Characterization of apical and basal thiol-disulfide redox regulation in human colonic epithelial cells

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1Graduate Program in Molecular and Systems Pharmacology, Department of Medicine, 2Division of Endocrinology, Metabolism and Lipids, and 3Division of Pulmonary, Allergy and Critical Care Medicine, Emory University, Atlanta, Georgia

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Mannery YO, Ziegler TR, Hao L, Shyntum Y, Jones DP. Characterization of apical and basal thiol-disulfide redox regulation in human colonic epithelial cells. Am J Physiol Gastrointest Liver Physiol 299: G523–G530, 2010. First published May 13, 2010; doi:10.1152/ajpgi.00359.2009.—Control of extracellular thiol-disulfide redox potential (Eh) is necessary to protect cell surface proteins from external oxidative and reductive stresses. Previous studies show that human colonic epithelial Caco-2 cells, which grow in cell culture with the apical surface exposed to the medium, regulate extracellular cysteine/cystine Eh Eh based on physiological values (approximately −80 mV) observed in vivo. The present study tested whether extracellular Eh regulation occurs on the basal surface of Caco-2 cells and investigated relevant mechanisms. Experiments were performed with confluent, differentiated cells grown on a permeable membrane surface. Cells were exposed to an oxidizing potential (0 mV) using a fixed cysteine-to-cystine ratio, and culture medium was sampled over time for change in Eh. Regulation of extracellular thiol-disulfide Eh on the basal domain was faster, and the extent of change at 24 h was greater than on the apical surface. Mechanistic studies showed that redox regulation on the basal surface was partially sodium dependent and inhibited by extracellular lysine, a competitive inhibitor of cystine transport by the xC_ system. Studies using the thiol-reactive alkylation agent 4-acetamido-4′-maleimidylstilbene-2,2′-disulfonic acid and the glutathione synthesis inhibitor buthionine sulfoximine showed that extracellular redox regulation was not attributable to plasma membrane cysteine/cystine interconversion or intracellular glutathione, respectively. Thus the data show that redox regulation occurs at different rates on the apical and basal surfaces of the polarized Caco-2 epithelial cell line and that the y^1 system and xC_ systems function in extracellular cysteine/cystine redox regulation on the basal surface.

THE CYSTEINE/CYSTINE (Cys/CySS) redox couple is the major low-molecular-weight thiol/disulfide redox system in most extracellular fluids in humans (24, 27). In plasma, the predominant species is the oxidized disulfide form, CySS, which is usually present at >40 μM; Cys is typically present at values of ~8–10 μM in the fasting state (3). The average redox potential (Eh) of this couple in young healthy individuals is −80 ± 9 mV (18), being more reduced following ingestion of Cys and more oxidized in a variety of pathophysiological states (22, 31). Systematic variation of Eh in culture media shows that the Cys/CySS redox couple regulates biological processes, such as cell proliferation, proinflammatory signaling, and apoptosis (17). However, major questions remain concerning how cells regulate extracellular Eh.

Culture medium for mammalian cells often contains high physiological concentration of CySS (200–400 μM) and no added Cys. The resulting Eh is relatively oxidizing (>0 mV). Jonas et al. (20) showed that Caco-2 cells in vitro regulated extracellular redox conditions from 0 mV to the physiological range of −80 mV observed in vivo. In these experiments, cells were grown on a solid surface so that the observed extracellular redox regulation probably reflected that occurring at the apical surface. Regulation of intraluminal thiol/disulfide redox state had been previously shown in a vascularity perfused rat small intestine preparation (9), and related studies also showed redox regulation in the vascular perfusate (11). Importantly, these studies suggested that the extracellular redox regulation occurred via a Cys/CySS shuttle mechanism in which CySS was transported into cells, reduced to Cys, and transported back into the extracellular fluid (20, 29).

In the intestine, numerous sodium-dependent and sodium-independent systems facilitate the transport of CySS and Cys, including b0,+ t X_Ac, ASC (apical surface), and L, y^1, and Asc (basal surface) (Table 1) (2, 13, 14), but studies to identify specific transporters involved in redox regulation and studies to examine contributions of other redox control mechanism are not available.

In the present study, Caco-2 cells were used as a model of human intestinal epithelial cells to study the polarity and mechanisms of extracellular redox regulation. Caco-2 cells are suitable for these studies because they differentiate to form an ileal-like polarized monolayer with brush border microvilli, tight junctions, intestinal transporters, and specific small intestinal enzymes and gene products (32). When grown on a permeable support, the polarized nature of these Caco-2 cell monolayers allows them to be used to study physiological functions on the apical and basal domains. Because no previous study has characterized basal, as opposed to apical, thiol-disulfide redox regulation in intestinal cells, the purpose of the present study was to determine whether the basal domain of Caco-2 cells can regulate the extracellular environment following exposure to CySS, and, if so, to determine whether the regulation occurs through function of known transport systems or involves cell membrane-associated thiol/disulfide exchange, or copper- or flavin-dependent oxidoreductases. The hypotheses were that the basal domain is capable of regulating the extracellular redox environment and that Cys/CySS transport mechanisms function in the reduction of the extracellular Eh CySS in Caco-2 cells.

MATERIALS AND METHODS

Chemicals. 4-Acetamido-4′-maleimidylstilbene-2,2′-disulfonic acid, di-sodium salt (AMS) was purchased from Molecular Probes (Eugene,
and basal surface of intestinal epithelial cells

Table 1. CySS and Cys transport mechanisms on the apical and basal surface of intestinal epithelial cells

<table>
<thead>
<tr>
<th>Transport System</th>
<th>Substrate</th>
<th>Sodium Dependent</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Apical surface</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>b0, +</td>
<td>Neutral and cationic L-amino acids; CySS</td>
<td>No</td>
</tr>
<tr>
<td>X_AG</td>
<td>Anionic amino acids; CySS</td>
<td>Yes</td>
</tr>
<tr>
<td>ASC</td>
<td>Cys and other neutral amino acids</td>
<td>Yes</td>
</tr>
<tr>
<td><strong>Basal surface</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>y' L</td>
<td>Neutral L-amino acids; cationic amino acids; CySS</td>
<td>Yes</td>
</tr>
<tr>
<td>L</td>
<td>Cys and other neutral amino acids</td>
<td>No</td>
</tr>
<tr>
<td>Asc</td>
<td>Small neutral L- and D-amino acids; Cys</td>
<td>No</td>
</tr>
</tbody>
</table>

CySS, cystine; Cys, cysteine. [Based on Ganapanath et al. (14).]

OR. All other chemicals and reagents were purchased from Sigma (St. Louis, MO).

Cell culture. The human colon carcinoma cell line Caco-2 was used. Cultures were grown to confluency at 37°C in 5% CO2 using Dulbecco’s modified Eagle’s high-glucose medium (DMEM) containing 4.5 g/l of L-glutamine. DMEM was supplemented with nonessential amino acids, penicillin (10 U/ml), streptomycin (10 μg/ml), l-glutamine, and 10% fetal bovine serum. Once flasks reached 80% confluence, cells were split and seeded onto coated, permeable tissue culture inserts in six-well culture plates, enabling treatment/sampling from both apical and basal domains of the monolayer. Experiments were performed after 21–24 days, with the criterion for use being that the transepithelial electrical resistance (TER) was between 200 and 400 Ohm/cm² (see below).

Integrity of the permeability barrier. Epithelial barrier permeability is a dynamic process that is sensitive to oxidative stress and can affect measured rates and steady-state characteristics of redox regulation on the opposite poles of the epithelial cell layer. Because the purpose of the present research was to test for redox regulation on the basal surface and not to study effects on barrier permeability, we used TER between 200 and 400 Ohm/cm² as a criterion for acceptability of monolayers for use in experiments. TER was measured using an EVOMX electrovolmeter with an STX2 electrode (World Precision Instruments, Sarasota, FL); as cells reached confluency, TER increased and provided an index of tight junction formation. As an independent test of barrier integrity following change in culture conditions, a limited number of experiments were also performed with 10 mg/ml fluorescein isothiocyanate (FITC)-dextran (average molecular weight, 4,000), with 1 mM H2O2 as a positive control. Following removal of serum-free medium and rinsing of cells, FITC-dextran was added to the apical side, and medium was sampled from the basal side for periods up to 24 h. FITC-dextran concentration was quantified by fluorometry (λexitation = 492 nm, λemission = 525 nm).

Cell treatment protocol. Caco-2 cells were serum and glutamine starved in MEM for 24 h, washed once with 1 ml PBS, and immediately exposed to the oxidizing condition (0 mV) on the apical and basal domains. Extracellular Eo of 0 mV was obtained by addition of 99.75 μM CySS and 0.5 μM Cys to CySS-free DMEM, as previously described (20, 33). These concentrations were derived from calculations using the Nernst equation; measured initial Eo values shown in results deviate from this calculated value due to oxidation during filter sterilization after addition and carryover of relatively reduced medium during medium change. Medium from apical and basal domains was collected, and cells were harvested for Eo determination, at time points as indicated in RESULTS.

Redox determination by HPLC. Aliquots of cell culture medium (200 μl) from apical and basal domains were added to ice-cold 10% perchloric acid, containing 0.2 M boric acid and 10 M γ-glutamylglutamate (γ-Glu-Glu) as the internal standard. These samples were centrifuged to remove precipitates and used for extracellular Eo determinations. Cells were rinsed twice with 1 ml PBS, and ice-cold 5% perchloric acid, containing 0.2 M boric acid and 10 mM γ-Glu-Glu as the internal standard, was added directly to each well. Cells were scrapped, placed in microcentrifuge tubes, and centrifuged to separate precipitated protein. Pellets were resuspended in 1 M NaOH, and protein concentrations were measured using the Bradford method with rabbit γ-globulin as the protein standard (BioRad Laboratories, Hercules, CA) (4). All samples were stored at −20°C until derivatization with iodoacetic acid and dansyl chloride (25). HPLC with fluorescence detection was used to quantify dansyl derivatives of Cys, CySS, glutathione (GSH), and GSSG. These concentrations were used with the Nernst equation to calculate Eo of each redox couple, as described (7, 23, 28).

Sodium dependence and inhibitor studies. To test for sodium dependence, sodium chloride in the cell culture medium on both the basal and apical sides was replaced with isoosmotic choline chloride. To test for possible involvement of specific transport systems in redox regulation, cells were exposed to oxidizing extracellular Cys/CySS in treatments and presence of 2.5 mM lysine (Lys), 300 μM quinacrine acid (QA), or 0.5 mM threo-β-hydroxy-aspartate (THA), inhibitors of the y' L, X_ AG, and X_ T systems, respectively. To test for possible involvement of thiol oxidation in intestinal basal membranes, the copper-specific chelator bathocuproine disulfonate was used at 0.5 μM (8). To test for possible involvement of flavin-dependent reductases, diphenyleneiodonium chloride (DPI) was used at 0.5 μM, a concentration known to provide general inhibition of flavoproteins (15). To test for possible dependence on GSH, cellular GSH was depleted by pretreatment with 1 mM buthionine sulfoxide (BSO), a concentration that causes extensive loss of GSH over 24 h (1). To test for possible involvement of thiol/disulfide exchange involving cell surface thiols, the cell-impermeant alkylating agent, AMS, was added at 0.5 mM, a concentration known to inhibit redox-dependent cell signaling (15, 33). In these experiments, cells were treated for 1 h, except for BSO experiments, in which cells were treated with BSO in serum-free medium for 32 h.

Statistical analysis. Differences were compared across groups and time using one-way analysis of variance. Specific differences between treatments or time and their interactions were compared post hoc using the Fishers protected least-significant difference test. Results are expressed as means ± SE, and differences were considered significant when P < 0.05.

RESULTS

Caco-2 cells regulate extracellular Cys/CySS Eo more efficiently on the basal domain. Caco-2 cells under standard tissue culture conditions are oriented with the apical surface exposed and regulate Cys/CySS Eo in the culture medium (20). Experiments were performed with cells grown on permeable support membranes to determine whether the basal and apical domains individually have the capacity to regulate extracellular Eo. In cells with Cys/CySS added to give 0 mV, basal extracellular Cys/CySS Eo was altered from 7.0 ± 5.5 mV at 0 h to −116 ± 3 mV after 24 h in culture (Fig. 1A). The apical domain also regulated Eo, but the change was less, from 5.0 ± 3.9 mV at 0 h to −34 ± 9 mV after 24 h (Fig. 1A). Basal CySS concentration decreased from 91 ± 1 μM at 0 h to 7 ± 1 μM after 24 h (Fig. 1B). The CySS concentration on the apical domain also decreased over time; however, the change was less, from 94 ± 2 μM at 0 h to 40 ± 3 μM at 24 h (Fig. 1B). Cys concentration on the basal domain significantly increased over time from 0.5 ± 0.3 μM at 0 h to 15.9 ± 0.3 μM at 24 h (Fig. 1C). Apical Cys concentration also increased over time, but to a lesser extent, from 0.6 ± 0.3 μM at 0 h to 1.6 ± 0.3 μM at 24 h (Fig. 1C). The difference between apical and basal CySS and Cys concentrations, as well as the
maintenance of TER during these experiments, indicated that the epithelial barrier was not disrupted during these experiments. Control experiments with FITC-dextran also showed that barrier function of Caco-2 cells was not lost during 24 h following exposure to 0-mV conditions (data not shown). Consequently, the results show that Caco-2 cells regulate extracellular \( E_h \) on both the apical and basal domains, and that the basal domain has a greater capacity for extracellular \( E_h \) regulation.

**Redox regulation by Caco-2 cells occurs at physiological plasma CySS concentrations.** Cell culture media often contains 200–400 \( \mu \)M CySS, while plasma concentrations are usually in the range of \(~50–100\) \( \mu \)M in humans (27). To determine whether redox regulation on the basal surface is dependent on extracellular CySS concentration in this range, studies were performed to compare redox regulation with total Cys equivalents at 100 \( \mu \)M, instead of 200 \( \mu \)M (i.e., CySS concentrations of \(~50 and 100 \( \mu \)M, respectively), and initial \( E_h \) at 0 mV. Results showed that, at the lower CySS concentration, which is characteristic of that for young, healthy adults, the extent of correction of \( E_h \) by 4 h was equivalent to that at the higher concentration, which is characteristic of older individuals (Fig. 2). After 24 h, the cells at 100 \( \mu \)M initial conditions showed a greater change, from 6 ± 3 mV at 0 h to −114 ± 1 mV compared with 50 \( \mu \)M, from −2 ± 1 mV at 0 h to −85 ± 3 mV (Fig. 2). Moreover, the data showed that cells at 100 \( \mu \)M initial conditions showed a greater decrease in extracellular CySS concentration and a greater increase in extracellular Cys concentration at 4 and 24 h (Supplemental Fig. S1, A and B; Supplemental material for this article is available online at the Journal website). Thus the results show that Caco-2 cells can regulate \( E_h \) on the basal surface over a concentration range typical of values found in human plasma and that the effect is greater at 24 h with 100 \( \mu \)M than with 50 \( \mu \)M CySS.

**Regulation of extracellular redox environment is \( Na^+ \) dependent.** Previous results suggest that regulation of extracellular \( E_h \) occurs by a Cys/CySS shuttle mechanism dependent on Cys and CySS transport (10). By this mechanism, CySS, which is found in greater concentrations in the extracellular space, is transported into the intracellular space and undergoes reduction to Cys, which is then released (10). To test whether extracellular \( E_h \) regulation is \( Na^+ \) dependent, experiments were performed with \( Na^+ \)-free medium in which NaCl was replaced with choline chloride. Results showed that cells treated with sodium-free medium at 4 h demonstrated a decreased capacity to regulate the extracellular environment (−35.0 ± 3.3 at 4 h) compared with sodium-containing medium (−63.5 ± 0.2 mV) (Fig. 3A). While extracellular CySS concentrations were significantly higher in cells treated with sodium-free medium (Fig. 3B), exposure of the basal domain to sodium-free medium significantly lowered extracellular Cys concentrations (Fig. 3C). Together, these data suggest that either a system(s) participating in CySS influx, and/or Cys efflux requires sodium for function.

To determine whether CySS transport was specifically affected by the absence of sodium, the concentration of CySS in the intracellular space was measured. Results showed that the intracellular CySS concentration was significantly lower in cells treated in a sodium-free culture medium (Fig. 4). These data show that \( Na^+ \)-dependent CySS uptake contributes to the mechanism for extracellular redox regulation.

**Lys decreases the concentration of extracellular Cys.** To test for involvement of transport systems, experiments were performed with selective inhibitors. Lys, a cationic amino acid, was tested as a competitive inhibitor of the \( \gamma \)L system for transport of CySS on the basal domain. In cells exposed to 2.5 mM Lys, extracellular Cys concentrations were lower com-

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**Fig. 1.** Extracellular redox regulation occurs on both apical and basal domains of Caco-2 cells. Caco-2 cells were grown on permeable membranes for 21–24 days to achieve a transcellular resistance >200 \( \Omega \).cm\(^2\) and treated with 0 mV extracellular cysteine (Cys)/cystine (CySS) redox potential (\( E_h \)) obtained by medium Cys and CySS concentrations, with a total of 200 \( \mu \)M in Cys equivalents. Media from the apical and basal domains were collected at 0, 4, and 24 h. A: Cys and CySS concentrations were determined by HPLC analysis and used with the Nernst equation to calculate respective \( E_h \) values shown. B and C: corresponding extracellular CySS and Cys concentrations, respectively. Data are expressed as means ± SE, with \( n = 4. * P < 0.05 \) vs. apical vs. basal domain; †\( P < 0.05 \) vs. time 0 for apical and basal domains.

**Fig. 2.** Effect of CySS concentration on basal redox regulation. Concentrations of Cys and CySS needed to provide Cys/CySS \( E_h \) conditions (0 mV, pH 7.4) with total equivalents of 100 and 200 \( \mu \)M Cys were calculated using the Nernst equation. Under these conditions, initial CySS concentration was \(~50 and 100 \( \mu \)M, respectively). After 0, 4, and 24 h, media was harvested at respective time points, and \( E_h \) was calculated using the measured Cys and CySS concentrations and the Nernst equation. Data are expressed as means ± SE, with \( n = 4. * P < 0.05 \) for 100 \( \mu \)M CySS vs. 50 \( \mu \)M CySS. Measured concentrations for Cys and CySS are available as online supplement in Supplemental Fig. S1, A and B.
pared with controls at 60 and 240 min (Fig. 5A). Results also showed that, at 240 min, cells exposed to Lys demonstrated less of a change in extracellular Eh (87 ± 0.8 mV at 4 h) compared with control (95 ± 0.6 mV) (Fig. 5B).

Because redox regulation was not completely inhibited following exposure of the basal domain to Lys, experiments were performed to determine whether other CySS transport systems contribute to the mechanism to normalize the extracellular redox environment on the basal domain. To test whether extracellular Eh regulation is affected by inhibition of sodium-independent transport mechanisms, experiments were performed with QA (300 μM), an inhibitor of system Xc\textsuperscript{+}. Results showed that cells treated with QA demonstrated a diminished capacity to regulate the extracellular environment (−37.7 ± 4.4 mV at 240 min) compared with control (−75.8 ± 0.4 mV) (Fig. 6). THA (0.5 mM), an inhibitor of the X\textsubscript{AG} system, was used as a negative control and showed that extracellular redox regulation was not affected by inhibition of the X\textsubscript{AG} system (data not shown). Although complete inhibition did not occur when cells were exposed to Lys or QA, the data indicate that the y\textsuperscript{+}L and x\textsubscript{c} transport systems function as components of the Cys/CySS shuttle mechanism, regulating extracellular Eh, contributing to the ability of Caco-2 cells to reduce the extracellular redox environment. GSH is a low-molecular-weight thiol that is present in millimolar concentrations in the cell and has been previously shown to support CySS utilization by isolated hepatocytes through a mechanism involving release and reaction with CySS to generate Cys (30). In contrast, Anderson et al. (1) showed that the depletion of GSH in HT29 cells had no effect on capacity to regulate extracellular Eh. To determine whether regulation of Eh in Caco-2 cells was dependent on

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cellular GSH, cells were exposed to 1 mM BSO. A substantial decrease in cellular GSH concentration and oxidation of the cellular GSH/GSSG Eh occurred (Fig. 7, A and B). However, there was no significant effect on the ability of the basal domain to correct extracellular redox environment (Fig. 7 C). Data are, therefore, consistent with previous evidence showing that GSH does not contribute to extracellular redox regulation in colonic epithelial cells.

Redox regulation is not dependent on extracellular surface protein thiols, flavoproteins, or cuproproteins. To test other possible mechanisms contributing to regulation of the extracellular redox environment, experiments were performed with inhibitors of oxidoreductase activities. AMS, a cell-impermeant thiol-reacting reagent, was used at a concentration (0.5 mM) known to inhibit redox-dependent cell signaling in Caco-2 cells (33). Results showed no significant effect on extracellular redox regulation (Fig. 8 A). Pretreatment with 5 μM DPI, an inhibitor of many flavoproteins, also resulted in no significant effect on redox regulation on the basal surface (Fig. 8 B). Similarly, treatment with 0.5 mM bathocuproine disulfonate, a nontoxic copper-specific chelator used to limit Cys oxidation in culture medium, had no effect on basal surface redox regulation (Supplemental Fig. S2). Thus the data provide evidence that thiol-, flavin-, and copper-dependent oxidoreductase mechanisms do not contribute significantly to the ability of the basal domain to regulate extracellular redox.

DISCUSSION

Previous research showed that the apical domain of Caco-2 cells regulates the extracellular redox environment toward physiological values observed in vivo (20). Because Caco-2 cells are polarized, the present data extend this study to show that the polarized intestinal epithelial cells independently regulate the extracellular redox environment on the basal surface, and that this occurs with a greater rate/capacity than on the apical surface. The data do not exclude the possibility that paracellular movement of Cys or CySS affects the absolute rates measured; however, measurements of TER and transepithelial FITC-dextran movement showed that barrier integrity did not substantially change during the experiments. Furthermore, the differences in concentrations of CySS and Cys on opposing sides under many conditions (e.g., Fig. 1, B and C) are inconsistent with the conclusion that regulation is an indirect consequence of paracellular movement. On the other hand, data for CySS loss and Cys appearance in Fig. 1 are consistent with a portion of CySS uptake from the apical side being released as Cys on the basal side. Such a transepithelial movement could explain an apparently lower rate of redox regulation on the apical side of cells grown on the semipermeable membrane compared with rates observed for cells grown on solid support (21), where the apical surface is exposed to the culture media.
The abundance of sodium in the cell culture medium increased this regulation is dependent on extracellular Na\(^{+}\) (Fig. 3A), compared with controls (Fig. 4). Furthermore, transport-inhibitor studies showed a partial effect of Lys on redox regulation (Fig. 5B). Partial inhibition of redox regulation by Lys suggested that other transporters may function in the Cys/CySS shuttle mechanism. Examination of sodium-independent transport systems showed that treatment with QA (Fig. 6), but not THA (data not shown), markedly decreased the ability of the basal domain to normalize the extracellular redox environment. Therefore, the results provide evidence that the y\(^{+}\)L and the \(x_{c}\) systems contribute to extracellular Cys/CySS redox regulation. The results do not support a contribution of \(x_{AG}\) to this regulation. Other inhibitor studies showed that this regulation is not dependent on cellular GSH (Fig. 7), surface thiols (Fig. 8A), membranal flavoproteins (Fig. 8B), or cuproproteins (Supplemental Fig. S2). While these results do not exclude other mechanisms, they support the interpretation that redox regulation on the basal surface depends on transport of redox-active molecules rather than transmembranal electron transfer mechanisms.

Available data suggest that there are at least six amino acid transport systems on the basal domain of intestinal epithelial cells, with three that participate in the transport of Cys or CySS (13, 14). The molecular identities of these have been extensively studied, and the expression of the mRNA for some, such as SLC7 (y\(^{+}\)L), has been confirmed in Caco-2 cells. However, we did not perform molecular characterization in the present study, so we have retained functional rather than molecular evidence that the y\(^{+}\)L and the \(x_{c}\) systems contribute to extracellular Cys/CySS redox regulation. The results do not support a contribution of \(x_{AG}\) to this regulation. Other inhibitor studies showed that this regulation is not dependent on cellular GSH (Fig. 7), surface thiols (Fig. 8A), membranal flavoproteins (Fig. 8B), or cuproproteins (Supplemental Fig. S2). While these results do not exclude other mechanisms, they support the interpretation that redox regulation on the basal surface depends on transport of redox-active molecules rather than transmembranal electron transfer mechanisms.

In a study of luminal reduction of GSSG, Dahm and Jones (10) proposed a Cys/CySS shuttle mechanism for luminal GSSG reduction based on the detection of luminal CySSG, in association with regulation of luminal GSH/GSSG. Jonas et al. (20) provided additional support for the function of a Cys/CySS shuttle in regulation of extracellular Cys/CySS in Caco-2 cells. They used radiolabeled CySS uptake in Caco-2 cells to provide evidence for this shuttle mechanism; however, specific intestinal systems that function in transport were not studied, and possible function of other mechanisms was not studied.

The present research confirms that redox regulation on the basal surface involves transport mechanisms by showing that this regulation is dependent on extracellular Na\(^{+}\) (Fig. 3A). The absence of sodium in the cell culture medium increased extracellular CySS concentrations (Fig. 3B) and decreased extracellular Cys concentrations (Fig. 3C) compared with control. While it is possible that proteolysis could alter intracellular Cys concentrations, previous studies have shown that cellular protein content in Caco-2 cells is stable after day 16 in culture (34). Therefore, changes in proteolysis are not likely to have a large effect on intracellular Cys concentrations under the conditions of the experiments. Taken together, the results show that a system participating in either CySS influx and/or Cys efflux requires sodium for function.

We determined that CySS transport was selectively affected by measuring the concentration of cellular CySS following treatment with sodium-free medium. Figure 4 showed that cellular CySS content was dramatically decreased in cells treated with sodium-free medium compared with controls (Fig. 4). Furthermore, transport-inhibitor studies showed a partial effect of Lys on redox regulation (Fig. 5B). Partial inhibition of redox regulation by Lys suggested that other transporters may function in the Cys/CySS shuttle mechanism. Examination of sodium-independent transport systems showed that treatment with QA (Fig. 6), but not THA (data not shown), markedly decreased the ability of the basal domain to normalize the extracellular redox environment. Therefore, the results provide evidence that the y\(^{+}\)L and the \(x_{c}\) systems contribute to extracellular Cys/CySS redox regulation. The results do not support a contribution of \(x_{AG}\) to this regulation. Other inhibitor studies showed that this regulation is not dependent on cellular GSH (Fig. 7), surface thiols (Fig. 8A), membranal flavoproteins (Fig. 8B), or cuproproteins (Supplemental Fig. S2). While these results do not exclude other mechanisms, they support the interpretation that redox regulation on the basal surface depends on transport of redox-active molecules rather than transmembranal electron transfer mechanisms.

Available data suggest that there are at least six amino acid transport systems on the basal domain of intestinal epithelial cells, with three that participate in the transport of Cys or CySS (13, 14). The molecular identities of these have been extensively studied, and the expression of the mRNA for some, such as SLC7 (y\(^{+}\)L), has been confirmed in Caco-2 cells. However, we did not perform molecular characterization in the present study, so we have retained functional rather than molecular

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**Fig. 8. Effect of thiol inhibitor 4-acetamido-4′-maleimidylstilbene-2,2′-disulfonic acid, disodium salt (AMS) or flavoprotein inhibitor diphenylphosphorone (DPI) pretreatment on extracellular redox regulation in Caco-2 cells. A: to test whether regulation of extracellular Cys/CySS \(E_h\) was dependent on cell surface thiols, cells were pretreated for 1 h with 0.5 mM AMS, a concentration known to inhibit redox-sensitive cell signaling by EGF receptor in Caco-2 cells. Medium was removed, cells were washed, and cells were exposed to 0-mV extracellular \(E_h\). The extracellular Cys/CySS \(E_h\) at indicated times was determined using the measured Cys and CySS concentrations and the Nernst equation. Results show no significant effect of AMS on redox regulation. B: to test for possible function of plasma membrane flavin-dependent oxidoreductases, cells were pretreated for 1 h with 5 \(\mu\)M DPI, a general flavoprotein inhibitor, and analyzed as in A. Data are expressed as means ± SE, with \(n = 4\). No significant effects were observed in comparisons of control vs. DPI treatment. Additional data showing no significant effect of the copper chelator bathocuproine disulfonate on extracellular redox regulation are provided as online supplement in Supplemental Fig. S2.**

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**Fig. 9. Transport systems functioning in Cys/CySS shuttle mechanism for regulation of extracellular \(E_h\) on the basal surface of Caco-2 cells. Top: results from studies with sodium-free medium show that redox regulation is ~50% sodium dependent; inhibitor studies with lysisine indicate that the sodium-dependent activity involves CySS uptake by y\(^{+}\)L. Inhibitor studies with QA indicate that the sodium-independent activity involves CySS uptake by \(x_{c}\). The mechanism for reduction of CySS to Cys is not known. Bottom: basolateral systems for Cys efflux include the L and Asc systems.**
descriptions. The Asc and L systems transport Cys and other neutral amino acids, such as glycine, alanine, serine, and threonine. The transport function of these systems is sodium independent (13, 14). In the Cys/CySS shuttle mechanism, the function of these transporters could be indirectly sodium dependent, if the uptake of CySS is sodium dependent (see Fig. 9). System y\textsuperscript{+}L is an amino acid transporter that mediates the sodium-dependent transport of neutral amino acids, including CySS, as well as sodium-independent transport of cationic amino acids (5, 14).

Previous studies show that transport of CySS by system y\textsuperscript{+}L occurs through the binding of sodium as a co-substrate, converting CySS to a suitable charge for transport (2). Cationic amino acids inhibit the uptake of the neutral amino acids by y\textsuperscript{+}L. Thus the characteristics of Na\textsuperscript{+} dependence, inhibition by Lys, and appearance of Cys in the culture medium are consistent with a redox regulatory mechanism in which Na\textsuperscript{+}-dependent uptake of CySS occurs by y\textsuperscript{+}L. CySS reduction occurs intracellularly, and Cys is released by the Asc or L systems. These findings are consistent with the earlier findings of Fernandez et al. (12) in a renal proximal tubule cell line, where CySS uptake on the apical surface occurred by y\textsuperscript{+}L resulted in increased cellular Cys and efflux of Cys by system L. The substantial dependence of redox regulation on Na\textsuperscript{+} (Fig. 3), but only minor inhibition by Lys (Fig. 5), in the present study may indicate that concentrations of Lys >2.5 mM are needed for effective inhibition of y\textsuperscript{+}L.

The redox regulation was only ~50% Na\textsuperscript{+} dependent (Fig. 3); the Na\textsuperscript{+}-independent component of the Cys/CySS shuttle could involve x\textsubscript{c}. System x\textsubscript{c} is an amino acid antiporter that mediates the sodium-independent transport of CySS via 1:1 exchange with glutamate (6). Burdo et al. (6) provided evidence that the x\textsubscript{c} transport system is present at the brush border in the intestine, but did not examine basal localization. Because cells treated with QA demonstrated a decreased capacity to regulate the extracellular environment at 20, 60, and 240 min (Fig. 6), the data suggest that system x\textsubscript{c} is also present at the basal domain and participates in the Cys/CySS shuttle mechanism (Fig. 9).

The physiological relevance of extracellular redox regulation is rapidly being clarified by studies using a redox-clamp model, in which extracellular E\textsubscript{h} is systematically varied, and cell signaling and related cell functions are measured (16), and by human studies associating E\textsubscript{h} with health outcomes (26). The redox clamp studies show that cell surface and extracellular proteins, including growth factor receptors, integrins, and metalloproteinases, contain thiol that is sensitive to oxidation and alter cell proliferation, sensitivity to apoptosis, and proinflammatory and profibrotic signaling. Human studies have shown associations of oxidation with cardiovascular disease risk, age-related macular degeneration, and persistent atrial fibrillation. The present studies provide the most convincing data available to date that the regulation of extracellular E\textsubscript{h}, which are known to differ in different body fluids, are controlled by transport mechanisms with different characteristics on opposite poles of epithelial cells.

In the intestines, this polarity could allow extracellular E\textsubscript{h} appropriate for absorption on the apical surface, independently of cell signaling on the basal surface. Our data show that the basal domain regulates extracellular E\textsubscript{h} to a greater extent than the apical domain (Fig. 1), which could indicate a greater redox sensitivity of receptors or transporters on the basal surface. Alternatively, the overcorrection to $-116 \pm 3.4$ mV by 24 h, which is beyond the physiological range observed in human plasma ($-80 \pm 9$ mV), may reflect the function of basal surface transport in amino acid absorption. Other responses to variations in extracellular E\textsubscript{h}, such as stimulation of cell proliferation (18, 21) and enhanced sensitivity to apoptosis (19), have been observed and suggest that different responses in the intestines could also be important during injury or growth signaling.

The in vitro cell culture model used for the current experiments has been widely used in many studies concerning intestinal absorption and transport. Growth of Caco-2 cells onto tissue culture plate inserts allows separate access to the apical and basolateral domain. While artificial in nature, this system more effectively mimics the physiology of the gut in vivo than does culture of cells on a nonpermeable surface. A potential limitation of the study is that inhibitors, such as Lys and QA, are not absolutely specific to single transport mechanisms and do not exclude the function of other systems. Thus more specific interpretations will require availability of more specific inhibitors and/or use of knockout mice with individual transport deficiencies.

In summary, the basal domain of Caco-2 cells regulates the extracellular redox environment toward the physiological range observed in vivo and does this more rapidly than the apical domain. This capability was sodium dependent and inhibited by Lys and QA, indicating that the y\textsuperscript{+}L and x\textsubscript{c} systems function in the Cys/CySS shuttle mechanism regulating extracellular E\textsubscript{h}. Inhibitor studies provided no evidence for function of other transport systems or oxidoreductase mechanisms. Together, the data show that the y\textsuperscript{+}L and x\textsubscript{c} systems provide important components of the Cys/CySS shuttle mechanism for maintenance of the extracellular redox environment on the basal surface of this epithelial cell line.

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**DISCLOSURES**

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

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