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Effect of nitric oxide donors on renal tubular epithelial cell-matrix adhesion

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Effect of nitric oxide donors on renal tubular epithelial cell-matrix adhesion.

Background. Nitric oxide (NO) and its metabolite, peroxynitrite ($ONOO^-$), are involved in renal tubular cell injury. We postulated that if NO/$ONOO^-$ has an effect to reduce cell adhesion to the basement membrane, this may contribute to tubular obstruction and may be partially responsible for the harmful effect of NO on the tubular epithelium during acute renal failure (ARF).

Methods. We examined the effect of the NO donors (z)-1-[2-(2-aminoethyl)-N-(2-ammonioethyl)amino]diazen-1-ium-1,2-diolate (DETA/NO), spermine NONOate (SpNO), and the $ONOO^-$ donor 3-morpholinosydnonimine (SIN-1) on cell-matrix adhesion to collagen types I and IV and fibronectin using three renal tubular epithelial cell lines: LLC-PK_1, BSC-1, and OK.

Results. In LLC-PK_1 cells, DETA/NO (500 μM) had no effect, and SpNO (500 μM) had a modest effect on cell adhesion compared with controls. Exposure to SIN-1 caused a dose-dependent impairment in cell-matrix adhesion. Similar results were obtained in the different cell types and matrix proteins. The effect of SIN-1 (500 μM) on LLC-PK_1 cell adhesion was not associated with either cell death or alteration of matrix protein and was attenuated by either the NO scavenger 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide, the superoxide scavenger superoxide dismutase, or the $ONOO^-$ scavenger uric acid in a dose-dependent manner.

Conclusions. These results therefore support the possibility that $ONOO^-$ generated in the tubular epithelium during ischemia/reperfusion has the potential to impair the adhesion properties of tubular cells, which then may contribute to the tubular obstruction in ARF.

The results of several studies have provided evidence that renal tubular obstruction plays an important role in the sustained decrease in the glomerular filtration rate (GFR) in acute ischemic renal injury [1–9]. It has been shown that urinary casts, which consist of Tamm-Horsfall mucoprotein, brush border membranes, and conglomerated tubular epithelial cells, contribute to the obstruction [10]. Racusen, Fivush, and Li demonstrated, by trypan blue dye exclusion and subsequent *in vitro* culture, that more than 30% of renal tubular cells recovered in urine from patients with acute tubular necrosis were viable [11]. Graber et al also reported viable tubular cells in the urine sediment in patients with acute renal failure (ARF) and other renal disorders [12]. Moreover, these exfoliated tubular cells have changes in their cytoskeleton and cell–cell and cell–matrix adhesion that may be responsible for their anatomical and functional abnormalities [13–15]. This cytoskeletal disruption and subsequent cell adhesion disassembly may lead to apoptosis in anchorage-dependent cells [16–19].

Previous studies from our laboratory and others have demonstrated that cytosolic calcium [20–24] and nitric oxide (NO) may play an important role in renal tubular cell injury *in vitro* [25, 26] and *in vivo* ischemic ARF [27, 28]. Although NO has been demonstrated to be beneficial in the vasculature by attenuating leukocyte-endothelial cell adhesion during postischemic reperfusion injury [29–32], it may play a damaging role in renal tubular epithelium by attenuating tubular cell adhesion and contributing to tubular obstruction. A similar effect has been shown in mesangial cell-matrix adhesion [33].

The NO donor S-nitroso-N-acetyl-*D*,*L*-penicillamine has been shown to accelerate cell migration in response to cell injury in a renal epithelial cell wound-healing model [34]. However, whether the motogenic effect in this model was due to NO or a metabolite of NO was not examined. In a model of cell injury in which oxygen radicals may be generated, such as wound healing or ischemia, it is reasonable to consider the role of NO metabolites, especially peroxynitrite ($ONOO^-$). In this regard, increased 3-nitrotyrosine, a footprint of $ONOO^-$, has been shown by Western blot analysis in the outer stripe of the medulla in ischemic mouse kidney [27], thus supporting the potential role of $ONOO^-$ in ischemia-

Key words: acute renal failure, collagen, uric acid, superoxide dismutase, LLC-PK_1 cells.

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induced renal injury. Prior to this study, there have been no studies examining the effect of $ONOO^-$ on renal tubular epithelial cell adhesion. Therefore, the purpose of our study was to compare the effects of NO and $ONOO^-$ on renal tubular epithelial cell adhesion to several matrix proteins, including collagen types I and IV and fibronectin.

METHODS

Cells and cell culture

Three types of renal tubular epithelial cell lines—porcine proximal tubular cells ($LLC\text{-}PK_1$), green monkey kidney (BSC-1) cells, and opossum kidney (OK) cells—were purchased from American Type Culture Collection (ATCC, Rockville, MD, USA). The cells were cultured in 100 mm diameter cell culture dishes in (1:1) Dulbecco's modified Eagle medium plus Ham's F12 (DMEM/F12), supplemented with 10% fetal bovine serum (containing 2 mM glutamine, 15 mM HEPES, 1.5 g $NaHCO_3$, 100 unit/ml penicillin G sodium, 100 μg/ml streptomycin sulfate, pH 7.4), and incubated in 95% air/5% CO_2 with 90% humidity at 37°C. Cells were grown to confluence and were allowed to become quiescent in serum-free medium for 24 hours before the experiments.

Coating cell culture plates with matrix proteins

Collagen type I in 0.02 M acetic acid solution from rat tail tendon (Upstate Biotechnology, Lake Placid, NY, USA), collagen type IV from basement membrane of Engelbreth-Holm-Swarm mouse sarcoma (Sigma Chemical Co., St. Louis, MO, USA), and human fibronectin (Sigma Chemical Co.) were coated on 96-well polystyrene cell culture plates overnight at 4°C. A stock solution of collagen type IV was dissolved in 0.25% glacial acetic acid, and both collagen types I and IV were diluted in calcium-free phosphate-buffered saline (PBS) to a final concentration of 20 μg/ml. Fibronectin was dissolved in calcium-free PBS and was used at a final concentration of 5 μg/ml. Fifty microliters of each were then coated to each well. Before the experiments were performed, the protein solution was aspirated, and wells were washed twice with calcium-free PBS. Nonspecific adhesion was blocked with 2% bovine serum albumin in calcium-free PBS at room temperature for two hours. Prior to cell adhesion, the blocking solution was removed, and the wells were washed twice with calcium-free PBS.

Cell adhesion

The cell monolayer was trypsinized with 0.1% trypsin/0.04% ethylenediamine tetraacetate (EDTA) in Hank's balanced salt solution and was allowed to recover for one hour in DMEM/F12 medium containing 10% fetal bovine serum. After recovery, the medium was removed, and the cells were washed three times with calcium-free PBS. Cells were counted and resuspended to 50,000 cell per ml in 0.1% bovine serum albumin in DMEM/F12 medium at 37°C, pH 7.4. Cells were pretreated with NO donors and/or scavengers for 30 minutes at 37°C. Two hundred microliters of cell suspension were then added to each well, and the cells were allowed to adhere for 90 minutes at 37°C. At the end of the 90 minutes, the supernatant, which contained nonadherent cells, was gently aspirated, and the cells were washed twice with calcium-free PBS. The number of adherent cells was determined by hexosaminidase enzyme assay as previously described [35].

Effect of nitric oxide donors and/or scavengers on cell adhesion

Nitric oxide was generated by (z)-1-[2-(2-aminoethyl)-N-(2-ammonioethyl)amino]diazen-1-ium-1,2-diolate (DETA/NO; 500 μM) or spermine NO (SpNO; 500 μM). NO and superoxide, which immediately interact to form $ONOO^-$, were simultaneously generated by 3-morpholinosydnonimine (SIN-1) at doses of 100, 300, and 500 μM. The NO scavenger 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (C-PTIO; 100 μM), the superoxide scavenger superoxide dismutase (SOD; 200 unit/ml), and the $ONOO^-$ scavenger uric acid (UA, 100 μM) were added simultaneously with SIN-1 (500 μM) during 30 minutes of pretreatment and 90 minutes of cell adhesion. All NO donors and scavengers were freshly prepared and dissolved in oxygen-free distilled water before pretreatment.

Control cells were treated exactly as the treated cells, but without addition of donors or scavengers. The adhesion of these untreated control cells was defined as 100% adhesion. Each experiment (N) represents the mean adhesion of three wells and is expressed as a percentage of control.

In our adhesion assay, SIN-1 showed no direct inhibitory effect on hexosaminidase activity. This was tested by incubating $LLC\text{-}PK_1$ cells with 500 μM SIN-1 for two hours and then measuring hexosaminidase activity of the total cell lysate. There was no difference in hexosaminidase activity (OD_{405} per 1000 cells = control, 0.045 ± 0.002 vs. SIN-1, 0.048 ± 0.001; P = NS, N = 6). In addition, the adhesion data determined by hexosaminidase activity were confirmed by cell counting. SIN-1 pretreatment reduced cell adhesion to 46.1 ± 4.1% by cell counting versus 43.4 ± 11.3% using the hexosaminidase method (P = NS, N = 3).

Effect of SIN-1 on the integrity of matrix proteins

Collagen type IV was coated on the plates as described earlier in this article. After washing of the well, SIN-1 (500 μM), which was dissolved in 0.1% bovine serum albumin in DMEM/F12 medium, was added to the coated wells and incubated for 90 minutes. The wells

were then washed twice with calcium-free PBS, and LLC-PK$_1$ cells were added to the wells. Cell adhesion was assessed as described earlier here.

Cell viability

Cell viability was determined by the trypan blue dye exclusion method. Cell adhesion in controls and after SIN-1 (500 μM) pretreatment was performed as usual; nonadherent cells were collected by aspiration and centrifugation and were then washed twice with calcium-free PBS. Adherent cells were trypsinized and collected, and then trypsin/EDTA was inactivated as described earlier in this article, using the cell adhesion method. Both nonadherent and adherent cells were stained with 0.4% trypan blue and counted. Cell viability was expressed as a percentage of the absolute viable cells.

Statistical analysis

To compare values of control and experimental groups analysis of variance (ANOVA) was used, followed by Dunnett's test (Instat2 software, version 2.04, GraphPad, San Diego, CA, USA). A P of less than 0.05 was considered statistically significant. Data are presented as means ± SEM.

RESULTS

Control LLC-PK$_1$, BSC-1, and OK cell adhesion to collagen types I and IV and fibronectin

According to our preliminary results (data not shown), cell adhesion determined at 90 minutes is when the plateau phase of cell adhesion begins. Control cell adhesion of LLC-PK$_1$, BSC-1, and OK cells to collagen type IV was 37.3 ± 1.6%, 51.5 ± 3.2% and 48.4 ± 2.4% of total cells plated, respectively. Control LLC-PK$_1$ cell adhesion to collagen type I and fibronectin was 48.8 ± 2.8% and 40.3 ± 1.2%, respectively.

Effect of nitric oxide donors on LLC-PK$_1$, BSC-1, and OK cell adhesion to collagen type IV

As shown in Figure 1, DETA/NO (500 μM) did not attenuate cell adhesion in any cell line (LLC-PK$_1$, 96.4 ± 3.1%; BSC-1, 96.3 ± 1.8%; OK, 95.4 ± 1.9% vs. control, P = NS, N = 7 in each cell type). SpNO (500 μM) slightly attenuated cell adhesion in LLC-PK$_1$ cell (83.6 ± 2.4% vs. control, $P < 0.01$, $N = 7$), but did not attenuate cell adhesion in BSC-1 cells (94.3 ± 1.5% vs. control, P = NS, $N = 7$) or OK cells (89.5 ± 1.9% vs. control, P = NS, $N = 7$). In contrast, SIN-1 (500 μM) dramatically attenuated cell adhesion in all cell lines (LLC-PK$_1$, 45.9 ± 4.2%; BSC-1, 63.6 ± 1.6%; OK, 78.3 ± 3.7% vs. control; $P < 0.001$, $N = 7$ in each cell type). The results with SIN-1 were confirmed by cell counting. SIN-1 pretreatment reduced cell adhesion to 46.1 ± 4.1% by cell counting versus 43.4 ± 11.3% using the hexosaminidase method (P = NS, $N = 3$).

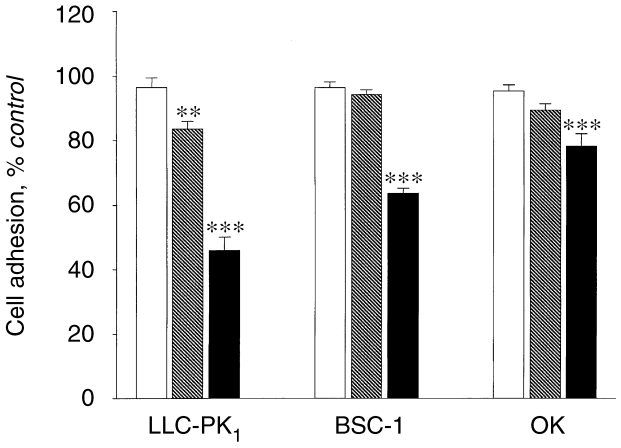

Fig. 1. Effect of nitric oxide (NO) donors on LLC-PK$_1$, BSC-1, and OK cell adhesion to collagen type IV (N = 7, in each cell type). Cells were pretreated with each NO donor for 30 minutes, plated in 96-well culture dishes, and allowed to adhere for 90 minutes. Adherent cells were measured by hexosaminidase enzyme assay. Cell adhesion was expressed as a percentage of control adhesion in each group. Symbols are: (□) DETA/NO 500 μM; (▨) SpNO 500 μM, and (■) SIN-1 500 μM. $^{**}P < 0.01$; $^{***}P < 0.001$ compared with control in each group.

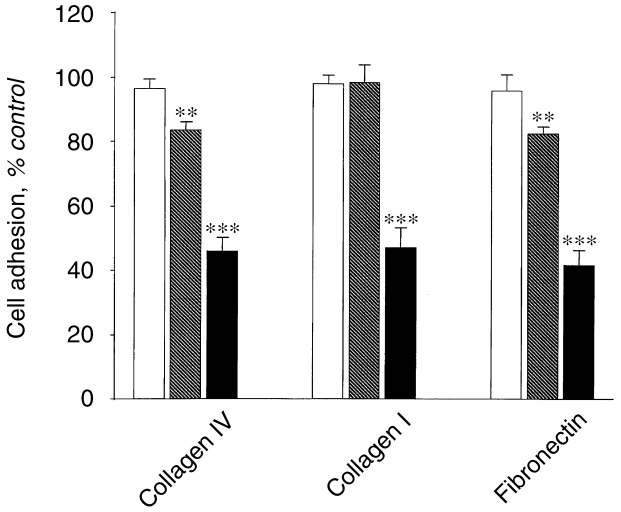

Fig. 2. Effect of nitric oxide (NO) donors on LLC-PK$_1$ cell adhesion to collagen type I (N = 4) and fibronectin (N = 6) in comparison to collagen type IV (N = 7). Cell adhesion was measured as described in Figure 1. Cell adhesion was expressed as a percentage of control adhesion in each group. Symbols are: (□) DETA/NO 500 μM; (▨) SpNO 500 μM; (■) SIN-1 500 μM. $^{**}P < 0.01$; $^{***}P < 0.001$ compared with control in each group.

Effect of nitric oxide donors on LLC-PK$_1$ cell adhesion to collagen type I and fibronectin as compared with collagen type IV

Cell adhesion experiments were also conducted with collagen type I and fibronectin in order to examine the effect of SIN-1 on cell adhesion to different matrix proteins (Fig. 2). DETA/NO (500 μM) had no effect on cell adhesion to any of the three matrices (collagen type I,

98.0 ± 2.5% vs. control, P = NS, N = 4; fibronectin, 95.7 ± 4.2% vs. control, P = NS, N = 6; collagen type IV, 96.4 ± 3.1% vs. control, P = NS, N = 7). SpNO (500 μM) also had no effect on cell adhesion to collagen type I (98.4 ± 5.4% vs. control, P = NS, N = 4), but had a modest effect on cell adhesion to fibronectin (82.3 ± 2.2% vs. control, $P < 0.01$, N = 6) similar to collagen type IV. SIN-1 (500 μM) dramatically attenuated LLC-PK_1 cell adhesion to collagen-type I (47.0 ± 6.0% vs. control, $P < 0.001$, N = 4) and fibronectin (41.6 ± 3.8% vs. control, $P < 0.001$, N = 6) in a manner similar to that observed with collagen type IV.

Dose-dependent effect of SIN-1 on LLC-PK_1, BSC-1, and OK cell adhesion to collagen types I and IV and fibronectin

The effect of SIN-1 on cell adhesion to collagen type IV was dose dependent in all cell lines (Fig. 3A). It was most prominent in the LLC-PK_1 cell (100 μM, 82.7 ± 2.5%; 300 μM, 53.3 ± 4.7%; 500 μM, 45.9 ± 4.2%, N = 7). A less prominent effect was seen in the BSC-1 cell (100 μM, 93.1 ± 3.7%; 300 μM, 79.0 ± 3.2%; 500 μM, 63.6 ± 1.6%, N = 7), and a modest effect was observed in the OK cell (100 μM, 93.0 ± 2.7%; 300 μM, 92.6 ± 4.0%; 500 μM, 78.3 ± 3.7%, N = 7). The effect of SIN-1 on LLC-PK_1 cell adhesion to collagen type I (100 μM, 94.1 ± 2.2%; 300 μM, 62.5 ± 5.1%; 500 μM, 47.0 ± 6.0%, N = 4) and fibronectin (100 μM, 84.9 ± 1.6%; 300 μM, 52.9 ± 4.5%; 500 μM, 41.6 ± 3.8%, N = 6) was also dose dependent (Fig. 3B), as occurred with collagen type IV.

Effect of SIN-1 on viability of adherent and nonadherent LLC-PK_1 cells

Cell viability was determined by trypan blue dye exclusion after a 120-minute exposure to SIN-1 (500 μM). There was no difference in viability between control and SIN-1–pretreated cells, either in adherent (SIN-1, 95.3 ± 1.7% vs. control, 93.8 ± 2.2%, P = NS, N = 4) or nonadherent cells (SIN-1, 79.8 ± 2.3% vs. control, 78.0 ± 4.0%, P = NS, N = 4) plated on collagen type IV (Fig. 4).

Effect of SIN-1 on coated collagen type IV

After SIN-1 (500 μM) was incubated in wells coated with collagen type IV (20 μg/ml, 50 μl/well), there was no difference in subsequent LLC-PK_1 cell adhesion to the SIN-1–exposed collagen type IV (98.0 ± 4.7%) versus unexposed collagen type IV (100%, P = NS, N = 4; Fig. 5).

Nitric oxide, superoxide, and $ONOO^-$ scavengers reversed the effect of SIN-1 on LLC-PK_1, BSC-1, and OK cell adhesion to collagen types I and IV and fibronectin

Simultaneous incubation of SIN-1 (500 μM) with either the NO scavenger C-PTIO (100 μM), the superoxide scavenger SOD (200 unit/ml), and the $ONOO^-$ scavenger uric acid (100 μM) reversed the effect of SIN-1 on cell adhesion in all cell lines and matrix proteins (Fig. 6). With cell adhesion to collagen type IV (N = 7), C-PTIO reversed the effect of SIN-1 on LLC-PK_1 (87.8 ± 1.6% vs. control, $P < 0.05$), BSC-1 (92.8 ± 2.0% vs. control, P = NS), and OK cell adhesion (89.2 ± 2.1% vs. control, P = NS). SOD reversed the effect of SIN-1 on LLC-PK_1 (95.8 ± 5.2% vs. control, P = NS), BSC-1 (99.0 ± 1.7% vs. control, P = NS), and OK cell adhesion (93.5 ± 8.0% vs. control, P = NS). Uric acid reversed the effect of SIN-1 on LLC-PK_1 (96.7 ± 3.1% vs. control, P = NS), BSC-1 (101.2 ± 3.4% vs. control, P = NS), and OK cell adhesion (98.5 ± 2.4% vs. control, P = NS).

The effect of SIN-1 on LLC-PK_1 cell adhesion to collagen type I (N = 4) was also reversed by C-PTIO (98.4 ± 2.4% vs. control, P = NS), SOD (103.4 ± 11.9% vs. control, P = NS), and uric acid (93.3 ± 3.1 vs. control, P = NS).

The scavengers demonstrated a similar pattern on SIN-1–treated LLC-PK_1 cell adhesion to fibronectin: C-PTIO (90.6 ± 2.6% vs. control, P = NS, N = 6), SOD (89.3 ± 3.5% vs. control, P = NS, N = 6), and uric acid (96.2 ± 2.5% vs. control, P = NS, N = 6).

Dose-dependent effects of nitric oxide, superoxide, and $ONOO^-$ scavengers on SIN-1 effect to decrease LLC-PK_1 cell adhesion to collagen type IV

Each scavenger reversed the effect of SIN-1 (500 μM) on LLC-PK_1 cell adhesion to collagen type IV in a dose-dependent manner. C-PTIO (100 μM; Fig. 7A) and uric acid (100 μM; Fig. 7C) completely reversed the effect of SIN-1. SOD at concentrations of 100 to 200 unit/ml reversed the effect of SIN-1 to approximately 80% of the control (Fig. 7B).

DISCUSSION

Nitric oxide is a very important molecule in biological systems, and its diversified effects have been extensively studied. Nevertheless, there are some controversial data about its beneficial and injurious effects. NO is a reactive radical that has a relatively short biological half-life [36] and reacts with many biological molecules such as oxygen and its radicals, transition metals, thiol groups, and antioxidants. In addition, NO influences a number of signal transduction pathways and biosynthetic systems that are essential for normal cellular function [37]. NO can be rapidly inactivated by oxyhemoglobin [38, 39]. Not only the diversified effects of NO, but also different NO synthase (NOS) isoforms, by which NO is produced *in vivo*, contribute to the ultimate effects of the molecule. The endothelial NOS and neuronal NOS, which generate NO at more physiological concentrations, seem to have mainly beneficial or protective effects. In contrast, induc-

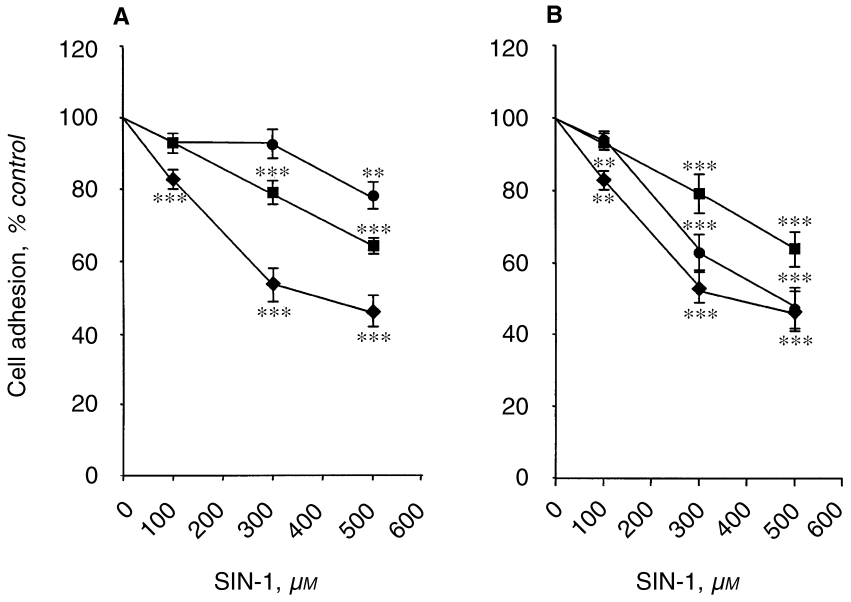

Fig. 3. Dose-dependent effect of SIN-1 on cell adhesion. (*A*) LLC-PK$_1$ (◆), BSC-1 (■), and OK (●) cell adhesion to collagen type IV ($N = 7$, in each cell type). (*B*) LLC-PK$_1$ cell adhesion on collagen type IV (◆; $N = 7$), collagen type I (●; $N = 4$), and fibronectin (■; $N = 6$). Cell adhesion was measured as described in Figure 1. Cell adhesion was expressed as a percentage of control adhesion in each group. $^{**}P < 0.01$; $^{***}P < 0.001$ compared with control in each group.

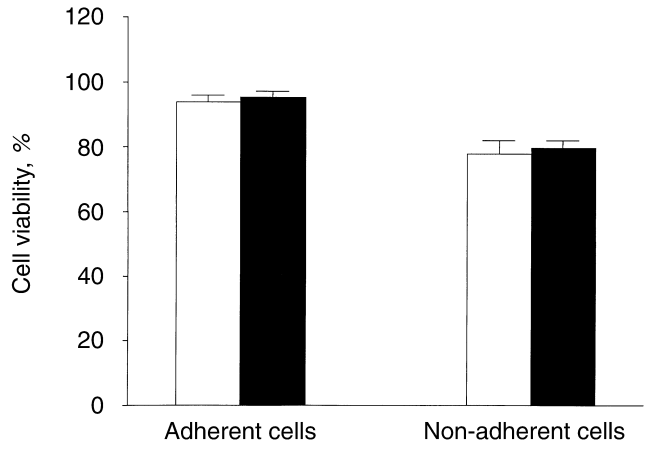

Fig. 4. Effect of SIN-1 (500 μM; ■) on cell viability of adherent ($N = 4$) and nonadherent LLC-PK$_1$ cells ($N = 4$) compared with control (□). Experiments were performed on collagen type IV-coated 96-well cell culture plates as described in Figure 1, and cell viability was determined by trypan blue dye exclusion.

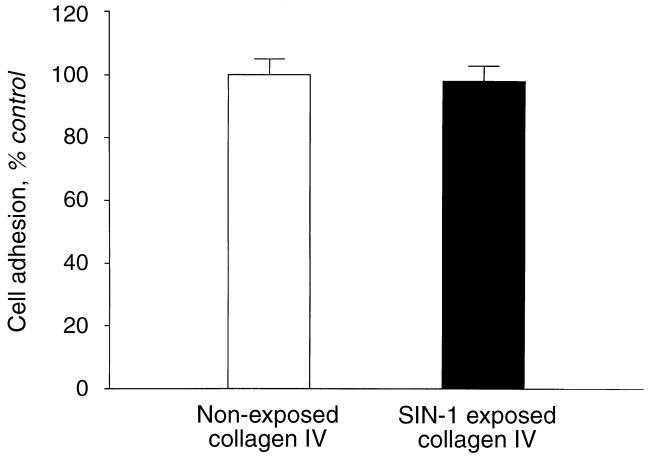

Fig. 5. Effect of SIN-1 on coated collagen type IV. Collagen type IV-coated culture wells were exposed to SIN-1 for 90 minutes and washed, and LLC-PK$_1$ cell adhesion was measured as described in Figure 2. Cell adhesion was expressed as the percentage of control adhesion. Symbols are: (■) non–SIN-1 exposed ($N = 4$); (□) SIN-1 (500 μM)–exposed coated collagen type IV ($N = 4$).

ible NOS, which generates greater amounts of NO during inflammation and injury, tends to have injurious effects. However, recent results revealed opposite effects of vascular smooth muscle NOS and macrophage NOS, both of which are inducible NOS isoforms, in postischemic ARF in rats [28]. In these studies, when rats were treated with antisense oligodeoxynucleotides specific for the macrophage NOS isoform, renal function was dramatically protected from ischemia/reperfusion injury. In contrast, animals treated with antisense oligodeoxynucleotides specific for vascular smooth muscle NOS showed markedly greater renal impairment than the untreated animals subjected to the same renal ischemia/reperfusion [28]. Thus, it is conceivable that the effects of NO and/or its metabolite in biological systems depend largely on the location and nature of the NOS isoform, surrounding reactive molecules, balance between NO generation and degradation, and other opposing mechanisms. Although NO may have a protective role on renal hemodynamics, in renal tubules, the effects of NO are less clear. Although an NO donor has been shown to have a motogenic effect on renal epithelial cells in culture [34, 40], whether this effect on cell adhesion is due to NO itself or its metabolite, $ONOO^-$ has not been examined.

In this study, the role of NO and $ONOO^-$ on renal

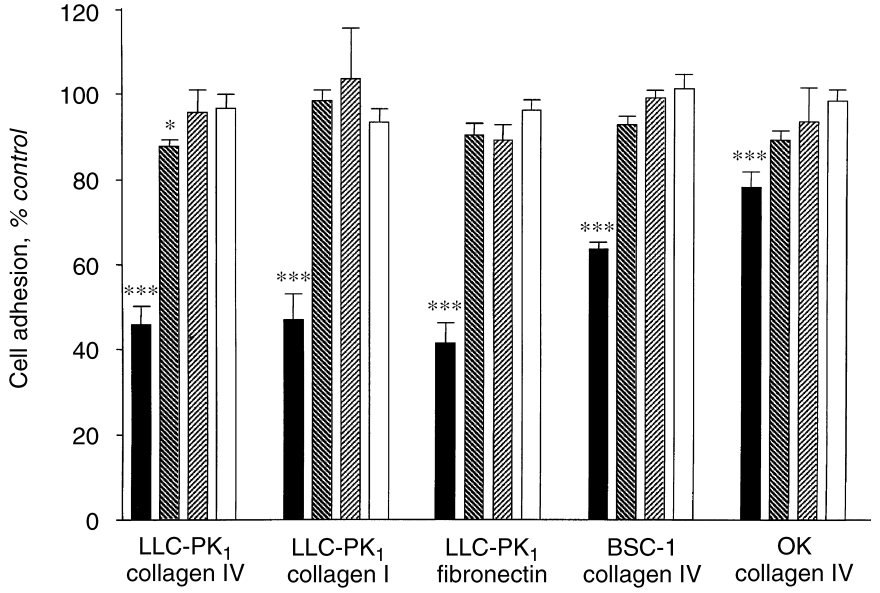

Fig. 6. Scavengers of either NO (C-PTIO), O_2^- (SOD), or $ONOO^-$ (UA) reversed the SIN-1 (500 μM) effect on LLC-PK_1, BSC-1, and OK cell adhesion to collagen type IV (N = 7), collagen type I (N = 4), and fibronectin (N = 6). Scavengers were added simultaneously with the SIN-1, and cell adhesion was measured as described in Figure 2. Cell adhesion was expressed as a percentage of control adhesion in each group. Symbols are: (■) SIN-1; (▨) SIN-1 + C-PTIO (100 μM); (▨) SIN-1 + SOD (200 unit/ml); (□) SIN-1 plus uric acid (100 μM). $*P < 0.05$; $***P < 0.001$ compared with control in each group.

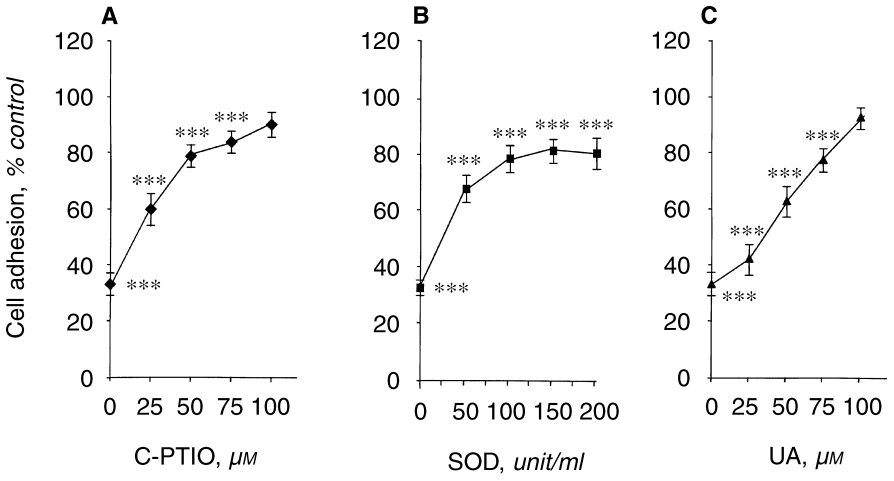

Fig. 7. Dose-dependent effect of scavengers on SIN-1–treated LLC-PK_1 cell adhesion to collagen type IV. (*A*) NO scavenger, C-PTIO, N = 4. (*B*) superoxide scavenger, SOD, N = 4. (*C*) $ONOO^-$ scavenger, UA, N = 4. Scavengers were added simultaneously with the SIN-1, and cell adhesion was measured as described in Figure 2. Cell adhesion was expressed as a percentage of control adhesion in each group. $***P < 0.001$ compared with control in each group.

tubular epithelial cell adhesion was examined. Only SIN-1, which generates both NO and superoxide, which rapidly react to form $ONOO^-$ [41], significantly and consistently decreased renal tubular epithelial cell adhesion in LLC-PK_1, BSC-1, and OK cell lines. In contrast, NO generated from DETA/NO and SpNO had virtually no effect on cell adhesion.

The amount of $ONOO^-$ generated from 500 μM SIN-1 at 37°C, pH 7.4, was estimated to be approximately 5 μM/min [41, 42]. In this regard, it has been reported that NO can increase to 1 to 5 μM during acute inflammation, ischemia/reperfusion, or sepsis [43]. Because the rate of $ONOO^-$ formation depends on the product of the superoxide and NO concentrations [44], it may therefore be possible for low micromolar concentrations of $ONOO^-$ (similar to those used in our study) to be generated *in vivo*.

Moreover, SIN-1 attenuated LLC-PK_1, BSC-1, and OK cell adhesion in a dose-dependent manner. The effect of SIN-1 on renal tubular cell adhesion was also demonstrated on different matrix proteins, that is, basement membrane (collagen type IV and fibronectin) and interstitium (collagen type I). In this regard, different stress situations may alter matrix proteins, and such alterations may affect cell-matrix adhesion [45]. However, in this study, 500 μM of SIN-1 was shown to have no effect on matrix protein integrity. Furthermore, cell viability with SIN-1 was not different from controls.

To confirm the effect of $ONOO^-$ in our study, scavengers of NO, superoxide, and $ONOO^-$ were individually added simultaneously with the SIN-1. C-PTIO was used to scavenge NO, SOD to scavenge superoxide, and uric acid to scavenge $ONOO^-$ [46, 47]; all three separately reversed the effect of SIN-1 on cell adhesion in a dose-

dependent manner. Because the NO donors DETA/NO and SpNO demonstrated little or no effect on renal tubular cell-matrix adhesion, the inhibition by C-PTIO and SOD of the SIN-1–mediated decrease in cell adhesion suggested that $ONOO^-$ and not NO itself was accountable for the effect.

This interpretation was supported by the effect of the $ONOO^-$ scavenger, uric acid, to block the effect of SIN-1.

During cell-matrix adhesion, adhesion molecules on cell membrane surfaces recognize their matrix protein receptors as an initial step of adhesion. After focal adhesion clustering, a cascade of events occurs, including actin microfilament/actin-binding protein assembly, cell–cell adhesion, cell membrane polarity establishment, and a variety of signal transductions. Once focal adhesion is initially established, more stable cell-matrix adhesion necessitates further events. If any of these steps are disturbed, fully developed cell-matrix adhesion will not occur. Although $ONOO^-$ is relatively nonreactive, its conjugate acid, peroxynitrous acid (HO-O-N = O), is an inorganic peroxyacid and is more reactive [48]. Presumably, at least 20% of $ONOO^-$ at pH 7.4, 37°C, in our experiment was already protonated to form peroxynitrous acid [48], because the pKa of $ONOO^-$ is 6.8 at 37°C [49]. $ONOO^-$ is capable of oxidizing a variety of biological molecules, including sulfides [50], thiols [51], deoxyribose [44], lipids [52], α-1 proteinase inhibitor [53], and ascorbate [54]. $ONOO^-$ also may cause nitrate tyrosine residues [55, 56] and may possibly cause protein fragmentation. Therefore, $ONOO^-$ is possibly capable of altering protein function [57, 58] and may affect renal tubular cell-matrix adhesion. However, the mechanism by which $ONOO^-$ attenuates renal tubular cell-matrix adhesion needs further study.

In conclusion, these results provide evidence that $ONOO^-$, not NO itself or superoxide, attenuates renal tubular cell-matrix adhesion in a dose-dependent manner. If $ONOO^-$ is produced during ischemia/reperfusion in ARF, it therefore may affect renal tubular cell-basement membrane adhesion and may contribute to tubular obstruction.

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APPENDIX

Abbreviations used in this article are: ARF, acute renal failure; C-PTIO, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide; DETA/NO, (z)-1-[2-(2-aminoethyl)-N-(2-ammonioethyl)amino]diazen-1-ium-1,2-diolate; DMEM/F12, Dulbecco's modified Eagle medium plus Ham's F12; EDTA, ethylenediamine tetraacetate; GFR, glomerular filtration rate; NO, nitric oxide; NOS, nitric oxide synthase; PBS, phosphate-buffered saline; $ONOO^-$, peroxynitrite; SIN-1, 3-morpholinosydnonimine; SOD, superoxide dismutase; SpNO, spermine NO; UA, uric acid.

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