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Tin protoporphyrin induces intestinal chloride secretion by inducing light oxidation processes

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Departments of [1]Pediatrics, [2]Internal Medicine, and [3]Radiation Oncology, [4]Veterans Administration Medical Center and The University of Iowa, Carver College of Medicine, Iowa City, Iowa; and [5]Department of Internal Medicine, Veterans Affairs Medical Center and University of Cincinnati College of Medicine, Cincinnati, Ohio

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Uc A, Reszka KJ, Buettner GR, Stokes JB. Tin protoporphyrin induces intestinal chloride secretion by inducing light oxidation processes. *Am J Physiol Cell Physiol* 292: C1906–C1914, 2007. First published January 10, 2007; doi:10.1152/ajpcell.00550.2006.—Heme induces Cl^- secretion in intestinal epithelial cells, most likely via carbon monoxide (CO) generation. The major source of endogenous CO comes from the degradation of heme via heme oxygenase (HO). We hypothesized that an inhibitor of HO activity, tin protoporphyrin (SnPP), may inhibit the stimulatory effect of heme on Cl^- secretion. To test this hypothesis, we treated an intestinal epithelial cell line (Caco-2 cells) with SnPP. In contrast to our expectations, Caco-2 cells treated with SnPP had an increase in their short-circuit currents (I_{sc}) in Ussing chambers. This effect was observed only when the system was exposed to ambient light. SnPP-induced I_{sc} was caused by Cl^- secretion because it was inhibited in Cl^--free medium, with ouabain or 5-nitro-2-(3-phenylpropylamino)benzoic acid (NPPB). The Cl^- secretion was not via activation of the CFTR, because a specific inhibitor had no effect. Likewise, inhibitors of adenylate cyclase and guanylate cyclase had no effect on the enhanced I_{sc}. SnPP-induced I_{sc} was inhibited by the antioxidant vitamins, α-tocopherol and ascorbic acid. Electron paramagnetic resonance experiments confirmed that oxidative reactions were initiated with light in cells loaded with SnPP. These data suggest that SnPP-induced effects may not be entirely due to the inhibition of HO activity but rather to light-induced oxidative processes. These novel effects of SnPP-photosensitized oxidation may also lead to a new understanding of how intestinal Cl^- secretion can be regulated by the redox environment of the cell.

heme oxygenase; electrolyte transport; carbon monoxide; cGMP; reactive oxygen species

INTESTINAL EPITHELIAL CELLS maintain fluid-electrolyte homeostasis by keeping a tight barrier and regulating ion channels (9, 32). Many factors, including microbial agents, toxins, drugs, or inflammation, can disrupt this intestinal barrier and induce secondary messengers such as cAMP and cGMP (15, 17, 33). These secondary messengers can modulate intestinal fluid-electrolyte balance by inducing epithelial Cl^- secretion (22, 42). We (45) have recently shown that heme and one of its degradation products, carbon monoxide (CO), can induce Cl^- secretion in intestinal epithelial cells via cGMP formation. The major source of endogenous CO comes from the degradation of heme via heme oxygenase (HO) (35).

Heme prosthetic groups are found ubiquitously in living organisms (hemoglobin, myoglobin, catalase, peroxidases, cytochrome *c*, guanylate cyclase, and nitric oxide synthase), and they participate in important physiological functions (34). Despite its vital functions, free heme can be a source of ROS and promotes several deleterious cellular processes (4, 18). Intracellular heme levels are tightly controlled by adjusting a fine balance between its biosynthesis and catabolism (34). Heme is degraded by HO with the formation of CO and biliverdin and the release of iron from the porphyrin ring (43). The HO enzyme exists in two major forms: HO-1 and HO-2 (1, 28). HO-1 protein is inducible by numerous stimuli including heavy metals, heme, cytokines, hypoxia, or heat shock; HO-2 is a constitutively synthesized enzyme (27).

Because our previous work (45) showed that heme and CO caused electrolyte secretion in intestinal epithelial cells, we hypothesized that an inhibitor of HO activity, tin protoporphyrin (SnPP) (12, 35), would inhibit heme-induced Cl^- secretion. Contrary to our expectations, SnPP did not inhibit the short-circuit current (I_{sc}) induced by hemin. SnPP caused a higher rate of Cl^- secretion than hemin itself, an effect that was light dependent and inhibited by antioxidants.

MATERIALS AND METHODS

Tissue culture. Caco-2 cells were kindly provided by Dr. Jeffrey Field (University of Iowa, Iowa City, IA) and used between *passages 30* and *55*. Stock cultures were grown to confluency at 37°C in 10% CO_2 using DMEM (Mediatech, Cellgro, Herndon, VA) containing 4.5 g/l glucose, 10% FCS, 50 U/ml penicillin, 50 μg/ml streptomycin, and 15 mM HEPES. T84 cells were obtained from the American Tissue Culture Collection and grown in a 1:1 mixture of DMEM and Ham's F-12 media, 5% FCS, 50 U/ml penicillin, 50 μg/ml streptomycin, 10 mM $NaHCO_3$, and 15 mM HEPES. Fresh medium was added every 2 days. Cells were released from stock plates by a brief trypsin-EDTA treatment and plated at 30,000 cells/cm^2 on Millipore Millicell PCF filters (Millipore, Billerica, MA). Development of a confluent monolayer was confirmed when transepithelial resistance (TER) was stable at ~1,300 Ω·cm^2 for 2 successive days (about 14–16 days after cells had been seeded).

Electrical measurements. Transmonolayer voltage, TER, and I_{sc} were measured in Ussing chambers constructed to accommodate Millicell filters (Jim's Instruments, Iowa City, IA) (23). For these measurements, cells were bathed in a Krebs-Ringer-bicarbonate solution consisting of (in mM) 115 NaCl, 25 $NaHCO_3$, 5 KCl, 5 Na-HEPES, 5 H-HEPES, 1.5 $CaCl_2$, 1 $MgCl_2$, 1 Na_2HPO_4, and 5 glucose. The Cl^-- and HCO_3^--free Na^+-HEPES solution contained (in mM) 1.5 $CaNO_3$, 5 K-gluconate, 1 $MgSO_4 \cdot 7H_2O$, 1 Na_2HPO_4, 5 Na-HEPES, 5 H-HEPES, 140 Na-isethionate, and 5 glucose. The Krebs-Ringer-bicarbonate solution was gassed with 5% CO_2 at 37°C to maintain pH at 7.4; the Cl^-- and HCO_3^--free Na^+-HEPES solution was gassed with air. A positive I_{sc} value represents a flow of positive

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charge from the luminal (apical) to the basolateral solution (absorption) or a flow of a negative charge from the basolateral solution to the apical solution (secretion).

Cell viability assay. Cell viability was determined by the 3-[4,5-demethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay. This assay relies on the production of formazan by the action of mitochondrial enzymes on MTT by living cells and correlates well with other measures of cell numbers (19). Confluent Caco-2 cells were treated with SnPP diluted in phenol red-free MEM (GIBCO-BRL Invitrogen, Carlsbad, CA) with L-glutamine in the apical or basolateral solution for 24 h at 37°C and 10% CO_2. At the end of the treatment, media containing SnPP were aspirated, and cells were maintained in Krebs-Ringer-bicarbonate solution. Cells were then exposed to ambient fluorescent light in the laboratory at room temperature for 30 min, 1 h, and 2 h. Following light exposure, cells were incubated with MTT (0.5 mg/ml, Sigma Aldrich, St. Louis, MO) at 37°C and 10% CO_2 for 2 h. The formazan formed was solubilized in 2-propanol and quantitated by measuring the absorbance at 550 nm.

Measurement of cell-associated SnPP. The SnPP content of Caco-2 cells was determined using the protocol previously described (4, 46). Media containing SnPP were aspirated, and cells were washed with HBSS three times. Cells were removed with 2×0.5 ml formic acid washes. The SnPP content of cells was determined spectrophotometrically (extinction coefficient at 409 nm = $2.8 \times 10^5\ M^{-1} \cdot cm^{-1}$) using a diode array UV/visible spectrophotometer (Agilent Technology, Palo Alto, CA). Cell-associated SnPP was expressed as nanomoles per filter over time.

Measurement of intracellular SnPP with confocal microscopy. We took advantage of SnPP's endogenous fluorescence and measured SnPP uptake by using confocal laser microscopy (Zeiss, Central Microscopy Facility, University of Iowa) and setting excitation at 543 nm and the emission at >560 nm (49). To help localize SnPP inside the cells, another fluorescent dye, To-Pro-3, was used to stain nuclear DNA (41).

Electron paramagnetic resonance measurements. Caco-2 cells were suspended in PBS (100,000 cells/ml) and transferred into a TM-flat cell for electron paramagnetic resonance (EPR) measurements. All EPR spectra were obtained at room temperature with a Bruker EMX spectrometer operating at 9.76 GHz. The EPR spectrometer settings were as follows: modulation frequency, 100 kHz; modulation amplitude, 1.0 G; microwave power, 40 mW; and receiver gain, 10^5–10^6. EPR spectra were recorded while the sample was being exposed to visible light (tungsten lamp) in the EPR cavity.

Chemicals. All chemicals were obtained from Sigma Aldrich unless otherwise stated. CFTR(inh)-72 (2) was a generous gift from Dr. Jonathan D. Kaunitz. SnPP was purchased from Frontier Scientific (Logan, UT) and diluted in 0.1 N NaOH. To-Pro-3 was purchased from Molecular Probes (Carlsbad, CA). Hemin (3 mM, Sigma-Aldrich) was prepared in 1 M NaOH and diluted to 0.5 mM in cell culture media and 10% FCS. The pH was adjusted to 7.4 by the slow addition of 12 M HCl. For convenience, we use heme (Fe^{2+}) and hemin (Fe^{3+}) interchangeably.

Statistics. Within-subject analyses (i.e., comparing I_{sc} at baseline and after intervention) were performed using paired-sample *t*-tests. Between-subject analyses (i.e., I_{sc} between control and SnPP groups) were performed using two-sample *t*-tests. Results are expressed as means ± SE. Statistical significance was defined as $P < 0.05$ (two-tailed analysis).

RESULTS

SnPP induces I_{sc} in Caco-2 cells. Because heme and the product of heme degradation CO can induce Cl^- secretion by Caco-2 cells (45), we hypothesized that a competitive inhibitor of HO, SnPP (12, 13), would inhibit heme-induced Cl^- secretion. To test this hypothesis, Caco-2 cells were treated with hemin (100 μM), SnPP (50 μM), or hemin plus SnPP from the basolateral side of Caco-2 cells for 24 h. Baseline I_{sc} was 1.5-fold higher in hemin-treated cells (Fig. 1, *A* and *B*). Contrary to our expectations, SnPP did not inhibit the I_{sc} increase caused by hemin. Interestingly, SnPP treatment alone increased I_{sc} gradually in Caco-2 cells. Cotreatment of SnPP with hemin inhibited the current induced by SnPP.

Basolateral but not apical SnPP induces I_{sc}. We (46) have previously shown that cells exposed to hemin from the basolateral surface demonstrate a higher HO-1 induction than cells exposed to hemin from the apical surface. In addition, hemin increases I_{sc} only if applied from the basolateral surface (45). To investigate if Caco-2 cells have a similar response to SnPP, we treated the cells with apical or basolateral SnPP (50 μM) for 24 h. Cells were then mounted in Ussing chambers and bathed with Krebs-Ringer solution that did not contain SnPP. Apical SnPP did not increase I_{sc} (Fig. 2). In contrast, basolateral SnPP increased I_{sc} significantly.

Cell-associated SnPP is higher following basolateral exposure. We postulated that the greater I_{sc} response to basolateral SnPP was related to SnPP having better access to the Caco-2 cell cytosol from this side. To test this hypothesis, we measured cell-associated SnPP levels in Caco-2 cells (4, 46). Confluent Caco-2 cells were treated with SnPP (5–50 μM) in

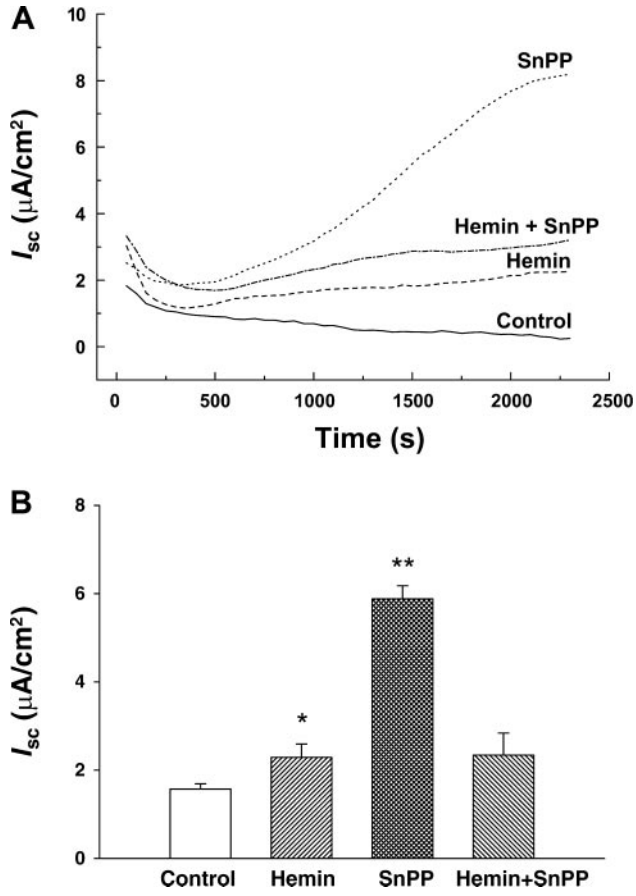

Fig. 1. Tin protoporphyrin (SnPP) induces short-circuit current (I_{sc}) in Caco-2 cells. *A*: confluent monolayers were treated with SnPP (50 μM), hemin (100 μM), or SnPP + hemin in the basolateral solution for 24 h. I_{sc} was measured in Ussing chambers. Baseline I_{sc} was higher in hemin-treated cells; I_{sc} increased gradually in SnPP treated cells. Cotreatment of SnPP and hemin inhibited the current induced by SnPP. *B*: summary of I_{sc} values. $^*P < 0.05$ and $^{**}P < 0.01$ compared with control; $n = 9$.

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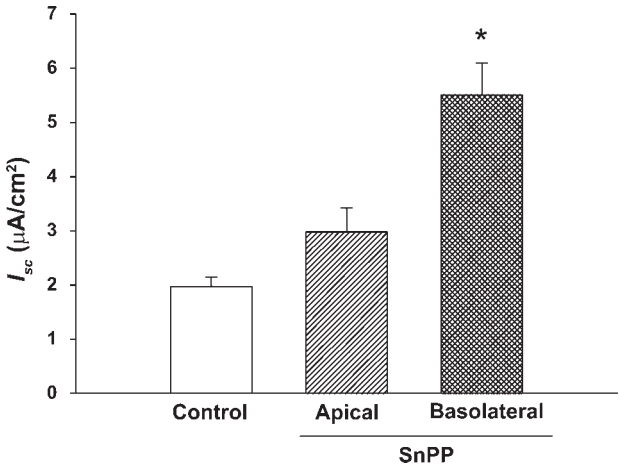

Fig. 2. Effect of SnPP on I_{sc}. Caco-2 cells were treated with SnPP (50 μM) in the apical or basolateral solution for 24 h. Apical SnPP had a minimal effect on I_{sc} [P = not significant (NS) compared with control; n = 6], whereas basolateral SnPP greatly enhanced I_{sc} (*$P < 0.01$ compared with control; n = 6).

the apical or basolateral solution for 24 h. Cell-associated SnPP was much higher after basolateral treatment compared with apical treatment at all concentrations (Fig. 3).

We also assessed intracellular localization with confocal laser microscopy. The SnPP signal inside the cell was barely detectable after apical SnPP application (data not shown). In contrast, after basolateral treatment, the fluorescent SnPP signal was abundant in the cytoplasm; the signal from the nucleus was minimal (Fig. 4). These results indicate that the greater cell-associated SnPP following basolateral exposure is due to cell uptake of SnPP and not to extracellular binding. The results suggest that SnPP must gain access to an intracellular pool to produce its effects on I_{sc}.

SnPP dose response in Caco-2 cells. To determine the effect of concentration on SnPP-induced I_{sc} in Caco-2 cells, we treated cells with SnPP (10, 25, and 50 μM) for 24 h and analyzed I_{sc} responses in Ussing chambers (Fig. 5, *A* and *B*). Maximum I_{sc} was reached much faster with 50 μM SnPP

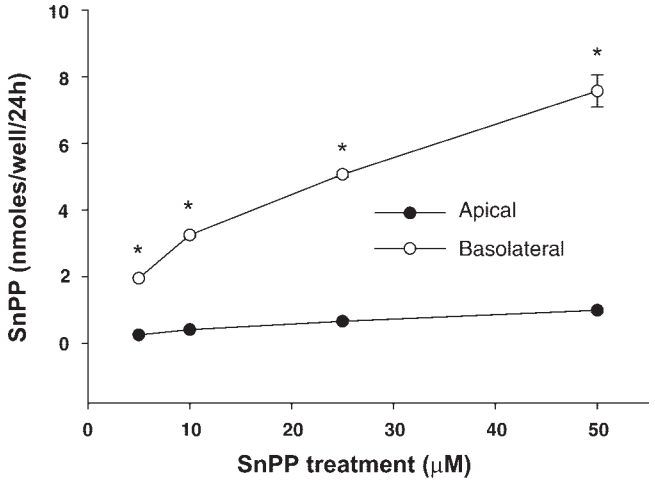

Fig. 3. Cell-associated SnPP. Caco-2 cells were treated with 5–50 μM SnPP from the apical or basolateral side for 24 h. Cells were removed with formic acid, and cellular SnPP content was measured spectrophotometrically. Cell-associated SnPP was much higher after basolateral exposure. *$P < 0.01$, apical vs. basolateral; n = 4.

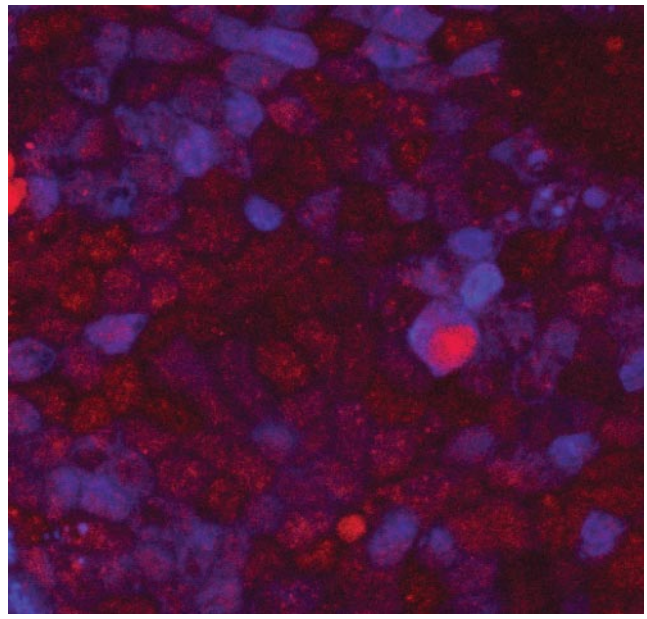

Fig. 4. Intracellular SnPP localization. Caco-2 cells were treated with 50 μM SnPP for 2 h and counterstained with To-Pro-3 after being fixed with 2% paraformaldehyde. SnPP was visualized by setting excitation at 543 nm and emission at >560 nm (red color); To-Pro-3 was visualized by setting the excitation at 633 nm and emission at 650 nm (blue color). SnPP was diffusely distributed inside the cells and did not localize to the nucleus. This image is representative of 3 different experiments.

compared with 10 and 25 μM SnPP (66.2 ± 4 min for 10 μM, 65.9 ± 4.2 min for 25 μM, and 42.9 ± 5.8 min for 50 μM; $P <$ 0.01, 50 vs. 10 or 25 μM SnPP), but peak current was not as great as that recorded from cells exposed to 10 or 25 μM SnPP. To determine if cell toxicity played a role in this response, we performed a MTT assay on cells treated with 50 μM SnPP for 24 h (basolateral) at 0, 10, 40, and 100 min (the time point when I_{sc} had declined) after starting the experiment. Evidence of cell toxicity was observed only at 100 min. The MTT assay was also done after cells were treated with 10 μM basolateral SnPP for 24 h and kept in Ussing chambers for similar time points; no cell toxicity was seen at any time point examined. Therefore, it is possible that the relatively lower peak I_{sc} response to 50 μM SnPP is the result of some cell toxicity.

SnPP dose response in T84 cells. To determine if the results were unique to Caco-2 cells, we examined the responses to SnPP in another intestinal epithelial cell line, T84 cells. Cells were treated with SnPP (10, 25, and 50 μM) for 24 h (Fig. 6, *A* and *B*). In contrast to Caco-2 cells, SnPP induced I_{sc} in a dose-dependent way in T84 cells. SnPP at 50 μM produced a consistent stimulation of I_{sc}, whereas 10 μM produced almost no stimulation and 25 μM had an intermediate effect. No cell toxicity was observed with SnPP at these concentrations at any time point, suggesting that T84 cells were more resistant to higher concentrations of SnPP than Caco-2 cells.

SnPP-induced I_{sc} is Cl^- secretion. To determine whether Na^+ absorption via the epithelial Na^+ channel (ENaC) could account for the SnPP-induced I_{sc}, we treated the cells with benzamil (10 μM) (29) in the apical solution of Ussing chambers. Benzamil had no effect on I_{sc}, suggesting that Na^+ absorption via ENaC did not have a role in the generation of this current (Fig. 7).

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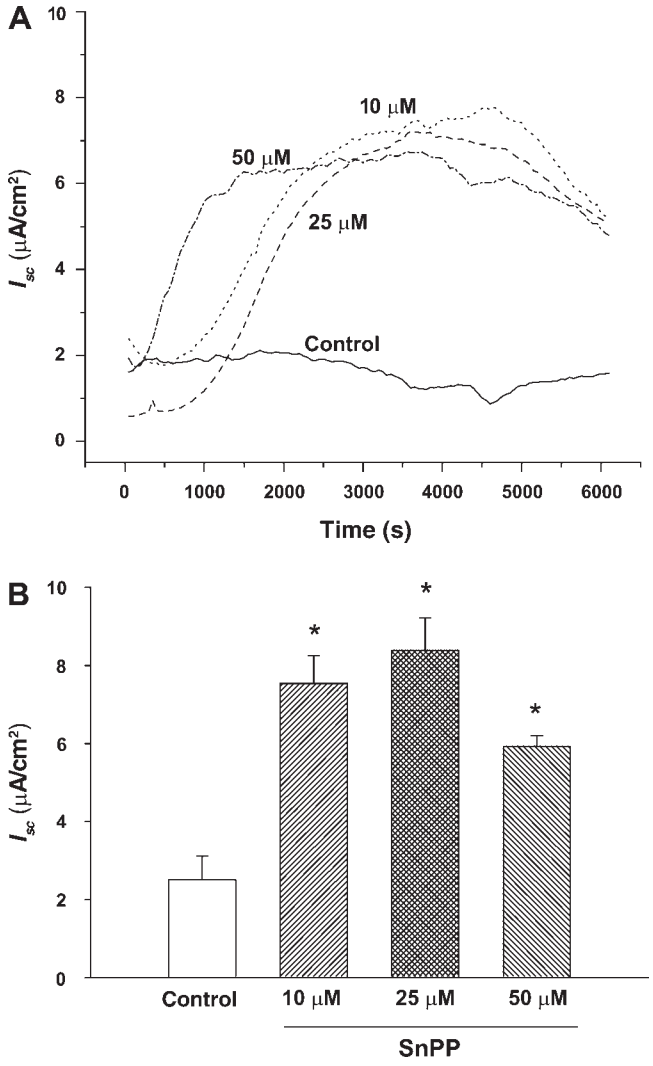

Fig. 5. SnPP dose response. *A*: representative time course. Caco-2 cells were treated with 10, 25, and 50 μM SnPP in the basolateral solution for 24 h. I_{sc} was lowest with 50 μM SnPP; the time to reach maximum I_{sc} was longest with 10 μM SnPP. *B*: summary of peak I_{sc} data. *$P < 0.01$, control vs. treatment; $n = 7$.

To determine the nature of the I_{sc} induced by basolateral SnPP exposure, Ussing chamber experiments were repeated in Cl^-- and HCO_3^--free Na^+-HEPES solution. The SnPP-induced current was significantly reduced if Cl^- and HCO_3^- were absent in Ussing chambers, suggesting that the I_{sc} increase caused by SnPP was due mostly to active Cl^- secretion (Fig. 8).

The nature of SnPP-induced I_{sc} was further examined by ouabain, an inhibitor of basolateral Na^+-K^+-ATPase, a pump needed to maintain low intracellular Na^+ levels (37). Ouabain inhibited SnPP-induced I_{sc} (Table 1), indicating that this stimulated current was dependent on the Na^+-K^+ pump. These results imply that this current does not represent an active transport process powered by a H^+-ATPase. We then tested the effect of a Cl^- channel inhibitor, 5-nitro-2-(3-phenylpropylamino)benzoic acid (NPPB) (5), on SnPP-induced I_{sc}. NPPB (100 μM) applied to the apical surface of the cells significantly inhibited I_{sc} caused by SnPP (Table 1). These results indicate that SnPP-induced I_{sc} was the result of active Cl^- secretion and not Na^+ absorption.

To investigate if the current induced by SnPP was caused by cGMP or cAMP, we used inhibitors. After being treated with SnPP (10 μM basolateral) for 24 h, cells were incubated in Ussing chambers with the soluble guanylate cyclase inhibitor [1,2,4]oxadiazolo-[4,3-a]quinoxalin-1-one (ODQ; 25 μM) or inhibitors of cAMP-dependent protein kinase Rp-cAMPS (50 μM) and H-89 (10 μM). Neither agent had any effect on SnPP-induced I_{sc}, suggesting that this current was not dependent on cAMP or cGMP.

The role of CFTR on SnPP-induced current was investigated by treating the monolayers with a potent and selective CFTR inhibitor, CFTR(inh)-72 (2). We observed no change in SnPP-related responses with CFTR(inh)-72 (2 μM). Therefore, it is unlikely that SnPP-induced Cl^- secretion is caused by CFTR activation.

SnPP-induced I_{sc} is light dependent. SnPP is an efficient photosensitizer that can generate singlet oxygen ($^1O_2$) after exposure to light (26). $^1O_2$ can react with electron-rich moieties

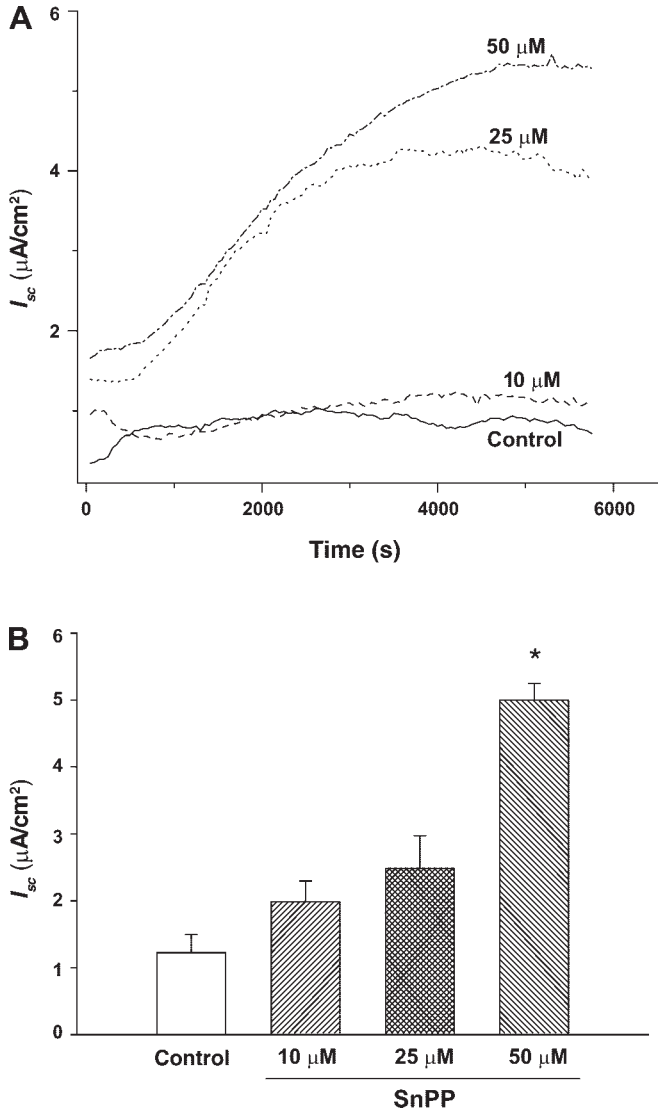

Fig. 6. SnPP dose response in T84 cells. *A*: representative time course of monolayers treated with 10, 25, and 50 μM SnPP in the basolateral solution for 24 h. The I_{sc} response to SnPP was dose dependent. *B*: summary of peak I_{sc} data. *$P < 0.01$ compared with control; $n = 5$.

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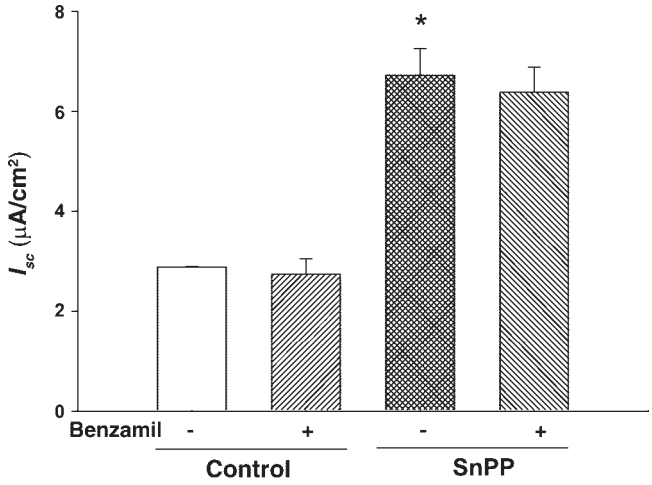

Fig. 7. SnPP-induced I_{sc} is not caused by electrogenic Na^+ absorption. Caco-2 monolayers were treated with 50 μM SnPP for 24 h. The epithelial Na^+ channel inhibitor benzamil (10 μM) had no effect on SnPP-induced I_{sc}. $*P <$ 0.01, control vs. SnPP; P = NS, SnPP vs. SnPP + benzamil; $n = 7$.

in lipids, amino acids, and nucleic acids, causing their oxidation (30). Because the SnPP effect required 45–60 min to develop, we thought that the photooxidation initiated by SnPP and ambient (fluorescent) light could be playing a role. To test this idea, we covered one control and one SnPP-treated (50 μM basolateral) set of Caco-2 cells with aluminum foil for the first 30 min of the experiment and then removed the covers (Fig. 9). I_{sc} increased in SnPP-treated cells only after they were exposed to light; light had no effect on cells not treated with SnPP.

To determine whether SnPP undergoes photodegradation during light exposure, we measured changes in its absorption spectrum. SnPP (10 μM) was diluted in the Krebs solution used to measure I_{sc}, and absorption spectra of the solution were obtained at time points up to 7 h while the solution was continuously exposed to ambient fluorescent light. There was no change in SnPP absorption after 30 min of light exposure. Starting at 1 h, the absorption of SnPP (407 nm) decreased, and isosbestic points appeared at 398 and 418.5 nm (Fig. 10).

Table 1. *SnPP-induced I_{sc} is Cl^- dependent*

	Baseline	NPPB	Ouabain
Control	100±9	75±9*	23±10*
SnPP	362±9†	186±21‡	42±10‡

Values are means ± SE [in % of short-circuit current (I_{sc})]; $n = 4$. Caco-2 cells were treated with tin protoporphyrin (SnPP; 50 μM) in the basolateral solution for 24 h, and I_{sc} was measured in Ussing chambers. Responses to 5-nitro-2-(3-phenylpropylamino)benzoic acid (NPPB; 100 μM apical) or ouabain (100 μM basolateral) were measured. $*P < 0.01$, baseline control vs. NPPB or ouabain; $†P < 0.01$, control vs. SnPP; $‡P < 0.01$, baseline SnPP vs. NPPB or ouabain.

These results indicate that SnPP underwent a chemical change upon light exposure.

We then asked if SnPP that had been exposed to light in solution could reproduce the effects on Caco-2 cells acutely. First, we added freshly prepared SnPP to Ussing chambers (10 μM) from the basolateral surface. This treatment caused no increase in I_{sc}, suggesting that a prolonged incubation and possibly intracellular accumulation were required to see an effect. Second, we prepared SnPP (10 μM) in Krebs solution and exposed to the ambient fluorescent light for 24 h. Caco-2 cells were then incubated with this solution in Ussing chambers from the basolateral surface. This treatment did not cause an increase in I_{sc}. Therefore, the end product of SnPP degradation upon light exposure did not seem to reproduce the effect of light on cells previously exposed to SnPP. These results suggest that the stable degradation product of SnPP is not the agent responsible for increasing Cl^- secretion. Thus, the action of light on intracellular SnPP must generate an intermediate product that induces Cl^- secretion.

SnPP-induced I_{sc} is caused by oxidative stress. Photooxidative effects of SnPP were initially described in animals who received this compound along with phototherapy (14, 21, 25). Further studies (10, 31) have suggested that the phototoxic reactions observed with SnPP were the results of lipid peroxidation. To examine the role of lipid peroxidation on SnPP-induced I_{sc}, we examined the effects of α-tocopherol, an

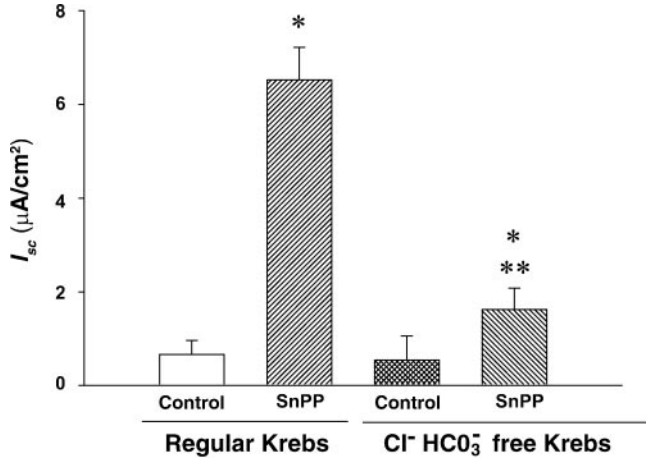

Fig. 8. SnPP-induced I_{sc} is Cl^- dependent. Caco-2 cells were treated with basolateral SnPP (50 μM) for 24 h. SnPP-induced I_{sc} was significantly lower if Cl^- and HCO_3^- were absent. $*P < 0.01$, control vs. SnPP; $**P < 0.01$, SnPP in Krebs-Ringer solution vs. SnPP in Cl^-- and HCO_3^--free HEPES; $n = 4$.

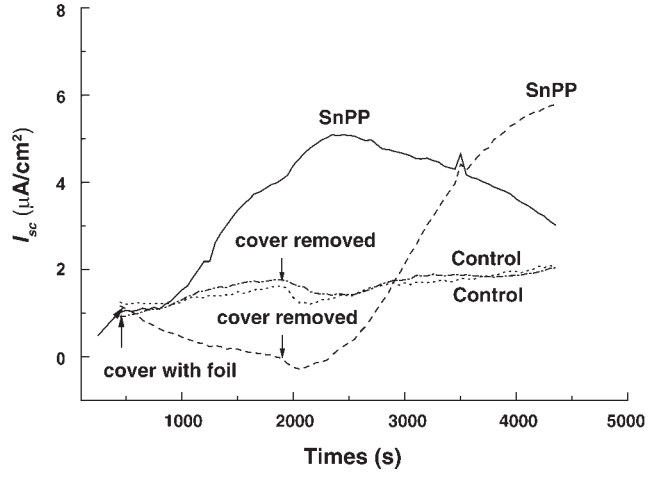

Fig. 9. SnPP-induced I_{sc} is light dependent. Caco-2 cells were treated with basolateral SnPP (50 μM) for 24 h. One control and one SnPP-treated well were covered with aluminum foil the first 30 min. I_{sc} increased only after SnPP-treated cells were exposed to ambient fluorescent lights. The time course is representative of 5 different experiments.

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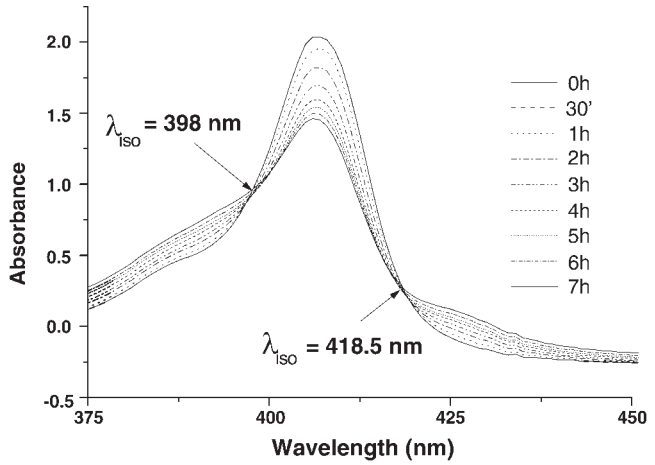

Fig. 10. Degradation of SnPP with ambient fluorescent light. SnPP was diluted in Krebs solution (10 μM), and spectra were obtained at 0 h, 30 min, and hourly for 7 h while SnPP was continuously exposed to ambient fluorescent light. SnPP absorption at 407 nm decreased over time with light exposure, and isosbestic points (λ_{ISO}) appeared at 398 and 418.5 nm. The results shown are representative of 3 separate experiments.

inhibitor of the free radical chain reactions of lipid peroxidation (7). Caco-2 cells were pretreated with α-tocopherol (10 μM) for 5 days prior to being treated with SnPP. α-Tocopherol significantly inhibited the effect of SnPP (Fig. 11, *A* and *B*), suggesting that lipid peroxidation was playing a role in Cl^- secretion induced by SnPP.

We then studied the effects of another antioxidant, ascorbic acid (7). Ascorbate has been reported to protect SnPP-treated rats against phototoxicity (24). Ascorbic acid (1 mM) was given to Caco-2 cells following 24 h treatment with SnPP (10 μM basolateral) at the time of mounting the cells in the Ussing chambers. Ascorbate abolished the increase in current caused by SnPP (Fig. 12, *A* and *B*). These results further confirm that the effect of SnPP + light is mediated via oxidation reactions.

To determine the role of oxidative stress in SnPP-induced Cl^- secretion, we used EPR. We took advantage of ascorbate's ability to react with oxidants with the subsequent formation of the ascorbate free radical ($Asc^{\cdot -}$). The level of $Asc^{\cdot -}$ is a real-time indicator of the flux of oxidants in a system (8). Caco-2 cells were treated with SnPP (10 μM) from the basolateral surface for 24 h and ascorbate (1 mM) for the last 2 h. Cells were then removed from the filters and analyzed by EPR. As expected, a background level of $Asc^{\cdot -}$ was observed (Fig. 13*A*). However, upon exposure to light, the steady-state level of $Asc^{\cdot -}$ was increased (Fig. 13*B*). These data support the idea that oxidative reactions can be initiated with light in a cell sample loaded with SnPP.

DISCUSSION

In this study, we used SnPP to inhibit the effects of hemin on intestinal epithelial cell Cl^- secretion. Contrary to our expectations, SnPP produced an increase in I_{sc}. We found that the SnPP effect on I_{sc} was light dependent and inhibited by antioxidants.

The I_{sc} increase caused by SnPP was the result of active Cl^- secretion: it was significantly inhibited by the absence of Cl^- in Ussing chamber solutions. It was not inhibited by benzamil, a specific inhibitor of ENaC, but was inhibited by NPPB, a nonselective inhibitor of anion transporters. The inhibition by ouabain, an inhibitor of Na^+-K^+-ATPase, which functions to maintain low intracellular Na^+ levels in cells (11), further supports the conclusion that the SnPP-stimulated I_{sc} is Cl^- secretion. A low intracellular Na^+ concentration is necessary to allow the entry of Na^+ and Cl^- across the basolateral membrane. Cl^- accumulated inside the cell is secreted across the apical membrane through apical Cl^- channels. The inhibitory effect of ouabain on this current demonstrates that SnPP-induced Cl^- secretion requires a Na^+ gradient maintained by basolateral Na^+-K^+-ATPase. This effect of ouabain greatly reduces the possibility that this current could have been powered by an H^+-ATPase.

Intestinal epithelial Cl^- secretion is dependent on the activation of apical Cl^- channels. One of the best-characterized apical Cl^- channel is CFTR, a channel that is activated via cAMP (6). In our experiments, two cAMP inhibitors and a specific CFTR inhibitor had no effect on SnPP-induced current. Therefore, it is unlikely that SnPP induces Cl^- secretion via CFTR and/or cAMP activation. On the other hand, SnPP-induced I_{sc} was reduced by NPPB, a nonspecific Cl^- trans-

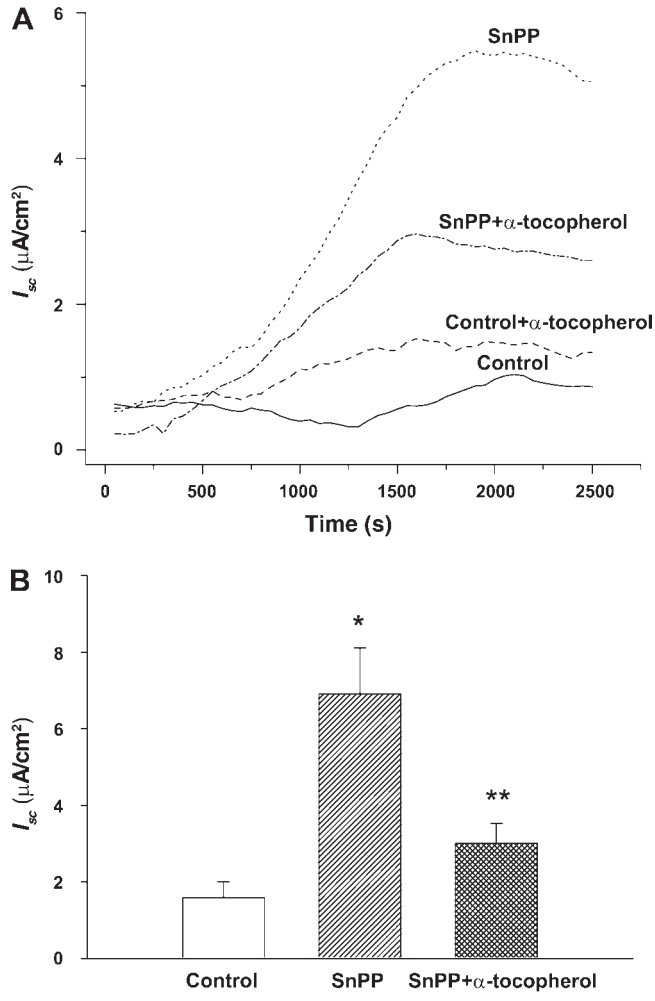

Fig. 11. α-Tocopherol inhibits SnPP-induced I_{sc}. Caco-2 cells were treated with basolateral SnPP (50 μM) for 24 h. *A*: time course of the I_{sc} response to SnPP. *B*: summary of the peak response to SnPP experiments: α-tocopherol inhibited SnPP-induced I_{sc}. *$P < 0.01$, control vs. SnPP; **$P < 0.05$, SnPP vs. SnPP + α-tocopherol; $n = 4$.

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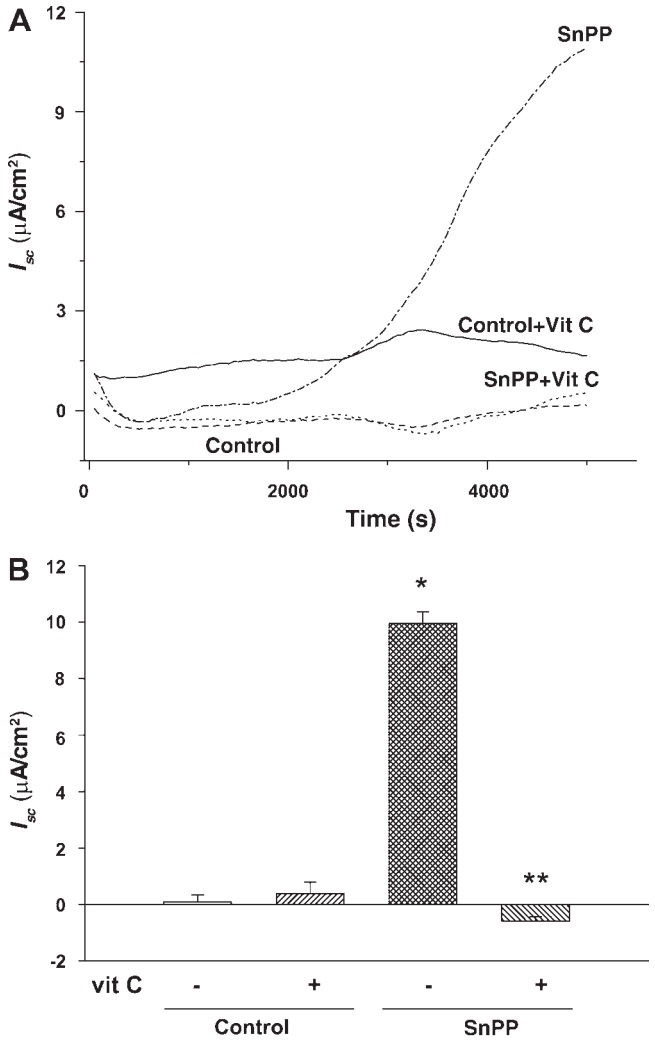

Fig. 12. Ascorbic acid [vitamin C (Vit C)] abolishes SnPP-induced I_{sc}. Caco-2 cells were treated with basolateral SnPP (10 μM) for 24 h. *A*: time course of the effect of Vit C (1 mM). *B*: summary of the peak response to SnPP. Vit C inhibited SnPP-induced I_{sc}. *$P < 0.01$, control vs. SnPP; **$P < 0.01$, SnPP vs. SnPP + Vit C; $n = 5$.

porter inhibitor. These results, taken together, suggest that the apical Cl^- channel responsible for SnPP-stimulated Cl^- secretion is not CFTR but another kind of Cl^- channel.

SnPP stimulated Cl^- secretion only if the cells were treated from the basolateral surface and not from the apical or lumen side. This differential effect was most likely the result of SnPP having better access to an intracellular compartment from the basolateral side. These observations, along with hemin (iron-protoporphyrin) inducing HO-1 more efficiently from the enterocyte basolateral surface (46), suggest that intestinal epithelial cells have a more efficient uptake and transport mechanisms for porphyrins from their basolateral membrane.

We observed that SnPP induced Cl^- secretion only when cells were exposed to ambient light. One possibility we considered was the generation of CO from the light degradation of SnPP, as demonstrated by Vreman and colleagues (47). CO induces Cl^- secretion in intestinal epithelial cells via cGMP, an effect we (45) have previously shown to be inhibited by the soluble guanylate cyclase inhibitor ODQ. However, CO generation was an unlikely intermediate of the SnPP-increased I_{sc} because ODQ had no effect on the current.

We have determined that Cl^- secretion in Caco-2 cells was due to oxidative events induced by SnPP and light. This deduction is based on the fact that the SnPP-induced current is inhibited by two antioxidants, lipid-soluble α-tocopherol (vitamin E) and water-soluble ascorbate (vitamin C). This conclusion is further supported by the observed increase in the steady-state level of $Asc^{\cdot -}$ in cells that were exposed to light after being treated with SnPP.

Another observation consistent with the conclusion that the light-mediated SnPP effect is related to oxidation is that coincubation of hemin and SnPP reduced the effect of SnPP alone on I_{sc}. Although hemin is capable of inducing Cl^- secretion in intestinal epithelial cells (45), it reduced the Cl^- secretory effects of SnPP. It seems possible that the inhibitory effect of hemin on SnPP-induced I_{sc} was secondary to HO-1 induction. The inhibitory effect of hemin occurred after 24 h of treatment, a time point when HO-1 is induced in these cells (44). Degradation of heme via HO-1 generates antioxidant products such as bilirubin and biliverdin (38, 39). These molecules might have inhibited the SnPP response by changing the redox environment of the cell. An extension of this idea is that these metabolic products of heme might mitigate an otherwise strongly oxidative state induced by heme itself.

Another important conclusion from this study is the role of SnPP in creating oxidative stress in vivo. SnPP is widely used in animal and cell culture experiments to inhibit the activity of HO-1. Interestingly, ambient laboratory light is sufficient to

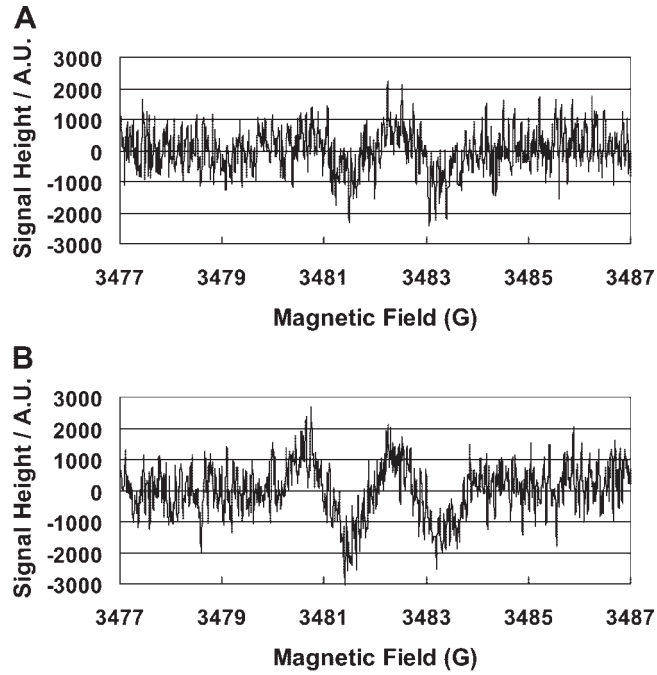

Fig. 13. Light increased the electron paramagnetic resonance (EPR) signal intensity of the ascorbate radical ($Asc^{\cdot -}$). Caco-2 cells (100,000 cells/ml) were treated with SnPP (10 μM) basolaterally for 24 h and ascorbate (1 mM) for 2 h. Cells were examined by EPR. *A*: in the absence of light, a small $Asc^{\cdot -}$ signal is seen, consistent with slow, background oxidation of ascorbate. *B*: visible light (tungsten lamp) increased the intensity of the $Asc^{\cdot -}$ signal ($n = 3$). This increase was not seen in the absence of SnPP (data not shown). AU, arbitrary units.

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initiate significant photooxidative reactions of SnPP in cell culture studies. Therefore, the effects observed with SnPP may not be entirely due to its inhibition of HO-1 but also the generation of photooxidative products by this porphyrin. It is not known if other metalloporphyrins, such as cobalt protoporphyrin (CoPP) or zinc protoporphyrin (ZnPP), would have photosensitizing effects similar to SnPP in this setting. In general, CoPP and ZnPP are considered to be ineffective photosensitizers (10, 36). The possible effects of these metalloporphyrins will be studied in the future.

Our data suggest that SnPP and light induce changes in the redox environment of the cell, which leads to Cl^- secretion. There is precedence for a role of the cellular redox status in the regulation of a Cl^- channel, CFTR. Reducing conditions increase the open state of CFTR; in contrast, oxidation of the CFTR decreases the open state of the channel (20). While it seems very unlikely that SnPP-induced Cl^- secretion in Caco-2 cells is mediated via CFTR, it does seem likely that changing the redox environment of the cell is intimately involved in regulating this Cl^- secretion.

These results underscore the important role that the redox environment of the cell may play in electrolyte secretion. Such a regulatory system may be responsible, in part, for the Cl^- secretion (and thus diarrhea) observed with intestinal inflammation. Toxic ROS and other oxidants can be generated during intestinal inflammation from macrophages and monocytes (40, 48). These molecules might contribute to tissue injury (3) and play an important role in the diarrhea observed in inflammatory conditions (16).

In summary, SnPP, a competitive inhibitor of HO, caused Cl^- secretion at a higher magnitude than hemin itself, an effect that was light dependent and inhibited by antioxidants. These data suggest that SnPP-induced effects may not be entirely due to the inhibition of HO activity but rather to light-induced oxidative processes. This study also underscores the concept that the redox environment of the cell may play a role in regulating Cl^- secretion.

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REFERENCES

1. **Abraham NG, Lin JH, Mitrione SM, Schwartzman ML, Levere RD, Shibahara S.** Expression of heme oxygenase gene in rat and human liver. *Biochem Biophys Res Commun* 150: 717–722, 1988.
2. **Akiba Y, Jung M, Ouk S, Kaunitz JD.** A novel small molecule CFTR inhibitor attenuates. HCO_3^- secretion and duodenal ulceration in rats. *Am J Physiol Gastrointest Liver Physiol* 289: G753–G759, 2005.
3. **Baker SS, Campbell CL.** Rat enterocyte injury by oxygen-dependent processes. *Gastroenterology* 101: 716–720, 1991.
4. **Balla G, Vercellotti GM, Muller-Eberhard U, Eaton J, Jacob HS.** Exposure of endothelial cells to free heme potentiates damage mediated by granulocytes and toxic oxygen species. *Lab Invest* 64: 648–655, 1991.
5. **Bens M, Van Huyen JP, Cluzeaud F, Teulon J, Vandewalle A.** CFTR disruption impairs cAMP-dependent Cl^- secretion in primary cultures of mouse cortical collecting ducts. *Am J Physiol Renal Physiol* 281: F434–F442, 2001.
6. **Berger HA, Anderson MP, Gregory RJ, Thompson S, Howard PW, Maurer RA, Mulligan R, Smith AE, Welsh MJ.** Identification and regulation of the cystic fibrosis transmembrane conductance regulator-generated chloride channel. *J Clin Invest* 88: 1422–1431, 1991.
7. **Buettner GR.** The pecking order of free radicals and antioxidants: lipid peroxidation, alpha-tocopherol, and ascorbate. *Arch Biochem Biophys* 300: 535–543, 1993.
8. **Buettner GR, Jurkiewicz BA.** Ascorbate free radical as a marker of oxidative stress: an EPR study. *Free Radic Biol Med* 14: 49–55, 1993.
9. **Davis GR, Santa Ana CA, Morawski S, Fordtran JS.** Active chloride secretion in the normal human jejunum. *J Clin Invest* 66: 1326–1333, 1980.
10. **Dennery PA, Vreman HJ, Rodgers PA, Stevenson DK.** Role of lipid peroxidation in metalloporphyrin-mediated phototoxic reactions in neonatal rats. *Pediatr Res* 33: 87–91, 1993.
11. **Donowitz M, Welsh MJ.** Ca^{2+} and cyclic AMP in regulation of intestinal Na, K, and Cl transport. *Annu Rev Physiol* 48: 135–150, 1986.
12. **Drummond GS, Kappas A.** Prevention of neonatal hyperbilirubinemia by tin protoporphyrin IX, a potent competitive inhibitor of heme oxidation. *Proc Natl Acad Sci USA* 78: 6466–6470, 1981.
13. **Drummond GS, Kappas A.** Chemoprevention of neonatal jaundice: potency of tin-protoporphyrin in an animal model. *Science* 217: 1250–1252, 1982.
14. **Fort FL, Gold J.** Phototoxicity of tin protoporphyrin, tin mesoporphyrin, and tin diiododeuteroporphyrin under neonatal phototherapy conditions. *Pediatrics* 84: 1031–1037, 1989.
15. **Forte LR, Thorne PK, Eber SL, Krause WJ, Freeman RH, Francis SH, Corbin JD.** Stimulation of intestinal Cl^- transport by heat-stable enterotoxin: activation of cAMP-dependent protein kinase by cGMP. *Am J Physiol Cell Physiol* 263: C607–C615, 1992.
16. **Gaginella TS, Kachur JF, Tamai H, Keshavarzian A.** Reactive oxygen and nitrogen metabolites as mediators of secretory diarrhea. *Gastroenterology* 109: 2019–2028, 1995.
17. **Guandalini S, Rao MC, Smith PL, Field M.** cGMP modulation of ileal ion transport: in vitro effects of *Escherichia coli* heat-stable enterotoxin. *Am J Physiol Gastrointest Liver Physiol* 243: G36–G41, 1982.
18. **Gutteridge JM, Smith A.** Antioxidant protection by haemopexin of haem-stimulated lipid peroxidation. *Biochem J* 256: 861–865, 1988.
19. **Hansen MB, Nielsen SE, Berg K.** Re-examination and further development of a precise and rapid dye method for measuring cell growth/cell kill. *J Immunol Methods* 119: 203–210, 1989.
20. **Harrington MA, Gunderson KL, Kopito RR.** Redox reagents and divalent cations alter the kinetics of cystic fibrosis transmembrane conductance regulator channel gating. *J Biol Chem* 274: 27536–27544, 1999.
21. **Hintz SR, Vreman HJ, Stevenson DK.** Mortality of metalloporphyrin-treated neonatal rats after light exposure. *Dev Pharmacol Ther* 14: 187–192, 1990.
22. **Hughes JM, Murad F, Chang B, Guerrant RL.** Role of cyclic GMP in the action of heat-stable enterotoxin of *Escherichia coli*. *Nature* 271: 755–756, 1978.
23. **Husted RF, Volk KA, Sigmund RD, Stokes JB.** Anion secretion by the inner medullary collecting duct. Evidence for involvement of the cystic fibrosis transmembrane conductance regulator. *J Clin Invest* 95: 644–650, 1995.
24. **Keino H, Mimura S, Nagae H, Banno T, Kashiwamata S.** Protection by L-ascorbic acid against phototoxicity in tin-protoporphyrin-treated suckling rats. *Biol Neonate* 63: 183–190, 1993.
25. **Keino H, Nagae H, Mimura S, Watanabe K, Kashiwamata S.** Dangerous effects of tin-protoporphyrin plus photoirradiation on neonatal rats. *Eur J Pediatr* 149: 278–279, 1990.
26. **Land EJ, McDonagh AF, McGarvey DJ, Truscott TG.** Photophysical studies of tin(IV)-protoporphyrin: potential phototoxicity of a chemotherapeutic agent proposed for the prevention of neonatal jaundice. *Proc Natl Acad Sci USA* 85: 5249–5253, 1988.
27. **Maines MD.** The heme oxygenase system: a regulator of second messenger gases. *Annu Rev Pharmacol Toxicol* 37: 517–554, 1997.
28. **McCoubrey WK, Maines MD.** The structure, organization and differential expression of the gene encoding rat heme oxygenase-2. *Gene* 139: 155–161, 1994.
29. **McCray PB Jr, Bettencourt JD, Bastacky J, Denning GM, Welsh MJ.** Expression of CFTR and a cAMP-stimulated chloride secretory current in cultured human fetal alveolar epithelial cells. *Am J Respir Cell Mol Biol* 9: 578–585, 1993.
30. **Michaeli A, Feitelson J.** Reactivity of singlet oxygen toward amino acids and peptides. *Photochem Photobiol* 59: 284–289, 1994.

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31. **Mimura S, Nagae H, Keino H, Watanabe K, Kashiwamata S.** Sn-protoporphyrin plus photoirradiation induces lipid peroxidation in vivo and in vitro in nonjaundiced Gunn rats. *Biol Neonate* 60: 39–44, 1991.
32. **Mourad FH, Turvill JL, Farthing MJ.** Role of nitric oxide in intestinal water and electrolyte transport. *Gut* 44: 143–147, 1999.
33. **Musch MW, Clarke LL, Mamah D, Gawenis LR, Zhang Z, Ellsworth W, Shalowitz D, Mittal N, Efthimiou P, Alnadjim Z, Hurst SD, Chang EB, Barrett TA.** T cell activation causes diarrhea by increasing intestinal permeability and inhibiting epithelial Na^+/K^+-ATPase. *J Clin Invest* 110: 1739–1747, 2002.
34. **Ponka P.** Cell biology of heme. *Am J Med Sci* 318: 241–256, 1999.
35. **Posselt AM, Kwong LK, Vreman HJ, Stevenson DK.** Suppression of carbon monoxide excretion rate by tin protoporphyrin. *Am J Dis Child* 140: 147–150, 1986.
36. **Scott J, Quirke JM, Vreman HJ, Stevenson DK, Downum KR.** Metalloporphyrin phototoxicity. *J Photochem Photobiol B* 7: 149–157, 1990.
37. **Smith JJ, Welsh MJ.** cAMP stimulates bicarbonate secretion across normal, but not cystic fibrosis airway epithelia. *J Clin Invest* 89: 1148–1153, 1992.
38. **Stocker R, McDonagh AF, Glazer AN, Ames BN.** Antioxidant activities of bile pigments: biliverdin and bilirubin. *Methods Enzymol* 186: 301–309, 1990.
39. **Stocker R, Yamamoto Y, McDonagh AF, Glazer AN, Ames BN.** Bilirubin is an antioxidant of possible physiological importance. *Science* 235: 1043–1046, 1987.
40. **Suematsu M, Suzuki M, Kitahora T, Miura S, Suzuki K, Hibi T, Watanabe M, Nagata H, Asakura H, Tsuchiya M.** Increased respiratory burst of leukocytes in inflammatory bowel diseases–the analysis of free radical generation by using chemiluminescence probe. *J Clin Lab Immunol* 24: 125–128, 1987.
41. **Suzuki T, Fujikura K, Higashiyama T, Takata K.** DNA staining for fluorescence and laser confocal microscopy. *J Histochem Cytochem* 45: 49–53, 1997.
42. **Takahashi T, Matsushita K, Welsh MJ, Stokes JB.** Effect of cAMP on intracellular and extracellular ATP content of Cl^--secreting epithelia and 3T3 fibroblasts. *J Biol Chem* 269: 17853–17857, 1994.
43. **Tenhunen R, Marver HS, Schmid R.** The enzymatic conversion of heme to bilirubin by microsomal heme oxygenase. *Proc Natl Acad Sci USA* 61: 748–755, 1968.
44. **Uc A, Britigan BE.** Does heme oxygenase-1 have a role in Caco-2 cell cycle progression? *Exp Biol Med (Maywood)* 228: 590–595, 2003.
45. **Uc A, Husted RF, Giriyappa RL, Britigan BE, Stokes JB.** Hemin induces active chloride secretion in Caco-2 cells. *Am J Physiol Gastrointest Liver Physiol* 289: G202–G208, 2005.
46. **Uc A, Stokes JB, Britigan BE.** Heme transport exhibits polarity in Caco-2 cells: evidence for an active and membrane protein-mediated process. *Am J Physiol Gastrointest Liver Physiol* 287: G1150–G1157, 2004.
47. **Vreman HJ, Gillman MJ, Downum KR, Stevenson DK.** In vitro generation of carbon monoxide from organic molecules and synthetic metalloporphyrins mediated by light. *Dev Pharmacol Ther* 15: 112–124, 1990.
48. **Williams JG, Hughes LE, Hallett MB.** Toxic oxygen metabolite production by circulating phagocytic cells in inflammatory bowel disease. *Gut* 31: 187–193, 1990.
49. **Yang G, Nguyen X, Ou J, Rekulapelli P, Stevenson DK, Dennery PA.** Unique effects of zinc protoporphyrin on HO-1 induction and apoptosis. *Blood* 97: 1306–1313, 2001.

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