Hydrogen peroxide stimulates chloride secretion in primary inner medullary collecting duct cells via mPGES-1-derived PGE2

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Soodvilai S, Jia Z, Yang T. Hydrogen peroxide stimulates chloride secretion in primary inner medullary collecting duct cells via mPGES-1-derived PGE2. Am J Physiol Renal Physiol 293: F1571–F1576, 2007. —We investigated the role and mechanism of H2O2 in regulation of NaCl transport in primary inner medullary collecting duct (IMCD) cells. IMCD cells were isolated from wild-type mice and grown onto semipermeable membranes, and short-circuit current (Isc) was determined by Ussing chamber. Exposure of IMCD cells to H2O2 at a range of 100–300 μM caused a rapid increase in Isc in a time- and dose-dependent manner. This increase was almost abolished by the cystic fibrosis transmembrane conductance regulator (CFTR) Cl− channel inhibitors diphenylamine-2-carboxylic acid (DPC) and CFTRinh-172. In contrast, the magnitude of stimulation was unaffected by the epithelial Na+ channel (ENaC) inhibitor amiloride. The H2O2-induced Cl− secretion was significantly inhibited by indomethacin, as well as by microsomal PGE synthase-1 (mPGES-1) deficiency. Like H2O2, PGE2 treatment induced a twofold increase in Isc that was reduced by the protein kinase A (PKA) inhibitors H-89 and KT5720. These data suggest that H2O2 stimulates CFTR Cl− channel-mediated Cl− secretion through cyclooxygenase- and mPGES-1-dependent release of PGE2 and subsequent activation of PKA.

hydrogen peroxide; microsomal prostaglandin E synthase-1; cystic fibrosis transmembrane conductance regulator

The collecting duct plays an important role in modifying urine concentration by regulation of water and electrolyte reabsorption. The major electrolytes controlled by this segment are Na+ and Cl− (1, 29). The general mechanism of Na+ reabsorption in collecting duct involves coordinated Na+ transport from lumen across apical membrane via amiloride-sensitive Na+ channel (ENaC), and Na+ exits from the cell to interstitial fluid across the basolateral membrane via Na+-K+-ATPase (22). Unlike Na+, Cl− could be both absorbed and secreted in the collecting duct where Cl− transport occurs through distinct mechanisms (25). First, Cl− could be actively reabsorbed by an electrogenic Cl−/HCO3− exchanger expressed on the apical membrane and then exits the cell via a Cl− channel. Second, Cl− could diffuse passively down an electrochemical gradient via a paracellular pathway. Third, Cl− could be secreted via a cystic fibrosis transmembrane conductance regulator (CFTR) Cl− channel expressed on the apical membrane (31).

The collecting duct is responsible for absorption of approximately 2–3% of the filtered fluid and is critically important for final adjustment of urinary Na+, K+, Cl−, H+, water, and urea excretion. This is consistent with the fact that the fluid reabsorption rate in the collecting duct is subjected to regulation by a number of hormones and autocrine/paracrine factors, including aldosterone, insulin, arginine vasopressin, PGE2, nitric oxide, and ANG II (1, 2, 4, 6, 10–12, 15, 17, 18, 26). Emerging evidence suggests that reactive oxygen species (ROS) might represent a novel class of regulators of tubular fluid reabsorption. It has been reported that ROS regulate NaCl reabsorption in isolated, perfused thick ascending limbs through protein kinase C (19–21, 27). Additionally, peroxynitrite (OONO−), a reaction product of superoxide and nitric oxide, inhibits Na+–K+–ATPase activity and paracellular permeability in proximal tubule cells (14). By contrast, the role of ROS in the collecting duct has been studied in much less detail. The observation that manipulation of redox state in the renal medulla influences urinary sodium excretion without an effect on glomerular filtration rate (16, 36) seems to suggest a potential role of ROS in regulation of collecting duct function. Therefore, the present study was undertaken to determine the effect of H2O2 on NaCl transport in inner medullary collecting duct (IMCD) cells.

MATERIALS AND METHODS

Materials. DMEM (ATCC), Snapwell (Costa), collagen type I (Worthington Biochemical), hyaluronidase, amiloride, diphenylamine-2-carboxylic acid (DPC), and epidermal growth factor (EGF) were purchased from Sigma; PGE2, H-89, CFTRinh-172, and KT-5720 were obtained from Calbiochem; and indomethacin was from Cayman Chemicals. All other chemicals were purchased from standard sources and were generally of the highest purity available.

Animals. mPGES-1 mutant mice were originally generated by Trebino et al. (30). This mouse colony was propagated at the University of Utah and maintained on a mixed DBA/1lacJ*C57BL/6 background. All protocols employing mice were approved by the University of Utah Institutional Animal Care and Use Committee and were conducted in accordance the guidelines and principles of this Committee.

Cell culture. Primary cultures of renal IMCD cells were generated with a modification of a previous study (9). In brief, mice were killed by cervical dislocation, and kidneys were quickly removed under sterile condition. The renal medulla was dissected, minced, and suspended in the modified medium (DMEM, 10% fetal bovine serum, 20 ng/ml EGF, and 100 U/ml penicillin G-streptomycin sulfate). Cells were seeded onto semipermeable membranes (12-mm-diameter Snapwell) for electrophysiological studies. After the cells...
membranes were mounted in Ussing chamber for a 60-min equilibration period. The basal $I_{sc}$, $V_{te}$, and $R_{te}$ were 2.37 ± 0.11 $\mu$A/cm$^2$, 7.16 ± 0.58 mV, and 2.563 ± 146 ohm/cm$^2$, respectively ($n = 50$). The $I_{sc}$ developed in this cell type appeared to be mainly due to amiloride-sensitive epithelial sodium channel (ENaC) since the final addition of 100 $\mu$M amiloride to the apical media decreased $I_{sc}$ by 72.0 ± 2.0%. To verify responsiveness of the isolated IMCD cells to a defined stimulus, we determined the effect of aldosterone on $I_{sc}$ in these cells. Exposure to 1 $\mu$M aldosterone for 24 h caused an increase of $I_{sc}$ from 2.53 ± 0.22 to 4.67 ± 0.22 $\mu$A/cm$^2$ ($n = 10$), and this stimulation was inhibited by apical administration of amiloride, indicating involvement of amiloride.

Effect of H$_2$O$_2$ on $I_{sc}$ in IMCD cell monolayers. We first examined the dose response of H$_2$O$_2$ on $I_{sc}$. Following the equilibrium period, H$_2$O$_2$ was added to the basolateral side of the IMCD cell monolayers. As shown in Fig. 1A, at 1 min, H$_2$O$_2$ dose-dependently increased $I_{sc}$ that was detectable at 100 $\mu$M and maximal at 300 $\mu$M (from 2.72 ± 0.22 to 2.95 ± 0.21 and to 3.60 ± 0.21 $\mu$A/cm$^2$). The stimulatory effect of H$_2$O$_2$ on $I_{sc}$ was reversible since after washout of H$_2$O$_2$ the $I_{sc}$ returned to the basal level. H$_2$O$_2$ at 300 $\mu$M was chosen as a concentration for subsequent experiments. Next, we determined the time course of H$_2$O$_2$-induced $I_{sc}$. After a 60-min equilibration period, IMCD cell monolayers were incubated with 300 $\mu$M H$_2$O$_2$ for various periods of time, and the $I_{sc}$ was recorded. As shown in Fig. 1B, H$_2$O$_2$ at 300 $\mu$M induced a rapid and transient increase in $I_{sc}$, peaking at 1 min. Apical administration of H$_2$O$_2$ had a similar stimulatory effect on the $I_{sc}$ (data not shown). A positive $I_{sc}$ may reflect the active transport of Na$^+$ transport from apical to basolateral side of the monolayer or Cl$^-$ transport from basolateral to apical side of the monolayer. Therefore, we next determined whether the increase of $I_{sc}$ in response to H$_2$O$_2$ was mediated by Na$^+$ or Cl$^-$ transport. The effect of H$_2$O$_2$ was determined when the ENaC was inhibited by amiloride. After the equilibration period, amiloride (final concentration at 100 $\mu$M) was added to the apical side of monolayers for 5 min to inhibit ENaC, followed by addition of 300 $\mu$M H$_2$O$_2$ to the basolateral side. As shown in Fig. 2, H$_2$O$_2$ increased the $I_{sc}$ in the presence of amiloride, indicating amiloride-insensitive currents. Next, we examined whether Cl$^-$ transport contributed to the H$_2$O$_2$-induced $I_{sc}$.

**RESULTS**

**Basal electrical parameters of primary IMCD cells.** The confluent monolayer of IMCD cells grown on semipermeable

Fig. 1. Effect of H$_2$O$_2$ on short-circuit current ($I_{sc}$) in primary inner medullary collecting duct (IMCD) cell monolayers. A: dose-response. B: time course. In experiment in A, the monolayers were equilibrated for 60 min, and then the incubated with various concentrations of H$_2$O$_2$ at 1 min; then the $I_{sc}$ was recorded. In the experiment in B, after equilibration period, the monolayers were incubated with 300 $\mu$M H$_2$O$_2$ at different time intervals. Each time point represents mean ± SE of $I_{sc}$ for 9 monolayers (A) and 7 monolayers (B). *Significantly different from control as determined by pair t-test ($P < 0.01$).

**Enzyme immunoassay.** Culture medium samples were centrifuged for 5 min at 10,000 rpm and diluted 1:5 with enzyme immunoassay buffer. Concentrations of PGE$_2$ were determined by enzyme immunoassay (Cayman Chemicals).

**Data analysis.** Data are summarized as means ± SE. Statistical analysis was performed using one-way ANOVA or Student’s t-test as appropriate.
stimulated $I_{sc}$ using the nonspecific (CFTR) Cl$^-$ channel blocker DPC and the specific CFTR inhibitor, CFTRinhibitor-172. Addition of 1 mM DPC or 20 μM CFTRinhibitor-172 on top of amiloride almost abolished the H$_2$O$_2$-stimulated $I_{sc}$. These results indicated that CFTR plays a major role in H$_2$O$_2$-stimulated amiloride-insensitive $I_{sc}$. To validate this result, we pretreated the IMCD cell monolayers with 1 mM DPC for 5 min, followed by application of 300 μM H$_2$O$_2$ at the basolateral side. In the presence of DPC, the H$_2$O$_2$-induced $I_{sc}$ was completely eliminated (Fig. 3).

**Role of PGE$_2$ in H$_2$O$_2$-stimulated $I_{sc}$**. H$_2$O$_2$ has been reported to stimulate PGE$_2$ release from proximal tubule and collecting duct cells (7, 35), raising a possibility that PGE$_2$ may mediate the H$_2$O$_2$-stimulated Cl$^-$ transport. To test this hypothesis, the monolayers were preincubated with 10 μM indomethacin, a nonselective cyclooxygenase (COX) inhibitor, for 30 min and then with 100 μM amiloride for 5 min, followed by a final addition of 300 μM H$_2$O$_2$ to the basolateral side. Indomethacin had no effect on basal $I_{sc}$ but significantly attenuated the H$_2$O$_2$-stimulated $I_{sc}$ (Fig. 4A), indicating the requirement of COX activity. PGE$_2$ is the major metabolite of arachidonic acid produced in the kidney by the activity of COX, which exists in two isoforms (COX-1 and COX-2). To examine involvement of a specific COX isoform, we determined the effect of SC-560, a COX-1 inhibitor, and Cay10404, a COX-2 inhibitor, on the H$_2$O$_2$-induced Cl$^-$ current. For COX inhibitors and H$_2$O$_2$ treatments, the $I_{sc}$ were recorded after the monolayers were incubated with 0.5 μM SC-560 or 1 μM Cay10404 for 30 min or 300 μM H$_2$O$_2$ for 1 min. For the COX inhibitors plus H$_2$O$_2$, the monolayer were preincubated with COX inhibitors for 30 min, followed by adding H$_2$O$_2$ for 1 min. As shown in Fig. 4B, exposure to SC-560 completely abolished the effect of H$_2$O$_2$. In contrast, exposure to Cay10404 only slightly affected H$_2$O$_2$-induced Cl$^-$ current. To evaluate the contribution from PGE$_2$, the effect of H$_2$O$_2$ was abolished the H$_2$O$_2$-stimulated $I_{sc}$ by twofold, indicating PGE$_2$-mediated PGE$_2$ release in the primary IMCD cell monolayers isolated from mPGES-1 $^{-/-}$ mice. The H$_2$O$_2$-stimulated $I_{sc}$ was significantly attenuated in mPGES-1 $^{-/-}$ IMCD cells compared with the $^{+/+}$ cells (Fig. 5A). To support the contention that H$_2$O$_2$ induced PGE$_2$ release mediated by mPGES-1, the effect of H$_2$O$_2$ on PGE$_2$ release was determined in mPGES-1 $^{-/-}$ IMCD cells compared with the $^{+/+}$ cells. As shown in Fig. 5B, H$_2$O$_2$ stimulated PGE$_2$ release in the $^{+/+}$ IMCD cells. This stimulation was diminished in mPGES-1 $^{-/-}$ IMCD cells. This result indicated that H$_2$O$_2$-induced PGE$_2$ release mediated by mPGES-1.

We examined the effect of PGE$_2$ on Cl$^-$ transport in IMCD cell monolayers. As shown in Fig. 6, 1 μM PGE$_2$ induced a significant increase in $I_{sc}$ in the presence of amiloride. Given the fact that cAMP is a major signaling pathway of PGE$_2$ (28, 32), we examined the effect of PKA inhibition on PGE$_2$-stimulated Cl$^-$ current. Before administration of H$_2$O$_2$, the cells were preincubated with vehicle, 5 μM H-89, or 5 μM KT5720 for 30 min, followed by 100 μM amiloride for 5 min. As shown in Fig. 6, H-89 and KT5720 alone produced no effect on Cl$^-$ current. PGE$_2$ stimulated Cl$^-$ current by twofold, and this effect was reduced by the PKA inhibitors H-89 and KT5720, indicating PGE$_2$-induced Cl$^-$ current mediated at least by PKA activity.

**Effect of PGE$_2$ on H$_2$O$_2$-stimulated $I_{sc}$**. Following the demonstration of PKA as a mediator of PGE$_2$-induced...
monolayers were isolated from mPGES-1 control. # P/H11021 monolayers for WT and 10 monolayers for KO. * As shown in Fig. 7, H2O2 significantly increased Cl− secretion in response to H2O2. Results are expressed as means ± SE. 

**DISCUSSION**

Although in vivo evidence supports a potential role of ROS in regulation of fluid metabolism in the renal medulla, direct evidence for redox-dependent regulation of NaCl transport in the collecting duct cells is still lacking. The present study investigated the role and mechanism of H2O2 in regulation of NaCl transport in primary IMCD cells using an Ussing chamber.

We provide evidence that H2O2 induces a direct stimulatory effect on Isc that was almost abolished by the CFTR Cl− channel inhibitors DPC and CFTRinh-172 but was insensitive to the ENaC inhibitor amiloride. We further provide evidence for involvement of mPGES-1-derived PGE2 in mediating the increased Cl− secretion in response to H2O2.

Although various immortal collecting duct cells are widely used for studying NaCl transport processes, the transformation with constitutively active oncogenes may make the cells significantly different from the native collecting duct cells. Therefore, we utilized the primary cultures of IMCD cells. The primary IMCD cell monolayers developed high resistance and negative luminal voltage. These cells exhibited responsiveness to aldosterone in terms of increases in amiloride-sensitive ISc and also expressed ENaC protein as assessed by immunoblotting (data not shown). These properties characterized the primary IMCD cells as a useful model for studying NaCl transport processes in the collecting duct despite some evidence that stimulation of Cl− current, we examined the involvement of PKA in H2O2-stimulated ISc in the presence of amiloride. For the PKA inhibitor plus H2O2, the IMCD cell monolayers were preincubated with 5 μM of H-89 or KT5720 for 30 min, and then with 100 μM amiloride for additional 5 min, followed by administration of a final 300 μM H2O2 to the basolateral side. As shown in Fig. 7, H2O2 significantly increased Cl− current, which was eliminated by exposure to H-89 or KT5720, whereas exposure to H-89 or KT5720 alone had no effect on Cl− current. These results support the contention that the H2O2-induced stimulation of Cl− transport required PKA activity.
IMCD may process relatively low Na⁺ transport capacity compared with the proximal part of the collecting duct.

The present study examined the effect of H₂O₂ on Iₑsc, which reflects NaCl transport in cultured collecting duct cells. We found that exposure to H₂O₂ increased Iₑsc in a dose- and time-dependent manner in primary IMCD cell monolayers. The dose-dependent effect was observed at a range of 100–300 μM H₂O₂, while a further increase above this range was associated with cytotoxicity (data not shown). The effect of H₂O₂ was transient, lasting shorter than 15 min possibly because of the activation of antioxidant systems such as catalase, which converts H₂O₂ to H₂O (33). The positive Iₑsc could be contributed by reabsorption of cations (e.g., Na⁺) or secretion of anions (e.g., Cl⁻). The stimulation of Iₑsc in response to H₂O₂ was unlikely to be mediated by ENaC as this phenomenon persisted in the presence of amiloride on the apical side. This leaves a possibility that the increased Iₑsc might be due to amiloride-insensitive Na⁺ reabsorption or Cl⁻ secretion. The fact that addition of the CFTR inhibitors DPC or CFTRinh-172 on the top of amiloride abolished the H₂O₂-stimulated Iₑsc suggests involvement of CFTR rather than amiloride-insensitive Na⁺ transport. These data are consistent with the observation that H₂O₂ stimulated the CFTR Cl⁻ channel in human airway epithelial cells (5). The lack of an effect of H₂O₂ on ENaC-mediated Na⁺ reabsorption was also consistent with a previous study showing that H₂O₂ had no effect on Na⁺ reabsorption in isolated perfused thick ascending limb (21).

We attempted to determine the intracellular mediators of H₂O₂-induced activation of CFTR in cultured IMCD cells with emphasis on prostaglandins. ROS have been reported to stimulate PGE₂ release from both collecting duct and proximal tubule cells (7, 35). We found that H₂O₂-stimulated Iₑsc was significantly attenuated by the nonselective COX inhibitor indomethacin, indicating a requirement of COX activity for the activation of CFTR. The involvement of specific COX isoforms was tested using the COX-1 inhibitor SC-560 and the COX-2 inhibitor Cay10404. Exposure of IMCD cells to SC-560 completely abolished the effect of H₂O₂, whereas Cay-10404 had no obvious effect. These results indicated that COX-1 but not COX-2 may play a role in H₂O₂-induced Cl⁻ secretion in collecting duct cells. This result is consistent with the observation that COX-1 predominates in the collecting duct, while COX-2 is restricted to renal medullary interstitial cells (3, 8).

The COX-1-derived product involved in H₂O₂-induced Cl⁻ secretion appeared to be PGE₂. This can be inferred from the observation that collecting duct cells lacking mPGES-1, a well-characterized PGE synthase, exhibited a significant attenuation of H₂O₂-stimulated Iₑsc in the presence of amiloride. In support of this finding, exposure to H₂O₂ induced a rapid release in wild-type IMCD cells but not in mPGES-1-deficient IMCD cells. Given the residual response in mPGES-1-deficient cells relative to the complete blockade with COX-1-selective or nonselective COX inhibitors, it seems reasonable to speculate that other PGs than the E series may play a minor role in H₂O₂-induced Cl⁻ secretion.

Concomurate with the role of endogenous PGE₂ derived from mPGES-1 is the observation that exposure to exogenous PGE₂ significantly elevated Iₑsc in the presence of amiloride, mimicking the effect of H₂O₂. These findings are consistent with the previous report that PGE₂ stimulated Cl⁻ secretion in the collecting duct cells (23, 32). Another possibility exists that mPGES-1-mediated Cl⁻ secretion may be relevant to the pathogenesis of cyst formation and enlargement in polycystic kidney disease (PKD). This possibility seems to warrant future investigation using mPGES-1 inhibitors or mPGES-1−/− mice.

mPGES-1 is a novel PGE synthase with an established role in pain and inflammatory responses via coupling to COX-2 (27). Emerging evidence suggests that mPGES-1 may play a physiological role in facilitating renal salt excretion (7). Within the kidney, mPGES-1 predominates in the distal nephron, a site for COX-1 but not COX-2 expression. These colocalization data seem to suggest coupling of mPGES-1 with COX-1 but not COX-2 in the collecting duct (3, 24, 34), clearly different from that in inflammatory cells. In support of this notion, the present study provides the first functional evidence for coupling of mPGES-1 with COX-1 but not COX-2 in renal collecting duct cells in the setting of ROS-induced Cl⁻ secretion.

The mechanism of PGE₂ action appeared to involve activation of PKA as PGE₂-stimulated Iₑsc was significantly reduced by the PKA inhibitors H-89 and KT5720. However, the incomplete inhibition of the effect of PGE₂ by PKA inhibitors indicated involvement of a PKA-independent pathway. The exact mechanism of PKA-dependent activation of CFTR is unknown. PKA may directly or indirectly phosphorylate CFTR, leading to trafficking of the channel to the cell membrane or increased open probability of the channel. It is unlikely, however, that the stimulatory effect of H₂O₂ involves increased synthesis of the channel given the rapid response to H₂O₂. The mechanism for PGE₂-dependent activation of the cAMP/PKA pathway, particularly the type of EP receptors involved, still remained uninvestigated in the present study. The biological action of PGE₂ is mediated by G protein-coupled E-prostanoid receptors designated EP₁, EP₂, EP₃ and EP₄, among which EP₂ and EP₃ act primarily via cAMP. Since EP₄ but not EP₂ is expressed in the collecting duct, it seems reasonable to speculate that EP₄ may be responsible for PGE₂-dependent activation of CFTR.

CFTR is abundantly expressed along the nephron with unclear physiological function. However, there is a rich literature documenting pathological roles of renal CFTR, particularly in PKD. It has been reported that CFTR mediates Cl⁻ secretion from the cyst epithelia, which appears to be a critical event for the fluid accumulation and enlargement of the cysts (13). The elucidation of the H₂O₂/PGE₂/CFTR pathway in regulation of Cl⁻ secretion from renal collecting duct cells may help understand the pathophysiology of PKD and may also offer a unique therapeutic opportunity for this devastating disease.

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