Nitric oxide induces apoptosis in renal tubular epithelial cells through activation of caspase-8

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Nitric oxide induces apoptosis in renal tubular epithelial cells through activation of caspase-8. Am J Physiol Renal Physiol 290: F1044–F1054, 2006. First published December 13, 2005; doi:10.1152/ajprenal.00341.2005.—The susceptibility or resistance of tubular epithelial cells (TEC) to apoptosis is pivotal to the long-term maintenance of kidney function following episodes of inflammation, such as graft rejection. TEC apoptosis can occur with ischemia as well as with proinflammatory cytokines and nitric oxide (NO), produced by infiltrating mononuclear cells. TEC can also produce abundant amounts of NO during inflammation but the role and regulation of NO-induced injury of TEC are not well understood. Apoptosis in TEC in vitro was determined by FACS analysis with annexin-V and propidium iodide staining. NO in culture supernatants was measured by Greiss reagent, and protein expression of inducible NO synthetase (NOS2/iNOS) and caspase-8 was examined by Western blot analysis. Here, we showed that murine TEC produced abundant amounts of NO in response to proinflammatory cytokines (IFN-γ/TNF-α) through upregulation of NOS2, and inhibition of endogenous NO production by l-NMMA reduced TEC apoptosis in cytokine-stimulated cultures. Addition of exogenous NO (sodium nitroprusside) induced TEC apoptosis as well as caspase-8 activation in a dose-dependent manner. The key role of caspase-8 in NO-induced TEC apoptosis was demonstrated by that NO-induced TEC apoptosis can be blocked by caspase-8 inhibition using z-IETD-fmk, caspase-8 silencing with shRNA or by overexpressing the endogenous caspase-8 inhibitor c-FLIP (cellular Flice-inhibitory protein). In conclusion, endogenous NO from NOS2 activity as well as exogenous NO can contribute to renal injury through apoptosis of TEC. Activation of caspase-8 plays a central role in NO-induced apoptosis and caspase-8 inhibition may be an important therapeutic target during renal inflammation.

Nitric oxide (NO) can be produced by any of NO synthases (NOS), including constitutive endothelial NOS (NOS3/eNOS), neuronal NOS (NOS1/nNOS), and inducible NOS (NOS2/iNOS) (19, 31). Among them only NOS2 is induced by immune or inflammatory stimuli, potentially resulting in prolonged production of large amounts of NO (micromolar ranges) by activated cells (30). In the tissue injury, NO can be either cytoprotective or cytotoxic, which may depend on its concentration within the microenvironment or targeting cell type. For example, NO has been demonstrated to inhibit cytokine- or FasL-induced apoptosis in colonic epithelial cells (13), hepatocytes (17, 22), and synovial cells (29); on the other hand, it has been characterized as a potent inflammatory mediator and is reported to induce apoptotic death in many types of cells including macrophages (1), pancreatic β-cells (2), and human tubular epithelial cells (TEC) (15), suggesting the importance of understanding the difference of NO-stimulated cellular signaling in different cell types.

Within the kidney, NOS2 is constitutively expressed in several segments of the renal tubule (medullary thick ascending limb, proximal and distal convoluted tubule) and glomeruli (23, 27). In response to the stress or inflammation, NOS2 is upregulated in glomerular, mesangial, smooth muscle, and tubular epithelial cells (26, 34, 35), suggesting NO produced by NOS2 may have a pathophysiological role in these conditions. Indeed, systemic inhibition of NOS2 reduces tubulointerstitial injury in renal allograft rejection (39) and prevents renal ischemia-reperfusion injury (7, 8, 32, 40), which is further supported by evidence showing protection from ischemic acute renal failure in NOS2 null mice (8, 25). However, the mechanisms or cellular signal pathways by which NO induces renal tubular epithelial injury remain unknown.

Recently, we demonstrated that activation of caspase-8 plays a critical role in TEC self-injury and apoptosis following exposure to cytokines (IL-2, IFN-γ, and TNF-α) (11, 12). TEC apoptosis in IFN-γ/TNF-α-stimulated cultures is Fas-FasL interaction-dependent fratricide (12). Loss of Fas or FasL function can prevent IFN-γ/TNF-α-induced TEC apoptosis. We also showed that IFN-γ activates caspase-8 in TEC, and inhibition of caspase-8 activation by its inhibitor or overexpression of c-FLIP reduces IFN-γ-induced TEC apoptosis (11). IFN-γ and TNF-α can induce the expression of NO as well as augment the expression of surface death receptors such as Fas, in which NO may augment TEC apoptosis by different or independent mechanisms from cytokines. In the present study, we use monolayer of TEC as an in vitro model of renal tubular epithelium to study the mechanisms by which NO induces tubular epithelial injury. The results demonstrate that IFN-γ can induce NOS2 expression in TEC leading to NO production, which is enhanced by the addition of TNF-α. We also show that TEC apoptosis by NO requires the activation of caspase-8, which can be blocked by overexpression of the endogenous caspase-8 inhibitor c-FLIP or by mRNA silencing of caspase-8. Inhibition of caspase-8 activation by c-FLIP or caspase-8 RNAi may represent therapeutic targets to protect TEC from NO-induced apoptosis as well as from proinflam-
matory cytokine- and receptor-dependent apoptosis during inflam-
mation or allograft rejection.

MATERIALS AND METHODS

Cells and reagents. Primary renal proximal TEC were isolated
from the kidney cortex of C3H-Hej mice as described previously (12).
The cloned proximal TEC, by immortalized with origin-deficient
SV40 DNA as described previously (20), were from C3H-Hej
(CS3.7), FasL mutant MRL-gld (MRM-gld), and Fas-deficient MRL-
lpr (M3.1-lpr) mice. All TEC expressed E-cadherin, CD13 (alanine
aminopeptidase), and CD26 (dipeptidyl peptidase) and were grown in
K1 medium, a mixture of DMEM and Ham’s F12 (50:50) (Invitrogen-
GIBCO, Burlington, ON), supplemented with 10% bovine calf serum,
hormone mix (5 H9262 g/ml of insulin, 34 pg/ml of triiodothyronine, 5
H9262 g/ml of transferrin, 1.73 ng/ml of sodium selenite, and 18 ng/ml of
hydrocortisone), and 25 ng/ml of EGF (Sigma, St. Louis, MO). Both
mrIFN-γ and mrTNF-α were purchased from BD Pharmingen (Mis-
sissauga, ON). Rabbit polyclonal anti-caspase-8 IgG (H-134) and
specific caspase-8 inhibitor (z-ITED-fmk) were obtained from Santa
Cruz Biotechnology (Santa Cruz, CA). Anti-NOS2 (inducible nitric
oxide synthase) antibody was from Upstate (Charlottesville, VA).
Sodium nitroprusside (SNP), NOS inhibitor
N\textsubscript{G}-monomethyl-L-arginine (L-NMMA), and its control
N\textsubscript{G}-monomethyl-D-arginine (D-NMMA) were from Sigma.

Confocal microscopy. The monolayer of CS3.7 TEC was charac-
terized by examination of tight junction formation using a confocal
microscope (Zeiss LSM510-META, Carl Zeiss International). CS3.7
cells, grown in Transwells (Transwell-COL, Corning, Corning, NY),
were fixed with 2% of parafomaldehyde, followed by permeabilization
with 0.25% of Triton X-100. The tight junction protein zonula
occludens 1 (ZO-1) was recognized by rabbit anti-ZO-1 polyclonal
IgG (H-300, Santa Cruz Biotechnology) and visualized with donkey
anti-rabbit IgG conjugated with Alexa Fluor-488 (Molecular Probes-
Invitrogen, Burlington, ON).

NO measurement. NO secreted from cells is rapidly oxidized to
nitrite in culture medium, therefore determinations of nitrite concen-
trations were used as a measurement of NO production. Fifty micro-
liters of culture supernatant were mixed with 50 l of Greiss reagent
(1% sulfanilamide, 0.1% naphthylethyline diamine dihydrochloride,
and 2.5% phosphoric acid) in triplicate in 96-well plates. After
incubation for 10 min at room temperature, absorbance was read at
550 nm and the level of NO was calculated using a standard curve
with known sodium nitrite concentrations.

Apoptosis analysis. Apoptotic cells were determined by FACS
(Fluorescence Activated Cell Sorter, or Flow Cytometry) analysis
using Annexin-V (early apoptosis) and propidium iodide (PI; late
apoptosis). TEC monolayers were released by a brief incubation with
Trypsin-EDTA solution (Sigma) and then incubated with Annexin-V
conjugated with fluorescein isothiocyanate (FITC) or phycoerythrin
(PE) in 1 binding buffer (BD Pharmingen) for 15 min, followed by
PI staining. The apoptotic cells in cytokine-treated TEC cultures were
also identified by terminal transferase dUTP nick end labeling

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Fig. 1. Induction of nitric oxide (NO) in tubular epithe-
lial cells (TEC) in response to stimulation of proinflam-
matory cytokines. A: monolayer of CS3.7, a proximal
TEC cell line, was characterized by formation of tight
junction detected by confocal microscopy. Data were
presented by a typical fluorescent image of ZO-1 protein.
B: monolayer of primary TEC, seeded at 0.25 \times 10^6
cells/well in 24-well plates overnight, was treated with
IFN-γ, TNF-α, or TNF-α/IFN-γ mixture (10 ng/ml of
each cytokine) in a volume of 0.5 ml/well for 24 h. The
molar concentrations of NO in the collected culture
supernatant (~500 l/sample) were determined in 50 l of
the sample using Greiss reagent. Monolayer of CS3.7
TEC was treated as above. C: monolayer of CS3.7 TEC
was treated with TNF-α/IFN-γ in the absence or presence
of 3 mM l-NMMA or d-NMMA (as control) for
24 h. Data are presented as means ± SD of triplicates in
1 typical experiment, which were repeated 4 times. *P < 0.01
(vs. medium control, n = 5); **P < 0.01 (vs. IFN-γ-treated;
n = 5); ***P < 0.01 (vs. l-NMMA-
treated; n = 5).
c-FLIPL was overexpressed in CS3.7 TEC as described previously using Western blotting. Expression of total protein was determined using Western blots. Briefly, whole cell lysates were homogenized in lysis buffer [10 mM Hepes (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 0.1% NP 40, 1 mM DTT, and a protease inhibitor cocktail (Roche)], followed by mixing with an equal volume of 2× SDS sample buffer [20 mM Tris-HCl (pH 6.8), 5% (v/v) SDS, 10% (vol/vol) mercaptoethanol, 2 mM EDTA, and 0.02% bromophenol blue] and then boiled for 5 min. The protein content of cell lysates from each sample were fractionated by SDS-PAGE and transferred to nitrocellular membranes (Bio-Rad Laboratories), blocked with 5% fat-free milk (Carnation) in TBS-T (20 mM Tris-HCl, pH 7.6, 137 mM NaCl, 0.1% Tween 20) for 1 h, and then probed with the appropriate antibodies in TBS containing 2.5% of milk at 4°C overnight. The specific protein binds recognized by the antibodies on the membrane were visualized by an ECL assay (Amersham Pharmacia Biotech, Buckinghamshire, UK). Blots were reprobed using anti-β-actin IgG (Sigma) for confirmation of equal protein loading if possible.

Overexpression of c-FLIP in TEC. Endogenous caspase-8 inhibitor c-FLIPs was overexpressed in CS3.7 TEC as described previously (11). Briefly, c-FLIPs cDNA was subcloned into a modified Herpes simplex virus (HSV) expression vector (pHEX6300) and regulated by hEF1α promoter with bGH polyA tail. This vector also contains a fused gene (ggeo) encoding Zeocin resistance (NH2 terminus) and a green fluorescence protein (GFP) (COOH terminus), controlled by EM7 promoter, to allow cell sorting and antibiotic selection (37). CS3.7 TEC were transfected with either pHEX6300 vector (control cells) or the vector containing the c-FLIPs cDNA insert (pHEX6300-FLIP) using Lipofectamine 2000 (Invitrogen-GIBCO) and following the manufacturer’s protocol. The stably transfected cell lines with either pHEX6300 vector (control cells) or the vector containing the c-FLIPs cDNA insert (pHEX6300-FLIP) using Lipofectamine 2000 (Invitrogen-GIBCO) and following the manufacturer’s protocol. The stably transfected cell lines with either pHEX6300 (CS3.7–6300) or pHEX6300-FLIP (CS3.7-FLIP) were grown under antibiotic selection (Zeocin) and sorted for GFP enrichment before passaging. GFP expression was stable in subsequent passages, and more than 95% of cells used for experiments showed strong green fluorescence by flow cytometry or microscopy.

RNA interference by stable expression of shRNA. A stable expression vector (pHEX-siRNA) for short hairpin RNA (shRNA) was developed from pHEX6300 vector by replacing the hEF1α promoter with the H1 RNA promoter, which is suitable for shRNA expression. A specific siRNA-targeting sequence of caspase-8 mRNA and nonspecific control (scrambled) sequence were previously described (10). The sequence of oligonucleotides encoding the shRNA targeting caspase-8 (5'-AAC CTC GGG GAT ACT GTC TGA) or control (5'-AAT CCG ATA GGC TAT GCC GTF-3') was synthesized by Integrated DNA Technologies (Corvalle, IA) and ligated into pHEX-siRNA to create pHEX-siRNA-Casp8 or pHEX-siRNA-control. pHEX-siRNA-Casp8 or control vector pHEX-siRNA-control was transfected into CS3.7 cells using Lipofectamine 2000 (Invitrogen-GIBCO) following the manufacturer’s protocol. Transfected cells were grown in the presence of Zeocin (up to 500 μg/ml; Invitrogen-GIBCO) and selected by cell sorting using FACS for GFP. More than 95% of control or siRNA-transfected cells showed strong green fluorescence by either flow cytometry or microscopy.

Statistical analysis. One-way ANOVA or t-tests (one-tailed distribution) using Statview statistical software (SAS, Cary, NC) were used as appropriate for comparisons between groups. Data of each group were collected from separate experiments for statistical analysis. A *P* value of ≤0.05 was considered significant.

RESULTS

**IFN-γ/TNF-α-induced NO production in TEC.** We tested the capacity of exogenous IFN-γ and TNF-α to induce NOS2 expression and NO production in murine TEC cultures. Monolayer of proximal cloned (CS3.7) (Fig. 1A) and primary TEC was treated with IFN-γ and/or TNF-α in K1 medium. As shown in Fig. 1B, there was a 15-fold increase in NO level in primary culture supernatants after 24 h of IFN-γ treatment (11.34 ± 1.5 μM) compared with the basal levels in untreated controls.
cultures (0.69 ± 0.4 μM). NO production further increased by 60-fold in cultures treated with both IFN-γ and TNF-α (55.25 ± 5.0 μM), demonstrating a synergistic response. Similar results were noted in CS3.7 TEC cultures (Fig. 1C).

However, TNF-α alone did not significantly induce NO production in both primary and cloned CS3.7 TEC. To confirm the increase in NO in the culture medium was due to the activity of NOS2, CS3.7 TEC cultures were stimulated with TNF-α

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**Fig. 3. Inhibition of NO production decreases cytokine-induced apoptosis in TEC.** Monolayer of CS3.7 TEC was treated with 3 mM l-NMMA alone, or TNF-α/IFN-γ mixture (10 ng/ml) in the absence or presence of 3 mM l-NMMA for 24 or 48 h. A: level of NO in the culture supernatant. B and C: apoptosis in TEC was analyzed using FACS. B: graph presented apoptosis indicated by annexin-V-FITC (top and bottom right quadrants) and PI staining (left and right top quadrants) in a typical experiment. C: graph indicated means ± SD of annexin-V positivity of 6 separate experiments. *P < 0.001 (vs. medium control, n = 6); **P < 0.001 (vs. IFN-γ/TNF-α treated only, n = 6). D and E: apoptosis in TEC was confirmed by detection of fragmenting nuclear DNA at the 3’-hydroxyl ends using TUNEL assay after 24 h of treatment. D: graph presented incorporation of dUTP-FITC in a typical experiment (thin line: background). E: graph indicated means ± SD of dUTP-FITC positivity of 2 separate experiments.
and IFN-γ (10 ng/ml each) in the presence of a NOS inhibitor (3 mM L-NMMA) or analog control (D-NMMA). As shown in Fig. 1D, NO levels (50.2 ± 4.1 μM) in cytokine-treated cultures were reduced to near basal levels (3.2 ± 1.2 μM) by L-NMMA, but not by D-NMMA (53.3 ± 6.2 μM), in support of an effect of cytokines on NOS2 induction.

To examine the correlation of NOS2 expression and NO production in the cytokine (IFN-γ and/or TNF-α)-stimulated TEC, NOS2 expression was examined by Western blot in TNF-α and/or IFN-γ-treated TEC cultures. As shown in Fig. 2A, NOS2 expression was induced in IFN-γ-treated TEC and maximally increased with TNF-α. Consistent with the data of NO in culture medium, NOS2 was unchanged in TNF-α alone treated TEC compared with untreated controls. The expression of NOS2 was time dependent with maximal levels after 12 h of exposure to TNF-α and IFN-γ (Fig. 2B). Collectively, these data confirm that inflammatory cytokines can stimulate NOS2 protein expression in TEC leading to abundant NO production.

TEC apoptosis induced by IFN-γ/TNF-α is decreased by inhibition of NO production. Cytokines (IFN-γ, TNF-α, and IL-2) can induce apoptosis in cloned or primary TEC (11, 12). As cytokines can also induce NOS2 with subsequent NO production, TEC death could result from the direct effect of NO as well as the Fas/FasL-mediated “self injury” we have described. We tested the effect of endogenous production of NO on TEC apoptosis. Confluent monolayer of CS3.7 TEC was treated with IFN-γ (10 ng/ml) and TNF-α (10 ng/ml) in the absence or presence of NOS inhibitor (3 mM L-NMMA). After 24 or 48 h, cells were assessed for apoptosis and supernatants for NO levels. As shown in Fig. 3A, NO levels were induced by the cytokine stimulation and reduced to basal levels by l-NMMA. Early and late apoptosis was assessed in the same cultures using annexin-V and PI staining, respectively. As demonstrated in Fig. 3B, apoptosis was induced by cytokines compared with untreated controls at 24 and 48 h. Annexin-V and PI positivity increased from 6.43% in medium controls to 21.21% with cytokines at 24 h and was reduced to 9.03% by l-NMMA. Similarly, there was a reduction of apoptosis by l-NMMA at 48 h, but was more modest despite near complete blockade of NO production. Further statistical analysis indicated that reduction of apoptosis by inhibition of endogenous NO production by l-NMMA was significant in the proinflammatory cytokine-stimulated TEC (Fig. 3C). The increase of apoptosis indicated by annexin-V/PI measurement in this experiment was confirmed by TUNEL assay (Fig. 3, D and E), in which IFN-γ/TNF-α-induced TEC apoptosis, identified by TUNEL assay, was reduced by l-NMMA from 32.32 to 21.79%. Collectively, these data suggest inflammatory cytokine-induced endogenous NO contributes to TEC apoptosis, which could be an independent effect of cytokines on TEC death.

NO-induced TEC apoptosis depends on activation of caspase-8. As there is considerable “crosstalk” between receptor-dependent and -independent pathways of caspase activation (42), we tested whether apoptosis of TEC in response to NO involves the activation of caspase-8. First, to isolate the effect of NO on TEC and avoid the use of cytokines, which can also activate caspases, we used SNP to provide NO to confirm apoptosis effect. CS3.7 TEC were treated with SNP in increasing concentrations (to 50 μg/ml) for 24 h and tested for apoptosis by FACS analyses. As illustrated in Fig. 4, A and B, early and late apoptotic cells increased in a dose-dependent manner.
manner with SNP in primary (top) and CS3.7 (bottom) TEC. In primary TEC, basal level of TEC apoptosis in untreated cultures was 11.18%, which increased to 53.71% with 50 \mu g/ml of SNP (Fig. 4A). Similar results were seen in SNP-treated CS3.7 TEC cultures (Fig. 4B). NO directly induced TEC apoptosis was further confirmed by statistical analysis (Fig. 4C). It was noticed that increase of SNP from 10 to 50 \mu g/ml shifted early apoptosis to late apoptosis in TEC. The reasons for that are unclear but may indicate that high concentrations of NO may accelerate the process of apoptosis in TEC. NO-induced TEC apoptosis was also confirmed in the CS3.7 cultures using NOC-18 as an NO donor molecule (21) (data not shown).

Caspase-8 can initiate apoptosis through surface receptors (Fas, TNF-R1) and also mediate receptor-independent apoptosis pathways such as ceramide (4, 41). Activation of caspase-8 in TEC by NO was assessed using Western blot analysis. CS3.7 TEC were stimulated with different concentrations of SNP and for various times. As shown in Fig. 5, cleavage of pro-caspase-8 protein occurred with SNP maximally after 3 h of incubation with 50 \mu g/ml of SNP. Addition of z-IETD-fmk, a specific peptide inhibitor of caspase-8 to prevent TEC apoptosis, confirmed the central role of caspase-8 activation in NO-induced TEC apoptosis. As shown in Fig. 5C, apoptosis decreased from 28.1 ± 4.5% in SNP (25 \mu g/ml)-treated TEC cultures to 14.8 ± 2.1% in cultures with 100 \mu M z-IETD, which was not different from basal apoptosis (15.1 ± 2.5%) noted in unstimulated TEC in these series of experiments. Compared with other untreated TEC controls described above, the relatively higher levels of basal apoptosis in vehicle controls noted in these particular experiments might be due to 0.5% of DMSO treatment.

Decreased expression of pro-caspase-8 by shRNA prevents NO-induced apoptosis in TEC. A siRNA sequence has been reported to specifically knockdown the expression of pro-caspase-8 (10). We developed a system for stable expression of this siRNA sequence as shRNA to disrupt caspase-8 mRNA in TEC and tested these transfected cells for resistance to NO-induced apoptosis. As shown in Fig. 6A, pro-caspase-8 was reduced in CS3.7-caspase-8 cells transfected with the pHEX-siRNA-Casp8 vector, compared with control cells transfected with pHEX-siRNA-control (CS3.7-control). Attenuation of pro-caspase-8 protein levels against \beta-actin protein was quantified by a densitometry (Fig. 6B), indicating 50.9% decrease of caspase-8 protein in CS3.7-caspase-8 compared with it in CS3.7-control cells (1.14 ± 0.21 in CS3.7-control; 0.56 ± 0.1 in CS3.7-caspase-8 cells). To test the effect of pro-caspase-8 deficiency on NO-induced apoptosis, CS3.7-control and CS3.7-caspase-8 cell cultures were treated with increasing concentrations of SNP (up to 50 \mu g/ml) for 24 h and apoptosis was assessed by FACS analysis. As demonstrated in Fig. 6C, apoptosis in CS3.7-control cells was induced by SNP in a concentration-dependent manner, indicated by increase in apoptosis from 14.94% of basal level in control cultures to 31.2% in 10 \mu g/ml of SNP-treated cells and further to 44% in 25 or 50 \mu g/ml of SNP-treated cultures. In contrast, CS3.7-caspase-8 cells were resistant to SNP-induced apoptosis up to 50 \mu g/ml of SNP (13.71% in controls vs. 16.39% in 50 \mu g/ml of SNP-treated cultures). These results were further confirmed by statistical analysis (Fig. 6D). Taken together, these data indicate that reducing expression of caspase-8 results in amelioration of NO-mediated TEC apoptosis, which further supports that caspase-8 activation plays a critical role in the NO-induced TEC apoptosis.

Fig. 5. Pro-caspase-8 is activated in NO-induced TEC apoptosis. A: monolayer of CS3.7 TEC was treated with various concentrations of SNP for 3 h. B: monolayer of CS3.7 TEC was treated with 50 \mu g/ml of SNP for various periods of time (up to 3 h). C: monolayer of CS3.7 TEC was pretreated with 100 \mu M IETD peptide or vehicle (DMSO, 0.5% in culture medium) for 3 h before exposure to 25 \mu g/ml of SNP for 24 h. The level of pro-caspase-8 protein (~54 kDa) in each sample was determined by Western blot with 10% SDS-PAGE, in which control for loaded protein (\beta-actin) was reprobed. The apoptosis was analyzed using FACS with annexin-V-FITC. Data are presented as means ± SD of 3 determinants in 1 typical experiment, which were repeated 4 times. *P < 0.01 (n = 5).
Overexpression of c-FLIP inhibits NO-induced TEC apoptosis. c-FLIP isoforms including c-FLIPL are considered to be key endogenous inhibitory proteins for caspase-8 activation in several cell types including lymphocytes and endothelium (24). Recently, we described the expression of FLIP in TEC and its central role in regulating cytokine-induced apoptosis (11). The requirement for caspase-8 activation in NO-mediated TEC apoptosis was evident as described above, and therefore we tested whether upregulation of c-FLIP in TEC could block NO-induced activation of pro-caspase-8. Stable enhanced expression of c-FLIP in CS3.7 TEC has been described previously (11). Monolayer of CS3.7-FLIP and CS3.7–6300 (“empty vector”-transfected CS3.7 cells) as well as parental CS3.7 TEC was treated with 50 μg/ml of SNP for 3 h. As demonstrated in Fig. 7A, in CS3.7–6300 TEC as well as in the parental CS3.7, pro-caspase-8 levels were decreased on addition of SNP, indicating activation of caspase-8. In contrast, pro-caspase-8 protein levels were unchanged in CS3.7-FLIP following 50 μg/ml SNP treatment compared with untreated cells which was confirmed by densitometry ratios using β-actin (0.49 in treated vs. 0.55 in untreated). In comparison, the density of the pro-caspase-8 band was decreased from 0.69 in CS3.7 and 0.50 in CS3.7–6300 to 0.26 in SNP-treated CS3.7 and 0.28 in SNP-treated CS3.7–6300. These data suggest that overexpression of the endogenous caspase-8 inhibitor c-FLIP prevents NO-activated pro-caspase-8 in TEC and is consistent with our results using synthetic inhibitors and shRNA.
The capacity of c-FLIP overexpression to protect TEC from NO-mediated apoptosis was then tested. As indicated in Fig. 7B, apoptosis was induced in CS3.7–6300 TEC from 1.7 ± 0.8% in media controls (basal levels) to 8.1 ± 1.9% in 5 μg/ml of SNP-treated, 15.5 ± 2.5% in 10 μg/ml of SNP-treated, and 8.2 ± 1.8% in 50 μg/ml of SNP-treated cultures. In marked contrast, apoptosis could not be induced in CS3.7-FLIP with SNP using up to 50 μg/ml of SNP. A slight decrease in activation of caspase-8 with a more substantial decrease in SNP-induced apoptosis was observed in CS3.7–6300 cells compared with treated parental CS3.7 cells. It is of note that an increase in basal c-FLIP was noted in stable empty vector CS3.7–6300 (11). It is possible that sorting and initial enrichment of TEC may have selected TEC with higher endogenous c-FLIP levels thus leading to enhanced resistance to caspase activation and apoptosis resistance noted here. Alternatively, the GFP may have had a modulating effect on apoptosis. In our unpublished observation, 100 μg/ml of SNP were able to upregulate anti-apoptotic survivin expression in CS3.7 TEC, and NO has been demonstrated to inhibit cytokine- or FasL-induced apoptosis in various cells (13, 17, 22, 29). All these might result in relatively less apoptosis in 50 μg/ml of SNP compared with 10 μg/ml of SNP-stimulated CS3.7–6300 cultures. Nevertheless, data demonstrated that the levels of caspase-8 activation and apoptosis were clearly higher in CS3.7–6300 compared with CS3.7-FLIP TEC supporting a key role for c-FLIP in regulating caspase-8-mediated NO-induced apoptosis in TEC.

No augmentation of Fas expression in NO-treated TEC. The tumor suppressor protein p53 has been shown to augment Fas expression by the trafficking of Fas from intracellular storage vesicles to the cell membrane in vascular smooth muscle cells and NO has been shown to cause accumulation of p53 in some cells (5, 9). As well we previously demonstrated that inflammatory cytokines (IFN-γ and TNF-α) mediate Fas/FasL-dependent TEC self-injury (“fratricide”) (12). We therefore tested whether NO enhanced Fas expression as subsequent apoptosis might then occur by Fas activation of caspase-8. CS3.7 monolayer was treated with SNP for 24 h and Fas protein expression was tested by Western blot analysis (Fig. 8A) and surface Fas expression measured by FACS analysis (Fig. 8B and C). Fas protein expression was not increased in SNP-treated TEC (up to 50 μg/ml of SNP), but rather decreased compared with untreated control TEC at 24 h. In Western blot analysis, the normalized density of the Fas protein band was 0.82 in control cells (medium only), 0.89 in 5 μg/ml of SNP-treated, 0.71 in 10 μg/ml of SNP-treated, and 0.62 in 50 μg/ml of SNP-treated TEC. Consistent with Fas protein results in Fig. 8A, FACS analysis indicated that there was a SNP concentration-related decrease in TEC surface expression of Fas from 11.85% in untreated cells, to 12.86% in 5 μg/ml of SNP-treated, 10.54% in 10 μg/ml of SNP-treated, and 8.77% in 50 μg/ml of SNP-treated cells (Fig. 8B). However, this decrease was not significant (Fig. 8C). To study kinetics of inhibition, CS3.7 cells were harvested 6 or 12 h after addition of 50 μg/ml of SNP. As shown in Fig. 8D, there was no change in Fas expression in SNP-treated TEC at these earlier time points confirmed by densitometry of Fas protein bands (1.01 in 0-h, 1.10 in 6-h, and 1.12 in 12-h cultures). FasL expression in these cells was not affected by SNP treatment at any time point as well. Although exposure to exogenous NO (50 μg/ml SNP) for 24 h can attenuate the surface and total protein expression of Fas, apoptosis is increased along with caspase-8 activation. To study this more, we tested the effect of NO on apoptosis of Fas-deficient TEC (M3.1-lpr) or FasL mutant TEC (MRM-...
Interestingly, these TEC were resistant to NO, suggesting the complete loss of Fas or FasL function was protective. It is possible that even low levels of Fas expression in SNP-treated TEC were sufficient for recruitment of caspase-8 to the DISC with subsequent apoptosis. Taken together, these data indicate that NO does not appear to initiate apoptosis by upregulating Fas expression in TEC, but the significance of NO in down-regulating the surface expression of Fas remains unclear.

**DISCUSSION**

Tubular epithelial injury can be a primary cause for nephron loss, and TEC represent a key functional component of kidneys. Therefore, TEC susceptibility to injury would be expected to direct long-term function of the inflamed kidney such as renal allografts, in which many forms of injury inevitably undergo in addition to ischemia (33). Posttransplant-like other tubulointerstitial nephritis, TEC death by apoptosis can also
occur through reactive oxidative metabolites, exposure to inflammatory mediator NO and cytokines (TNF-α, IL-1) as well as with direct contact with cytotoxic T cells (FasL/Fas, Perforin/Granzyme B) during rejection. We previously made the observation that TEC by expressing both Fas and FasL can undergo “self injury” and apoptosis following activation with cytokines (IFN-γ, TNF-α). Furthermore, this apoptosis can be prevented by blocking Fas expression or by the enhanced expression of an endogenous caspase-8 inhibitory protein (c-FLIP), which is downregulated by IL-2 in TEC (11, 12). In this study, we demonstrated that while NO can be produced by TEC in response to inflammatory cytokines including IFN-γ, NO can also induce apoptosis in TEC independently. The key regulatory step in NO-mediated TEC apoptosis appears to be the activation of caspase-8 and that inhibition of caspase-8 activation by c-FLIP or by RNA silencing can markedly block tubular epithelial death. These studies together indicate that augmenting the endogenous capacity of TEC to resist injury would be a useful strategy in treatment of tubular epithelial injury in the kidney diseases including renal allograft rejection.

In the rejecting kidneys as an example of tubular epithelial injury, there is augmented expression of proinflammatory cytokines, including IL-1, IFN-γ, and TNF-α, as well as enhanced production of NOS2/NO (14, 36). Previous studies suggest that in acute rejection, high levels of NOS2 expression within the kidney are accounted for by infiltrating cells (macrophages/monocytes) in the renal interstitium, which can produce abundant amounts of NO (6, 16). However, as NOS2 is expressed in many renal parenchymal cells including tubular epithelium, smooth muscle cells, and glomeruli, both host and donor cells may contribute to NO production in allograft (26, 34, 35). In this study, we further confirmed the increasing expression of NOS2 in renal TEC with production of very high levels of NO in culture supernatants following proinflammatory mixed cytokine stimulation, which is similar with previous results in human renal proximal tubular cells (28, 34). This TEC-derived NO could contribute to inflammation in the kidney. During acute rejection and other forms of inflammation within the kidney, TEC would be exposed to a spectrum of several proinflammatory cytokines from infiltrates, resulting in TEC NO production, and our data suggest NO production from resident TEC as well as infiltrating cells could promote renal injury.

The mechanisms by which NO as an inflammatory mediator damages renal tubular epithelium are not clear. In our study, data showed that activation of caspase-8 was required for NO-induced apoptosis in TEC in vitro. The importance of caspase-8 in this process was clear as apoptosis could be blocked by the addition of caspase-8-specific inhibitor (IETD peptide), knockdown of caspase-8 expression by shRNA, and overexpression of caspase-8 endogenous inhibitory protein (c-FLIP), which can prevent functional FADD-pro-caspase-8 interactions (38). There are several possible mechanisms by which NO is able to activate pro-caspase-8 in TEC. In previous studies, we demonstrated Fas/Fasl interactions mediate apoptosis (fratricide) in cytokine-stimulated TEC (12) and were associated with activation of caspase-8 and augmented expression of Fas. In the present study, NO exposure resulted in caspase-8 activation in TEC without enhancing the expression of Fas or Fasl suggesting a direct effect of NO on caspase-8. However, NO-induced apoptosis in TEC appears to require some degree of Fas/Fasl interaction, as apoptosis is not induced in NO-treated M3.1-lpr (Fas deficient) or MRM-gld (Fasl mutant) TEC. It is possible that a certain level of Fas/Fasl interaction is required for efficient recruitment of pro-caspase-8 to the DISC (3). Consistent with this, addition of an NO donor (0–50 μg/ml SNAP) to smooth muscle cells leads to apoptosis via activation of the Fas-Fasl pathway as blocking Fasl with antibody prevents SNAP-induced apoptosis (18) (Fig. 9). Alternatively, NO may activate pro-caspase-8 by a mitochondria-dependent mechanism through release of cytochrome c and activation of caspase-3, from which the active caspase-3 can cleave pro-caspase-8. Several signaling pathways, such as disruption of mitochondrial electron transfer chain, activation of JNK/SAPK and p38 kinase, induction of p53 accumulation and ceramide generation, which could alter mitochondrial membrane potential resulting in cytochrome c release have been reported to be activated by NO in various experimental systems (9). Although further studies to clarify the mechanism(s) by which NO activates pro-caspase-8 in TEC are required, the central role of caspase-8 in NO-induced tubular injury is demonstrated in this study.

In conclusion, we demonstrated that in addition to producing substantial amounts of NO by upregulation of NOS2 in response to proinflammatory cytokines, TEC undergo apoptosis by endogenous as well as exogenous NO, and activation of caspase-8 plays a key role. Taken with our previous studies showing caspase-8 activation through Fas/Fasl interaction leads to TEC fratricide which contributes to renal allograft injury (11, 12), inhibition of caspase-8 appears to be an attractive therapeutic target in the prevention of tubular epithelial injury in allograft rejection or other tubulointerstitial nephritis. Enhanced expression of c-FLIP through endogenous regulators or by genetic manipulation, as well as specific inhibition by siRNA, may augment TEC survival and promote renal allograft function in vivo.

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