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Peroxynitrite inhibits sodium uptake in rat colonic membrane vesicles

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Peroxynitrite ($ONOO^-$) is a potent oxidizing agent that initiates lipid peroxidation and sulfhydryl oxidation and may be responsible for a portion of the cytotoxicity attributed to superoxide anion ($\cdot O_2^-$). We quantified the extent to which $ONOO^-$, xanthine plus xanthine oxidase (XO) and hydrogen peroxide (H_2O_2), decreased sodium (Na^+) uptake into membrane vesicles derived from colonic cells of dexamethasone-treated rats. Carrier-free $^{22}Na^+$ uptake into vesicles was measured in the presence of an inside negative membrane potential, produced by the addition of the potassium ionophore valinomycin (10 μM) after removal of all external potassium by cation exchange chromatography. Preincubation of vesicles with either 100 μM or 1 mM $ONOO^-$ for 30 s decreased the amiloride-blockable fraction of Na^+ uptake by 27 ± 7% and 55 ± 2%, respectively ($\bar{X} \pm$ S.E.; $n \geq 5$; $P < 0.05$ from control). However, the amiloride-insensitive part of Na^+ uptake was not affected, indicating that there was no overt destruction of these vesicles by these $ONOO^-$ concentrations. Decomposed $ONOO^-$, hydrogen peroxide (1 μM–10 mM), or xanthine (500 μM) plus XO (10–30 mU/ml), either in the absence or in the presence of 100 μM FeEDTA, did not decrease Na^+ uptake. These data suggest that $ONOO^-$ is a potent injurious agent that can compromise Na^+ uptake across epithelial cells, possibly by damaging Na^+ channels.

Introduction

Partially reduced oxygen species, which include superoxide ($\cdot O_2^-$), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($\cdot OH$), can be generated both inside and outside of cells and are known to damage cells by causing lipid peroxidation, sulfhydryl oxidation, DNA strand scission, and by compromising vital cell functions [1]. Superoxide and H_2O_2 are produced by cytoplasmic proteins, as well as mitochondrial, endoplasmic reticular and nuclear membrane electron transport processes of cells during normal metabolism. The rates of production of O_2^- and H_2O_2 are enhanced following exposure of organisms to elevated concentrations of oxygen or xenobiotics [1,2]. Systemic injury to the skin or liver releases substantial amounts of xanthine oxidase (XO) into the circulation [3], which oxidizes xanthine to urate while converting molecular oxygen to H_2O_2 and $\cdot O_2^-$. In the presence of iron, $\cdot O_2^-$ and H_2O_2 may combine to form $\cdot OH$, one of the most reactive species known.

Recently, a second pathway for the generation of strong oxidants with the reactivity of $\cdot OH$ has been described. The entry of calcium into endothelium [4] and neurons [5] triggers the synthesis and release of the stable free radical nitric oxide ($NO\cdot$) as a secondary messenger which mediates vasodilatior and reduces platelet adhesion [6]. Stimulated macrophages and neutrophils also produce both $\cdot O_2^-$ and $NO\cdot$ [7]. Because $\cdot O_2^-$ and $NO\cdot$ contain unpaired electrons, they rapidly react to form peroxynitrite ($ONOO^-$) [8], a reactive and potentially toxic species:

$$\cdot O_2^- + NO\cdot \rightarrow ONOO^-$$

Peroxynitrite has a half-life of under 1 s at pH 7.4 at 37°C, because peroxynitrous acid ($ONOOH$) sponta-

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neously decomposes to give ·OH and nitrogen dioxide (·NO_2):

$$ONOO^- + H^+ \rightleftharpoons ONOOH \rightarrow \cdot OH + \cdot NO_2$$

In spite of its reactivity, the modest rate of decomposition under physiological conditions allows $ONOO^-$ to diffuse for up to several cell diameters. Peroxynitrite and its by-products initiate lipid peroxidation and oxidize sulfhydryl groups, and therefore may contribute significantly to partially reduced oxygen species injury [9,10]. In addition, $ONOO^-$ is a substrate for Cu, Zn superoxide dismutase (SOD), which catalyzes the formation of a potent nitrating agent with the reactivity of nitronium ion (NO_2^+) [11]. The physiological relevance of $ONOO^-$ was recently established by the demonstration that stimulated rat alveolar macrophages produce significant amounts of $ONOO^-$ [12].

Increased concentrations of reactive oxygen species compromise both active (transcellular) and passive (paracellular) solute transport across a variety of epithelial tissues [13–15]. For example, it has been demonstrated that exposure to hyperoxia or xanthine and XO decreases the short circuit current, an index of sodium (Na^+) transport, across the toad bladder [15] and ventral toad skin [13], respectively. However, these studies did not address the underlying mechanisms. Since the entry of Na^+ across the apical membrane of epithelial cells is the rate-limiting step in transcellular Na^+ transport, and amiloride-blockable Na^+ channels are the principal proteins regulating this process in most epithelial cells [16], we hypothesize that injury to these proteins by reactive oxygen species may contribute to the observed decrease in Na^+ transport.

We quantified the extent to which $ONOO^-$, xanthine plus XO, and H_2O_2 decreased Na^+ uptake into membrane vesicles derived from colonic epithelial cells of dexamethasone-treated rats. Previous studies have shown that 80–83% of Na^+ uptake into these vesicles occurs through amiloride-blockable Na^+ channels [17]. Our results indicate that incubation of vesicles with $ONOO^-$ results in concentration-dependent inhibition of the amiloride-blockable Na^+ uptake. We performed additional experiments to identify the reactive species involved ($ONOO^-$, ·OH, ·NO_2, NO_2^+) and possible mechanisms responsible for this injury. These findings demonstrate potential toxicity of both $ONOO^-$ and its by-products to a biological system and shed new insight as to the mechanisms of reactive oxygen species injury to epithelial tissues.

Methods

Animals and tissue preparation. Vesicles were prepared from dexamethasone-treated rats according to the methods of Garty et al. [18] and Bridges et al. [19]. In brief, female Sprague-Dawley rats (225–275 g body wt) were injected subcutaneously with dexamethasone suspended in corn oil (6 mg/kg), 68,42, and 18 h prior to being killed by decapitation. Dexamethasone injection in the manner described, causes a 25-fold increase in the magnitude of electrogenic $^{22}Na^+$ uptake across membrane vesicles [17]. More than 80% of this $^{22}Na^+$ uptake is inhibited by 1 μM amiloride, indicating that it occurs through amiloride-blockable Na^+ channels [17]. Cells from the distal portion of the colonic mucosa were isolated in a Ca^{2+}-free medium and washed three times at 4°C with 50 ml of a 145 mM K_2SO_4, 5 mM K_2HPO_4, 5 mM EGTA solution; pH = 7.5 (referred to as 'isolation buffer'). After the final wash, the cells were suspended in 10 ml of isolation buffer and incubated for 1 h at 37°C. The cells were cooled and then homogenized for 15 s with a Polytron homogenizer. The homogenate was diluted 2-fold and centrifuged for 10 min at $1000 \times g$. The pellet was discarded and the supernatant was centrifuged for 1 h at $40000 \times g$. The membrane vesicle pellet was resuspended in approx. 300 μl of isolation buffer per rat ($\approx$ 1 mg vesicle protein/ml).

Generation of reactive oxygen species. Membrane vesicles were incubated with $ONOO^-$ (1, 10, 100 and 1000 μM for 30 s), H_2O_2 (1, 100, 1000 and 10000 μM for 30 s, 10 min, or 30 min), or xanthine (500 μM) and XO (10, 20 or 30 mU/ml; 4 min or 8 min). In additional experiments, we added 100 μM FeEDTA ($FeCl_3$/EDTA, 1 : 1.1) into the xanthine + XO solution to enhance the generation of ·OH via the $\cdot O_2^-$ driven Fenton reaction. To test whether production of NO_2^+ would increase the peroxynitrite-induced injury to Na^+ transport, 1 mg/ml of Cu, Zn SOD was added to some vesicular solutions prior to the addition of 1 mM $ONOO^-$. At the end of the incubation periods intravesicular $^{22}Na^+$ uptake was then measured as described below.

Sodium uptake procedure. Carrier free $^{22}Na^+$ uptake into vesicles was measured in the presence of an inside negative membrane potential, produced by the addition of the potassium ionophore valinomycin (10 μM) after all external potassium was removed by cation exchange chromatography [17]. A chemical gradient for K^+ was imposed by applying 100–150 μl ($\approx$ 100 μg protein) of the vesicle solution to a cation-exchange column (Dowex 50- X8–100, Tris-form) and eluting the vesicles with 1.5 ml 8.5% sucrose into a test tube containing 5–7 μl 1 M Tris-base to ensure a final pH of 7.4. Aliquots of vesicles were then added to vials containing carrier-free $^{22}Na^+$ (New England Nuclear, final concentration 50 nM; approx. $5 \cdot 10^5$ cpm) in a small volume (150 μl) of 8.5% sucrose. Depending on the experimental requirements, the radioactive solution also contained valinomycin (10 μM) and/or amiloride (1 μM; both from Sigma Chemicals, St.

Louis, MO). Samples were removed from the radioactive suspension at designated times and placed on a second cation exchange column previously washed with 1 mg/ml bovine serum albumin (Sigma Chemical, St. Louis, MO) in 8.5% sucrose. The vesicles were eluted with 1.5 ml of 8.5% sucrose into scintillation vials for counting. Following a 24 h dark adaptation period, the vials were counted with a HRB-1214 Rack Beta Liquid Scintillation Counter. All uptake studies were conducted at room temperature (22°C). Protein concentration was assayed in the vesicles eluted from the first column by the method of Bradford, using bovine serum albumin as the standard [20]. To assess the degree of non-specific binding of $^{22}Na^+$ to the vesicular membranes in a single control experiment Triton X-100 (final concentration: 0.05%) was added to vesicles 30 s prior to elution through the second cation-exchange column.

Initial results under all experimental conditions indicated that $^{22}Na^+$ uptake was maximal at 5 min after the addition of radioactivity and remained stable for at least 15 min thereafter (Fig. 1). Therefore, in all measurements reported here intravesicular $^{22}Na^+$ uptake was measured 5 min after instillation of $^{22}Na^+$ into the vesicular solution.

Chemicals and biochemical assays. Stock solutions of $ONOO^-$ (≈ 700 mM) were prepared in 1 M NaOH (pH 14–15) as previously described [21]. Concentration was determined spectrophotometrically by measuring the absorption of the stock solution at 302 nm (ϵ_{302nm} = 1670 M^{-1} cm^{-1}). Small aliquots (5 μl) of $ONOO^-$, serially diluted with water, were added to 100-μl aliquots of vesicular solution pretreated with 10 μl of 200 mM KH_2PO_4 to prevent any change in the extravesicular pH. To evaluate any non-specific effects of $ONOO^-$ diluent on Na^+ uptake, $ONOO^-$ was first allowed to decompose by incubating it with 200 mM phosphate buffer (pH 7.4) for two min prior to its addition to the vesicular solution. Peroxynitrite concentration, assessed spectrophotometrically after 2 min decomposition, was equal to zero.

Incubation of vesicles with H_2O_2 consisted of addition of small aliquots of reagent grade H_2O_2 (Sigma, St. Louis, MO; 30%) serially diluted with water into 100-μl aliquots of vesicle solution.

Xanthine oxidase (grade III Calbiochem, dissolved in 2.3 M ammonium sulfate) was desalted by chromatography on a Sephadex G-25 column (Pharmacia; PD-10) equilibrated with isolation buffer. Its activity was determined spectrophotometrically by measuring the rate of urate production at 295 nm in 50 μM xanthine, 50 mM KPO_4, pH 7.4 at 25°C (ϵ_m = $1.1 \cdot 10^{-4}$ M^{-1} cm^{-1}) [21]. 2 μl each of 25 mM xanthine and 500 mU/ml XO were added into 100 μl vesicle solution.

The concentration of H_2O_2 in the vesicular solution was measured as an index of the partially reduced oxygen species concentration, as previously described [22]. Briefly, 200-μl aliquots were removed at time 0, just prior to the addition of XO, and at 5, 10, 20, 30, 45, and 60 min later. These aliquots were mixed with 800 μl of a 100 mM potassium phosphate (pH 7.0), which contained 3000 U of horseradish peroxidase (type II, 200 U/mg; Sigma), 1.5 mM 4-aminoantipyrine and 0.11 M phenol. Allopurinol (100 μM) was also added to the reagent to prevent the generation of additional H_2O_2 after sampling.

Production of $^{\cdot}OH$ by xanthine and XO, in the absence or presence of 100 μM FeEDTA, was quanti-

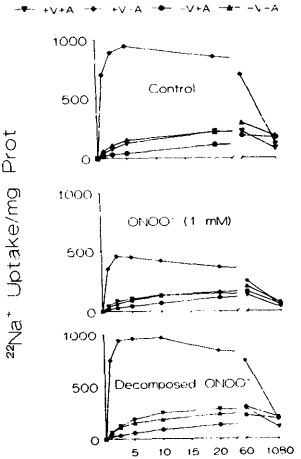

Fig. 1. Sodium uptake time course. Membrane vesicles were pretreated with phosphate buffer (Control), 1 mM $ONOO^-$ ($ONOO^-$ 1 mM), or 'decomposed' $ONOO^-$ (Decomposed $ONOO^-$) for 30 s. After elution through a Tris⁺-base column the intravesicular uptake of $^{22}Na^+$ was measured under the following conditions: {+V+A} = 10 μM valinomycin (V) and 1 μM amiloride (A); {−V−A} = no V or A present; {+V−A} = 10 μM V and no A. {−V+A} = no V and 1 μM A. $^{22}Na^+$ uptake was calculated as follows:

$$^{22}Na^+ \text{ uptake/mg protein} = \frac{\text{Intravesicular } ^{22}Na^+ \text{ (cpm)} \times 100}{\text{Total } ^{22}Na^+ \text{ (cpm) in solution} \times \text{mg protein}}$$

Typical values from a single experiment. Similar results were obtained with five additional vesicle preparations.

Production of $^{\cdot}OH$ by xanthine and XO, in the absence or presence of 100 μM FeEDTA, was quanti-

fied by measuring the rate of formaldehyde formation in the presence of dimethylsulfoxide (100 mM) as previously described [21].

Gel electrophoresis and Western blotting. Discontinuous sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method of Laemmli [23], using a Bio-Rad mini-gel apparatus. Approx. 1 mg/ml of solubilized vesicle protein from vesicles incubated with isolation buffer or $ONOO^-$ was loaded per lane on a 6% acrylamide gel. Western blotting was carried out according to the method of Towbin [24], as modified by Burnette [25], using a Bio-Rad mini-transblot apparatus. The transfer buffer contained 192 mM glycine and 25 mM Tris (pH 8.3) and was carried out for 1 h at 100 V. The immunoblots were processed and developed as previously described by Sorscher et al. [26]. The nitrocellulose strips were blocked in a 5% solution of dried milk in Tris-buffered saline, 20 mM Tris (pH 7.5), and 500 mM NaCl, and then incubated with either the primary antibody (rabbit polyclonal IgG, raised against highly purified Na^+ channel protein isolated from beef kidney papilla [26]), or nonspecific rabbit IgG, at a concentration of 10 μg/ml (in Tris-buffered saline and 0.05% Tween 20) and 1% dried milk. The secondary antibody (alkaline phosphatase-conjugated donkey anti-rabbit IgG; Jackson Immuno Research) was used at a dilution of 1:5000. The blots were color developed with nitroblue tetrazolium chloride (15 mg), dissolved in 500 μl of 70% dimethylformamide (DMF; Bio-Rad) with BCIP (bromochlorindolyl phosphate 7.5 mg, dissolved in 500 μl DMF) and added to 50 ml of 0.1 M $NaHCO_3$, 1 mM $MgCl_2$ (pH 9.8) for color development.

Results

In the presence of valinomycin, intravesicular Na^+ uptake increased rapidly and reached a plateau value by 3–5 min. At 5 min, valinomycin enhanced Na^+ uptake was 7-fold higher than in the absence of valinomycin and corresponded to approx. 62 pmol $^{22}Na^+$/mg vesicle protein (Fig. 1). Furthermore, 85% of valinomycin enhanced Na^+ uptake was blocked by 1 μM amiloride, strongly suggesting that a large fraction of the transport occurred though amiloride-blockable Na^+ channels [17]. After 5 min, valinomycin enhanced Na^+ uptake declined slowly, due to the decrease of the electrical potential, and at 24 h its value was 2-fold higher than in the absence of valinomycin. In Triton-X-100 disrupted vesicles, $^{22}Na^+$ radioactivity was at background levels (data not shown). The transient nature of the uptake, its increase in the presence of valinomycin and a potassium diffusion potential, and its virtual disappearance when the vesicles were ruptured with Triton-X-100 indicate that the measured radioactivity

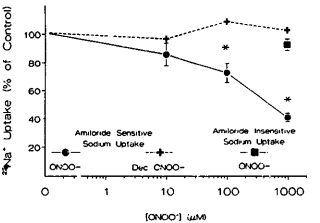

Fig. 2. Effects of $ONOO^-$ on $^{22}Na^+$ uptake. Amiloride-sensitive $^{22}Na^+$ uptake was calculated as the difference in uptake between valinomycin-treated vesicles in the absence and presence of 1 μM amiloride ((+V−A)−(+V+A)). Amiloride-insensitive $^{22}Na^+$ uptake was the measured uptake value in the presence of valinomycin and 1 μM amiloride (+V+A). Membrane vesicles were incubated with the indicated [$ONOO^-$] for 30 s. All values were expressed as a percent of the corresponding control values ($ONOO^-$ = 0). Values are means ± 1 S.E.; $n \geq 3$. * $P < 0.05$ compared to control (two-tailed paired t-test).

was due to intravesicular $^{22}Na^+$ accumulation and could not be accounted for by non-specific binding of $^{22}Na^+$ to the vesicular membranes.

The time course of intravesicular Na^+ uptake after exposure to $ONOO^-$ was identical to control with a plateau value occurring by 5 min (Fig. 1). Similar equilibrium values for $^{22}Na^+$ uptake in the presence or absence of $ONOO^-$ were observed (Fig. 1). This similarity in time courses enabled us to quantify the effects of $ONOO^-$ by measuring intravesicular $^{22}Na^+$ uptake at 5 min. Peroxynitrite, in concentrations above 100 μM, inhibited the amiloride-blockable portion of Na^+ uptake (Figs. 2 and 3). However, the amiloride-insensitive component of $^{22}Na^+$ uptake was not affected by

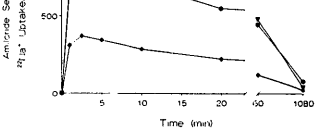

Fig. 3. Time course of amiloride-sensitive sodium uptake. $^{22}Na^+$ uptake in the presence of valinomycin and 1 μM amiloride (+V+A); vesicles were pretreated with 1 mM active or decomposed peroxynitrite for 30 s, or no peroxynitrite at all. The results of a single experiment. Similar results were obtained in three additional vesicle preparations.

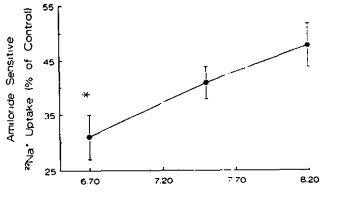

Fig. 4. $^{22}Na^+$ uptake inhibition by $ONOO^-$: dependence on extravesicular pH. Amiloride-blockable $^{22}Na^+$ uptake into membrane vesicles, pretreated with 1 mM $ONOO^-$ at the indicated extravesicular pH values. Values are means ± 1 S.E.; $n \geq 5$. * $P < 0.05$ compared to corresponding pH = 8.2 value (one way analysis of variance using the least significant difference test).

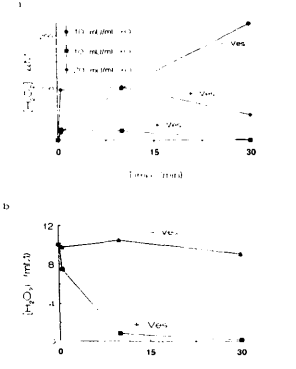

Fig. 5. $[H_2O_2]$ vs. time. Concentration of H_2O_2 at different times following the instillation of (a) 500 μM xanthine and either 10 or 20 mU/ml XO or (b) 10 mM H_2O_2 into isolation buffer in the presence or absence of vesicles (1 mg/ml). Mean values, $n \geq 2$.

$ONOO^-$. These data suggest that the $ONOO^-$ inhibition of $^{22}Na^+$ uptake was due to decreased flux through conductive pathways and not due to overt vesicle destruction.

Peroxynitrite has a pK_a of 6.8 at 37°C and rapidly decomposes once protonated (half-life < 0.9 s at pH 7.4) to form ·OH and $\cdot NO_2$ [21]. In order to identify the species responsible for the decrease in Na^+ transport, we incubated vesicles with 1 mM $ONOO^-$ at an external pH of 6.7, 7.5 and 8.2. We found no statistically significant difference between Na^+ uptake in extravesicular pH 7.5 compared to either 6.7 or 8.2. However, Na^+ uptake was inhibited to a greater extent by 1 mM $ONOO^-$ at pH 6.7 compared to pH 8.2 (Fig. 4).

Coincubation of membrane vesicular protein with Cu, Zn SOD (1 mg/ml) prior to the addition of 1 mM $ONOO^-$ did not further decrease amiloride-blockable Na^+ uptake in vesicles incubated with Cu, Zr SOD and $ONOO^-$, as compared to Na^+ uptake in vesicles incubated with $ONOO^-$ alone [33 ± 4% of control ($n = 7$) vs. 31 ± 4% of control ($n = 3$), respectively]. These results indicate that the nitrating agent formed by SOD plus $ONOO^-$ did not exacerbate $ONOO^-$ induced injury to Na^+ channels in this experimental model.

Instillation of xanthine plus XO into isolation buffer resulted in significant time-dependent increase in H_2O_2 concentration (Fig. 5a). Reagent grade H_2O_2 concentration remained constant in isolation buffer (Fig. 5b) but decreased significantly in a time-dependent manner when vesicular protein (1 mg/ml) was present (Figs. 5a and b), indicating the presence of catalatic activity in the membrane vesicles. Neither H_2O_2 nor xanthine plus XO had any effect on Na^+ transport (Table I). Addition of FeEDTA (final concentration 100 μM) to vesicular protein, prior to instillation of xanthine (500 M) plus XO (20 mU/ml), resulted in significant generation of ·OH (final concentration after 8 min: 10 μM) but also failed to decrease vesicular Na^+ uptake (Table I).

Western blot analysis of our vesicle protein (Fig. 6) revealed polypeptides of molecular masses of 248, 188, 114, 100 and 63 kDa, which compare favorably with reported values for the major subunits of purified Na^+ channel protein from bovine renal papilla [26,27].

TABLE I

Effect of reactive oxygen species on amiloride-blockable $^{22}Na^+$ uptake into membrane vesicles

Time = incubation time. $^{22}Na^+$ uptake values are given as means ± S.E., (n) = number of measurements. None of the values are significantly different from control.

Radical generating compounds			Time (min)	$^{22}Na^+$ uptake (% control)
Xanthine (μM)	XO (mU/ml)	FeEDTA (μM)		
500	10	–	4	96 ± 15 (5)
500	10	–	8	97 ± 11 (8)
500	20–30	–	8	114 ± 4 (2)
500	20	100	8	92 ± 8 (3)
H_2O_2 (μM)				
10000	–	–	30	98 ± 8 (5)

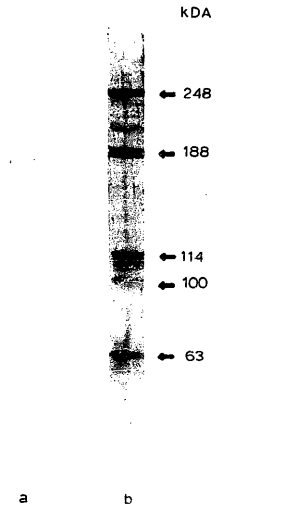

Fig. 6. Immunoblot of rat colonic membrane vesicles probed with a rabbit polyclonal antibody raised against the amiloride-sensitive Na^+ channel of the bovine kidney. Total vesicle protein was run out on a 6% SDS-PAGE gel and blotted onto nitrocellulose. The nitrocellulose strips were overlaid with the primary antibody and subsequently with an alkaline-phosphatase-conjugated donkey anti-rabbit IgG. Lane a, non-specific IgG; Lane b: Protein A purified anti-Na^+ channel IgG.

Non-specific rabbit IgG showed no immunological activity against our vesicle protein. Incubation of the membrane vesicles with 1 mM $ONOO^-$ had no effect on subunit composition, indicating no overt destruction of the Na^+ channel protein.

Discussion

Under our experimental conditions, $ONOO^-$ produced a concentration-dependent decrease of Na^+ uptake into epithelial membrane vesicles, apparently by inhibiting Na^+ movement through amiloride-blockable Na^+ channels. Two observations support our conclusion that the $ONOO^-$ inhibition of $^{22}Na^+$ uptake was due to decreased flux through these conductive pathways and not vesicular destruction. First, the amiloride-insensitive component of $^{22}Na^+$ uptake was not affected by $ONOO^-$ (Fig. 2). It might be argued that amiloride-insensitive uptake is too small to allow detection of decreased uptake. However, vesicles preincubated with peroxynitrite demonstrated a 28% decrease in amiloride-blockable sodium uptake in the absence of a valinomycin induced increase in the potassium diffusion potential ($-V+A$; fig. 1), demonstrating that even at low uptake levels, inhibition can be observed. Second, the observation of similar equilibrium values for $^{22}Na^+$ uptake in the presence or absence of $ONOO^-$ argues against disruption of vesicle integrity.

Both $ONOO^-$ and its protonated form (peroxynitrous acid) are strong oxidizing agents but with different selectivities. Peroxynitrite itself has been shown to mediate oxidation of both non-protein and protein sulfhydryls. The data of Radi et al. [9] indicate that $ONOO^-$ oxidized cysteine to cystine and bovine serum albumin-thiol groups with rate constants that were, respectively, 1200- and 2600-times faster than H_2O_2 oxidation. Oxidation of key cysteine groups may play a pivotal role in the inactivation of Na^+ channels. Previous studies have demonstrated that organic mercurials added to the luminal side of epithelial tissues react with sulfhydryl groups and reduce Na^+ conductance, prevent the inhibition of Na^+ transport by amiloride, and abolish the cation selectivity of the Na^+ channels [28]. In our experiments, $ONOO^-$ did not abolish the amiloride inhibition of Na^+ transport, suggesting an alternate locus of action of $ONOO^-$ than the mercurial compounds. Furthermore, the observation that incubation with $ONOO^-$ did not change protein subunits of the Na^+ channel on Western blot suggests that neither aggregation nor degradation of protein subunits occurred.

The greater decrease in Na^+ uptake observed at acidic extravesicular pH suggests that either ONOOH or its immediate decomposition products inhibited Na^+ transport. In the absence of $ONOO^-$, Na^+ uptake was not altered by pH because the extravesicular pH was adjusted to 7.5 before the addition of $^{22}Na^+$. Even if the internal pH of the vesicles was altered by our incubation conditions, it would not have affected $^{22}Na^+$ uptake [29]. Radi et al. [10] have demonstrated that coincubation of $ONOO^-$ with phosphatidylcholine liposomes at acidic or neutral pH values resulted in a significant degree of lipid peroxidation. Both $\cdot OH$ and $\cdot NO_2$, the two main by-products of peroxynitrous acid, are capable of initiating lipid peroxidation by abstraction of a hydrogen from polyunsaturated fatty acids. In our system, generation of large amounts of $\cdot OH$ by the action of XO on xanthine in the presence of saturating levels of FeEDTA did not affect Na^+ transport. In addition, the yield of $\cdot OH$ and $\cdot NO_2$ from $ONOO^-$ is maximal at acidic pH. Therefore, these data indicate

that ·OH, was not responsible for the decrease in Na^+ transport. Thus direct attack by ONOOH or $\cdot NO_2$ may have impaired Na^+ transport.

At first glance, the concentrations of $ONOO^-$ needed to substantially inactivate Na^+ transport seem high, but inactivation must be considered in terms of *concentration* × *time* to compensate for the rapid decomposition of $ONOO^-$. Because spontaneous decomposition of $ONOO^-$ obeys first-order kinetics with a rate constant of k_1, the concentration of $ONOO^-$ at time t is given by $[ONOO^-]_0 e^{-k_1 t}$ were $[ONOO^-]_0$ is the initial $ONOO^-$ concentration. After this expression is integrated from time zero to infinity, the *concentration* × *time* exposure to $ONOO^-$ equals $\{[ONOO^-]_0/k_1\}$. At pH 7.4 and 37°C, we have measured k_1 to be 0.65 s^{-1} which implies that the exposure to a 1 mM concentration is equivalent to a steady state concentration of 26 μM $ONOO^-$ for 1 min or 2.6 μM $ONOO^-$ for 10 min. Thus, a slow rate of $ONOO^-$ generation for a period of a few minutes could substantially inactivate Na^+ transport.

These rates of $ONOO^-$ formation could potentially be achieved under pathological conditions where tissues are stimulated to produce NO· and $\cdot O_2^-$. Simultaneous production may occur during activation of macrophages and neutrophils, or when ischemic tissue is reperfused [6]. Since the rate of $ONOO^-$ formation is determined by the product of $\cdot O_2^-$ and NO· concentrations, each 10-fold increase of the reactants will amplify the rate of $ONOO^-$ formation by a factor of 100. Using activated rat alveolar macrophages, we have observed the production of 0.1 nmol $ONOO^-/10^6$ cells per min [12], which would produce around 1 mM $ONOO^-$ per min in the lung epithelial lining fluid.

In a previous study [13], we have shown that short circuit current (I_{sc}) across the ventral toad skin was inhibited by the addition of 500 μM xanthine and two boluses of 15 mU/ml of XO to the apical side of this epithelium. However, significant changes in I_{sc} were not seen till 25–35 min from the instillation of XO. These differences in the appearance of injury may be attributed to the much lower reactivity of H_2O_2 as compared to $ONOO^-$. Although we measured a decrease in H_2O_2 concentration over time in the presence of vesicles in isolation buffer, the decrease is unlikely to mask an inhibition of the Na^+ channel because of the prodigious concentrations of H_2O_2 used. The calculated exposure to H_2O_2 under our experimental conditions was 5700 μM min. The observed decrease in H_2O_2 concentration suggests that H_2O_2 neither interferes with the uptake of Na^+ through the apical membrane Na^+ channel nor disrupts vesicle membrane integrity. However, these levels of H_2O_2 are known to cause significant injury to other epithelial functions [30,31] indicating different susceptibilities of cellular targets.

We have previously shown that the reaction of $ONOO^-$ with transition metals can generate the positively charged and highly reactive NO_2^+, which is capable of nitrating a wide range of molecules [11]. Since NO_2^+ is positively charged, it could be drawn into the binding site of Na^+ and then destroy an essential amino acid required for Na^+ transport. To test this hypothesis, we added Cu, Zn SOD to the vesicle aliquot prior to incubation with $ONOO^-$. There was no change in Na^+ uptake inhibition, suggesting that the secondary formation of NO_2^+ from $ONOO^-$ was not an important factor in this injury model.

In summary, we have demonstrated that physiological concentrations of both $ONOO^-$ and ONOOH inhibit Na^+ uptake into epithelial cell membrane vesicles by either damaging or inactivating Na^+ channels. On the other hand, Na^+ uptake was not affected by supraphysiological concentrations of $\cdot O_2^-$ H_2O_2 and ·OH radicals. These findings demonstrate the potential toxicity of $ONOO^-$ to a biological system and shed new insight as to the mechanisms of reactive oxygen species injury to epithelial tissues.

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