Endogenous flow-induced superoxide stimulates Na/H exchange activity via PKC in thick ascending limbs

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Endogenous flow-induced superoxide stimulates Na/H exchange activity via PKC in thick ascending limbs. Am J Physiol Renal Physiol 307: F800–F805, 2014. First published July 30, 2014; doi:10.1152/ajprenal.00260.2014.—Luminal flow stimulates Na reabsorption along the nephron and activates protein kinase C (PKC) which enhances endogenous superoxide (O$_2^-$) production by thick ascending limbs (TALs). Exogenously-added O$_2^-$ augments TAL Na reabsorption, a process also dependent on PKC. Luminal Na/H exchange (NHE) mediates NaHCO$_3$ reabsorption. However, whether flow-stimulated, endogenously-produced O$_2^-$ enhances luminal NHE activity and the signaling pathway involved are unclear. We hypothesized that flow-induced production of endogenous O$_2^-$ stimulates luminal NHE activity via PKC in TALs. Intracellular pH recovery was measured as an indicator of NHE activity in isolated, perfused rat TALs. Increasing luminal flow from 5 to 20 nl/min enhanced total NHE activity from 0.104 ± 0.031 to 0.167 ± 0.036 pH U/min, 81%. The O$_2^-$ scavenger tempol decreased total NHE activity by 0.066 ± 0.011 pH U/min at 20 nl/min but had no significant effect at 5 nl/min. With the NHE inhibitor EIPA in the bath to block basolateral NHE, tempol reduced flow-enhanced luminal NHE activity by 0.029 ± 0.010 pH U/min, 30%. When experiments were repeated with staurosporine, a nonselective PKC inhibitor, tempol had no effect. Because PKC could mediate both induction of O$_2^-$ by flow and the effect of O$_2^-$ on luminal NHE activity, we used hypoxanthine/xanthine oxidase to elevate O$_2^-$, hypoxanthine/xanthine oxidase increased luminal NHE activity by 0.099 ± 0.020 pH U/min, 137%. Staurosporine and the PKCα/β1-specific inhibitor Gö6976 blunted this effect. We conclude that flow-induced O$_2^-$ stimulates luminal NHE activity in TALs via PKCα/β1. This accounts for part of flow-stimulated bicarbonate reabsorption by TALs.

LUMINAL FLOW IN THE NEPHRON is highly variable. Acutely it changes due to glomerular filtration rate (29), tubuloglomerular feedback (17, 28), peristalsis of the renal pelvis (8, 44), and fluid reabsorption (29, 50). Hypertension (4, 6), volume status (3, 42), and diabetes (40) can change luminal flow chronically. Increases in luminal flow enhance Na reabsorption in the proximal tubule (7, 46), thick ascending limb of the loop of Henle (39), and cortical collecting duct (10, 43). However, the mechanisms involved are not fully understood.

The thick ascending limb is important in salt, water, and acid/base homeostasis (15, 16, 35). Na is reabsorbed by thick ascending limbs as NaCl and Na bicarbonate via Na-K-2Cl cotransport (35) and Na/H exchange (NHE) (15, 16), respectively. Luminal flow in this segment may be as high as 20 nl/min during conditions of diuresis (4, 30, 42) and may stop due to peristalsis of the renal pelvis (8, 42, 44). The effects of luminal flow on thick ascending limb NHE have not been thoroughly studied.

Superoxide (O$_2^-$) is a physiological regulator of renal function (47, 51). Elevated O$_2^-$ levels can cause salt retention (32, 51), kidney damage (34), and hypertension (33, 34). O$_2^-$ enhances Na reabsorption in thick ascending limbs. We showed that exogenously-added O$_2^-$ stimulates net NaCl reabsorption in this segment via a protein kinase C (PKC)-dependent pathway (45) and this is at least partly due to activation of Na-K-2Cl cotransport (25). Exogenously-added O$_2^-$ also stimulates luminal NHE activity (26), but the signaling cascade involved is unknown.

We (18) and others (1) showed that increases in luminal flow stimulate O$_2^-$ production in thick ascending limbs and this process is due to activation of PKC (20). However, the effects of luminal flow-enhanced O$_2^-$ production on thick ascending limb transport have not been thoroughly studied. We showed that the O$_2^-$ scavenger tempol reduces net NaCl reabsorption by isolated, perfused thick ascending limbs (38). It is likely that stimulation of endogenous O$_2^-$ production by luminal flow accounts for this effect, but this has not been directly measured. Additionally, the effects of endogenously-produced, flow-stimulated O$_2^-$ on thick ascending limb NHE activity and the signaling cascades involved have not been studied.

We hypothesized that luminal flow-stimulated O$_2^-$ production enhances luminal NHE activity via PKC. To test this hypothesis, we examined the effect of tempol on the recovery rate of intracellular pH (pHi) after an acid load in rat thick ascending limbs perfused at either 5 or 20 nl/min. We then repeated the experiments at 20 nl/min in the absence and presence of PKC inhibitors. Finally, we used an exogenous source of O$_2^-$ in conjunction with PKC inhibitors to further assess the role of PKC. Our findings indicate that PKCα and/or PKCβ1 mediate(s) stimulation of luminal NHE by endogenous flow-induced O$_2^-$ production in thick ascending limbs.

MATERIALS AND METHODS

Chemicals and solutions. The pH-sensitive fluorescent dye 2′,7′-bis-(2-carboxyethyl)-5′-(and-6′)-carboxyfluorescein acetoxymethyl ester (BCECF-AM) was obtained from Life Technologies (Eugene, OR). 4-Hydroxy-TEMPO (tempol), hypoxanthine (HX), and xanthine oxidase (XO) were purchased from Sigma (St. Louis, MO). The NHE inhibitor EIPA was obtained from Cayman Chemical (Ann Arbor, MI). Staurosporine was from Roche Applied Science (Indianapolis, IN) and Gö6976 was from Enzo Life Sciences (Farmingdale, NY). The physiological saline used to perfuse and bathe thick ascending limbs contained (in mM) 130 NaCl, 2.5 NaH$_2$PO$_4$, 4 KCl, 1.2 MgSO$_4$, 6 t-alanine, 1 Na$_2$citrate, 5.5 glucose, 2 Ca(lactate)$_2$, and 10 HEPES, pH 7.4 at 37°C. The composition of the basolateral bath used to acid-load cells (acetate solution) was the same, except that 20 mM sodium acetate was added and NaCl was decreased from 130 to 110...
RESULTS

We first tested whether increases in luminal flow rate had an effect on basal pH$_i$ recovery rate (Fig. 1). We measured basal pH$_i$ recovery rates of thick ascending limbs perfused at 5 and 20 nl/min. Figure 1A is a representative experiment that shows the fitted regression lines used to calculate pH$_i$ recovery rates. Figure 1B represents the means of these experiments. At 5 nl/min, the basal rate of pH$_i$ recovery was 0.104 ± 0.031 pH$_i$ U/min. When luminal flow was increased to 20 nl/min in the same tubule, the basal pH$_i$ recovery rate rose by 81 ± 24% to 0.167 ± 0.036 pH$_i$ U/min (P < 0.02; n = 6). These data suggest that increased luminal flow enhances basal NHE activity.

Next, we studied the effect of tempol on total NHE activity in the presence of different luminal flow rates in thick ascen-
When thick ascending limbs were perfused at 5 nl/min, the basal recovery rate of pH was 0.131 ± 0.027 pH units/min, while in the presence of the O₂ scavenger tempol (100 μM) it was 0.128 ± 0.027 pH units/min, not significantly different (Δ = −0.003 ± 0.004; n = 5; Fig. 2A). In contrast, when tubules were perfused at 20 nl/min, the basal rate of pH recovery was 0.168 ± 0.035 pH units/min, ~30% greater than at 5 nl/min. Treatment with tempol caused a 39% decrease in pH recovery rate to 0.103 ± 0.026 pH units/min (Δ = −0.066 ± 0.013 pH units/min; P < 0.01; n = 4; Fig. 2B). Taken together, these data suggest that luminal flow stimulates endogenous O₂ production which enhances NHE activity.

We previously showed that exogenously-added O₂ stimulates luminal NHE activity in thick ascending limbs. Therefore, we next studied whether endogenously-produced O₂ that is induced by luminal flow had a similar effect. Figure 3 shows that in the presence of the NHE inhibitor EIPA (100 μM) in the basolateral bath so that pH recovery only reflects luminal NHE activity, tempol caused a 30% decrease in pH recovery rate (from 0.097 ± 0.018 to 0.068 ± 0.015 pH units/min; Δ = −0.029 ± 0.013 pH units/min; P < 0.04; n = 6) in thick ascending limbs perfused at 20 nl/min. These data indicate that endogenous flow-induced O₂ stimulates luminal NHE activity in thick ascending limbs. Control experiments performed with NHE inhibitors in both the bath and lumen blocked pH recovery, thus confirming that the pH recovery rate measured in experiments performed with EIPA in only the bath reflected luminal NHE activity.

PKC mediates the effect of O₂ in many cell types including thick ascending limb cells. We previously showed that PKC activation is required for the stimulatory effect of O₂ on NaCl absorption in thick ascending limbs (45). Therefore, we next studied the role of PKC in mediating the stimulatory effect of flow-induced O₂ on luminal NHE activity by testing the effect of tempol with the general PKC inhibitor staurosporine in the basolateral bath (Fig. 4). In the presence of staurosporine (10 nM), the effect of tempol on pH recovery rate was abolished (0.136 ± 0.018 vs. 0.133 ± 0.007 pH units/min; Δ = −0.003 ± 0.013 pH units/min; n = 4). These data may suggest that the stimulatory effect of flow-induced endogenous O₂ on NHE activity is PKC-dependent; however, they may also simply indicate that PKC activation is required for flow to stimulate O₂ production (20).

Fig. 2. Effect of the superoxide (O₂⁻) scavenger tempol (100 μM) at different flow rates on total Na/H exchange activity as measured by pH recovery after an acid load in thick ascending limbs. Con, control. A: luminal flow rate = 5 nl/min (N.S., nonsignificant; n = 5). B: luminal flow rate = 20 nl/min (*P < 0.01; n = 4).

Fig. 3. Effect of tempol (100 μM) on luminal Na/H exchange activity as measured by pH recovery rate after an acid load in flow-stimulated thick ascending limbs. The NHE inhibitor EIPA (100 μM) was present in basolateral bath throughout the experiment. *P < 0.04; n = 6. Luminal flow rate = 20 nl/min.

Fig. 4. Effect of tempol (100 μM) in the presence of the general protein kinase C (PKC) inhibitor staurosporine (Stauro; 10 nM) on luminal Na/H exchange activity as measured by pH recovery rate after an acid load in flow-stimulated thick ascending limbs. The NHE inhibitor EIPA (100 μM) was present in the basolateral bath throughout the experiment. n = 4. Luminal flow rate = 20 nl/min.
To address this issue, we used HX (0.5 mM) and XO (1 mU/ml) to exogenously generate $O_2^-$ and examined the role of PKC in the stimulatory effect of $O_2^-$ on luminal NHE activity (Fig. 5). As shown in Fig. 5A, HX/XO increased pH$_i$ recovery rate in perfused thick ascending limbs by 118% (from 0.140 ± 0.047 to 0.305 ± 0.053 pH$_i$ U/min; $\Delta = 0.165 ± 0.042$ pH$_i$ U/min; $P < 0.02; n = 5$). In the presence of staurosporine (Fig. 5B), the effect of HX/XO was completely abolished (0.134 ± 0.043 vs. 0.125 ± 0.030 pH$_i$ U/min; $\Delta = -0.009 ± 0.014$ pH$_i$ U/min; $n = 3$). Thus, stimulation of luminal NHE activity by $O_2^-$ is PKC-dependent.

PKC has several isoforms; therefore, we next examined which isoform might mediate the effect of $O_2^-$ on luminal NHE. Gö 6976 is a selective PKC inhibitor that blocks PKC$_\alpha$ and PKC$_\beta_1$. We tested the ability of this isoform-specific inhibitor to block the effect of HX/XO-generated $O_2^-$ on luminal NHE activity (Fig. 6). Figure 6A shows that HX/XO increased pH$_i$ recovery rate by 137% (from 0.072 ± 0.023 to 0.170 ± 0.035 pH$_i$ U/min; $\Delta = 0.099 ± 0.020$ pH$_i$ U/min; $P < 0.008; n = 5$). In contrast, after Gö 6976 (100 nM) treatment, HX/XO enhanced pH$_i$ recovery rate by only 17% (0.116 ± 0.029 vs. 0.137 ± 0.025 pH$_i$ U/min; $\Delta = 0.020 ± 0.015$ pH$_i$ U/min; $n = 5$; Fig. 6B). These data indicate that virtually all of the stimulatory effect of $O_2^-$ on luminal NHE activity is mediated by PKC$_\alpha$ and/or PKC$_\beta_1$.

Taken together, our data suggest that endogenous, flow-induced $O_2^-$ stimulates PKC$_\alpha$/β1 to affect NHE activity. However, other factors can affect PKC activity, such as changes in intracellular calcium. Increases in luminal flow can trigger a rise in intracellular calcium in renal cells (23, 48). Therefore, it is possible that flow-related changes in intracellular calcium may be directly activating calcium-dependent PKC$_\alpha$ and PKC$_\beta_1$ and thus directly mediating the stimulatory effect on NHE activity. To address this issue, we tested the effect of increasing luminal flow on total NHE activity in the presence of tempol throughout the experiment to scavenge $O_2^-$. We found that basal pH$_i$ recovery rate did not change significantly (0.164 ± 0.043 vs. 0.189 ± 0.049 pH$_i$ U/min; $\Delta = 0.025 ± 0.015$ pH$_i$ U/min; 19 ± 7%; $n = 6$) when luminal flow rate was increased from 5 to 20 nl/min. These data suggest that flow-induced increases in $O_2^-$ mediate most, if not all, of the flow-induced stimulation of NHE activity.

Fig. 5. Effect of $O_2^-$ generated from hypoxanthine (HX; 0.5 mM) and xanthine oxidase (XO; 1 mU/ml) in the absence and presence of the general PKC inhibitor Stauro (10 nM) on luminal Na/H exchange activity as measured by pH$_i$ recovery rate after an acid load in thick ascending limbs. Stauro and the NHE inhibitor EIPA (100 μM) were present in the basolateral bath throughout the experiment. A: effect of HX/XO. *$P < 0.02; n = 5$. B: effect of HX/XO in the presence of Stauro. n = 3. Luminal flow rate = 20 nl/min.

Fig. 6. Effect of $O_2^-$ generated from HX (0.5 mM) and XO (1 mU/ml) in the absence and presence of 100 nM Gö 6976, a PKC$_\alpha$- and β-selective inhibitor, on luminal Na/H exchange activity as measured by pH$_i$ recovery rate after an acid load in thick ascending limbs. Gö 6976 and the NHE inhibitor EIPA (100 μM) were present in the basolateral bath throughout the experiment. A: effect of HX/XO. *$P < 0.008; n = 5$. B: effect of HX/XO in the presence of Gö 6976. n = 5. Luminal flow rate = 20 nl/min.
DISCUSSION

The role of endogenously-produced O$_2^-$ in the stimulation of luminal NHE activity by luminal flow and the signaling pathway involved are unclear. We showed that luminal flow enhances endogenously-produced O$_2^-$ which stimulates luminal NHE activity in thick ascending limbs. This is the first study to show that flow-induced O$_2^-$ stimulates luminal NHE activity. Our data also suggest that this process is mediated by PKC and/or PKCbI. In addition, we saw that exogenously-added O$_2^-$ also enhances luminal NHE activity via a PKC$\alpha$/$\beta$I-dependent mechanism.

Our findings are consistent with reports that enhanced luminal flow stimulates Na reabsorption in the nephron. In mouse proximal tubules, increases in luminal flow stimulate bicarbonate reabsorption due to changes in both NHE and Na$^+$-ATPase activity. In rabbit cortical collecting ducts, luminal flow enhances net Na absorption via regulation of apical epithelial Na channels. Increases in luminal flow also enhance Na and Cl transport in avian thick ascending limbs.

The sodium reabsorption brought about by luminal NHE activity causes reabsorption of bicarbonate. Although not directly tested, our data along with our earlier findings suggest that exogenously-added O$_2^-$ stimulates bicarbonate reabsorption via increased luminal NHE activity. This in conjunction with our data on O$_2^-$-induced stimulation of Na-K-2Cl cotransport may account for the Na-retaining effects of renal O$_2^-$.

Our current findings support our earlier studies on the effect of O$_2^-$ on ion transporters in thick ascending limbs. We previously demonstrated that exogenously-added O$_2^-$ stimulates luminal NHE (26). Thus, endogenous and exogenous O$_2^-$ affect luminal NHE in a similar fashion. Additionally, our data are comparable with our earlier reports in which we demonstrated that endogenous and exogenous O$_2^-$ increase sodium and chloride absorption primarily through enhanced Na-K-2Cl cotransport.

Our current data are consistent with our previous findings that increases in luminal flow stimulate O$_2^-$ production in thick ascending limbs. Used flow rates that fall within the physiological range of 0 to 20 nl/min in the thick ascending limb (8, 30, 42, 44). We previously characterized the relationship between flow rate and O$_2^-$ production using rates covering this range. We found that O$_2^-$ production increases linearly with flow at rates 5 nl/min and above. However, O$_2^-$ production at 5 nl/min was no different from basal conditions. It was unclear whether 5 nl/ml represented an actual point at which there was no distinguishable increase in O$_2^-$ production in response to flow or instead was due to limitations in the method used to measure O$_2^-$

Our data are also consistent with reports in other cell types that PKC mediates the effects of O$_2^-$ In mesangial cells, O$_2^-$ activates PKC in response to high glucose. O$_2^-$ causes vasoconstriction via activation of PKC in the vasculature. In endothelial cells, O$_2^-$-induced activation of nuclear factor-$\kappa$B is PKC-dependent. In addition, many effects of O$_2^-$ are mediated by PKC in the central nervous system.

Additionally, our finding that PKC is involved in the effect of O$_2^-$ on luminal NHE is consistent with reports that PKC affects many different transporters in thick ascending limbs, and in some cases that PKC$\alpha$ is the isoform involved. We showed that PKC$\alpha$ mediates the stimulatory effect of O$_2^-$ on NaCl reabsorption. PKC is required for angiotensin II-stimulated Na-K-2Cl cotransporter activity and indirectly stimulates Na bicarbonate absorption. In addition, a PKC-dependent pathway mediates inhibition of apical K$^+$ channel activity caused by high concentrations of prostaglandin E$_2$.

Our data provide strong evidence that flow-induced increases in endogenous O$_2^-$ mediate the stimulatory effect on NHE activity and that it is PKC$\alpha$/$\beta$I-dependent. Although increased flow can cause a rise in intracellular calcium in renal tubules (23, 48) and thereby directly activate calcium-dependent PKC and stimulate NHE in the absence of O$_2^-$, we found that this was not the case. Enhanced O$_2^-$ resulting from increased flow was necessary for the PKC-dependent stimulatory effect on NHE activity.

Changes in luminal flow are the result of variations in glomerular filtration rate, tubuloglomerular feedback, and fluid absorption along the nephron. Mechanical constriction of the renal pelvis can halt and even reverse flow. The significance of variations in luminal flow is evident in conditions in which luminal flow is acutely or chronically enhanced, such as high-salt diet, volume expansion, and the osmotic diuresis associated with hypertension and diabetes.

In summary, we found that flow-induced O$_2^-$ stimulates luminal NHE via a PKC-dependent mechanism. This may account for part of flow-stimulated bicarbonate reabsorption in this segment. Regulation of thick ascending limb Na and bicarbonate absorption by O$_2^-$ may play an important role in acid/base disturbances and the pathogenesis of several forms of hypertension and other diseases associated with Na retention.

GRANTS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: N.J.H. performed experiments; N.J.H. analyzed data; N.J.H. and J.L.G. interpreted results of experiments; N.J.H. prepared figures; J.L.G. drafted manuscript; N.J.H. and J.L.G. edited and revised manuscript; J.L.G. conception and design of research; J.L.G. approved final version of manuscript.

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