TNF induces caspase-dependent inflammation in renal endothelial cells through a Rho- and myosin light chain kinase-dependent mechanism

Xiaoyan Wu, Rongqing Guo, Peili Chen, Quan Wang, and Patrick N. Cunningham

Section of Nephrology, University of Chicago, Chicago, Illinois

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Wu X, Guo R, Chen P, Wang Q, Cunningham PN. TNF induces caspase-dependent inflammation in renal endothelial cells through a Rho- and myosin light chain kinase-dependent mechanism. Am J Physiol Renal Physiol 297: F316–F326, 2009. First published May 6, 2009; doi:10.1152/ajprenal.00089.2009.—The pathogenesis of LPS-induced acute kidney injury (AKI) requires signaling through tumor necrosis factor-α (TNF) receptor 1 (TNFR1), which within the kidney is primarily located in the endothelium. We showed previously that caspase inhibition protected mice against LPS-induced AKI and in parallel significantly inhibited LPS-induced renal inflammation. Therefore we hypothesized that caspase activation amplifies TNF-induced inflammation in renal endothelial cells (ECs). In cultured renal ECs, TNF induced apoptosis through a caspase-8-dependent pathway. TNF caused translocation of the p65 subunit of NFκB to the nucleus, resulting in upregulation of inflammatory markers such as adhesion molecules ICAM-1 and VCAM-1. However, the broad-nucleus, resulting in upregulation of inflammatory markers such as adhesion molecules ICAM-1 and VCAM-1. Therefore we hypothesized that caspase activation amplifies TNF-induced inflammation in renal endothelial cells (ECs). In cultured renal ECs, TNF induced apoptosis through a caspase-8-dependent pathway. TNF caused translocation of the p65 subunit of NFκB to the nucleus, resulting in upregulation of inflammatory markers such as adhesion molecules ICAM-1 and VCAM-1. However, the broad-spectrum caspase inhibitor Boc-d-fmk reduced NFκB activation as assessed by gel shift assay, reduced phosphorylation of subunit IκBα, and significantly inhibited TNF-induced expression of ICAM-1 and VCAM-1 as assessed by both real-time PCR and flow cytometry. Broad-spectrum caspase inhibition markedly inhibited neutrophil adhesion to the TNF-activated endothelial monolayer, supporting the functional significance of this effect. Specific inhibitors of caspases-8 and -3, but not of caspase-1, reduced TNF-induced NFκB activation. Caspase inhibition also reduced TNF-induced myosin light chain (MLC)-2 phosphorylation, and activation of upstream regulator RhoA. Consistent with this, MLC kinase (MLCK) inhibitor ML-7 reduced TNF-induced NFκB activation. Thus caspase activation influences NFκB signaling via its effect on cytoskeletal changes occurring through RhoA and MLCK pathways. These cell culture experiments support a role for caspase activation in TNF-induced inflammation in the renal endothelium, a key event in LPS-induced AKI.

adhesion molecules; cytokines; sepsis; acute kidney injury; neutrophil

Sepsis is one of the leading causes of mortality in the intensive care unit, and it continues to be a challenge to clinical investigators. In patients with severe sepsis the incidence of acute renal injury ranges from 24% to 51%, and the combination of severe sepsis and acute renal injury is associated with 70% mortality (67). Despite advances in medical practice in the past decades, the pathophysiology of sepsis-induced acute renal injury is incompletely understood, and the mortality remains high.

Many of the clinical findings of sepsis, including acute kidney injury (AKI), may be replicated in animal models by the administration of lipopolysaccharide (LPS), a component of the outer cell membrane of gram-negative bacteria. A key mediator of sepsis is tumor necrosis factor-α (TNF). In prior work, mice deficient in its main receptor TNFR1 were found to be strongly resistant to LPS-induced AKI, and renal cross-transplantation experiments showed that much of the effect of TNF signaling was operative within the kidney (11). TNF is well known to mediate many inflammatory events, such as the expression of cytokines, chemokines, and adhesion molecules, through the activation of the NFκB pathway (24). The translocation of the p65 subunit of NFκB to the nucleus leads to the transcription of many inflammatory genes and plays a key part in the recruitment of leukocytes to the kidney.

Independently, TNF may also lead to apoptosis through the receptor-mediated pathway of apoptosis, in which the TNFR1 adaptor molecule FADD allows activation of caspase-8 and its downstream effector, caspase-3. While apoptosis within the kidney has been detected in models of AKI including LPS, ischemia-reperfusion, and cisplatin, the extent of apoptosis is modest, perhaps insufficient to explain the associated loss of renal function (13, 14, 22, 57). Pharmacological caspase inhibitors have been found to be protective against both endotoxemic and ischemia-reperfusion-mediated AKI, as well as in other models of organ injury (13, 22). A consistent feature found in these studies is that caspase inhibition not only blocks apoptosis as expected, but also had strong anti-inflammatory effects. This is somewhat surprising, because apoptosis has classically been understood to be a noninflammatory process, at least relative to necrosis (42).

In considering the possible mechanisms of the effects of TNF in LPS-induced AKI, it should be noted that most TNF is found in the kidney is predominantly expressed in the endothelium (1, 2). The fundamental function of the kidney, generation of glomerular filtrate, is obviously dependent on intact flow through the renal vasculature, and several authors have argued for the importance of the renal endothelium in AKI (48, 64). In prior work (22), we noted that LPS induces apoptosis not only within renal tubules but also in the renal endothelium. Other evidence of injury within the renal endothelium in sepsis includes the adhesion of neutrophils to the activated endothelium, under the influence of adhesion molecules such as ICAM-1 (56, 70), and an increase in vascular permeability (71). Given the occurrence of both apoptosis and inflammation in the renal endothelium during AKI, the endothelium is a highly relevant site to study the interaction between these two important phenomena. We had previously hypothesized that much of the protective effect of caspase inhibition is due to its anti-inflammatory, as opposed to its antiapoptotic, effects. Thus in the studies described below we test the hypothesis that caspase inhibition in renal endothelial cells (ECs) inhibits TNF-induced adhesion molecule expression.
MATERIALS AND METHODS

Primary culture of renal cells. As a modification of a previously reported protocol (20), kidneys from C57BL/6 mice were minced, collagenase digested, and incubated with biotinylated rat anti-mouse CD31 MAb (Abcam, Cambridge, MA), followed by additional incubation with MACS beads coated with anti-CD31 Ab (Miltenyi Biotec, Auburn, CA). After washing, bead-bound cells were selected by passing cells through a MACS separation column (Miltenyi Biotec) under a magnetic field. ECs were grown first on coated (2% gelatin, for freshly isolated cells) and then uncoated plastic flasks in DMEM (GIBCO BRL, Grand Island, NY) supplemented with 20% Fetal bovine serum (FBS), 1% nonessential amino acids, 2 mM sodium pyruvate, 0.5 g/l L-glutamine, heparin (100 μg/ml), endothelial cell growth support (100 μg/ml), 100 U/ml penicillin, and 100 mg/ml streptomycin. These cells were characterized as ECs by various morphological and biochemical characteristics, including formation of tubelike structures when grown in Matrigel and uptake of oxidized LDL (not shown). All experiments were performed on cells from passages 2–10. As controls in some experiments, a previously characterized line of proximal tubular cells (PTECs, a generous gift of Dr. John Schwartz, Boston University) were grown in DMEM supplemented with 10% FBS, 100 U/ml penicillin, and 100 mg/ml streptomycin (Cellgro, Kansas City, MO) at 37°C (61). All experiments with caspase inhibitors used a concentration of 50 μM, other than the dose-response experiments shown in Fig. 5. Caspase inhibitors were given 2 h before TNF exposure in all experiments. Harvest of endothelial and bone marrow cells from mice was done according to a protocol that was reviewed and approved by the University of Chicago Institutional Animal Care and Use Committee.

Flow cytometry. Cells (5 × 10⁵) were detached with Accutase (Innovative Cell Tech, San Diego, CA) and suspended in buffer (PBS, 1% BSA, 0.1% sodium azide) at 4°C. Without permeabilization, cells were incubated with primary Abs [hamster anti-mouse ICAM-1 (clone 3E2) or rat anti-mouse VCAM-1 (clone 429), both from BD Pharmingen (San Diego, CA)] at a 1:100 dilution for 30 min. After subsequent washing, samples were subjected to FACSCanto (Becton Dickinson, San Jose, CA) analysis immediately. Signals from 10,000–30,000 cells were gated on forward scatter/side scatter parameters to exclude cell aggregates and debris. Data analysis was performed with FlowJo software (Ashland, OR) and displayed as one-parameter histograms, with fluorescence intensity on the logarithmic x-axis versus percentage of total cell counts on the y-axis. In double-staining experiments in which apoptosis and VCAM-1 expression were examined simultaneously, cells were first stained with anti-VCAM-1 as described above followed by annexin V in binding buffer, for 15 min each. Data are displayed as a two-parameter dot plot displaying FL1-FITC (annexin V) on the x-axis and FL2-APC (VCAM-1) on the y-axis, using logarithmic scales.

Isolation of neutrophils. Neutrophils were collected from mouse bone marrow via titanium. Cells were isolated with a discontinuous Percoll step gradient (52%/65%/75%) and centrifuged at 1,200 g for 40 min at room temperature (RT) without braking. An enriched neutrophil population was recovered at 55%/75% interface. These dyes are taken up and retained by live cells throughout the cytoplasm (15, 69). ECs and isolated neutrophils were labeled separately with red or green calcein-AM (2 μg/ml, Molecular Probes, Eugene, OR) at RT for 30 min in the dark. These cells were then washed separately in HBSS-1% BSA-0.1% Na azide. For each coverslip, 1 × 10⁶ calcein green-stained neutrophils were plated onto the calcein red-stained endothelial monolayer. After 2 h, the coverslips were gently washed with HBSS for 10 min, and the binding of neutrophils to the endothelial monolayer was determined by taking 10 photomicrographs at random with an Olympus 1x81 DSU spinning disk confocal microscope (magnification ×100) and counting the number of green cells. Neutrophils overlapping the rare areas where the ECs were not confluent were excluded.

Real-time PCR. Total RNA was isolated and purified from cells with the RNeasy mini RNA purification kit (Qiagen, Valencia, CA). Five micrograms of renal EC RNA was used to generate cDNA with random hexamer primers and the SuperScript first-strand synthesis kit (Invitrogen, Carlsbad, CA). PCR was performed in a Prism 7700 Sequence Detection System (Applied Biosystems, Carlsbad, CA) at 95°C (5 min) followed by 45 cycles at 95°C (15 s)/60°C (30 s). SYBR Green intercalating dye was used for signal detection. For each sample, the number of cycles required to generate a given threshold signal (Ct) was recorded. With a standard curve generated from serial dilutions of sample cDNA, the ratio of ICAM-1 or VCAM-1 expression relative to GAPDH expression was calculated for each experimental group and normalized relative to an average of ratios from the control group. Sequences of the primers used in this study were as follows: ICAM-1 forward primer 5'-GCC AAG TTC TCA AAC CAC GGA-3'; ICAM-1 reverse primer 5'-CAG AGC GGC AGA GCA AAA G-3'; VCAM-1 forward primer 5'-CTG GAA CCT CTT GCA CTC TA-3'; VCAM-1 reverse primer 5'-TGT GCC TCC ACC AGA CTG TA-3'; and GAPDH forward primer 5'-GCC AAA TTC AAC GGC ACA GT-3', GAPDH reverse primer 5'-AGA TGG TGA TGG GCT TCC C-3'.

Ligase-mediated-PCR. Genomic DNA (0.5 μg) from renal ECs was isolated and purified with a commercial kit (Zymo Research, Orange, CA). The DNA was annealed to primer targets via T4 DNA ligase as previously described (11). The ligated DNA template was amplified by PCR for 26 cycles at 94°C (1 min)/72°C (3 min) to better show aptoptic DNA laddering. The reaction product was resolved on a 1.3% agarose gel, and ethidium bromide-stained bands were detected under UV light. To standardize the amount of DNA template that was used, standard PCR to the gene En-2 was performed with specific primers (not shown).

Electrophoretic mobility shift assay. ECs were grown on 10-cm² petri dishes until 90% confluence. Cells were treated with TNF (20 ng/ml, 4 h) with or without pretreatment with caspase inhibitors (50...
In a separate experiment, ECs were given myosin light chain (MLC) kinase (MLCK) inhibitor ML-7 (100 nM) 1 h before TNF stimulation. Nuclear proteins were extracted as previously described (26). Briefly, cells were suspended in lysis buffer A (mM: 10 HEPES, pH 7.9, 10 KCl, 0.1 EDTA, 0.1 EGTA, and 1 DTT, with 0.6% NP-40) with Roche Protease Inhibitor Cocktail tablet for 30 min at 4°C. After vigorous vortexing the mixture was spun down for 30 min at 14,000 g and 4°C, and the pellets were resuspended in 100 μl of protein extraction buffer C (mM: 20 HEPES, pH 7.9, 1 EDTA, 1 EGTA, and 1 DTT, with 0.4 M NaCl) with protease inhibitors for 1 h at 4°C. After centrifugation, the supernatant was collected and protein concentration was measured with bicinchoninic acid (BCA) protein assay reagent (Pierce, Rockford, IL). Double-stranded NF-κB consensus oligonucleotide (Santa Cruz Biotechnology, Santa Cruz, CA) was 3'-biotinylated with a commercial biotin 3'-end labeling kit (Pierce). Standard electrophoretic mobility shift assay (EMSA) reactions were conducted with an EMSA accessory kit (Novagen, Gibbstown, NJ) with 10 μg of cell nuclear extract and 30 fmol of biotin end-labeled DNA in a 18-μl volume binding reaction per sample, with 5 μl of EMSA buffer (mM: 400 KCl, 80 HEPES, and 0.1 EDTA, with 80% glycerol, pH 8.0, 1 μl of poly(dI-dC) (0.01 U/μl), and 1 μl of sonicated salmon sperm DNA (500 ng/μl)). EMSA reactions were carried out at RT for 30 min, terminated by adding 5 μl of 5× nucleic acid sample loading buffer (0.2% bromophenol blue, 0.2% xylene cyanol FF, 25% glycerol, 50 mM Tris-HCl, pH 8.0, 5 μM EDTA), and then separated on a 6% polyacrylamide gel. The gel was prerun at 100 V for 30 min and subsequently run at 100 V for 1 h after sample loading in 0.5× TBE buffer (89 mM Tris-acetate, 89 mM boric acid, 2 mM EDTA). Reaction products were then transferred to a Biodyne B nylon membrane (Pierce) at 100 V for 1 h and fixed by UV cross-linking. The biotin-labeled reaction products were then visualized by incubation with streptavidin-horseradish peroxidase (HRP) conjugate and subsequent incubation with SuperSignal West Pico chemiluminescence reagents (Pierce).

IκBα ELISA. Phosphorylation of IκBα in whole cell lysate was measured with the IκBα ActiviELISA Kit (Imgenex, San Diego, CA) according to the manufacturer's instructions. After treatment with TNF and/or Boc-d-fmk, cells were lysed in complete lysis buffer provided by the manufacturer. Lysates and standards were applied to a 96-well plate previously coated with MAb to phosphorylated IκBα (Ser32/36) for 4 h at 4°C followed by blocking in 1% BSA at RT. After washing in Tris-buffered saline-Tween 20 (TBST), a biotinylated detection MAb (1:200) was added for 1 h at RT, followed by washing and streptavidin-HRP (1:1,000) for 1 h at RT. After further washing, luminescent substrate was added at RT for 20 min. The plate was read on a microplate luminometer (BioTek Instruments, Winooski, VT). The standard curve was generated with serial dilutions of phosphorylated IκBα from the manufacturer ranging from 0 to 10 ng/ml.

Western blotting. After treatment with TNF and/or caspase inhibitors, ECs were scraped from plastic six-well plates in lysis buffer (PBS, 1% 0.5% sodium deoxycholate, 0.1% SDS) supplemented with protease inhibitors (Roche Complete Protease Inhibitor Cocktail tablets) and phosphatase inhibitors (100 mM cayculin, 1 μM microcystine, 10 mM NaF, 1 mM glycerol phosphate) at 4°C. Protein concentration for each sample was determined by the BCA method. Forty micrograms of proteins was run in each lane on an 8% SDS-PAGE gel and transferred onto polyvinylidene difluoride (PVDF) membrane at 100 V for 2 h at 4°C. Membranes were then blocked in 5% milk-TBST and probed with rabbit anti-human MLC, rabbit anti-human diphospho-MLC (Thr18/Ser19) (see Fig. 7A), or mouse anti-phospho-MLC (Ser19) MAb (see Fig. 7B) (Cell Signaling, Danvers, MA; 1:500, 1:500, and 1:1,000, respectively) overnight at 4°C and then with HRP-anti-rabbit IgG or anti-mouse IgG (1:5,000) at RT for 1 h after washing. Blots were visualized by chemiluminescence with the SuperSignal West Pico system (Pierce). In parallel, Western blotting to β-actin was used as a control to ensure equal protein loading.

Immuno-fluorescent staining. Renal EC were cultured to confluence on glass coverslips. After treatment with TNF and/or caspase inhibitors, EC monolayers were fixed in 3% paraformaldehyde for 20 min, washed with PBS-Tween (0.1%), and permeabilized with 0.2% Triton X-100 for 2 min, followed by blocking with 2% BSA for 1 h. Cells were stained with Texas red-phalloidin (1:40) (Invitrogen) for 45 min at room temperature, washed with PBS-Tween (0.1%), and stained with DAPI (500 nM) for another 5 min. The coverslips were mounted on slides and examined with a Leica SP2 AOBs confocal microscope (oil-immersion, ×60 objective).

Rho activation assay. RhoA activity was measured by a pulldown of GTP-bound RhoA using the Rho activation assay kit (Millipore, Billerica, MA), in which a fusion protein consisting of the RhoA-binding domain (RBD) of rhoetkin fused to glutathione S-transferase (GST) was used to affinity precipitate GTP-bound RhoA. Briefly, ECs were serum starved for 2 h in 1% FBS medium, followed by stimulation with TNF (20 ng/ml) at time periods of 1 min to 3 h before rapid lysis in MLB buffer at 4°C (mM: 25 HEPES pH 7.5, 150 NaCl, 10 MgCl2, and 1.0 EDTA, with 1% Igepal CA-630 and 2% glycerol, supplemented with protease inhibitors). In parallel, Boc-d-fmk (50 μM) was applied 2 h before TNF stimulation. Cell lysates were centrifuged at 14,000 g for 10 min at 4°C, and the supernatants were collected for measurement of protein via the BCA method. For each sample, 450 μl of supernatant was incubated with 25 μl of GST-RBD beads at 4°C for 45 min to precipitate GTP-bound RhoA. After centrifugation and washing with MLB lysis buffer three times, samples were resuspended in 30 μl of protein sample buffer (50 mM Tris-Cl pH 6.8, 200 mM mercaptoethanol, 2% SDS, 10% glycerol, 0.1% bromophenol blue) and boiled for 5 min to release bound RhoA. Samples were run on an 8% SDS-PAGE gel and transferred to a PVDF membrane. RhoA was probed by Western blotting using a monoclonal anti-RhoA Ab provided by the manufacturer. To adjust for equal loading, the membranes were blotted for β-actin in parallel.

Statistical analysis. Data were analyzed with Minitab software (State College, PA). Unless noted otherwise, data are given as means ± SE. Groups were compared by two-tailed t-test, or ANOVA when more than two groups were compared. A P value ≤0.05 was considered significant.

RESULTS

Induction of apoptosis within ECs. To determine the sensitivity of renal ECs to TNF, we tested the induction of apoptosis. TNF induced DNA laddering in renal ECs, as detected by ligase-mediated PCR (Fig. 1A). This occurred in a time-dependent fashion, peaking at ≈8 h after TNF exposure. As expected, renal EC apoptosis after 4 h of TNF exposure was strongly inhibited by pretreatment with the broad-spectrum caspase inhibitor Boc-d-fmk, as well as caspase-3 inhibitor z-DQMD-fmk. A partial decrease in TNF-induced apoptosis was seen with both caspase-8 inhibitor z-IETD-fmk and z-DQMD-fmk. A partial decrease in TNF-induced apoptosis was observed in prior animal work, we measured expression of adhesion molecules ICAM-1 and VCAM-1 in renal ECs. Both ICAM-1 and VCAM-1 mRNA were detected at a low level at baseline in unstimulated cells, as measured by real-time PCR.
mRNA expression of both adhesion molecules was markedly increased by TNF, beginning at 4 h (Fig. 2) and increasing further by 24 h (not shown). Pretreatment with broad-spectrum caspase inhibitor Boc-D-fmk significantly inhibited TNF-induced ICAM-1 and VCAM-1 mRNA expression (52% and 27% decrease vs. TNF vehicle group, respectively, \( P < 0.05 \); Fig. 2A). Cell surface expression of ICAM-1 and VCAM-1 was measured by flow cytometry to see whether this effect was seen at the level of protein expression. Indeed, while TNF induced an increase in ICAM-1 and VCAM-1 expression on renal ECs, this was significantly inhibited by broad-spectrum caspase inhibition (Fig. 2B). Thus TNF-induced adhesion molecule expression in cultured renal ECs exhibited the same caspase dependence as was observed in vivo.

**Caspase inhibitors block TNF-induced neutrophil adhesion.** We previously demonstrated (70) a possible role for influx of neutrophils into the kidney in the setting of LPS-induced AKI. To determine whether caspase inhibition was able to functionally inhibit neutrophil adhesion, we performed an assay of static neutrophil-EC monolayer adhesion, modeled after Hauser et al. (23). Neutrophils were obtained from mouse bone marrow by Percoll gradient centrifugation, and the purity of this population was confirmed by their strong staining with MAbs 7/4 and Gr-1 (Fig. 3A). After neutrophils and confluent ECs were marked for 4 h, TNF exposure is shown relative to baseline cells without TNF or caspase inhibitor. B: caspase inhibition also significantly inhibited TNF-induced EC surface expression of ICAM-1 and VCAM-1 at 24 h, as determined by flow cytometry (log scale). TNF-treated cells are shown in blue and cells treated with TNF and Boc-D-fmk are shown in green. Control group (red) was untreated with either TNF or Boc-D-fmk. A group treated with Boc-D-fmk alone is omitted for clarity but was similar to the control group. *\( P < 0.05 \) compared with TNF vehicle group. \( n = 6 \) per group for RT-PCR, \( n = 5 \) per group for flow cytometry.
with green and red calcein cytoplasmic dyes, respectively, neutrophils were placed onto the confluent renal EC monolayer for 2 h and then washed, as previously described (23). In this assay, prior TNF administration to the endothelial monolayer strongly increased neutrophil-EC adhesion (Fig. 3, B and C). This TNF-induced increase in neutrophil adhesion was significantly inhibited by pretreatment of the renal ECs with the broad-spectrum caspase inhibitor Boc-D-fmk (69% decrease vs. TNF/vehicle group, \( P < 0.01 \); Fig. 3D). Similarly, anti-ICAM-1 neutralizing MAb YN1.1 almost completely blocked TNF-induced neutrophil adhesion (97% decrease vs. TNF + vehicle group, \( P < 0.001 \); Fig. 3E), while anti-VCAM-1 neutralizing MAb had a weaker, nonsignificant effect (\( P = 0.16 \)). This confirmed that it is principally TNF-mediated ICAM-1 expression mediating neutrophil attachment in this assay, lending functional relevance to the partial caspase dependence of TNF-mediated ICAM-1 expression.

**Coincidence of apoptosis and adhesion molecule expression.**

One key transcription factor mediating the expression of adhesion molecules is NF-κB, which also inhibits apoptosis by inducing the transcription of multiple antiapoptotic genes such as IAP and XIAP (16, 62). Thus we asked the question whether two distinct renal EC subpopulations were undergoing either adhesion molecule expression or apoptosis. As an index of inflammation, we measured VCAM-1 expression by flow cytometry, which although less relevant than ICAM-1 for neutrophil adhesion, is similarly regulated by NF-κB and was more potently induced by TNF as detected by flow cytometry. Simultaneously we measured apoptosis by staining with FITC-labeled annexin 5. At baseline, relatively few cells were apoptotic, although there was a bimodal distribution of VCAM-1 intensity, suggesting some expression of preformed VCAM-1 before TNF stimulation. After 2 h of TNF stimulation, VCAM-1 expression was more frequent than apoptosis, as detected by annexin 5 labeling. By 6 h after TNF expression, most cells were positive for both VCAM-1 expression and apoptosis, with relatively few cells undergoing apoptosis without VCAM-1 expression, or vice versa (Fig. 4). Thus we found little evidence that these two phenotypic responses of cultured renal ECs to TNF were distinct. Presumably individual cells first expressed greater adhesion molecules, and subsequently became apoptotic under continued TNF exposure.

**Effect of caspase inhibition on NF-κB activation.** Because multiple inflammatory genes such as ICAM-1 and VCAM-1 are induced through the NF-κB transcription pathway, we hypothesized that caspase inhibition influences NF-κB activity. To address this, EMSA (gel shift assay) was performed on nuclear protein extracts of renal ECs in response to TNF.
This confirmed that TNF induced a rapid if relatively brief B activation at this proximal step, whether caspases affect NF-κB where it acts as a transcription factor (7, 21, 51). To determine and degradation, freeing the p65 subunit to enter the nucleus, once phosphorylated IκB is targeted for polyubiquitination and degradation, freeing the p65 subunit to enter the nucleus, where it acts as a transcription factor (7, 21, 51). To determine whether caspases affect NF-κB activation at this proximal step, we measured phosphorylated IκBα by ELISA in renal ECs. This confirmed that TNF induced a rapid if relatively brief increase in the phosphorylated form of IκBα in renal ECs (Fig. 6). However, pretreatment with broad-spectrum caspase inhibitor significantly prevented this increase in phosphorylated IκBα, showing that caspase inhibition limits TNF-induced IKK activity.

**Relationship between caspases, cytoskeletal changes, and NF-κB activation.** Although other authors have noted that NF-κB signaling is dependent on caspase-8, they have described an effect that appears independent of enzymatic activity (30, 60, 63), which is not consistent with our results above. However, Wadgaonkar et al. (66) have reported that TNF-induced NF-κB activation in bovine pulmonary ECs is dependent on the nonmuscle isoform of MLCK (nmMLCK), likely due to phosphorylation of its target MLC and subsequent changes in the endothelial cytoskeleton. In turn, TNF has been shown to cause increased MLC phosphorylation, through pathways involving the activation of both MLCK and monomeric G protein RhoA (52, 54). Consistent with this possible mechanism, we confirmed by Western blot that TNF induced an increase in the phosphorylated form of MLC in renal ECs (Fig. 7A), occurring in parallel with actin stress fiber formation, as has been described by others (Fig. 7E) (54). Treatment with a broad-spectrum caspase inhibitor strongly inhibited this increase in phosphorylated MLC (Fig. 7B). In addition to phosphorylation by nmMLCK, MLC phosphorylation in bovine pulmonary ECs has also been shown to be driven by inhibition of MLC phosphatase by Rho kinase, downstream of RhoA(33(475,886),(501,907), 53). To assess involvement of these upstream regulators of TNF-induced MLCK activation, a pulldown assay of active RhoA was performed. This confirmed rapid activation of RhoA by TNF, which was inhibited by caspase inhibitor Boc-d-fmk (Fig. 7, C and D). Consistent with its effect on MLC phos-

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**Fig. 4.** Coexpression of adhesion molecules and apoptosis. Flow cytometry shows that at baseline there was moderate VCAM-1 expression and a small extent of apoptosis in cultured renal ECs, as assessed by annexin 5 staining (control; left). Two hours after exposure to TNF (20 ng/ml), VCAM-1 expression increased more than apoptosis (center). By 6 h after TNF (20 ng/ml), most ECs simultaneously expressed VCAM-1 and stained with annexin 5, with relatively few cells positive for VCAM-1 or annexin 5 alone (right). Gates were defined by control ECs where primary Abs and annexin V were omitted.

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**Fig. 5.** Effect of caspase inhibition on TNF-induced NF-κB activation. Renal ECs were treated with TNF for 4 h, with or without pretreatment with broad-spectrum caspase inhibitor Boc-d-fmk (50 μM). Nuclear protein was extracted and subjected to gel shift assay using a labeled probe for the NF-κB p65 subunit of NF-κB at serine 32 and 36 by IκB kinase (IKK). Use of caspase-8 inhibitor z-IETD-fmk at a range of concentrations showed a dose-response effect, supporting the specificity of caspase-8 and -3.

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**Fig. 6.** An upstream event leading to translocation of the NF-κB p65 subunit to the nucleus is phosphorylation of the IκBα subunit of NF-κB by TNF, which was inhibited by caspase inhibitor Boc-d-fmk. In contrast, TNF markedly increased nuclear translocation of the p65 subunit of NF-κB. This NF-κB activation was partially but significantly attenuated by broad-spectrum caspase inhibition (Fig. 6A). Interestingly, PTECs also showed NF-κB activation on gel shift assay when incubated with TNF, but caspase inhibition attenuated this to a negligible degree, unlike in renal ECs (Fig. 6B). Use of several specific caspase inhibitors showed that TNF-induced NF-κB activation in renal ECs was also reduced by caspase-3 and -8 inhibitors (Fig. 6C). Use of caspase-8 inhibitor z-IETD-fmk at a range of concentrations showed a dose-response effect, supporting the specificity of this agent (Fig. 6D). In contrast, NF-κB activation was not strongly affected by Ac-YVAD-CHO, an inhibitor of caspase-1 (Fig. 6B). This is consistent with the fact that caspase-1 has a weaker role in activating downstream effector caspases or inducing apoptosis (38, 39). Thus the influence of caspase activation on the NF-κB pathway in the setting of TNF signaling seems to be mediated through caspase-8 and its target caspase-3.

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**Fig. 7.** Sequence of events leading to augmentation of Rho and myofilament changes in the endothelial cytoskeleton. **A:** TNF induced strong NF-κB activation, with no clear dose-response effect. In contrast, use of caspase-8 inhibitor z-IETD-fmk at various doses did show a dose-response effect, with greatest potency at and above 50 μM.
phorylation, caspase inhibition also strongly inhibited TNF-induced aggregation of actin stress fibers in renal ECs as visualized by phalloidin staining, the functional event immediately downstream of MLC activation (Fig. 7E). Demonstrating this linkage of TNF-induced cytoskeletal changes to TNF-induced NF-κB signaling, use of ML-7, a specific inhibitor of MLCK, partially prevented TNF-induced NF-κB activation after 1 h (Fig. 7F), similar to what was seen with caspase inhibition. Together, these findings suggest that TNF-induced caspase activation leads to activation of RhoA and MLCK, MLC phosphorylation, and subsequent cytoskeletal changes, which ultimately influence TNF-induced activation of NF-κB.

DISCUSSION

Caspases have a well-demonstrated role in the initiation and execution of apoptosis, but their role in inflammation has been only appreciated more recently. Apoptosis has classically been thought to be a noninflammatory process. Unlike the disordered process of necrosis, in which cell membranes are broken, discharging cell contents into the surroundings, apoptosis is an orderly, energy-dependent process whereby cells shrink and are quickly taken up by neighboring nonapoptotic cells (28). Generally, apoptosis has a role in growth and development, as well as in homeostasis of the adaptive immune response. In fact, apoptosis of neutrophils and other leukocytes has a clear role in turning off the process of inflammation (5). However, a variety of studies, including models of ischemic as well as endotoxic AKI, have shown that inhibition of caspases not only protects against end-organ damage, but also offers considerable protection against the process of inflammation (10, 12–14, 22). Our data show that the inhibition of adhesion molecule expression and neutrophil adherence seen in vivo occurs within the signaling apparatus of renal ECs, independent of leukocytes or other intercellular interactions within the intact organism.

Expression of adhesion molecules such as ICAM-1 on the endothelium is a key event that leads to the attraction and adherence of leukocytes to areas of inflammation. The importance of ICAM-1 to the recruitment of neutrophils in renal ischemia-reperfusion and in endotoxin-induced AKI has been demonstrated in animal models, so the relevance of this phenomenon is high (32, 70). Arguably the most important mechanism by which cytokines such as TNF lead to inflammation is activation of the NF-κB pathway. TNFR1 signals through its adapter proteins TRADD, TNF receptor-associated factor (TRAF)-2, and receptor-interacting protein (RIP) to cause...
phosphorylation of the IkBα subunit of NF-κB, leading to its polyubiquitination and degradation. This frees the p65 subunit to translocate to the nucleus, where it induces the transcription of a wide host of genes including ICAM-1 as well as VCAM-1 (7, 21). While the fact that TNF activates caspases is well known, the NF-κB pathway was classically thought to be independent of caspase activation. Here we have conclusively shown in renal ECs that caspase enzyme activity enhances TNF-induced ICAM-1 and VCAM-1 expression, at the level of both transcription as well as cell surface expression. Furthermore, caspase inhibition not only reduced renal EC ICAM-1 expression but strongly prevented adherence of neutrophils to the activated endothelial monolayer, justifying the relevance of this pathway to leukocyte adhesion to renal endothelium. Blockade of this with anti-ICAM-1 Ab showed that this adherence was largely ICAM-1-dependent. While some reports have described VCAM-1-mediated neutrophil adhesion through its ligand very late antigen-1 (58), VCAM-1 is typically thought to mediate monocyte and lymphocyte adhesion, and thus the lack of effect on neutrophils with anti-VCAM-1 Ab was unsurprising. This finding that caspases affect endothelial-leukocyte adhesion may help provide a mechanism for observations in animal studies that caspase inhibitors reverse LPS-induced impairment in peritubular capillary perfusion, as has been reported by Tiwari et al. (65).

Although other authors have shown a stimulatory role for caspase-8, as well as caspases-10 and -2, in lymphocyte proliferation and/or NF-κB activation in response to activation of the T cell receptor (TCR) and/or stimulation by TNF or LPS, the mechanism has remained unclear (30, 36, 60, 63). Consistent with this, humans lacking caspase-8 have impaired proliferation and activation of T, B, and NK cells (30). Many of these reports show that this effect occurs with minimal or no caspase-8 cleavage or activity, and it has been proposed that the prodomain of inactive caspase-8 has a role in aggregating the IKK/NF-κB signaling complex near the TCR, Toll-like receptor (TLR) 4, or TNFR1 receptor complexes (30, 34, 37, 59, 63). Our findings in ECs that demonstrated less NF-κB activation with the use of caspase inhibitors shows that the effect on IKK activity depends not merely on caspase expression but on caspase enzymatic activity, through a TNF/caspase-8/caspase-3-dependent pathway. Nonspecific effects of pharmacological inhibitors are always a potential concern, but we observed a significant reduction in NF-κB activation with broad-spectrum caspase inhibitors as well as both caspase-8 and caspase-3 inhibitors. Although programmed cell death is a complex process, we observed effects on IkBα phosphorylation within 5 min, likely too soon for the downstream morphological events of apoptosis to occur, so it appears this effect is truly dependent on caspase activation per se.

The evidence described above that caspases may enhance NF-κB signaling in ECs is all the more notable considering that other investigators have shown that caspase-mediated cleavage of TRAF-1, NIK, and IKK complex components may serve to turn off NF-κB signaling and push the cell toward apoptosis in other cell types (19, 25, 43, 44). While various reports have previously noted that TNF can activate RhoA in the setting of changes in morphology of endothelial cells, the mechanism has been poorly characterized. We found that TNF induced rapid (1 min) but transient activation of RhoA, consistent with the time course described in several other reports (27, 31, 47). Our results are consistent with a role for TNF-induced caspase activation upstream of RhoA activation. Notably, the protein GDP dissociation inhibitor D4-GDI may serve as an inhibitor of Rho activity and has been reported to be a caspase substrate (17, 35). Caspase-induced cleavage and inactivation of this or related accessory proteins could possibly explain the mechanism whereby TNF activates RhoA. RhoA activation leads to activation of Rho kinase, which phosphorylates MLC phosphatase, inactivating it and thereby increasing MLC phosphorylation. Cleavage by caspase-3 has also been shown to activate Rho kinase directly (8). Phosphorylated MLC is able to use its ATPase activity to drive important cytoskeletal changes including the reorganization of cytoplasmic actin into stress fibers. The other major agent of MLC phosphorylation, MLCK, is also activated by TNF. The mechanism for this is not clearly known, but may be dependent on tyrosine phosphorylation of the nonmuscle isoform of MLCK by p60-cSrc by TNF (4). It is also possible that MLCK activity may be directly enhanced by activated caspase-3 (52). Together, both MLCK and RhoA/Rho kinase lead to enhanced phosphorylation of its substrate MLC, which drives multiple complex changes in the actin cytoskeleton. Consistent with the above mechanisms, we observed that caspase blockade strongly prevented this TNF-induced aggregation of actin stress fibers in renal ECs, which is the likely mechanism connecting caspases to NF-κB signaling. Wadgaonkar et al. (66) have previously shown in bovine pulmonary ECs that MLCK activity enhances TNF-induced NF-κB activation in parallel with changes in the actin cytoskeleton, leading to an increase in IKK activity as described above. It was proposed that this could occur via an improvement in trafficking of TNFR1 from the golgi to the cell membrane, as has been described by Jin et al. (29), and/or alterations in NF-κB trafficking, since IkBα contains ankyrin repeats that could mediate interactions with actin stress fibers. Alternatively, the p65 subunit has also been shown to interact with actin-containing structures (3). A similar process in which cytoskeletal events enhance upstream apoptotic TNFR1-mediated signaling has also been described in bovine pulmonary ECs (53, 54). It is not clear why we found that caspase activation influences NF-κB signaling in renal ECs, but not in PTECs. This could possibly be due to a selective role for nmMLCK, which is mainly expressed in ECs (66), or due to differences in the cytoarchitectures of these two cell types. Our paradigm for the possible mechanisms by which caspase activation could enhance TNF-induced NF-κB signaling is summarized in Fig. 8.

Our findings do not rule out other possible mechanisms by which caspases could enhance inflammatory signaling. As one example, it has been demonstrated that caspase-3 may mediate cleavage of upstream MAP kinase MEKK1, leading to a constitutively active fragment that not only activates p38-MAPK, but also may play a role in NF-κB signaling (5, 40). As another example, a role for DNA repair enzyme poly(ADP-ribose) polymerase-1 (PARP-1) in NF-κB signaling has been shown in multiple studies. The cleavage of PARP-1 by caspase-3 not only inactivates the DNA repair machinery in the context of apoptotic DNA cleavage, but, in addition, caspase cleavage fragments of PARP-1 may serve as a coactivator at the NF-κB binding sequence. It is unclear whether enzymatic activity of PARP-1 may contribute to
this event (9, 49, 50, 55). However, our data showing an increase in IKK activity cannot be explained by this mechanism. Quite unlike the role that most caspases have in apoptosis, caspase-1 mainly serves to cleave IL-1β and IL-18 from their inactive precursors to their active forms (6, 39). Therefore caspase-1 could theoretically contribute to NF-κB activation though the autocrine action of IL-1β and IL-18 on cell surface receptors. However, we observed only weak reduction of TNF-induced NF-κB activation with caspase-1 inhibition; therefore this mechanism does not appear to explain the effect we observed. Although caspase-1−/− mice are partially resistant to LPS-induced AKI, the mechanism for this protection was not entirely clear (68). Consistent with this, we previously observed minimal activation of caspase-1 after LPS administration to mice (22). Since there are numerous cellular targets of caspase cleavage, there could easily be yet other possible ways in which caspase activation may interact with TNF-induced NF-κB activation (18) as well.

Flow cytometry experiments showed that TNF initially induced adhesion molecule expression on ECs within 2 h, with apoptosis occurring in the majority of cells by 6 h. After 6 h, the majority of cells expressed VCAM-1 while simultaneously staining positive for annexin 5, with little evidence for subpopulations undergoing adhesion molecule expression without apoptosis, or vice versa. Therefore the intracellular “decision” to undergo apoptosis versus adhesion molecule expression seems to favor adhesion molecule expression initially, assisted by the prosurvival affects of NF-κB signaling, but under persistent TNF stimulation results in programmed cell death. In these experiments there was some degree of preformed VCAM-1 expression on unstimulated cells, which may explain why the effect of caspase inhibition on adhesion molecule expression in flow cytometry was less dramatic than what was observed at the RNA level. Adhesion molecule expression on apoptotic ECs could provide a possible mechanism of endogenous clearance of injured cells by phagocytes. Of course, a caveat in any cell culture experiment is that the behavior of cultured cells may exaggerate or distort characteristics of the same cell type in the living organism. Similarly, longstanding exposure of relatively high concentrations of TNF over many hours may not be entirely relevant to events occurring within the vasculature in vivo.

In conclusion, this report provides a cell signaling-level explanation of effects of caspase inhibitors on tissue inflammation that were previously noted in animal experiments. This effect on inflammation explains in part the potency of caspase inhibition in protecting against functional AKI, given that the extent of apoptosis noted in LPS-induced AKI was modest. It is potentially highly relevant that this interaction between these two pathways occurs within renal ECs, consistent with an emerging appreciation of the importance of the renal vasculature in AKI (48, 64). While we chose to examine TNF signaling in ECs given the previously demonstrated importance of TNF in LPS-induced AKI, other pathways relevant to sepsis, such TLR4 and IL-1β, have also been shown to simultaneously activate caspases and NF-κB (34, 41). It is likely that this interaction between caspases and NF-κB signaling is not unique to the kidney and may play an important systemic role in the vasculature in sepsis generally. As an example of this, animal studies using small interfering RNA (siRNA) to downregulate caspases-8 and -3 have been found to strongly reduce mortality in septic shock (45). It is hoped that this knowledge may ultimately lead to more effective pharmacological strategies to improve patient outcomes in sepsis and AKI.

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