Indoxyl sulfate induces complex redox alterations in mesangial cells

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Submitted 30 July 2004; accepted in final form 12 January 2006

Gelasco, Andrew K., and John R. Raymond. Indoxyl sulfate induces complex redox alterations in mesangial cells. Am J Physiol Renal Physiol 290: F1551–F1558, 2006.—Indoxyl sulfate is a protein metabolite that is concentrated in the serum of patients with chronic renal insufficiency. It also is a uremic toxin that has been implicated in the progression of chronic renal disease in rodent models. We have shown previously that mesangial cell redox status is related to glomerular damage. We used three methods to examine the ability of indoxyl sulfate to alter mesangial cell redox as a possible mechanism for its toxicity. Indoxyl sulfate increases mesangial cell reduction rate in a concentration-dependent manner as demonstrated by redox microphysiometry. Alterations occurred at concentrations as low as 100 μM, with more marked alterations occurring at higher concentrations associated with human renal failure. We demonstrated that indoxyl sulfate induces the production of intracellular reactive oxygen species (ROS) in mesangial cells (EC50 = 550 μM) by using the ROS-sensitive fluorescent dye CM-DCF. ROS generation was only partially (∼50%) inhibited by the NADPH oxidase inhibitor diphenylethylene iodonium at low (∼300 μM) indoxyl sulfate concentrations. Diphenylethylene iodonium was without effect at higher concentrations of indoxyl sulfate. We also used electron paramagnetic spin resonance spectroscopy with extracellular and intracellular spin traps to show that indoxyl sulfate increases extracellular SOD-sensitive O₂⁻ production and intracellular hydroxyl radical production that may derive from an initial O₂⁻ burst. These results document that indoxyl sulfate, when applied to renal mesangial cells at pathological concentrations, induces rapid and complex changes in mesangial cell redox.

electron paramagnetic spin resonance; fluorescence; microphysiometry; superoxide; NADPH oxidase; reactive oxygen species; uremic toxins

END-STAGE RENAL DISEASE (ESRD) affects over 300,000 people in the U.S. at an annual cost of nearly $22 billion (41). One of the hallmarks of chronic renal insufficiency is the seemingly inexorable progression to ESRD. It is possible that accumulation of circulating toxins in chronic renal insufficiency could accelerate the progression to ESRD. Substances that accumulate in patients with moderate to severe renal failure and that could mediate toxicity are collectively referred to as uremic toxins (30). In many forms of progressive renal disease, the renal mesangial cell serves as a key target for toxicity. The mesangial cell can respond to toxic stresses in several potentially deleterious ways, including proliferation, contraction, release of inflammatory mediators, and alterations in the glomerular basement membrane, leading to irreversible fibrosis. Thus it is possible that some of the manifestations of chronic renal diseases on the renal glomerulus may be due to the actions of uremic toxins on mesangial cells.

Indoxyl sulfate, which also is known as metabolic indican or urinaric indican, is a naturally occurring metabolite of tryptophan. Because indoxyl sulfate levels are elevated in the serum of patients with chronic renal disease, it has been implicated as a putative uremic toxin. Indoxyl sulfate is produced in the liver by modification of indole, a tryptophan derivative that is generated by bacteria in the large intestine. Indoxyl sulfate levels are elevated in patients with chronic renal disease as a result of poor urinary clearance. In addition, indoxyl sulfate has been shown to bind efficiently to albumin and therefore is not effectively cleared by hemodialysis (15, 38).

Indoxyl sulfate and similar putative uremic toxins such as hippuric acid, indole-3-carboxylic acid, and 3-carboxy-4-methyl-5-propyl-2-furanpropionate have been shown to displace drugs from serum albumin (3, 5, 33, 37–39) and, more recently, to act as substrates for organic anion transporters (Oat1 and Oat3) in proximal tubules (6, 7, 13, 14, 28, 34), although the significance of those effects on progressive renal disease is not certain. Indoxyl sulfate and similar compounds are only now being recognized for their potential pathological roles in cell signaling, such as the production of reactive oxygen species (ROS), stimulation of proliferation, or alterations in extracellular matrix. Both ROS-mediated cell proliferation and oxidative stress in renal glomeruli can lead to glomerular hypertrophy, glomerulosclerosis, diminished renal function, and, eventually, chronic renal failure (18). For uremic toxins such as indoxyl sulfate, one may envision a vicious cycle in which deteriorating renal function and reduced glomerular filtration contribute to progressive elevations of serum levels of indoxyl sulfate. In turn, indoxyl sulfate causes additional proliferative and fibrotic glomerular damage (30). Thus it will be worthwhile to better understand the effects of indoxyl sulfate on native glomerular cells such as mesangial cells.

Investigators in our laboratory (23, 24) and others (20–22, 25, 27) have previously demonstrated a role for the generation of ROS in mesangial cell proliferation and activation of mitogenic kinase cascades. In this study, we used three methods to establish that indoxyl sulfate, at concentrations (26, 31) found in serum of patients with ESRD (100–300 μM), can induce the complex production of ROS in rat mesangial cells in culture. Specifically, we wanted to address the role that NADPH oxidase may play in the production of ROS that are generated, because superoxide production via NADPH oxidase has been implicated in numerous redox signaling pathways (19–22, 27). ROS may also play key roles in the signaling effects of indoxyl sulfate and other uremic toxins.

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EXPERIMENTAL PROCEDURES

Materials. RPMI 1640 medium, penicillin-streptomycin, phosphate-buffered saline (PBS), Hanks’ balanced salt solution (HBSS), and fetal calf serum were obtained from Gibco BRL (Gaithersburg, MD). 2-Ethoxy carbonyl-2-methyl-3,4-dihydro-2H-pyrrole-1-oxide (EMPO) and 1-hydroxy-3-carboxy-2,2,5,5-tetramethylpyrrolidine (CP-H) were obtained from Alexis Biochemical (San Diego, CA) and were used without further purification. Indoxyl sulfate, potassium ferricyanide, potassium ferrous cyanide, menadione, N-acetylcysteine (NAC), and Chelex 100 were obtained from Sigma (St. Louis, MO). 5(6)-Chloromethyl-2′,7′-dichlorodihydrofluorescein (CM-DCFH_2) diacetate acetyl ester was obtained from Molecular Probes (Eugene, OR).

Cell culture. Primary rat renal mesangial cells were obtained from cortices of kidneys from young (100–150 g) male Sprague-Dawley rats by using standard sieving techniques as described previously (24). The kidneys were harvested in accordance with a protocol reviewed and approved by the Institutional Animal Care and Use Committee of the Medical University of South Carolina. Cells were incubated at 37°C in a humidified atmosphere of 95% air and 5% CO_2 and were incubated every 1–2 wk by trypsinization until pure cultures of mesangial cells were obtained. These cells were plated at a density of 2–5 × 10^4 cells/ml in RPMI medium supplemented with 20% heat-inactivated fetal bovine serum and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin). Cells used were from passages 6–16.

Potentiometric measurement of cellular reduction rate. A Cytosensor microphysiometer (Molecular Devices, Sunnyvale, CA) was modified to probe the redox state of mesangial cells via an electrochemical potentiometric method as described previously (24, 29). In these experiments, the standard silicon sensors were replaced with customized modified gold electrodes (Molecular Devices), and a ferricyanide-ferrocyanide mixture was added to the extracellular perfusate to act as a redox sensor. The procedure has been validated for several cell types, including mesangial cells (24, 29). Before each experiment, the chambers and electrodes were equilibrated with experimental redox medium consisting of PBS (145 mM Na^+), 4 mM KCl, 1 mM Mg^2+, 1 mM Ca^2+, 143 mM Cl^−, and 10 mM phosphate, pH 7.4) supplemented with 10 mM glucose, 20 mM HEPES, ±1 mg/ml BSA, and 100 μM each of ferricyanide and ferrocyanide. We have typically added an electron transfer mediator such as menadione to the perfusate to enhance the redox state of the electrode. A nA digital current–voltage controller was used to drive the gold electrodes to equilibrate with the extracellular ferricyanide-ferrocyanide redox pair. Data are presented as percent change from basal values, which were calculated as the mean values of three consecutive readings obtained before the readings taken using the test compounds.

Electron paramagnetic resonance spectroscopy and spin trapping. Mesangial cells were trypsinized to release them from the culture dishes and were washed three times in PBS/diethylthiuram monosulfide (DTPA). Washed mesangial cells were then suspended in PBS/DTPA to a final concentration of 10,000 cells/μl. Cell suspensions were incubated with the appropriate spin trap, either CP-H (500 μM) or EMPO (20 mM), and indoxyl sulfate before being transferred to a capillary tube sample holder (50 μl). For diphenylene iodonium (DPI) inhibition experiments, cell suspensions were pretreated with DPI for 20 min before the addition of CP-H and indoxyl sulfate. Electron paramagnetic resonance (EPR) spectra were collected within 3 min of indoxyl sulfate treatment on a Bruker ELEXSYS E500 CW-EPR spectrometer equipped with a 9908SHQ cavity. Typical spectroscopic conditions were as follows: microwave frequency, 9.779 GHz; microwave power, 20 mW; modulation amplitude, 2 gauss; time constant, 655 ms; sweep time, 163 s (for CP-H/02/326 s (for EMPO-OH); sweep width, 64 gauss; receiver gain, 74 dB. EPR spectra were simulated using the WinSim 2000 program (12).

Confocal microscopy and fluorometric laser imaging plate reader analyses of H_2O_2 production. Mesangial cells were plated on 24-mm collagen-coated glass coverslips in six-well culture dishes for live cell confocal microscopy. The culture medium was changed to RPMI 1640 serum-free medium for 48 h before the experiment. Before experimentation, the medium was removed and the cells were washed twice with HBSS/HEPES buffer. Cells were loaded with 10 μM CM-DCFH_2 (an H_2O_2-sensitive fluorescent dye) in HBSS/HEPES, pH 7.4, for 30 min. After cells take up CM-DDCFH_2, it is subsequently modified by intracellular esterases such that it is trapped in the cell. Therefore, cells were next washed three times with HBSS/HEPES and the coverslips were placed on a temperature-controlled perfusion chamber with 1 ml HBSS/HEPES maintained at 37°C. Live cell images were acquired using an Olympus microscope UltraView LCI high-resolution workstation equipped with a laser scanning confocal unit (Ochrohne, Chino, CA), a 15-mW krypton three-line laser head, and a UAPO 340 × 20 (1.35 numerical aperture) oil-immersion objective. Fluorescence was excited using the 488-nm argon laser emission line and collected using a standard fluorescein isothiocyanate filter set (530 ± 30 nm). Images were analyzed using the proprietary software of the microscope workstatation (UltraView; Olympus America).

For fluorometric laser imaging plate reader (FLIPR; Molecular Devices) experiments, cells were seeded in 96-well plates, loaded with CM-DCFH_2, and washed as described above. Plates were placed in the FLIPR instrument and equilibrated to 37°C. The rate and extent of ROS production was measured as the increase of fluorescence of the oxidized dye. Measurements were taken every 1.5 s for 180 s and every 30 s up to 900 s, and the rate of ROS production was determined as either the function (slope) of total fluorescence vs. time over the first 180 s or the maximum value obtained either during the first 180 s or over the entire data-acquisition period (900 s).

RESULTS

Indoxyl sulfate alters reducing rates in rat renal mesangial cells. Redox microphysiometry experiments (Fig. 1) demonstrated that indoxyl sulfate increased the reducing rate of rat mesangial cells. Figure 1A contains representative reduction rate tracings from cell monolayers exposed to increasing concentrations of indoxyl sulfate for five pump cycles (shaded region of the plot). As shown in this plot, the reducing rate did not change upon the switch to control perfusate (buffer). In contrast, switching to perfusate containing indoxyl sulfate caused concentration-dependent increases in the reducing rate, with significant increases at indoxyl sulfate concentrations as low as 50 μM (Fig. 1B). Figure 1B also shows two positive controls for increased mesangial cell reducing rate. Both sodium azide (5 μM), which causes ROS “leakage” by interrupting mitochondrial respiration (oxidative phosphorylation), and serotonin (5-HT), which has been demonstrated previously to induce ROS production in mesangial cells (23, 24), increased the reducing rate.
Because indoxyl sulfate has been shown to bind avidly to albumin in human serum (3), we were concerned that albumin might interfere with the cellular actions of indoxyl sulfate. Figure 1C demonstrates that the addition of 0.5% BSA to the perfusate did not alter the ability of indoxyl sulfate to increase the reducing rate at a pathophysiological concentration (500 μM). Similar results were obtained at higher concentrations (not shown). Thus the presence of albumin in serum is unlikely to affect the ability of indoxyl sulfate, in concentrations demonstrated to occur in patients with ESRD (15, 38), to alter mesangial cell redox status.

**Indoxyl sulfate induces ROS formation in rat mesangial cells.** We next examined the extent of ROS production caused by indoxyl sulfate treatment of mesangial cells by using a fluorescent H₂O₂ indicator and a FLIPR. Cells were seeded into 96-well plates, loaded with CM-DCFH₂, washed, and placed into the FLIPR. Cells were then treated with increasing indoxyl sulfate concentrations from 30 μM to 6 mM, and fluorescence was measured. Figure 2 shows concentration-response relationships of the fluorescence data derived from either the slope of fluorescence vs. time over the first 180 s of exposure or the maximum fluorescence value obtained over the first 180 s. Both curves are sigmoidal in shape, suggesting that a saturable process is involved in the production of H₂O₂. Kinetic curve fitting of both plots gave similar EC₅₀ values of ∼540–555 μM. Moreover, the data show that measurable amounts of H₂O₂ were generated in mesangial cells by concentrations of indoxyl sulfate as low as 100 μM, which is within the range of concentrations of indoxyl sulfate reported in patients with chronic renal disease (15, 38).

NADPH oxidase previously has been shown to be a major source of mesangial cell ROS (23, 24). NADPH oxidase could be involved in the production of H₂O₂, because superoxide can be converted into H₂O₂ through the actions of superoxide dismutase (SOD). To examine a potential role for NADPH oxidase in the effects of indoxyl sulfate, we pretreated mesangial cells with medium containing either 100 μM apocyanin, or vehicle (DMSO) during CM-DCFH₂ loading, after which we exposed cells to increasing indoxyl sulfate concentrations. After pretreatment, the cells were exposed to three different concentrations (150, 300, and 1,000 μM) of indoxyl sulfate in the presence of 100 μM DPI, 300 μM apocyanin, or 20 μg/ml DMSO (vehicle). Similar experiments have demonstrated that DMSO at concentrations up to 25 μg/ml does not affect the response to indoxyl sulfate (data not shown). As shown in Fig. 3, pretreatment of mesangial cells with DPI significantly reduced (>50%) ROS production at pathophysiological concentrations of indoxyl sulfate (<300 μM). This effect was apparent whether maximum fluorescence was measured at 180 or 900 s. In contrast, there was little effect of DPI on ROS production induced by 1 mM indoxyl sulfate.

**Fig. 1.** Indoxyl sulfate alters the reducing rate of rat mesangial cells in culture as measured by redox microphysiometry. A: indoxyl sulfate at 250 μM, 500 μM, and 1 mM increased the extracellular reducing rates of mesangial cells compared with untreated controls, expressed as a percentage of a stable baseline. Cells were treated with indoxyl sulfate for 6 min (4 cycles) as indicated by the shaded box. B: indoxyl sulfate increased mesangial cell reducing rates in a concentration-dependent manner. Positive controls of 5 mM sodium azide (oxidative phosphorylation inhibitor) and 1 μM serotonin (5-HT; a 5-HTA receptor agonist) are shown for comparison.

**Fig. 2.** Indoxyl sulfate increases intracellular production of H₂O₂ as measured by oxidation of 5(6)-chloromethyl-2',7'-dichlorodihydrofluorescein (5,6-CM-DCF) on a FLIPR (fluorometric laser imaging plate reader). Indoxyl sulfate causes an increase in intracellular H₂O₂ production measured as maximum fluorescence 180 s after stimulation (○) or rate of fluorescence change after stimulation (●). Bars indicate means ± SE compared with control (unstimulated) cells based on 3 experiments.
Described in EXPERIMENTAL PROCEDURES. Histogram represents the maximum with either 100 μM DPI at pathophysiological indoxyl sulfate concentrations after pretreatment for 30 min with either 100 μM DPI, 300 μM apocyanin (apo), or vehicle (DMSO), as described in EXPERIMENTAL PROCEDURES. Histogram represents the maximum stimulation during the first 180 s or during the entire 900-s incubation. At low concentrations of indoxyl sulfate (150 or 300 μM), the fluorescence signal was significantly reduced by 69 and 74% at 150 μM, and by 49 and 56% at 300 μM, with DPI treatment. At both 300 μM and 1 mM indoxyl sulfate, apocyanin attenuated the fluorescent signal. *P < 0.01 vs. matched values without inhibitor pretreatment. Bars indicate means ± SE based on at least 3 experiments. NS, no significant difference; t, time in seconds.

DPI inhibits a number of flavin-containing enzymes, most notably NADPH oxidase. Another NADPH oxidase inhibitor, apocyanin, attenuated ROS production at all concentrations of indoxyl sulfate, and this attenuation was sustained throughout the study. The most likely explanation for the DPI-insensitive component of the fluorescence signal at 1 mM indoxyl sulfate is that there are two pathways leading to H2O2 production, one of which requires a flavin-sensitive enzyme such as NADPH oxidase.

**Intracellular H2O2 production can be inhibited by NAC and DPI.** We used confocal microscopy with the fluorescent dye CM-DCHF2 to demonstrate that H2O2 is produced in response to indoxyl sulfate in mesangial cells (Fig. 4). Figure 4 shows photomicrographs of quiescent mesangial cells loaded with CM-DCHF2 for 30 min, during which time the cells were incubated with vehicle, DPI, or the reducing agent NAC and then subsequently treated with either indoxyl sulfate (300 μM) or vehicle. Confocal microscopic images composed of super-imposed Z-axis scans were taken just before [time (t) = 0 min] and 10 min after (t = 10 min) exposure to indoxyl sulfate. The 10-min time point was chosen as representative of the near-maximal response time. Figure 4A shows vehicle-treated cells, whereas Fig. 4B shows a representative image of cells treated with 300 μM indoxyl sulfate at t = 10 min. As shown in Fig. 4C, mesangial cells that were treated with NAC (10 μM) during the final 20 min of CM-DCHF2 loading had a significantly reduced response to 300 μM indoxyl sulfate. In contrast, Fig. 4D shows that the basal level of fluorescence in NAC-treated cells is similar to that seen in control cells (compare with Fig. 4A). NAC is an antioxidant that primarily scavenges HO- and H2O2 but that can indirectly scavenge O2⋅−, as well.

We then used DPI to investigate a potential role of NADPH oxidase in ROS production in indoxyl sulfate-stimulated cells. In a manner similar to NAC treatment, the cells were treated with DPI during the final 20 min of CM-DCHF2 loading. Figure 4, E (t = 0 min) and F (t = 10 min), shows the nearly complete attenuation of dichlorofluorescein oxidation by 100 μM DPI upon treatment with 300 μM indoxyl sulfate. A lower concentration of DPI (10 μM) also significantly attenuated indoxyl sulfate-induced increases in fluorescence (Fig. 4H, compare with 4F), although a residual signal remained at 10 min after exposure (Fig. 4G).

**Indoxyl sulfate increases extracellular ROS production in rat mesangial cells.** EPR spectroscopy is a useful tool for measuring ROS production because it directly probes free...
Integration of the EPR signals from three different experiments shows that indoxyl sulfate-induced CP· formation is concentration dependent and that it can be blocked at pathophysiological concentrations of indoxyl sulfate by NADPH oxidase inhibitors. As shown in Fig. 6, 300 μM indoxyl sulfate increased extracellular production of \( \text{O}_2^\cdot \) about twofold. The amplitude of the CP· signal was very high at 1 mM indoxyl sulfate, increasing approximately sixfold over the basal signal for untreated cells. At this concentration of indoxyl sulfate, pretreatment with 100 μM DPI caused no significant attenuation of \( \text{O}_2^\cdot \) production. Given that CP· formation has been reported to be due exclusively to the reaction of CP-H with \( \text{O}_2^\cdot \) (8–11), we expected that pretreatment of the cells with DPI would block NADPH oxidase and subsequently reduce the rate of \( \text{O}_2^\cdot \) production significantly. This observation indicates that indoxyl sulfate can cause production of extracellular \( \text{O}_2^\cdot \) through a non-DPI (flavin enzyme)-inhibitable mechanism.

Again, in contrast, when the cells were treated with either 10 or 300 μM indoxyl sulfate, increasing approximately sixfold over the basal signal for untreated cells. At this concentration of indoxyl sulfate, pretreatment with 100 μM DPI caused no significant attenuation of \( \text{O}_2^\cdot \) production. Given that CP· formation has been reported to be due exclusively to the reaction of CP-H with \( \text{O}_2^\cdot \) (8–11), we expected that pretreatment of the cells with DPI would block NADPH oxidase and subsequently reduce the rate of \( \text{O}_2^\cdot \) production significantly. This observation indicates that indoxyl sulfate can cause production of extracellular \( \text{O}_2^\cdot \) through a non-DPI (flavin enzyme)-inhibitable mechanism.

Radicals and can be used to both quantitate and identify specific free radical species. We used \( \text{O}_2^\cdot \)-selective spin trap, CP-H, to investigate the extracellular production of \( \text{O}_2^\cdot \) in indoxyl sulfate-treated mesangial cells. CP-H has been reported to react directly with \( \text{O}_2^\cdot \) to form a nitroxide radical species (CP·), which can be identified by its distinctive three-line EPR spectrum (9, 11). Because the trap is present in excess of the \( \text{O}_2^\cdot \), the amplitude of the EPR signal is directly proportional to the extracellular \( \text{O}_2^\cdot \) concentration. Mesangial cells were suspended in HBSS/HEPES, at 10,000 cells/μl, and were treated with increasing concentrations of indoxyl sulfate for 5 min at room temperature. EPR spectra were measured within 3 min after incubation. Figure 5 shows representative spectra from one of these experiments. The consistency of shapes of the EPR spectra indicates that a single radical species (CP·) is being rapidly produced with increasing concentrations of indoxyl sulfate from 300 μM to 1 mM. To confirm that the ROS trapped by CP-H was exclusively extracellular \( \text{O}_2^\cdot \), we pretreated mesangial cells with polyethylene glycol-SOD, which rapidly metabolizes \( \text{O}_2^\cdot \) to \( \text{H}_2\text{O}_2 \), before exposure to 1 mM indoxyl sulfate. The resulting EPR signal (Fig. 5D) was <20% of the intensity of that for the untreated control cells (Fig. 5E) even at this elevated concentration of indoxyl sulfate. Non-glycosylated SOD had a similar effect, suggesting that \( \text{O}_2^\cdot \) is, in fact, the extracellular ROS that is produced in response to indoxyl sulfate and which is trapped by CP-H.

Fig. 5. Indoxyl sulfate increases extracellular production of reactive oxygen species (ROS), causing oxidation of the electron paramagnetic resonance (EPR) spin probe 1-hydroxy-3-carboxy-2,2,5,5-tetramethylpyrroline (CP-H) to CP·. Rat mesangial cells in PBS at 10,000 cells/μl were treated with the spin trap CP-H (500 μM) for 1 min and then with 1 mM (A), 500 μM (B), or 300 μM (C) indoxyl sulfate for 1 min. Indoxyl sulfate at all concentrations showed increased production of CP· (with increasing toxin concentration) compared with control cells treated with PBS (E), as measured using EPR spectroscopy. The EPR spectrum (D) shows the effect of 10 units (U) of superoxide dismutase (SOD) on CP· production induced by indoxyl sulfate, supporting the identity of the trapped ROS as \( \text{O}_2^\cdot \). IS, indoxyl sulfate.
100 μM DPI before exposure to 300 μM indoxyl sulfate, the extent of CP-H formation was greatly attenuated, a result consistent with both of the results observed for intracellular ROS production (Fig. 4) and a second mechanism of O₂⁻ production at high indoxyl sulfate concentrations.

Indoxyl sulfate induces intracellular hydroxyl radical production in rat mesangial cells. Because CP-H is poorly transported across the cell membrane (9–11, 17, 36), we used the more cell-permeable spin trap, EMPO, to investigate the identity of the intracellular ROS observed in the FLIPR fluorescence experiments. EMPO reacts with both O₂⁻ and HO₂ radicals and exhibits EPR spectral characteristics that distinguish the two radical adducts (35, 40, 42). Figure 7A shows an EPR spectrum that indicates the presence of EMPO spin-trapped radical species in indoxyl sulfate-treated mesangial cells. This “four-line” spectrum is characteristic of trapped oxygen-based radical species (O₂⁻ and HO₂) and was present only in cells treated with both indoxyl sulfate and 20 mM EMPO. Under these experimental conditions, in which the cell suspension was treated with EMPO and then washed with HBSS/HEPES before indoxyl sulfate stimulation, only intracellular ROS species should be detected. Figure 7C shows the EPR spectrum for control cells pretreated with EMPO, washed, and then exposed to buffer. Computer simulation of the spectrum observed in Fig. 7A with WinSim 2000 (12) can be used to identify the radical species trapped, because the spectral parameters are unique for each type of EMPO-trapped radical. Simulation of the spectrum was best fit using the parameters listed in Table 1, which are consistent with those obtained from spectra of EMPO-OH⁻ (35, 40). The simulated spectrum is shown in Fig. 7B. The spectral parameters and distribution of the cis/trans-EMPO adduct isomers are not only consistent with those previously reported for the EMPO-OH⁻ adduct but are significantly different from those expected for the EMPO-OOH⁻ adduct (35, 40, 42). The presence of intracellular HO⁻ is consistent with the results observed in the FLIPR experiments, in that intracellular H₂O₂ is generated upon indoxyl sulfate stimulation and can be converted to HO⁻ in the presence of iron through the Fenton (16) reaction.

**Table 1. EPR simulation parameters for EMPO-trapped indoxyl sulfate-induced radical formation in rat mesangial cells**

<table>
<thead>
<tr>
<th>Composition</th>
<th>A₀, mT</th>
<th>A₀H₀, mT</th>
<th>A₀Hᵥ, mT</th>
</tr>
</thead>
<tbody>
<tr>
<td>EMPO-OH isomer 1</td>
<td>47.7</td>
<td>1.40</td>
<td>1.51</td>
</tr>
<tr>
<td>EMPO-OH isomer 2</td>
<td>52.3</td>
<td>1.39</td>
<td>1.27</td>
</tr>
</tbody>
</table>

A₀, A₀H₀, and A₀Hᵥ are hyperfine coupling constants for 2-ethoxycarbonyl-2-methyl-3,4-dihydro-2H-pyrrole-1-oxide (EMPO) isomers in electron paramagnetic resonance (EPR) simulations.

**DISCUSSION**

The role of indoxyl sulfate as a uremic toxin is not completely understood, but it has been shown to inhibit binding of drugs to serum albumin (37, 38), to be elevated in sera of ESRD patients (100–300 μM vs. 0–3 μM in normal patients) and in animal models of renal disease (31, 32), to cause
progression of renal disease in rats (1, 2, 32), and to be a substrate for, and an inhibitor of, human organic anion transporters in proximal tubules (6, 7, 13, 14). Recently, indoxyl sulfate was implicated in increased carbonyl stress in hemodialysis patients (26). Work in our laboratory has previously shown that there is a relationship between altered redox status and activation of mitogenic kinases and production of the profibrotic factor transforming growth factor (TGF)-β in mesangial cells (4, 23, 24). The purpose of this study was to identify a potential role for indoxyl sulfate as a mediator of oxidative stress in mesangial cells.

What is new about this work is that we have used three methods to demonstrate that indoxyl sulfate, when applied at concentrations seen in patients with chronic renal insufficiency, rapidly induces the production of intracellular and extracellular ROS in cultured mesangial cells. Investigators in our laboratory previously used redox microphysiometry to demonstrate that hormones can alter the redox states of various cell lines, including mesangial cells (24) and transfected fibroblasts (29). In the current study, increasing concentrations of indoxyl sulfate (up to 1 mM) resulted in an increased reducing rate (an indicator of ROS production) to a maximum of ~250% of basal levels. When mesangial cells were exposed to concentrations of indoxyl sulfate similar to those that are manifested in patients with ESRD (100–300 μM), the reducing rate was increased to ~150% of basal levels. Redox microphysiometry can be used to detect ROS that are produced extracellularly or those produced intracellularly and transported to the extracellular space. Although redox microphysiometry can serve as a screening tool for cellular redox effects on cells, it does not provide significant insight into the nature of the ROS being produced.

Accordingly, we used two different methods to examine intracellular and extracellular ROS formation in response to indoxyl sulfate. We used the fluorescent dye CM-DCFH2 to measure selective intracellular H2O2 production and EPR spin trapping to identify both intracellular and extracellular ROS. Studies with CM-DCFH2 showed that indoxyl sulfate increased H2O2 production, and at concentrations of indoxyl sulfate ≤300 μM, this effect was sensitive to DPI and apocyanin (Figs. 3 and 4), suggesting that NADPH oxidase was involved in the process. This is plausible in that O2•- (the product of NADPH oxidase) can be converted to H2O2 by SOD. Nevertheless, there was a significant DPI-insensitive component of H2O2 production that became predominant at indoxyl sulfate concentrations >300 μM, suggesting that indoxyl sulfate induces production of H2O2 in mesangial cells through two pathways. The relevance of this high concentration of indoxyl sulfate effect in light of the known pathophysiological levels of the uremic toxin can be debated, but it is possible that these high concentrations could exist, however fleetingly, in localized intercellular space in the compromised nephron.

We also found that indoxyl sulfate could induce the production of intracellular hydroxyl radical (HO•) by using an intracellular spin trap (EMPO) for EPR studies (Fig. 7). The relationship between the pathways of production of O2•- and HO• is not clear, but H2O2 can be converted to H2O by glutathione peroxidase or to H2O and O2 by catalase, or it can be converted to HO• in the presence of iron via the Fenton (16) reaction. Thus we have provided evidence that indoxyl sulfate can induce the formation of three types of intracellular ROS in mesangial cells: O2•-, H2O2, and HO•.

We also examined the possibility that indoxyl sulfate could induce the production of extracellular ROS in mesangial cells by using CP-H, a spin trap that does not readily cross the plasma membrane of intact cells. CP-H has been reported to react exclusively with O2•- (9, 11). We showed a concentration-dependent increase in a three-line EPR spectrum, consistent with O2•- formation, in the extracellular media from mesangial cells exposed to indoxyl sulfate (Figs. 5 and 6). The ability of exogenously applied SOD to completely block the development of this spectrum verifies that the ROS trapped by CP-H is O2•- Again, we observed different effects at supra- and pathophysiological concentrations. The development of the three-line spectrum was insensitive to DPI and apocyanin at 1 mM indoxyl sulfate, suggesting that NADPH oxidase was not involved in the production of extracellular O2•-. However, DPI and apocyanin proved effective at blocking CP-H formation at 300 μM indoxyl sulfate, supporting a role for an NADPH oxidase-like enzyme in this process. The role of this extracellular O2•- is unclear, but it likely contributes to the redox microphysiometry signal observed during indoxyl sulfate treatment.

In conclusion, indoxyl sulfate stimulates the production of intracellular and extracellular ROS in rat mesangial cells as summarized in Fig. 8. Indoxyl sulfate also results in increases in the production of intracellular O2•-, HO•, and H2O2 through a pathway that most likely involves NADPH oxidase. The complex and evanescent nature of the production of intracellular ROS makes them ideal candidates to serve as cellular signaling molecules and also makes them candidates for mediating mesangial cell toxicity. The finding that indoxyl sulfate also induces production of extracellular O2•- through a pathway that does not appear to involve NADPH oxidase was unexpected, although this finding suggests that indoxyl sulfate may induce mesangial cells to release or produce extracellular ROS, which could serve paracrine functions with other mesangial cells, glomerular podocytes, or native cells of the glomerular vasculature.

ACKNOWLEDGMENTS

We are indebted to Dr. Maria Garnovskaya for helpful discussions regarding redox microphysiometry.

GRANTS

This work was supported in part by National Institutes of Health (NIH) Grants DK-59950 (to A. K. Gelasco) and DK-54720 (to J. R. Raymond), a Department of Veterans Affairs Merit Award (to J. R. Raymond), and a Research Enhancement Award Program (to A. K. Gelasco and J. R. Raymond), as well as a laboratory endowment jointly supported by the Medical University of South Carolina (MUSC) Division of Nephrology and Dialysis Clinicals, Incorporated (to J. R. Raymond). The FLIPR is a shared MUSC resource obtained with NIH Grant S10RR-13005. The microphysiometer is a shared Veterans Affairs resource obtained with a Veterans Affairs large equipment grant. The EPR spectrometer is a shared MUSC resource obtained with NIH Grant S10RR-13656.

REFERENCES

ALTERATION OF CELLULAR REDOX BY INDOXYL SULFATE


