The Glutathione Precursor L-2-Oxothiazolidine-4-Carboxylic Acid Protects Against Liver Injury due to Chronic Enteral Ethanol Exposure in the Rat

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L-2-oxothiazolidine-4-carboxylic acid (OTC) is a cysteine prodrug that maintains glutathione in tissues. Here, its effect on alcohol-induced liver injury in an enteral alcohol feeding model was investigated. Male Wistar rats were given control high-fat or ethanol containing diets enterally for 4 weeks. Treated rats received 500 mg/kg/d of dietary OTC. Ethanol delivery, weight gain, and the cyclic pattern of ethanol in the urine were not different between the OTC-ethanol and ethanol groups. After 4 weeks, serum aspartate transaminase (AST), necrosis and inflammation were elevated significantly by ethanol compared with appropriate high-fat controls, effects blocked by OTC. Moreover, ethanol elevated hepatic tumor necrosis factor α (TNF-α) messenger RNA (mRNA) and the nuclear transcription factor nuclear factor κB (NF-κB) 2-3 fold. NF-κB in isolated Kupffer cells was also increased by ethanol. These effects were all blocked by OTC treatment. Additionally, superoxide production was higher in Kupffer cells isolated from ethanol-treated rats, an effect blunted by OTC. OTC also increased circulating glutathione (GSH) levels about 2-fold; however, GSH levels were not affected by ethanol or OTC in livers from the groups studied. Surprisingly, GSH was elevated by ethanol and OTC treatment in isolated Kupffer cells about 2-fold. Moreover, GSH (Ki-10 μM/L) and cysteinylglycine, but not oxidized glutathione (GSSG) or OTC, blunted the LPS-induced increase in calcium in isolated Kupffer cells, possibly by activating a glycine-gated chloride channel due to their structural similarity with glycine. Collectively, it is concluded that GSH is protective, in part, by increasing circulating GSH, which blunts activation of Kupffer cells via the glycine-gated chloride channel. (HEPATOLOGY 2000;31:391-398.)

Abbreviations: GdCl3, gadolinium chloride; TNF-α, tumor necrosis factor α; GSH, glutathione; OTC, L-2-oxothiazolidine-4-carboxylic acid; AST, aspartate transaminase; HBSS, Hanks’ balanced salt solution; EGTA, ethylene glycol bis (β-aminoethyl ether)-N,N,N’,N’-tetraacetic acid; cDNA, complementary DNA; PCR, polymerase chain reaction; TBE, Tris-borate-ethylene diaminetetraacetic acid; mRNA, messenger RNA; NF-κB, nuclear factor κB; LPS, lipopolysaccharide.

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MATERIALS AND METHODS

Animals and Sample Collection. Male Wistar rats (300-320 g) used in this study were housed in a facility approved by the Association for the Accreditation and Assessment for Laboratory Animal Care. An intragastric cannula was surgically implanted as described by Tsukamoto et al.,11 and rats were given a high fat liquid diet for 4 weeks. The diet consisted of corn oil (37% of calories), protein (23%), carbohydrate, minerals, vitamins, and ethanol (ethanol diet) or isocaloric dextrin-maltose (control diet) supplemented with lipotropes.12,13 OTC (a kind gift from Cynthia Leaf, Transcend Therapeutics) was added to the diet at a dose of 500 mg/kg/d.10 The ethanol concentration ranged between 7% and 9% vol/vol depending on the demonstrated degree of intoxication and the urine alcohol concentration. Rats were given 10 to 14 g/kg/d ethanol. Each
morning, 24-hour urine samples were collected from the vessel below each metabolism cage, and the ethanol concentration was determined enzymatically. Blood was sampled weekly from the tail vein and centrifuged, and serum was stored frozen before determination of aspartate transaminase (AST) using Sigma analytical kits (St. Louis, MO).

**Histology.** Liver biopsy samples were collected at 2-week intervals for histological evaluation. Rats were anesthetized using Metofane, and livers were harvested and fixed in 10% buffered formalin. Tissue was stained with hematoxylin and eosin to assess inflammation, steatosis, and necrosis and was scored using the method described by Nanji et al.15 The number of cells containing fat was estimated as follows: <25% = 1; <50% = 2; <75% = 3; >75% = 4.

Areas of inflammation and necrosis were scored as 1 for 1 foci per low magnification field or 2+ for 2 or more foci per field. Slides were evaluated by one author blind to the treatments as well as by an outside expert in rodent liver pathology.

**Kupffer Cell Isolation and Culture.** Kupffer cells were isolated from rats by collagenase digestion and differential centrifugation using density gradients of Percoll (Pharmacia, Uppsala, Sweden) as described elsewhere with slight modifications. Briefly, the liver was perfused in situ through the portal vein with Ca2+- and Mg2+-free Hank’ balanced salt solution (HBBS) containing 0.5 mmol/L ethylene glycol bis (β-aminoethoxy) ether-N,N,N’,N’-tetraacetic acid (EGTA) at 37°C for 5 minutes at a flow rate of 26 mL/min. Subsequently, perfusion was with HBBS containing 0.025% collagenase IV (Sigma Chemical Co., St. Louis, MO) at 37°C for 5 minutes. After the liver was digested, it was excised and cut into small pieces in collageanase buffer. The suspension was filtered through nylon gauze, and the filtrate was centrifuged twice at 50g at 4°C for 3 minutes to remove parenchymal cells. The nonparenchymal cell fraction was washed with buffer and centrifuged on a density cushion of Percoll at 1,000g for 15 minutes to obtain the Kupffer cell fraction, followed by washing with buffer again. The viability of isolated Kupffer cells was determined by trypan blue exclusion and routinely exceeded 90%. Cells were seeded onto 24-well culture plates (Corning Inc., Corning, NY) or 25-mm glass coverslips at a concentration of 5 × 10³ and cultured in Dulbecco’s modified Eagle medium ( Gibco Laboratories Life Technologies Inc., Grand Island, NY) supplemented with 10% fetal bovine serum and antibiotics (100 U/mL of penicillin G and 100 mg/mL of streptomycin sulfate) at 37°C with 5% CO2. Nonadherent cells were removed after 1 hour by replacing the culture medium. All adherent cells phagocytosed latex beads, indicating that they were Kupffer cells. Cells were cultured for 24 hours before experiments.

**Measurement of Intracellular Ca2+.** Intracellular Ca2+ in individual Kupffer cells was measured fluorometrically using the calcium indicator dye fura-2 and a microspectrofluorometer (Photon Technology International, South Brunswick, NJ) interfaced with an inverted microscope (Diaphot, Nikon, Japan). Kupffer cells cultured on coverslips were incubated in modified Hank’s buffer (115 mmol/L NaCl, 5 mmol/L KCl, 0.3 mmol/L NaH2PO4, 0.4 mmol/L KH2PO4, 5.6 mmol/L glucose, 0.8 mmol/L MgSO4, 1.26 mmol/L CaCl2, 15 mmol/L N-2-hydroxyethylpipperazine-N’-2-ethanesulfonic acid, pH 7.4) containing 5 mmol/L fura-2/acetoxymethyl ester (Molecular Probes Inc., Eugene, OR) and 0.03% Pluronic F127 (BASF Wyandotte, Wyandotte, MI) at room temperature for 60 minutes. Coverslips plated with Kupffer cells were rinsed and placed in chambers with buffer at room temperature. Changes in fluorescence intensity of aspartate transaminase (AST) using Sigma analytical kits (St. Louis, MO) at room temperature. Changes in fluorescence intensity of AST were monitored in individual Kupffer cells. Each dotter, Wyandotte, MI) at room temperature for 60 minutes. Coverslips plated with Kupffer cells were rinsed and placed in chambers with buffer at room temperature. Changes in fluorescence intensity of AST were monitored in individual Kupffer cells. Each dotter, Wyandotte, MI) at room temperature for 60 minutes. Coverslips plated with Kupffer cells were rinsed and placed in chambers with buffer at room temperature. Changes in fluorescence intensity of AST were monitored in individual Kupffer cells. Each cover slip was covered with a wax gem (Perkin Elmer, Cetus, Norwalk, CT), and 0.5 mg oligo d(T) 12-18 primer (BRL). After the reaction mixture reached 42°C, 400 U of reverse transcriptase (BRL) was added to each tube and the sample was incubated for 30 minutes at 42°C. Reverse transcription was stopped by denaturing the enzyme at 99°C. The reaction mixture was diluted with distilled water to a final volume of 50 µL. Commericially available polymerase chain reaction (PCR) primers for β-actin were purchased from Clontech Laboratories, Inc. (Palo Alto, CA). Primers for TNF-α (Stratagene, La Jolla, CA), contained the following sequences: TNF-α sense (5'AGAGCAGACAAGAGCATGATG-3') and antisense (5'-TACAGGCCTGTCACTCGAAATTT-3'). The size of amplified PCR products for TNF-α was 692 base and 281 base pairs for β-actin. Aliquots (5 µL) of synthesized cDNA were added to 45 µL of PCR mix containing 5 µL of 10 × PCR buffer, 1 µL of each deoxynucleotide (1 µmol/L), 0.5 µL of sense and antisense primers (0.15 µmol/L), and 0.25 µL of DNA polymerase (Gene Amp PCR kit; Perkin Elmer Cetus).

The reaction mixture was covered with a wax gem (Perkin Elmer Cetus), and amplification was initiated by 1 minute of denaturation at 94°C for 1 cycle, followed by multiple (20 to 35) cycles at 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 1 minute using a GeneAmp PCR system 9800 DNA Thermal Cycler (Perkin Elmer Cetus). After the last cycle of amplification, samples were incubated for 7 minutes at 72°C. Dilutions of cDNA were amplified for 20, 25, 28, 30, 33, and 35 cycles to define optimal conditions for linearity to permit semiquantitative analysis of signal strength. When appropriate, the specificity of the PCR bands was confirmed by restriction site analysis of the amplified cDNA, which generates restriction fragments of the expected size (data not shown). The amplified PCR products were subjected to electrophoresis at 75 volts through 2% agarose gels (BRL) for 1 hour using the X714 Hae III digest for molecular weight markers (Sigma Chemical Co.). Agarose gels were stained with 0.5 mg/mL ethidium bromide Tris-borate-ethylene dianimintetraacetic acid (TBE) buffer (ICN, Costa Mesa, CA) and photographed with type 55 Polaroid positive/negative film. The relative amount of messenger RNA (mRNA) transcript was determined using Eagle Eye 11 Still Video System (Stratagene Inc., La Jolla, CA). Densitometric analysis of the captured image was performed on a Macintosh computer using NIH
remained constant. Daily urine alcohol concentrations fluctuated even though the alcohol concentration in the diet received 12 g/kg/d after 1 week of treatment with ethanol (11.9 g/kg/d) during the 4 weeks of this study. On average, rats in the OTC groups received increasing amounts of ethanol (10.5-12.8 g/kg/d) between the groups. Rats in the ethanol and ethanol plus OTC groups gained body weight throughout the 4 weeks of this study at a rate of 6.9 g/day. There were no significant differences in body weight at the end of the study (Fig. 2). Rats placed on the Tsukamoto-French protocol maintained their body weight throughout the 4 weeks of this study at a rate of 6.9 g/day. There were no significant differences in body weight at the end of the study (Fig. 2).

Binding conditions for nuclear factor κB (NF-κB) were characterized, and electrophoretic mobility shift assays were performed as described in detail elsewhere. Briefly, 8 to 40 µg of nuclear extract from cells or liver tissue were preincubated for 10 minutes on ice with 1 µg poly (dl-dc) and 20 µg bovine serum albumin (BSA) (both from Pharmacia Biotech, Piscataway, NJ) in a buffer containing 1 mmol/L HEPES (pH 7.6), 40 mmol/L MgCl₂, 0.1 mol/L NaCl, 8% glycerol, 0.1 mmol/L dithiothreitol (DTT), 0.05 mmol/L ethylenediaminetraacetic acid, and 2 µL of a 21P-labeled DNA probe (10,000 cpm/µL, Cerenkov) containing 0.4 ng of double-stranded oligonucleotide. Mixtures were incubated 20 minutes on ice and resolved on 5% polyacrylamide (29:1 cross-linking) and 0.4 × TBE gels. After electrophoresis, gels were dried and exposed to Kodak film. Specificity of NF-κB binding was verified by competition assays and ability of specific antibodies to supershift protein/DNA complexes. In competition assays, 100-fold excess of unlabeled oligonucleotide was added 10 minutes before addition of the labeled probe. In supershift experiments, 1 µL of rabbit antisera against p50 protein (Santa Cruz Biotech, Santa Cruz, CA) was added to the reaction mixture after incubation with labeled probe, which was further incubated at 25°C for 30 minutes. Labeled and unlabeled oligonucleotides contained the consensus sequence for NF-κB (top strand: 5'-GCAGAGGGATTCCGGAG-3'; bottom strand: 5'-GTCTGCGAAGGTCCCTCTGG-3'). Data were quantitated by scanning autoradiograms with GelScan XL (Pharmacia LKB, Uppsala, Sweden).

**Measurement of Superoxide (O₂⁻) Production by Kupffer Cells.** Kupffer cells O₂⁻ production was measured by the superoxide dismutase-inhibitable reduction of ferricytochrome c. Cells were plated in 24-well tissue culture plates at 10⁶ cells/well and cultured at 37°C for 24 hours in Dulbecco's Modified Eagle Medium-High Glucose (DMEM-H) with 10% fetal bovine serum. Supernatant was replaced with HBSS containing Mg²⁺ and Ca²⁺ supplemented with ferricytochrome c (0.8 mg/mL, final concentration). LPS (10 µg/mL) was added to stimulate Kupffer cells. The reduction of ferricytochrome c was measured both in the presence and absence of superoxide dismutase (85 U/mL). The difference in absorbance of ferricytochrome c, measured at 550 mmol/L, was used to calculate O₂⁻ concentration, using a molar extinction coefficient of 17,500.

**Statistics.** Student's t test or ANOVA was used for determination of statistical significance as appropriate. Comparison of pathological scores was determined using Mann-Whitney rank sum test. Significance was defined as a P value of less than .05.

**RESULTS**

**In Vivo Experiments.** Ethanol and ethanol plus OTC–treated rats placed on the Tsukamoto-French protocol maintained their body weight throughout the 4 weeks of this study at values around 300 g. There were no significant differences between the groups. Rats in the ethanol and ethanol plus OTC groups received increasing amounts of ethanol (10.5-11.9 g/kg/d) during the 4 weeks of this study. On average, rats received 12 g/kg/d after 1 week of treatment with ethanol diet. As reported previously, the daily urine alcohol concentration cycled even though the alcohol concentration in the diet remained constant. Daily urine alcohol concentrations fluctuated between 0 and 390 mg/dL alcohol with a periodicity of 5 to 6 days similar to previous reports. This cyclic pattern was not affected by treatment with OTC; however, average urine alcohol concentrations were the same (ethanol, 203 ± 28; and ethanol plus OTC, 211 ± 30 mg/dL).

Serum AST release in the 3 groups studied is summarized in Fig. 1. There were no differences between the control and ethanol plus OTC groups. However, AST levels were elevated significantly in the ethanol group at 4 weeks. Histological evaluation of steatosis of liver at 2 and 4 weeks indicated that ethanol treatment increased fat deposition over the 4-week period as expected (Figs. 2 and 3). OTC treatment did not alter this pattern of fat accumulation. Moreover, inflammation caused by ethanol treatment was detected at 2 weeks and the inflammation score was increased 4-fold by 4 weeks; however, necrosis was not detected until the fourth week of ethanol treatment in this study. The addition of OTC significantly blunted the increase in inflammation by about 50% and decreased necrosis significantly at 4 weeks (Fig. 3).

**In Vitro Experiments.** OTC does not elevate glutathione but rather maintains tissue levels. Indeed, hepatic glutathione levels were not altered significantly in the groups studied here (control, 1,972 ± 128; control plus OTC, 2,329 ± 567; ethanol, 2,142 ± 214; ethanol plus OTC, 1,917 ± 344 nmol/g liver). However, plasma GSH levels of 4.6 ± 0.5 µmol/L after 4 weeks of enteral ethanol were nearly doubled (8.0 ± 2.3 µmol/L) when rats were given OTC with ethanol (P < .05) but were not different in the control groups (data not shown).

Four weeks of enteral ethanol caused a large increase in...
TNF mRNA (lanes 5-7, upper panel Fig. 4) compared with appropriate high-fat controls (lanes 2-4). However, in rats given OTC with ethanol, (lanes 8-10), TNF mRNA was not elevated. Indeed, when data are presented as a fraction of the housekeeping gene, β-actin, ethanol significantly increased TNF-α about 3-fold, an effect that was blocked totally by OTC (Fig. 4, lower panel).

Because TNF-α synthesis requires NF-κB, the effect of ethanol and OTC on the transcription factor NF-κB was examined (Fig. 5, upper). In nuclear extracts from chow and high-fat control animals (lanes 2-5), NF-κB was detectable but not different. However, much more intense staining was observed in samples from ethanol-treated rats (lanes 6 and 7). This increase was blunted by OTC (lanes 8-9). Densitometric analysis of the mobility shift assay showed that ethanol increased NF-κB over 2-fold, and effect largely blunted by OTC (Fig. 5, lower). Furthermore, nearly identical results were obtained in isolated Kupffer cells harvested from rats treated for 4 weeks. In these experiments, NF-κB in isolated Kupffer cells was increased over 2-fold by ethanol treatment, an effect that was blunted to control levels in cells isolated from ethanol plus OTC treated rats (data not shown). In isolated Kupffer cells, superoxide production was increased significantly by ethanol and blunted by OTC (control, 0.18 ± 0.02; ethanol, 0.27 ± 0.03; ethanol plus OTC, 0.18 ± 0.02 nmol/min/10^6 cells; P < .05).

Recently, a glycine-gated chloride channel has been de-
scribed in Kupffer cells which, when activated, blunts the increase in calcium caused by endotoxin by hyperpolarizing the cell membrane.23 Because glycine is one of the amino acids of the tripeptide GSH, the effect of GSH on Kupffer cell intracellular calcium was examined (Fig. 6A). When lipopolysaccharide (LPS) was added to isolated Kupffer cells, intracellular calcium increased rapidly reaching values between 300 and 400 nmol/L in less than 1 minute then slowly returning to basal levels. In the presence of GSH (1 mmol/L), however, values only increased about half as high. The dose-response for GSH on intracellular calcium is depicted in Fig. 6B. GSH blunted the response of Kupffer cells to LPS at very low concentrations. Further, the GSH derivative cysteinyl-glycine had similar effects (Fig. 6C); however, neither oxidized glutathione (GSSG) (100 µmol/L) or OTC (2 mmol/L) added to isolated Kupffer cells affected the stimulation of calcium by LPS. Furthermore, the increase in intracellular calcium was also blunted about 50% by cysteinyl-glycine (Fig. 6C and 6D).

**DISCUSSION**

**OTC Prevents Early Alcohol-Induced Liver Injury.** Here, ethanol increased serum transaminases, hepatic fat, inflammation, and necrosis confirming earlier work (Fig. 3).3 Although OTC had no effect on hepatic fat accumulation, it significantly blunted the increase in transaminases, inflammation and necrosis caused by ethanol. Therefore, it is concluded that GSH is beneficial in early alcohol-induced liver injury. What is its mechanism of action?

**OTC and Glutathione.** The protective effect of GSH has been studied and characterized in models of oxidative stress and cellular injury.24,25 Glutathione is synthesized by the γ-glutamyl cycle and γ-glamylcysteine synthetase produces glutamyl l-cysteine. The subsequent addition of glycine catalyzed by glutathione synthetase generates glutathione. OTC is metabolized intracellularly to cysteine, and because γ-glamylcysteine synthetase is limiting, glutathione synthesis is increased.26 By providing cysteine, L-2-oxothiazolidine-4-carboxylic acid has been shown in many studies to replete glutathione (see Results).10 In this study, OTC did not affect absolute levels of GSH; however, it most likely increases GSH turnover. However, OTC was administered slowly over 24 hours most likely explaining why tissue GSH levels were not elevated here (see Results). Alternatively, OTC could change the properties of the sinusoidal GSH transport.

OTC is protective in hepatocytes.27 It inhibited increases in serum alanine transaminase caused by dichloroethylene by about 10-fold. Recently, OTC was shown to increase GSH in normal cells and to increase sensitivity to chemotherapeutic agents.28 Liver GSH increased 2-fold after a bolus dose of OTC.29

**Kupffer Cells and Endotoxin Are Involved in Alcohol-Induced Liver Disease.** Considerable evidence has accumulated supporting the hypothesis that gut-derived endotoxin is involved in early alcohol-induced liver injury. For example, sterilization of the gut with nonabsorbable antibiotics4 and decreasing endotoxin with lactobacillus feeding15 minimized injury caused by alcohol in the enteral feeding model. Further, destruction of Kupffer cells with GdCl3 or hyperpolarization of the Kupffer cell membrane with glycine also diminished injury caused by enteral alcohol.30 It is becoming clear that gut-derived endotoxin activates Kupffer cells to produce toxic free radicals and cytokines in alcohol-induced liver disease.8 Indeed, α-hydroxethyl radical has been detected in the Tsukamoto-French model and is most likely of Kupffer cell origin since it was diminished by GdCl3.31 In this study, GSH levels were increased in plasma and Kupffer cells after treatment with ethanol and OTC. GSH is decreased in whole liver by ethanol;32 however, ethanol increased GSH about 2-fold in Kupffer cells by a mechanism not currently understood. Because ethanol increases GSH in Kupffer cells in the
presence and absence of OTC, increased GSH in Kupffer cells alone cannot explain the protective effect of OTC observed here. Plasma GSH was elevated about 2-fold after OTC treatment (see Results). Based on the data in Kupffer cells, plasma, and whole liver, we hypothesize that elevated plasma GSH binds to the glycine-gated chloride channel receptor on the Kupffer cell and blunts the effect of circulating endotoxin caused by ethanol treatment (Fig. 7). Alternatively, one possible action of OTC in this study is to diminish free radicals caused by the nucleophilic actions of GSH. Indeed, ethanol increased and OTC decreased superoxide formation by isolated Kupffer cells (see Results). In further support of this idea, the transcription factor NF-κB, which is activated by oxidants and was increased by ethanol in this study, was not elevated in the presence of OTC in liver (Fig. 5) and in Kupffer cells (see Results). Therefore, it is likely that activation of NF-κB leads to transcription of TNF-α, which is involved in alcohol-induced liver injury. OTC blocks this inflammatory cascade most likely because GSH directly down-regulates IκB phosphorylation. LPS transduces signals through CD14 and TLR4 or TLR2, and the subsequent cascade includes TRAF6, NIK, and IKKs. Therefore, if GSH down-regulates IκB phosphorylation, NF-κB activation would be blocked and TNF production by non-parenchymal cells would be reduced.

Serum TNF-α levels were a good predictor of survival in alcohol-induced hepatitis, and anti-TNF-α antibody minimized injury in the enteral alcohol feeding model of Tsukamoto and French. In this study, OTC prevented the increase in TNF-α mRNA due to alcohol supporting the hypothesis that it interferes with an endotoxin-Kupffer cell-TNF-α mechanism (Fig. 4).

Role of GSH in Alcohol-Induced Liver Injury. In an important study, Hirano et al. showed that chronic enteral feeding with ethanol decreased hepatic GSH. Further, they showed that the small mitochondrial GSH pool was particularly sensitive to ethanol, and suggested that it may have particular importance in alcohol-induced liver injury. The lack of effect of ethanol on GSH in this study is not understood but may be caused by the fact that subcellular pools were not examined. Alcohol-induced liver injury is blocked in the TNF-receptor-1 knockout mouse supporting the hypothesis that TNF is essential. Furthermore, TNF stimulates mitochondrial production of reactive oxygen species, which can be reversed by GSH. In this study, however, there were not remarkable effects of ethanol and OTC on hepatic GSH levels (see Results). This does not rule out an effect, however, on GSH turnover. On the other hand, OTC increased plasma GSH in this study. Moreover, here it was shown that GSH and

![Graph](image1)

**Fig. 5.** The effect of ethanol and OTC on NF-κB. Nuclear extracts from whole liver of chow-fed, ethanol, or ethanol plus OTC-treated rats were incubated with 32P-labeled double-stranded oligonucleotide encompassing the κB motif to detect NF-κB DNA binding activity. Upper panel: representative autoradiogram of electrophoretic mobility shift assay. Lower panel: densitometry. Representative experiments.

![Graph](image2)

**Fig. 6.** Effect of glutathione and cysteinyl-glycine on intracellular calcium in isolated Kupffer cells. Intracellular calcium was measured in isolated Kupffer cells fluorometrically using fura-2 as described in the Materials and Methods. (A) Changes in intracellular calcium after the addition of LPS in the presence and absence of GSH. (B) A dose-response of GSH on peak intracellular calcium is depicted. (C) The effect of cysteinyl-glycine on intracellular calcium in isolated Kupffer cells. (D) Average peak calcium data in presence and absence of cysteinyl-glycine. Data are from 3 to 6 individual measurements. *P < .05 using Student’s t test.
cysteinyl-glycine blunted the increase in Ca\(^{2+}\) in Kupffer cells caused by endotoxin (Fig. 6). Evidence has accumulated that glycine blunts the increase in Ca\(^{2+}\) and cytokine production by activating a glycine-gated chloride channel in Kupffer cells (Fig. 7). It has been shown that other amino acids such as glycine, \(\beta\)-alanine, and taurine also blunt the increase in intracellular calcium by LPS via a similar mechanism.\(^{17,36}\) In support of this hypothesis, these phenomena were antagonized by strychnine, dependent on extracellular chloride, and associated with a large influx of radiolabeled chloride.\(^{10}\) Because GSH and cysteinyl-glycine also blunted the increase in Ca\(^{2+}\) due to LPS, it is proposed here that a part of the protective action of OTC is via actions of circulating GSH and GSH derivatives on Kupffer cells (Fig. 7). This is a likely hypothesis because the IC\(_{50}\) for GSH is very low (10 \(\mu\)mol/L) and OTC doubled circulating GSH in this physiological concentration range. This, in turn, prevents production of toxic cytokines such as TNF-\(\alpha\) and minimizes early alcohol-induced liver injury. On the other hand, these data do not rule out an important role for mitochondrial GSH or a change in susceptibility to TNF-\(\alpha\) in early alcohol-induced liver injury.

**Fig. 7.** Working hypothesis for the protective action of OTC. OTC is metabolized by 5-oxoprolinase to glutathione (GSH) in the hepatocyte. Data from this study supports the hypothesis that circulating GSH and cysteinyl-glycine (cys-gly) blunts the increase in intracellular calcium in Kupffer cells caused by endotoxin. It is known that there is a glycine-gated chloride channel in liver that stimulates influx of chloride causing hyperpolarization caused by endotoxin. It is known that there is a glycine-gated chloride channel in the Kupffer cell membrane making calcium channels more difficult to open.\(^{17}\) Thus, calcium-sensitive pathways such as TNF-\(\alpha\) synthesis are inhibited by OTC treatment.

**REFERENCES**