ABSTRACT

F₂-isoprostanes are the newly identified reactive oxygen species–catalyzed peroxidation products of arachidonate. 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-phenylenediamine. 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

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 radlolabeling. 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 6.6%/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 cells 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.

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

21-Aminosteroid significantly and in a dose-dependent manner prevented the cytolyis of 1.5 mM H2O2 (Figure 2, upper). 21-Aminosteroid attenuated the cytolyis induced by 0.5 to 1.5 mM concentrations of H2O2 and was particularly effective against 0.5 mM H2O2 (Figure 2, lower).

**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 cytolyis; specific 51Cr release was reduced from 55.0 ± 2.4 to 24.7 ± 0.5% by 21-aminosteroid.
Effect of 21-Aminosteroid on H₂O₂-Induced Lipid Peroxidation

H₂O₂ induced an over twofold increase in TBARS that was effectively inhibited by the preincubation of LLC-PK₁ cells with 21-aminosteroid (Figure 4).

DISCUSSION

We provide evidence for the first time in cell culture that H₂O₂ directly induces an excessive production of F₂-isoprostanes and that it can be effectively inhibited by 21-aminosteroid and other lipid peroxidation inhibitors. This study additionally demonstrates that 21-aminosteroid inhibits H₂O₂-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 H₂O₂ on proximal tubular cells. Elevated H₂O₂ 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 F₂-isoprostanes production, did not demonstrate total abrogation of H₂O₂-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), and in this regard, this report provides additional information on the ability of 21-aminosteroid to limit oxidative renal cell injury in vitro.

In summary, in addition to directly compromising cellular structure and function, lipid peroxidation by generating F₂-isoprostanes through a nonenzymatic 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