Extracellular cysteine/cystine redox regulates the p44/p42 MAPK pathway by metalloprotease-dependent epidermal growth factor receptor signaling

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Submitted 30 June 2004; accepted in final form 21 February 2005

ABUNDANT EVIDENCE DEMONSTRATES that reversible redox reactions of thiol/disulfide couples have important roles in the regulation of fundamental cellular processes such as cell proliferation, differentiation, and apoptosis (19, 27, 32). Most of the available research on thiol/disulfide couples has focused on intracellular glutathione (GSH), the most abundant low molecular weight thiol in cells. However, recent evidence suggests that the extracellular thiol/disulfide redox environment may also be important. The predominant low molecular weight thiol/disulfide pool found in plasma consists of the cysteine (Cys)/cystine (CySS) couple (16). The redox state (Eh) of plasma Cys/CySS differs considerably between individuals, from about −130 to −20 mV (16), and is oxidized in association with aging (17) and smoking (26). Oral intake of Cys results in a more reduced redox state (36).

In vitro studies showed that bromodeoxyuridine (BrdU) incorporation in colon carcinoma (Caco-2) cells cultured at −150 mV was twice the rate at 0 mV and occurred without an associated change in intracellular GSH (15). In Caco-2 cells that were allowed to undergo spontaneous differentiation, extracellular redox was more oxidized in differentiated, nondifferentiated cells than in proliferative cells (27). Other studies, focused on chemicals that determine Cys/CySS redox, i.e., Cys or CySS, have clearly demonstrated the involvement of Cys and/or CySS in cellular proliferation. For example, exogenous Cys was shown to promote proliferation in Caco-2 cells (29) and in intestinal lamina propria T-lymphocytes (35) without increasing intracellular GSH (29). However, the mechanism by which Cys or reduced Cys/CySS redox conditions increase proliferation is not clear.

The intestine produces peptide growth factors that are important mediators of intestinal epithelial growth (8). Several lines of evidence support the concept of thiol-mediated regulation of early mitogenic signaling events associated with the plasma membrane. For instance, increased kinase activity of the insulin and epidermal growth factor (EGF) receptors (EGFRs) was observed in the presence of sulfhydryl agents (6). Also, reduction of disulfides influences receptor autophosphorylation and, consequently, phosphorylation of downstream substrates (10). Similarly, GSH is required for growth stimulation by pancreatic spasmolytic peptide in Colo-357 cells (30). EGF and other EGF-like peptides stimulate proliferation in intestinal cells by binding to the EGFR (39). Ligand binding results in receptor dimerization and activation of an intracellular tyrosine kinase domain. This results in both autophosphorylation and phosphorylation of various intracellular effector proteins, such as proteins of the mitogen-activated protein kinase (MAPK) pathway (25). The EGFR has two cysteine-rich extracellular domains, making it a candidate for sensitivity to extracellular thiol/disulfide redox.

Understanding thiol-dependent control is complicated by the presence of reactive oxygen species (ROS) signaling. Many extracellular stimuli, including EGF, induce the production of ROS in cells, which act as second messengers (22, 3). Activation of downstream p44/p42 MAPK by ROS (5, 11, 18) has been shown to be mediated by Src (11, 41), inhibition of
protein tyrosine kinases (21), and glutathiolation of Ras (1). However, ultraviolet light (UVA) and EGFR, through overproduction of ROS, inhibited EGFR activation as well as p44/p42 MAPK; this was rescued by the antioxidant vitamin E (24). These studies define intracellular effects of ROS but have not clarified the role of extracellular thiols and disulfides.

Ligands of the EGFR [EGF, transforming growth factor (TGF)-α, heparin-bound EGF, amphiregulin, betacellulin, and epiregulin] are synthesized as transmembrane precursors that must be cleaved by metalloproteinases to release mature ligands (23). The two closely related metalloproteinase families, matrix metalloproteinases and metallocarboxypeptidase-disintegrins, both contain cysteine residues (38, 40) and are also redox sensitive (34, 37). Thus, in addition to the possibility that thiol/disulfide redox could directly alter the EGFR, extracellular redox could affect the activity of metalloproteinases and thereby alter release of surface-bound EGFR ligands.

The aim of the present study was to determine whether changes in the extracellular Cys/CySS redox could affect signaling through the p44/p42 MAPK pathway signaled by EGFR. For this purpose, we utilized Caco-2 cells, a human colon carcinoma cell line in which cell proliferation rate changes in response to altered Cys/CySS redox (15). Redox conditions of 0, −80, and −150 mV were used to treat the cells, and the phosphorylation of EGFR and p44/p42 MAPK was determined. Our results show a redox-dependent phosphorylation of the EGFR, with increased phosphorylation at more reducing conditions. This was followed by a significant increase in p44/p42 MAPK phosphorylation. Effects of inhibition of metalloproteinases and TGF-α release were also studied. The results show that the activation of this mitogenic pathway by extracellular redox involves metalloproteinases but does not exclude additional direct effects of extracellular thiol/disulfide redox state on the EGFR.

MATERIALS AND METHODS

Chemicals. Mouse anti-human phospho-EGFR antibody, phospho-p44/p42 MAPK antibody, rabbit anti-human EGFR antibody, and p44/p42 antibody were purchased from Cell Signaling Technologies (Beverly, MA). Horseradish peroxidase-labeled goat anti-mouse and goat anti-rabbit IgG were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The matrix metalloproteinase inhibitor GM6001, the p44/p42 MAPK kinase inhibitor U0126, the TGF-α ELISA kit, and the Diphtheria toxin CRM mutant (CRM197) were purchased from Calbiochem (San Diego, CA). The alkylating agents 4-acetamido-4’-maleimidylstilbene-2, 2’-disulfonic acid (AMS), and monobromotrimethyl-ammonio-bromide (qBBr) were purchased from Molecular Probes (Eugene, OR). Anti-TGF-α antibody was purchased from Oncogene (San Diego, CA). MEM and FBS were purchased from Life Technologies (Grand Island, NY). Chemiluminescence detection reagents were purchased from Amersham Pharmacia Biotech (Piscataway, NJ). Bio-Rad dye reagent was purchased from Bio-Rad laboratories (Richmond, CA). The cell proliferation ELISA kit was purchased from Roche Molecular Biochemicals (Indianapolis, IN). All other chemicals were purchased from Sigma (St. Louis, MO).

Cell culture. The human colon cancer cell line Caco-2 was obtained from the American Type Culture Collection (Rockville, MD). Cells were cultured between passages 21 and 25. For all experiments, cells were cultured in MEM, supplemented with 2 mM glutamine, 10 μg/ml streptomycin, 10 U/ml penicillin, and 1% fetal calf serum, at 37°C, 95% air-5% CO2. Cells were plated at low density (8 × 10^4 cells/well) in six-well plates and cultured until 90% confluent. Cells were then cultured without serum for 48 h and harvested (zero time) or treated with culture medium with Cys/CySS redox at −150, −80, and 0 mV for the indicated times. To generate the desired redox states, concentrations of Cys/CySS [0.5 μM/99.5 μM (0 mV), 14 μM/93 μM (−80 mV), 180 μM/10 μM (−150 mV)], calculated using the Nernst equation (7), were added to cyst(e)ine-free DMEM, containing 4 mM glutamine, 10 μM ascorbic acid, and 10 μg/ml streptomycin (12): Eh = −250 + 30 log [(CySS)/(Cys)] for pH 7.4 in culture medium, where respective concentrations are expressed in molarity. For EGFR phosphorylation studies, cells were treated for 2, 5, 7, and 10 min. For MAPK phosphorylation studies, cells were treated with redox-controlled media for 5, 10, 15, and 30 min before harvest. Cells were seeded in 96-well plates at a density of 10^5 cells/well for measurement of 5-bromo-2-deoxyuridine incorporation into cellular DNA. For all other experiments, the −150-mV condition and the 5-min time point were used.

For collection of samples, medium was aspirated, cells were washed twice with PBS, and cells were lysed with 200 μl of lysis buffer containing 50 mM HEPES, 150 mM NaCl, 0.1 mM PMSF, 1% Triton X-100, and 10% glycerol. These extracts were incubated for 20 min at 4°C and spun at 10,000 g for 10 min. The supernatant was collected, the concentration of protein was determined by the Bradford method (4), and the samples were analyzed for EGFR and p44/p42 MAPK phosphorylation by Western blotting.

p44/p42 MAPK phosphorylation. Cell lysates were resolved by electrophoresis with a 7.5% resolving gel. Proteins were transferred onto nylon membranes (Amersham Pharmacia Biotech), and the membranes were blocked with 5% nonfat milk for 1 h. The membranes were incubated with primary phospho-p44/p42 MAPK antibody at 4°C overnight, incubated with horseradish peroxidase-conjugated anti-mouse secondary antibody for 1 h, and developed according to the chemiluminescence system. Densitometry was performed for quantification. The levels of total MAPK were used to ensure equal protein loading. The same membranes were stripped using a stripping buffer containing 62.5 mM Tris·HCl (pH 6.8), 2% SDS, and 100 mM β-mercaptoethanol. The stripped membranes were washed three times with PBS, blocked with nonfat dry milk for 1 h, and incubated with rabbit anti-MAPK overnight at 4°C. Membranes were then incubated with goat anti-rabbit secondary antibody for 1 h and developed according to the chemiluminescence system.

EGFR phosphorylation. To determine whether the phosphorylation of the EGFR was responsible for the downstream phosphorylation of MAPK, cells were treated with the above Cys/CySS redox conditions for 2, 5, 7, and 10 min, which were time points earlier than the maximal phosphorylation of p44/p42 MAPK. Briefly, cellular protein was harvested as above and resolved by electrophoresis. Western blotting using an antibody specific for phospho-EGFR (Tyr1068) was used to determine the phosphorylation of the EGFR in response to the extracellular redox conditions. Densitometry was performed for quantification. Levels of total EGFR were used to control for protein loading. Membranes were stripped and reprobed with rabbit anti-EGFR at 4°C overnight, incubated with goat anti-rabbit secondary antibody for 1 h, and developed according to the chemiluminescence system.

Treatments with inhibitors/alkylating agents. Cells were pretreated for 30 min with either GM6001, anti-TGF-α, or CRM197, or for 1 h with either AMS, qBBr, U0126, or AG1478, washed, and then harvested (zero-time controls) or treated with the −150-mV condition for 5 min. Controls were treated with the −150-mV condition alone. The cellular protein was collected, and the phosphorylation of p44/p42 MAPK as well as EGFR were determined as described.

BrdU incorporation. Cells were plated in 96-well plates for 24 h and then treated for 24 with DMEM under the −150-mV condition, without or with AG1478, GM6001, or U0126. To measure DNA synthesis, cells were incubated in the presence of 100 μM BrdU for 2 h. After labeling, BrdU incorporation was measured by colorimetric

http://ajpgi.physiology.org/ by 10.20.33.1 on June 26, 2017
immunoassay using a commercially available cell proliferation ELISA kit.

Statistical analysis. Where appropriate, tests for statistically significant differences ($P < 0.05$) were performed by ANOVA. Student’s $t$-test was used to test for differences between treatment (plus inhibitor/alkylating agent) and control (−150 mV) conditions. Data are given as means ± SE; $P$ values of <0.05 were considered significant.

RESULTS

Effect of extracellular Cys/CySS redox on p44/p42 MAPK phosphorylation. Because our laboratory’s previous studies showed that cell proliferation at −150 mV was greater than at −80 or 0 mV (15), we used these conditions to determine whether phosphorylation of the p44/p42 MAPK pathway was redox dependent. The results show that p44/p42 MAPK was phosphorylated by a more reduced extracellular redox in a time-dependent manner (Fig. 1, A and B). At 10 min, there was a significant increase in phosphorylation of both p44 and p42 at −150 and −80 mV compared with 0 mV. To confirm the phosphorylation of p44/p42 MAPK by extracellular Cys/CySS redox, we investigated its inhibition by U0126, a specific inhibitor for MAPK kinase, which is upstream of p44/p42 MAPK in this signaling pathway (Fig. 1C). The results show that the phosphorylation of p44/p42 MAPK by Cys/CySS redox was inhibited by pretreatment with U0126.

Phosphorylation of EGFR in response to extracellular Cys/CySS redox. Changes in phosphorylation of the EGFR at Tyr1068 were measured as a function of Cys/CySS redox. Tyr 1068 is a direct binding site for Grb2/SH2 domain, which results in Ras activation and downstream activation of p44/p42 MAPK (31). The results (Fig. 2, A and B) showed that the most reduced Cys/CySS redox state (−150 mV) caused a significant increase in phosphorylation of the EGFR, which was maximal at 5 min. A similar pattern of phosphorylation with time was seen at −80 mV, and this was maximal at 5 min.

An inhibitor of EGFR phosphorylation, AG1478, was used to confirm that redox-dependent phosphorylation of EGFR was upstream of p44/p42 MAPK phosphorylation (Fig. 2C) under the same conditions. Cells were incubated at −150 mV without or with AG1478 and analyzed after 5 min. The results show that AG1478 inhibited EGFR phosphorylation at Tyr 1068 and that this inhibition of EGFR phosphorylation was associated with a dramatic inhibition of p44/p42 MAPK phosphorylation (Fig. 3). Thus the results establish that, under these redox conditions, EGFR phosphorylation was upstream of MAPK phosphorylation.

Involvement of extracellular thiols. To determine whether the phosphorylation of EGFR and the subsequent downstream phosphorylation of MAPK induced by reducing extracellular redox conditions was dependent on extracellular thiols, we used the nonpermeant alkylating agents qBBr and AMS. The results show that treatment with the negatively charged alkylating agent AMS significantly attenuated both EGFR phosphorylation (Fig. 4A) as well as its downstream phosphorylation of MAPK (Fig. 4B). In contrast, the positively charged qBBr had no significant effect. The AMS results suggest that the effect of extracellular Cys/CySS redox on the EGFR-MAPK pathway involves interaction of extracellular thiols. This conclusion is consistent with the previous finding that extracellular Cys/CySS affected cell proliferation without altering cellular GSH concentration (15).

Metalloproteinases are involved in Cys/CySS redox phosphorylation of EGFR and p44/p42 MAPK by Cys/CySS redox. Metalloproteinase action is required for ectodomain shedding of cellular EGFR ligands, which can activate the EGFR and p44/p42 MAPK signaling. To determine whether phosphorylation of EGFR by Cys/CySS redox involved metalloproteinases and subsequent release of membrane-bound EGFR ligands into cell medium, we used the general metalloproteinase inhibitor GM6001. Results (Fig. 5A) showed a significant inhibition of EGFR phosphorylation. General inhibition of metalloproteinases also significantly attenuated the phosphorylation of p44/p42 MAPK (Fig. 5B). Thus one mechanism for extra-
cellular Cys/CySS redox-dependent phosphorylation of EGFR and p44/p42 MAPK involves activation of one or more metalloproteinases.

TGF-α is involved in the phosphorylation of p44/p42 MAPK by Cys/CySS redox. The metalloproteinase inhibitor (GM6001) used also inhibits TGF-α release, and TGF-α is an EGFR ligand released by ectodomain shedding. Thus we evaluated the role of TGF-α release in redox signaling in Caco-2 cells by using a TGF-α antibody that blocks TGF-α activity. The results (Fig. 6A) show that blocking TGF-α with anti-TGF-α led to partial inhibition of the redox-dependent phosphorylation of p44/p42 MAPK. In contrast, CRM197, an inhibitor of the EGFR ligand heparin-bound EGF, had no effect on p44/p42 MAPK activation (Fig. 6B). Measurement of TGF-α in the culture medium by ELISA showed a significant increase in the amount of TGF-α released into culture medium at the more reduced Cys/CySS redox states (Fig. 6C). Thus Cys/CySS redox regulates metalloproteinase action to release EGFR ligand(s), and release of TGF-α is at least partially responsible for redox-signaled phosphorylation of the p44/p42 MAPK pathway.

BrdU incorporation into cellular DNA. Because EGFR and p44/p42 MAPK phosphorylation leads to their activation and signaling in cell proliferation, we investigated the effect of inhibiting EGFR and p44/p42 MAPK phosphorylation on BrdU incorporation induced by Cys/CySS redox. The results (Fig. 7) show significant inhibition of BrdU incorporation on treatment with the metalloproteinase inhibitor GM6001, the EGFR inhibitor AG1478, and the MAPK kinase inhibitor U0126. These data show that one of the mechanisms by which extracellular Cys/CySS redox controls cell proliferation is...
through the phosphorylation of the EGFR resulting in the downstream phosphorylation of p44/p42 MAPK in a metalloproteinase-dependent manner.

DISCUSSION

The present data add to the understanding of redox signaling in growth control, which now appears to include at least three different redox-dependent processes. One of these involves cellular GSH, which must be maintained at high concentrations with a reduced GSH/GSSG redox state for rapid proliferation (12–14, 28, 33, 42). The second involves ROS, which are generated during growth signaling by receptor tyrosine kinases, apparently mediated by mitogenic NADPH oxidases (9, 20). A third involves Cys but, unlike the other two processes, appears to be extracellular and mediated by the redox state of the Cys/CySS pool (15, 27). Cys is a precursor of GSH, and Cys or CySS can independently affect cell proliferation by altering the available Cys/H₂S/CySS pool. Consequently, experiments were performed with variation in Cys/CySS redox state under

Fig. 4. Effect of alkylating agents monobromotrimethyl-ammoniobimane bromide (qBBr) and 4-acetamido-4′-maleimidystilbene-2, 2′-disulfonic acid (AMS) on redox-dependent phosphorylation of the EGFR and p44/p42 MAPK. A: Caco-2 cells were harvested (zero time), treated with the −150-mV condition for 5 min, or preincubated for 1 h with 0.5 mM qBBr or AMS and then treated with the −150-mV condition (5 min). Cellular protein was resolved by SDS-PAGE, and phosphorylation of EGFR was detected by Western blotting. Blots at top show phosphorylation of EGFR and are representative of 4 different experiments. Middle panels show corresponding Western blots for EGFR, and the bottom panel provides densitometric analysis of phospho-EGFR. B: cells were treated as in A, and phosphorylation of p44/p42 MAPK was detected as previously described. Blots shown are a representative of 4 different experiments as in A. Densitometric analyses of the Western blots shown at top are shown at bottom. *Significant difference of AMS vs. control (−150 mV without AMS; P < 0.05).

Fig. 5. Metalloproteinase inhibition attenuates redox-dependent phosphorylation of the EGFR and p44/p42 MAPK. A: Caco-2 cells were either harvested (zero time), treated with the −150-mV condition for 5 min, or preincubated for 30 min with 5 μM GM6001 and then treated with the −150-mV condition for 5 min as outlined in MATERIALS AND METHODS. Phosphorylation of EGFR was detected by Western blots (top), representative of 3 different experiments, with total EGFR shown at middle. Bottom: densitometric analyses of Western blots shown at top. *Significant difference for GM6001 vs. −150 mV without GM6001 (P < 0.01). B: cells were treated as in A, and phosphorylation of p44/p42 MAPK was detected as outlined. Blots shown at top are representative of 3 different experiments. Densitometric analysis of the Western blots at top are at the bottom. *Significant difference for GM6001 vs. −150 mV without GM6001 (P < 0.01).
conditions where the total Cys + CySS pool size was maintained constant. Under these conditions, varying extracellular Cys/CySS redox did not alter the concentration of cellular GSH or redox state of cellular GSH/GSSG (15). Thus the results indicated that the growth stimulation due to extracellular Cys/CySS was not due to effects on cellular GSH. The present data support this interpretation by providing evidence that the response is blocked by a nonpermanent alkylating agent. Various growth factor receptors are sensitive to thiol content (5, 9) and redox (11). We therefore hypothesized that Cys/CySS redox could affect the mitogenic signaling pathway via an extracellular effect on a receptor tyrosine kinase. The variation of extracellular Cys/CySS redox did not alter the intracellular milieu maintained by the GSH/GSSG redox state (15). To determine whether signaling occurred on the basal or apical surface, we used transwell plates to grow Caco-2 cells, treated them with the different redox conditions, and looked at EGFR and p44/p42 MAPK phosphorylation. In cells with a resistance of ≥200 Ω/cm², indicating the presence of tight junctions, there was very little EGFR or p44/p42 phosphorylation and there was no significant difference in extent of phosphorylation between −150 and 0 mV. Subconfluent and confluent cells differ in the integrity of the epithelial barrier and the rate of cell proliferation. Thus the data could indicate that the extracellular Cys/CySS redox state is important only under conditions where the barrier function is compromised. Alternatively, p44/p42 MAPK activity dramatically decreases in postconfluent cells (2), and this decrease in activity could limit the ability to detect changes. Thus the results observed in the present study may not apply to normal tissue but rather be of importance only under conditions of epithelial injury or regeneration.

The present data show that changes in extracellular Cys/CySS redox affect phosphorylation of p44/p42 MAPK by an upstream effect on EGFR phosphorylation. In principle, this could occur by a direct effect involving Cys residues on the extracellular surface of EGFR or by an indirect effect on other systems that activate EGFR. The extracellular domain of the EGFR contains 51 Cys residues (39), making it a good candidate for thiol redox sensitivity. Moreover, EGFR does...
not require ligand binding for tyrosine kinase activity and dimerization (20), and numerous studies have demonstrated the transactivation of EGFR by nonligands. However, because of the complexity of the system, we were unable to evaluate this direct effect. In contrast, the use of a general metalloproteinase inhibitor demonstrated the involvement of metalloproteinase. Metalloproteinases contain critical cysteine residues and have been shown to be redox sensitive (35, 38). Thus the results provide evidence for an indirect mechanism for redox sensitivity but do not exclude direct effects.

A proposed scheme representing the sensitivity of the EGFR signaling pathway to extracellular Cys/CySS redox is given in Fig. 8. The nearly complete inhibition by GM6001 indicates that a metalloproteinase could represent the major redox-sensitive event. However, because GM6001 inhibits multiple metalloproteinases, this experiment does not define the specific redox sensor, and additional research will be needed to identify the redox-sensitive protease. The EGFR inhibitor AG1478, as well as the MAPK kinase inhibitor U0126, completely blocked p44/p42 MAPK phosphorylation. These inhibitors, as well as GM6001, also dramatically inhibited cell proliferation measured by BrdU incorporation into cellular DNA. Extracellular redox control of cell proliferation by this pathway could have important influences on tissue homeostasis. The Cys/CySS redox varies from −20 to −130 mV in humans, with most reduced values found in young, healthy individuals (17). Cys/CySS redox is oxidized in smokers (26) and is reduced after an oral load of Cys (36). Cys/CySS redox may be sufficient to contribute to variation in cell proliferation and thereby affect critical processes of tissue repair and maintenance under different pathophysiological conditions.

In summary, these studies in human gut epithelial cells are the first to demonstrate that alteration of the extracellular Cys/CySS redox is sufficient to alter phosphorylation of p44/p42 MAPK, with the greatest phosphorylation observed at the most reducing conditions (−150 mV). The data show that EGFR is phosphorylated under these conditions and that
such phosphorylation signals MAPK phosphorylation. The data also demonstrate that this redox sensitivity involves extracellular thiols and metalloproteinase activation, with at least partial signaling through TGF-α. These studies therefore indicate that extracellular Cys/CySS redox can signal cellular proliferation through the mitogenic MAPK pathway. Due to the existence of multiple metalloproteinases and signaling ligands, further studies are needed to determine which metalloproteinases and EGFR ligands are involved in EGFR phosphorylation as well as which Cys residues of the metalloproteinases or EGFR are responsive to Cys/CySS redox.

GRANTS

This work was supported by National Institutes of Health grants DK-55850 (T. R. Ziegler) and ES-011195 (D. P. Jones).

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