The ubiquitin-proteasome system is responsible for cysteine-responsive regulation of cysteine dioxygenase concentration in liver

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The ubiquitin-proteasome system is responsible for cysteine-responsive regulation of cysteine dioxygenase concentration in liver. Am J Physiol Endocrinol Metab 286: E439–E448, 2004. First published November 25, 2003; 10.1152/ajpendo.00336.2003.—Hepatic cysteine dioxygenase (CDO) activity is a critical regulator of cellular cysteine concentration and availability of cysteine for anabolic processes and is markedly higher in animals fed diets containing excess sulfur amino acids compared with those fed levels at or below the requirement. Rat hepatocytes responded to a deficiency or excess of cysteine in the culture medium with a decrease or increase in CDO level but no change in CDO mRNA level. The cysteine analog, cysteamine, but not cysteine metabolites or thiol reagents, was also effective in increasing CDO. Inhibitors of the 26S proteasome blocked CDO degradation in cysteine-deficient cells but had little or no effect on CDO concentration in hepatocytes cultured with excess cysteine. High-molecular-mass CDO-ubiquitin conjugates were observed in cells cultured in cysteine-deficient medium, whether or not proteasome inhibitor was present, but these CDO-ubiquitin conjugates were not observed in cells cultured in cysteine-supplemented medium with or without proteasome inhibitor. Similar results were observed for degradation of recombinant CDO expressed in human hepatocarcinoma cells cultured in cysteine-deficient or cysteine-supplemented medium. CDO is an example of a mammalian enzyme that is robustly regulated via its substrate, with the presence of substrate blocking the ubiquitination of CDO and, hence, the targeting of CDO for proteasomal degradation. This regulation occurs in primary hepatocytes in a manner that corresponds with changes observed in intact animals.

Cysteine Dioxygenase (CDO; EC 1.13.11.20) is an Fe²⁺ metalloenzyme that adds molecular oxygen to the thiol group of cysteine to form cysteinesulfinate. CDO is a unique and highly conserved protein that has a limited tissue distribution, being expressed predominantly in liver, with lower levels in kidney, lung, and brain (23, 39). By catalyzing the first step in the oxidative metabolism of cysteine, CDO plays a key role in cysteine catabolism, in provision of cysteine carbon for gluconeogenesis or oxidative metabolism, in taurine synthesis, and in supply of inorganic sulfur for sulfation reactions. Furthermore, because of its robust regulation in response to cysteine intake or supply, CDO plays a central role in controlling cellular and body cysteine concentrations (3, 4, 40).

Hepatic CDO is regulated in response to diet (1–4, 10, 40). Marked increases in CDO level of ~35-fold occur when the amount of dietary protein or sulfur amino acids is increased from below-requrement levels to above-requrement levels (1–4, 40). CDO activity is barely detectable in liver of rats fed low-protein diets, but CDO activity increases up to ~6 nmol·min⁻¹·mg protein⁻¹ in rats fed diets containing high levels of protein, methionine, or cysteine. Hepatic cysteine concentration, measured in fasted rats, increased stepwise with increments in dietary protein, from 0.02 µmol/g in rats fed a 100 g casein/kg diet to 0.08 µmol/g in rats fed a 400 g casein/kg diet, but it remained below 0.1 µmol/g (26, 40). Changes in CDO level have a large physiological impact on the capacity to catabolize cysteine to taurine and sulfate/pyruvate, as demonstrated by differences in rates of product formation by isolated hepatocytes and by different tissue and urinary taurine and sulfate levels in intact rats adapted to diets with varying levels of protein or sulfur amino acids (1–3).

The liver is uniquely positioned to remove sulfur amino acids due to its first-pass metabolism of dietary amino acids in the portal blood draining the intestine (20). Additionally, the high cysteine-to-cystine ratio maintained by the intestine facilitates the uptake of cysteine by the liver via neutral amino acid transport systems (11, 12). The robust response of hepatic CDO in response to dietary intake, along with the high Kₘ of the liver isozyme of methionine adenosyltransferase, provides for rapid removal of excess sulfur amino acids along with maintenance of a sufficiently high cellular cysteine level to ensure adequate rates of synthesis of glutathione, coenzyme A, and proteins.

Normal regulation of cysteine metabolism appears to play an important role in health, because high levels of cysteine and low levels of sulfate, and reportedly low CDO activity, have been associated with the occurrence of rheumatoid arthritis and several neurological diseases (7, 8, 13, 16, 22). Elevated levels of cysteine have been shown to be both cytotoxic and neurotoxic (18, 27, 30), and high levels of total plasma cysteine have recently been found to be associated with increased risk for cardiovascular disease, adverse pregnancy outcome, and a more oxidative redox environment (14, 15). Evidence also exists that low levels of CDO can result in low rates of inorganic sulfate release from sulfur amino acids and, hence, impaired sulfation reactions (7, 8, 13), and low or absent CDO activity is clearly the basis of the requirement of some species for dietary taurine (38).

Our previous work has demonstrated that hepatic CDO mRNA levels and the association of CDO mRNA with polynomials do not change with increases in sulfur amino acid intake, whereas CDO concentration and activity increase in...
parallel with each other, suggesting that CDO degradation is the point of regulation (3–5, 26, 40). Although either methionine or cyst(e)ine was effective in both dietary and cell culture studies, cysteine or a closely related molecule is necessary for the regulation of CDO concentration at the cellular level; precursors of cysteine were dispensable, and metabolites of cysteine, including glutathione, were ineffective by themselves (10, 25).

To further define the mechanisms involved in the response of hepatic CDO to dietary protein or sulfur amino acids, we used primary rat hepatocytes to investigate the time course of changes in CDO during culture in cysteine-deficient or cysteine-supplemented medium, the ability of various cysteine analogs and thiol reagents to mimic the effect of cysteine on CDO levels, and the effect of inhibitors of protein synthesis and protein degradation on CDO levels. Finally, we evaluated the effect of cysteine on the accumulation of ubiquitinated CDO in primary rat hepatocytes and in a human hepatocarcinoma cell line.

Our results clearly indicate that regulation of CDO concentration involves regulation of CDO degradation by the ubiquitin-26S proteasome system. Cysteine appears to function in a cysteine-specific manner to impair the ubiquitination of CDO and, hence, its recognition by the 26S proteasome.

**EXPERIMENTAL PROCEDURES**

**Materials.** t-Cysteine, d-cysteine, S-methyl-L-cysteine, L-cysteine methyl ester, t-cysteinesulfinate, 2-mercaptoethylamine (cysteamine), 2-mercaptoethanol, bathocuproine disulfonate, α-2-propargylglycine, cycloheximide, N-acetylemaleimide (NEM), leupeptin (N-acetyl-Leu-Leu-Arginyl), chloroquine, and 3-methylenediamine were obtained from Sigma. Proteasome inhibitor I [PI1; carboxybenzoxyl-ile-Glu(orrubu)-Ala-leucinal], lactacystin, MG132 (carboxybenzoxyl-Leu-Leu-leucinal), epoxomicin, bestatin [(2S,3R)-3-amino-2-hydroxy-4-phenylbutanoyl-L-leucine], ALLN (N-acetyl-Leu-Leu- norleucinal), ALLM (calpain inhibitor II, N-acetyl-Leu-Leu-methioninal), EST [(2S, 3R)-trans-epoxysuccinyl-L-leucylamido-3-methylbutane ethyl ester], and E64 (t-trans-3-carboxyoxiran-2-carbonyl-L-leucylamidine) were obtained from Calbiochem. Dithiothreitol was from United States Biochemical. AEBSF [4-(2-aminoethyl)-benzenesulfonyl fluoride] was obtained from Acros Organics/Fisher.

*Cultured rat hepatocytes.* Male Sprague-Dawley rats were purchased from Harlan Sprague Dawley (Indianapolis, IN) and housed as described previously (40). Rats were fed either a high-protein (400 g/kg diet) or low-protein (100 g casein/kg diet) diet for ≥1 wk. The composition of the diets was based on the AIN-93 formulation (35) and has been described (40). Rats had free access to diet and water. Rats weighed 200–300 g when they were used as a source of hepatocytes. The experimental protocol was approved by the Cornell University Institutional Animal Care and Use Committee.

Hepatocytes were isolated and cultured on collagen-coated dishes in modified Williams’ medium E, as described previously (4, 25). The basal medium was made by supplementing sulfur amino acid-free Williams’ medium E (GIBCO/Invitrogen) with (per liter) 5 mg transferrin, 1 g albumin, 3 mmol sodium selenite, 10 mg insulin, 50 μg EGF, 50 μmol dexamethasone, 1 × 10⁵ units penicillin G, 100 μg streptomycin sulfate, 0.25 mg amphotericin B, 2.2 g sodium bicarbonate, 0.05 mmol bathocuproine disulfonate (to minimize disulfide formation), 0.20 mmol L-cysteine, and 0.15 mmol l-methionine. Thus the basal medium was a low-sulfur amino acid medium that contained a total of 0.35 mM cysteine equivalents compared with 0.65 mM in standard Williams’ medium E. Cysteine-supplemented medium was prepared by adding an additional 1 mM of L-cysteine to give a total of 1.35 mM of cysteine equivalents. Other additions to the medium were made as described in Figs. 1–5 for specific experimental treatments.

Hepatocytes were established in cysteine-supplemented or basal medium, as indicated in Figs. 1–5, for 18 h. Medium was then changed to the treatment medium, and treatment medium was replaced with fresh medium after 24 h for time points longer than 24 h. For Western blot analysis, cells were harvested into TNESV lysis solution, which contained 50 mM Tris, pH 7.5, 1% (vol/vol) Nonidet P-40, 2 mM EDTA, 100 mM NaCl, and 10 mM sodium orthovanadate, supplemented with 0.1 mM AEBSF, 25 mM leupeptin (10 μg/ml aprorex), 10 mM NEM, and 20 μg MG132 (28, 29). A 100-mm-diameter dish of cells was washed twice with 5 ml of ice-cold PBS. Then 1 ml of TNESV lysis solution was added, and the dishes were incubated on a rocking platform in a 4°C cold room for 20 min. The contents of each dish were scraped into a 1.5-ml Eppendorf tube and centrifuged at ~14,000 g for 20 min at 4°C. The cleared lysate was frozen and stored at −70°C until Western blot analysis was done.

For measurement of intracellular thiol concentrations, cells were washed three times with 5 ml of ice-cold PBS and then scraped off the dish into a microfuge tube containing 0.4 ml of ice-cold PBS. A portion of the cell suspension was added to an equal volume of 0.4 M perchloric acid, 2.5 mM γ-gluamylglutamate (internal standard for HPLC), 7.5 mM bathocuproine disulfonate, and 7.5 mM bathocuproine disulfonate. This mixture was sonicated and centrifuged to obtain the supernatant, which was frozen and stored at −70°C until HPLC analysis was done.

All cell culture experiments were repeated at least three times with separate hepatocyte preparations to ensure that results were reproducible. Within each experiment, all measurements were made on single plates of cells. Vehicle controls were run for all additions; the volume of vehicle (DMSO, methanol, or glycerol) was always <0.1% of the total volume of medium.

*Cultured C3A/HepG2 cells.* C3A/HepG2 cells stably transfected with the pTet-On regulator plasmid were plated in 6-well tissue culture plates in cysteine-free or cysteine-supplemented Dulbecco’s modified Eagle’s medium (DMEM). Cysteine-free DMEM was prepared by supplementing sulfur amino acid-free DMEM with 0.05 mM bathocuproine disulfonate, 0.1 mM L-methionine, 2 mM L-glutamine, 0.1 mM nonessential amino acid solution (GIBCO/Invitrogen), 1 mM sodium pyruvate, and 10% (vol/vol) Tet-free fetal bovine serum (HyClone). Cysteine-supplemented treatment medium also contained 1 mM L-cysteine. At 24 h after plating, these cells were transiently transfected with pTRE2 (BD Biosciences/Clontech) into which the cDNA for rat liver CDO had been cloned. Transfection was done using FuGENE 6 reagent (Roche). Three hours after transfection, the medium was changed to medium supplemented with 2 μg/ml doxycycline to turn on CDO expression. Cells were cultured in the presence of doxycycline for 36 h, with a medium change at 24 h, and then were cultured in medium without doxycycline for assessment of CDO degradation. Cells were harvested at 0, 6, 12, or 24 h after the removal of doxycycline with the lysis buffer described above.

*Analysis of CDO level.* CDO in cell lysates was measured by SDS-PAGE and immunoblotting, as described previously (3, 4). To ensure linearity and accurate quantification, a standard curve (2–10 or 8–30 ng CDO) was generated on each gel/membrane by use of recombinant HsCO-CDO (39). Rabbit anti-CDO was raised against recombinantly expressed rat liver CDO (Cornell University Center for Research Animal Resources), and the immunoglobulin (IgG) fraction was purified using Affi-Gel (Bio-Rad) and used as the primary antibody (1:2,000 dilution). Supersignal Pico (Pierce) was used as the secondary antibody. Protein in the supernatant fractions was measured by the method of Smith et al. (36). Amounts of CDO were expressed on the basis of total soluble protein in the samples.

Immunoaffinity precipitation of CDO-ubiquitin conjugates. Lysate containing 1 mg of total protein was incubated with ~15 μg of rabbit anti-rat CDO primary antibody (500 μl final volume) for 2 h at 4°C, followed by mixing overnight with 70 μl of
a mixture of protein A- and protein G-agarose (Invitrogen). On the following day, the agarose was washed four times with lysis solution, after which the agarose was resuspended in SDS-PAGE sample buffer, heated at 95°C for 5 min, and centrifuged to obtain the supernatant. The proteins in the supernatant were separated on an SDS-PAGE gel (12% wt/vol polyacrylamide) and transferred to a polyvinylidene difluoride membrane (Immobolin-P, Millipore). Immunoblotting was performed using the Supersignal West Dura Substrate (Pierce), with a 1:20,000 dilution of mouse monoclonal anti-ubiquitin IgG (Santa Cruz Biotechnology) as the primary antibody and a 1:150,000 dilution of HRP-conjugated goat anti-mouse IgG (Pierce) as the secondary antibody, or using a 1:30,000 dilution of rabbit polyclonal anti-rat CDO antibody as the primary antibody and a 1:150,000 dilution of HRP-conjugated goat anti-rabbit IgG (Pierce) as the secondary antibody. The membrane was immunoblotted with anti-ubiquitin first, and then the membrane was stripped and rebotted for CDO.

Cysteine and glutathione levels. Acid supernatants from hepatocytes were neutralized and treated with dithiothreitol to reduce disulfides. Total cysteine and glutathione were measured by formation of S-carboxymethyl derivatives followed by chromatophore derivatization of primary amines with 1-fluoro-2,4-dinitrobenzene and separation of these derivatives by reversed-phase ion-exchange HPLC by use of a 3-aminopropyl column (Brownlee Labs, Santa Clara, CA), as described previously (4, 17, 40).

Statistical analysis. Quantitative results were analyzed using ANOVA (one-way) and Dunnett’s comparison with control values (Minitab, release 13.1).

RESULTS

Changes in CDO level in rat hepatocytes cultured in basal or cysteine-supplemented medium. Primary hepatocytes underwent similar changes in CDO concentration in response to cysteine supplementation in the medium as have been observed in liver of intact rats fed diets varying in protein level (40). When hepatocytes with a high initial CDO concentration were switched to basal low-cysteine medium, CDO concentration gradually declined (Fig. 1A). This decline in CDO level was prevented by cysteine supplementation of the medium. Hepatocytes with a low initial level of CDO were able to accumulate CDO when cells were switched to a cysteine-supplemented medium, demonstrating that cultured hepatocytes continue to synthesize new CDO (Fig. 1B). Total number of cells, as judged by total protein per dish, remained constant (∼1.5 mg/60-mm dish) over 30 h of culture and was not affected by cysteine supplementation at any time point. As reported previously (3–5, 25, 40), CDO mRNA level was not affected by cysteine supplementation (data not shown). In cells cultured for 48 h or longer, a substantial decrease in CDO concentration was observed, even in the presence of supplemental cysteine, consistent with downregulation of CDO1 gene expression in rat hepatocytes maintained in culture. Thus CDO level in cultured hepatocytes responds to cysteine availability in the same manner as does CDO in liver of intact rats (1–5, 25, 40). Our previous observations of no change in CDO mRNA level in cultured hepatocytes or in liver of intact rats and no evidence of an effect of sulfur amino acids on CDO mRNA translation suggest that cysteine acts by decreasing CDO degradation rather than by increasing CDO synthesis.

Effect of inhibition of protein synthesis on CDO level. We further explored the role of protein synthesis in the regulation of CDO concentration by culturing cells in the presence of cycloheximide or emetine to block synthesis of new protein. Either cycloheximide (Fig. 1C, lane 3) or emetine (not shown) caused a higher, rather than the predicted lower, CDO level compared with no treatment (lane 2). Most likely, this was due to an increase in intracellular cysteine concentration, secondary to the block in incorporation of amino acids into protein. In hepatocytes cultured in the presence of supplemental methionine or cysteine plus cycloheximide, CDO concentration was less than that achieved by addition of methionine or cysteine alone (lanes 6 and 7 vs. 4 and 5). These results indicate that, although new CDO synthesis obviously contributed to the increased levels of CDO over time in cultured hepatocytes, CDO levels were higher in hepatocytes cultured with excess methionine or cysteine in the medium, even when protein synthesis was blocked. These results are consistent with regulation via a cysteine-responsive degradation pathway. These observations also demonstrate that the regulation of CDO in response to an increase in cysteine concentration does not require synthesis of a short-lived protein.

Effect of L-cysteine, cysteine analogs, and thiol reagents on CDO level in cultured hepatocytes. The specificity of cysteine in the regulation of CDO concentration was evaluated by testing the effect of several cysteine analogs and thiol reagents. A representative Western blot is shown in Fig. 1D, but slight variations were observed among experiments. On the basis of the results of eight experiments, L-cysteine was consistently the most effective of the compounds tested, but equimolar amounts of L-cysteine methyl ester and cysteamine (2-mercaptoethylamine) were both nearly equally effective (lanes 3, 6, and 7 vs. lane 2). These two compounds retain intact cysteiny1 side-chains. D-Cysteine had a notable effect and S-methyl-L-cysteine gave a slight response in CDO concentration (lanes 8 and 5 vs. lane 2); these effects could be due to limited conversion to L-cysteine. The thiol reagents, dithiothreitol and 2-mercaptoethanol, and the oxidized derivative of cysteine, cysteine-sulfinate, were ineffective (lanes 4, 9, and 10 vs. lane 2). Thus correct stereochemistry, an intact sulfhydryl group, and an unsubstituted side-chain all appeared to be critical. The mechanism of cysteine’s action in regulation of CDO turnover seems to be substrate specific and not a general effect of the thiol redox state.

Effect of protease inhibitors on CDO level. To further elucidate the role of protein degradation pathways in regulation of CDO concentration, we tested the ability of various cell-permeable protease inhibitors with differing specificities for the 26S proteasome NH2-terminal threonine hydrolases, lysosomal proteases, or calpains to block degradation of CDO (Fig. 2). Compared with hepatocytes that received no addition or vehicle only, addition of E64 and leupeptin, which are effective cysteine protease inhibitors, had no effect. Addition of other protease inhibitors (lactacystin, MG132, P11, AEBSF, and ALLM) significantly increased CDO level compared with control levels. Addition of lactacystin and P11, inhibitors of the 26S proteasome, resulted in the highest levels of CDO. Chloroquine, a lysosomotropic agent, and methyladenine, an inhibitor of macroautophagy, were much less effective than lactacystin and P11. Both AEBSF and chloroquine decreased hepatocyte viability (decreased total protein per dish, $P < 0.05$), but addition of the other inhibitors had no effect on hepatocyte recovery.

In addition to the inhibitors and concentrations shown in Fig. 3, several others were tested. Addition of epoxomicin (10 μM)
was equally as effective as lactacystin or PI1. ALLN (2–50 μM) and bestatin (10–100 μM) were less effective than PI1, even at the highest concentrations. EST (10 μM) had no effect. No effect of ALLM on CDO concentration was observed until concentrations above 100 μM were used, whereas an effect was observed with as little as 10 μM ALLN. These results, collectively, suggest that CDO degradation in hepatocytes is regulated by the ATP-dependent ubiquitin-proteasome system.

Effect of proteasome inhibitor in cells cultured with cysteine, 2-mercaptoethylamine, cysteinesulfinate, or 2-mercaptoethanol. Quantitative results for the effect of cysteine, 2-mercaptoethylamine, cysteinesulfinate, and 2-mercaptoethanol on the CDO level in hepatocytes are shown in Fig. 3A. Both cysteine and 2-mercaptoethylamine maintained high levels of CDO over 16 h, whereas cysteinesulfinate and 2-mercaptoethanol were ineffective and yielded CDO levels comparable to those obtained in cells cultured in basal medium with no addition. All cells cultured with PI1 maintained CDO levels similar to those in the cysteine-supplemented cells, regardless of addition of cysteine, cysteine analog, or thiol. The effect of cysteine or 2-mercaptoethylamine on CDO level and the effect of proteasome inhibitor on CDO level were not additive.

Cysteine and glutathione levels in cells cultured with cysteine, 2-mercaptoethylamine, cysteinesulfinate, or 2-mercaptoethanol. The concentration of cysteine in hepatocytes cultured in cysteine-supplemented medium was 4 ± 0.4 nmol/mg protein (~0.7 vs. μmol/g cells) vs. 2.5 ± 0.2 nmol/mg (~0.4 μmol/g cells) for those cultured in the basal medium (Fig. 3B). The cysteine concentration in cells cultured with 2-mercaptoethylamine, cysteinesulfinate, or 2-mercaptoethanol remained low and similar to that in cells cultured in basal medium. The total glutathione concentration in hepatocytes cultured in the various treatment media was ~60 ± 6 nmol/mg protein (~10 μmol/g cells) and was not different for the various groups.

Fig. 1. Representative Western blots for cysteine dioxygenase (CDO) in soluble extracts of cultured hepatocytes. A: hepatocytes were obtained from a rat fed a high-protein (HP) diet and cultured in basal medium (−) or cysteine-supplemented (+) medium for up to 24 h. Equal amounts (25 μg) of total soluble protein were loaded in each lane. B: hepatocytes were obtained from a rat fed a low-protein diet and cultured in cysteine-supplemented medium (+) for up to 30 h. Equal amounts (50 μg) of total soluble protein were loaded in each lane. C: hepatocytes were obtained from a rat fed an HP diet, established in cysteine-supplemented medium, and then cultured for 18 h in basal medium supplemented with 20 μM cycloheximide (CHX), 1 mM l-cysteine (Cys), 1 mM l-methionine (Met), or combinations of these, as indicated. Each lane was loaded with 30 μg of total soluble protein. D: hepatocytes obtained from a rat fed an HP diet were established in cysteine-supplemented medium and then cultured for 18 h in basal medium supplemented with 1 mM l-cysteine or 1 mM cysteine analog or thiol, as indicated. Each lane was loaded with 25 μg of total soluble protein.
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**Fig. 2. Effect of protease inhibitors on CDO level in hepatocytes.** Hepatocytes were obtained from a rat fed an HP diet and were established in cysteine-supplemented medium and then cultured for 18 h in basal medium supplemented with 1 mM L-cysteine, various protease inhibitors (as defined in text), or vehicle (DMSO), as indicated. Inhibitors were used at the following concentrations: 5 μM lactacystin, 20 μM MG132, 1 μM P11, 1 μM E64, 100 μM leupeptin, 1 mM AEBSF, 1 mM chloroquine, 10 mM methyladenine, and 5 μM ALLM. Results of quantitative Western blotting are shown in the bar graph. Values are means ± SE for 3 or 4 experiments. *Value is significantly greater (P ≤ 0.05) than that for cells cultured with no addition. **Value is significantly greater (P ≤ 0.05) than value for cysteine-supplemented group. A representative Western blot is shown at top; each lane was loaded with 20 μg of total soluble protein.

Clearly, the effect of 2-mercaptoethanolamine in maintenance of CDO concentration was not due to a secondary effect on intracellular cysteine, or glutathione, concentration.

**Effect of a thiol-blocking reagent on recovery of ubiquitinated CDO.** Addition of NEM to the lysis buffer has been reported to facilitate the recovery of ubiquitinated proteins (6, 28, 29). All known members of the family of isopeptidases that hydrolyze the ubiquitin chain from ubiquitinated proteins are dependent on active-site cysteinyl residues and can, hence, be inactivated with thiol-blocking reagents such as NEM (21, 29). When NEM was included in the lysis buffer, several higher-molecular-mass bands were detected with anti-CDO compared with recovery of only a small amount of one higher-mass band when lysis buffer without NEM was used (Fig. 4A). Overexposure of the CDO band itself was necessary to detect the ubiquitinated CDO bands, and the tri- and tetra-ubiquitinated bands still appeared very light, which is consistent with the rapid hydrolysis of polyubiquitinated CDO by the 26S proteasome. Nevertheless, these bands had molecular masses that corresponded to the predicted masses for mono-, di-, tri-, and tetra-ubiquitinated CDO, with each ubiquitin adding a mass of ∼8.6 kDa. The appearance of the ubiquitin ladder and its sensitivity to NEM provide strong evidence for a role of ubiquitination in regulation of the degradation of CDO. The relative absence of ubiquitinated CDO with more than four attached ubiquitin molecules is consistent with the high affinity of the proteasome for the tetra-ubiquitin conjugate (21).

**Effect of supplemental cysteine and proteasome function on the accumulation of ubiquitinated CDO.** Using the NEM-containing lysis buffer, we further examined the effect of cysteine and P11 on the accumulation of ubiquitinated forms of CDO in cultured hepatocytes. Hepatocytes were cultured for 5, 10, or 16 h in basal medium alone or with added cysteine, P11, or both. CDO decreased over time in cells cultured in basal medium and was notably less than for other treatments at 10 and 16 h, consistent with more rapid degradation of CDO (Fig. 4C). Despite a dramatic loss of total CDO, some ubiquitinated CDO bands were still evident at 16 h (Fig. 4B). Addition of P11 to the basal medium resulted in somewhat denser bands for ubiquitinated CDO, especially at 16 h, consistent with inhibition of degradation of ubiquitinated CDO by the 26S proteasome. In contrast, ubiquitinated CDO did not accumulate in cells cultured with supplemental cysteine nor in cells cultured with both supplemental cysteine and P11, even though CDO was stable and not readily degraded in either case (Fig. 4C). Thus CDO degradation via the 26S proteasome appeared to be regulated in response to cysteine at the level of ubiquitination: at low cellular cysteine levels, CDO was ubiquitinated and rapidly degraded by the 26S proteasome, whereas at higher cellular cysteine levels, CDO was not ubiquitinated and thus did not become a substrate for the 26S proteasome.

**Effect of supplemental cysteine, 2-mercaptoethanolate, or 2-mercaptoethanol and proteasome inhibitors on the accumulation of ubiquitinated CDO in cultured hepatocytes.** Hepatocytes were obtained from a rat fed an HP diet and were established in cysteine-supplemented medium and then cultured for 18 h in the presence of 1 mM cysteine, 2-mercaptoethanolate, cysteinesulfinylate, 2-mercaptoethanol, or no cysteine addition and with or without 0.5 μM proteasome inhibitor-1 (P11). Values are means ± SE for 4 experiments. *Value is statistically significantly less (P ≤ 0.05) than the value for the cysteine-supplemented group.
lation of ubiquitinated CDO. To further verify that CDO-ubiquitin conjugates accumulated in hepatocytes cultured in medium with low levels of sulfur amino acids, we immuno-precipitated CDO from hepatocytes cultured under various conditions and did Western blot analyses using anti-CDO and anti-ubiquitin. Cysteine, 2-mercaptoethylamine, lactacystin, or PI1 was effective in maintaining high levels of CDO (Fig. 5A).

With longer exposure, CDO-ubiquitin conjugates were visible at molecular masses consistent with the attachment of 1, 2, or 3 ubiquitin molecules per CDO molecule (Fig. 5B). When the same membrane was probed with anti-ubiquitin, bands were visualized that corresponded to CDO with 2 or 3 ubiquitin molecules attached, as well as higher-molecular-mass poly-ubiquitin conjugates indicated by CDO-Ubn in Fig. 5C. The amount of protein loaded was not sufficient for visualization of higher-molecular-mass CDO-ubiquitin conjugates with anti-CDO at the exposure used (Fig. 5B). Figure 5D shows cell lysates from each treatment group. It is clear that addition of a proteasome inhibitor gave more ubiquitin bands compared with no proteasome inhibitor, demonstrating the general effect of proteasome inhibitors on blocking the degradation of many ubiquitinated proteins. On the other hand, the presence or absence of cysteine or 2-mercaptoethylamine had no general effect on the accumulation of ubiquitinated proteins, demonstrating that the effect of these compounds on CDO is specific and not a general effect on many proteins.

Effect of cysteine on accumulation of CDO-ubiquitin conjugates in HepG2 cells transfected with CDO cDNA. The levels of CDO and CDO-ubiquitin conjugates (1 and 2 ubiquitin molecules per CDO molecule) in HepG2/C3A cells that had expressed recombinant CDO were similar to our observations in primary rat hepatocytes, in that higher levels of CDO-ubiquitin conjugates were observed in cells cultured without cysteine, whereas higher levels of CDO were observed in cells cultured with cysteine (Fig. 6). Ubiquitin conjugates of CDO in the cells cultured in cysteine-free medium were particularly apparent at the 12-h and 24-h time points, because more sample was loaded on the gel for the 12- and 24-h time points (twice as much as for the 0- and 6-h time points).

A lower level of sulfur amino acids in the basal medium was used in this experiment than in experiments with primary hepatocytes. This was necessary to sufficiently lower the cellular cysteine level, because HepG2 cells do not express CDO and thus tend to have relatively high cysteine levels, even in sulfur amino acid-restricted medium.

DISCUSSION

Evidence presented in this study demonstrates that hepatic CDO is robustly regulated by ubiquitination in response to the cellular cysteine level. When cysteine availability is low, CDO is readily ubiquitinated and, hence, becomes substrate for proteolysis by the 26S proteasome. This allows rapid degradation of CDO to proceed, resulting in very low hepatic CDO concentration. The low CDO activity, in turn, spares cysteine for anabolic purposes such as synthesis of protein, glutathione, and coenzyme A. On the other hand, when cysteine is present in excess of cellular needs, the ubiquitination of CDO is blocked, and CDO accumulates in the cell. The higher level of CDO activity rapidly oxidizes excess cysteine to cysteinesulfinate, allowing rapid disposal of cysteine as taurine or pyruvate/sulfate.
Results of these studies with cultured hepatocytes and HepG2 cells are consistent with our previous reports of the regulation of CDO in liver of intact rats fed diets with varying levels of protein or sulfur amino acids (19, 20, 23). Furthermore, the time course data for changes in CDO in cultured hepatocytes or HepG2 cells compare favorably with our estimates of the rate of CDO degradation in liver of rats fed a low-protein ($t_{1/2} = 8$ h) vs. a high-protein ($t_{1/2} = 20$ h) diet (23). Although the response of CDO in hepatocytes to sulfur amino acid supply was similar to that observed in liver of intact rats, the intracellular cysteine levels we observed for hepatocytes were notably higher than those previously reported for rat liver (26, 40), and the relation between intracellular cysteine concentration and regulation of CDO degradation remains to be elucidated.

In studies with hepatocytes or HepG2 cells, we have found that maximal levels of CDO can be obtained by using medium that contains a total of 1 mmol/l sulfur amino acids, with methionine being equally as effective as a combination of methionine and cysteine (32). Because of oxidation of cysteine to cystine, as well as utilization of cysteine by the cells, the medium cysteine concentration decreases to $\approx 0.2$ mmol/l by 24 h, the time when medium is replaced with fresh medium. The latter level is not far from the concentration of cysteine in the portal blood (20). No evidence of low cell viability or oxidative stress was observed. Intracellular cystine was unde-

![Fig. 5. Accumulation of CDO-ubiquitin conjugates in hepatocytes cultured in the absence of excess cysteine or the presence of proteasome inhibitor. A-C: representative Western blots of immunoprecipitates of CDO. Hepatocytes were obtained from a rat fed an HP diet and were established in cysteine-supplemented medium and then cultured in medium with 1 mM cysteine (Cys), 1 mM 2-mercaptoethanol (ME), 1 mM 2-mercaptoethylamine (MEA), 1 uM PI1, or 5 uM lactacystin (LC), as indicated. Lysate containing 1 mg total protein was immunoprecipitated with anti-CDO. A and B: membrane was immunoblotted with anti-CDO (A, 10-s exposure; B, 2-min exposure). C: the same membrane shown in A and B was immunoblotted with anti-ubiquitin (3-min exposure). The immunoglobulin heavy-chain (50 kDa) and light-chain (25 kDa) bands in A-C are due to reaction with the secondary antibody. D: representative Western blot of cell lysates with anti-ubiquitin. Each lane was loaded with 15 ug of total protein. The presence of numerous ubiquitinated proteins in cell lysates causes the immunoblot to appear as a smear.](http://ajpendo.physiology.org/doi/10.1152/ajpendo.00673.2003)
Cysteine appears to act in a substrate-like manner in down-regulating the ubiquitination of CDO. Although general effects of relatively high levels of thiols and thiol reagents on the ubiquitin 26S proteasome system have been reported (24, 31, 33), these effects are all mediated through the thiol-dependent ubiquitin-activating enzymes (E1s), ubiquitin-conjugating enzymes (E2s), and deubiquitination enzymes, and would have general effects on intracellular protein degradation. The effect of cysteine on CDO degradation does not appear to be a general thiol effect but rather a specific response to cysteine availability. Thiol reagents such as dithiothreitol and 2-mercaptoethanol were ineffective compared with cysteine, and previous studies in which the synthesis of glutathione, the major cellular thiol, was blocked by buthionine sulfoximine showed that depletion of cellular glutathione did not negate the ability of cysteine to increase the cellular CDO level (25). On the other hand, analogs of cysteine that retain the side-chain structure of cysteine were effective in upregulating CDO to levels comparable to those obtained with cysteine itself. Of these, mercaptoethyamine (cysteamine) was unique in that it was nearly as effective as cysteine in blocking ubiquitination of CDO, but it is not capable of being converted to cysteine by cellular enzymes and did not increase the intracellular cysteine (or glutathione) concentration.

Our data suggest that CDO degradation is regulated at the level of recognition of CDO as substrate by the E2/E3 complex responsible for its ubiquitination. It is possible that conformational changes brought about by binding of cysteine or a cysteine analog prevent efficient polyubiquitination of CDO. A change in conformation could mask a degradation signal recognized by the E2/E3 complex or an accessory protein or preclude the ubiquitination of a specific lysyl residue in the CDO by this complex. Another possibility is that the E2/E3 complex recognizes an oxidized form of CDO as a damaged protein to be degraded, and that the Fe³⁺ or a critical thiol group of CDO is protected from oxidation in the presence of cysteine or cysteamine. Both studies with rat liver CDO (42) and studies in our laboratory with recombinantly expressed CDO demonstrate that cysteamine (J. E. Dominy and M. H. Stipanuk, unreported observations) does not act as either a substrate or an inhibitor of CDO, suggesting that binding of cysteine to the active site is not necessary for blocking CDO ubiquitination. Future studies will be directed at elucidating the molecular mechanisms involved in regulation of CDO turnover.

Most of the known native substrates of the ubiquitin-proteasome pathway are short-lived transcriptional activators, growth cycle regulators, and some unstable membrane proteins (16). Damaged or misfolded proteins are also targets for ubiquitination and proteasomal degradation. Few mammalian enzymes involved in intermediary metabolism have been shown to be targeted by the ubiquitin-proteasomal system, and even fewer have been shown to be targeted in a regulated manner in response to substrate (9, 16, 21, 34, 37). Coleman and Pegg (9) recently demonstrated that binding of polyamine analogs prevented the efficient ubiquitination of spermidine/spermine N⁴-acetyltransferase in a cell-free system. The regulation of spermidine/spermine N⁴-acetyltransferase activity is known to be complex, occurring via changes in the amount of enzyme protein, which is mediated through effects at several levels, including gene transcription, mRNA translation, and protein turnover (9, 19, 34, 41). The importance of regulated degradation of spermidine/spermine N⁴-acetyltransferase by the ubiquitin-proteasome system in response to the natural substrates (spermidine and spermine) in intact cells or intact animals has not yet been demonstrated. A second example of a mammalian enzyme regulated by its substrate is type 2 iodothyronine deiodinase, which catalyzes the deiodination of thyroxine (T₄) to 3,5,3’-triiodothyronine (T₃). Steinsapir et al. (37) demon-

![Representative Western blot of CDO in C3A Tet-On cells transfected with E446 CYSTEINE-RESPONSIVE REGULATION OF CYSTEINE DIOXYGENASE](image-url)
strated that this deiodinase is regulated by a substrate (T₄ or rT₃), with substrate accelerating its degradation via the ubiquitin-proteasome system. In this case, ubiquitination of the enzyme apparently requires interaction with the catalytic center of the protein.

Our results add an important new example of an enzyme that is regulated via the ubiquitin-proteasomal degradation system in intact mammalian cells in response to its natural substrate. Furthermore, the regulatory mechanism appears to function in intact rats with enzyme concentration varying >20-fold over the physiological range of sulfur amino acid intake. The regulation of CDO also appears to be rather simple in that regulation of degradation by the ubiquitination-proteasome system seems to be the dominant, if not only, mechanism involved in maintaining the appropriate steady-state level of CDO (3, 4, 40). Further understanding of the precise interactions involved in the regulation of CDO ubiquitination by its cognate E2/E3 complex is of interest, not only because of the importance of understanding the biological complexities of the ubiquitin-proteasomal system, but also because of the important metabolic roles of cellular thiols, including the role of the thiol/disulfide redox environment, in regulation of many aspects of cellular function.

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GRANTS

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REFERENCES


