The Effect of CYP2E1-Dependent Oxidant Stress on Activity of Proteasomes in HepG2 Cells

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ABSTRACT

A reduction in proteasome activity and accumulation of oxidized proteins may play a role in alcoholic liver disease. The current study assessed proteasome peptidase activities and oxidative modifications of proteasomes during oxidative stress generated by CYP2E1. The model of toxicity by arachidonic acid (AA) and iron [ferric-nitrilotriacetate (Fe-NTA)] in HepG2 cells overexpressing CYP2E1 (E47 cells) and control C34 cells was used. AA/Fe-NTA treatment decreased trypsin-like (T-L) activity of the proteasome in E47 cells but not in C34 cells. This inhibition was abolished by antioxidants. Chymotrypsin-like activity of the proteasome was increased in E47 cells, and activity was not altered by AA/Fe-NTA treatment. There were no changes in content of subunits of 20S proteasomes or 19S regulator ATPase subunits S4 and p42 by AA/Fe-NTA treatment. An increased content of the PA28α subunit of the 11S regulator of proteasomes was detected in E47 cells. In proteasome pellets, the decline of T-L activity was accompanied by increased content of carbonyl adducts, suggesting oxidative modification of proteasomes. Higher levels of ubiquitinated, 3-nitrotyrosine- and 4-hydroxynonenal-modified proteins and lower levels of free ubiquitin were detected in untreated E47 cells in comparison with C34 cells. Accumulation of protein cross-linked, detergent-insoluble aggregates was increased with AA/Fe-NTA treatment in E47 cells. Thus, reactive oxygen species generated upon CYP2E1-dependent oxidative stress mediated a decline in T-L proteasome function, increased carbonyl adducts in proteasomes, and promoted protein aggregate formation; this may alter the balance among protein oxidation, ubiquitination, and degradation.

ROS production as a consequence of oxidative stress is one of the mechanisms that contribute to the modification and misfolding of proteins (Goldberg, 1995). The extent of accumulation of oxidatively modified proteins depends on both the rate of production and the efficiency of the removal of the modified proteins. The degradation of oxidatively modified proteins is believed to occur by proteasomes present largely in the cytoplasm. Some studies indicate that the ATP-independent 20S proteasome is the form that recognizes and selectively degrades oxidatively modified proteins (Shringarpure et al., 2001), whereas others (Ciechanover and Schwartz, 1994) suggest the involvement of both ATP/ubiquitin-independent (20S proteasome) and ATP/ubiquitin-dependent (26S proteasome) pathways in degrading oxidatively modified proteins. The 20S proteasome is the catalytic core of the 26S proteasome and comprises four heptameric rings of noncatalytic α-subunits and catalytic β-subunits. Both forms of the proteasome cleave substrate proteins at the carboxyl end of basic (trypsin-like), hydrophobic (chymotrypsin-like), and acidic (peptidylglutamyl-peptide hydrolase) amino acids. The 20S proteasome can associate at one or both ends with the 19S regulatory proteasome activator (also known as PA700) containing ATPase activity and ubiquitin or with the 11S regulatory proteasome activator (also known as PA28) containing PA28α and PA28β subunits (Orlowski and Wilk, 2000; Shringarpure et al., 2001).

The ubiquitin-proteasome system is the intracellular proteolytic machinery that is involved in cell cycle regulation, transcriptional factor regulation, cell differentiation, antigen processing, apoptosis, and turnover of normal and misfolded proteins (Rivett and Hearn, 2004; Zhang et al., 2004). The ethanol-inducible CYP2E1 is a catalyst of ROS production and lipid peroxidation in vitro (Ekstrom and Ingelman-Sundberg, 1989). CYP2E1 induction by ethanol is associated with increased oxidative stress in the liver of ethanol-fed rodents.

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ABBREVIATIONS: ROS, reactive oxygen species; ChT-L, chymotrypsin-like; AA, arachidonic acid; Fe-NTA, ferric-nitrilotriacetate; LLVY-MCA, Suc-Leu-Leu-Val-Tyr-7-amido-4-methylcoumarin; LSTR-MCA, Boc-Leu-Ser-Thr-Arg-7-amido-4-methylcoumarin; MCA, 7-amino-4-methylcoumarin; BHT, butylated hydroxytoluene; BHA, butylated hydroxyanisole; DPPD, N,N'-diphenyl-p-phenylenediamine; MEM, minimal essential medium; MTT, 3-[4,5-dimethylthiazol-2-yl]2,5-diphenyltetrazolium bromide; PGP, peptidylglutamyl-peptide hydrolase; T-L, trypsin-like; PAGE, polyacrylamide gel electrophoresis; HNE, 4-hydroxynonenal; 3NT, 3-nitrotyrosine; Z-LLE-2, Z-Leu-Leu-Glu-7-amido-4-methylcoumarin.
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(Nanji et al., 1994; Patacchioli et al., 1999). Intragastric administration of ethanol caused a 35 to 40% decline in the ChT-L proteasome activity in rat liver (Patacchioli et al., 1999). Decreased proteasome activity was associated with increased severity of liver pathology and oxidative stress in alcohol-treated rats (Donohue et al., 2004). Reduction of this ChT-L activity was detected in wild-type mice-fed ethanol but not in CYP2E1 knockout mice, suggesting an important role for CYP2E1 in decreasing the proteasome ChT-L activity (Bardag-Gorce et al., 2000). The addition of various proteasome inhibitors potentiated toxicity in HepG2 cells overexpressing CYP2E1 (E47 cells) by AA/Fe-NTA treatment, and this was associated with accumulation of oxidized and nitrated proteins (Perez and Cederbaum, 2003). CYP2E1 turnover is mediated by the proteasome, because several low-molecular weight inducers of CYP2E1 such as ethanol, dimethyl sulfoxide, and pyrazole elevate CYP2E1 by stabilizing the enzyme against proteasome-catalyzed degradation (Roberts, 1997; Yang and Cederbaum, 1997; Huan et al., 2004). Moreover, the compromised proteasome function is believed to be involved in the formation of Mallory bodies, a hallmark of alcohol-induced liver disease (Bardag-Gorce et al., 2004). In view of the importance of the proteasome in removing oxidized/damaged proteins and in regulating CYP2E1 levels and the possibility that the decrease in proteasome activity may play a role in the development of alcoholic liver injury, the current study was carried out to assess proteasome peptidase activities in HepG2 cells overexpressing CYP2E1 (E47 cells) as compared with control cells transfected with the empty pCI vector (C34 cells).

Materials and Methods

Chemicals. Genetin (G418) was from Invitrogen (Carlsbad, CA). Proteasome peptide substrates LLVY-MCA, LSTR-MCA, and MCA, inhibitors lactacystin and leupeptin, monoclonal anti-2,4-dinitrophenylhydrazine antibody, butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), and DPPD were purchased from Sigma-Aldrich (St. Louis, MO). Proteasome inhibitor Z-Leu-Leu-Leu-CHO (MG132) was from BIOMOL Research Laboratories (Plymouth Meeting, PA). Polyclonal antibodies to 20S proteasome (2C subunit) were from A.G. Scientific (San Diego, CA). Antiubiquitin antibody was from StressGen Biotechnologies (San Diego, CA). The 20S proteasome subunit 11S regulator subunit PA28, 19S regulator ATPase subunits S4 (Rpt2) and p42 (Rpt4), and agarose-immobilized monoclonal antibody to body 20S proteasome subunit α4 were from Affinity BioReagents (Golden, CO). Easy TAG expression protein α-S-labeling mixture was from PerkinElmer Life and Analytical Sciences (Boston, MA). The iron-NTA complex (1:3 Fe/NTA) was prepared as described previously (Sakurai and Cederbaum, 1998). Protein concentration was measured using the Bio-Rad DC protein assay (Bio-Rad, Hercules, CA).

Culture and Treatment of Cells. Two human hepatoma HepG2 cell sublines (Chen and Cederbaum, 1998) were used as models in this study: 1) E47 cells, which constitutively express human CYP2E1, and 2) C34 cells, which are HepG2 cells transfected with the empty pCI vector. All cell lines were grown in minimal essential medium (MEM) containing 10% fetal bovine serum and 0.5 mg/ml G418 supplemented with 100 units/ml penicillin and 100 μg/ml streptomycin in a humidified atmosphere in 5% CO2 at 37°C. Cells were subcultured at a 1:10 ratio once a week. For the experiments, cells were plated at a density of 30,000 cells/ml and incubated for 12 h in MEM supplemented with 5% fetal bovine serum and 100 units/ml penicillin and 100 μg/ml streptomycin. After this period, the medium was replaced with MEM supplemented with or without 20 μM arachidonic acid. After 16 h of incubation at 37°C, the medium was removed and the cells were washed once with phosphate-buffered saline to remove unincorporated arachidonic acid. Then, 25 μM Fe-NTA was added and the cells were incubated for 0, 1, 3, and 5 h before the biochemical analyses. For determination of an effect of antioxidants on proteasome activity, E47 and C34 cells were incubated with or without 50 μM α-tocopherol or 50 μM BHA or 50 μM BHT or 20 μM DPPD for 2 or 12 h before the addition of Fe-NTA.

Isolation of Cytosolic and Proteasome Pellet. Cells were harvested by scraping and homogenized in 100 mM Tris-HCl buffer and 5 mM MgCl2, pH 7.5. Cytosolic fractions were prepared by differential centrifugation of the post supernatant fraction (12,000g) at 100,000g for 1 h at 4°C. To isolate the proteasome pellet, cytosolic fractions from six experiments were combined and centrifuged at 160,000g for 4.5 h at 4°C (Vigouroux et al., 2003).

Cytotoxicity Measurements. Cells were plated onto 24-well plates. After the AA/Fe-NTA treatment, the medium was removed and cell viability was evaluated by the MTT test. To the cells in each well, 250 μl of a 1 mg/ml solution of MTT in MEM was added and the plate was incubated for 1 h at 37°C. At the end of this period, the medium was removed and 500 μl of 1-propanol was added to each well and incubated for 30 min at 37°C. The absorbance of the blue formazan dye was measured at a wavelength of 570 nm with background subtraction at 630 nm. Control refers to incubations in the absence of arachidonic acid and Fe-NTA and was considered as the 100% viability value.

Peptidase Activities of the Proteasome. Chymotrypsin-like, trypsin-like, and peptidylglutamyl-peptide hydrolase activities of the proteasome were assayed using the fluorogenic peptides 50 μM LLVY-MCA, 50 μM LSTR-MCA, and 100 μM Z-Leu-Leu-Glu-7-amido-4-methylcoumarin, respectively. Assays were carried out using 5 to 20 μg of cytosolic protein and 1 to 10 μg of proteasome pellet protein in a total volume of 200 μl. The assay buffer was composed of 100 mM Tris-HCl buffer and 5 mM MgCl2, pH 7.5, and contained the specific peptide substrate. After incubation at 37°C for 30 min, the reaction was quenched by the addition of 500 μl of ice-cold ethanol (Rivett et al., 1994). A 2.0-ml aliquot of H2O was then added, and the fluorescence of the samples was evaluated using a Bio-Tek Kontron SFM 25 spectrofluorometer. The excitation/emission wavelengths were 370/430 nm for amidomethylcoumarin and 333/450 nm for β-naphthylamide products, respectively. Peptidase activities were measured in the absence and presence of the proteasome-specific inhibitor lactacystin (25 μM) for ChT-L and peptidylglutamyl-peptide hydrolase (PGPH) activities and 20 μM leupeptin for T-L activity; the difference between the two rates was attributed to the proteasome activity. Specific peptidase activities were determined by comparison with a standard curve generated using pure fluorescent product 7-amino-4-methylcoumarin. As determined in control experiments, peptidase activities were linear for 30 min under the conditions of the assays.

Western Blot and Immunoprecipitation Analysis. SDS-polyacrylamide gel electrophoresis (PAGE) and immunoblot analysis were carried out by the method of Towbin et al. (1982). Immunoreactive bands were revealed by chemiluminescence using the ECL kit (GE Healthcare, Little Chalfont, Buckinghamshire, UK). The 20S proteasome from the proteasome pellet was immunoprecipitated with agarose-immobilized monoclonal antibody to 20S proteasome subunit α4 (Hendil et al., 1995). Cells were homogenized by sonication for 5 s in 5 volumes of 50 mM Tris-HCl and 17% glycerol, pH 7.4. Homogenates were cleared by centrifugation at 12,000g for 5 min, and equal amounts of protein of each proteasome pellet sample (200–500 μg) were incubated on the rocker for 2 h with 20 to 60 μl of Sepharose with immobilized α4 antibodies. The Sepharose beads were washed three times in 20 mM Tris-HCl, 20 mM NaCl, 0.1 mM Na2EDTA, 1 mM MgCl2, 0.5% NP40, 0.1% SDS, and 17% glycerol, pH 7.5, with centrifugations at 40g for 5 min. The final pellet was suspended in Laemmli buffer. The proteasome content in aliquots of
each sample was first estimated by Western blot using antiproteasome antibody. Equal amounts of precipitated proteasomes were then loaded for SDS-PAGE, and specific protein adducts were detected by using the corresponding antibodies. For immunohistochemical determination of protein carbonyl groups, polyvinylidene difluoride membranes were used for the transfer of proteins, which were sequentially treated with 2,4-dinitrophenylhydrazine and with primary antibody specific for the 2,4-dinitrophenylhydrazine group (Robinson et al., 1999).

Fig. 1. The effect of AA/Fe treatment on viability of C34 and E47 cells. C34 (open circles) and E47 (solid circles) cells were incubated for 16 h in MEM supplemented with or without 20 μM arachidonic acid. After washing, cells were cultured for variable periods (0–5 h) in MEM with 25 μM Fe-NTA. The loss of viability (assessed by MTT reduction activity) in the E47 cells treated with AA/Fe is significantly higher than in the C34 cells at all time points evaluated (*, p < 0.05). Results are from five experiments.

Fig. 2. Detection of oxidative modifications and ubiquitination of cytosolic proteins in C34 and E47 cells. Cells were incubated in MEM with 20 μM AA/25 μM Fe-NTA for 0 to 5 h. Western blot analysis was performed with polyclonal antibody specific to ubiquitinated proteins and to free ubiquitin (10 kDa) (A and B), anti-HNE Michael adduct polyclonal antibody (C), polyvalent 3-nitrotyrosine antibody (D), and monoclonal anti-2,4-dinitrophenylhydrazine antibody (E). The data shown are representative of three separate experiments.

Results

Cell Viability, Oxidative Modification, and Ubiquitination of Cytosolic Proteins. In this study, the model of CYP2E1-dependent synergistic toxicity by arachidonic acid and iron in E47 cells and control C34 cells was used to evaluate proteasome activity and content (Caro and Cederbaum, 2001). As shown in Fig. 1, there was a time-dependent loss of viability when E47 cells were treated with Fe-NTA. Smaller decreases in cell viability were found with the C34 cells. For example, 5 h of AA/Fe-NTA treatment caused cell viability loss of 60% with E47 cells compared with a cell viability loss of only 20% with C34 cells.

Immunoblot analysis of the samples showed a significant rise in high-molecular weight ubiquitinated conjugates in the cytosol of E47 cells before treatment with AA/Fe-NTA as compared with C34 cells (Fig. 2A). The addition of AA/Fe-NTA increased high-molecular weight ubiquitin conjugates in the C34 cells without any further increase over the already elevated conjugates in the E47 cells. A large decrease in levels of free ubiquitin (10 kDa) was found in the E47 cells (Fig. 2B); this could be due to a high rate of its utilization for protein ubiquitination. Basal levels of HNE adducts as well as 3NT adducts in cytosolic proteins in E47 cells were higher before treatment with AA/Fe-NTA than in C34 cells (0-h lanes) and remained elevated at all time points evaluated after the addition of AA/Fe-NTA but were not further increased over the basal level (Fig. 2, C and D). Protein carbonyls were not different between the two cell lines with or without treatment with AA/Fe-NTA (Fig. 2E).

Formation of Detergent-Insoluble Oxidized Protein Aggregates. Because increased levels of ubiquitinated and oxidized proteins were detected in cytosol of E47 cells, we assayed for the presence of protein accumulation and formation of protein-insoluble aggregates as a result of covalent cross-linking and subsequent loss of solubility. Newly synthesized proteins were metabolically labeled with [35S]methionine/cysteine in methionine/cysteine-free minimal essential Eagle's medium plus 10% dialyzed fetal bovine serum for 16 h at 37°C. This method was used to allow determination of the formation of newly produced protein aggregates rather than assaying for the accumulation of aggregates during cell passages. After incubation, the labeling mixture was removed, cells were washed three times with phosphate-buffered saline, and complete MEM was added. Cells were collected in detergent-buffered solution consisting of 1% Triton X-100, 0.5% sodium deoxycholate, and 0.1% SDS in 10 mM Tris-HCl and 1 mM EDTA, pH 8.0, and homogenized by passing through 22-gauge needles attached to syringes. After cell lysis (15 min at 4°C followed by vigorous stirring), samples were centrifuged at 13,000 g for 10 min. The supernatant contained detergent-soluble proteins, whereas the pellet contained detergent-insoluble proteins. The pellet was washed three times and dissolved in 1 N NaOH and subjected to scintillation counting. The formation of protein aggregates is expressed as a percentage of [35S] counts in the aggregates compared with [35S] counts per milligram of total cell protein (Demasi and Davies, 2003).

Statistics. Data are expressed as mean ± S.E. The number of experiments is indicated in the legends to figures. One-way analysis of variance followed by Student-Newman-Keuls post hoc test was used for the determination of the statistical significance.
online/cysteine for 16 h before Fe-NTA treatment. Labeled proteins that could not be solubilized in detergent were recovered as the detergent-insoluble protein pellet after centrifugation at 13,000g. The amount of material in the detergent-insoluble protein pellet was higher in E47 cells compared with C34 cells (Fig. 3), consistent with the increase in ubiquitinated conjugates and protein adducts. Insoluble aggregates increased after 3 h of AA/Fe-NTA treatment with both cell lines, although the increase was greater with the E47 cells (Fig. 3). As a positive control, MG132 increased the amount of material in detergent-insoluble protein pellets in both cell lines and equalized the amount in the two cell lines (Fig. 3).

AA/Fe-Induced Alterations in Proteasome Peptidase Activities. To investigate whether CYP2E1-derived ROS may change proteasome activities, the fluorogenic peptide substrates LLVY-MCA, LSTR-MCA, and Z-LLE-2 were used to measure the chymotrypsin-like, trypsin-like, and peptidylglutamyl-peptide hydrolase activities, respectively. All of the assays were performed in the absence and presence of the proteasome inhibitors lactacystin or leupeptin to reflect proteasome-dependent activity. As shown in Fig. 4, AA/Fe-NTA treatment significantly decreased in a time-dependent manner the T-L activity of the proteasome present in the cytosolic fractions of E47 cells as compared with C34 cells. A 25% loss of T-L activity already occurred after 1 h of AA/Fe-NTA treatment and activity decreased continuously to 44% at 3 h and 69% at 5 h. The basal level of ChT-L activity before the addition of AA/Fe-NTA was higher in the E47 cells and was not significantly altered until the cell viability had declined considerably after 5 h of treatment; the ChT-L activity was much less sensitive to the AA/Fe treatment than the T-L activity in the E47 cells. The peptidase activities in C34 cells did not decrease with AA/Fe-NTA treatment. PGPH activity was low and did not change with the various treatments.

The presence of protein substrates and/or inhibitors or

![Fig. 3. [35S]Methionine/cysteine protein labeling and determination of detergent-insoluble protein aggregates. The content of protein aggregates are expressed as a percentage of [35S] counts in aggregates compared with [35S] counts per milligram of total cell protein. Results are from six experiments.

![Fig. 4. Peptidase activities of the proteasome in cytosolic fractions of C34 and E47 cells. ChT-L, T-L, and PGPH activities of the proteasome were evaluated utilizing the fluorogenic peptides LLVY-MCA, LSTR-MCA, and Z-LLE-2, respectively. Peptidase activities were measured in the absence and presence of the proteasome-specific inhibitors lactacystin (25 μM) for ChT-L and PGPH and leupeptin (20 μM) for T-L activity; the difference between the two rates was attributed to the proteasome activity. Data represent the mean ± S.E. values of four independent analyses.

![Fig. 5. Effect of CYP2E1 oxidative stress on the proteasome. T-L, ChT-L, and PGPH activities of the proteasome were evaluated utilizing the fluorogenic peptides LLVY-MCA, LSTR-MCA, and Z-LLE-2, respectively. Peptidase activities were measured in the absence and presence of the proteasome-specific inhibitors lactacystin (25 μM) for ChT-L and PGPH and leupeptin (20 μM) for T-L activity; the difference between the two rates was attributed to the proteasome activity. Data represent the mean ± S.E. values of four independent analyses.

# p < 0.05 compared with control for C34 cells; §, p < 0.05 compared with control for C34 cells.

**p < 0.01 compared with control for C34 cells; ***p < 0.001 compared with control for C34 cells.

**p < 0.01 compared with control for C34 cells; ***p < 0.001 compared with control for C34 cells.

# p < 0.05 compared with control for C34 cells; §, p < 0.05 compared with control for C34 cells.

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![Effect of CYP2E1 Oxidative Stress on the Proteasome](https://example.com/proteasome.png)
modulators of proteasomes in the cytosolic fractions may affect the measurable activity of proteasomes. To evaluate whether cytosolic factors contribute to the decline in T-L activity of proteasomes in the cytosol of E47 cells by AA/Fe-NTA treatment (the peptide substrates are not absolutely specific for the proteasome), proteasome-enriched pellets were isolated and proteasome peptidase activities were measured in the original cytosols, supernatants (from the high-speed centrifugation), and the proteasome pellets. Table 1 shows the total activity, the activity in the presence of either lactacystin or leupeptin, and the net activities; i.e., lactacystin- or leupeptin-sensitive ChT-L and T-L. All peptidase activities were highly enriched in the proteasome pellets compared with the original cytosols. The ChT-L activities in the pellets were inhibited more than 74% by lactacystin, whereas the T-L activities were inhibited more than 90% by leupeptin, validating that the most of the hydrolytic activities were due to the proteasome. The remaining high-speed supernatant displayed very low, barely detectable activities. An approximate 20% increase in E47 cell basal ChT-L activity found in the cytosol was also found in the proteasome pellets compared with the ChT-L activity in the proteasome pellet of C34 cells. Treatment with AA/Fe-NTA for 3 h caused a 25% decrease in ChT-L activity in the E47 cell proteasome pellets and a 10% nonsignificant decrease in the C34 cell proteasome pellets. The decline in T-L activity after 3 h of AA/Fe-NTA treatment in the cytosolic fraction of E47 cells (approximately 50%) was replicated in the proteasome pellets (approximately 50% loss of activity) (Table 1). A small decrease (approximately 20%) in T-L activity was observed in the C34 cell proteasome pellet after AA/Fe-NTA treatment. PGPH activities essentially were similar between the C34 and E47 cells and relatively resistant to the AA/Fe-NTA treatment.

Kinetic experiments showed that the $V_{max}$ of T-L activity in the proteasome pellet from the E47 cells was decreased with AA/Fe-NTA treatment; $V_{max}$ did not change in C34 cells (approximately 14 nmol/min/mg protein) (Fig. 6A). The $V_{max}$ for T-L activity for the E47 proteasome pellet was 15.1 nmol/min/mg protein for basal time and 11.7, 7.9, and 4.5 nmol/min/mg protein after 1, 3, and 5 h of AA/Fe-NTA treatment (Fig. 6B). The apparent Michaelis constants for LSTR-MCA, the peptide substrate used to assay T-L activity, was 133 (C34 cells) and 333 (E47 cells) $\mu$M; there were no changes in the $K_m$ in both cell lines by AA/Fe-NTA, with the exception of T-L activity in E47 cells at 5 h of treatment when cell viability appreciably declines.

**AA/Fe Effect on the Content of Proteasome Subunits.** To evaluate whether the observed effects of the AA/Fe-NTA treatment on proteasome activity may be caused by changes in the total content of the 20S $\alpha$ structural core subunits, catalytic $\beta$-subunits, as well as in the content of the modulatory 11S and 19S subunits, Western blot analysis using the corresponding antibodies to these proteasome subunits was performed (Fig. 7). The $\beta$-subunits, XYZ ($\beta_5$, $\beta_1$, and $\beta_2$ are the analogous subunits in yeast), contain the catalytic sites associated with ChT-L, PGPH, and T-L activities, respectively. The comparison of proteasome $\alpha$- and $\beta$-subunits contents did not reveal any differences in content in basal levels or any significant alterations during 3 h of AA/Fe-NTA treatment (Fig. 7). After 5 h of treatment, when the level of toxicity and loss of viability were high, some reduction in contents of subunits was observed. The contents of 19S regulator ATPase subunits S4 (Rpt2) and p42 (Rpt4) were also not altered in E47 cells compared with C34 cells with or without AA/Fe-NTA treatment. The basal level of the 11S regulator subunit PA28$\alpha$ was 67% higher in the cytosol of E47 cells compared with C34 cells ($p < 0.05$); treatment with AA/Fe-NTA caused a small insignificant decline (not anticipated increase) in PA28$\alpha$ in the E47 cells (please see legend to Fig. 7 for arbitrary densitometry units for the PA28$\alpha$ results).

**Oxidative Modifications of Proteasomes.** To evaluate possible mechanisms by which the AA/Fe-NTA treatment in
TABLE 1

Effect of 20 μM AA and 25 μM Fe-NTA treatment on proteasome activities in cytosolic, supernatant, and pellet fractions of C34 and E47 cells

Proteasome pellets were isolated from combined cytosolic fractions of six experiments after centrifugation at 160,000g for 4.5 h at 4°C. Proteasome activities are expressed as nanomoles per minute per milligram of protein. Results show that the 3-h treatment with AA/Fe lowered the T-L activity in pellets from the E47 cells to a greater extent than in pellets from C34 cells.

<table>
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<th>E47, 0 h</th>
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<tr>
<td>Leupeptin-sensitive</td>
<td>0.04 ± 0.00</td>
<td>0.03 ± 0.00</td>
<td>0.04 ± 0.00</td>
<td>0.03 ± 0.00</td>
</tr>
<tr>
<td><strong>Pellet</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>4.78 ± 0.11</td>
<td>3.82 ± 0.12</td>
<td>3.36 ± 0.12</td>
<td>1.65 ± 0.12</td>
</tr>
<tr>
<td>Leupeptin</td>
<td>0.24 ± 0.02</td>
<td>0.18 ± 0.04</td>
<td>0.16 ± 0.04</td>
<td>0.06 ± 0.04</td>
</tr>
<tr>
<td>Leupeptin-sensitive</td>
<td>4.54 ± 0.11</td>
<td>3.60 ± 0.12</td>
<td>3.24 ± 0.12</td>
<td>1.59 ± 0.12</td>
</tr>
<tr>
<td><strong>PGPH</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Cytosol</td>
<td>0.01 ± 0.000</td>
<td>0.01 ± 0.000</td>
<td>0.01 ± 0.000</td>
<td>0.01 ± 0.000</td>
</tr>
<tr>
<td>Supernatant</td>
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<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Pellet</td>
<td>0.21 ± 0.001</td>
<td>0.20 ± 0.01</td>
<td>0.24 ± 0.01</td>
<td>0.19 ± 0.01</td>
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</tbody>
</table>

*P < 0.01 compared with control C34 cells.

E47 cells caused the reduction of T-L activity of the proteasome, the possible occurrence of HNE-, carbonyl-, and 3NT-derived adducts in isolated proteasomes was determined. Proteasomes were immunoprecipitated with Sepharose CL-4B-immobilized monoclonal antibody to the 20S proteasome subunit α4. Equal amounts of precipitated proteasomes, as estimated by Western blot, were loaded for SDS-PAGE, and protein adducts were detected by using the corresponding antibodies. Basal levels of carbonyl adducts were similar for the proteasome isolated from C34 and E47 cells; however, an increase in content of carbonyl adducts in proteasomes from the E47 cells was found after 3 h of AA/Fe-NTA treatment, suggesting the occurrence of oxidized modifications of proteasomes (Fig. 8A). Increased content of carbonyl adducts was also detected in proteasome pellets as measured by Western blots (data not shown). Basal levels of HNE adducts were higher in the proteasome pellet from the E47 cells, but these levels were not changed with treatment (Fig. 8B). 3NT adducts in proteasomes from the two cell lines were not different with or without treatment (Fig. 8C).

Discussion

Toxicity by ethanol in the intragastric infusion model required the presence of polyunsaturated fatty acids in the diet and was increased by iron (Castillo et al., 1992; Nanji et al., 1994; Tsukamoto et al., 1995). In developing an in vitro model to mimic the toxicity of ethanol, we found that the addition of AA plus iron caused strong toxicity to HepG2 cells expressing CYP2E1 (Sakurai and Cederbaum, 1998; Caro and Cederbaum, 2001). Lipid peroxidation is a major contributor to the developing toxicity in E47 cells exposed to Fe-NTA and arachidonic acid (Caro and Cederbaum, 2001).

Higher levels of HNE Michael adducts and 3NT protein adducts were found in cytosolic proteins of E47 cells compared with C34 cells, with or without AA/Fe-NTA treatment. Similarly, increased levels of high-molecular weight ubiquitinated proteins and decreased levels of free ubiquitin (10 kDa) were found in the cytosol of E47 cells with and without AA/Fe treatment compared with C34 cells. Covalent binding of ubiquitin to proteins is believed to be an important mechanism by which proteins are marked for subsequent degradation by ubiquitin/ATP-dependent 26S proteasomes (Ciezhanower and Schwartz, 1994). The appearance of high levels of ubiquitinated proteins and HNE and 3NT protein adducts in the E47 cells probably reflects the effects of increased ubiquitination or adduct modification of oxidized proteins caused by CYP2E1-generated ROS. It is not likely that diminished rates of degradation play an important role in accumulation of these conjugates under basal conditions, because T-L activity was similar in C34 and E47 cell cytosol and ChT-L activity was higher with the E47 cells under basal conditions. However, decreased T-L activity caused by the AA/Fe-NTA treatment may contribute to the maintenance of the elevated accumulation of ubiquitinated or HNE or 3NT conjugates in E47 cells.

The presence of high-molecular weight ubiquitinated proteins and various modified protein adducts could lead to the formation of cross-linked detergent-insoluble aggregates in the E47 cells. Indeed, enhanced protein aggregate accumulation was found in untreated E47 cells compared with C34 cells, and AA/Fe-NTA treatment further increased accumulation of these protein aggregates. The failure of AA/Fe-NTA treatment to further elevate levels of ubiquitinated proteins and various protein adducts may suggest that accumulation of these modified proteins does not play a critical role in the...
developing toxicity produced by AA/Fe-NTA. Lack of accumulation may be due to the ChT-L activity of the proteasome, which is not decreased by AA/Fe-NTA. Alternatively, modified proteins may, under conditions of severe oxidative stress produced by AA/Fe-NTA, accumulate as insoluble aggregates. The involvement of free radicals and cross-linking reactions by aldehydic lipid peroxidation products or carbohydrates has been postulated as one of the initial steps in formation of oxidized cross-linked aggregates (Nakano et al., 1995; Grune et al., 1997). Heavily oxidized and cross-linked proteins are poor substrates for the proteasome, and such aggregates are able to inhibit the proteasome (Grune and Davies, 2003). Most aggregated proteins, particularly cross-linked aggregates, may no longer fit into the proteasome and therefore clog them up (Kisselev et al., 2002). Using mutant aggregation-prone proteins, Bence et al. (2001) showed that protein aggregates caused nearly complete inhibition of the ubiquitin-proteasome system, cell cycle arrest, and cell death. Mallory bodies (often a hallmark of alcoholic liver disease) are believed to be aggregates composed of cytoker-
at, ubiquitinated proteins, and various other proteins that form in diseased liver because of disruption in the ubiquitin-proteasome protein degradation pathway (Riley et al., 2003). It is interesting to speculate that CYP2E1-dependent ROS production and oxidant stress may play role in Mallory body formation via formation of protein aggregates and inhibition of proteasome activity.

To evaluate whether the rise in levels of ubiquitinated, oxidatively modified proteins and accumulation of protein aggregates in E47 cells could be a result of proteasome failure to degrade these oxidatively modified proteins, ChT-L, T-L, and PGPH activities of proteasomes were determined. The T-L activity of the proteasome was the most sensitive to inhibition by CYP2E1-dependent oxidative stress. Higher sensitivity of T-L activity of proteasome to ROS production during coronary occlusion/reperfusion was also observed (Bulteau et al., 2001). The inhibition of T-L activity was detected in cytosol as well as in high-speed proteasome pellets of the E47 cells. V\textsubscript{max} for T-L activity was lower after AA/Fe-NTA treatment of E47 cells, whereas K\textsubscript{m} for LSTR-MCA was higher compared with the C34 cells. It is noteworthy that alcohol consumption caused a similar decrease of V\textsubscript{max} and increase of K\textsubscript{m} (289 versus 171 \textmu M for controls) for LSTR-MCA in cytosolic fractions of rat livers (Donohue, 2002), as found with the E47 cells.

That the reduction of T-L proteasome activity produced by AA/Fe-NTA treatment of E47 cells is due to oxidative changes is supported by the prevention of the loss of the T-L activity by the antioxidants α-tocopherol, BHA, BHT, and DPPD. α-Tocopherol prevented the increase in lipid peroxidation and the loss in cell viability produced by AA/Fe-NTA treatment in E47 cells (Caro and Cederbaum, 2001). Despite the decline of T-L activity in E47 cells by AA/Fe-NTA treatment, there was no loss in content of proteasomes as estimated by measuring levels of several main α- and β-proteasome subunits as well as the 19S regulator ATPase subunits. It is not known whether the decline in T-L activity plays a role in the development toxicity by AA/Fe-NTA to the E47 cells. Time courses for both are similar, and treatment with antioxidants prevents both. In addition, proteasome inhibitors, including lactacystin, increase the toxicity of AA/Fe-NTA to the E47 cells (Perez and Cederbaum, 2003). However, the ChT-L activity was still functional during the period of toxicity produced by AA/Fe-NTA.

An increased content of the PA28\alpha subunit of the 11S regulator of proteasomes was detected in the cytosol of E47 cells before AA/Fe-NTA treatment and remained elevated with treatment. Based on information obtained from genetic, biochemical, and crystal structure analysis, the 20S proteasome activity is largely controlled by gating of the central opening within the outer α-subunits rings of the 20S proteasome and the regulated import of proteolytic substrates (Kohler et al., 2001). PA28 subunits are positive allosteric effectors of proteasome activity, and the binding of PA28 to the 20S proteasome causes conformational changes of α- and β-subunits to facilitate substrate access to or product release from proteasome active sites (Knowlton et al., 1997; Voges et al., 1999). Therefore, the necessity for increase of proteolysis of oxidatively modified protein by the opening state of proteasomes may explain the increase of PA28\alpha content in E47 cells. Whether this contributes to the elevated ChT-L activity is not known. We speculate that this increase and perhaps the increase in basal ChT-L activity in E47 cells reflect an adaptation to CYP2E1-generated oxidant stress.

It was shown that decreases of peptidase activities of proteasomes after HNE treatments or during occlusion/reperfusion procedures were accompanied by free radical-derived modifications of the 20S proteasomes (Reinheckel et al., 2000; Bulteau et al., 2001). We found an increase in the protein-associated carbonyl adducts in proteasomes of E47 cells after 3 h of AA/Fe-NTA treatment. Carbonyls can be generated in response to a wide variety of oxidizing agents, including alkoxy and peroxy radicals (Griffiths et al., 2002).

In summary, increased levels of endogenous ubiquitinated, HNE-modified proteins, and N\textsubscript{T}NT adducts and accumulation of detergent-insoluble protein aggregates were found in E47 cells compared with C34 cells. Accumulation of oxidized and ubiquitinated proteins may stimulate endogenous ChT-L activity, perhaps by promoting opening of the gate of 20S proteasomes by increasing PA28\alpha levels. AA/Fe-NTA treatment caused a further accumulation of aggregates and oxidative modifications of proteasomes that seem to cause a decline of T-L proteasome activity. We suggest that the accumulation of ubiquitinated and HNE-modified cytosolic proteins and insoluble protein aggregates, as well as proteasome T-L activity impairment, may contribute to the toxic effect of AA/Fe-NTA treatment on E47 cells and speculate that such interactions between CYP2E1 and the proteasome may contribute to the decline in protease activity found after alcohol treatment.

References


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