Molecular Pharmacology Fast Forward. Published on November 8, 2005 as doi:10.1124/mol.105.017954
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**MOL 17954**

# Rapid Modulation of P-glycoprotein-Mediated Transport at the Blood-Brain Barrier by TNF-α and LPS

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Molecular Pharmacology Fast Forward. Published on November 8, 2005 as DOI: 10.1124/mol.105.017954
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**Running Title: Modulation of P-glycoprotein During Inflammation**

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**29 Text pages**

**0 Tables**

**9 Figures**

**29 References**

**Abstract: 195 words**

**Introduction: 744 words**

**Discussion: 1157 words**

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## ABSTRACT

At the blood-brain barrier, p-glycoprotein, an ATP-driven drug efflux pump, selectively limits drug access to the brain parenchyma, impeding pharmacotherapy of a number of CNS disorders. We previously used confocal imaging to demonstrate in isolated rat brain capillaries that endothelin-1 (ET-1), acting through an $ET_B$ receptor, NO synthase and protein kinase C, rapidly and reversibly reduces p-glycoprotein transport function. Here we define a link between the brain's innate immune response and functional regulation of p-glycoprotein. We show that exposing brain capillaries to the inflammatory cytokine, tumor necrosis factor-α (TNF-α), activated a TNF-R1 receptor, released ET-1, activated $ET_B$ receptor signaling and essentially abolished p-glycoprotein-mediated transport. Bacterial lipopolysaccharide (LPS), a potent activator of the brain's innate immune response, reduced p-glycoprotein activity through TNF-α release, ET-1 release and $ET_B$ receptor signaling. TNF-α and LPS effects had a rapid onset (minutes), were reversible and did not involve changes in tight junctional permeability. These findings define a signaling pathway through which p-glycoprotein activity is acutely modulated. They show that this key component of the selective/active blood-brain barrier is an early target of cytokine signaling during the innate immune response and suggest ways to manipulate the barrier for improved CNS pharmacotherapy.

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One primary function of capillaries within the brain parenchyma is to provide the blood-brain barrier, which restricts and regulates movements of water and solutes into and out of the CNS. This highly effective barrier contributes to CNS homeostasis and protects against neurotoxins, but also greatly limits entry of drugs used to treat CNS disorders (Konsman et al., 2004). Barrier function reflects two properties of the non-fenestrated, brain capillary endothelium: a) extremely low paracellular permeability and low rate of transcytosis (physical barrier), and b) expression of ATP-driven drug efflux pumps (active/selective barrier). These pumps limit uptake of lipophilic xenobiotics that would otherwise cross the physical barrier and penetrate into the brain parenchyma (Begley, 2004; Fricker and Miller, 2004).

Because of its luminal membrane location, high expression level, potency and ability to transport therapeutics, p-glycoprotein, is considered a primary obstacle to drug penetration of the blood-brain barrier. Mice in which the p-glycoprotein gene has been disrupted exhibit substantially increased brain levels of administered chemotherapeutics, HIV protease inhibitors, anticonvulsants, antipsychotics and glucocorticoids, all p-glycoprotein substrates (Goralski et al., 2003; Schinkel et al., 1996). Thus, altering p-glycoprotein activity has the potential to selectively open the barrier to many CNS active drugs. Indeed, inhibiting p-glycoprotein dramatically increases both brain levels of the chemotherapeutic, taxol, and the drug's effectiveness against an intracerebrally implanted human glioblastoma (Fellner et al., 2002).

Certainly, an understanding of how blood-brain barrier p-glycoprotein is modulated would aid in devising strategies to treat CNS disorders. We recently demonstrated rapid and transient downregulation of p-glycoprotein activity in rat brain capillaries signaled by endothelin-1 (ET-1) acting through $ET_B$ receptor, nitric oxide synthase (NOS) and protein kinase C (PKC) (Hartz et al., 2004). The low concentrations of ET-1 used did not affect capillary

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junctional permeability or specific transport mediated by multidrug resistance-associated proteins (Mrps, also drug efflux pumps). Since activation of the brain's innate immune response is known to cause ET release (Nie and Olsson, 1996; Schinelli, 2002), our results suggested that transiently reduced p-glycoprotein function might be an early consequence of mild inflammation (Hartz et al., 2004). The present report is concerned with the relationship between activation of the brain's innate immune response and modulation of brain capillary p-glycoprotein activity.

The innate immune response is triggered by a variety of stimuli, including infection, trauma, disease and cell stress and is characterized by the release of proinflammatory cytokines (Nguyen et al., 2002; Rivest, 2003). Blood vessels within the brain are directly exposed to mediators of inflammation released during peripheral inflammation and during the brain's innate immune response. 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 and Herkenham, 2005)), and are capable of responding to inflammatory stimuli and of amplifying inflammatory signals by producing additional mediators of inflammation (Nguyen et al., 2002; Rivest, 2003). Thus, brain capillaries are both a target for and an active participant in the innate immune response. Indeed, 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. Moreover, recent evidence also shows that inflammation can alter p-glycoprotein expression in several tissues (including brain) and in brain capillary endothelial cells in particular (Goralski et al., 2003; Tan et al., 2002; Theron et al., 2003). These studies have

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focused on changes taking place over periods of hours to days and it is not clear whether p-glycoprotein activity is also altered in the short term.

The present experiments link the brain's innate immune response to rapid changes in blood-brain barrier p-glycoprotein activity. We show that low levels of the cytokine, TNF-α, released ET-1, activated $ET_B$ signaling and rapidly reduced p-glycoprotein-mediated transport in rat brain capillaries. We also demonstrate that the potent inflammogen, LPS, acted through toll-like receptor 4 (TLR4) and TNF-α release, to elicit the same response. As with ET-1 (Hartz et al., 2004), the present experiments show that capillary tight junctional permeability was not affected and that the effects of TNF-α and LPS were rapidly reversed when the compounds were removed. These findings demonstrate for the first time that p-glycoprotein, a critical component of the selective/active blood-brain barrier, is an early target of cytokine signaling during the innate immune response. Moreover, they define an intracellular signaling pathway through which inflammogens and mediators of inflammation acutely modulate p-glycoprotein activity.

## MATERIALS and METHODS

### Chemicals

Endothelin-1, RES-701-1, JKC-301 and anti-TNF-α antibody were purchased from Calbiochem-Novabiochem (LaJolla, CA). Bisindolylmaleimide I (BIM) was from Molecular Probes (Eugene, OR) and phosphoramidon was from A.G. Scientific (San Diego, CA). The TLR4 blocker (receptor antagonist derived from a mutant E. coli strain) was obtained from Sigma. Monoclonal antibodies to ET-1, H398, and HM102 were from Alexis-Axxora (San Diego, CA). The antibody for TNF-R1 immunostaining was from Stressgen (Victoria, BC, Canada), the antibody for TNF-R1 Western blotting was from USBiological (Swampscott, MA). C219 antibody was purchased from Signet (Dedham, MA) and isotype control mouse IgG1 was

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from Abcam (Cambridge, MA). NBD-CSA was custom-synthesized by R. Wenger (Basel, CH) (Schramm et al., 1995). PSC833 (Valspodar) was a kind gift from Novartis (Basel, CH). All other chemicals were obtained from Sigma (St. Louis, MO).

**Isolation of brain capillaries**

Rat brain capillaries were isolated as described previously (Hartz et al., 2004; Miller et al., 2000). For each preparation, 3-10 male Sprague-Dawley rats (retired breeders, 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 5800 g 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). Capillaries were washed three times in BSA-free PBS buffer and immediately used for transport experiments, RNA isolation, immunostaining or plasma membrane isolation.

**Transport**

Freshly isolated capillaries were transferred to glass cover slips, preincubated for 30 min with the effectors and then incubated for 1 hour at room temperature with 2 μM of a fluorescent-labeled cyclosporine A derivative (NBD-CSA (Hartz et al., 2004; Miller et al., 2000)). In some experiments, capillaries were first loaded with NBD-CSA to steady state, and then modulators were added. In other experiments, capillaries were incubated with 2 μM Texas Red

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(sulforhodamine 101 free acid), a fluorescent organic anion and Mrp substrate. The aim of these latter experiments was to monitor tight junctional permeability (for rationale, see Results).

For each treatment, confocal fluorescence images of 7-15 capillaries were acquired (Zeiss 510 meta laser scanning confocal microscope, 40x water immersion objective, NA=1.2) and luminal fluorescence intensity was measured using Zeiss Image Examiner software as described previously (Hartz et al., 2004; Miller et al., 2000). Each experiment was carried out 2-4 times. Results of representative transport experiments are shown; data are presented as mean ± SEM. Differences between means were considered to be statistically significant when $P<0.05$, using an unpaired t-test.

**RT-PCR**

Total capillary RNA was isolated using TRIzol® reagent (Invitrogen, Carlsbad, CA) and purified using the RNeasy Mini kit (Qiagen, Valencia, CA). Reverse transcription was performed using the GeneAmp RNA PCR kit (Applied Biosystems, Foster City, CA). PCR of RT product was done with *Taq* DNA Polymerase from Promega (Madison, WI) using primers for rat TNF-R1 (forward: 5'-CTGCCACGCAGGATTCTTTCTAAGC-3', reverse: 5'-GGATATCGGCA CAGTAGACTGATGC-3'), rat TLR4 (forward: 5'-CATGTCCATCGGTTGATCTTGGGAG-3', reverse: 5'-ACTTGGCAGAGCCAACTGACCAAAG-3') and rat TACE (forward: 5'-GAGCCATCTGAAGAGTTTGTCCGTC-3', reverse: 5'-CCACGAGGTGTTCCGGTATATG-TCA-3'). All primers were custom-synthesized by Operon (Alameda, CA).

**Immunohistochemistry**

Freshly isolated rat brain capillaries adhering to glass cover slips were fixed for 15 minutes with 3% paraformaldehyde/0.2% glutaraldehyde at room temperature. After washing

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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. Then, capillaries were incubated for 1 hour at 37°C with polyclonal primary anti-TNF-R1 rabbit antibody (1:100, 9 µg/ml). After washing (PBS, 1% BSA), capillaries were incubated with anti-rabbit Alexa Fluor® 488-conjugated secondary IgG (1:1,000, 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. TNF-R1 was visualized using a Zeiss 510 meta laser scanning confocal microscope.

**Western blot analysis**

Freshly isolated rat brain 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 in PBS containing protease inhibitor cocktail 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, membranes were incubated overnight with a 1:100 dilution (1 µg/ml) of monoclonal C219 primary antibody (Signet, Dedham, MA). TNF-R1 was detected by incubating membranes with a 1:500 dilution (1 µg/ml) of primary antibody against TNF-R1 (USBiological, Swampscott, MA). Membranes were washed and incubated for 1 h with the corresponding 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).

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Protein bands were visualized and recorded using a BioRad Gel Doc 2000™ gel documentation system (BioRad, Hercules, CA).

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## RESULTS

We have developed a simple, but powerful method to assess p-glycoprotein transport activity in intact brain capillaries. It is based on measuring the accumulation of a fluorescent cyclosporine A derivative (NBD-CSA) in capillary lumens using confocal microscopy and quantitative image analysis (Hartz et al., 2004; Miller et al., 2000). NBD-CSA accumulation in brain capillary lumens is concentrative, specific and reduced by inhibitors of metabolism, p-glycoprotein substrates and by the p-glycoprotein-specific inhibitor, PSC833; cellular accumulation is not affected. We previously showed that NBD-CSA accumulation is not altered by inhibitors of MRPs (Bauer et al., 2004). However, brain capillary endothelial cells express another multispecific, drug efflux pump at the luminal plasma membrane, breast cancer resistance protein (BCRP (Cooray et al., 2002; Eisenblatter et al., 2003)). BCRP and p-glycoprotein have several substrates in common, but PCS833 is at best a low affinity inhibitor of BCRP (Chen et al., 2000) and it does not affect the blood to brain transport of BCRP substrates (Cisternino et al., 2004). In preliminary experiments with rat brain capillaries, the BCRP inhibitor, fumitremorgin C, did not reduce the luminal accumulation of NBD-CSA (not shown).

At steady state, p-glycoprotein-mediated and metabolism-dependent transport accounts for 50-60% of luminal NBD-CSA accumulation; the remainder is not affected by PSC833, CSA or NaCN; it appears to be due to passive diffusion and non-specific binding (Bauer et al., 2004; Hartz et al., 2004; Miller et al., 2000). Importantly, steady state luminal accumulation of NBD-CSA increases when p-glycoprotein expression is upregulated by activation of the nuclear receptor, PXR, and decreases when p-glycoprotein activity is reduced by ET-1 (Bauer et al., 2004; Hartz et al., 2004). Thus, measurement of luminal accumulation of NBD-CSA provides a means to assess p-glycoprotein function in intact brain capillaries.

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Exposing capillaries to TNF-α reduced steady state luminal NBD-CSA accumulation in a concentration-dependent manner (Fig. 1A, 1B and 1C). Significant effects were found with TNF-α concentrations as low as 0.01 ng/ml and the maximal effect was found with 1 ng/ml. The effects of 1-10 ng/ml TNF-α were comparable to those seen with a blocking concentration of the specific p-glycoprotein inhibitor, PSC833 (not shown), suggesting near-complete inhibition of transporter function. As with ET-1 (Hartz et al., 2004), TNF-α effects on transport had a rapid onset and were reversible. Figure 1D shows the results of an experiment in which capillaries were first incubated to steady state in medium with 2 μM NBD-CSA and then exposed to 1 ng/ml TNF-α. Within 30 min, TNF-α significantly reduced luminal NBD-CSA. After removal of TNF-α from the medium, luminal NBD-CSA increased, returning to control levels within about 90 min. In contrast, capillaries that were not exposed to TNF-α (controls) showed no change in luminal fluorescence over the 5 h time course of the experiment.

The decrease in luminal NBD-CSA accumulation caused by TNF-α could have resulted from reduced p-glycoprotein function or from opening of tight junctions followed by leakage of pumped dye out of the lumen. Indeed, we recently showed that mannitol and sucrose (100 mM), used to osmotically open tight junctions at the blood-brain barrier (Kroll and Neuwelt, 1998), rapidly and reversibly reduce steady state, luminal NBD-CSA fluorescence to about 50% of control levels, an effect comparable to that seen with PSC833 and ET-1 (Hartz et al., 2004). Thus, osmotic opening of the tight junctions can elicit the same pattern of effects as seen with TNF-α.

Clearly, in isolated brain capillaries, opening of the tight junctions would reduce accumulation of all substrates pumped into the lumen. We previously used altered luminal uptake and efflux of Texas Red (sulforhodamine 101 free acid), a rhodamine-based, fluorescent

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organic anion, to experimentally distinguish between opening of tight junctions and reduced pumping by p-glycoprotein (Hartz et al., 2004). Texas Red is a substrate for Mrp2 (and possibly other MRPs), another xenobiotic efflux pump located in the luminal membrane of the rat brain capillary endothelium (Miller et al., 2000). Consistent with this, steady state, concentrative, luminal accumulation of Texas Red in rat brain capillaries is reduced by the Mrp inhibitor $LTC_4$, but is not affected by the p-glycoprotein inhibitor, PSC833 (Hartz et al., 2004; Miller et al., 2000). Although osmotic opening of capillary tight junctions with mannitol and sucrose reduces steady state luminal accumulation of Texas Red and accelerates efflux of Texas Red from the lumens of preloaded capillaries (Hartz et al., 2004), ET-1 at 100 nM alters neither Texas Red uptake nor efflux (Hartz et al., 2004). Figures 2A and 2B show that steady state Texas Red accumulation in capillary lumens was reduced to the same extent by 0.3 μM $LTC_4$ (inhibits Mrps) and by 100 mM mannitol (opens tight junctions), but it was not affected by 1-10 ng/ml TNF-α. This range of TNF-α concentrations caused maximal reduction in NBD-CSA accumulation in capillary lumens (Fig. 1C). The absence of effect of TNF-α on concentrative Texas Red accumulation (Fig. 2B) indicates that TNF-α, like ET-1 (Hartz et al., 2004), neither affected transport of the organic anion into the capillary lumen nor increased tight junctional permeability.

TNF-α signals through type 1 (TNF-R1, p55) and type 2 (TNF-R2, p75) receptors and both are expressed in the brain vasculature (Nadeau and Rivest, 1999). RT-PCR detected a signal for TNF-R1 mRNA in our rat brain capillary extracts (Fig. 3A). Western blots of capillaries and capillary membranes showed two bands, one at the correct molecular weight for TNF-R1 and the second at the correct molecular weight for the TNF-R1 precursor protein (Fig. 3B). Immunostaining capillaries with the antibody to TNF-R1 showed immunoreactivity on both the

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luminal and abluminal surfaces (Fig. 3C). Thus, the capillaries were likely sensitive to TNF-α exposure from both the brain and blood sides. Consistent with TNF-α acting through a TNF-R1 receptor, H398, a monclonal antibody that acts as a specific TNF-R1 antagonist, blocked the effects of TNF-α on NBD-CSA accumulation (Fig. 3D). In contrast, HM102, a specific TNF-R2 agonist, was without effect (not shown).

To determine whether TNF-α activated $ET_B$ receptor signaling, we exposed capillaries to TNF-α without (control) and with JKC-301 ($ET_A$ receptor antagonist), RES-701-1 ($ET_B$ receptor antagonist), L-NMMA (NOS inhibitor) or BIM (PKC inhibitor) and measured steady state luminal NBD-CSA accumulation. In agreement with previous experiments (Hartz et al., 2004), at the concentrations used here, none of these drugs by themselves affected NBD-CSA transport (not shown). However, the $ET_B$ receptor antagonist and the NOS and PKC inhibitors blocked the effects of TNF-α; the $ET_A$ receptor antagonist was without effect (Fig. 4A).

Having the $ET_B$ receptor antagonist block the effects of TNF-α strongly suggests that TNF-α caused release of ET from the capillaries. Two experiments were carried out to verify this supposition. First, we exposed capillaries to TNF-α in the absence and presence of an antibody to ET-1 and measured steady state luminal NBD-CSA accumulation. In preliminary experiments, we determined that the concentration of antibody used was sufficient to block the effects of 1 nM ET-1 on NBD-CSA transport (not shown) and that immunoglobulins that were not specific for ET-1 did not alter the response to TNF-α. As shown in Fig. 4B, the anti-ET-1 antibody by itself did not affect NBD-CSA transport. In contrast, pretreating capillaries with the antibody abolished the effects of 1 ng/ml TNF-α. Second, ET-1 is released from cells as a pro-hormone that is rapidly converted to the active hormone by an ET converting enzyme (ECE, (Schiffrin and Touyz, 1998)). We pretreated capillaries with phosphoramidon, a specific

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inhibitor of ECE and found that the effects of 1 ng/ml TNF-α on luminal NBD-CSA accumulation were abolished (Fig. 4C). Thus, TNF-α stimulated ET-1 release from the capillaries and ET-1 then reduced p-glycoprotein-mediated transport by signaling through the $ET_B$ receptor, NOS and PKC.

The reaction to LPS, a cell wall component of gram negative bacteria, is probably the best characterized example of innate recognition that leads to a comprehensive inflammatory response and one consequence of the brain's innate immune response to LPS is release of TNF-α (Hawiger, 2001). RT-PCR revealed a signal for TLR4 mRNA in isolated rat brain capillaries (Fig. 5A). Exposing isolated brain capillaries to LPS caused a concentration-dependent decrease in steady state luminal accumulation of NBD-CSA (Fig. 5B). LPS concentrations as low as 0.01 ng/ml significantly reduced accumulation and maximal effects were found with 1 ng/ml. The effects of 1 ng/ml LPS were comparable to those seen with a blocking concentration of the specific p-glycoprotein inhibitor PSC833 (not shown). As with TNF-α, 10-100 ng/ml LPS had no effect on Texas Red uptake by the capillaries (Fig. 2B), indicating that in this concentration range LPS did not reduce luminal NBD-CSA accumulation through increased tight junctional permeability. LPS did appear to reduce NBD-CSA transport by acting through a TLR4 receptor. Exposing capillaries to a TLR4 receptor antagonist abolished the LPS effect (Fig. 5C).

We next determined whether LPS signaled the change in p-glycoprotein-mediated transport by acting through TNF-α and ET-1. Blocking TNF-R1 with H398 (Fig. 6A) or pretreating capillaries with the antibody to TNF-α (Fig. 6B) significantly attenuated the effects of 1 ng/ml LPS. TNF-α, like ET, is released from cells in an inactive pro-form and converted extracellularly to the active peptide. RT-PCR showed that isolated capillaries expressed mRNA for TNF-α converting enzyme (TACE, not shown). Blocking TACE with TIMP3 significantly

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reduced the effects of LPS on luminal NBD-CSA accumulation (Fig. 6C). Consistent with LPS acting through ET-1 signaling, blocking the $ET_B$ receptor, NOS or PKC substantially reduced the effects of LPS (Fig. 7A). As with TNF-α, LPS effects on transport were also significantly reduced when ECE was inhibited with phosphoramidon (Fig. 7B).

Interestingly, when capillaries were exposed to antibodies to TNF-α or ET-1 or to inhibitors of TNF-R1, TACE, the $ET_B$ receptor, ECE or PKC, a small but significant effect of LPS on luminal NBD-CSA accumulation was still evident (Figs. 6 and 7). In contrast, transport was completely restored when NOS was inhibited (Fig. 7A). Thus, the TLR4 receptor appeared to activate two signaling pathways: a major one that was shared with TNF-α and ET-1 and a minor one that involved NOS activation, but not the other elements of TNF-α and ET-1 signaling.

To determine whether changes in transporter expression contributed to the decrease in p-glycoprotein-mediated transport found in brain capillaries exposed to LPS, TNF-α and ET-1, we measured immunoreactive protein in capillary membranes using Western blots. Figure 8A shows no change in p-glycoprotein signal from control levels in membranes from capillaries exposed for 1 h to LPS, TNF-α or ET-1. Finally, inhibition of protein synthesis by cycloheximide did not attenuate the effects of 1 ng/ml LPS or 1 ng/ml TNF-α (Fig. 8B). Thus, it appears that the decrease in p-glycoprotein transport in response to LPS, TNF-α and ET-1 signaling neither changed plasma membrane transporter level nor required synthesis of new protein.

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## DISCUSSION

We previously demonstrated that ET-1, signaling through an $ET_B$ receptor, NOS and PKC, rapidly and reversibly reduces p-glycoprotein-mediated transport in isolated rat brain capillaries (Hartz et al., 2004). The present results link the brain's innate immune response to ET-1 signaling and to its effect on p-glycoprotein at the blood-brain barrier. They define for the first time 1) an early response of the selective blood-brain barrier to bacterial infection and to a primary inflammatory cytokine, and 2) a signaling system that rapidly modulates p-glycoprotein activity in brain capillary endothelial cells.

We found rapid (15-30 min) and reversible loss of p-glycoprotein transport function in brain capillaries exposed to low concentrations of the proinflammatory cytokine, TNF-α. As with ET-1, the effect of TNF-α was specific in that neither organic anion transport on Mrps (another family of drug efflux pumps expressed on the luminal plasma membrane of the endothelial cells) nor tight junctional permeability were altered. TNF-α acted through a TNF-R1 receptor. TNF-α signaling to p-glycoprotein was blocked when capillaries were exposed to an antagonist of the $ET_B$ receptor, to an antibody to ET-1 itself or to a drug that inhibited the ECE. Thus, TNF-α stimulated ET-1 release from the capillaries and released hormone signaled through the $ET_B$ receptor, NOS and PKC to reduce p-glycoprotein activity. Importantly, signaling to p-glycoprotein also could be initiated by low levels of LPS, a potent trigger for the innate immune response. LPS acted through two signaling pathways. With 1 ng/ml LPS, most of the signaling occurred via the TLR4 receptor, TNF-α release, TNF-R1, ET-1 release, the $ET_B$ receptor, NOS and PKC. However, about 20% of the total response bypassed TLR4 and activated NOS, but not PKC. This was a surprise, since we previously found that the NO generator, sodium nitroprusside (SNP), reduced p-glycoprotein-mediated transport, but that SNP

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effects were blocked when PKC was inhibited (Hartz et al., 2004). All of these events occurred when protein synthesis was inhibited by cycloheximide. Thus, all elements of the signaling pathway were constitutively expressed in freshly isolated brain capillaries. Figure 9A shows the full sequence of events.

Several aspects of LPS/ET–1/TNF-α signaling require further comment. First, visual inspection of our isolated capillaries show little contamination by other structures or cells. We certainly recognize that contaminating microglia could contribute to signaling, but have drawn a detailed signaling mechanism shown in Fig. 9B as capillary-based. In this, we propose that signaling originated from the abluminal (brain) surface of the capillaries. In our experiments, capillaries were exposed to LPS, ET –1 and TNF-α added to the bath. Since the tight junctional barrier would limit access of these compounds to the lumen, certainly over the short-term, signaling most likely originates from receptors located at the abluminal surface of the vessels. Similarly, it is unlikely that the antibodies used to block TLR4 during LPS exposure and to bind released ET-1 during LPS and TNF-α exposure could access capillary lumens. Thus, TNF-α and ET-1 released from the capillaries must have acted at the abluminal surface. Note that release of ET-1 or TNF-α from the abluminal side of the endothelium implies local (autocrine or paracrine) action, since release is from a small mass of capillaries into an essentially infinite bath. Note also that immunostaining showed that the $ET_B$ receptor and TNF-R1 were present on both sides of the capillary endothelium ((Hartz et al., 2004) and present study), so it is possible that signaling could also be initiated from the luminal surface in response to vascular ET-1, LPS or TNF-α. If signaling from the luminal membrane is wired similarly to that from the abluminal membrane, systemic inflammation in its early stages would be expected to affect blood-brain barrier p-glycoprotein activity in a manner similar to CNS inflammation.

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Second, several aspects of $ET_B$ receptor signaling remain uncertain. We have yet to identify the NOS and PKC isoforms involved and the mechanism by which signaling modifies p-glycoprotein function. With regard to the latter, our experiments show rapid onset and reversal of LPS, ET -1 and TNF-α effects ((Hartz et al., 2004) and present study). Moreover, these experiments were carried out at room temperature, so we would expect to see even faster responses at 37º C. The rapidity of response and the lack of change in transporter expression levels suggest that transporter function may be modified in situ. Thus, PKC may alter p-glycoprotein transport activity by direct phosphorylation of the transporter or through phosphorylation of an accessory protein that itself modulates p-glycoprotein function in the membrane. Alternatively, PKC may alter trafficking of the protein between the luminal plasma membrane and intracellular compartments, i.e., by increasing retrieval of transporter from the membrane to a vesicular compartment. Regulated insertion/retrieval has been proposed as a means of rapidly modulating efflux pump activity in liver (Kipp and Arias, 2002) and renal proximal tubule (Miller, 2002; Terlouw et al., 2003). As in brain capillaries, protein kinase-based signaling (PKA and PKC) has been implicated in kidney and liver. Experiments are currently underway to distinguish among the possibilities.

Third, the brain's innate immune response is triggered by a variety of pathogen-associated molecular patterns (PAMPs) acting through TLRs, e.g., LPS through TLR4, double stranded (viral) RNA through TLR3, gram-positive cell wall constituents through TLR2, and by a number of proinflammatory cytokines acting through their own receptors, e.g., IL-1 and Il-6 (Nguyen et al., 2002; Rivest, 2003). Release of TNF-α is a common aspect of PAMP and cytokine signaling. It is not yet clear whether PAMPs other than LPS or cytokines other then

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TNF-α directly or indirectly (through TNF-α) activate $ET_B$ receptor signaling and thus also rapidly alter p-glycoprotein activity.

Finally, the present results demonstrate how the activity of one of the blood-brain barrier's drug efflux pumps, p-glycoprotein, can be rapidly and specifically modulated. Because of the important role that p-glycoprotein plays in determining the entry of therapeutic drugs into the CNS, reduced activity could have profound consequences in those situations where p-glycoprotein protects against neurotoxicity, e.g., ivermectin treatment for river blindness (Schinkel et al., 1996) or impedes CNS pharmacotherapy, e.g., chemotherapy for glioblastoma (Fellner et al., 2002). At present, it is not clear what physiological/pathophysiological role reduced blood-brain barrier p-glycoprotein activity would play in the brain's innate immune response. With regard to pharmacotherapy, we previously argued that ET-1 signaling, which caused rapid, but fully reversible loss of transport activity and no change in tight junctional permeability, provided just the sequence of events needed to overcome p-glycoprotein-based CNS drug resistance (Hartz et al., 2004). However, we also pointed out that the use of ETs or their agonists for this purpose would be impractical. It now appears that the same rapid, reversible reduction in brain capillary p-glycoprotein activity can be achieved through the brain's innate immune response. Current experiments are focused on defining the conditions under which a similar reduction can be induced in animal models in vivo. It remains to be seen to what extent this response can be manipulated to be of practical use in the clinic.

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## FOOTNOTES

This research was supported in part by the Intramural Research Program of the NIH, National Institute of Environmental Health Sciences and by grant DFG FR1211/8-1 (to GF) from the German Research Foundation.

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## FIGURE LEGENDS

**Figure 1.** TNF-α reduces p-glycoprotein-mediated transport in isolated rat brain capillaries. (A) Representative confocal image showing steady state (60 min) NBD-CSA (2 μM) accumulation in a rat brain capillary. (B) Corresponding image for capillary exposed to 1 ng/ml TNF-α. (C) Concentration-dependent decrease of steady state luminal NBD-CSA fluorescence in capillaries exposed to TNF-α. (D) Time course of TNF-α effects on steady state luminal NBD-CSA accumulation. Capillaries were first loaded to steady state (60 min) in medium with 2 μM NBD-CSA. Then (time zero on graph), 1 ng/ml TNF-α was added to the medium; 150 min later, TNF-α was removed. In both controls and TNF-α- treated capillaries, NBD-CSA was present in the medium throughout the experiment. From 30-210 min, mean luminal fluorescence in TNF-α-treated capillaries was significantly lower than time-paired controls ($P<0.001$). Each point represents the mean value for 10-15 capillaries from a single preparation; variability is given by SEM bars. Units are arbitrary fluorescence units (scale 0-255). Statistical comparisons: ** significantly lower than controls, $P<0.01$; *** significantly lower than controls, $P<0.001$.

**Figure 2.** TNF-α and LPS do not alter tight junctional permeability in isolated rat brain capillaries (A) Effects of 0.3 μM $LTC_4$ (blocks Mrp-mediated transport) and 100 mM mannitol (opens tight junctions) on steady state (60 min) luminal accumulation of 2 μM Texas Red. Each point represents the mean value for 10-15 capillaries from a single preparation; variability is given by SEM bars. Units are arbitrary fluorescence units (scale 0-255). (B) Lack of effect of TNF-α and LPS on steady state (60 min) luminal accumulation of 2 μM Texas Red. Pooled data

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from 9 experiments (3-10 rats per preparation). Each point represents the mean value in ± SEM for 40-90 capillaries. Statistical comparisons: *** significantly lower than controls, $P<0.001$.

**Figure 3.** TNF-α signals through a TFN-R1 receptor to reduce p-glycoprotein activity. (A) RT-PCR showing presence of mRNA for TNF-R1 in various rat tissues, including brain capillaries. (B) Westen blot demonstrating signal for TNF-R1 and its precursor. (C) TNF-R1 immunostaining showing immunoreactive product (green) on both the luminal and abluminal plasma membranes of a rat brain capillary. Capillaries not exposed to primary antibody showed no staining. The nucleus is stained with propidium iodide (red). The scale bar indicates 5 μm. (D) H398 (10 μg/ml), a TNF-R1 antagonist, abolishes TNF-α effects. Each point represents the mean value for 10-15 capillaries from a single preparation; variability is given by SEM bars. Units are arbitrary fluorescence units (scale 0-255). Statistical comparisons: *** significantly lower than controls, $P<0.001$.

**Figure 4.** TNF-α signals through the $ET_B$ receptor, NOS and PKC to reduce p-glycoprotein activity. (A) RES-701-1 (10 nM), an $ET_B$ receptor antagonist, L-NMMA (10 μM), an NOS inhibitor, and BIM (10 nM), a PKC inhibitor, block TNF-α signaling; JKC-301 (10 nM), an $ET_A$ receptor antagonist, has no effect. (B) An anti-ET-1 antibody (0.25 μg/ml) abolishes TNF-α effects. (C) Phosphoramidon (PA, 2.5 μM), an inhibitor of ECE, abolishes TNF-α effects. Each point represents the mean value for 10-15 capillaries from a single preparation; variability is given by SEM bars. Units are arbitrary fluorescence units (scale 0-255). Statistical comparisons: *** significantly lower than controls, $P<0.001$.

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**Figure 5.** LPS activates TNF-α signaling to p-glycoprotein. (A) RT-PCR showing presence of mRNA for TLR4 in various rat tissues, including brain capillaries. (B) Concentration-dependent decrease of luminal NBD-CSA fluorescence with LPS. (C) Blocking TLR4 (receptor antagonist at 1 μg/ml) abolishes the LPS effect. Each point represents the mean value for 10-15 capillaries from a single preparation; variability is given by SEM bars. Units are arbitrary fluorescence units (scale 0-255). Statistical comparisons: *** significantly lower than controls, $P<0.001$.

**Figure 6.** LPS signals through the TNF-α to reduce p-glycoprotein activity. (A) Blocking TNF-R1 with H398 (10 μg/ml) attenuates LPS signaling. (B) Anti-TNF-α antibody reduces LPS effects. (C) Blocking TACE with TIMP3 (10 nM) reduces the effects of LPS on luminal NBD-CSA accumulation. Each point represents the mean value for 10-15 capillaries from a single preparation; variability is given by SEM bars. Units are arbitrary fluorescence units (scale 0-255). Statistical comparisons: * significantly lower than controls, $P<0.05$; *** significantly lower than controls, $P<0.001$.

**Figure 7.** LPS signals through the $ET_B$ receptor, NOS and PKC. (A) Blocking $ET_B$ (10 nm RES-701-1) or PKC (10 nM BIM) significantly reduced the effects of LPS; blocking NOS (10 μM L-NMMA) abolished LPS effects. (B) Blocking ECE with phosphoramidon (PA, 2.5 μM) reduced LPS effects, indicating release of ET-1. Each point represents the mean value for 10-15 capillaries from a single preparation; variability is given by SEM bars. Units are arbitrary

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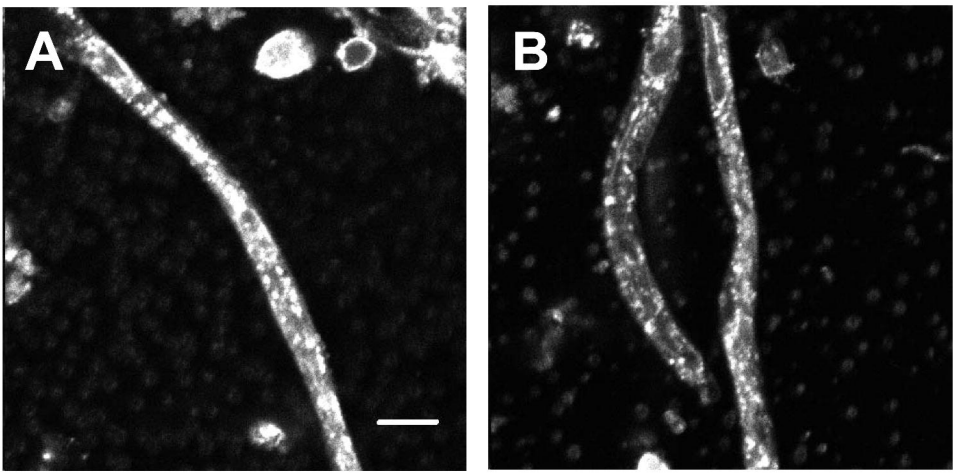
fluorescence units (scale 0-255). Statistical comparisons: * significantly lower than controls, $P<0.05$; *** significantly lower than controls, $P<0.001$.

**Figure 8.** (A) Western blot showing that neither 1 ng/ml TNF-α, 100 nM ET-1 nor 1 ng/ml LPS (1 hour exposure) alters p-glycoprotein expression level in capillary plasma membranes. The negative control (-) is whole brain homogenate; the positive control (+) is rat renal brush border membrane. (B) Inhibition of protein synthesis by 100 μg/ml cycloheximide does not attenuate the effects of LPS or TNF-α on p-glycoprotein-mediated transport. Each point represents the mean value for 10-15 capillaries from a single preparation; variability is given by SEM bars. Units are arbitrary fluorescence units (scale 0-255). Statistical comparisons: *** significantly lower than controls, $P<0.001$.

**Figure 9.** Relationships between LPS, TNF-α and ET-1 signaling and reduced p-glycoprotein activity. (A) Signaling sequence as demonstrated in (Hartz et al., 2004) and present study. (B) Proposed capillary-based sequence. The primary LPS signaling pathway is through TLR4. It involves release of TNF-α, which acts through TNF-R1 to release ET-1. ET-1 activates in turn an $ET_B$ receptor, NOS and PKC, causing rapid but reversible loss of p-glycoprotein transport function. LPS also signals to p-glycoprotein through NOS by a pathway (broken line) that does not involve TLR4, TNF-R1, the $ET_B$ receptor or PKC.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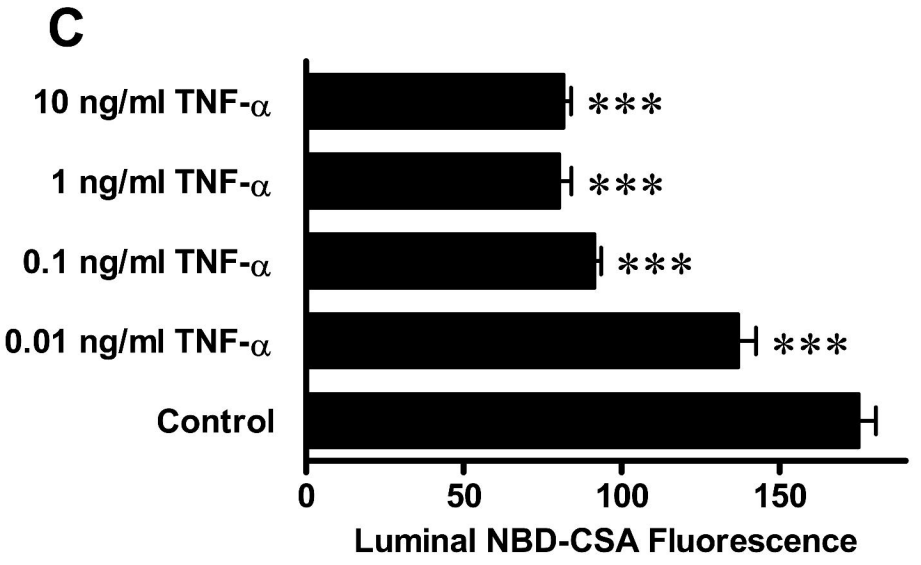
B


C
10 ng/ml TNF-α
1 ng/ml TNF-α
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Control
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***
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***
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50
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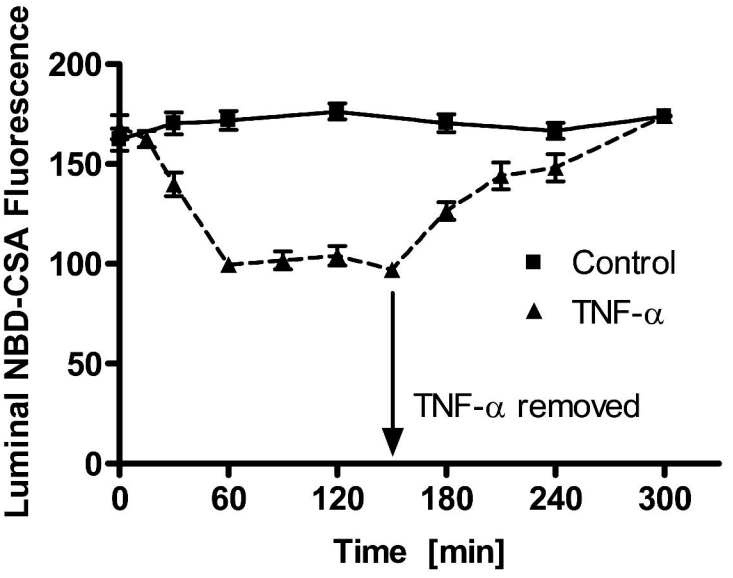
Luminal NBD-CSA Fluorescence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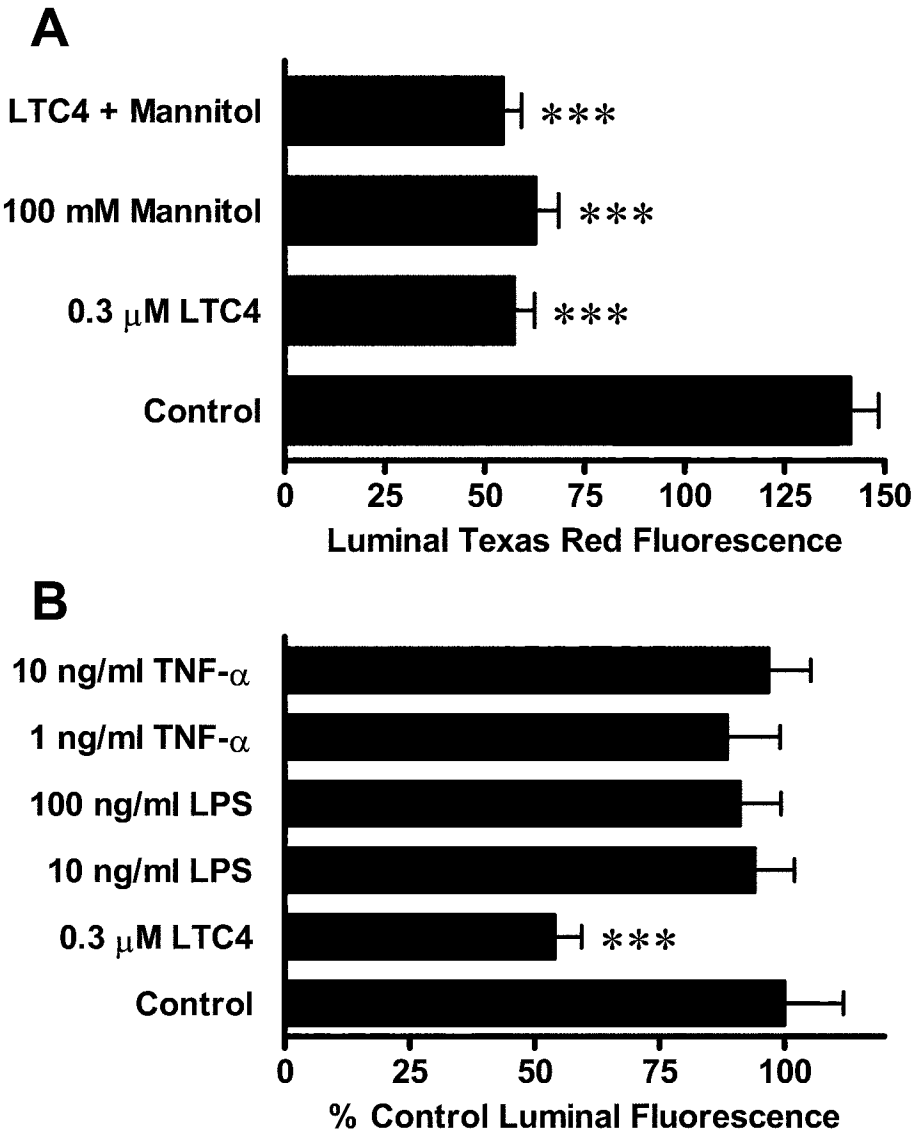

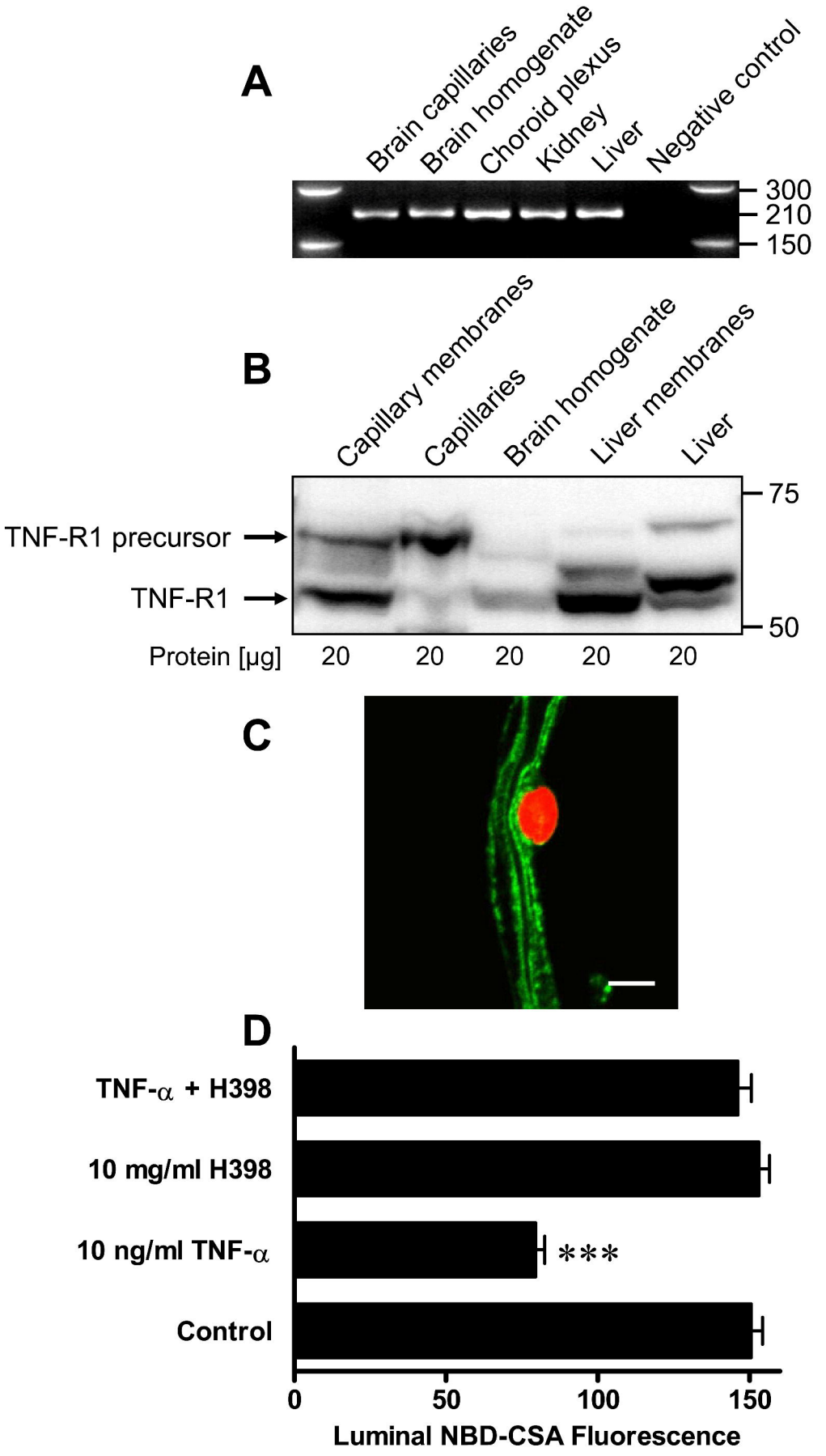
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Brain homogenate
Choroid plexus
Kidney
Liver
Negative control
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210
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Capillary membranes
Capillaries
Brain homogenate
Liver membranes
Liver
75
50
TNF-R1 precursor
TNF-R1
Protein [µg]
20
20
20
20
20
C
D
TNF-α + H398
10 mg/ml H398
10 ng/ml TNF-α
***
Control
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Luminal NBD-CSA Fluorescence

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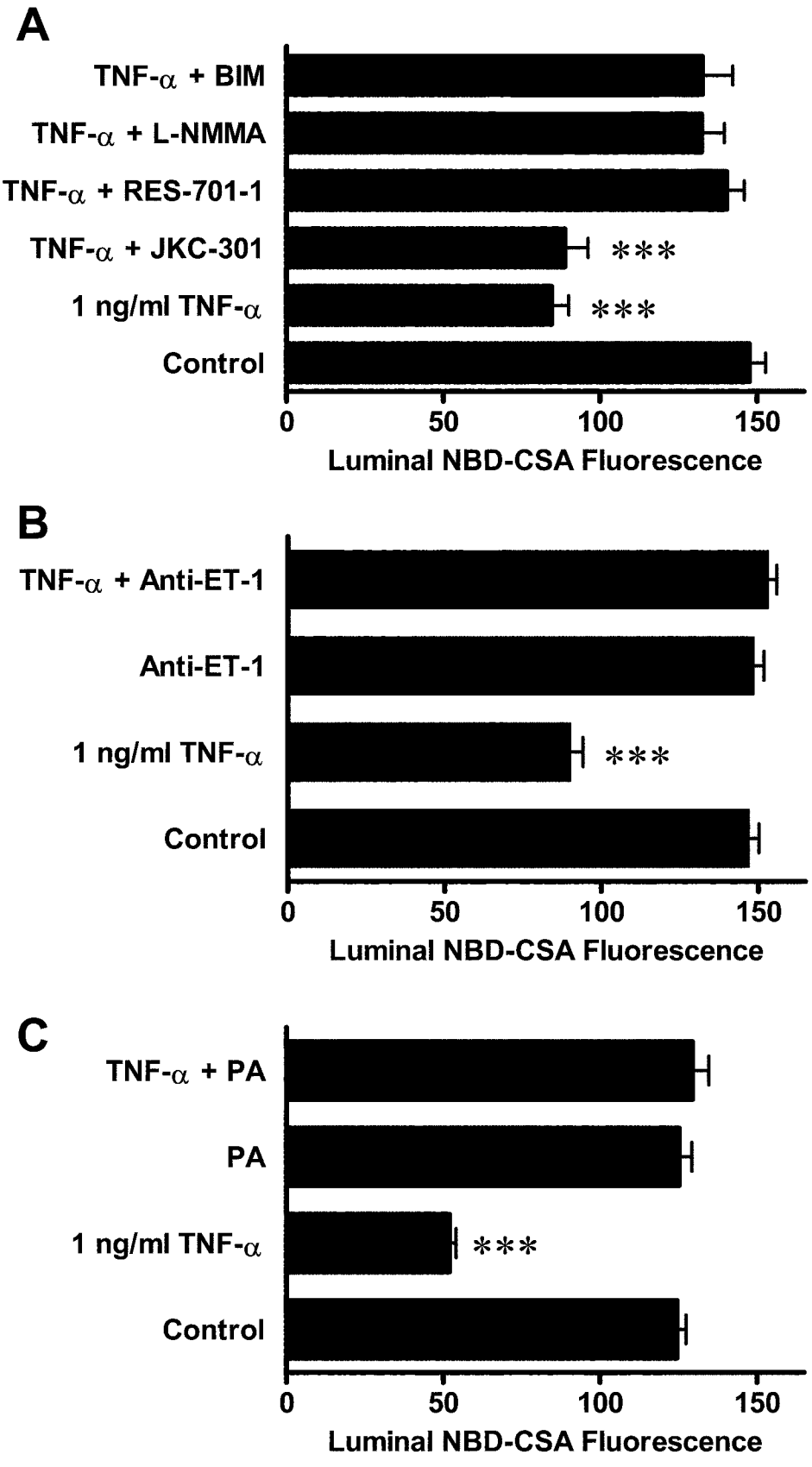

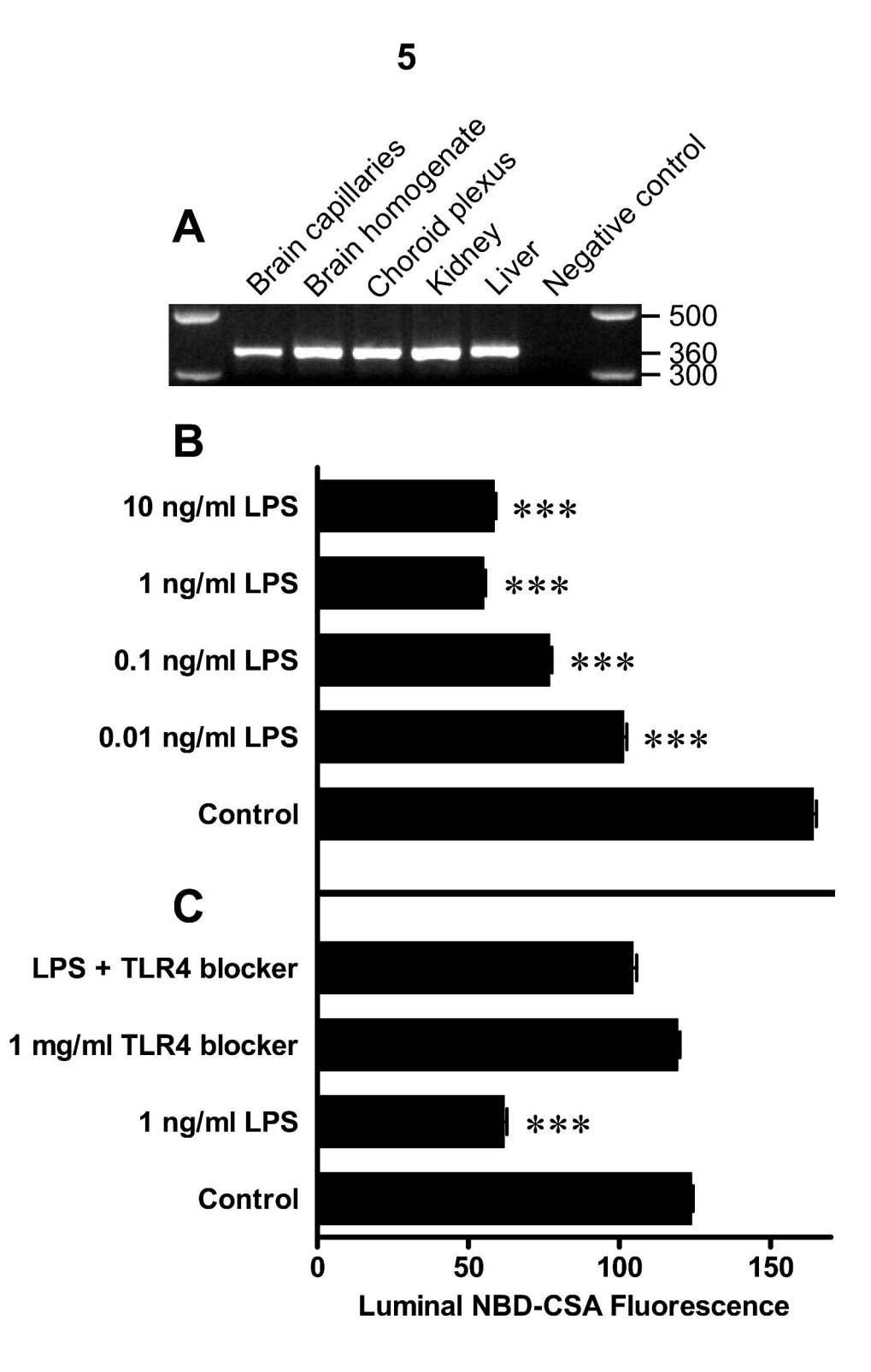
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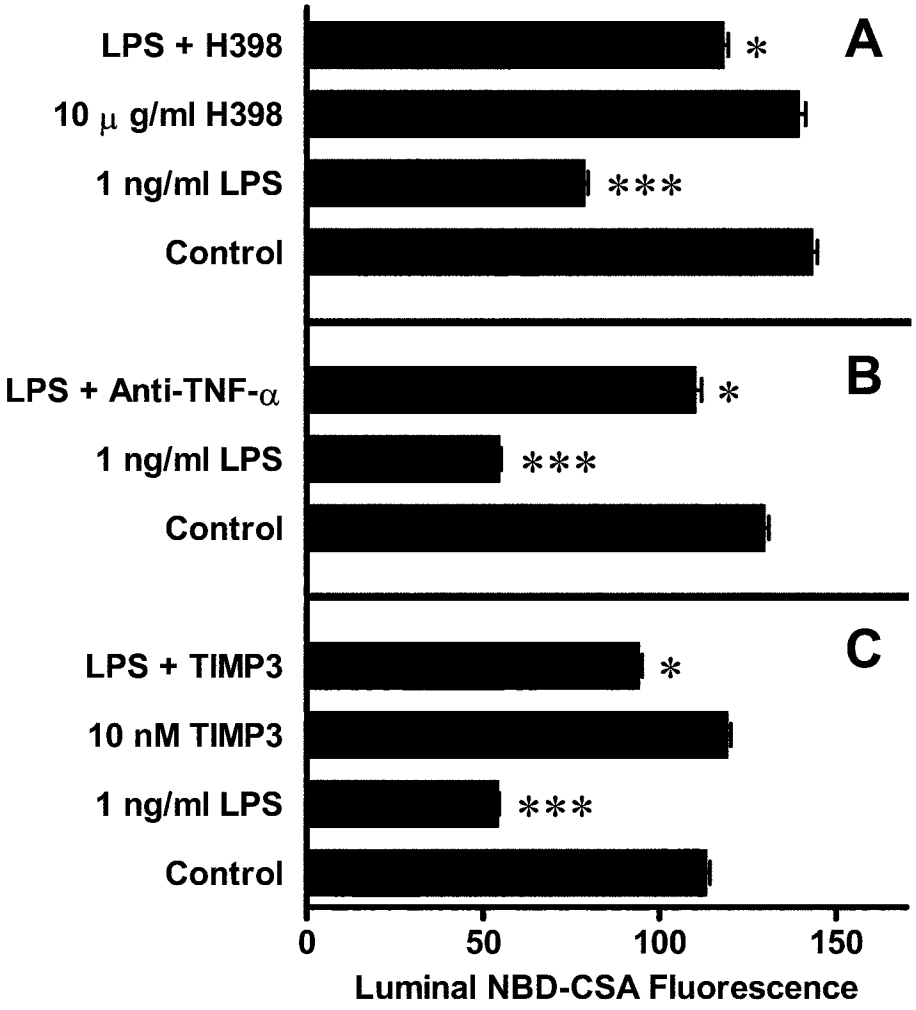

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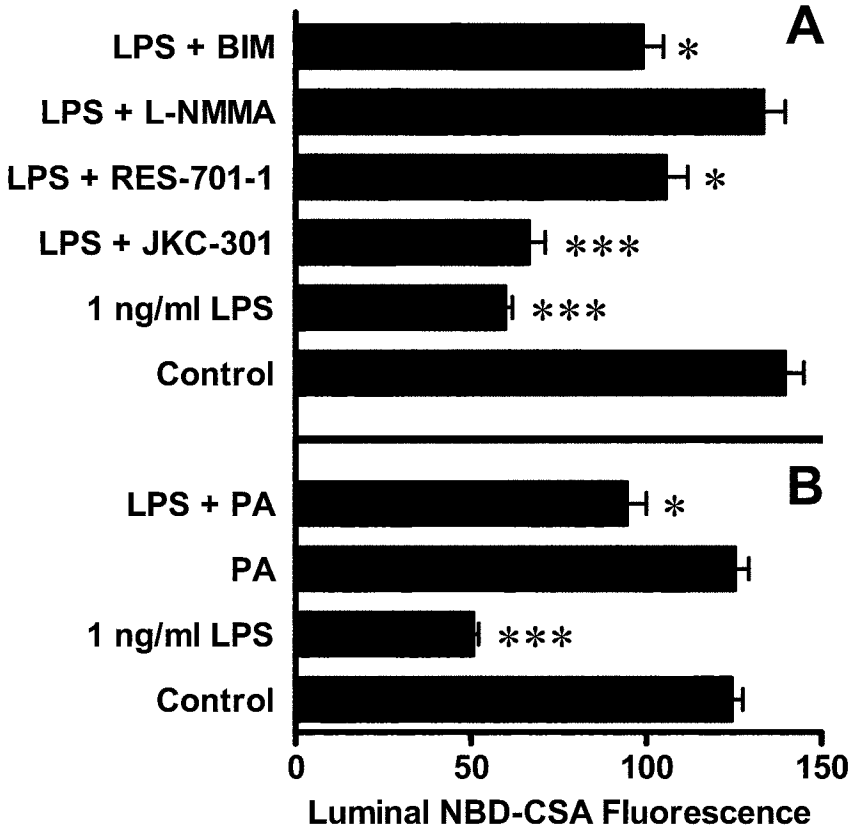

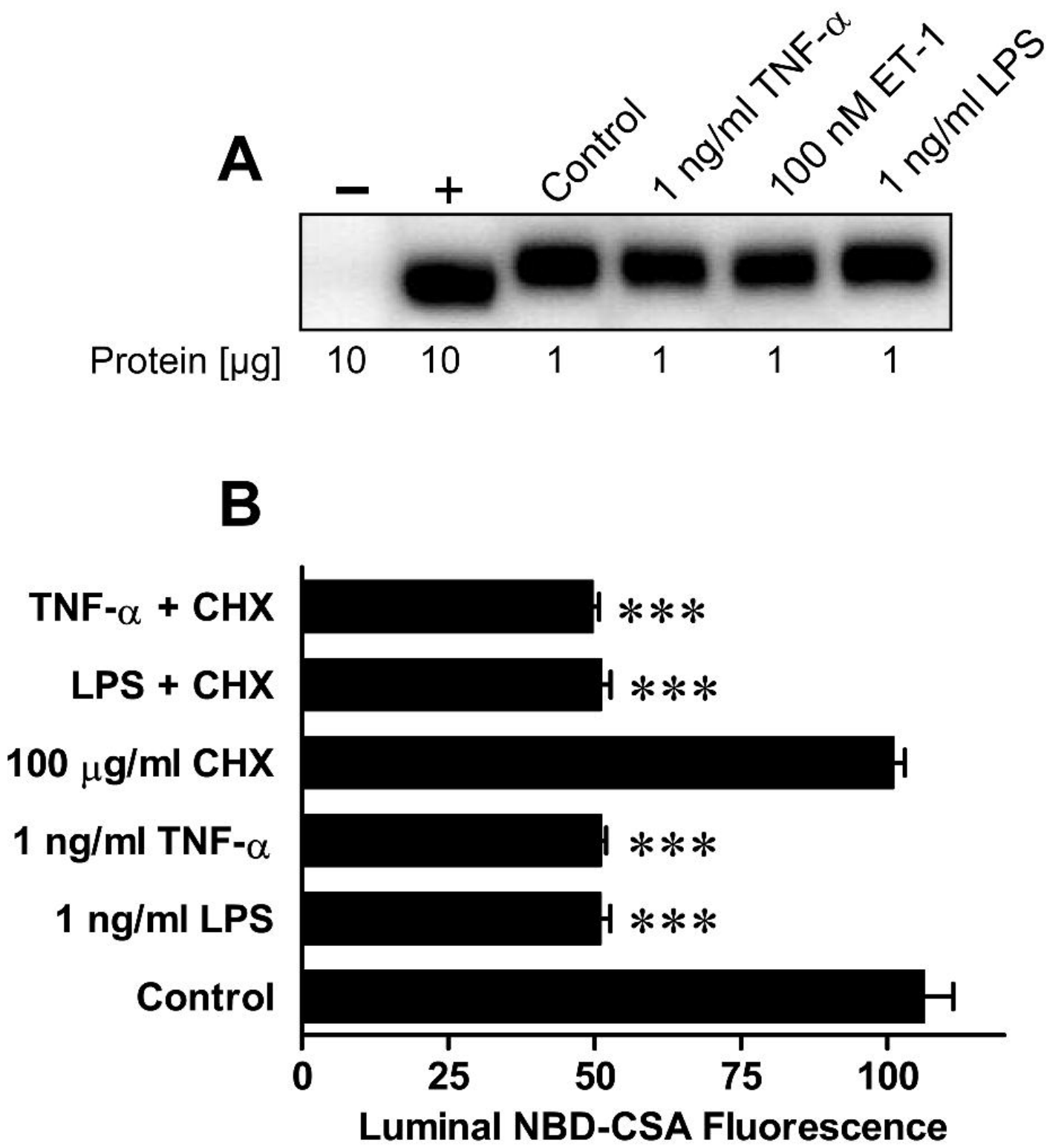
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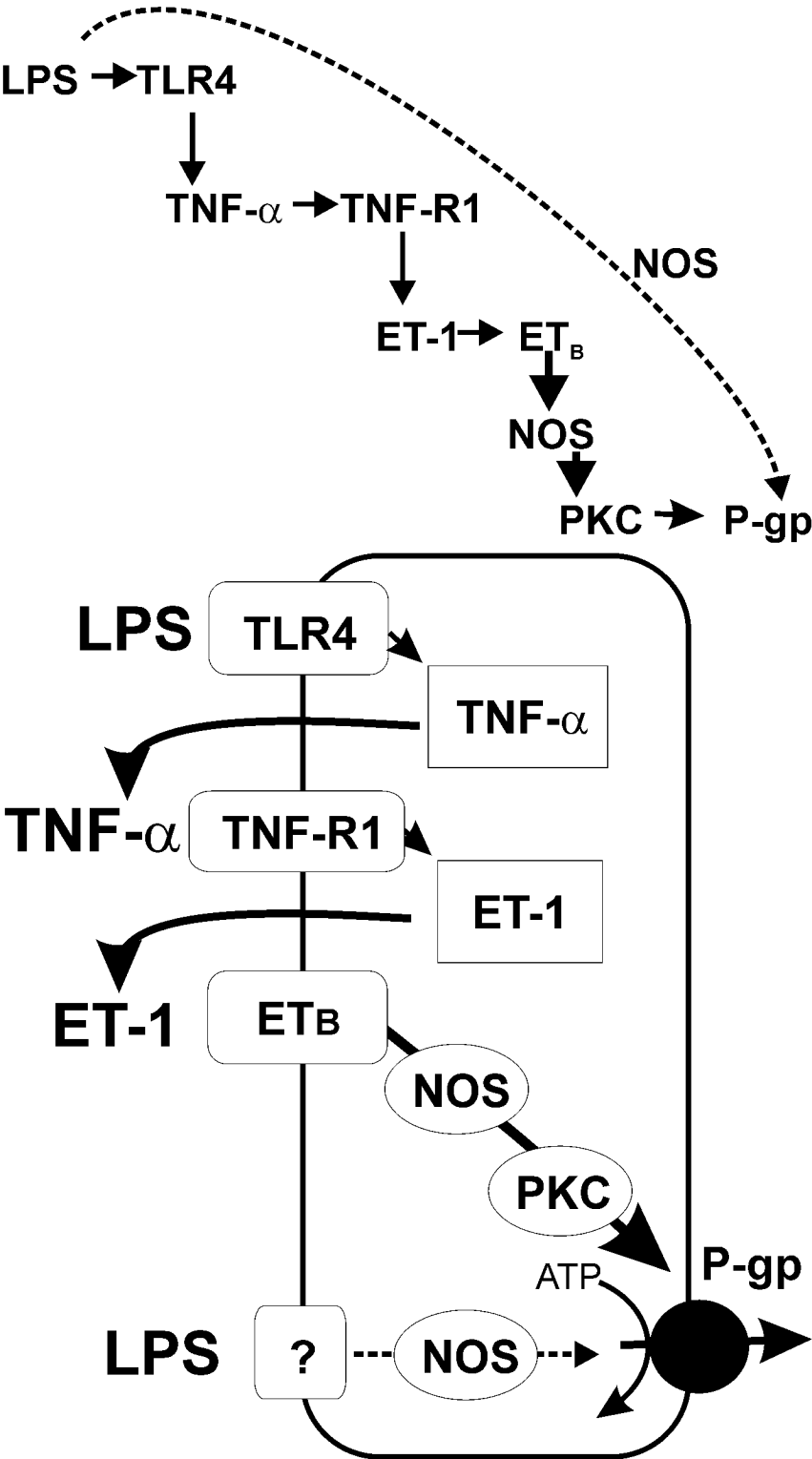
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TNF-R1
ET-1
ET-1
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ATP
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