
A.

Competition of 2 nM [^{3}H]enalaprilat binding to HUVECs by ACE substrates: natural kinin sequences (*n = 4-5*)



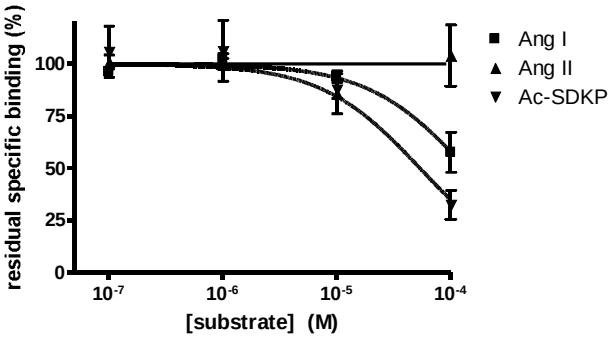
B.

synthetic kinin receptor ligands (*n = 3*)

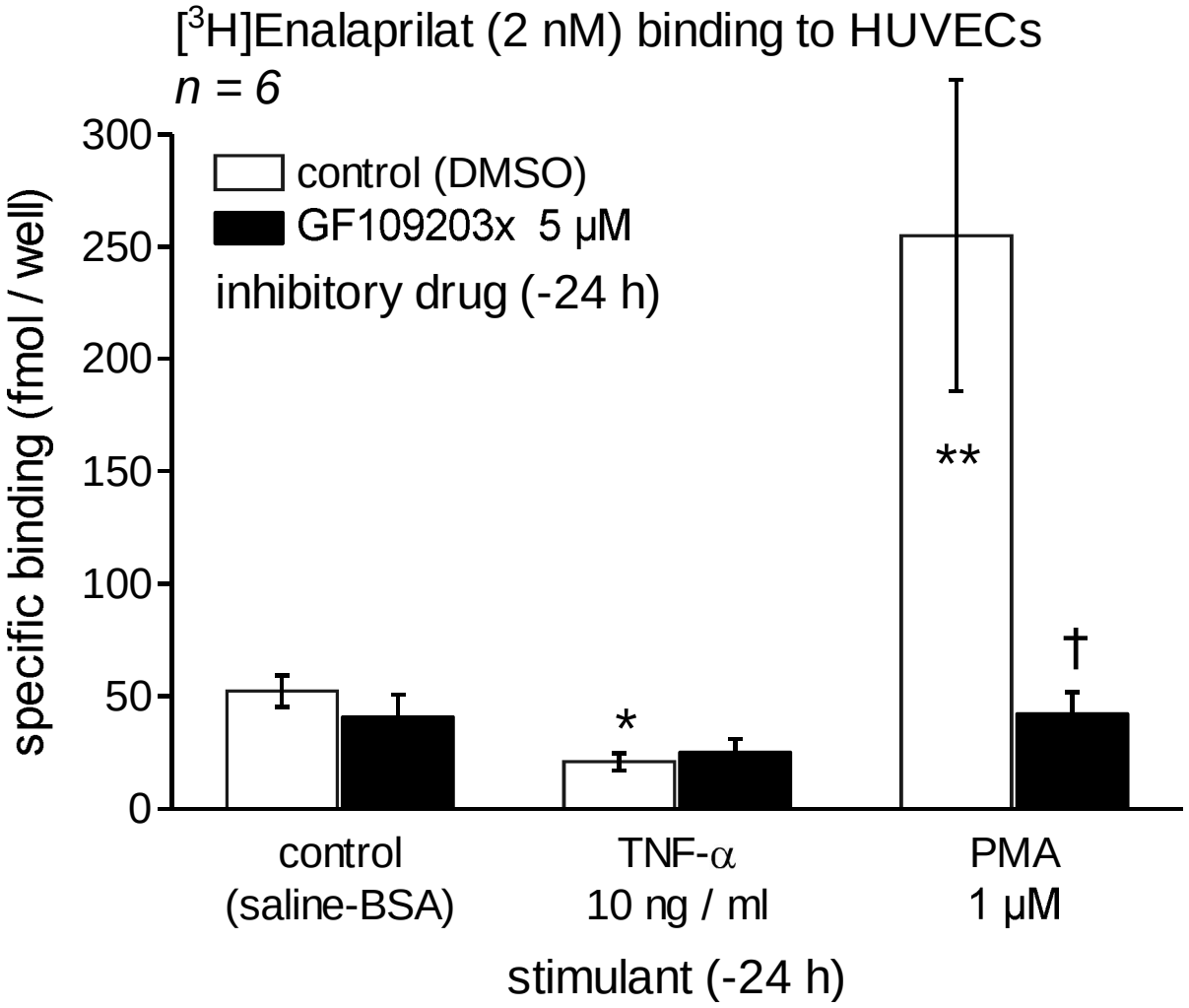


C.

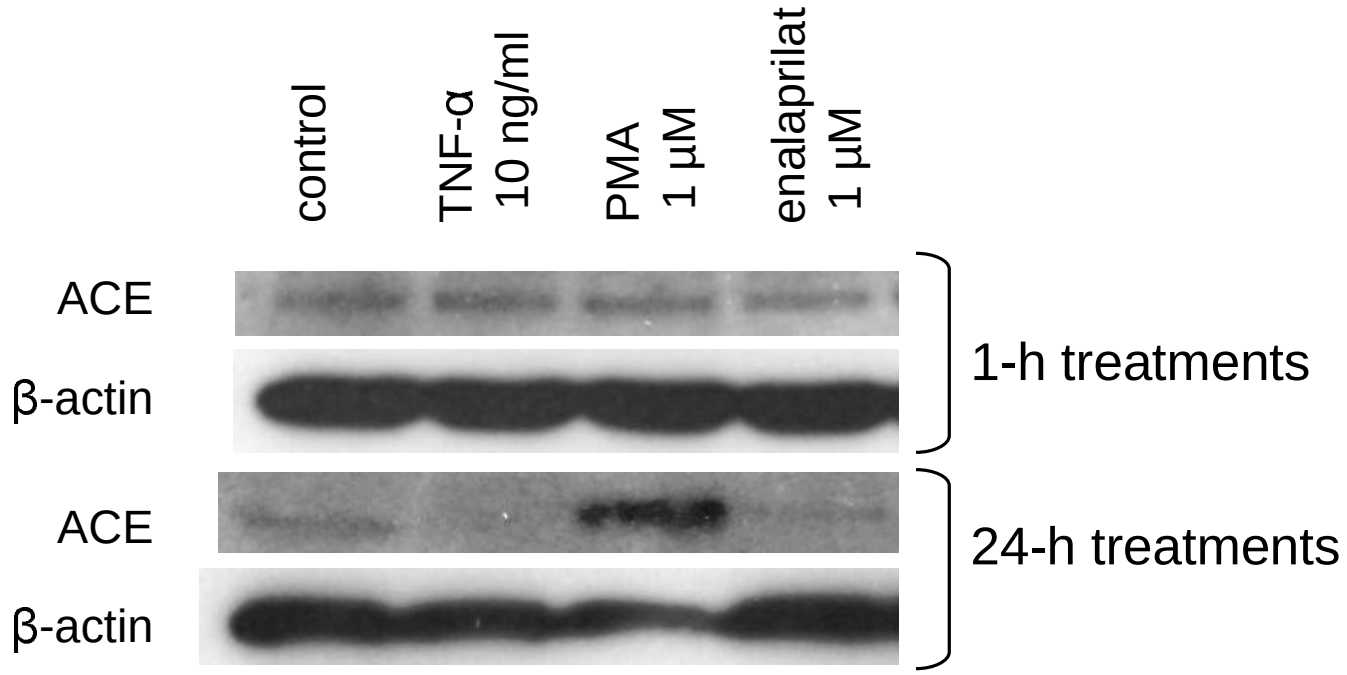
others (*n = 3*)

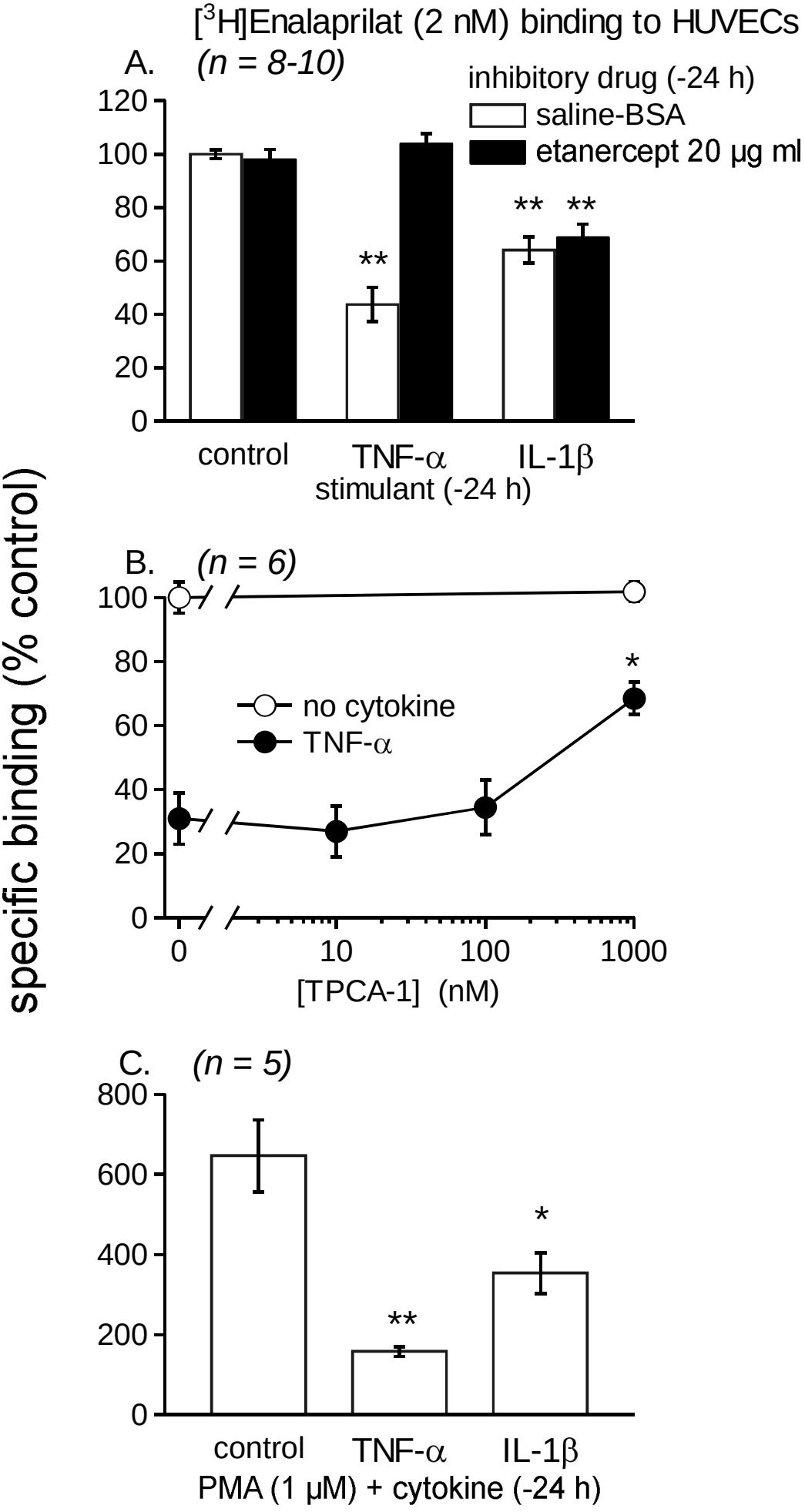


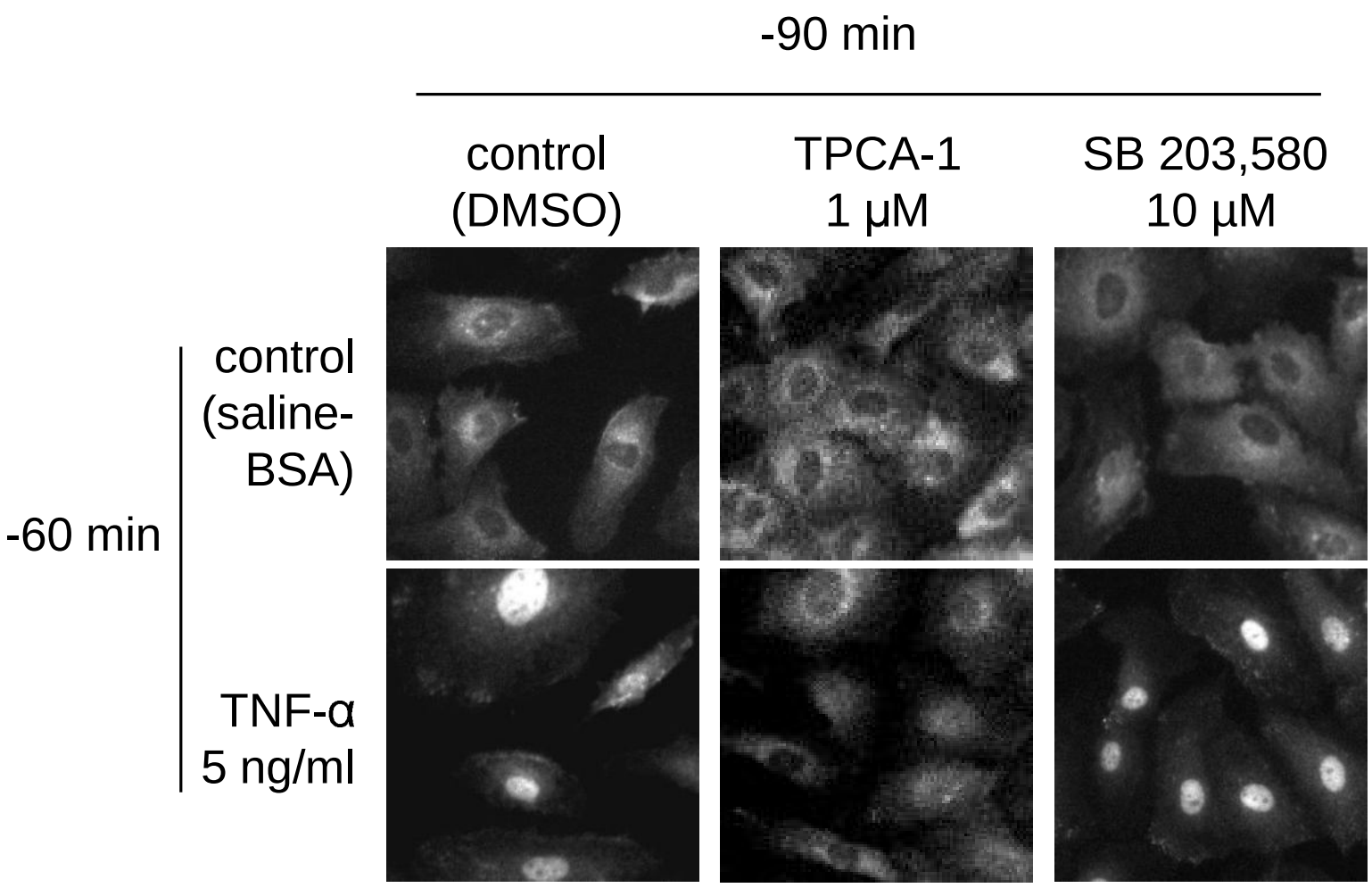
A.

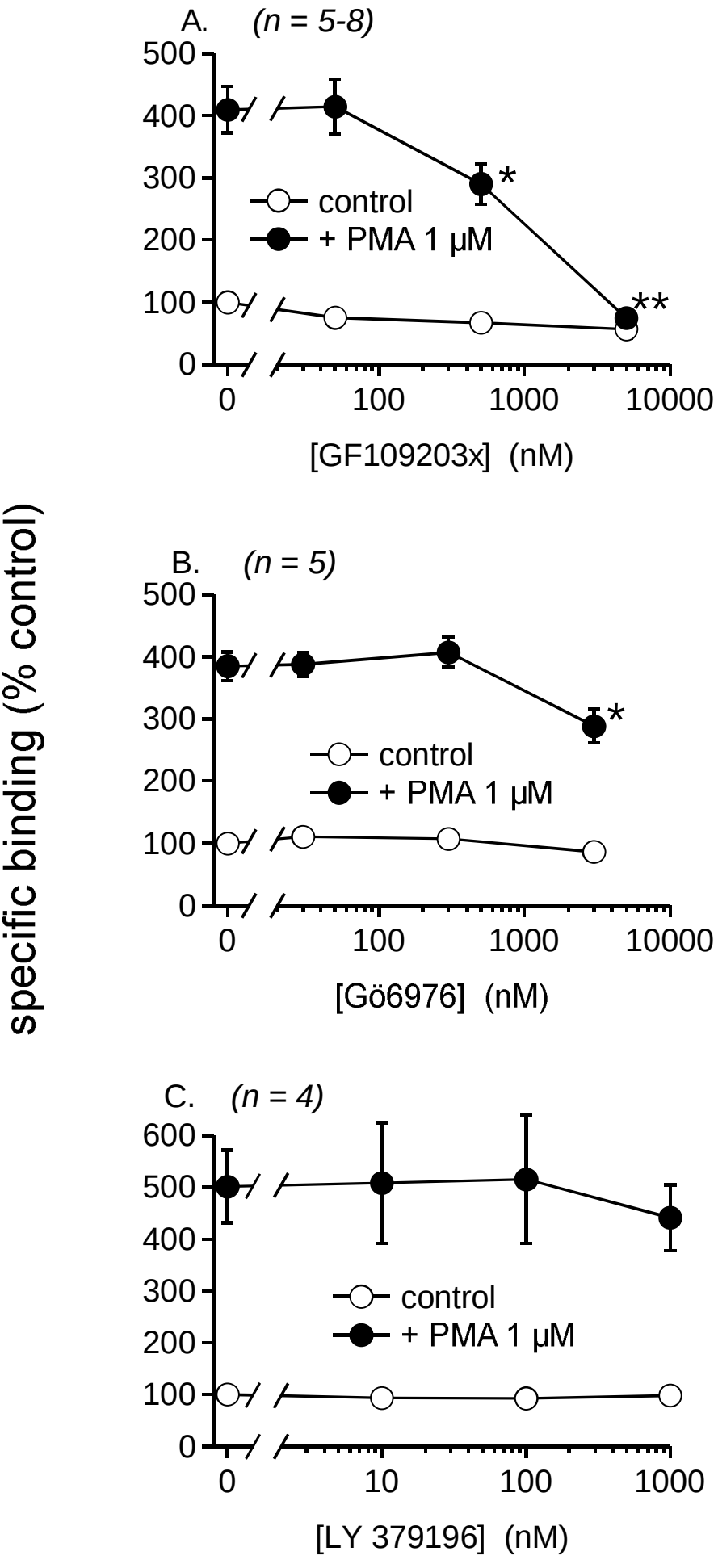

[3H]Enalaprilat (2 nM) binding to HUVECs
n = 6
control (DMSO)
GF109203x 5 µM
inhibitory drug (-24 h)
specific binding (fmol / well)
300
250
200
150
100
50
0
*
**
†
control
(saline-BSA)
TNF-α
10 ng / ml
PMA
1 µM
stimulant (-24 h)

B.

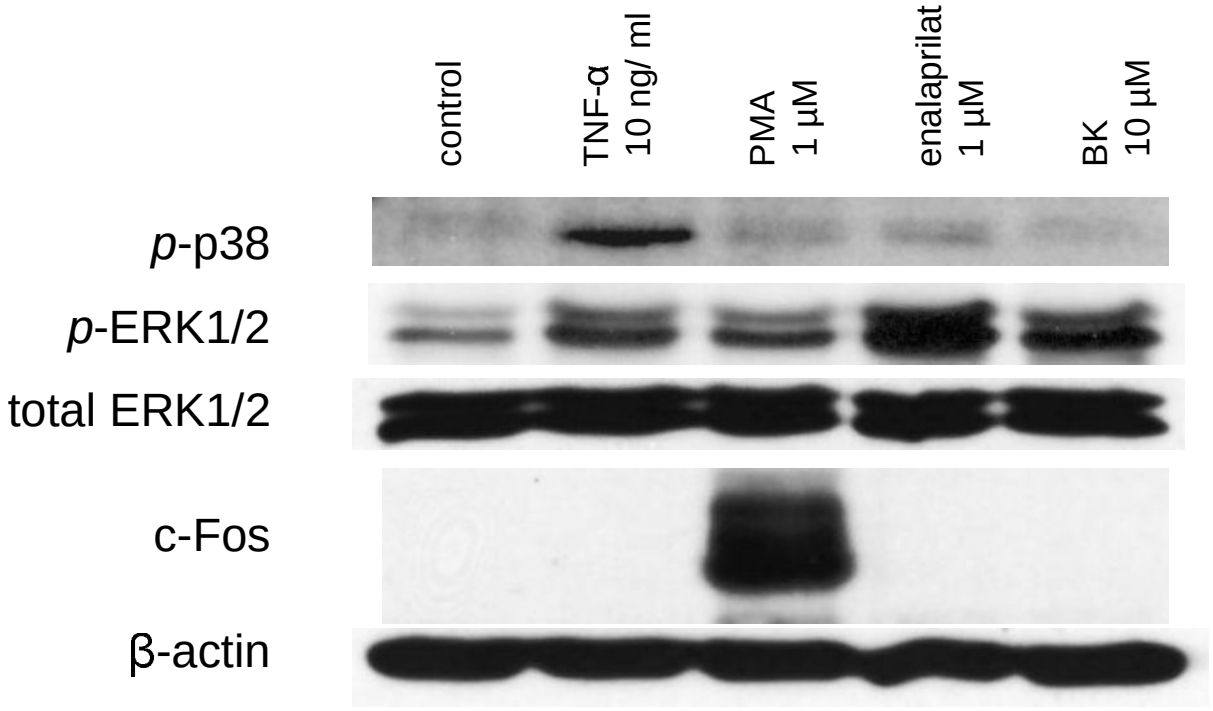

control
TNF-α
10 ng/ml
PMA
1 µM
enalaprilat
1 µM
ACE
β-actin
1-h treatments
ACE
β-actin
24-h treatments


[3H]Enalaprilat (2 nM) binding to HUVECs
A.
(n = 8-10)
inhibitory drug (-24 h)
saline-BSA
etanercept 20 µg ml
120
100
80
60
40
20
0
**
**
**
control
TNF-α
IL-1β
stimulant (-24 h)
B.
(n = 6)
specific binding (% control)
100
80
60
40
20
0
no cytokine
TNF-α
*
0
10
100
1000
[TPCA-1] (nM)
C.
(n = 5)
800
600
400
200
0
**
*
control
TNF-α
IL-1β
PMA (1 µM) + cytokine (-24 h)

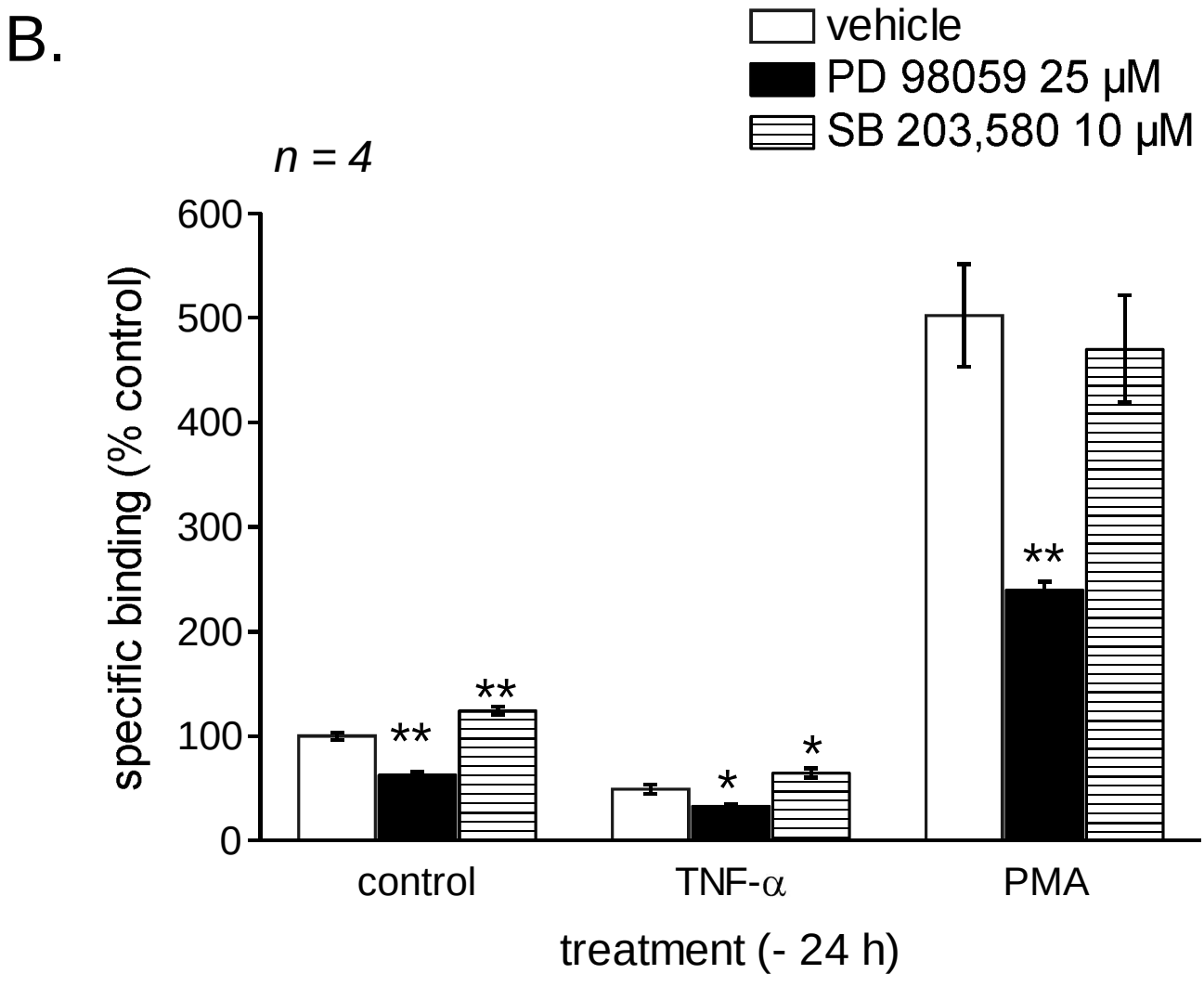

-90 min
control (DMSO)
TPCA-1 1 µM
SB 203,580 10 µM
-60 min
control (saline-BSA)
TNF-α 5 ng/ml

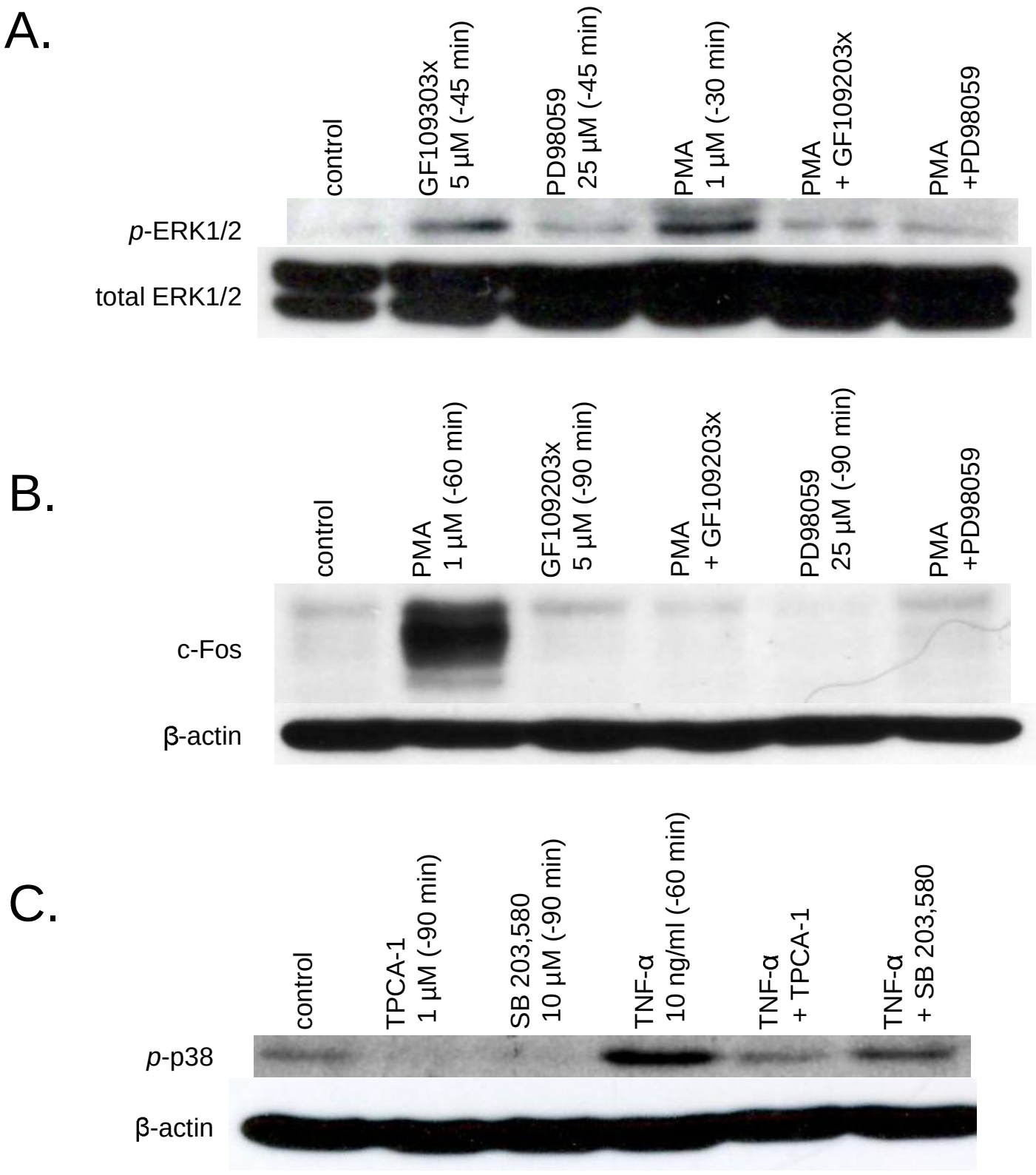

A. (n = 5-8)
500
400
300
200
100
0
control
+ PMA 1 µM
*
**
0
100
1000
10000
[GF109203x] (nM)
specific binding (% control)
B. (n = 5)
500
400
300
200
100
0
control
+ PMA 1 µM
*
0
100
1000
10000
[Gö6976] (nM)
C. (n = 4)
600
500
400
300
200
100
0
control
+ PMA 1 µM
0
10
100
1000
[LY 379196] (nM)

A.

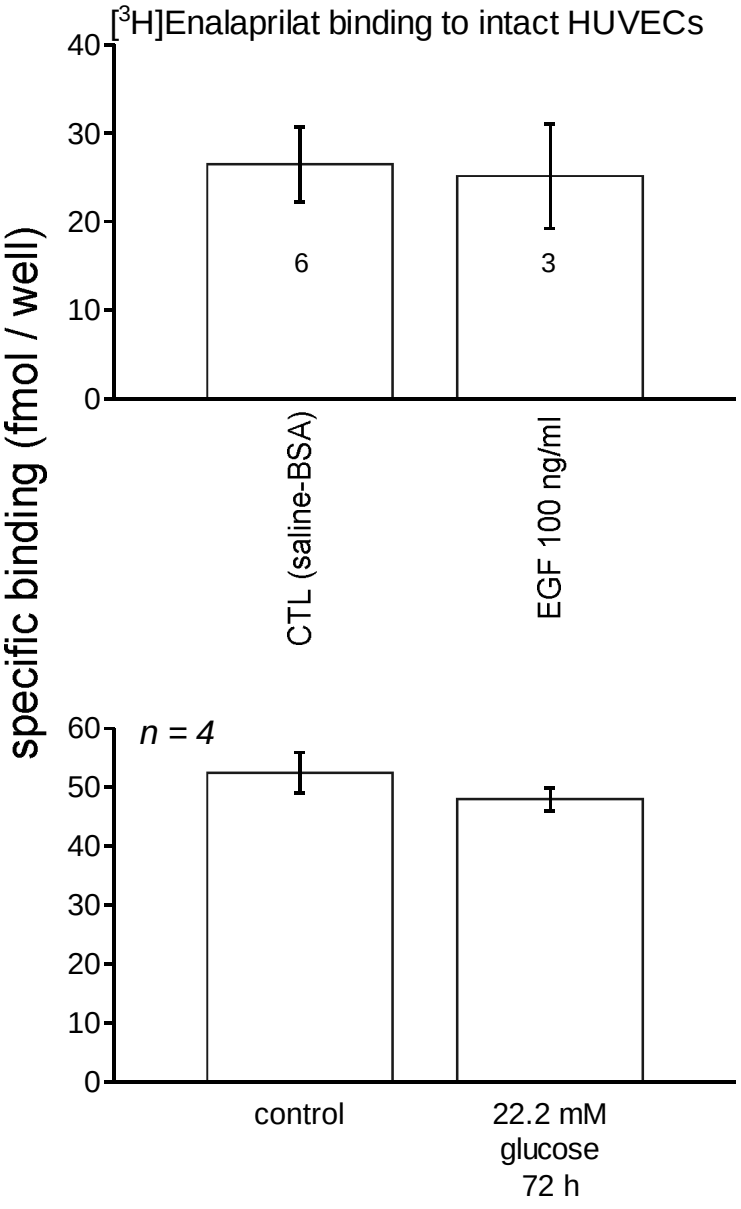

control
TNF-α 10 ng/ ml
PMA 1 µM
enalaprilat 1 µM
BK 10 µM
p-p38
p-ERK1/2
total ERK1/2
c-Fos
β-actin

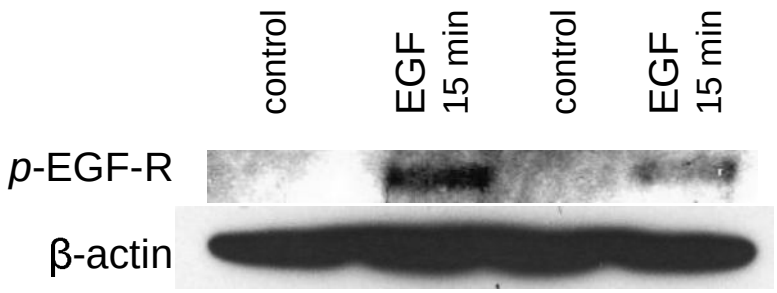
B.


vehicle
PD 98059 25 µM
SB 203,580 10 µM
n = 4
specific binding (% control)
600
500
400
300
200
100
0
**
**
*
*
**
control
TNF-α
PMA
treatment (- 24 h)

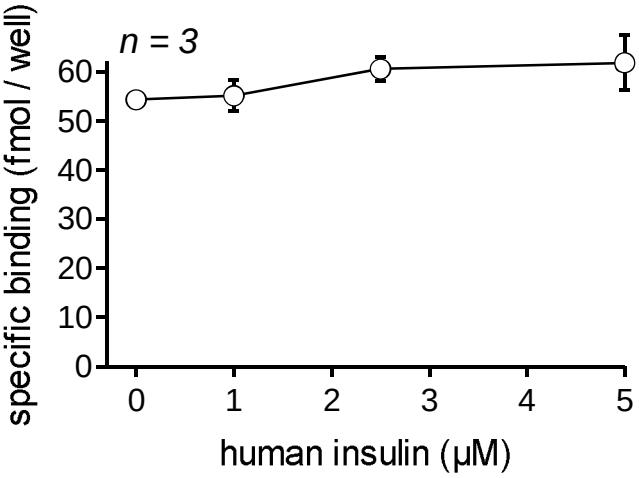

A.
control
GF109303x 5 µM (-45 min)
PD98059 25 µM (-45 min)
PMA 1 µM (-30 min)
PMA + GF109203x
PMA +PD98059
p-ERK1/2
total ERK1/2
B.
control
PMA 1 µM (-60 min)
GF109203x 5 µM (-90 min)
PMA + GF109203x
PD98059 25 µM (-90 min)
PMA +PD98059
c-Fos
β-actin
C.
control
TPCA-1 1 µM (-90 min)
SB 203,580 10 µM (-90 min)
TNF-α 10 ng/ml (-60 min)
TNF-α + TPCA-1
TNF-α + SB 203,580
p-p38
β-actin

A. [^{3}H]Enalaprilat (2 nM) binding to HUVECs





B. Insulin signaling in HUVECs



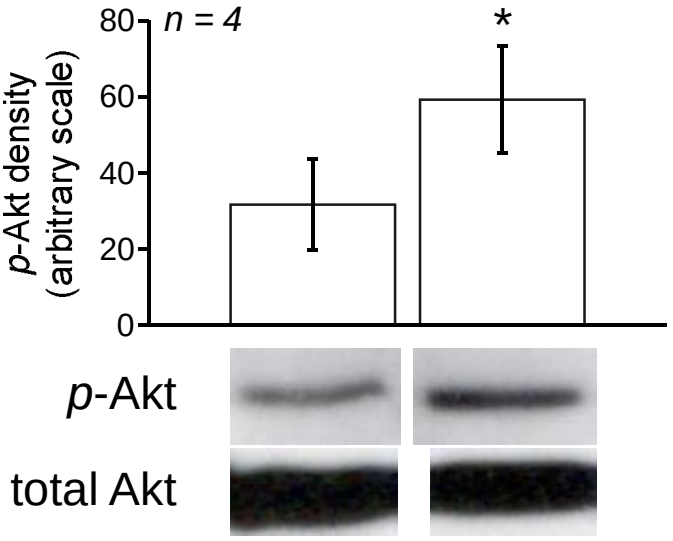


Figure 10

Fig. 10