

## Research Article

# Regulation of P-Glycoprotein in Renal Proximal Tubule Epithelial Cells by LPS and TNF- $\alpha$

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Received 2 July 2009; Revised 13 November 2009; Accepted 8 December 2009

Academic Editor: Xue-Ru Wu

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During endotoxemia, the ATP-dependent drug efflux pump P-glycoprotein (*Abcb1*/P-gp) is upregulated in kidney proximal tubule epithelial cells. The signaling pathway through which lipopolysaccharide (LPS) or tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) regulates P-gp expression and activity was investigated further in the present study. Exposure of rat kidney proximal tubule cells to TNF- $\alpha$  alone or TNF- $\alpha$  and LPS increased P-gp gene and protein expression levels and efflux activity, suggesting de novo P-gp synthesis. Upon exposure to TNF- $\alpha$  in combination with LPS, P-gp activity in renal proximal tubule cells is increased under influence of nitric oxide (NO) produced by inducible NO synthase. Upon exposure to TNF- $\alpha$  alone, P-gp upregulation seems to involve TLR4 activation and nuclear factor kappaB (NF- $\kappa$ B) translocation, a pathway that is likely independent of NO. These findings indicate that at least two pathways regulate P-gp expression in the kidney during endotoxemia.

## 1. Introduction

P-glycoprotein (*Abcb1*/P-gp) is a member of the ATP binding cassette (ABC) superfamily and is able to transport a broad range of uncharged and cationic compounds. The apical localization of P-gp in renal proximal tubule epithelial cells is consistent with its importance in excretory transport into urine [1]. In agreement with the detoxifying role of P-gp, we recently found an upregulation of P-gp in rat kidney during endotoxemia [2] and in mouse kidney after ischemia reperfusion injury [3]. P-gp is likely to be regulated by nitric oxide (NO) produced by renal inducible NO-synthase (iNOS), because co-administration of an iNOS-inhibitor attenuated the endotoxin-induced effects on its expression in rats [2]. An upregulation of important efflux pumps, like P-gp, may diminish the accumulation of toxic compounds and serves a protective function in acute kidney injury.

Using killifish renal proximal tubules, we found previously that NO has a regulatory role in the transport activity of multidrug resistance protein 2 (*Abcc2*/Mrp2) via

an intracellular signaling pathway in response to the in vitro action of several nephrotoxic chemicals [4]. This pathway involved at least endothelin (ET) release, binding to the basolateral ET<sub>B</sub> receptor, and activation of NOS, soluble guanylyl cyclase (sGC) and protein kinase C (PKC) [5]. A similar regulatory pathway was found for P-gp in rat brain capillaries [6], where it was recently shown to be correlated with an innate immune response [7, 8]. The inflammatory mediator, tumor necrosis factor—alpha (TNF- $\alpha$ ) signalled through the TNF-receptor 1 (TNFR1) to increase P-gp expression and transport activity by triggering the ET-NOS-PKC pathway, finally activating the nuclear factor kappaB, NF- $\kappa$ B [7].

As NF- $\kappa$ B was previously shown to be involved in P-gp increase during renal toxicity [2, 3, 9], we hypothesized that the same signaling pathway is responsible for the upregulation of P-gp in the kidney during endotoxemia and that NO is the central player in the field. To test this hypothesis, we used a spontaneously immortalized rat kidney proximal tubular cell line and determined P-gp expression and activity

after treating cells with endotoxin (lipopolysaccharide; LPS) or the most important pro-inflammatory cytokine, TNF- $\alpha$ . Our findings indicate that exposure to TNF- $\alpha$  in combination with LPS increases P-gp activity in renal proximal tubule cells under influence of NO produced by iNOS. Upon exposure to TNF- $\alpha$  alone, P-gp upregulation seems to involve TLR4 activation and NF- $\kappa$ B translocation, a pathway that is likely independent of NO. These findings indicate that at least two different pathways regulate P-gp expression during endotoxemia.

## 2. Materials and Methods

**2.1. Chemicals.** Dulbecco's modified eagle's medium (DMEM), Hanks' balanced salt solution (HBSS), insulin-transferrin-selenium, and TRIzol reagent were from Invitrogen Life Sciences (Breda, The Netherlands), fetal calf serum from MP Biomedicals (Asse-Relegum, Belgium), 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (Hepes) from Roche diagnostics (Almere, the Netherlands), calcein-acetoxymethylester (calcein-AM) from Molecular Probes (Eugene, OR), bisindolylmaleimide (BIM, Bruschwig chemie, Amsterdam, the Netherlands), H398 from Alexis (Lausen, Switzerland), antihuman Toll-like receptor (TLR)4 CD284/MD complex from Biolegend (San Diego, USA), and 1H-[1,2,4]oxadiazolo[4,3,-a]quinoxalin-1-one (ODQ) and SN50 from Calbiochem (Nottingham, U.K.). The P-gp inhibitor, PSC-833, was a gift from Novartis Pharma (Valspodar, Arnhem, The Netherlands). All other chemicals were of analytical grade and purchased from Sigma-Aldrich (Zwijndrecht, The Netherlands) or Merck (Darmstadt, Germany).

**2.2. Cell Culture and Analysis of P-gp Transport Activity.** The spontaneously immortalized epithelial cell line isolated from rat kidney proximal tubule (GERP cells) was a kind gift of the department of Veterinary Pharmacology, Pharmacy and Toxicology of the University of Utrecht, The Netherlands. These cells (passages 53201374) were cultured in collagen coated culture flasks in DMEM supplemented with 5% fetal calf serum and 1% insulin-transferrin-selenium. We previously demonstrated that P-gp activity in these cells was highest just after reaching confluency and decreased with culturing time [10]. Cells were plated at a density of 40,000–60,000 cells/well for 3 days in a 24-well plate.

Culture medium was replaced for medium alone (control group) or medium supplemented with the tested compounds: lipopolysaccharide (10–100  $\mu$ g/mL LPS, *Escherichia coli* 0127:B8), or TNF- $\alpha$  (1–100 ng/mL), or the NO-donor sodium nitroprusside (0.1–0.5 mM SNP), or to a combination of LPS and TNF- $\alpha$ , with the NO-scavenger 2-phenyl-4,4,5,5-tetramethylimidazole-1-oxyl 3-oxide (0.01–0.5 mM PTIO). The concentration TNF- $\alpha$  relates to plasma levels that can be reached in patients with septic conditions [11]. Cells were also exposed to TNF- $\alpha$  in combination with an inhibitor of sGC, ODQ, (0.01 mM), an inhibitor of PKC, BIM, (100 nM), an inhibitor of TNFR1, H398, (10  $\mu$ g/mL), the anti-human TLR4, CD284/MD

complex, (5  $\mu$ g/mL), the selective I kappa B kinase (IKK) $\beta$  inhibitor, IMD-0354, (10–50  $\mu$ M), or the peptide inhibitor for NF- $\kappa$ B nuclear translocation SN50 (1–1.8  $\mu$ M).

After treatment the transport activity of P-gp was determined with the calcein accumulation assay [12], as previously described [10]. The activity was determined by calculating the ratio of cellular accumulation of fluorescent calcein in the presence and absence of the P-gp inhibitor, PSC 833, and control cells were set to 100%.

**2.3. Determination of mRNA Expression.** Cells were exposed to medium (control), LPS, TNF- $\alpha$  or to a combination of LPS and TNF- $\alpha$  for various time periods (2, 6, 24 hours) and were, subsequently, harvested for RNA isolation (for 5 minutes at 1300 g at room temperature). Cell pellets were transferred in ice cold TRIzol reagent (Invitrogen, Breda, the Netherlands) and RNA was isolated as described previously [13]. Quantitative Real Time-PCR (RQ-PCR) on cDNA was performed according to the TaqMan protocol in optical tubes using either the ABI PRISM 7700 single reporter Sequence Detection System ( $n = 6$ , Applied Biosystems, Zwijndrecht, The Netherlands) or the ABI PRISM 7900HT Gene Expression Micro Fluidic Card Sequence Detection System (3 pooled samples from 9 different rats, Applied Biosystems) according to the manufacturer's instructions. All experiments were performed in triplicate. In rodents, two genes (*Abcb1a* and *Abcb1b*) encode for P-gp, however, during endotoxemia only *Abcb1b* was found to be differentially expressed [2]. Different rat genes (*GAPDH*: (Rn99999916\_S1), *Abcb1b*: (Rn00561753\_m1), *NOSII*: (Rn00561646\_m1), or *Ednrb/endothelin receptor B*: Rn00569139\_m1) were amplified with a pre-developed Gene Expression Assay, provided by Applied Biosystems. The relative expression of genes in control cells were normalized for the average cycle threshold ( $C_T$ ) value for the housekeeping gene, *GAPDH* ( $C_T = 15.9 \pm 1.0$ ) and set to 1.

For the analysis of TNF- $\alpha$  receptor 1 (TNFR1), TNFR2, and toll-like receptor-4 (TLR4) mRNA expression in GERP cells exposed for 24 hours to TNF- $\alpha$ , PCR amplification was performed as previously described [14]. Gene-specific primers were purchased from Biolegio (Nijmegen, The Netherlands): rat *TNFR1* (M63122; nt.983-1382), forward primer: gggattcagctcctgcaaa, reversed primer (atgaactcctccagcgtgt; (M63122, nt.704-1902) forward primer: tccctgtaaggagaacagaa, reversed primer: gcttttctccacaatcctc [15]. Rat *TNFR2*: (AF498039, nt. 579-1034), forward primer: gtctctgacaccatcatcc, reversed primer: gtcaatggtgctgctgttcaa; (AF498039, nt.541-1242), forward primer: aatgaaacgtgatatgcagtg, reversed primer: gctacagcgttcagatgc [15]. Rat *TLR4*: (NM\_019178, nt.2418-2546), forward primer: gagccgaaagtattgtgg, reversed primer: agcaag-gacttctcactttct [16]. Rat  $\beta$ -*actin*: forward primer: ctatgagct-gctgacggc, reversed primer: agtttcatggatgccacagg [14]. The expected PCR products are: TNFR1-primer set 1: 400 bp, TNFR1-primer set 2: 1199 bp, TNFR2-primer set 1: 456 bp, TNFR2-primer set 2: 702 bp, TLR4: 129 bp. MilliQ was used as a negative control. The expression of the housekeeping gene  $\beta$ -*actin* was measured as a control for the performed PCR reaction.

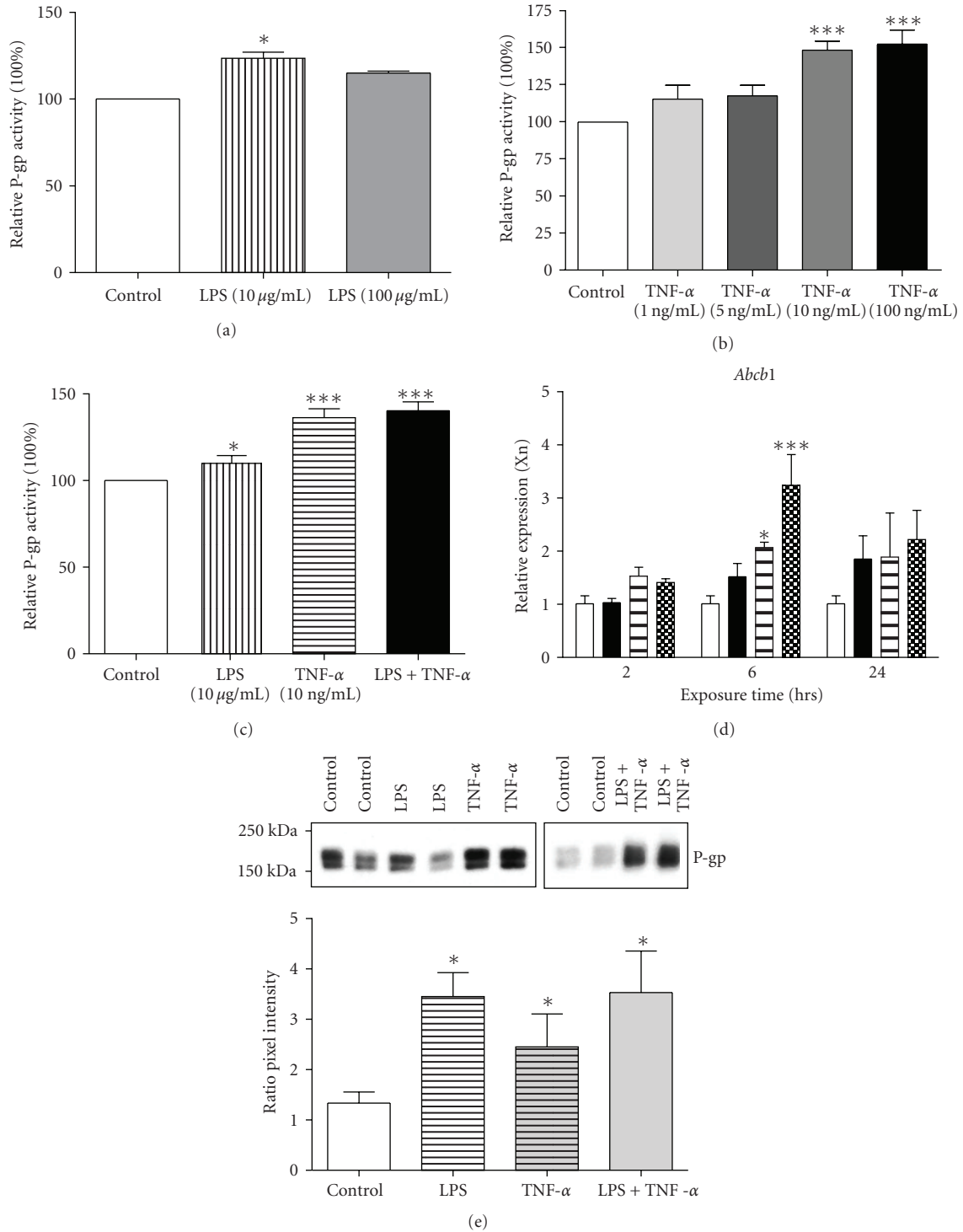


FIGURE 1: P-gp activity and expression after exposure to TNF- $\alpha$  and/or LPS in GERP cells. (a)–(c) P-gp transport activity in GERP cells after exposure to different concentrations of TNF- $\alpha$  ((a)  $n = 4$ –6) or LPS ((b)  $n = 4$ ) for 24 hours compared to control (cells exposed to culture medium). ((c)  $n = 12$ ) Optimal concentrations of both sepsis mediators were used for exposure to GERP cells for 24 hours. P-gp activity was determined using the calcein accumulation assay. ((d)  $n = 4$ ) *Abcb1b* expression in GERP cells (control; open bars) after exposure to either 10  $\mu\text{g/mL}$  LPS (closed bars), 10 ng/mL TNF- $\alpha$  (striped bars) or both 10  $\mu\text{g/mL}$  LPS and 10 ng/mL TNF- $\alpha$  (chequered bars) for 2, 6 or 24 hours. mRNA levels were determined with RQ-PCR and expression was normalized for the GAPDH  $C_T$  value ( $15.9 \pm 1.0$ ). ((e)  $n = 4$ ) P-gp protein expression in GERP cells (control; lanes 1, 2, 7 and 8) after exposure to either 10  $\mu\text{g/mL}$  LPS (lanes 3 and 4), 10 ng/mL TNF- $\alpha$  (lanes 5 and 6) or to both 10  $\mu\text{g/mL}$  LPS and 10 ng/mL TNF- $\alpha$  (lanes 9 and 10) for 24 hours. Total cell lysate fractions of GERP cells were used and expression of P-gp was determined by Western blotting. Relative pixel intensities (ratio P-gp/ $\beta$ -actin) were determined through image analysis. Data are expressed as mean  $\pm$  SEM. Significantly different compared to control cells (\*:  $P < .05$ , \*\*\*:  $P < .001$ ).

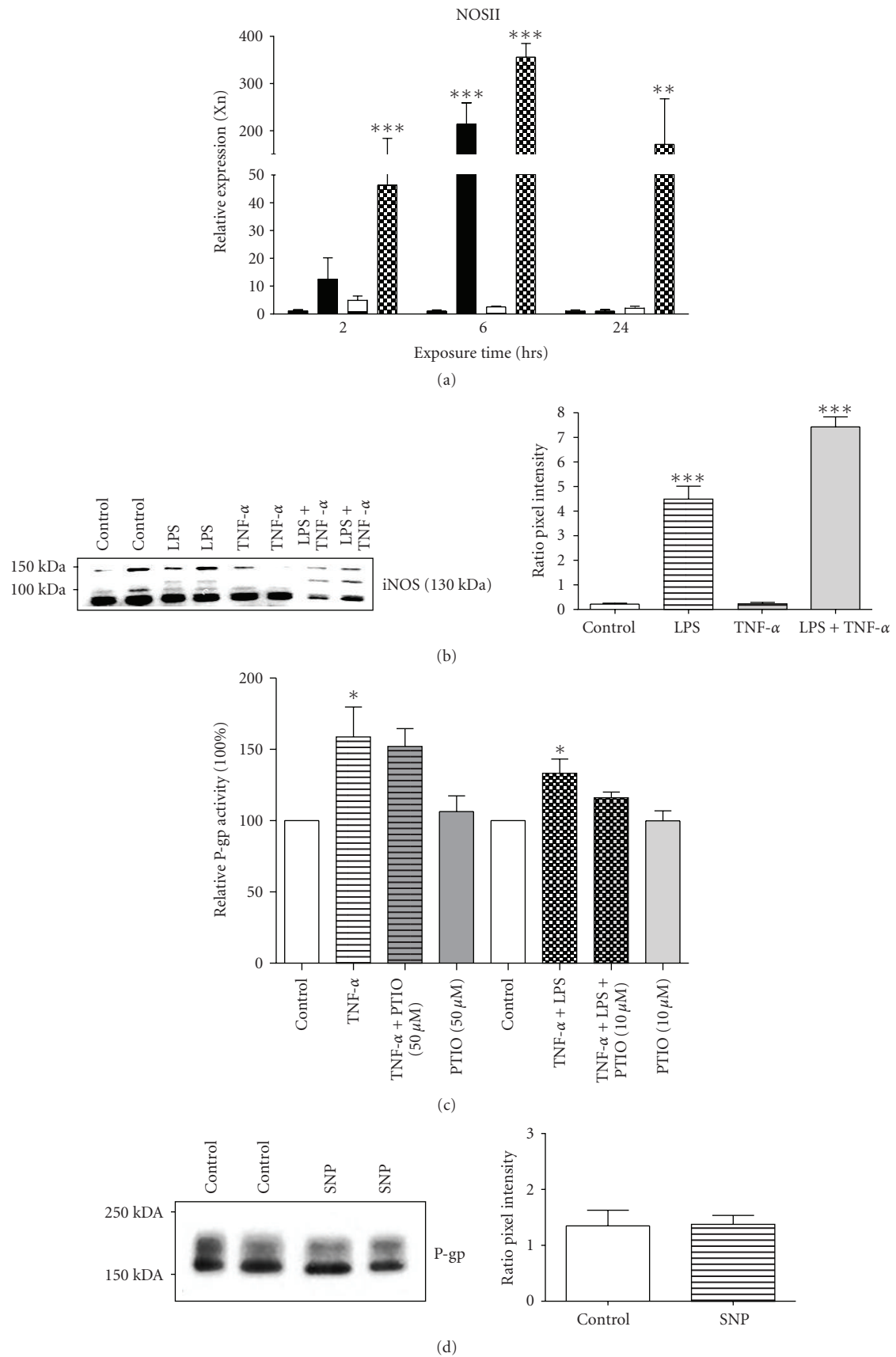


FIGURE 2: Continued.

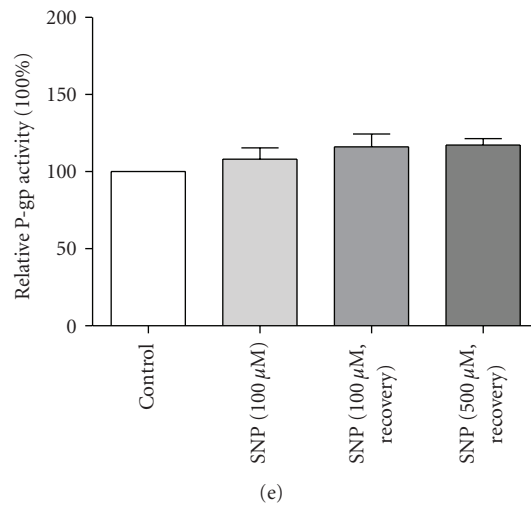


FIGURE 2: Expression of iNOS and effect of NO on Abcb1/P-gp activity in GERP cells. ((a)  $n = 4$ ) *NOSII* expression in GERP cells after exposure to either 10  $\mu$ g/mL LPS (closed bars), 10 ng/mL TNF- $\alpha$  (striped bars) or both 10  $\mu$ g/mL LPS and 10 ng/mL TNF- $\alpha$  (chequered bars) for 2, 6 or 24 hours ( $n = 4$ ). mRNA levels were determined with RQ-PCR and expression was normalized for the GAPDH  $C_T$  value. ((b)  $n = 4$ ) iNOS protein expression in GERP cells (control; lanes 1 and 2) after exposure to either 10  $\mu$ g/mL LPS (lanes 3 and 4), 10 ng/mL TNF- $\alpha$  (lanes 5 and 6) or both 10  $\mu$ g/mL LPS and 10 ng/mL TNF- $\alpha$  (lanes 7 and 8) for 6 hours. ((c)  $n = 3-5$ ) P-gp transport activity in GERP cells after exposure to 10 ng/mL TNF- $\alpha$  or both 10  $\mu$ g/mL LPS and 10 ng/mL TNF- $\alpha$  in combination with 50 or 10  $\mu$ M of the NO-scavenger PTIO, respectively, for 24 hours compared to control (cells exposed to culture medium). ((d)  $n = 4$ ) P-gp protein expression in GERP cells (control; lanes 1 and 2) after exposure to 100  $\mu$ M SNP (lanes 3 and 4) for 1 hour with 5 hours recovery. ((e)  $n = 4$ ) P-gp transport activity in GERP cells, compared control, after exposure to 100 or 500  $\mu$ M SNP for 6 hours or for 1 hour with a subsequent recovery period of 5 hours. Total cell fractions of GERP cells were used and expression of iNOS (b) or P-gp (d) was determined by Western blotting. Relative pixel intensities (ratio iNOS/ $\beta$ -actin (b) and ratio P-gp/ $\beta$ -actin (d)) were determined through image analysis. Data are expressed as mean  $\pm$  SEM. Significantly different compared to control (\*:  $P < .05$ , \*\*:  $P < .01$ , \*\*\*:  $P < .001$ ).

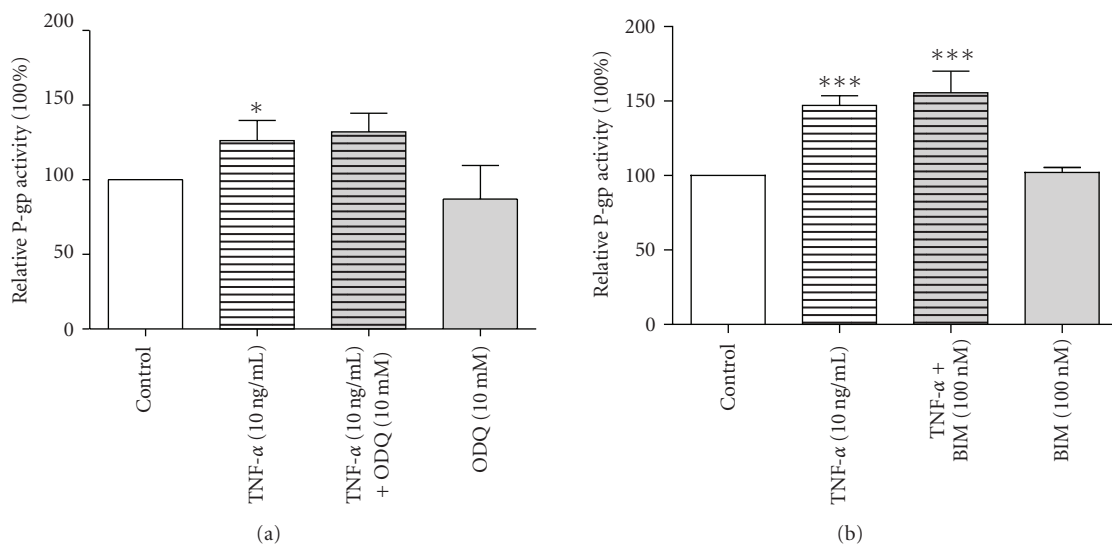


FIGURE 3: Lack of involvement of cGMP and PKC in P-gp regulation. ((a)  $n = 3-6$ ) P-gp transport activity in GERP cells after exposure to 10 ng/mL TNF- $\alpha$  in combination with 10  $\mu$ M of the sGC inhibitor ODQ for 24 hours compared to control (cells exposed to culture medium). ((b)  $n = 3$ ) P-gp transport activity in GERP cells after exposure to 10 ng/mL TNF- $\alpha$  in combination with 100 nM of the PKC inhibitor BIM for 24 hours compared to control. Data are expressed as mean  $\pm$  SEM. Significantly different compared to control (\*:  $P < .05$ , \*\*\*:  $P < .001$ ). Treatment with ODQ or BIM alone showed no significant difference compared to control.

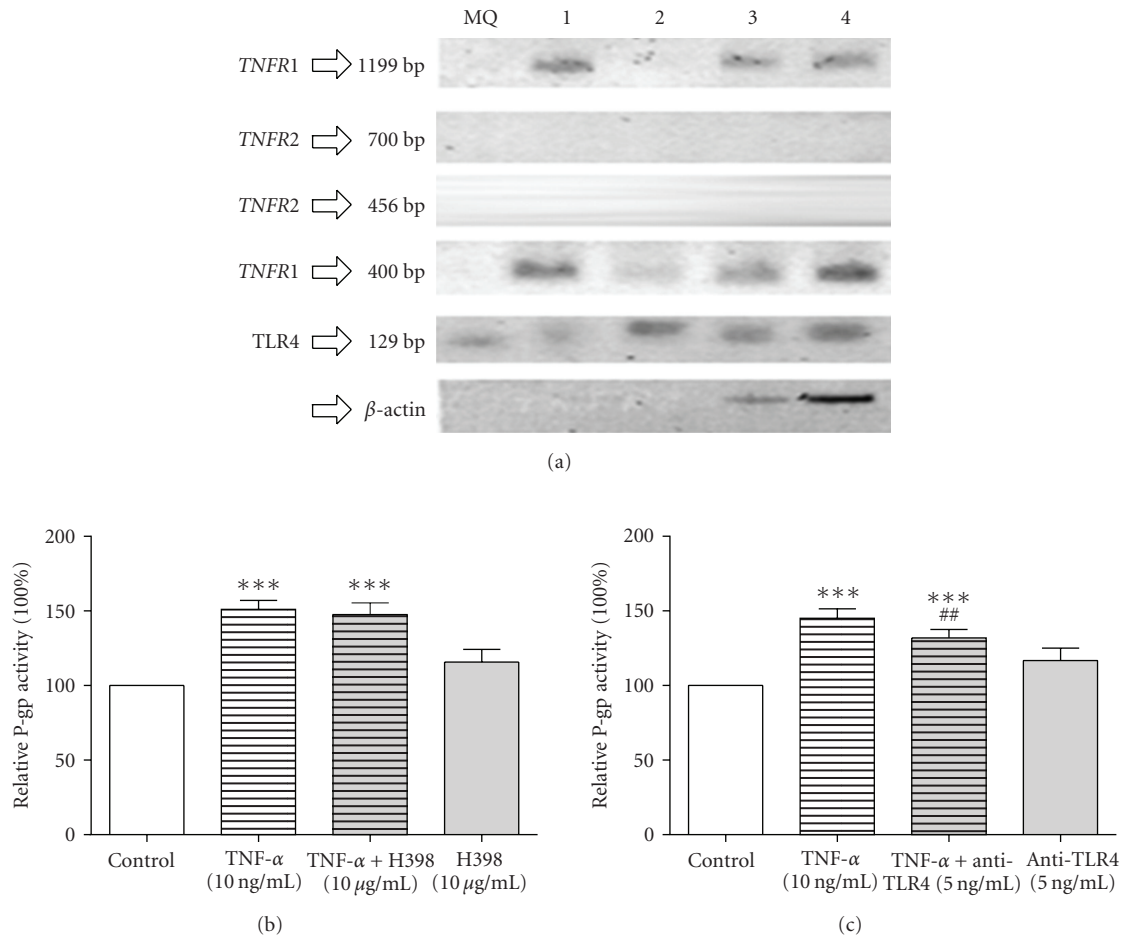


FIGURE 4: Role of TNF receptors and toll-like receptor 4 in modulation of P-gp activity in GERP cells. (a) *TNFR1*, *TNFR2* and *TLR4* mRNA expression was analyzed in GERP cells (control; lane 1 and 2) exposed for 24 hours to TNF- $\alpha$  (lanes 3 and 4) using PCR. For *TNFR1* and *TNFR2* two different primer sets were used, for *TLR4* only one primer set was used. MilliQ (MQ) was used as a negative control. For both experiments the expression of the housekeeping gene  $\beta$ -actin was performed as a control for the performed PCR reaction. ((b)  $n = 3$ ) P-gp transport activity in GERP cells after exposure to 10 ng/mL TNF- $\alpha$  in combination with 10 mg/mL of the TNFR1 inhibitor H398 for 24 hours compared to control (cells exposed to culture medium). ((c)  $n = 7$ ) P-gp transport activity in GERP cells after exposure to 10 ng/mL TNF- $\alpha$  in combination with 5 ng/mL of the antibody against human TLR4 for 24 hours compared to control. Data are expressed as mean  $\pm$  SEM. Significantly different compared to control (\*\*\*:  $P < .001$ ) or to TNF- $\alpha$  (##;  $P < .01$ ). Treatment with H398 or anti-TLR4 alone showed no significant difference compared to control.

**2.4. Western Blotting.** Cells were harvested after exposure to different inflammatory mediators and lysed with 0.1% Triton X-100 supplemented with protease inhibitors (1 mM PMSE, 10  $\mu$ M E64, 1  $\mu$ g/mL pepstatin, 5  $\mu$ g/mL leupeptin and 1  $\mu$ g/mL aprotinin) during 30 minutes on ice. The amount of protein in cell lysates was determined with the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA) using bovine serum albumin as standard and samples were subjected to Western blotting as described previously [17].

Samples were separated on a 6% sodium dodecylsulfate polyacrylamide gel and transferred to Hybond-C pure nitrocellulose membrane (Amersham, Buckinghamshire, UK). The membrane was incubated overnight at 4°C with the primary antibodies against iNOS (1 : 1000, according to [18]), ABCB1/P-gp (1 : 200, C219, DakoCytomation,

Denmark), or  $\beta$ -actin (1 : 10000, Sigma-Aldrich) in Tris-buffered saline supplemented with 0.1% Tween-20 (TBS-T) and 1% non-fat dried milk.

Subsequently, the membranes were washed three times in TBS-T, blocked in TBS-T containing 5% non-fat dried milk, washed three times in TBS-T again, and incubated with affinity-purified horseradish peroxidase-conjugated goat antirabbit IgG (Sigma-Aldrich) for iNOS or goat antimouse IgG (Sigma-Aldrich) for P-gp and  $\beta$ -actin diluted 1 : 5000 in TBS-T for 1 hour at room temperature. The washing steps were repeated, after which the membranes were visualized with enhanced chemiluminescence (Pierce Chemical, Rockford, IL). For semiquantification, the pixel intensity of the bands was measured using Scion Image version beta 4.02 for Windows (Scion Corporation, Frederick, MD).



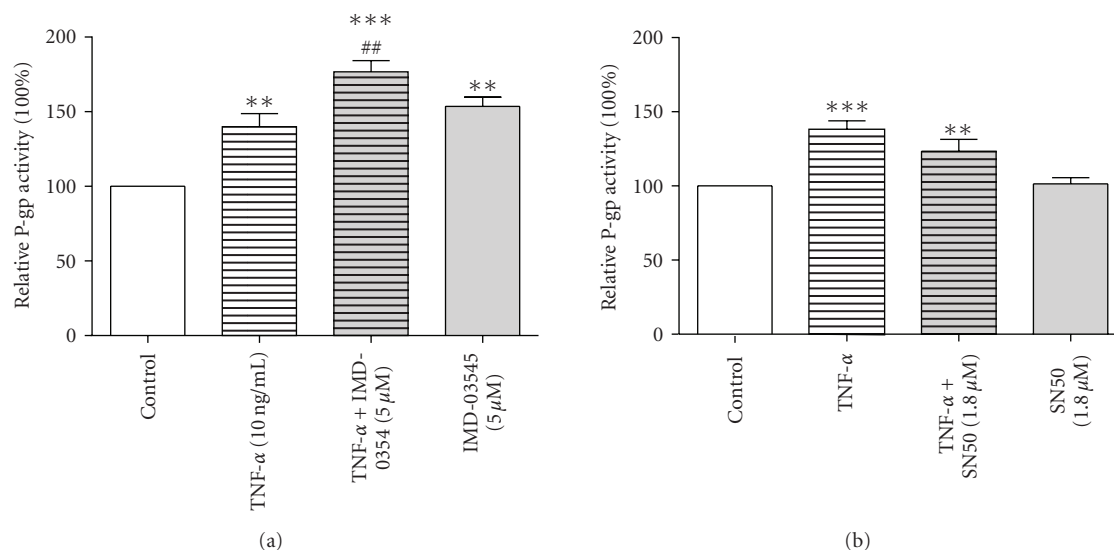


FIGURE 5: Role of NF- $\kappa$ B in modulation of P-gp activity in GERP cells. ((a)  $n = 4$ ) P-gp transport activity in GERP cells after exposure to 10 ng/mL TNF- $\alpha$  in combination with 5  $\mu$ M of the selective IKK $\beta$  inhibitor IMD-0354 compared to control (cells exposed to culture medium). ((b)  $n = 7$ ) P-gp transport activity in GERP cells after exposure to 10 ng/mL TNF- $\alpha$  in combination with 1.8  $\mu$ M of a cell-permeable inhibitor peptide that interferes with NF- $\kappa$ B nuclear translocation, SN50, for 24 hours compared to control. Data are expressed as mean  $\pm$  SEM. Significantly different compared to control cells (\*\*;  $P < .01$ , \*\*\*;  $P < .001$ ) or to TNF- $\alpha$  (##;  $P < .001$ ). Treatment with SN50 alone showed no, but with IMD-0354 alone did show significant difference compared to control.

**2.5. Data Analysis.** Values are given as mean  $\pm$  SEM. Analysis was performed using GraphPad Prism 4.03 for Windows (Graphpad Software Inc., San Diego, CA, USA) and SPSS (version 12.0.1 for Windows, Chicago, IL). Differences between the experimental groups were tested using one-way ANOVA with Bonferroni's correction or two-way repeated measurements ANOVA. A two-sided  $P$  value  $< .05$  was considered significantly different.

### 3. Results and Discussion

**3.1. Effect of Inflammatory Mediators on P-gp Expression and Activity.** Treatment of GERP cells with 10  $\mu$ g/mL LPS and/or 10–100 ng/mL TNF- $\alpha$  for 24 hours resulted in an increase in P-gp activity (Figures 1(a)–1(c),  $P < .05$ ). As 10 ng/mL is a clinically relevant concentration [11] and a 10-fold higher TNF- $\alpha$  concentration or combination of TNF- $\alpha$  and LPS did not further increase P-gp activity, we used a concentration of 10 ng/mL TNF- $\alpha$  for additional experiments. In accordance, we also observed an upregulation in *Abcb1b* expression after exposure to TNF- $\alpha$ , LPS, or a combination of TNF- $\alpha$  and LPS (Figure 1(d)), which was accompanied by an increase in P-gp protein expression (Figure 1(e)), suggesting *de novo* P-gp synthesis. This finding is in agreement with previous studies in mice and rats in which the upregulation of renal P-gp was demonstrated after in vivo LPS exposure [2, 19, 20].

**3.2. P-gp Expression and Activity Also Independent of NO.** We previously suggested that P-gp is likely to be regulated by NO produced by iNOS, as coadministration of aminoguanidine reversed the induction in iNOS, reduced renal damage and

attenuated the endotoxin-induced effects on its expression in rats [2]. In agreement, we show here that both iNOS mRNA (Figure 2(a),  $P < .001$ ) and protein (Figure 2(b)) are upregulated when cells are exposed to LPS, either alone or in combination with TNF- $\alpha$ . Treatment with TNF- $\alpha$  alone, however, only marginally induced NOSII mRNA, but had no effect on iNOS protein (Figures 2(a), and 2(b)).

To determine the NO-dependency of the upregulation of P-gp, GERP cells were co-treated with the NO-scavenger PTIO or exposed to the NO donor SNP. Indeed, the P-gp efflux activity was not significantly different from control anymore after co-treatment with PTIO (Figure 2(c)). Remarkably, SNP did not affect *Abcb1b* (data not shown) and P-gp protein expression (Figure 2(d)) and its activity was slightly, though not significantly, upregulated after 1 hour exposure and 5 hours recovery (Figure 2(e)). Prolonged exposure times to SNP resulted in cytotoxicity (data not shown). This is in contrast with previous findings demonstrating that NO, released by the NO donor S-nitroso-N-acetylpenicillamine, was able to upregulate P-gp expression in the human CaCo2 cell line [21]. However, SNP produces besides NO also iron and cyanide [22], so it is not clear whether only effects of NO on P-gp expression and activity were measured. In contrast to LPS, our findings indicate that P-gp signaling seems largely NO-independent after exposure to TNF- $\alpha$ . In addition, this signaling pathway may be dependent on the type of cytokine as NO mediated increased P-gp activity after stimulation with interferon- $\gamma$  [21]. Furthermore, it was recently demonstrated that in vivo inhibition of NOS and subsequent NO production with L-NAME did not affect blood-brain barrier P-gp function during endotoxemia [23], which contradicts with

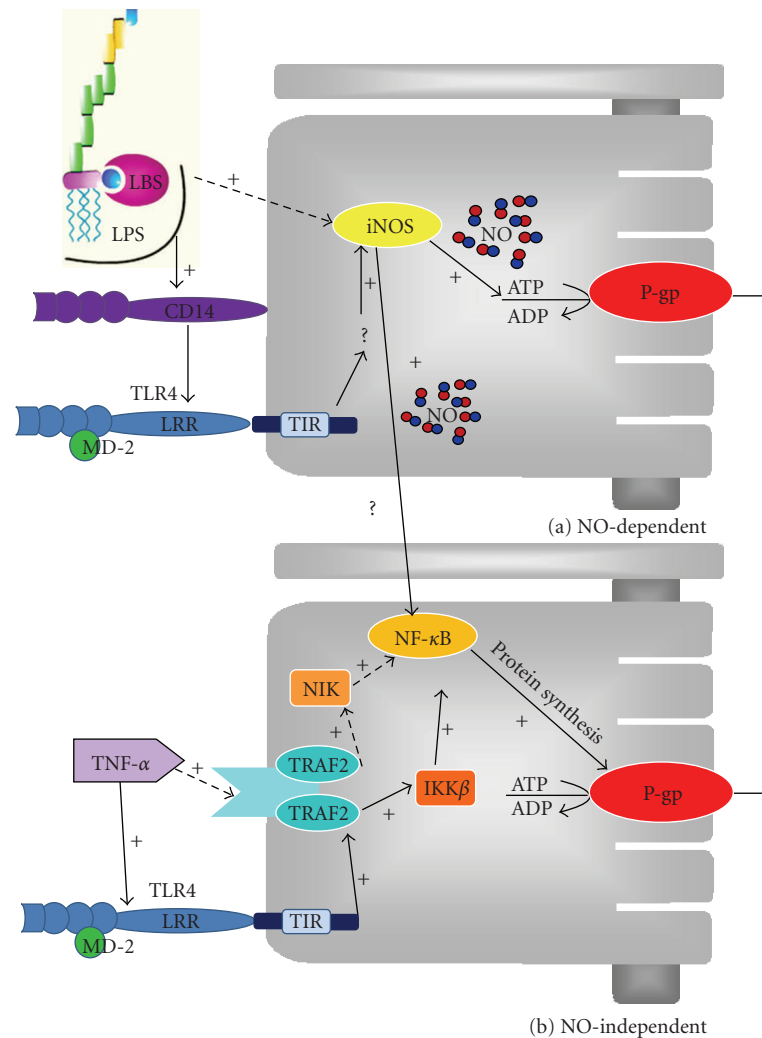


FIGURE 6: Scheme illustrating the proposed sequence of events by which inflammatory mediators increase P-gp-mediated transport in GERP cells. (a) NO-dependent pathway after exposure to LPS directly or indirectly via TLR4. (b) NO-independent pathway after exposure to TNF- $\alpha$ . This pathway involves the activation of NF- $\kappa$ B inducing kinase (NIK) by TNF receptor associated factor family (TRAF) 2 and, consequently, the activation of NF- $\kappa$ B or the activation of TLR4 leading to the activation of IKK $\beta$  by TRAF2 and consequently the activation of NF- $\kappa$ B. TIR; toll-IL-1R domain in TLR, LRR, leucine rich repeats domain in TLR.

previous in vitro findings in isolated rat brain capillaries [7, 8].

**3.3. Regulation of P-gp Activity by TNF- $\alpha$ .** To dissect the signaling pathway through which exposure to TNF- $\alpha$  increased P-gp expression and activity we used various pharmacological agents. Since NO is a well-known activator of sGC, which is in turn involved in the PKC signalling pathway [5], the effects of the sGC inhibitor ODQ (Figure 3(a)) and the PKC inhibitor BIM (Figure 3(b)) on P-gp activity were examined. Importantly, none of the two substances could prevent the upregulation of P-gp caused by TNF- $\alpha$ , suggesting that a novel pathway mediates P-gp activity in GERP cells and/or that the NO-sGC-PKC pathway is not activated in our cell line. This finding is in contrast with previous studies using killifish renal proximal tubules and brain capillary membranes [4–7].

LPS signals through toll-like receptor-4 (TLR-4) and TNF- $\alpha$  mediates their effect by binding to TNFR1 (p55) and TNFR2 (p75), which are known to be expressed in the kidney and their expression patterns are modulated during immune-mediated and ischemic renal injury [24, 25]. Figure 4(a) shows that *TNFR1* and *TLR4* are expressed in the GERP cell line, whereas *TNFR2* expression could not be detected. To confirm the results of the PCR analysis, we used inhibitors of and/or antibodies against TNFR1 (H398, Figure 4(b)) and TLR4 (anti-TLR4, Figure 4(c)) and determined P-gp activity. Exposure to H398 could not prevent the upregulation of P-gp by TNF- $\alpha$ , suggesting that although TNFR1 is expressed in GERP cells it may not be involved in P-gp regulation (Figure 4(b)). Many cell types require cooperation between TNFR1 and TNFR2 to generate TNF responses [26], and the absence of TNFR2 in our cells could explain why TNFR1 did not affect the regulation of P-gp activity by TNF- $\alpha$ . On the other hand,



inhibition of TLR4 resulted in an attenuation in P-gp activity (Figure 4(c)), and an involvement of this toll-like receptor is in agreement with previous findings in brain capillaries [8].

Since the transcription factor NF- $\kappa$ B is the downstream effector of TNF- $\alpha$  signalling in brain capillaries [7], we wondered whether the same holds true for the regulation of P-gp activity by TNF- $\alpha$  in this proximal tubular cell line. GERP cells were exposed for 24 hours to the selective I kappa B kinase (IKK) $\beta$  inhibitor, IMD-0354 (Figure 5(a)), or SN50 (Figure 5(b)). Remarkably, IMD-0354 alone gave a significant upregulation of P-gp activity compared to control cells (Figure 5(a),  $P < .01$ ), indicating that the compound itself has an effect on P-gp. An alternative explanation would be that IMD-0354 may have potentiated the responses to TNF- $\alpha$  and, therefore, NF- $\kappa$ B inhibits P-gp activity and blockade of nuclear translocation of this transcription factor leads to enhancement of TNF- $\alpha$  induction. Exposing the cells to the combination of SN50, a cell-permeable inhibitor peptide that interferes with NF- $\kappa$ B nuclear translocation, and TNF- $\alpha$  slightly reversed the increase in P-gp activity upon TNF- $\alpha$  treatment (Figure 5(b) NS), suggesting a role for the transcription factor. Although higher SN50 concentrations have been used before [7] this was not feasible in our cell system due to solubility problems. Hence, a role for NF- $\kappa$ B was demonstrated previously after detrimental stimuli. Thevenod et al. showed that an upregulation of P-gp by NF- $\kappa$ B is potentially important for protecting renal proximal tubule cells from apoptosis induced by cadmium- and reactive oxygen species (ROS) [9]. In addition, an increase in P-gp expression and/or activity by TNF- $\alpha$  was reported for primary rat hepatocytes [27], a rat hepatoma cell line [28] and mouse liver [29], which are also NF- $\kappa$ B dependent [28]. Furthermore, an NF- $\kappa$ B binding site was found in the promoter region of the Abcb1 gene [30]. Therefore, we suggest that NF- $\kappa$ B might have a central role in the signal transduction mechanism and could be involved in two distinct signalling pathways of P-gp. This nuclear factor is probably activated directly by proinflammatory cytokines and NO, and indirectly via TLR4. However, more research is needed to further unravel the regulation mechanism of P-gp in the GERP cell line and the inflammatory response on the activity of the efflux pump.

#### 4. Conclusions

In accordance with previous rat endotoxemia studies, the upregulation of P-gp expression and activity after exposure to LPS in combination with TNF- $\alpha$  in the cultured renal proximal tubule cells are under influence of NO produced by iNOS. On the other hand, the signaling pathway of TNF- $\alpha$  alone leading to P-gp upregulation seems to involve TLR4 activation and NF- $\kappa$ B translocation, which results in *de novo* synthesis of P-gp. Figure 6 summarizes the sequence of events. Our findings indicate that at least two different pathways regulate P-gp in renal proximal tubule cells during endotoxemia.

#### Acknowledgment

The first and last authors were supported by a grant from the Netherlands Organisation for Scientific Research (NWO).

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