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ALTERATION OF RAT LIVER ENDOPLASMIC RETICULUM Ca^{2+}-ATPase THIOL INTEGRITY BY CIPROFIBRATE, A PEROXISOME PROLIFERATOR

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Abstract—Ciprofibrate (CP), a peroxisome proliferator, has been shown to reduce rat liver endoplasmic reticulum (ER) Ca^{2+}-ATPase activity both *in vitro* and *in vivo*. The ER Ca^{2+}-ATPase is highly susceptible to thiol reactivity, and maintenance of maximal enzyme activity is critically dependent upon the integrity of these thiol groups. We therefore investigated whether CP alters ER Ca^{2+}-ATPase thiol groups as a possible mechanism of enzyme inhibition. Using a thiol immunoblot technique, free thiol groups specifically on the ER Ca^{2+}-ATPase were localized. Exposure of freshly isolated rat liver microsomes to CP (500 μM) resulted in a loss of sulfhydryl reactivity on the ER Ca^{2+}-ATPase protein at 107 kDa, as identified using the thiol immunoblot assay. However, when rat liver microsomes were exposed to CP in the presence of reduced glutathione (GSH), thiol groups on the ER Ca^{2+}-ATPase were protected. Also, the reduction of ER Ca^{2+}-ATPase activity by CP could be ameliorated by co-incubation of rat liver microsomes with GSH. These observations indicate that CP reduces rat liver ER Ca^{2+}-ATPase activity through interactions with free thiol groups located on this enzyme.

Peroxisome proliferating agents (PPA)† are a diverse class of non-genotoxic rodent hepatocarcinogens that induce the proliferation of peroxisomes within the liver and increase peroxisomal enzyme activity [1]. Acyl CoA oxidase, an enzyme of the β-oxidation pathway, is induced by up to 20-fold in response to a peroxisome proliferator [2]. The induction of this enzyme leads to elevated levels of hydrogen peroxide (H_2O_2), the generation of which has been postulated as being the source of oxidant-induced DNA damage [3]. The loss of oxidative homeostasis following exposure to a PPA is further compounded by the ability of the PPA to inhibit H_2O_2 detoxifying enzyme systems such as glutathione peroxidase and glutathione-*S*-transferase [4]. The PPA induce other metabolic alterations in rodent liver, including the induction of Cu^{2+}, Zn^{2+} superoxide dismutase [5], cytochrome P450IVA1 [6], and alkaline phosphatase [7]. We have demonstrated previously that the PPA, ciprofibrate (CP), also inhibits a Ca^{2+} transporting enzyme of rat liver endoplasmic reticulum (ER) [8]. This enzyme, the ER Ca^{2+}-ATPase, which is responsible for the maintenance of intracellular Ca^{2+} pools, is inhibited both *in vitro* and transiently *in vivo* during the first 24 hr following exposure to CP.

The inhibition of the liver ER Ca^{2+}-ATPase has also been observed with other structurally diverse agents such as 2,5-di-*tert*-butylbenzohydroquinone [9], thapsigargin [10], phenobarbital and DDT [11]. Furthermore, it has been established that the inhibition of this enzyme by thapsigargin and 2,5-di-*tert*-butylbenzohydroquinone leads to an increase in hepatocyte free intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) [9, 10]; this has also been observed for CP [8]. Despite these observations, the mechanism(s) of CP-induced ER Ca^{2+}-ATPase inhibition remains unclear. The nature of both the sarcoplasmic reticulum (SR) and the ER Ca^{2+}-ATPase is such that these enzymes are highly susceptible to thiol interactions [12–15]. Furthermore, the integrity of these thiol groups is essential for maximal enzyme activity, since inhibition of enzyme activity can be achieved after modification of as little as two thiol groups [16]. We therefore investigated whether CP inhibits ER Ca^{2+}-ATPase activity as a function of thiol group modification. Using a technique referred to as a thiol immunoblot assay, which employs the use of 3-(*N*-maleimido-propionyl) biocytin (MPB), a biotin-linked sulfhydryl reactive label [17], we directly assessed the effects of CP specifically on the ER Ca^{2+}-ATPase thiol groups. This study demonstrated that the mechanism of CP-induced ER Ca^{2+}-ATPase inhibition occurs as a result of a loss and/or modification of free thiol groups specifically on the ER Ca^{2+}-ATPase.

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† Abbreviations: PPA, peroxisome proliferating agents; CP, ciprofibrate; ER, endoplasmic reticulum; SR, sarcoplasmic reticulum; MPB, 3-(*N*-maleimido-propionyl) biocytin; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; BSA, bovine serum albumin; and TBS, Tris-buffered saline.

MATERIALS AND METHODS

Materials. MPB was purchased from the Sigma Chemical Co. (St. Louis, MO). CP was a gift from the Sterling Research Institute (Rensselaer, NY). ATP was purchased from Boehringer Mannheim (Indianapolis, IN) and $^{45}CaCl_2$ was supplied by the Amersham Corp. (Arlington Heights, IL). The cardiac SR Ca^{2+}-ATPase C4 polyclonal antibody

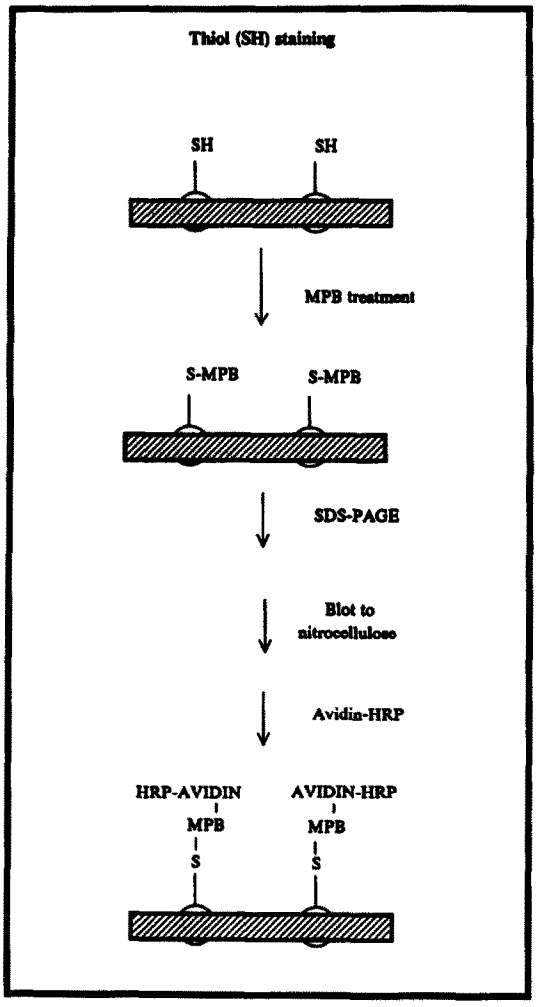

Fig. 1. Schematic representation of the thiol immunoblot assay. Depicted in hatches is the ER with the membrane bound ER Ca^{2+}-ATPase protein displaying free thiol groups (-SH). Rat liver microsomes were reacted with the thiol specific MPB probe which binds covalently to the free thiol groups. Next, microsomal proteins were separated on an SDS-polyacrylamide gel and transferred to nitrocellulose; free thiol groups were then visualized with an avidin-linked horse radish peroxidase antibody.

(Ab C4) was a gift from Dr. David MacLennan and Dr. Jonathan Lytton [18]. Western blot analysis was carried out using the Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA), and molecular weight markers were obtained from Bio-Rad (Richmond, CA). All other chemicals were of the highest purity commercially available.

Thiol immunoblot assay. The thiol immunoblot assay which is depicted schematically in Fig. 1, was carried out according to Bayer *et al.* [17] with minor modifications. Liver microsomes were prepared from fed male F344 rats as previously described [8] and preincubated with 0.5 mg protein in 1.0 mL of 100 mM KCl, 20 mM HEPES-KOH (pH 7.0), 10 mM $MgCl_2$, 50 μM $CaCl_2$, and 90 μM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N'*,*N'*-tetraacetic acid (EGTA) for 5 min at 37°. CP was dissolved in dimethyl sulfoxide (DMSO) and added to microsomal homogenate preparations at a final concentration of 500 μM [0.5% (v/v)]; controls received DMSO at 0.5% (v/v). The microsomal homogenate was incubated at 37° with CP for 0–10 min, after which the reaction was stopped by rapid centrifugation followed by aspiration of the supernatant. The microsomal pellet was then washed twice by centrifugation with phosphate-buffered saline (PBS) and resuspended in 1.0 mL PBS to which 30 μg/mL of MPB was added, vortexed vigorously, and allowed to incubate at room temperature for 30 min with constant agitation. After labeling with MPB, microsomal protein was spun-down, resuspended in loading buffer [9% (w/v) sodium dodecyl sulfate (SDS), 30% (v/v) glycerol, 2% (v/v) 2-mercaptoethanol and 0.2% bromophenol blue] and separated on a 7.5% SDS-polyacrylamide gel. Following electrophoresis, proteins were transferred to nitrocellulose membrane using a Mini Trans-Blot electrophoretic transfer cell (Bio-Rad). Membranes were subsequently blocked with 5% bovine serum albumin (BSA) in PBS for 1 hr, then rinsed three times in PBS, and incubated for 30 min with an avidin-linked horseradish peroxidase antibody (Vectastain ABC). Membranes were finally developed after washing three times in PBS with 0.02% H_2O_2 and 4-chloro-1-naphthol as the substrate.

Western blot analysis and immunoprecipitation of MPB-labeled ER Ca^{2+}*-ATPase.* Western blots were carried out using rat liver microsomal protein (10 μg) that was separated on a 7.5% SDS-polyacrylamide gel and electrophoretically transferred to nitrocellulose membrane. After blocking with 0.05% (w/v) Tween-20 in PBS, membranes were probed with a 1:500 dilution of Ab C4 serum [18] for approximately 1 hr at room temperature. Following incubation with Ab C4, membranes were washed three times with PBS and incubated with a rabbit IgG biotinylated secondary antibody, which was then visualized by coupling to an avidin-linked horseradish peroxidase antibody.

For immunoprecipitation experiments, microsomal protein (2 mg) was resuspended in 1.0 mL of 100 mM KCl, 20 mM HEPES-KOH (pH 7.0), 10 mM $MgCl_2$, 50 μM $CaCl_2$, 90 μM EGTA and incubated with 15 μg/mL of MPB for approximately 30 min at room temperature. MPB-labeled microsomal protein was then spun-down and resuspended in 0.5 mL of Tris-buffered saline (TBS) to which Ab C4 (1:125) coupled to protein A-sepharose (Oncogene Science, Manhasset, NY) was added and incubated overnight at 4°. Immunoprecipitates were collected by centrifugation, washed and incubated at 100° for 5 min, and separated on a 7.5% SDS-polyacrylamide gel. Immunoprecipitated protein was then transferred to nitrocellulose membrane and subsequently detected using an avidin-linked horseradish peroxidase antibody.

Microsomal Ca^{2+} *accumulation assay.* Active accumulation of Ca^{2+} by the ER Ca^{2+}-ATPase was assessed by the uptake of $^{45}Ca^{2+}$ using a filtration method described by Moore *et al.* [19]. Rat liver microsomes (0.5 mg) were preincubated for 5 min at

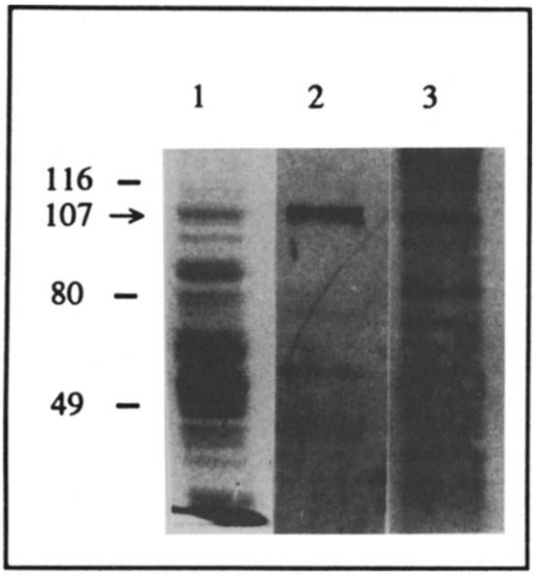

Fig. 2. Identification of the rat liver ER Ca^{2+}-ATPase as a thiol rich 107 kDa protein. Lane 1: Coomassie Blue stained gel of microsomal protein separated on a 7.5% SDS-polyacrylamide gel; lane 2: Western blot of the ER Ca^{2+}-ATPase using the polyclonal antibody Ab C4; lane 3: immunoprecipitation of the MPB-labeled ER Ca^{2+}-ATPase. Molecular weight markers (Bio-Rad) in kilodaltons (kDa) are shown on the left.

37° in 1.0 mL of 100 mM KCl, 20 mM HEPES-KOH (pH 7.0), 10 mM $MgCl_2$, 50 μM $CaCl_2$, 90 μM EGTA and 0.2 μCi of $^{45}Ca^{2+}$. Microsomes were then treated with CP (500 μM) alone or with CP plus GSH (2 mM) for 5 min at 37° prior to the initiation of active Ca^{2+} accumulation by the addition of 2.5 mM ATP. The reaction was allowed to proceed for 10 min at 37°, after which the incorporation of $^{45}Ca^{2+}$ into microsomes was assessed following rapid filtration of 50-μL aliquot samples and liquid scintillation counting. The incorporation of $^{45}Ca^{2+}$ into microsomes was subsequently corrected for non-specific binding.

RESULTS

The rat liver ER Ca^{2+}-ATPase protein has been identified as a 107 kDa protein [20] which can be discerned easily on Coomassie Blue stained gels from microsomal preparations as seen in Fig. 2 (lane 1). To demonstrate that this 107 kDa polypeptide was definitely the ER Ca^{2+}-ATPase protein, Western blots were carried out. The rat liver ER Ca^{2+}-ATPase possesses sufficient cross-reactive immunogenicity to be recognized by a polyclonal antibody generated from the rabbit cardiac SR Ca^{2+}-ATPase (Ab C4) [18], as shown in Fig. 2 (lane 2). The polyclonal Ab C4 recognized a single polypeptide with a molecular mass of 107 kDa, thus confirming that the 107 kDa protein identified in Coomassie Blue stained gels was indeed the ER Ca^{2+}-ATPase protein.

To ascertain whether the thiol immunoblot assay could definitively detect ER Ca^{2+}-ATPase thiols, microsomal proteins were labeled with the MPB thiol probe, and the ER Ca^{2+}-ATPase immunoprecipitated with Ab C4. Immunoprecipitated protein was then separated on a 7.5% SDS-polyacrylamide gel, transferred to nitrocellulose, and visualized with a biotin-linked horseradish peroxidase antibody. As shown in Fig. 2 (lane 3), the predominant immunoprecipitated band was detected at 107 kDa which, as expected, co-migrated with the Western blot of microsomal protein using Ab C4. This demonstrates that the ER Ca^{2+}-ATPase contains detectable sulfhydryl groups using this technique.

Isolated rat liver microsomes were then exposed to CP at 500 μM for 0, 2 and 5 min and examined by thiol immunoblot analysis. CP exposure resulted in a loss of detectable MPB thiol labeling on the 107 kDa protein band (Fig. 3, lane 4) after 5 min as compared with the control at 0 min (Fig. 3, lane 2) and that observed for untreated MPB-labeled ER Ca^{2+}-ATPase protein (Fig. 2, lane 3). Despite the apparent loss of thiol reactivity on the ER Ca^{2+}-ATPase protein at 107 kDa, other microsomal proteins did not appear to be affected, indicating that the loss of thiol labeling on the ER Ca^{2+}-ATPase was a specific effect. Furthermore, this observation also confirms the phenomenon of the susceptibility of the ER Ca^{2+}-ATPase towards thiol reactivity. The specificity of the MPB thiol label was further demonstrated in the control sample (Fig. 3, lane 1), where MPB labeling was omitted from microsomal preparations; in this case, negligible background labeling was detected following analysis with the biotin-linked horse radish peroxidase antibody.

If the loss of MPB labeling on the ER Ca^{2+}-ATPase protein following treatment with CP was directly associated with interactions on free sulfhydryl groups, then co-incubation of CP with GSH would presumably diminish CP interactions with the free ER Ca^{2+}-ATPase thiols. This hypothesis was tested by isolating rat liver microsomes and incubating them with CP alone or with CP plus GSH (2 mM) for 0, 5 and 10 min. Microsomal proteins were then immediately labeled with MPB and visualized by the thiol immunoblot assay. In these experiments, the loss of sulfhydryl reactivity was again observed at the 107 kDa protein band, corresponding to that of the ER Ca^{2+}-ATPase protein (Fig. 4A). When CP-treated microsomal proteins were co-incubated with 2 mM GSH, ER Ca^{2+}-ATPase thiol groups were protected and MPB thiol reactivity was preserved (Fig. 4A). The preservation of thiol labeling on the ER Ca^{2+}-ATPase in the presence of GSH suggested that the inhibition of enzyme activity by CP was occurring through modification of critical thiol groups on the ER Ca^{2+}-ATPase. This was confirmed by the experiment shown in Fig. 4B, which demonstrates that the inhibition of ER Ca^{2+}-ATPase activity by CP, measured as a function of microsomal Ca^{2+} uptake, could be significantly protected in the presence of 2 mM GSH ($P < 0.05$).

DISCUSSION

The ER Ca^{2+}-ATPase is a thiol rich Ca^{2+}

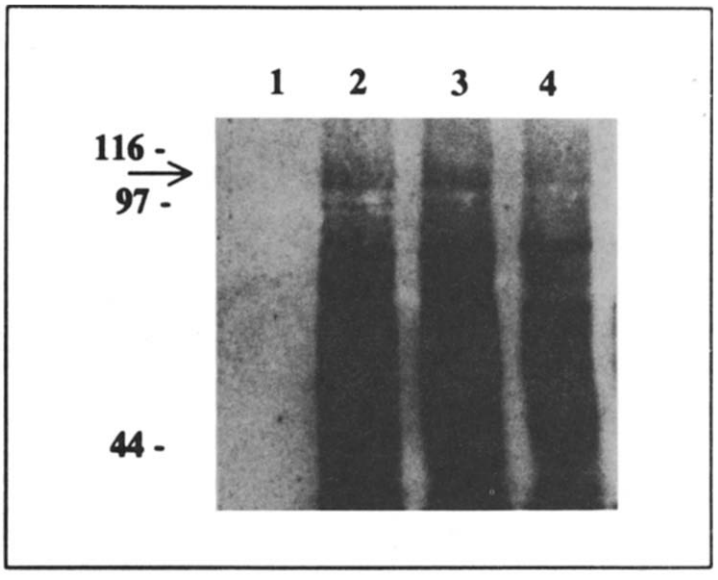

Fig. 3. Effect of CP on total microsomal thiol integrity assessed by application of the thiol immunoblot assay. Lane 1 represents microsomal proteins in which the MPB labeling step was omitted; lanes 2, 3 and 4 show the thiol profile of rat liver microsomes following treatment with CP (500 μM) for 0, 2 and 5 min, respectively. The arrow indicates the position of the ER Ca^{2+}-ATPase at 107 kDa.

transporting enzyme, whose activity is dependent upon the integrity of several critical thiols [16, 21]. Using a thiol immunoblot assay, we have assessed the effects of CP on the ER Ca^{2+}-ATPase thiol groups. These studies have revealed that the ER Ca^{2+}-ATPase undergoes a loss of thiol reactivity when exposed to CP and that subsequent incubation of CP with GSH protects against the loss of free thiol groups and also ameliorates CP-induced ER Ca^{2+}-ATPase inhibition. Collectively, the data support the conclusion that the reduction of ER Ca^{2+}-ATPase activity by CP occurs as a result of modification to critical thiol groups on this enzyme.

The inhibition of the ER Ca^{2+}-ATPase as a function of thiol group modification can occur through either direct or indirect mechanisms. One of the more extensively reported mechanisms is that of Ca^{2+}-ATPase thiol oxidation, an indirect mechanism, which has been shown to occur for both SR and ER Ca^{2+}-ATPases [12, 13, 22]. As a result of thiol oxidation, disulfide bonds are formed between two closely juxtaposed sulfhydryl groups. A second possibility of Ca^{2+}-ATPase inhibition occurs through direct interactions with free thiols as seen with alkylating agents [15] as well as through direct interactions with heavy metals [23]. Another potential direct mechanism may also involve the production of protein thioether linkages by 4-hydroxynonenal which is generated as a result of lipid peroxidation [24]. Several groups have demonstrated that the PPA have the ability to elevate lipofuscin and conjugated diene levels which are indicative of lipid peroxidation [25, 26]. However, other groups have failed to demonstrate PPA-induced lipid peroxidation [27, 28]. Based on the experiments described here, coupled with the propensity of CP to induce oxidative stress, an indirect method involving oxidation of thiol groups appears likely. Nonetheless, a direct mechanism of thiol interaction with CP cannot be ruled out, since these experiments merely demonstrate that free thiol groups are the end-point effector of this interaction. Interestingly, other PPA, perfluorodecanoic acid (PFDA) and perfluorooctanoic acid (PFOA), were found to covalently bind cellular proteins in a manner that appears to involve thiol groups also [29]. The mechanism(s) of PPA thiol interactions remains to be elucidated.

The loss of thiol reactivity on the ER Ca^{2+}-ATPase does not appear to be a function of protein mixed disulfide formation, since the thiol staining of the ER Ca^{2+}-ATPase protein remained at 107 kDa. We observed a complete abolition of thiol reactivity using the thiol-specific MPB probe when microsomal proteins were treated with CP. However, this did not correlate with the degree of ER Ca^{2+}-ATPase inhibition at the same concentration of CP. This discrepancy can be explained on the basis of the sensitivity of the thiol immunoblot assay. Other techniques, such as [^{14}C]*N*-ethylmaleimide labeling of purified ER Ca^{2+}-ATPase protein, would provide a higher magnitude of sensitivity. The thiol immunoblot technique, nonetheless, achieves specificity rather than absolute quantification of thiol groups.

Alteration of thiol status has been demonstrated to affect Ca^{2+} homeostasis as a result of the inhibition of the ER Ca^{2+}-ATPase [12]. Recent evidence also indicates that oxidized glutathione and subsequently altered GSH/GSSG ratios cause spontaneous release of intracellular Ca^{2+} stores and enhance the efficacy of inositol 1,4,5-trisphosphate to release Ca^{2+}

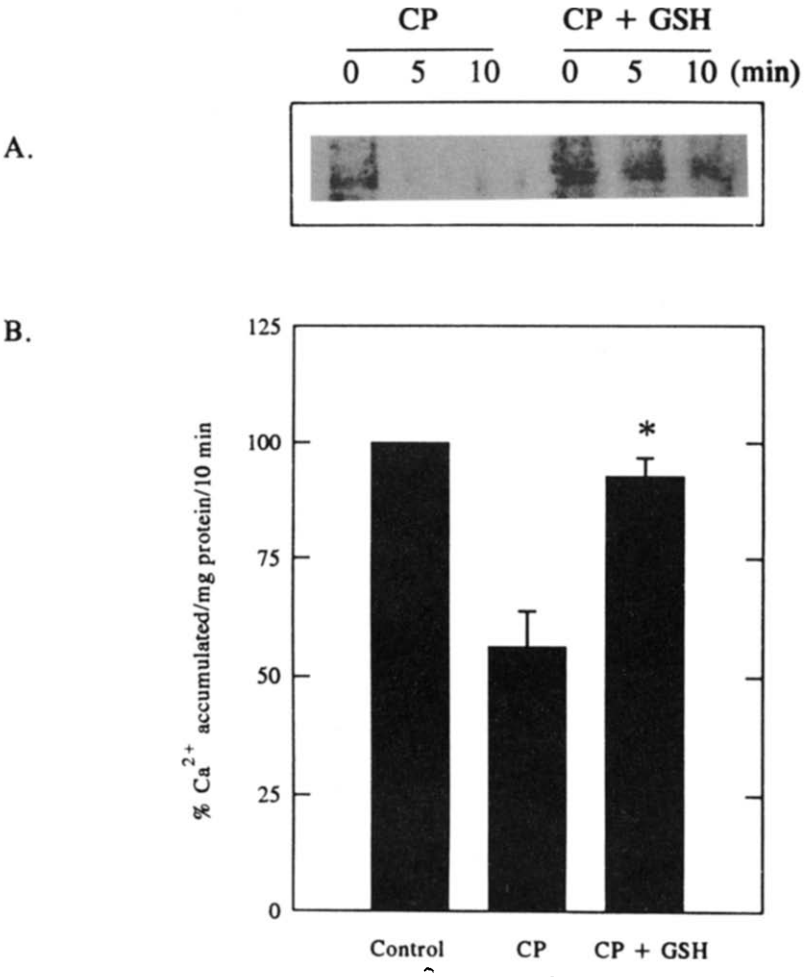

Fig. 4. (A) Thiol immunoblot assay showing the effect of ER Ca^{2+}-ATPase thiol reactivity following treatment with CP (500 μM) or CP plus GSH (2 mM) after 0, 5 and 10 min. (B) Ca^{2+} accumulation in microsomal fractions exposed to CP or CP plus GSH as in (A), assessed as described in Materials and Methods. Data represent the means ± SEM of three separate experiments. Key (*) statistically significant from CP at $P < 0.05$ (Student's t-test).

[30, 31]. The ability of CP potentially to modulate thiol status, together with our previous observation that CP rapidly increases hepatocyte $[Ca^{2+}]_i$ [8], suggests that the initial rapid release of $[Ca^{2+}]_i$ by CP may occur as a result of perturbation of hepatocyte GSH/GSSG ratios and that the subsequent prolonged phase of $[Ca^{2+}]_i$ elevation occurs as a result of thiol interactions, presumably oxidative, on the ER Ca^{2+}-ATPase. This is indirectly supported by the observation that clofibrate, which is a less potent PPA than CP [32], only produces a transient rise in hepatocyte $[Ca^{2+}]_i$ [33].

The ability of CP to modify thiol integrity on the ER Ca^{2+}-ATPase has been described. Potentially, these data may also offer some explanation to another PPA-related toxic effect, that being the inhibition of hepatic glutathione *S*-transferase (GST) [4, 34]. In a manner similar to the ER Ca^{2+}-ATPase, hepatocellular GST enzyme activity has also been shown to be critically dependent upon thiol integrity [35]. It appears, therefore, that the propensity of CP and, possibly other PPA, to modify thiol groups offers another potential toxicological mechanism for this class of compounds.

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