IN VIVO REGULATION OF CYSTEINE DIOXYGENASE VIA THE UBIQUITIN-26S PROTEASOME SYSTEM

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1. INTRODUCTION

The intracellular free amino acid pool of cysteine is tightly regulated in the mammalian liver. In rats, for instance, intracellular cysteine is maintained between 20 and 100 μmol/g even when dietary protein or sulfur amino acid intake is varied from sub-requirement to above-requirement levels for this species (Lee *et al.*, 2004). The narrow range of permissible cysteine concentrations is the consequence of two homeostatic requirements. Liver tissue must keep cysteine levels sufficiently high to meet the needs of protein synthesis and the production of other essential molecules like glutathione, coenzyme A, taurine, and inorganic sulfur. At the same time, however, cysteine concentrations must also be kept below the threshold of cytotoxicity.

An important enzyme that contributes to the regulation of steady-state intracellular cysteine levels is cysteine dioxygenase (CDO, EC 1.13.11.20). Expressed at high levels in the liver with lower levels in the kidney, brain, and lung, this Fe^{2+} metalloenzyme catalyzes the addition of molecular oxygen to the sulfhydryl group of cysteine, yielding cysteinesulfinate. The oxidative catabolism of cysteine to cysteinesulfinate represents an irreversible loss of cysteine from the free amino acid pool; cysteinesulfinate is shuttled into numerous metabolic pathways including hypotaurine/taurine synthesis, inorganic sulfur production, and use of the carbon backbone as pyruvate for gluconeogenesis or oxidative decarboxylation and cellular respiration. *In vivo* data suggest that the liver, the organ with the highest amount of CDO expression, uses CDO as a means of disposing excess cysteine obtained through the diet as well as to provide the essential metabolites sulfate, hypotaurine, and taurine (Garcia and Stipanuk, 1992).

Steady-state levels of hepatic CDO protein are exquisitely regulated by dietary sulfur amino acids. Hepatic CDO activity is barely detectable in rats fed low-protein (i.e.,

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sulfur amino acid poor) diets, but increases as much as 35-fold in rats fed diets enriched with methionine, cystine, or total protein (Bella *et al.*, 1999a,b). The regulation of CDO appears to be specifically associated with changes in intracellular cysteine concentration; other non-sulfur amino acids have no effect on CDO levels (Kwon *et al.,* 2001). Cysteine's ability to regulate CDO levels is rather unique in that it is an exclusively post-translational phenomenon (Bella *et al.*, 2000). As demonstrated in rat primary hepatocyte cultures, high levels of cysteine significantly prolong the half-life of CDO by decreasing its ubiquitination and subsequent degradation via the 26S proteasome system (Stipanuk *et al.*, 2004).

Because previous work describing the cysteine-dependent regulation of CDO by the ubiquitin-26S proteasome system has been limited to cell culture models, we decided to explore whether this same system is responsible for the regulation of hepatic and kidney CDO protein *in vivo*. We accomplished this by pharmacological inhibition of the 26S proteasome complex. We also evaluated whether this inhibition had any effect on hypotaurine/taurine metabolism as would be predicted by a perturbation in steady-state CDO levels.

2. MATERIALS AND METHODS

2.1. Animal Feeding Studies

Male Sprague-Dawley rats (170-210 g) were purchased from Harlan Sprague Dawley (Indianapolis, IN). Rats were housed in polycarbonate cages containing paper bedding in a room maintained at 20°C and 60-70% humidity with light from 18:00 h to 06:00 h. These animals had *ad libitum* access to water but had access to food only during the dark cycle 06:00 h to 18:00 h, wherein food was provided in ceramic cups. To ensure high initial levels of hepatic CDO protein, all rats were fed a high protein diet (HP) for one week prior to the treatment day. This diet, prepared by Dyets, Inc. (Bethlehem, PA), contained 40% casein by weight. On the treatment day, rats were randomly assigned to the following experimental groups: maintenance on high protein (HP) diet, switch to a low protein (LP, 10% casein by weight) diet, switch to a low protein diet supplemented with 8.12 g/kg diet cysteine (LP+CYS), or switch to a low protein diet (LP+PII) plus an intraperitoneal injection of the specific proteasome inhibitor, proteasome inhibitor I (PII, 17 mg/kg in DMSO). The LP diet mix, made by Dyets, Inc., was prepared with 25 g/kg of sucrose excluded from it. For the LP and LP+PII diets, all of this sucrose was added back in. For the LP+CYS diet, 16.88 g/kg sucrose was added along with 8.12 g/kg cysteine to fully reconstitute the diet. All diets were prepared as gel cubes by the addition of a hot 3% (w/v) agar solution, followed by casting in containers at 4°C and cutting into easily managed cubes.

At the end of the fasting light cycle on treatment day (time = 0 h), 3 rats were killed to establish baseline values and the remaining rats were switched to their assigned dietary treatments (6 rats/treatment). Animals were subsequently killed 6 h and 10 h after the diet switch (3 rats from each group per time point). Animals in the LP+PII group received an IP injection of PII 2.5 h after the diet switch. At the appropriate time point, rats were weighed, and anesthetized using sodium pentobarbital (30 mg/ml in 15% v/v ethanol) at a dose of 90 mg/kg. Ventricular blood was collected into heparinized (180 U/syringe) syringes by cardiac puncture, transferred to microfuge tubes, and centrifuged

for 3 minutes at 3,000xg. The plasma was removed and frozen in liquid nitrogen. Whole livers and kidneys were removed, rinsed with ice-cold saline, and immediately frozen in liquid nitrogen. The experimental protocol used in this study was approved by the Cornell University Institutional Animal Care and Use Committee.

2.2. Western Blot Analysis

Western blot analysis was conducted as previously described but with some minor modifications (Bella *et al.,* 1999b). Briefly, livers were homogenized in a lysis buffer (20% w/v) containing 50 mM Tris-Cl, 150 mM NaCl, 1 mM EDTA, 0.5% v/v NP-40, 10 mM ortho-vanadate, 1x protease inhibitor cocktail (Sigma, St. Louis, MO), 10 mM N-ethylmaleimide, and 20 μM MG-132 (Boston Biochem, Boston, MA), pH=7.4. Homogenates were centrifuged at 16,500xg for 20 min. Supernatant proteins were separated by one-dimensional SDS-PAGE (either 12% or 15% w/v acrylamide) and then electroblotted overnight onto 0.45 μm Immobilin-P PVDF membranes (Millipore Corporation, Medford, MA). Immunoreactive protein was detected by chemiluminescence using rabbit anti-rat CDO polyclonal antibody (Stipanuk *et al.,* 2004a) and HRPO-conjugated goat anti-rabbit secondary antibody (Supersignal Pico, Pierce) with exposure to Kodak X-OMAT film. Developed films were scanned using a desktop scanner. With the obtained electronic images, two-dimensional quantitative densitometric analysis was performed on areas of interest using AlphaEase software (Alpha Innotech, San Leandro, CA). The apparent molecular weights of native CDO (which runs as a double band on SDS-PAGE with a molecular weight of ~23 kDa) and ubiquitinated CDO (~23 kDa + n•8 kDa, where n = the number of attached ubiquitin moieties) were consistent with previously published values (Yamaguchi *et al.*, 1978; Stipanuk *et al.,* 2004b).

2.3. Analysis of Tissue Hypotaurine and Taurine Content by High Performance Liquid Chromatography (HPLC)

Acid extracts of tissue homogenates were prepared by homogenizing frozen liver samples in 4 volumes of 5% (w/v) sulfosalicylic acid (SSA). Homogenates were then centrifuged at 10,000xg for 10 min. One-half milliliter of acid supernatant was removed and added to 50 μl of 0.2 mM m-cresol purple with mixing. While mixing the sample, 0.48 ml of 2 M KOH/2.4 M $KHCO_3$ and subsequently 50 μl of 50 mM dithiothreitol were added. The mixture was incubated at 37°C for 30 min. After the incubation, 50 μl of 200 mM iodoacetate was added with mixing, and the mixture was placed in the dark for 10 min to alkylate free thiols.

Chromatography of derivatized samples was conducted on a 4.6 x 150 mm column packed with Nova-Pak C_{18} 4 μm spherical packing material (Waters Corp., Milford, MA) equipped with a C_{18} guard cartridge (5 μm spherical particles; Alltech Associates, Inc., Deerfield, IL). Samples and standards were derivatized with o-phthalaldehyde (OPA) prior to injection onto the column using an automatic sample injector (WISP Model 712, Waters Corp., Milford, MA). Under conditions of no-flow, 75 μl of OPA reagent was injected, and this was followed by injection of 50 μl of the standard or sample solution. The OPA reagent was made fresh each day by mixing 3.5 mg OPA with 50 μl 95% ethanol, 5 ml 100 mM borate buffer (pH = 10.4), and 10 μl 2-mercaptoethanol. A

programmed 3-minute delay before the initiation of flow allowed sufficient time for amines to react with the OPA.

Amino acids were separated by gradient elution using two buffers. Buffer A was 100 mM potassium phosphate buffer plus 3% (v/v) tetrahydrofuran (THF), pH=7.0, and buffer B was 100 mM potassium phosphate buffer plus 3% (v/v) tetrahydrofuran (THF) and 40% (v/v) acetonitrile, pH=7.0. Buffers were filtered through 0.45 μm filters before use. Flow rate was 1.0 ml/min and column temperatures were maintained at room temperature. Mobile phase was started isocratically for the first minute at 3% B and then gradients were run by increasing to 30% B over 6 min and to 55% buffer B over 13 min. The column was washed by increasing Buffer B to 100% over 2 min and holding at 100% B for 3 minutes. At 25 min into the run, the mobile phase was decreased to 3% B over 10 min and the column was allowed to equilibrate for another 11 min before the next sample injection.

Detection of OPA-derivatized amino acids was done using a Spectra/glo Filter Fluorometer (Gilson Medical Electronics, Middleton, WI) equipped with a 5 μl flow cell and filters for excitation and emission peaks at 360 and 455 nm, respectively. The fluorometer was connected to a personal computer equipped with Peak Simple Chromatography Data System version 3.21 (LabAlliance, State College, PA) for the integration of chromatographic peaks.

2.4. Statistics

All quantitative data are expressed as means ± standard deviations. Statistical analyses were conducted by ANOVA and Tukey's post-test procedure using Prism 3 (GraphPad Software, Inc., San Diego, CA). Differences were considered significant at $P \leq 0.05$. Unless otherwise stated, fold changes are relative to 0 h values.

3. RESULTS

Fig. 1 shows the effect of each treatment on the expression of hepatic CDO protein. Rats switched from the HP diet to the LP diet exhibited a significant decrease in CDO protein over the time course of the study; by the 10 h time point, CDO levels had fallen by more than 80%. CDO levels were stabilized, however, by maintaining animals on the HP diet, supplementing the LP diet with cysteine, or providing an injection of PII. Although the stabilization of CDO by PII was comparable to that of rats maintained on the HP diet or given the LP+CYS diet when measured at 6 h, the ability of PII to attenuate CDO degradation was diminished by approximately 50% between 6 and 10 h. Nevertheless, animals fed the LP diet and receiving a PII injection retained significantly more CDO protein than did animals fed the LP diet alone.

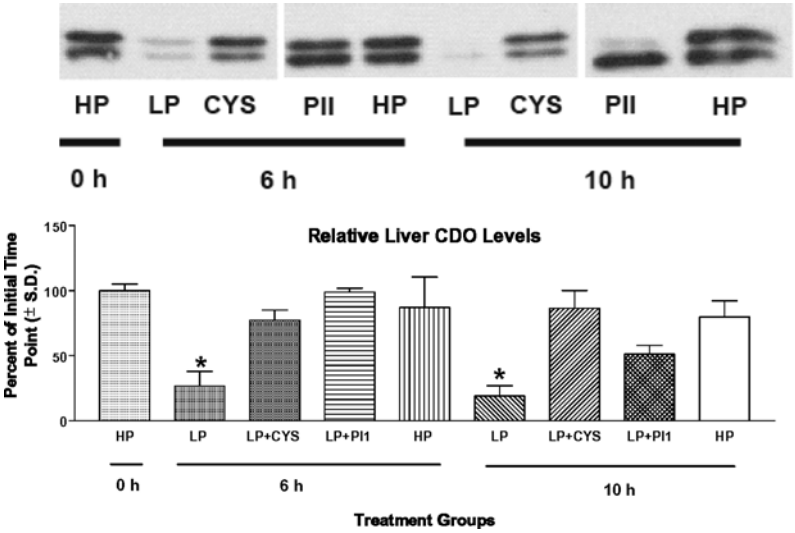

Figure 1. The effects of dietary manipulation and proteasome inhibition on levels of liver CDO protein in rats. For SDS-PAGE analysis of CDO, 60 μg of soluble liver protein was loaded per lane on a 12% polyacrylamide gel. A representative Western is shown for one such analysis (*Top*). To determine the relative changes in protein levels for all samples, the optical density of each CDO band was measured by densitometry. Optical densities were then normalized by HP 0 h control values (run on each gel) and expressed as a percent of these controls in the bar graph (*Bottom*). HP, high protein diet; LP, low protein diet; LP+CYS, low protein diet + 8.12 g cysteine/kg diet; LP+PII, low protein diet + IP injection of proteasome inhibitor I (17 mg/kg). * $P<0.01$ *vs*. 0 h HP group.

In the kidney, there was a slight decrease in the expression of CDO following a switch to the LP diet (Fig. 2). This effect was not significant. Supplementing the LP diet with cysteine produced a significant 2.5-fold increase in kidney CDO levels that was maintained up to at least 10 h after the diet switch. Proteasome inhibitor I also increased kidney CDO levels by ~2.0-fold over the course of the experiment, though due to sample variability this effect was not significantly different from either the LP or LP+CYS group.

Primary hepatocyte culture studies have shown that a reduction in cysteine availability causes an increase in the steady state levels of ubiquitinated CDO (Stipanuk *et al.,* 2004a). These ubiquitinated species, which constitute a relatively small fraction of the total CDO protein pool, are seen as a higher molecular weight ladder of bands on Western blots. The ubiquitinated CDO migrated with apparent molecular weights that differed from that of CDO by integer multiples of approximately 8 kDa. As seen in Fig. 3, mono- and di-ubiquitinated forms of CDO were present in the livers of rats regardless of the treatment group. There was a marked accumulation of these species in the LP+PII-treated animals, particularly at the 10 h time point, as would be expected from conditions wherein CDO ubiquitination is enhanced and proteasome activity is inhibited. Higher molecular weight forms of ubiquitinated CDO are also apparent at the 10 h time point, further demonstrating the accumulation of modified CDO following proteasome inhibition.

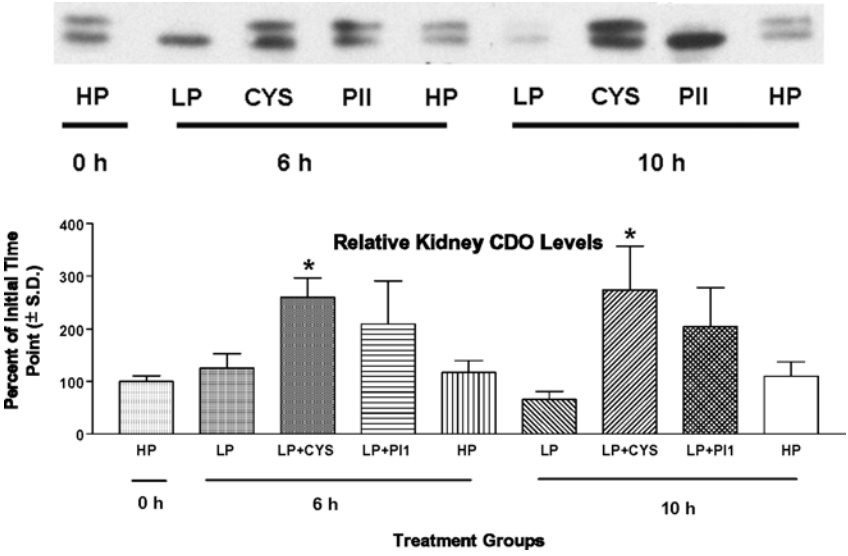

Figure 2. The effects of dietary manipulation and proteasome inhibition on levels of kidney CDO protein in rats. For SDS-PAGE analysis of CDO, 100 μg of soluble kidney protein was loaded per lane on a 12% polyacrylamide gel. A representative Western is shown for one such analysis (*Top*). To determine the relative changes in protein levels for all samples, the optical density of each CDO band was measured by densitometry. Optical densities were then normalized by HP 0 h control values (ran on each gel) and expressed as a percent of these controls in the bar graph (Bottom). * $P<0.05$ *vs*. the initial (0 h HP) value.

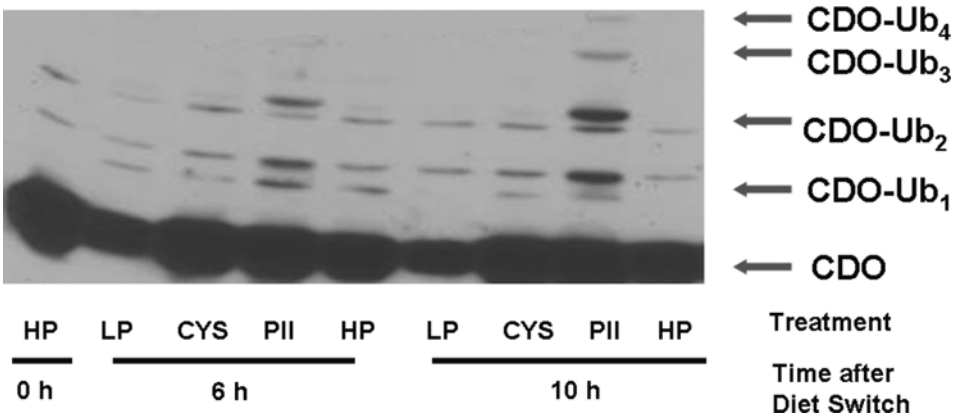

Figure 3. A Western blot showing the effects of dietary manipulation and proteasome inhibition on steady state levels of ubiquitinated liver CDO. 125 μg of liver homogenate was loaded into each lane. Ubiquitinated species are designated by CDO-Ub, with the total number of attached ubiquitin moieties indicated by a numeral subscript.

Normalization of the amount of ubiquitinated CDO by the relative quantity of unmodified CDO protein present in each sample enables a clearer depiction of the treatment-specific effects on steady state ubiquitination (Fig. 4). In the LP+CYS and HP groups, the relative amount of ubiquitinated CDO did not change significantly. Switching to the LP diet, by contrast, produced a significant increase in the relative amount of ubiquitinated CDO: 3.7-fold after 6 h and 5.9-fold after 10 h. At 6 h, 4.5 h after the PII injection, there was no enhancement of the relative amount of ubiquitinated CDO by PII above that achieved by the LP diet alone. By 10 h, however, there was a significant 9.3-fold increase in the relative amount of accumulated ubiquitinated CDO.

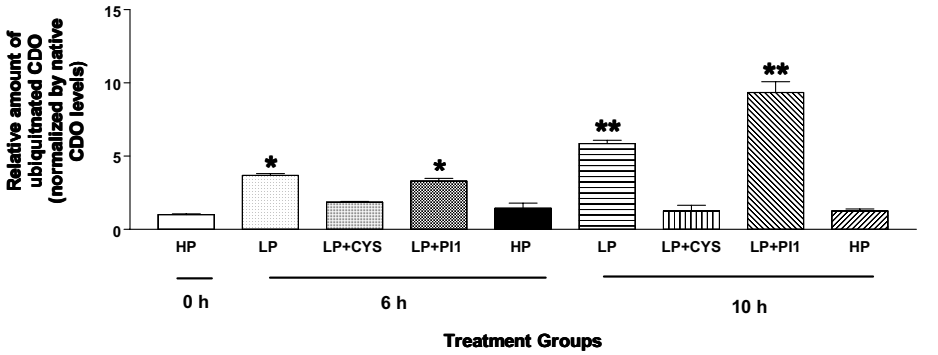

Figure 4. Changes in ubiquitinated hepatic CDO levels, normalized by the relative amount of unmodified (native) CDO in each sample. Normalization of ubiquitinated CDO by the relative amount of CDO provides a clearer representation of ubiquitination kinetics. This is due to the fact that the total quantity of ubiquitinated protein, the product of a first-order reaction, will be related in part to the amount of unmodified protein available for ubiquitination. Therefore, to determine changes in the steady-state kinetics of ubiquitination under conditions in which the pool of unmodified target protein is changing, normalization by the unmodified protein pool is needed. * $P<0.01$ *vs*. initial (0 h HP) value; * $P<0.001$ *vs*. initial (0 h HP) value.

Given the effectiveness of proteasome inhibition in stabilizing CDO levels *in vivo*, we asked whether there was an accompanying alteration in the downstream synthesis of hypotaurine and taurine. In the pathway leading to hypotaurine/taurine synthesis from cysteine, CDO catalyzes the first step by producing cysteine sulfinate. Cysteinesulfinate is then decarboxylated by cysteinesulfinate decarboxylase (CSD) to yield hypotaurine. Hypotaurine is finally oxidized to taurine by a process that has yet to be understood. The principal regulator of flux through this pathway has been alternately ascribed to either CDO or CSD, depending upon the model organism employed in the study (De la Rosa *et al.,* 1985, 1987; Drake *et al.*, 1987). However in the rat it is clear that, while intracellular cysteinesulfinate levels are very low (frequently beneath the limits of detection), the decarboxylation of cysteine sulfinate does not appear to be the rate limiting step in the overall flux of cysteine to hypotaurine/taurine (i.e., the flux of cysteine to hypotaurine/taurine is more affected by synthesis of cysteinesulfinate by cysteine dioxygenase than it is by changes in cysteinesulfinate decarboxylase activity *in vivo*) (Drake *et al.,* 1987). Thus, the production of cysteinesulfinate by CDO appears to be the rate-limiting step in the generation of hypotaurine/taurine under physiological conditions (Stipanuk, 2004).

Concordant with the idea that CDO is an important enzyme in the control of hypotaurine synthesis, there was a clear association between organ CDO protein levels and organ hypotaurine content in this study. In the liver, decrements in CDO protein within the LP group were associated with a sharp decline in hypotaurine content (Table 1). The LP+CYS, LP+PII, and HP groups, which had elevated levels of CDO protein relative to the LP groups, displayed elevated levels of liver hypotaurine. A similar trend was observed in the kidney. LP+CYS and LP+PII rats, which expressed higher levels of renal CDO than the LP or HP groups, tended to accumulate larger amounts of hypotaurine than the other dietary treatment groups. In the plasma compartment, hypotaurine values followed a slightly different pattern from those seen for the liver and kidney. Plasma hypotaurine levels were not elevated in rats continued on the HP diet but were elevated in rats switched to the LP+CYS diet. Elevated plasma hypotaurine was observed for rats in the LP+PII treatment group at 6 h, but hypotaurine had returned to initial (0 h HP) levels by 10 h.

Table 1. Effects of experimental diets and proteasome inhibition on hypotaurine levels in liver, kidney, and plasma

Time (h)	0	6			
	[Fasting]	*[Fed]*			
Diet	*HP*	*LP*	*LP+CYS*	*LP+PII*	*HP*
Liver	158±25[a]	37±3[b]	701±247[c]	685±123[c]	405±121[c]
Kidney	179±27[a]	102±17[a]	492±102[b]	432±17[b]	166±16[a]
Plasma	0.5±0.2[a]	0.4±0.2[a]	2.7±0.9[b]	2.8±0.6[b]	0.4±0.2[a]
Time *(h)*		10			
		[Fed]			
Diet		*LP*	*LP+CYS*	*LP+PII*	*HP*
Liver		37±10[b]	999±516[c]	249±191[ac]	337±75[ac]
Kidney		91±24[a]	685±218[b]	315±147[ab]	183±29[a]
Plasma		0.2±0.2[a]	3.5±1.4[b]	0.3±0.4[a]	0.2±0.2[a]

The unit for liver and kidney values is pmol/mg protein. The unit for plasma data is µmol/dl. Within a given tissue, values with different superscripts are significantly different ($P \leq 0.05$).

Unlike the case with hypotaurine, there was a poor association between CDO protein and taurine levels in this study. In the liver, taurine levels fell significantly in LP rats. This was expected as CDO levels were depressed by the LP diet. What was not anticipated, however, was a gradual but significant decline in taurine from the livers of HP control rats by 10 h. This decline occurred in spite of high levels of hepatic CDO. While significant decreases in taurine were occurring in the liver, no significant changes were noted in the kidney for either the LP or HP groups. LP+CYS rats, which maintained high levels of hepatic CDO and elevated levels of kidney CDO, showed no change in either liver or plasma taurine but did exhibit a trend towards increasing taurine levels in the kidney. Taurine levels were significantly reduced in the LP+PII animals relative to both the LP and HP groups at all time points, despite having CDO levels comparable to the HP rats. The largest drop in taurine within this group (63%) occurred by 6 h, with no further decrease by 10 h. This decrease was accompanied by a significant increase in plasma and kidney taurine levels at 6 h that was eventually stabilized by 10 h.

Table 2. Effects of experimental diets and proteasome inhibition on taurine levels in liver, kidney, and plasma

	Time (h)	0 *[Fasting]*	6 *[Fed]*			
	Diet	*HP*	*LP*	*LP+CYS*	*LP+PII*	*HP*
Liver		13±0.9[a]	8±0.1[bd]	11±0.4[af]	4.5±1.7[cd]	11±0.4[af]
Kidney		8.4±0.4[a]	7.5±0.4[a]	8.7±0.5[a]	12±0.7[b]	8.5±0.2[a]
Plasma		66±4[a]	38±6[a]	62±7[a]	147±13[b]	45±10[a]
	Time *(h)*		10 [Fed]			
	Diet		*LP*	*LP+CYS*	*LP+PII*	*HP*
Liver			6.5±1[d]	10±0.3[af]	4.2±1[d]	9.7±1[f]
Kidney			8.4±0.6[a]	10.3±0.6[c]	8.7±0.2[a]	7.4±0[a]
Plasma			40±4[a]	55±12[a]	51±17[a]	38±9[a]

The unit for liver and kidney values is nmol/mg protein. The unit for plasma data is μmol/dl. The unit for plasma data is μmol/dl. Within a given tissue, values with different superscripts are significantly different ($P \leq 0.05$).

4. DISCUSSION

Although the ubiquitin-26S proteasome pathway is thought to be responsible for the half life of most if not all intracellular mammalian proteins, direct evidence for targeted ubiquitination and degradation has been gathered for only a handful of proteins - the majority being short-lived transcription factors and signal transduction molecules (Ciechanover, 2005; Ciechanover and Ben-Saadon, 2004). Studies involving these proteins have relied heavily upon evidence derived from *in vitro* cell-free or intact cell culture models, with no demonstration of physiological regulation of the queried protein by the ubiquitin-26S proteasome system in a living mammal. In this paper, however, we have shown that CDO - a protein involved in intermediary amino acid metabolism - is robustly regulated by the ubiquitin-26S proteasome system *in vivo* and that this regulation has a significant impact on flux through the cysteine sulfinate pathway.

Rats on the LP diet rapidly down-regulated CDO protein levels in response to low cysteine availability. This down-regulation was prevented by pharmacological inhibition of the proteasome. These results are consistent with what we have previously seen in cultured primary hepatocytes and transfected HepG2/C3A cells, which rapidly ubiquitinate and degrade CDO under conditions of low cysteine availability (Stipanuk *et al.,* 2004a). Ubiquitinated species of CDO were elevated in the livers of rats on the LP diet as well as rats on a LP diet that received a specific proteasome inhibitor, though the degree of ubiquitinated CDO accumulation was greater in rats receiving PII. The degradation of liver CDO was also prevented by increasing the dietary availability of cysteine. Again, this parallels nicely with the results of our previous cell culture studies, wherein hepatocyte CDO degradation was markedly attenuated upon incubation in a high cysteine medium. In the cell culture studies, ubiquitination of CDO was down-regulated in response to cysteine. We found a similar phenomenon occurring *in vivo*. The fraction of hepatic CDO as ubiquitinated species was significantly less in rats on the LP +CYS or HP diets compared to their LP or LP+PII cohorts for a given time point. We are currently researching how cysteine signals a change in the ubiquitination status of CDO. Additional work remains in identifying the ubiquitin ligase that docks with CDO and covalently modifies it with ubiquitin.

Prior to this study, efforts to understand the regulation of CDO have focused almost exclusively upon the liver. There are two principal reasons for this investigational bias: the liver is the first organ to be directly exposed to intestinally absorbed cysteine and the liver expresses CDO at concentrations that are orders of magnitude higher than other organs. An earlier study from our lab that evaluated the effects of a long-term (2 week) manipulation of sulfur amino acid intake in rats indicated no long-term changes in CDO protein within the kidney or other nonhepatic tissues (Stipanuk *et al.,* 2002). Our current results, however, suggest that CDO levels in the kidney can be acutely upregulated in response to ingestion of high cysteine (<12 h). The increase in CDO above what was observed in the HP groups may be attributable to the fact that the major sulfur amino acid in casein is methionine, supplementation of which will not increase intracellular cysteine levels as much as directly feeding cysteine to the animal. The modest increase in CDO levels following proteasome inhibition also suggests that renal CDO is regulated via its turnover by the ubiquitin-proteasome system. Because of the low levels of native CDO in the kidney, however, we were unable to directly confirm this hypothesis by detecting the less abundant ubiquitinated species of CDO for any of the treatment groups.

Catalyzing the first step in the cysteine→taurine pathway, CDO is strategically positioned for regulating the synthesis of hypotaurine and taurine. We predicted that an increase in CDO protein, either through the administration of cysteine or PII, would increase flux through the cysteinesulfinate pathway and result in the accumulation of these two amino acids. We did not look for the accumulation of cysteinesulfinate due to the fact that endogenous levels are kept low *in vivo* by the combined actions of cysteinesulfinate/aspartate aminotransferase and cysteinesulfinate decarboxylase (unpublished results). Hypotaurine levels responded in accordance with our prediction, increasing in liver and kidney. Intracellular taurine levels, on the other hand, were not always directly associated with tissue CDO levels. Most notably, taurine levels were actually decreased by proteasome inhibition. The marked increase in taurine plasma levels associated with PII suggests that it may have activated taurine efflux pathways. Variability in taurine levels within the HP groups, however, coupled with the competing use of taurine for osmotic balance and bile acid synthesis (Huxtable, 1992) suggests that using steady-state levels of taurine to estimate cysteine sulfoxidation flux over the time-scale of this study may not be appropriate. Steady-state levels of hypotaurine, which is found at concentrations almost 100-fold lower than taurine and is not involved in any known physiological process apart from taurine synthesis, appear to be a more accurate reflection of flux. Indeed, the levels of this cysteine metabolite have been previously shown to be closely tied to sulfoxidation flux (Kasai *et al.,* 1992).

In summary, our results show that CDO levels within the liver and kidney are actively regulated by the 26S proteasome system. Altering the level of CDO by manipulation of the ubiquitin-proteasome system dramatically affected steady-state levels of hypotaurine, demonstrating that *in vivo*, CDO is an important regulator of cysteine sulfoxidation flux.

5. ACKNOWLEDGMENTS

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