Hydrogen Peroxide Induces 21-Aminosteroid-Inhibitable 
F2-Isoprostane Production and Cytolysis in Renal Tubular 
Epithelial Cells1

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ABSTRACT

F2-isoprostanes are the newly identified reactive oxygen species–catalyzed peroxidation products of arachidionate. The infusion of these prostaglandin F2-like prostanooids into the rat kidney induces profound parallel reductions in RBF and GFR, suggesting that these metabolites may be partly responsible for the hemodynamic alterations seen in free radical–linked acute renal injury models. The present study examined directly in renal proximal tubular (LLC-PK1) cells whether hydrogen peroxide, a reactive oxygen species implicated in many models of acute renal injury, induces F2-isoprostane production and whether its production can be inhibited by the recently synthesized lipid peroxidation inhibitor 21-aminosteroid (lazaroid U-74389G). The incubation of LLC-PK1 cell layers with hydrogen peroxide for 3 h resulted in a dose-related six-fold increase in F2-isoprostane production, measured by the gas chromatographic–mass spectroscopic method. The preincubation of cells with 21-aminosteroid prevented hydrogen peroxide–induced F2-isoprostane production, a finding also demonstrable with other lipid peroxidation inhibitors, e.g., 2-methyl aminochroman (U-83836E) and diphenyl-p-phenylene diamine. Besides inhibiting isoprostane production, 21-aminosteroid reduced hydrogen peroxide–induced lipid degradation and peroxidation, and protected the cells against hydrogen peroxide–induced cytolyis. The novel finding that hydrogen peroxide induces 21-aminosteroid–inhibitable F2-isoprostane production in renal epithelial cells supports the in vivo report that its levels are elevated in reactive oxygen species–linked renal injury models such as ischemia-reperfusion. Besides direct cell injury, lipid peroxidation by generating F2-isoprostanes may further contribute to renal dysfunction through a vasoconstrictive mechanism. Thus, the inhibition of excess F2-isoprostane production may be one of the additional mechanisms, besides cytoprotection, by which antioxidants ameliorate renal dysfunction in experimental models of acute renal injury.

Key Words: Lipid peroxidation, oxygen free radicals, kidney, renal injury, prostaglandins

Besides being one of the early and critical events preceding renal cell injury (1), reactive oxygen species (ROS)-induced membrane lipid peroxidation may further contribute to renal dysfunction for the following reasoning. It was recently discovered that ROS, by inducing arachidonic acid peroxidation, can generate a novel class of 8-epi-prostaglandin F2α-like prostaglandins, termed F2-isoprostanes (2). Its levels were found to be markedly elevated in a renal ischemia/reperfusion model, and its infusion into the renal artery of normal rats was accompanied by a striking reduction in RBF and GFR (3). Thus, these newly identified prostanooids may account for some of the undesirable hemodynamic alterations associated with acute renal injury. In this study, we examined directly whether hydrogen peroxide (H2O2), an ROS implicated in models of renal injury including renal ischemia/reperfusion, induced F2-isoprostane production in proximal renal epithelial cells and determined whether a recently synthesized potent lipid peroxidation inhibitor, 21-aminosteroid (lazaroid U-74389G) (4), in addition to limiting H2O2-induced lipid peroxidation and cell injury, was able to inhibit H2O2-induced F2-isoprostane production.

METHODS

H2O2-Induced F2-Isoprostane Production: Effect of 21-Aminosteroid

LLC-PK1 cells, an analog of proximal tubular cells, were grown by standard techniques (1). H2O2-induced irreversible cell injury was quantitated by the standard method of pre-loaded cellular 51Cr release (1).

Confluent cell layers were incubated with 0, 0.5, or 1.5 mM H2O2 for 3 h. Cell layers in additional wells preloaded with 20 μM each of 21-aminosteroid or other inhibitors of lipid peroxidation such as 2-methyl aminochroman or diphenyl-p-phenylene diamine (DPPD) were also incubated with 1.5
mM \text{H}_2\text{O}_2\). 2-Methyl aminochroman was dissolved in normal saline, and DPPD was dissolved in absolute ethanol. Scraped cells in the supernatant were sonicated briefly and immediately snap frozen and kept frozen until \text{F}_2\text{-isoprostane} determination at Vanderbilt University. Free \text{F}_2\text{-isoprostanes} in cells were measured after purification and derivatization by using gas chromatography/negative ion chemical ionization-mass spectrometry with \textsuperscript{3}H\text{prostaglandin F}_2\text{ as the internal standard as described previously (2).}

**H\textsubscript{2}O\textsubscript{2}-Induced Cell Injury: Effect of 21-Aminosteroid**

Confluent cell layers were loaded with 0 to 50 \text{mM} 21-aminosteroid during 3 h of \textsuperscript{5}Cr radlolabeling. Washed cell layers were then incubated for 3 h in the presence and absence of 1.5 mM \text{H}_2\text{O}_2, and \textsuperscript{5}Cr release was determined. Also examined was the effect of 20 \text{mM} 21-aminosteroid on graded concentrations of \text{H}_2\text{O}_2. 21-Aminosteroid was first dissolved in absolute ethanol and then diluted in fetal calf serum. The spontaneous \textsuperscript{5}Cr release (percentage of total) in the control was always determined in the same plate and subtracted from the \textsuperscript{5}Cr release (percentage of total) in experimental wells to provide the percent specific \textsuperscript{5}Cr release, which was 4.5 to 8.6%/h.

**Effect of 21-Aminosteroid on \text{H}_2\text{O}_2\text{-Induced Cytolysis and Lipid Degradation**

We used the release of \textsuperscript{3}H\text{arachidonic acid} from prelabeled LLC-PK\textsubscript{1} cells as an indirect measure for \text{H}_2\text{O}_2\text{-induced lipid degradation. Confluent cells were dual labeled with \textsuperscript{3}H\text{arachidonic acid for 18 h and with \textsuperscript{5}Cr for 3 h (1). 21-Aminosteroid (20 \text{mM})} was also included in one group of wells during the last 3 h of radlolabeling. Washed cell layers were then subjected to 3 h of 1.5 mM \text{H}_2\text{O}_2\text{ incubation.} \text{H}_2\text{O}_2\text{ radioactivity in 10 \text{\mu L}} of the supernatant was determined at 1.5 and 3 h of incubation, and \textsuperscript{5}Cr release was determined at 3 h. The \textsuperscript{3}H\text{arachidonic acid release, corrected for spontaneous release, was factored for 10^6 \text{cpm of \textsuperscript{5}Cr to account for cell content.**}

**Effect of 21-Aminosteroid on \text{H}_2\text{O}_2\text{-Induced Lipid Peroxidation**

Cells with and without 21-aminosteroid were incubated with 1.5 mM \text{H}_2\text{O}_2. At the end, butylated hydroxytoluene (0.01%) was added to minimize procedural lipid peroxidation. Malondialdehyde was measured as an index of lipid peroxidation in the cell-supernatant suspension as thiobarbituric acid-reactive substances (TBARS) by the standard thiobarbituric acid reaction (1).

**Statistical Methods**

Results are presented as mean ± SE. Data were analyzed by analysis of variance with Tukey's correction for multiple comparison. Significance was set at the \( P < 0.05 \) level.

**RESULTS**

**H\textsubscript{2}O\textsubscript{2}-Induced \text{F}_2\text{-Isoprostane Production: Effect of 21-Aminosteroid**

\text{H}_2\text{O}_2\text{ induced a dose-related six-fold increase in \text{F}_2\text{-isoprostane production that was effectively inhibited by 20 \text{mM} 21\text{-aminosteroid (Figure 1). Other lipid peroxida-}**

![Figure 1. The effect of 21-aminosteroid on \text{H}_2\text{O}_2-induced \text{F}_2\text{-isoprostane production. Cell layers were incubated with 0, 0.5, or 1.5 mM \text{H}_2\text{O}_2 for 3 h. Additional cell layers preloaded with 20 \text{mM} each of 21-aminosteroid (AS), 2-methyl aminochroman (MAC), or DPPD were also incubated with 1.5 mM \text{H}_2\text{O}_2. \text{F}_2\text{-isoprostanes were measured in cell-supernatant suspension by the gas chromatographic-mass spectroscopic method. Two experiments in duplicate at least, mean ± SE; * P < 0.05 versus control, ** P < 0.05 versus 1.5 mM \text{H}_2\text{O}_2.**}

**Effect of 21-Aminosteroid on \text{H}_2\text{O}_2\text{-Induced Cytolysis and Lipid Degradation**

21-Aminosteroid significantly and in a dose-dependent manner prevented the cytosis of 1.5 mM \text{H}_2\text{O}_2 (Figure 2, upper). 21-Aminosteroid attenuated the cytosis induced by 0.5 to 1.5 mM concentrations of \text{H}_2\text{O}_2 and was particularly effective against 0.5 mM \text{H}_2\text{O}_2 (Figure 2, lower).

**Concurrent Effect of 21-Aminosteroid on \text{H}_2\text{O}_2\text{-Induced Cytolysis and Lipid Degradation**

\text{H}_2\text{O}_2\text{ induced a time-dependent release of prelabeled \textsuperscript{3}H\text{arachidonic acid that was prevented or reduced by the presence of 21-aminosteroid (Figure 3). The reduction in arachidonic acid release by 21-aminosteroid was accompanied by a concomitant reduction but not prevention in \text{H}_2\text{O}_2\text{-induced cytosis; specific \textsuperscript{5}Cr release was reduced from 55.0 ± 2.4 to 24.7 ± 0.5% by 21-aminosteroid.**}
Effect of 21-Aminosteroid on H$_2$O$_2$-Induced Lipid Peroxidation

H$_2$O$_2$ induced an over twofold increase in TBARS that was effectively inhibited by the preincubation of LLC-PK$_1$ cells with 21-aminosteroid (Figure 4).

DISCUSSION

We provide evidence for the first time in cell culture that H$_2$O$_2$ directly induces an excessive production of F$_2$-isoprostanes and that it can be effectively inhibited by 21-aminosteroid and other lipid peroxidation inhibitors. This study additionally demonstrates that 21-aminosteroid inhibits H$_2$O$_2$-induced lipid alterations in renal epithelial cells and limits cell injury.

The model used here, albeit simple, allows one to directly examine the effect of H$_2$O$_2$ on proximal tubular cells. Elevated H$_2$O$_2$ levels, probably in the low micromolar concentrations, have been demonstrated directly or indirectly in acute renal injury models, and the bulk of the renal injury often occurs at the proximal tubular level (5–8). Lipid peroxidation accompanies models of renal injury and, with the attendant membrane damage, can potentially invoke a whole host of functional and structural derangements (1,9). Consistent with a role for lipid peroxidation in renal injury is our in vitro finding that lipid peroxidation inhibition by 21-aminosteroid was attended by cytoprotection. Although, by TBARS measurements, there was complete inhibition of lipid peroxidation with 21-aminosteroid, two other probably more sensitive measures of lipid alterations, [3H]-arachidonic acid release and F$_2$-isoprostanes production, did not demonstrate total abrogation of H$_2$O$_2$-induced lipid alterations with 21-aminosteroid. The finding that lipid alterations even in the mild form may initiate the injury processes of cell injury may be a possible explanation to account for the incomplete cytoprotection seen here with 21-aminosteroid. Alternatively,
membrane peroxidation is one of the several mechanisms involved in the injury process, and therefore, the inhibition of lipid peroxidation can only be expected to offer partial protection (9).

The new finding that oxidants can induce marked increases in 21-aminosteroid-inhibitable F₂-isoprostanes brings forth the intriguing possibility that these vasoactive F₂-isoprostanes may be involved in the renal functional derangements of acute renal injury, particularly in models where renal injury is accompanied by renal lipid peroxidation such as ischemia-reperfusion, rhabdomyolysis, and toxic nephropathies (7,10–12). The glomeruli, although structurally spared, have reduced GFR, which at least at the beginning, is partly due to reduced glomerular blood flow. The finding that peroxidative injury of tubular cells induces marked production of F₂-isoprostanes raises the possibility that the local diffusion of these arachidonate metabolites into the renal vasculature, particularly glomeruli, may account partly for the renal vasoconstriction accompanying acute renal injury. The previous finding that membrane blebbing and lipid decompositions are one of the earliest tubular lesions during renal ischemia and that lipid peroxidation occurs after reperfusion both in animals and in renal transplant patients is consistent with such a theory (10,11,13,14). Besides a vasoactive role, whether F₂-isoprostanes themselves directly perpetuate cellular injury remains to be determined. Furthermore, the generation of F₂-isoprostanes can be speculatively expected of other cell types exposed to oxidative attack, irrespective of the oxygen species involved, provided that there is peroxidation of arachidonic acid.

21-aminosteroids, the nonglucocorticoid lipophilic synthetic compounds, concentrate in the lipid bilayer and effectively limit the lipid peroxidation chain reactions, primarily by scavenging lipid radicals (4). They are effective in limiting experimental central nervous system injury (15), and clinical trials with these agents are underway in human subarachnoid hemorrhage. The utility of lazaroids in renal injury models is beginning to be explored (1,12,16–18). Besides a vasoactive role, such a theory (10,11,13,14). Besides a vasoactive role, whether F₂-isoprostanes themselves directly perpetuate cellular injury remains to be determined. Furthermore, the generation of F₂-isoprostanes can be speculatively expected of other cell types exposed to oxidative attack, irrespective of the oxygen species involved, provided that there is peroxidation of arachidonic acid.

In summary, in addition to directly compromising cellular structure and function, lipid peroxidation by generating F₂-isoprostanes through a nonenzymic process may further contribute to the pathophysiology of acute renal injury. These findings also raise the possibility that potential benefits from chain-breaking antioxidants in ROS-linked renal injury may be partly related to the inhibition of vasoactive F₂-isoprostane production. Detailed studies are needed in vivo to examine the pathogenic relevance of F₂-isoprostanes in those settings.

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REFERENCES