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TNF-α AND ENDOTHELIN-1 INCREASE P-GLYCOPROTEIN EXPRESSION AND TRANSPORT ACTIVITY AT THE BLOOD-BRAIN BARRIER

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Running Title: Upregulation of P-glycoprotein in Brain Capillaries

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Nonstandard Abbreviations:

BCRP, breast cancer resistance protein; BIM, bisindolylmaleimide I; BSA, bovine serum albumin; ECE, endothelin converting enzyme; ET-1, endothelin-1; ET_A, endothelin receptor A; Glut-1, glucose transporter 1; IL-1, interleukin-1; L-NMMA, N^G-Monomethyl-L-arginine; LPS, lipopolysaccharide; LTC_4, leukotriene C4; Mrp, multidrug resistance-associated protein; NBD-CSA, [*N*-ε-(4-nitrobenzofurazan-7-yl)-D-Lys^8]cyclosporin A; NF-κB; nuclear factor- κB; NO, nitric oxide; NOS, nitric oxide synthase; PBS, phosphate buffered saline; PKC, protein kinase C; PMA, phorbolmyristate acetate; ROS, reactive oxygen species; SNP, sodium nitroprusside; TLR4, toll-like receptor 4; TNF-α, tumor necrosis factor-α; TNF-R1, tumor necrosis factor receptor 1; ZO-1, zonula occludens protein 1.

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ABSTRACT

The ATP-driven drug efflux pump, P-glycoprotein, is a critical and selective element of the blood-brain barrier and a primary impediment to pharmacotherapy of CNS disorders. Thus, an understanding how P-glycoprotein function is regulated has the potential to improve CNS therapy. We recently demonstrated rapid (minutes) and reversible inactivation of P-glycoprotein in rat brain capillaries signaled through TNF-α and ET-1, components of the brain's innate immune response. Here we examine the longer-term consequences of continuous exposure of rat brain capillaries to low levels of TNF-α and ET-1. Exposing brain capillaries to TNF-α or ET-1 caused a rapid decrease in P-glycoprotein transport activity with no change in transporter protein expression. This was followed by a 2-3 h plateau at the low activity level and then by a sharp increase in both transport activity and protein expression. After 6 h, transport activity and transporter protein expression had increased to twice control levels. TNF-α signaled through TNF-R1, which caused in turn ET release and action through ET_A and ET_B receptors, NOS, PKC and NF-κB and finally increased P-glycoprotein expression and transport activity. Assuming similar effects occur in vivo, the present results imply a tightening of the selective blood-brain barrier with chronic inflammation and thus reduced efficacy of CNS-acting drugs that are P-glycoprotein substrates. Moreover, involvement of NF-κB raises the possibility that other effectors acting through this transcription factor may have similar effects on this key blood-brain barrier transporter.

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INTRODUCTION

The blood-brain barrier, which resides within the brain capillary endothelium, is a formidable obstacle to the transfer of xenobiotics from blood to brain. Barrier function reflects the low paracellular permeability of the endothelium (tight junctions), a low rate of transcytosis and high expression of certain multispecific, ATP-driven xenobiotic efflux pumps (Begley, 2004b). Luminal plasma membrane location, high expression level, transport potency and affinity for a large number of therapeutics make one of these pumps, P-glycoprotein, a primary impediment to blood-brain barrier penetration of drugs and thus a major determinant of CNS efficacy (Begley, 2004a; Schinkel et al., 1996). Indeed, mice with disrupted P-glycoprotein genes exhibit substantially increased brain levels of administered P-glycoprotein substrates, including chemotherapeutics, HIV protease inhibitors, anticonvulsants, antipsychotics and glucocorticoids (Goralski et al., 2003; Schinkel et al., 1996).

Although the influence of blood-brain barrier P-glycoprotein on CNS pharmacotherapy is well-documented, little is known about mechanisms that regulate its expression and function in that tissue. Such an understanding would be important in devising strategies to treat CNS disorders that involve altered barrier function, e.g., epilepsy (Loscher and Potschka, 2005) or that require barrier modification for therapy, e.g., gliobastoma (Fellner et al., 2002). In this regard, we have been investigating mechanisms that regulate P-glycoprotein activity in intact rat brain capillaries and have recently linked the brain's innate immune response to rapid functional inactivation of blood-brain barrier P-glycoprotein (Hartz et al., 2004; Hartz et al., 2006). Brain capillary endothelial cells, like endothelial cells throughout the body, express receptors for cytokines, e.g., tumor necrosis factor-α (TNF-α (Nadeau and Rivest, 1999)) and interleukin-1 (IL-1 (Konsman et al., 2004)) and inflammogens, e.g., lipopolysaccharide (LPS (Chakravarty

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and Herkenham, 2005)). They both respond to inflammatory stimuli and amplify inflammatory signals (Nguyen et al., 2002; Rivest, 2003). Thus, brain capillaries are both targets for and active participants in the innate immune response. Our experiments show that exposing isolated rat brain capillaries to the proinflammatory cytokine, TNF-α, caused a rapid and reversible loss of P-glycoprotein transport activity (Hartz et al., 2004; Hartz et al., 2006). This occurred through the following sequence of events: TNF-α acting through TNF receptor 1 (TNF-R1) released endothelin-1 (ET-1), which signaled through an ET_B receptor to activate nitric oxide synthase (NOS), and then protein kinase C (PKC); activation of PKC reduced P-glycoprotein transport activity. The inflammogen, LPS, acting through toll-like receptor 4 (TLR4) activated this pathway and rapidly reduced P-glycoprotein activity (Hartz et al., 2006). In these short-term experiments, neither capillary tight junctional permeability nor P-glycoprotein expression (protein) was changed.

The present report addresses the longer-term consequences of TNF-α exposure on blood-brain barrier P-glycoprotein expression and function. Available information on how extended exposure to TNF-α affects blood-brain barrier P-glycoprotein is limited and inconsistent. For example, in mice, Shiga-like toxin II increases P-glycoprotein expression in whole brain by a TNF-α- dependent mechanism (Zhao et al., 2002). In rat brain capillary endothelial cell lines, TNF-α has been found to increase P-glycoprotein mRNA and decrease transport function but not affect protein expression (Mandi et al., 1998; Theron et al., 2003).

Here we show in isolated, intact rat brain capillaries that continuous exposure to low levels of TNF-α and ET-1 affected P-glycoprotein transport activity and protein expression in a complex, time-dependent manner. In these experiments, we chose to measure expression as protein, since this correlates with transport function. The initial rapid reduction in transport

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activity first described by Hartz et al (Hartz et al., 2004; Hartz et al., 2006), was followed by a 2-3 h plateau at the reduced activity level and then by a rapid increase. After 6 h, both transport activity and transporter protein expression had increased to twice control levels. This increase involved signaling through the TNF-R1 receptor, ET_A and ET_B receptors, NOS, PKC and the transcription factor, nuclear factor-κB (NF-κB). These findings disclose a novel signaling pathway through which chronic inflammation can upregulate P-glycoprotein expression and activity and thereby tighten the blood-brain barrier to CNS-acting drugs that are P-glycoprotein substrates.

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MATERIALS AND METHODS

Chemicals

ET-1, RES-701-1, JKC-301, NF-κB transcriptional activation inhibitor and the NF-κB nuclear translocation inhibitor, SN50, were purchased from Calbiochem-Novabiochem (LaJolla, CA). H398 was from Alexis-Axxora (San Diego, CA), bisindolylmaleimide I (BIM) was from Molecular Probes (Eugene, OR), and phosphoramidon and phorbolmyristate acetate (PMA) were from A.G. Scientific (San Diego, CA). C219 antibody was purchased from Signet (Dedham, MA). NBD-CSA was custom-synthesized by R. Wenger (Basel, CH) (Schramm et al., 1995). PSC833 was a kind gift from Novartis (Basel, CH). All other chemicals were obtained from Sigma (St. Louis, MO).

Rat Brain Capillary Isolation

Rat brain capillaries were isolated as described previously (Hartz et al., 2004; Hartz et al., 2006). Briefly, Sprague-Dawley male retired rats (Taconic, Germantown, NY) were euthanized by CO_2 inhalation and decapitated. Brains were taken immediately and kept at 4°C in PBS buffer (2.7 mM KCl, 1.46 mM KH_2PO_4, 136.9 mM NaCl, 8.1 mM Na_2HPO_4, supplemented with 5 mM D-glucose and 1 mM Na-pyruvate, pH 7.4). Rat brains were dissected and homogenized in PBS. After addition of Ficoll® (final concentration 15%, Sigma, St. Louis, MO), the homogenate was centrifuged at 5,800g for 20 min at 4°C. The pellet was resuspended in PBS containing 1% BSA and passed over a glass bead column. Capillaries adhering to the glass beads were collected by gentle agitation in PBS (1% BSA) and washed three times in BSA-free PBS. Freshly isolated capillaries were used for transport experiments, immunostaining and plasma membrane isolation followed by Western blotting.

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P-glycoprotein Transport Activity

After 1-6 h exposure to effectors, capillaries were transferred to confocal chambers and incubated for 1 hour at room temperature with 2 μM of the fluorescent P-glycoprotein substrate, NBD-cyclosporin A (NBD-CSA) (Hartz et al., 2004; Hartz et al., 2006). In some experiments, capillaries were loaded to steady state (60 min) with NBD-CSA before exposing them to effectors. For each treatment, confocal fluorescence images of 7-15 capillaries were acquired (Zeiss LSM 510 meta confocal microscope or Zeiss LSM 410 inverted confocal microscope, 40x water immersion objective, NA=1.2) and luminal fluorescence intensity was measured using Zeiss Image Examiner software or Scion Image software as described previously (Hartz et al., 2004; Hartz et al., 2006). Specific, NBD-CSA fluorescence was taken as the difference between total luminal fluorescence and fluorescence in the presence of PSC833 or NaCN.

Immunohistochemistry

Capillaries adhering to glass cover slips were fixed for 15 minutes with 3% paraformaldehyde/0.2% glutaraldehyde at room temperature. After washing with PBS, capillaries were permeabilized for 30 minutes with 0.1% (v/v) Triton X-100 in PBS, and subsequently blocked with 1% BSA in PBS. Capillaries were then incubated for 1 hour at 37°C with monoclonal primary anti-P-glycoprotein C219 antibody (1 μg/ml; Signet, Dedham, MA), washed (PBS, 1% BSA) and incubated with Alexa Fluor® 488-conjugated secondary IgG (2 μg/ml; Molecular Probes, Eugene, OR) for 1 hour at 37°C. Negative controls were incubated with secondary antibody only; nuclei were counterstained with 5 μg/ml propidium iodide for 15 min. Immunostaining was visualized and measured using a Zeiss LSM 510 meta confocal microscope (Bauer et al., 2004).

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Brain Capillary Membrane Isolation and Western Blot Analysis

Capillaries were homogenized and lysed in mammalian tissue lysis buffer (Sigma, St. Louis, MO) containing protease inhibitor cocktail (Roche, Mannheim, FRG). Samples were centrifuged at 10,000g for 30 min. Denucleated supernatants were centrifuged at 100,000g for 90 min. Pellets were resuspended and protein concentrations were determined. Western blots were performed using the Invitrogen NuPage™ Bis-Tris electrophoresis system and conducted according to the manufacturer's protocol. To detect P-glycoprotein, PVDF membranes were incubated overnight with 1 µg/ml of monoclonal C219 primary antibody (Signet, Dedham, MA). Membranes were washed and incubated for 1 h with horseradish peroxidase-conjugated ImmunoPure® secondary IgG (1:15,000; Pierce, Rockford, IL). Membranes were again washed and P-glycoprotein was detected using SuperSignal® West Pico Chemoluminescent Substrate (Pierce, Rockford, IL). Protein bands were visualized and recorded using a BioRad Gel Doc 2000™ gel documentation system (BioRad, Hercules, CA). In preliminary experiments, we measured immunoreactive P-glycoprotein signal (integrated band intensity) as a function of the amount of membrane protein applied and found an approximately linear relationship.

Statistical Analysis

Data are presented as mean ± SEM. Differences between mean values for controls and treated capillaries were considered to be statistically significant when $P < 0.05$ using Student's t-test.

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RESULTS

We have developed a method to determine P-glycoprotein transport activity in living, intact brain capillaries (Bauer et al., 2004; Hartz et al., 2004; Miller et al., 2000). It involves measurement of the steady state, concentrative accumulation of the fluorescent, P-glycoprotein substrate, NBD-cyclosporin A (NBD-CSA) in capillary lumens using confocal microscopy and quantitative image analysis. In brain capillaries from rat, luminal NBD-CSA accumulation is inhibited maximally (50-60%) by 1 mM NaCN, a metabolic inhibitor, or 5 μM PSC833, a specific inhibitor of P-glycoprotein. Inhibitors of other xenobiotic efflux pumps, e.g., multidrug-resistance associated proteins (Mrps; MK571 and leukotriene C_4, LTC_4) and breast cancer resistance protein (BCRP; fumitremorgin C) are without effect (Bauer et al., 2004; Hartz et al., 2004; Hartz et al., 2006; Miller et al., 2000). Thus, in brain capillaries, luminal NBD-CSA accumulation is both ATP-driven and P-glycoprotein-specific. Remaining luminal fluorescence after inhibition with PSC833 or NaCN represents simple diffusion and unspecific binding of NBD-CSA to capillary tissue (Bauer et al., 2004; Hartz et al., 2004; Hartz et al., 2006; Miller et al., 2000). Figure 1A shows steady state, total luminal accumulation of NBD-CSA in rat brain capillaries, measured at various times over a 6 h time course. In control capillaries, luminal accumulation did not change with time. This result agrees with previous findings indicating that transport in isolated capillaries remains constant over at least 8 h of incubation (Bauer et al., 2004). Consistent with this, Western blots of plasma membranes from capillaries incubated in control medium showed no change in P-glycoprotein expression over 6 h (Figure 1B).

Luminal NBD-CSA accumulation was not constant in capillaries exposed continuously to 1 ng/ml TNF-α or 100 nM ET-1 (Figure 1A). Initially, it declined rapidly, so that after 1 h, luminal accumulation was about half of controls. This result agrees with our previous

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experiments focused on modulation of P-glycoprotein activity over the short-term (Hartz et al., 2004; Hartz et al., 2006). Since about half of substrate accumulation in control capillaries represents specific, P-glycoprotein-mediated transport (Hartz et al., 2004; Hartz et al., 2006), the decrease measured at 1 h reflects near-total loss of specific transport activity. This short-term loss of activity is not accompanied by any change in P-glycoprotein expression (Hartz et al., 2004; Hartz et al., 2006). Luminal NBD-CSA accumulation remained at this low level for 2 additional hours. After 3 h of exposure, accumulation began to increase. It reached control levels after about 4 h and with 5 and 6 h of exposure, luminal accumulation was significantly higher than controls ($P<0.001$; Figure 1A). For capillaries exposed to TNF-α or ET-1, Western blots showed a small increase in P-glycoprotein expression after 3 h and a substantial increase after 6 h (Figure 1C). Consistent with this, immunostaining of intact capillaries for P-glycoprotein showed an obvious increase following 6 h exposure to TNF-α or ET-1 (Figure 1D). Quantitation of luminal plasma membrane immunofluorescence indicated that P-glycoprotein expression had doubled in exposed capillaries.

For both TNF-α and ET-1, the increase in total luminal accumulation of NBD-CSA measured after 6 h of exposure was concentration-dependent (Figures 2A and 2C). Importantly, the component of accumulation that was insensitive to PSC833 and NaCN did not change (Figure 2A). Since PSC833 is a specific inhibitor of P-glycoprotein, the increase in luminal NBD-CSA accumulation reflects only a change in specific transport activity. As before (Bauer et al., 2004; Hartz et al., 2004; Hartz et al., 2006) we operationally defined the PSC833-sensitive component of NBD-CSA accumulation as P-glycoprotein-specific transport and used this as a measure of P-glycoprotein transport activity. By these criteria, 6 h exposure to 1-5 ng/ml TNF-α

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or 10-100 nM ET-1 roughly doubled P-glycoprotein transport activity in brain capillaries (Figures 2B and 2D).

The data presented in Figures 1 and 2 for single experiments agree well with the increases in P-glycoprotein activity and protein expression (Western blots) averaged over 8-10 individual experiments. For these pooled experiments, 6 h exposure to 1 ng/ml TNF-α increased P-glycoprotein-specific transport activity by 122 ± 11% and protein expression (integrated blot density) by 67 ± 11% and 6 h exposure to 100 nM ET-1 increased specific transport activity by 119 ± 11% and protein expression by 71 ± 8%. These data suggest that both TNF-α and ET-1 increased transport activity to a greater extent than protein expression. This could mean that TNF-α and ET-1 not only increase the amount of transporter protein but also the percentage of total transporter protein that is functionally active.

Additional experiments were focused on defining the mechanisms through which TNF-α and ET-1 increased P-glycoprotein expression and specific transport activity. In the basic experiment, we exposed isolated brain capillaries to 1 ng/ml TNF-α or 100 nM ET-1 without or with specific pharmacological agents. After 6h, we measured specific P-glycoprotein transport activity as PSC833-sensitive NBD-CSA accumulation in capillary lumens and P-glycoprotein expression using Western blots of capillary membranes. Preliminary experiments showed that at the concentrations used here none of the pharmacological agents used affected P-glycoprotein activity or expression after 1 h or 6 h of exposure ((Hartz et al., 2006) and our unpublished experiments). Using this protocol, it was clear that inhibiting protein synthesis with cycloheximide abolished the increases in P-glycoprotein transport activity and expression caused by 6 h exposure to TNF-α and ET-1 (Figure 3).

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Figure 4 shows the TNF-R1 antagonist, H398, abolished the increases in transport activity and protein expression seen after 6 h exposure to TNF-α, but not to ET-1. The increases in P-glycoprotein transport activity and expression with exposure to TNF-α were also abolished when ET_A and ET_B receptors were blocked with JKC-301 and RES-701-1, respectively (Figure 5A). Both JKC-301 and RES-701-1 also abolished the effects of 100 nM ET-1 on transport and protein expression (Figure 5B). Thus, blocking either the ET_A or the ET_B receptor disrupted signaling through TNF-α and ET-1.

These results imply that TNF-α signaling through TNF-R1 released ET from the capillaries and that ET then signaled through both of its receptors (autocrine/paracrine signaling). ETs are stored within cells as preprohormones, which are first cleaved intracellularly to yield prohormones, big-ETs (Turner and Murphy, 1996). The prohormones are released from the cells and cleaved to their active forms by a surface membrane bound ET converting enzyme (ECE). To test whether TNF-α signaled ET release, we first exposed capillaries to phosphoramidon, a specific ECE inhibitor, and measured the effects of TNF-α and ET-1 on P-glycoprotein activity and protein expression. Inhibiting ECE blocked the TNF-α-induced increases in transport activity and expression (Figure 6A), but phosphoramidon did not affect the action of ET-1 added to the incubation medium (Figure 6B). Thus, TNF-α acted through TNF-R1 to release ET which could signal increased P-glycoprotein expression through either ET_A or ET_B receptors.

We previously showed that TNF-α and ET-1 signaled the short-term (1 h) decrease in P-glycoprotein activity in rat brain capillaries by activating NOS and PKC in sequence (Hartz et al., 2004; Hartz et al., 2006). In the present longer-term experiments, blocking NOS with N^G-monomethyl-L-arginine (L-NMMA) or blocking PKC with bisindolylmaleimide (BIM) abolished the increase in P-glycoprotein activity and protein expression induced by exposing

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capillaries to TNF-α or ET-1 for 6 h (Figure 7). Consistent with this, exposing capillaries to the nitric oxide (NO) donor, sodium nitroprusside (SNP), or the PKC activator, phorbolmyristate acetate (PMA), increased P-glycoprotein activity and expression (Figures 8A and 8B). SNP effects were blocked by BIM (Figure 8C), but PMA effects were not affected by L-NMMA (Figure 8D), indicating signaling through NOS and NO preceded PKC activation. As with TNF-α and ET-1, cycloheximide abolished the effects of SNP and PMA (not shown).

The transcription factor, NF-κB, is a key downstream element of TNF-R1 signaling (Liu, 2005). Activation of NF-κB causes its translocation from cytoplasm to nucleus, increasing expression of a number of genes, including, P-glycoprotein (Bentires-Alj et al., 2003; Hayashi et al., 2005; Thevenod et al., 2000; Zhou and Kuo, 1997). In rat brain capillaries, blocking NF-κB transcriptional activation abolished the increase in P-glycoprotein activity and expression caused by 6 h exposure to TNF-α and ET-1 (Figures 9A and 9B). Identical effects were found with SN50, which blocks nuclear translocation of NF-κB (Figures 9C and 9D); SN50M, an inactive control peptide, was without effect. However, SN50 did not block the transient reduction in P-glycoprotein activity caused by 1 h exposure to TNF-α (not shown). Consistent with NF-κB being the downstream effector of TNF-α signaling in brain capillaries, blocking its ability to activate transcription or its nuclear translocation abolished the effects of SNP and PMA on P-glycoprotein activity and expression (Figure 10). Thus, in these 6 h experiments, TNF-α and ET-1 signaled the increase in P-glycoprotein expression by activating in turn NOS, PKC and NF-κB.

The present results raise the question of how the expression of other plasma membrane proteins essential for blood-brain barrier function is affected by TNF-α and ET-1. Western blots of plasma membranes from capillaries exposed for 6 h to 1 ng/ml TNF-α or 100 nM ET-1 showed reduced expression of the drug efflux pumps, multidrug resistance associated protein 2

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(Mrp2) and Mrp4 (Figure 11A). In contrast, expression of glucose transporter 1 (Glut-1), Na^+/K^+-ATPase and TNF-R1 increased (Figure 11A) and expression of three tight junction-associated proteins, zonula occludens protein 1 (ZO-1), occludin and claudin-5, did not change (Figure 11B). Note that for all of the above proteins as well as P-glycoprotein, exposure to TNF-α and ET-1 had the same effect on expression. This was not the case for Mrp1 and BCRP, where TNF-α had no effect, but ET-1 decreased expression. This suggests that ET-1 can signal changes in transporter protein expression independently of TNF-α signaling.

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DISCUSSION

Inflammation accompanies most CNS diseases and severe inflammation profoundly affects the blood-brain barrier (Huber et al., 2001). Inflammatory mediators, such as, TNF-α, IL-1β and interferon-γ, can increase junctional permeability and cause leakage of plasma constituents into the CNS. We previously defined an early response of the selective blood-brain barrier to low levels of bacterial endotoxin (LPS), the inflammatory cytokine, TNF-α, and the polypeptide hormone, ET-1 (Hartz et al., 2004; Hartz et al., 2006). This involved rapid (minutes) and reversible loss of P-glycoprotein activity signaled through TLR4, TNF-R1, ET_B receptor, NOS and PKC. In these studies, neither transporter expression nor capillary tight junctional permeability was changed. The present results extend the time courses of action for TNF-α and ET-1, disclosing complexity in both temporal response and signaling.

After 1 h of TNF-α or ET-1 exposure, we found a sharp decline in P-glycoprotein transport activity. This corresponds to the early, reversible events previously reported by Hartz et al (Hartz et al., 2004; Hartz et al., 2006). With continuous exposure to TNF-α or ET-1, transport activity remained depressed for an additional 2-3 h. Thereafter, transport activity increased, passing through control levels after about 4 h and significantly exceeding control levels after 5-6 h. At 6 h, transport activity was more than twice controls. At that time, P-glycoprotein expression (quantitative immunostaining of intact capillaries and Western blots of capillary membranes) had roughly doubled. These increases in transport activity and transporter expression above control levels were abolished when protein synthesis was inhibited by cycloheximide. They are however not indicative of a general increase in plasma membrane protein expression, since Western blots show both increases (P-glycoprotein, TNF-R1, Glut-1, Na^+/K^+-ATPase) and decreases (Mrp2, Mrp4) in expression of specific plasma membrane

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proteins after 6 h exposure to TNF-α or ET-1. At present, it is not clear whether these changes in expression are signaled through a common pathway.

The overall pattern of changes in brain capillary P-glycoprotein activity and protein expression in response to TNF-α and ET-1 is certainly complex, with transporter activity reduced over the short-term and transporter protein expression and activity increased over the long-term. A similar pattern has been found for Mrp2 in renal proximal tubules exposed to ET-1 or to certain tubular nephrotoxicants (Terlouw et al., 2002).

Mediators of inflammation have been shown to increase tight junctional permeability of the brain capillary endothelial cell monolayers in vitro and brain microvessels in vivo (Huber et al., 2001; Trickler et al., 2005). In this regard, we previously showed no change in capillary tight junctional permeability after 1 h exposure to low levels of TNF-α or ET-1 (Hartz et al., 2004; Hartz et al., 2006). Similarly, the present study shows that 6 h exposure to TNF-α or ET-1 neither altered expression of tight junctional proteins nor reduced concentrative luminal accumulation of NBD-CSA, again indicating no impairment of tight junctional function in our rat brain capillaries.

At a minimum, three separate processes account for the changes in P-glycoprotein transport activity and expression induced by TNF-α and ET-1. As proposed previously (Hartz et al., 2004; Hartz et al., 2006), the initial decrease in activity with no change in transporter expression and tight junctional permeability reflects changes in the dynamics of transporter trafficking (retrieval from the plasma membrane into a vesicular compartment) or transporter inactivation in situ (altered phosphorylation state of the transporter or an accessory protein). Given the resolution of confocal microscopy (~0.25 μm) and the extremely thin cytoplasmic compartment in the endothelial cells (~1 μm), we cannot experimentally distinguish the two

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possibilities. Clearly, P-glycoprotein can be maintained in this internalized/inactive state for at least 2 h (present study). The recovery to control levels with 3-4 h of exposure and the overshoot with 5-6 h of exposure likely involve two processes: release from internalization/inactivation and increased synthesis of transporter protein. The clearest evidence for the former comes from experiments with an inhibitor of protein synthesis. Hartz et al. (Hartz et al., 2004; Hartz et al., 2006) showed that the initial (1 h) decrease in transport activity caused by TNF-α and ET-1 still occurs in capillaries treated with cycloheximide. We show here that after 6 h exposure to TNF-α or ET-1 plus cycloheximide both transport activity and transporter expression were no different from untreated controls. This indicates that transporter release from the internalized/inactive state is independent of protein synthesis. Finally, the increase in transport activity above control levels after 6 h of TNF-α or ET-1 exposure correlated well with the increase in transporter protein expression. We do not yet know whether the release of transporter from the internalized/inactive state and the increase in transporter expression occurred simultaneously or sequentially.

Using pharmacological tools, we dissected the signaling pathway through which prolonged exposure to TNF-α increased P-glycoprotein activity and expression. The results are consistent with the following sequence of events: 1) TNF-α binds to TNF-R1, 2) ET is released and binds to ET_A and ET_B receptors, 3) NOS is activated, 5) PKC is activated, 6) NF-κB is activated and translocates from cytoplasm to nucleus, and 7) P-glycoprotein expression and activity increase (Figure 12A). As before (Hartz et al., 2006), we suggest that the first three steps occur at the basolateral surface of the endothelium. The rationale for this is based on basolateral receptor immunolocalization and the expected limited access of polypeptide and protein effectors and reagents to the luminal compartment of isolated capillaries with an intact junctional barrier (Hartz et al., 2006).

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Figure 12B shows the emerging picture of P-glycoprotein regulation derived from our experiments with intact rat brain capillaries. It is obvious from Figures 12A and 12B that the signaling scheme disclosed in the present study resembles the one previously proposed for the short-term loss of P-glycoprotein activity induced by TNF-α- and ET-1 (Hartz et al., 2004; Hartz et al., 2006). There are however two significant differences. First, short-term signaling to P-glycoprotein is blocked by an ET_B receptor antagonist but not by an ET_A receptor antagonist (Hartz et al., 2004; Hartz et al., 2006). In contrast, the longer-term increase in transporter activity and expression could be blocked by either an ET_B or an ET_A receptor antagonist (present study). This suggests that both receptors must be active for ET-1 signaling over the long-term. In this regard, ET_B and ET_A, like other G-protein-coupled receptors, can form heterodimers that influence both receptor trafficking and possibly signaling (Gregan et al., 2004; Harada et al., 2002). It remains to be determined to what extent this occurred in the present experiments.

Second, during long-term exposure, TNF-α and ET-1 signaled through NF-κB, a transcription factor that is activated by a number of stress-related signals, e.g., cytokines, hypoxia, reactive oxygen species (ROS), heat shock, heavy metals (Ali and Mann, 2004; Chen and Shi, 2002; Martindale and Holbrook, 2002). NF-κB plays an important role in cellular protection, but also has been implicated in cell death pathways. At the blood-brain barrier, NF-κB protects against damage initiated by for example, hypoxia/reoxygenation, stroke and traumatic brain injury (Crack and Taylor, 2005; Nonaka et al., 1999; Sullivan et al., 1999; Taylor and Crack, 2004; Yang et al., 1995). In brain capillary endothelial cells, NF-κB has been implicated in the increase in P-glycoprotein expression caused by exposure to hydrogen peroxide (Nwaozuzu et al., 2003) and to HIV-Tat protein (Hayashi et al., 2005), which is released into the CNS of AIDS patients. By implicating NF-κB in the induction of P-glycoprotein expression

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caused by exposure of brain capillaries to TNF-α- and ET-1, the present results suggest that other signals that activate this transcription factor may also upregulate P-glycoprotein and that other NF-κB targets could be changed as well. In this regard, P-glycoprotein is only one of several plasma membrane proteins with altered expression in response to TNF-α or ET-1 (Figure 11A). It remains to be determined to what extent signaling through NF-κB affects expression of the other proteins.

Together, the present findings show upregulation of P-glycoprotein expression and transport activity in brain capillaries following exposure to TNF-α and ET-1, both of which are active participants in the brain's innate immune response. If similar events occur in vivo, the present results imply a tightening of the selective blood-brain barrier with chronic, low level inflammation. This could be of significance, because both inflammation and altered barrier function are known to be associated with a number of neurological diseases (assuming similar responses in rats and humans). In epilepsy, increases in blood-brain barrier P-glycoprotein expression occurs in regions of the brain exhibiting seizure activity (Loscher and Potschka, 2005). Also, a recent report shows that P-glycoprotein expression in brain capillaries is increased in an animal model of ischemic stroke (Spudich et al., 2006). In that model, inhibition of P-glycoprotein transport function potentiates the neuroprotective effects of FK506 and rifampin, two drugs that are P-glycoprotein substrates. At present, the events that connect epileptic seizures and cerebral ischemia with increased P-glycoprotein expression are unknown. Nevertheless, it is tempting to speculate that, inflammatory signaling, a factor common to many CNS pathologies, is involved and that such signaling can be a target of pharmacotherapy.

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FOOTNOTES

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FIGURE LEGENDS

Figure 1. (A) Time course of TNF-α and ET-1 effects on steady state luminal NBD-CSA fluorescence. First, capillary lumens were loaded to steady state (60 min) in buffer with 2 μM NBD-CSA. Then they were exposed to 1 ng/ml TNF-α or 100 nM ET-1. Controls were incubated in TNF-α and ET-1-free medium and NBD-CSA was present throughout the experiment in all three groups. Each data point represents the mean value for 7-10 brain capillaries from a single capillary preparation (pooled tissue from 5 rats); variability is given by SEM bars. Units are arbitrary fluorescence units (scale 0-255). (B) Western blot showing time course of P-glycoprotein expression in membranes from control brain showing no change in P-glycoprotein expression over the time course of the experiment. Negative control is total brain homogenate; positive control is renal brush border membranes. Na^+/K^+-ATPase and β-actin bands indicate consistency of loading. (C) Western blots showing changes in P-glycoprotein expression in capillary membranes after 3h or 6h exposure to 1 ng/ml TNF-α or 100 nM ET-1. (D) Western blots showing changes in P-glycoprotein expression in capillary membranes after 6h exposure to 1 ng/ml TNF-α or 100 nM ET-1; β-actin bands indicate consistency of loading. (E) Representative images showing P-glycoprotein immunofluorescence (green) in capillaries exposed to 1 ng/ml TNF-α or 100 nM ET-1 for 6 hours (bar =10 μm). Nuclei were stained with propidium iodide (red). Numbers under the images give mean fluorescence intensities for immunostained capillaries. Statistical comparisons: *** significantly higher than controls, $P<0.001$.

Figure 2. Concentration-dependent increase of P-glycoprotein transport activity in rat brain capillaries exposed to TNF-α or ET-1. (A) Continuous exposure of brain capillaries to 0.5-5

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ng/ml TNF-α for 6 hours increased steady state total luminal NBD-CSA fluorescence. NBD-CSA accumulation in capillary lumens was reduced by the specific P-glycoprotein inhibitor, PSC833, and the metabolic inhibitor, NaCN, but TNF-α exposure only increased the PSC833- and NaCN-sensitive component of accumulation. (B) Specific luminal NBD-CSA fluorescence, taken as the difference between total luminal NBD-CSA fluorescence and fluorescence in the presence of PSC833 or NaCN. (C) Continuous exposure of brain capillaries to 1-100 nM ET-1 for 6 hours increased steady state total luminal NBD-CSA fluorescence. (D) Specific luminal NBD-CSA fluorescence taken as the difference between total luminal NBD-CSA fluorescence and fluorescence in the presence of PSC833. Each data point represents the mean value for 10-15 capillaries from a single preparation (pooled tissue from 3-10 rats); variability is given by SEM bars. Units are arbitrary fluorescence units (scale 0-255). Statistical comparison: *** significantly higher than controls, $P<0.001$.

Figure 3. Cycloheximide, an inhibitor of protein synthesis, abolished both the TNF-α-induced (A) and the ET-1-induced (B) increase in P-glycoprotein expression (Western blot) and P-glycoprotein transport activity (figure showing specific luminal NBD-CSA fluorescence). For specific luminal NBD-CSA fluorescence, data represents the mean value for 10-15 capillaries from a single preparation (pooled tissue from 3-10 rats); variability is given by SEM bars. Units are arbitrary fluorescence units (scale 0-255). Statistical comparisons: *** significantly higher than controls, $P<0.001$.

Figure 4. TNF-α induces P-glycoprotein expression and transport activity by acting through TNF-R1. (A) H398, a specific blocker for TNF-R1, abolished the TNF-α-induced increase of P-glycoprotein expression (Western blot) and P-glycoprotein transport activity (specific luminal

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NBD-CSA fluorescence). (B) H398 did not alter ET-1-induced increases in expression or transport. For specific luminal NBD-CSA fluorescence, data represents the mean value for 10-15 capillaries from a single preparation (pooled tissue from 3-10 rats); variability is given by SEM bars. Units are arbitrary fluorescence units (scale 0-255). Statistical comparison: *** significantly higher than controls, $P<0.001$.

Figure 5. TNF-α and ET-1 act through ET receptors to increase P-glycoprotein expression and transport activity. Both RES-701-1, a specific ET_B blocker, and JKC-301, a specific ET_A blocker, abolished TNF-α (A) and ET-1 (B) stimulation of P-glycoprotein expression (Western blot) and P-glycoprotein transport activity (specific luminal NBD-CSA fluorescence). For specific luminal NBD-CSA fluorescence, data represents the mean value for 10-15 capillaries from a single preparation (pooled tissue from 3-10 rats); variability is given by SEM bars. Units are arbitrary fluorescence units (scale 0-255). Statistical comparisons: *** significantly higher than controls, $P<0.001$.

Figure 6. TNF-α signaling to P-glycoprotein is blocked when endothelin converting enzyme is inhibited. (A) Phosphoramidon, a blocker of endothelin converting enzyme, abolished the TNF-α-induced increase of P-glycoprotein expression (Western blot) and transport activity (specific luminal NBD-CSA fluorescence). (B) Phosphoramidon did not affect the ET-1-induced increase of P-glycoprotein expression and transport activity. In both graphs showing specific luminal NBD-CSA fluorescence, data represents the mean value for 10-15 capillaries from a single preparation (pooled tissue from 3-10 rats); variability is given by SEM bars. Units are arbitrary fluorescence units (scale 0-255). Statistical comparisons: *** significantly higher than controls, $P<0.001$.

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Figure 7. TNF-α and ET-1 signal through NOS and PKC to increase P-glycoprotein expression and transport activity. L-NMMA, an inhibitor of NOS, blocked the effects of TNF-α (A) and ET-1 (B) on P-glycoprotein expression (Western blot) and transport activity (specific luminal NBD-CSA fluorescence). BIM, an inhibitor of typical PKC isoforms, blocked the effects of TNF-α (C) and ET-1 (D) on P-glycoprotein expression and transport activity. For specific luminal NBD-CSA fluorescence, data represents the mean value for 10-15 capillaries from a single preparation (pooled tissue from 3-10 rats); variability is given by SEM bars. Units are arbitrary fluorescence units (scale 0-255). Statistical comparisons: *** significantly higher than controls, $P<0.001$.

Figure 8. (A) SNP, a NO donor, increased P-glycoprotein expression (Western blot) and transport activity (specific luminal NBD-CSA fluorescence) in a concentration-dependent manner. (B) PMA, an activator of conventional and novel PKC isoforms, also induced P-glycoprotein expression and transport activity. (C) Blocking PKC with BIM abolished the SNP effect on P-glycoprotein expression and transport activity. (D) Blocking NOS with L-NMMA did not abolish the PMA-mediated induction of P-glycoprotein expression and transport activity. For specific luminal NBD-CSA fluorescence, data represents the mean value for 10-15 capillaries from a single preparation (pooled tissue from 3-10 rats); variability is given by SEM bars. Units are arbitrary fluorescence units (scale 0-255). Statistical comparisons: *** significantly higher than controls, $P<0.001$.

Figure 9. TNF-α and ET-1 act through NF-κB to increase of P-glycoprotein expression and transport activity. An inhibitor of NF-κB transcriptional activation abolished TNF-α (A) and ET-1 (B) effects on P-glycoprotein expression (Western blot) and transport activity (specific luminal

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NBD-CSA fluorescence). Blocking NF-κB nuclear translocation with SN50 abolished the TNF-α- (C) and ET-1- (B) induced increases of P-glycoprotein expression and transport activity. SN50M, an inactive control peptide mimicking SN50, had no effect. For specific luminal NBD-CSA fluorescence, data represents the mean value for 10-15 capillaries from a single preparation (pooled tissue from 3-10 rats); variability is given by SEM bars. Units are arbitrary fluorescence units (scale 0-255). Statistical comparison: *** significantly higher than controls, $P<0.001$.

Figure 10. Blocking NF-κB abolishes induction of P-glycoprotein expression and transport activity by SNP (A, B) and PMA (C, D). SN50M, an inactive control peptide mimicking SN50, had no effect. For specific luminal NBD-CSA fluorescence, data represents the mean value for 10-15 capillaries from a single preparation (pooled tissue from 3-10 rats); variability is given by SEM bars. Units are arbitrary fluorescence units (scale 0-255). Statistical comparison: *** significantly higher than controls, $P<0.001$.

Figure 11. Effects of exposing brain capillaries to 1 ng/ml TNF-α or 100 nM ET-1 (6 h) on expression of plasma membrane proteins (A) and tight junction-associated proteins (B).

Figure 12. Regulation of P-glycoprotein activity by inflammation-related signaling in rat brain capillaries. (A) Model of long-term signaling from TNF-α to P-glycoprotein (P-gp). (B) Signaling pathways affecting P-glycoprotein activity in rat brain capillaries. Activity is directly reduced through the two time-dependent pathways shown (short-term: 1 hour; long-term: 6 hours). For LPS, most of its effects are signaled through the TLR4-dependent pathway. Figure reflects findings from our present and previous studies (Bauer et al., 2004; Hartz et al., 2004; Hartz et al., 2006). Broken lines are used to indicate possible, additional intermediate steps.

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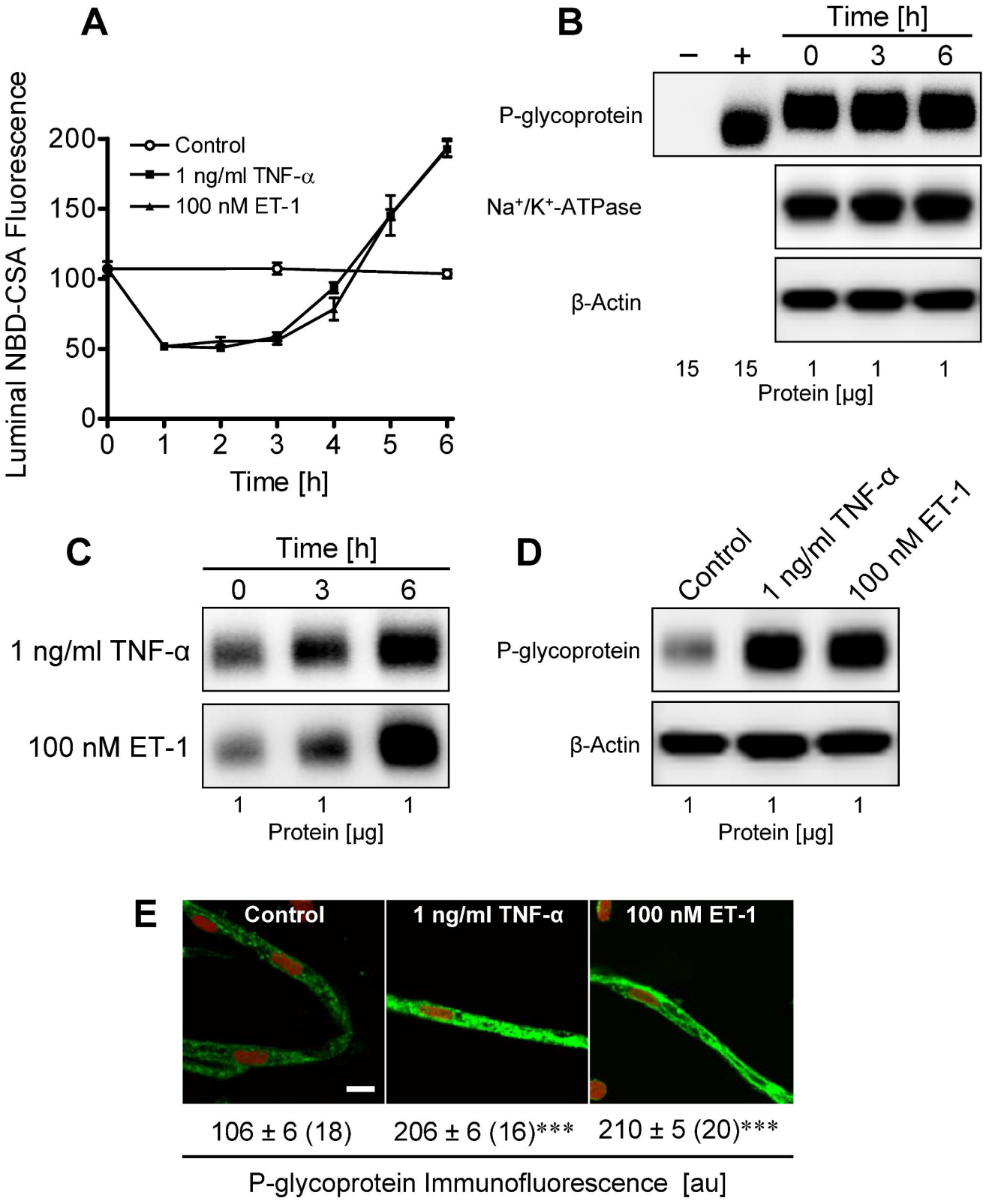
Figure 1


A
Luminal NBD-CSA Fluorescence
200
150
100
50
0
Control
1 ng/ml TNF-α
100 nM ET-1
0 1 2 3 4 5 6
Time [h]
B
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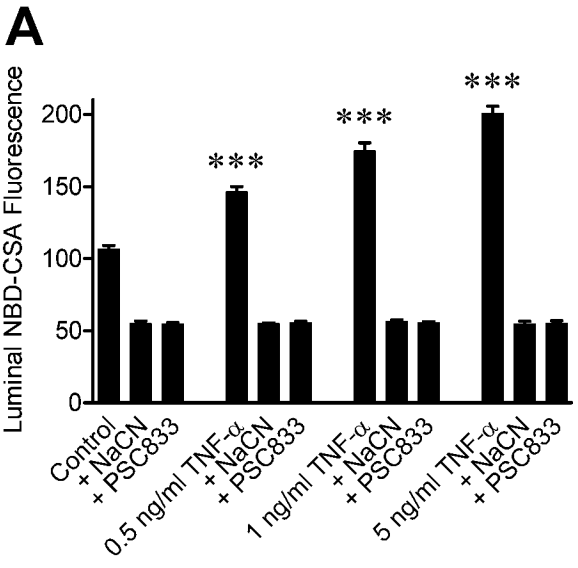
– + 0 3 6
P-glycoprotein
Na+/K+-ATPase
β-Actin
15 15 1 1 1
Protein [µg]
C
Time [h]
0 3 6
1 ng/ml TNF-α
100 nM ET-1
1 1 1
Protein [µg]
D
Control
1 ng/ml TNF-α
100 nM ET-1
P-glycoprotein
β-Actin
1 1 1
Protein [µg]
E
Control
1 ng/ml TNF-α
100 nM ET-1
106 ± 6 (18)
206 ± 6 (16)***
210 ± 5 (20)***
P-glycoprotein Immunofluorescence [au]

Figure 2


A
Luminal NBD-CSA Fluorescence
200
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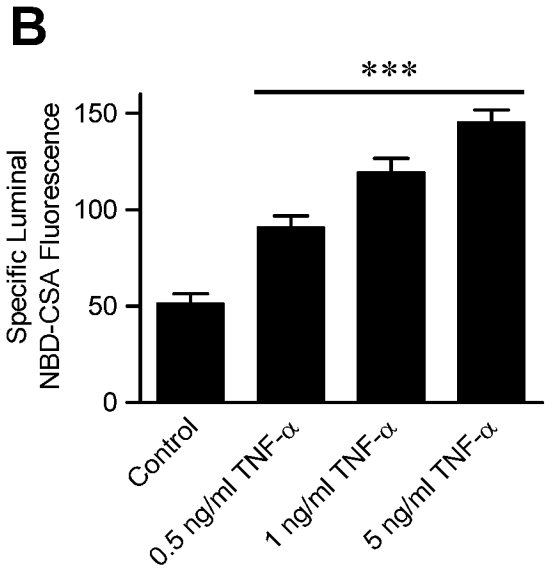
Control
+ NaCN
+ PSC833
0.5 ng/ml TNF-α
+ NaCN
+ PSC833
1 ng/ml TNF-α
+ NaCN
+ PSC833
5 ng/ml TNF-α
+ NaCN
+ PSC833


B
***
Specific Luminal
NBD-CSA Fluorescence
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100
50
0
Control
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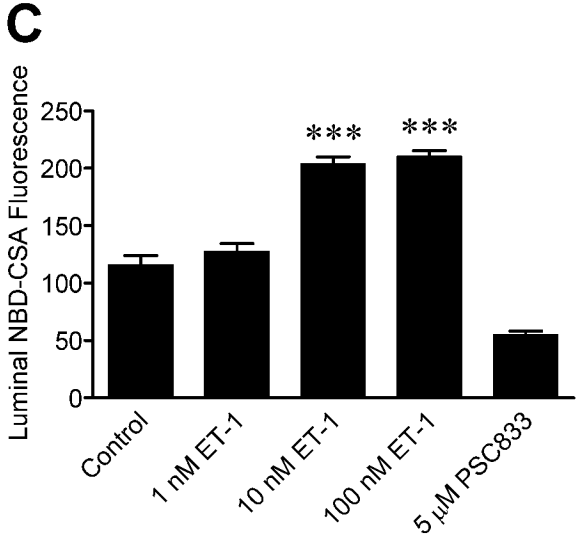
1 ng/ml TNF-α
5 ng/ml TNF-α


C
Luminal NBD-CSA Fluorescence
250
200
150
100
50
0
***
***
Control
1 nM ET-1
10 nM ET-1
100 nM ET-1
5 μM PSC833

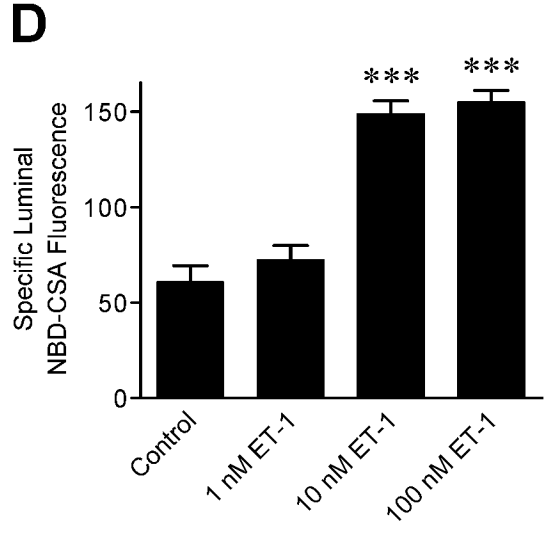

D
***
***
Specific Luminal
NBD-CSA Fluorescence
150
100
50
0
Control
1 nM ET-1
10 nM ET-1
100 nM ET-1

Figure 3

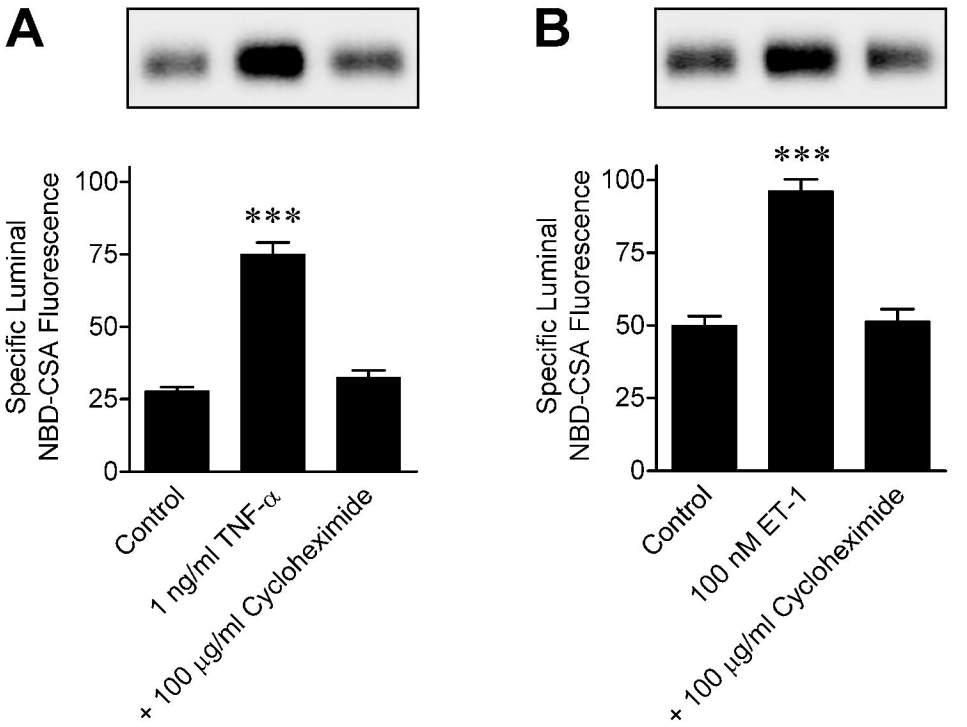

A
B
Specific Luminal NBD-CSA Fluorescence
100
75
50
25
0
***
Control
1 ng/ml TNF-α
+ 100 μg/ml Cycloheximide
100 nM ET-1

Figure 4

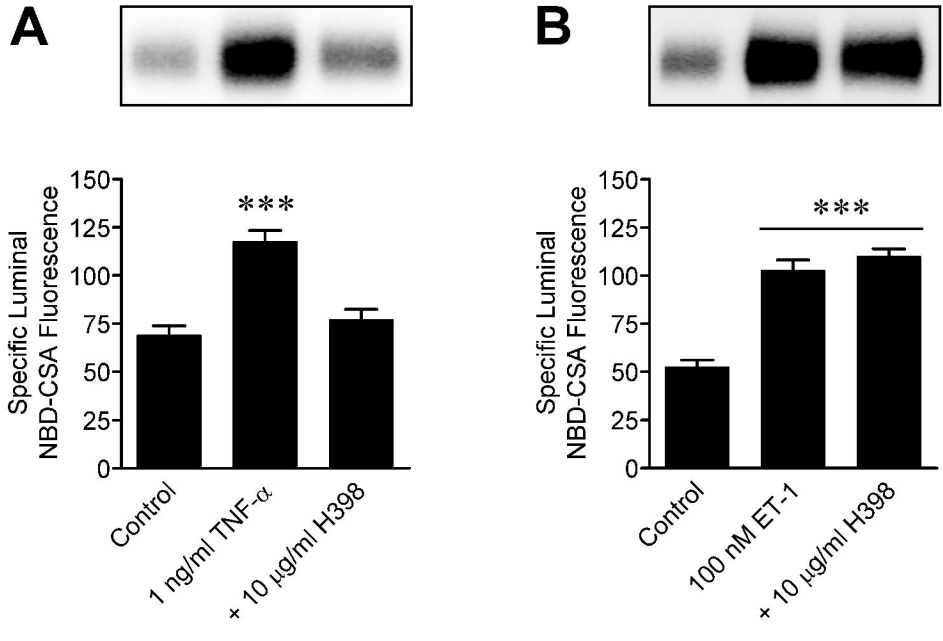

A
B
Specific Luminal NBD-CSA Fluorescence
150
125
100
75
50
25
0
***
***
Control
1 ng/ml TNF-α
+ 10 μg/ml H398
Control
100 nM ET-1
+ 10 μg/ml H398

Figure 5

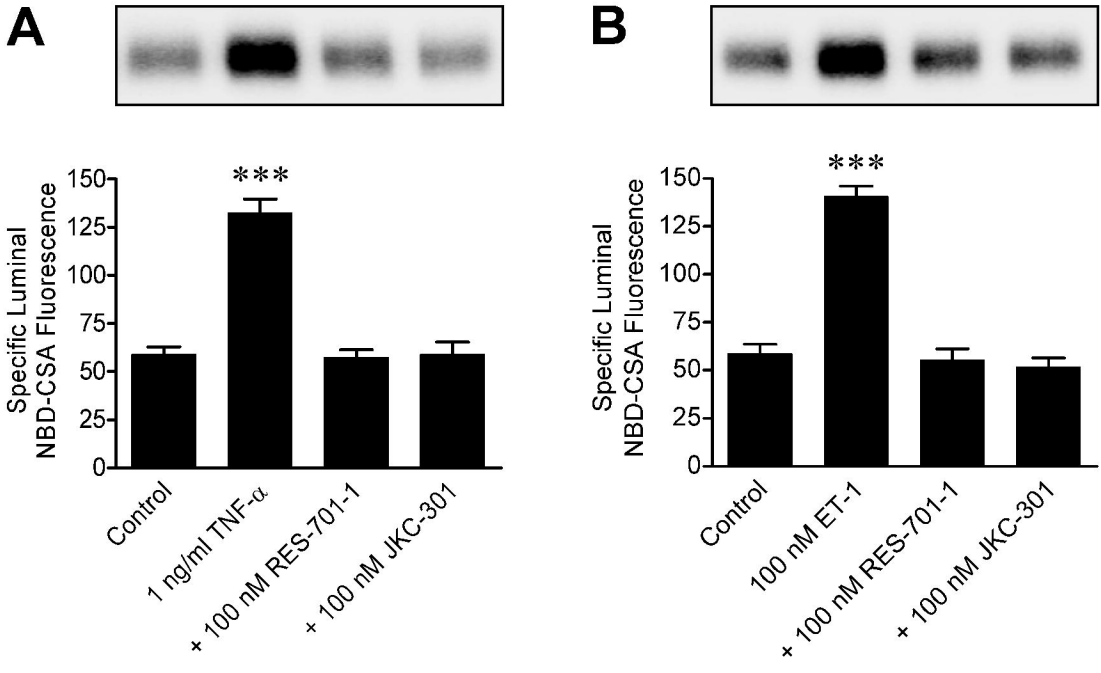

A
B
Specific Luminal NBD-CSA Fluorescence
150
125
100
75
50
25
0
***
Control
1 ng/ml TNF-α
+ 100 nM RES-701-1
+ 100 nM JKC-301
100 nM ET-1

Figure 6

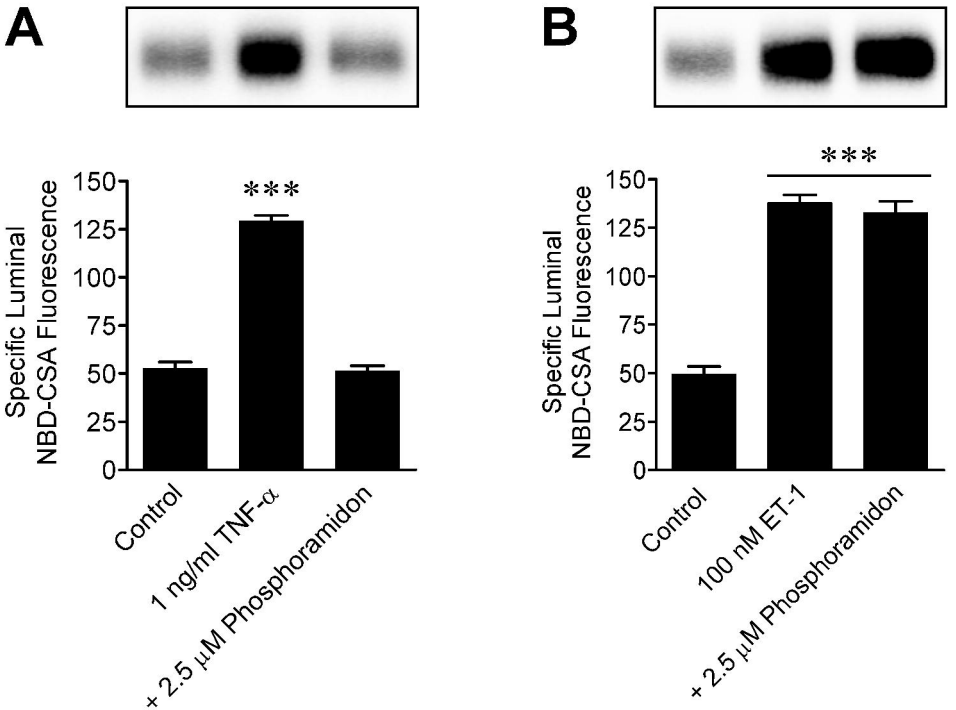

A
B
Specific Luminal NBD-CSA Fluorescence
150
125
100
75
50
25
0
***
Control
1 ng/ml TNF-α
+ 2.5 μM Phosphoramidon
100 nM ET-1

Figure 7

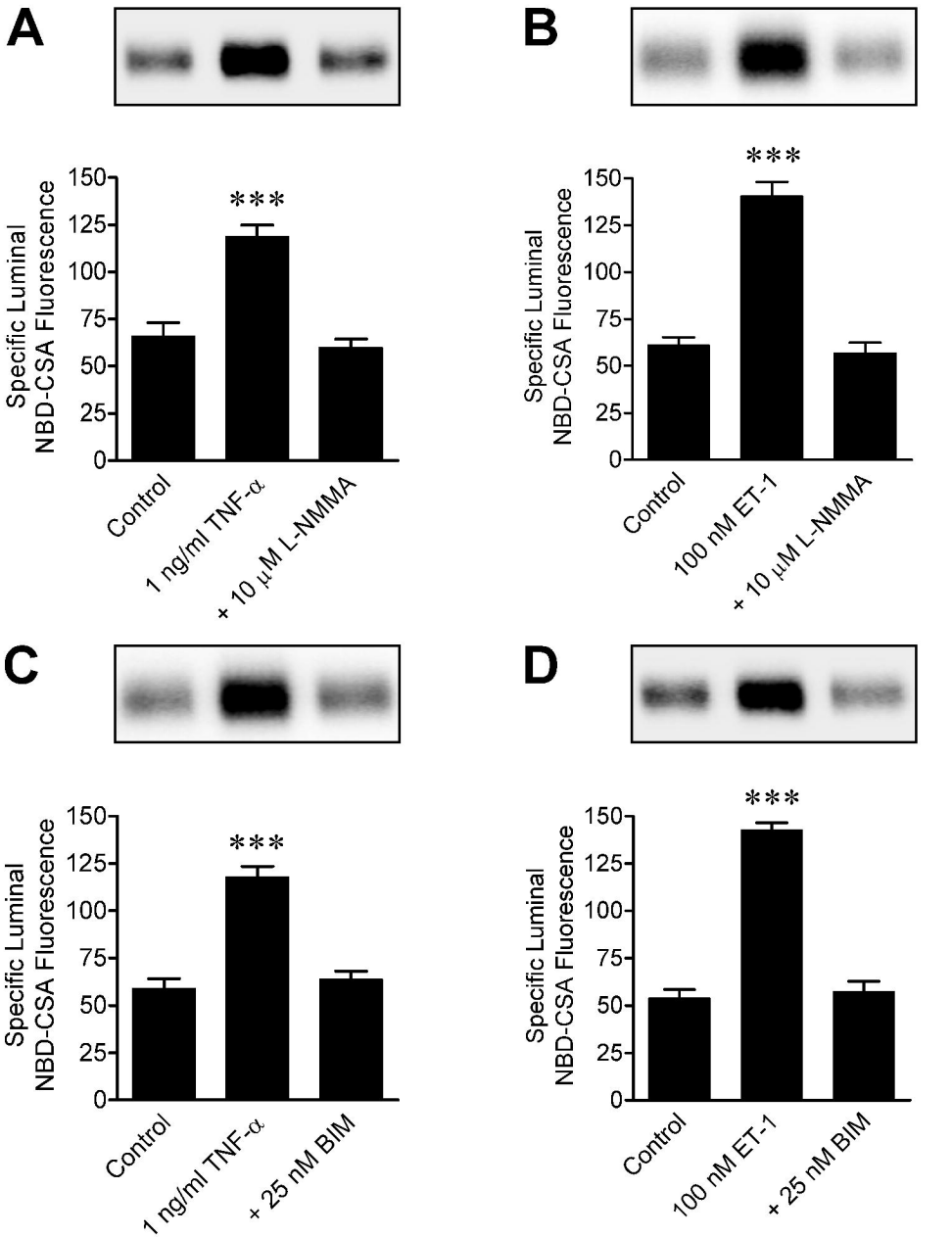

A
B
C
D
Specific Luminal NBD-CSA Fluorescence
150
125
100
75
50
25
0
***
Control
1 ng/ml TNF-α
+ 10 μM L-NMMA
100 nM ET-1
+ 25 nM BIM

Figure 8

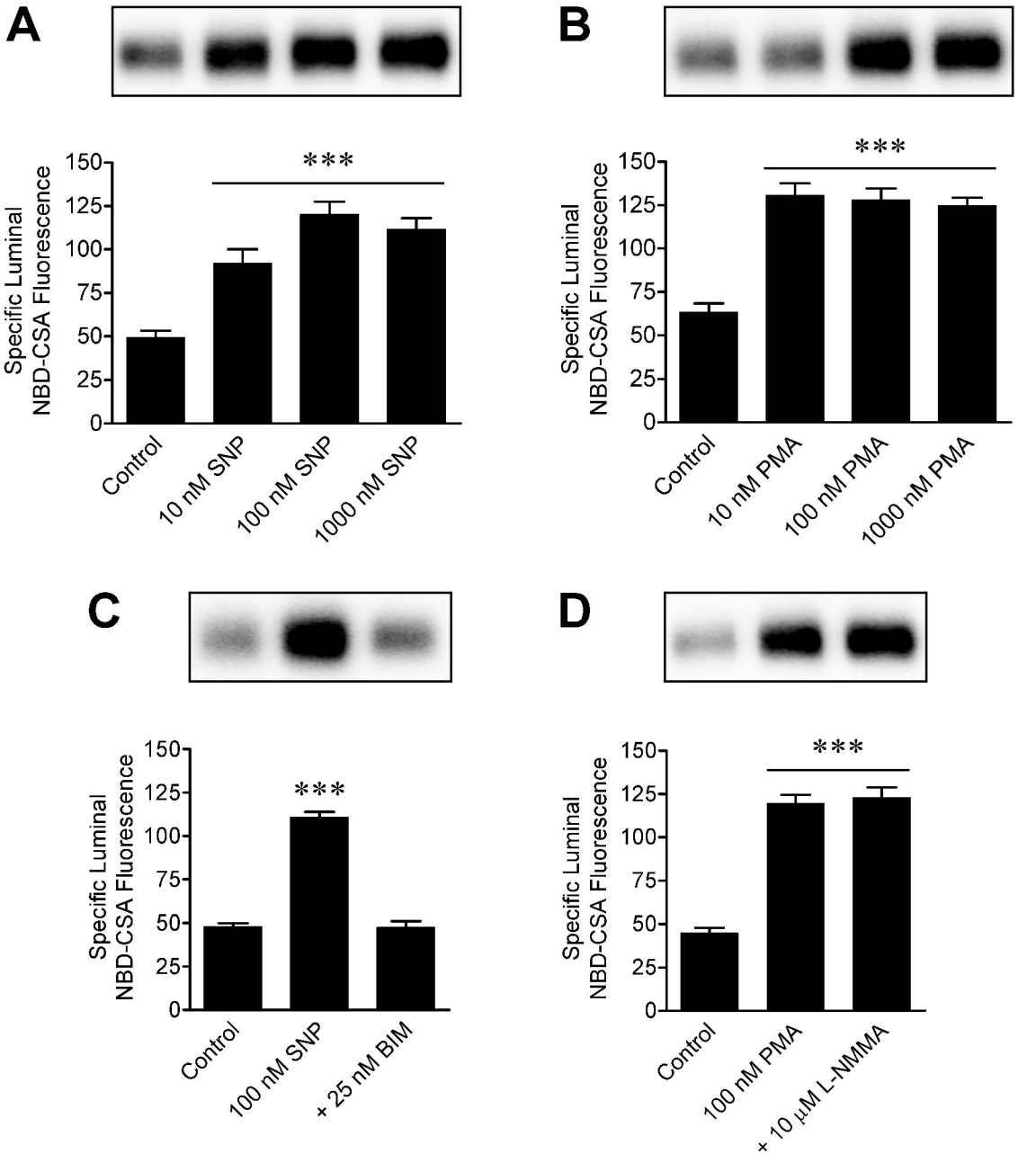

A
B
C
D
Specific Luminal NBD-CSA Fluorescence
150
125
100
75
50
25
0
***
Control
10 nM SNP
100 nM SNP
1000 nM SNP
10 nM PMA
100 nM PMA
1000 nM PMA
+ 25 nM BIM
100 nM PMA
+ 10 μM L-NMMA

Figure 9

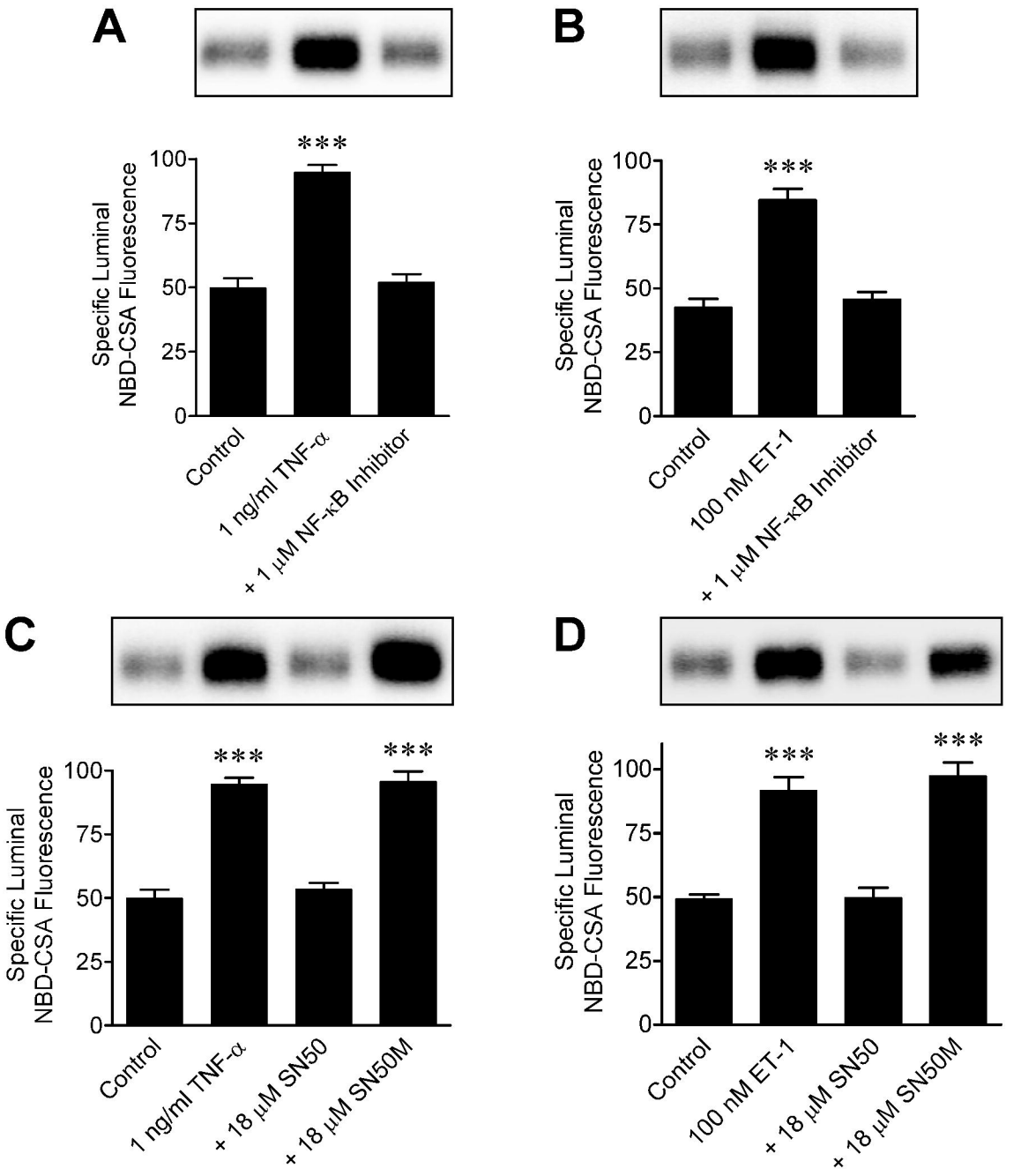

A
B
C
D
Specific Luminal NBD-CSA Fluorescence
100
75
50
25
0
***
Control
1 ng/ml TNF-α
+ 1 μM NF-κB Inhibitor
100 nM ET-1
+ 18 μM SN50
+ 18 μM SN50M

Figure 10

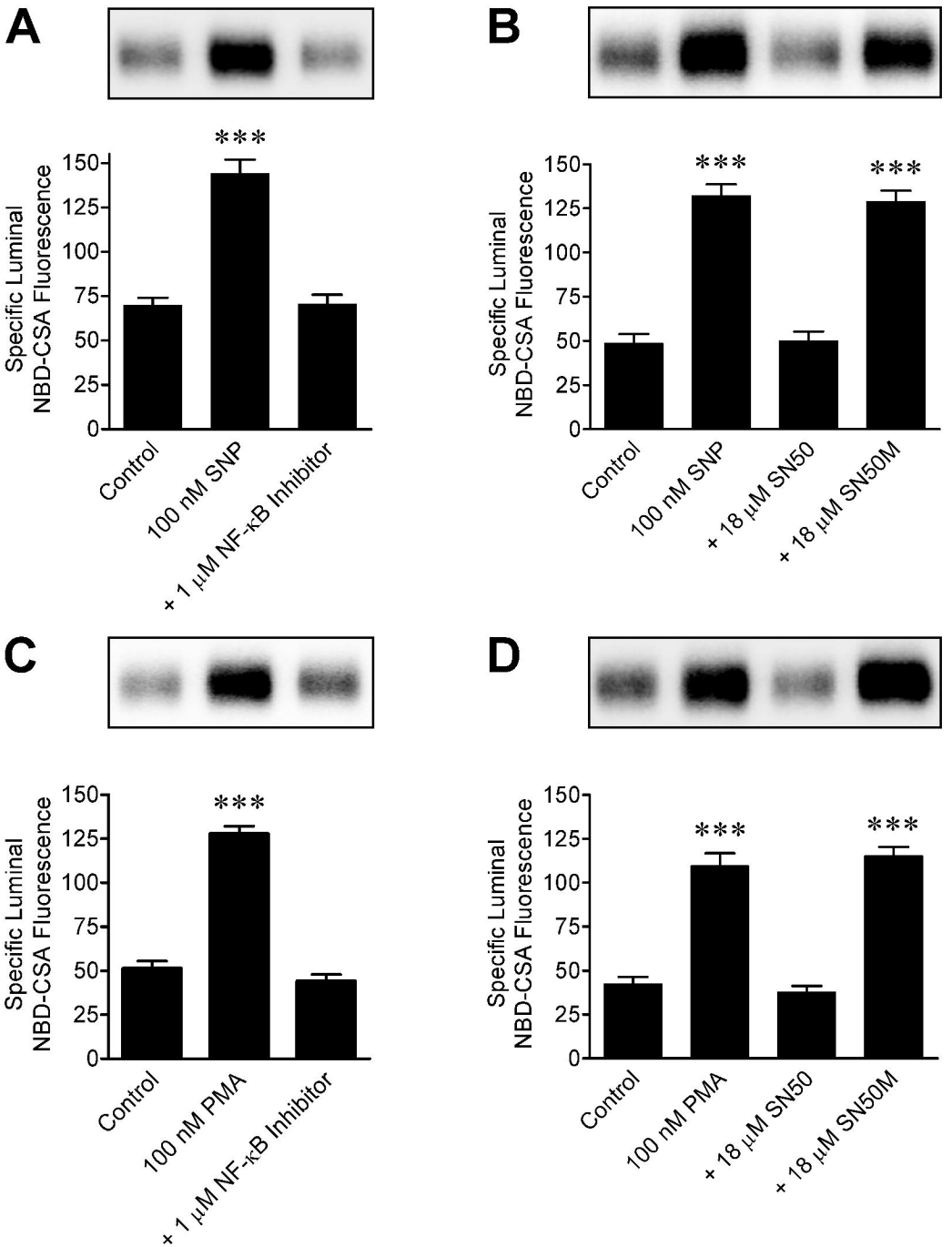

A
B
C
D
Specific Luminal NBD-CSA Fluorescence
150
125
100
75
50
25
0
***
Control
100 nM SNP
+ 1 μM NF-κB Inhibitor
+ 18 μM SN50
+ 18 μM SN50M
100 nM PMA

Figure 11

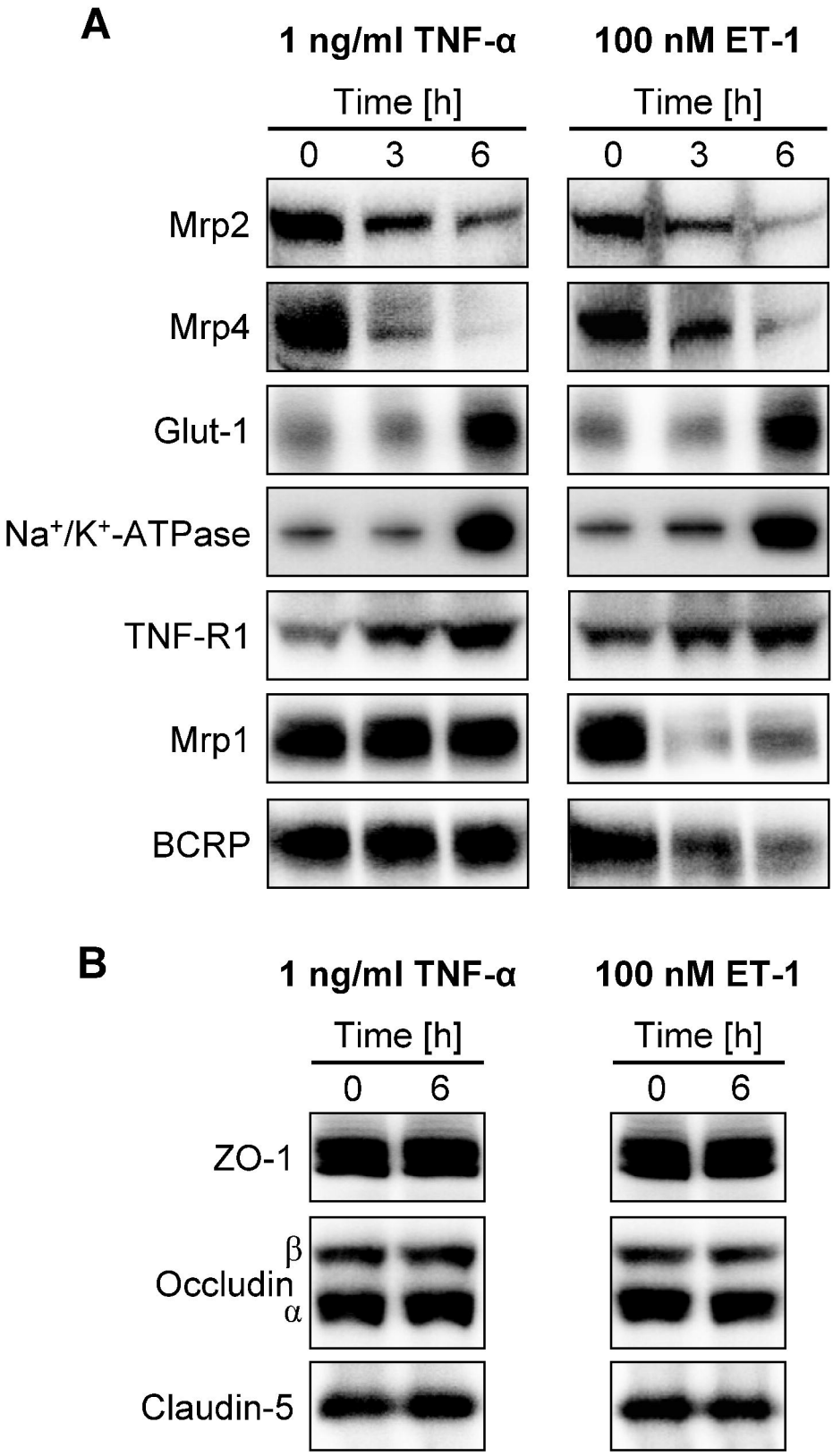
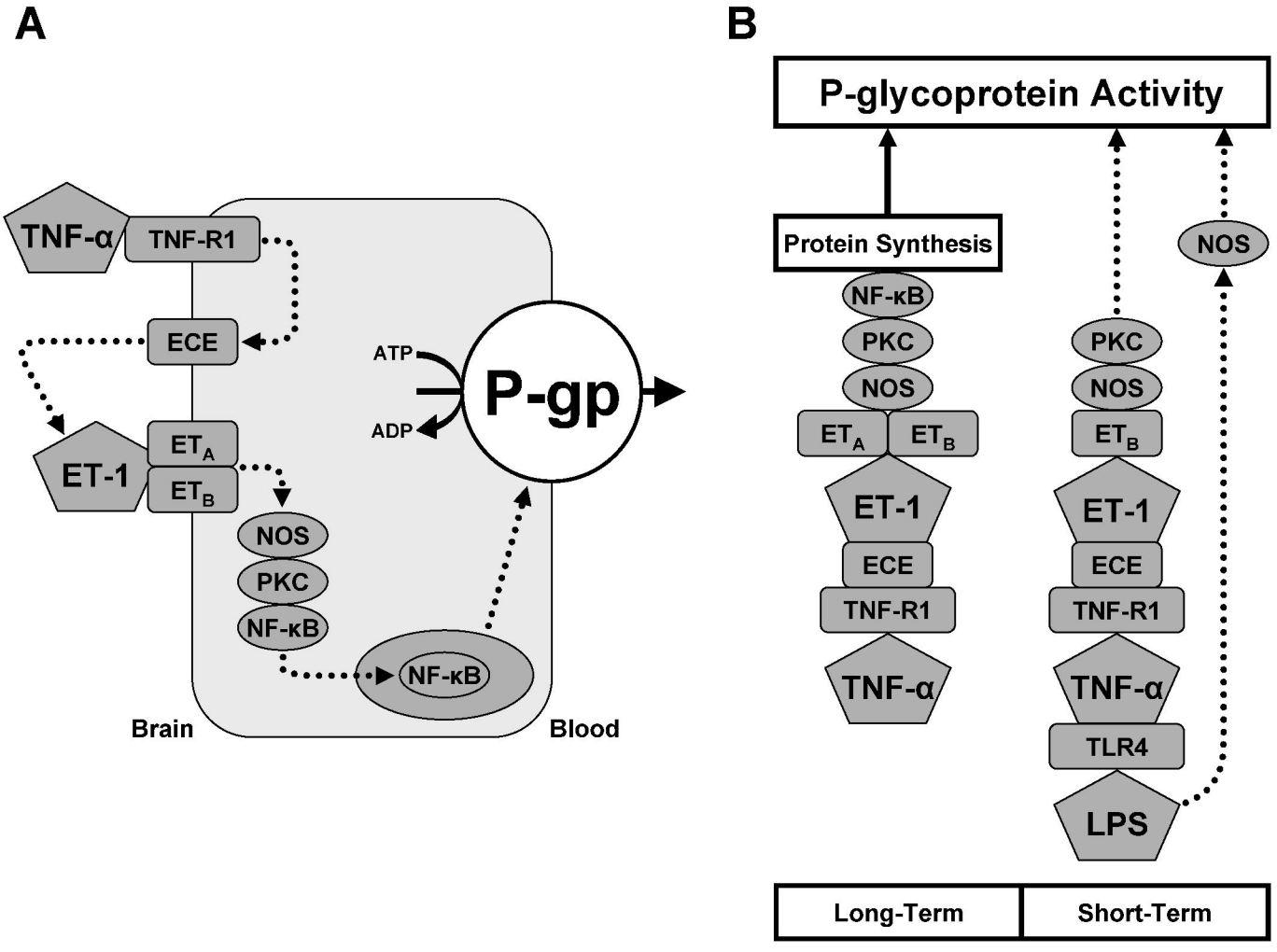

A
1 ng/ml TNF-α
100 nM ET-1
Time [h]
0
3
6
Mrp2
Mrp4
Glut-1
Na+/K+-ATPase
TNF-R1
Mrp1
BCRP
B
1 ng/ml TNF-α
100 nM ET-1
Time [h]
0
6
ZO-1
Occludin
β
α
Claudin-5

Figure 12


A
TNF-α
TNF-R1
ECE
ET-1
ET_A
ET_B
NOS
PKC
NF-κB
ATP
ADP
P-gp
NF-κB
Brain
Blood
B
P-glycoprotein Activity
Protein Synthesis
NF-κB
PKC
NOS
ET_A
ET_B
ET-1
ECE
TNF-R1
TNF-α
PKC
NOS
ET_B
ET-1
ECE
TNF-R1
TNF-α
TLR4
LPS
NOS
Long-Term
Short-Term