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

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ABSTRACT

F₂-isoprostanes are the newly identified reactive oxygen species-catalyzed peroxidation products of arachidonate. The infusion of these prostanoids 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 (lazarold 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-phenylenediamine. Besides inhibiting isoprostane production, 21-aminosteroid reduced hydrogen peroxide-induced lipid degradation and peroxidation, and protected the cells against hydrogen peroxide-induced cytology. 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 prostanoi 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 preloaded 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-phenylenediamine (DPPD) were also incubated with 1.5
mM H₂O₂, 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 F₂-isoprostane determination at Vanderbilt University. Free F₂-isoprostanes in the sonicated cell and supernatant mixture were determined after purification and derivatization by using gas chromatography/negative ion-chemical ionization-mass spectrometry with [³H]prostaglandin F₂ as the internal standard as described previously (2).

**H₂O₂-Induced Cell Injury: Effect of 21-Aminosteroid**

Confluent cell layers were loaded with 0 to 50 μM 21-aminosteroid during 3 h of ⁵¹Cr radiolabeling. Washed cell layers were then incubated for 3 h in the presence and absence of 1.5 mM H₂O₂, and ⁵¹Cr release was determined. Also examined was the effect of 20 μM 21-aminosteroid on graded concentrations of H₂O₂. 21-Aminosteroid was first dissolved in absolute ethanol and then diluted in fetal calf serum. The spontaneous ⁵¹Cr release (percentage of total) in the control was always determined in the same plate and subtracted from the ⁵¹Cr release (percentage of total) in experimental wells to provide the percent specific ⁵¹Cr release, which was 4.5 to 8.6%/h.

**Effect of 21-Aminosteroid on H₂O₂-Induced Cytolysis and Lipid Degradation**

We used the release of [³H]arachidonic acid from prelabeled LLC-PK₁ cells as an indirect measure for H₂O₂-induced lipid degradation. Confluent cell layers were dual labeled with [³H]arachidonic acid for 18 h and with ⁵¹Cr for 3 h (1). 21-Aminosteroid (20 μM) was also included in one group of wells during the last 3 h of radiolabeling. Washed cell layers were then subjected to 3 h of 1.5 mM H₂O₂ incubation. ³H radioactivity in 10 μL of the supernatant was determined at 1.5 and 3 h of incubation, and ⁵¹Cr release was determined at 3 h. The [³H]arachidonic acid release, corrected for spontaneous release, was factored for 10⁶ cpm of ⁵¹Cr to account for cell content.

**Effect of 21-Aminosteroid on H₂O₂-Induced Lipid Peroxidation**

Cells with and without 21-aminosteroid were incubated with 1.5 mM H₂O₂. 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**

**H₂O₂-Induced F₂-isoprostane Production: Effect of 21-Aminosteroid**

H₂O₂ induced a dose-related six-fold increase in F₂-isoprostane production that was effectively inhibited by 20 μM 21-aminosteroid (Figure 1). Other lipid peroxidation inhibitors (2-methyl aminochroman and DPPD) were also effective in inhibiting F₂-isoprostane production. 2-Methyl aminochroman, the most potent lipid peroxidation inhibitor (1), completely inhibited F₂-isoprostane production, even below the values in normal controls.

**Concurrent Effect of 21-Aminosteroid on H₂O₂-Induced Cytolysis and Lipid Degradation**

H₂O₂ induced a time-dependent release of prelabeled [³H]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 H₂O₂-induced cytolysis; specific ⁵¹Cr 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 ischemiareperfusion, 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), particularly glomeruli, which account partly for the renal vasoconstriction accompanying acute renal injury. These findings also raise the possibility that the local diffusion of these arachidonic 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.

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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