Pergamon

0006-2952(95)00214-6

Biochemical Pharmacology, Vol. 50, No. 7, pp. 929–935, 1995.

0006-2952/95 $9.50 + 0.00

DIQUAT-DEPENDENT PROTEIN CARBONYL FORMATION

IDENTIFICATION OF LIPID-DEPENDENT AND LIPID-INDEPENDENT PATHWAYS

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(*Received* 13 *December* 1994; *accepted* 24 *March* 1995)

Abstract—In a previous report on diquat-dependent oxidative damage in rat hepatic microsomes, protein oxidation, as measured by protein carbonyl (PC) formation, was observed in addition to lipid peroxidation (LP). Both phenomena were antioxidant sensitive. Inhibition of PC formation was somewhat surprising given the proposed mechanism of metal-catalyzed protein oxidation. Studies reported here examined diquat-dependent PC formation in greater detail. In rat hepatic microsomes, diquat-dependent thiobarbituric acid-reactive substances (TBARS) and PC formation were time and concentration dependent. In this system, LP was inhibited completely by U-74006F or U-78517G, whereas PC formation was inhibited only partially by these antioxidants. In an essentially lipid-free system consisting of purified rat hepatic cytochrome P450 reductase, BSA and an NADPH-generating system, PC formation was also observed, but was not antioxidant-sensitive. Under these conditions, minimal diquat-dependent TBARS formation was observed. The observation of relative antioxidant insensitivity is consistent with H_2O_2 (generated during the diquat redox cycle) catalyzing protein oxidation via a site-specific, metal-catalyzed mechanism. Thus, different pathways would appear to be involved in diquat-dependent PC formation in lipid-containing and lipid-free systems. Carbon tetrachloride induces LP following reductive activation to the trichloromethyl free radical, a pathway not directly involving H_2O_2 generation. In the microsomal system, CCl_4 induced TBARS and PC formation, both of which were completely inhibitable by antioxidants. Taken together, these data suggest that diquat induces PC formation by lipid-dependent (antioxidant-sensitive) and lipid-independent (antioxidant-insensitive) pathways. In microsomes, both pathways contribute to diquat-dependent PC formation. Data for the lipid-independent pathway are consistent with the mechanism of metal-catalyzed protein oxidation proposed by Stadtman and colleagues (reviewed in *Free Radic Biol Med* **9**: 315–325, 1990), while the lipid-dependent pathway is likely secondary to LP itself—via a Michael-type addition reaction between hydroxyalkenals and protein sulfhydryl groups, amino groups or other protein nucleophiles. The latter pathway is also responsible for carbon tetrachloride-dependent PC formation. Additional studies are in progress to further characterize the lipid-independent mechanism.

Key words: protein oxidation; lipid peroxidation; TBARS; protein carbonyl; antioxidant; cytochrome P450 reductase; rat; hepatic microsomes; albumin; *in vitro*

Diquat is a bipyridyl herbicide, the toxicity of which is thought to be mediated through induction of LP‡ [1, 2]. However, oxidative damage to cellular protein, although less well studied, may also contribute to a wide range of conditions in which roles for oxidative injury have been invoked, including aging, ischemia-reperfusion injury, oxygen toxicity and neurodegenerative disorders [3]. In a previous report, we demonstrated that diquat induces protein oxidation, as measured by PC formation, in addition to lipid peroxidation and suggested the possibility that protein oxidation may contribute to diquat-induced hepatotoxicity [4]. Smith and colleagues [5] have reported biliary efflux of PCs following diquat administration, further supporting a possible role in diquat-dependent hepatotoxicity. Stadtman [6] has proposed a mechanism of protein oxidation wherein H_2O_2 oxidizes protein-bound ferrous iron in a Fenton-type reaction. Dehydration of the protein-ferric-hydroxyl complex yields an α-carbon-centered radical that undergoes a second dehydration to a ferrous-imine. This imine complex hydrolyzes spontaneously to a PC moiety, with release of ferrous iron and ammonia (Fig. 1, Mechanism 1). In our previous report, both diquat-induced LP and PC formation were inhibited by the proprietary antioxidants U-74006F and U-78517G. On reflection, the ability of antioxidants to inhibit diquat-dependent PC formation was somewhat surprising given the nature of the pathway proposed by Stadtman, where the reaction environment largely excludes access by free radical scavengers. Studies reported here were designed to further characterize diquat-induced PC formation.

MATERIALS AND METHODS

U-74006F (21-[4-(2,6-di-1-pyrrolidinyl-4-pyrimidinyl)-1-piperazinyl]-16α-methyl-pregna-1,4,9(11)-triene-3,20 dione monomethane sulfonate; tirilazad mesylate) and U-78517G (2-[[4-(2,6-di-1-pyrrolidinyl-4-pyrimidinyl)-1-piperazinyl]-methyl]-3,4-dihydro-2,5,7,8-tetramethyl-2*H*-1-benzopyran-6-ol, 2-hydroxy-1,2,3-propanetricarboxylate) were synthesized within The Upjohn Co.

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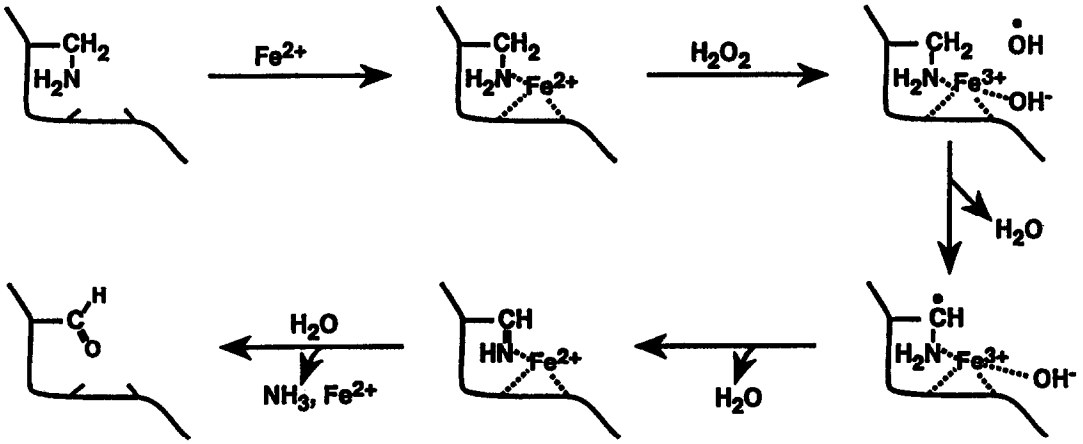
‡ Abbreviations: LP, lipid peroxidation; PC, protein carbonyl; TBA, thiobarbituric acid; TBARS, thiobarbituric acid-reactive substances; DNPH, 2,4-dinitrophenylhydrazine; and TCA, trichloroacetic acid.

Mechanism 1



Mechanism 2

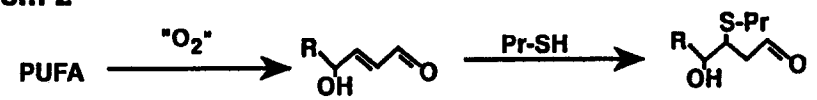


Fig. 1. Schematic representation of proposed mechanisms for protein oxidation. Mechanism 1–site-specific metal-catalyzed pathway proposed by Stadtman [6]; Mechanism 2—Michael-type addition reaction involving products secondary to lipid peroxidation proposed by Esterbauer and Zollner [7]. "O_2" indicates lipid peroxidation reactions; Pr-SH = protein sulfhydryl groups. Note in Mechanism 2 that the aldehyde functional group of the hydroxyalkenal is retained in the product. Adapted from Ref. 6.

Diquat dibromide was obtained from Chem Service (Washington Crossing, DE). Cytochrome *c* (horse heart, Type IV), DNPH, NADPH, BSA (essentially fatty acid free), bromelain, DEAE Sepharose 6B, 2′,5′-ADP agarose, ADP, 2′-AMP and ferrous chloride were purchased from the Sigma Chemical Co. (St. Louis, MO). All other chemicals and reagents were of analytical grade.

Washed hepatic microsomes were prepared from male Fischer 344 rats by differential centrifugation. Protein estimations were performed by the bicinchoninic acid method (BCA; Pierce Chemical Co., Rockford, IL) using BSA as a reference standard. Purification of bromelain-solubilized NADPH cytochrome P450 reductase was performed by a modification of the method of Yasukochi and Masters [8]. Briefly, hepatic microsomal pellets were resuspended in 50 mM Tris-1 mM EDTA, pH 8.0 (Tris-EDTA). Bromelain was added to the suspension (0.1 mg/mL final concentration) and allowed to incubate at 4° for 3.5 hr, with slow stirring. Following centrifugation at 300,000 *g*, supernatants were applied to a DEAE Sepharose 6B column (2 × 16.5 cm). The column was washed with three bed volumes of Tris-EDTA buffer, supplemented with 100 mM KCl. Cytochrome P450 reductase was eluted using a 1.1 L KCl gradient (100–400 mM). Fractions containing the majority of the reductase activity were pooled and concentrated 5-fold using Amicon Centriprep-30 microconcentrators (W. R. Grace & Co., Beverly, MA). The concentrated sample was diluted with an equal volume of 10 mM potassium phosphate-20% glycerol-0.02 mM EDTA (pH 7.7; phosphate-glycerol-EDTA) and applied to a 2′-5′-ADP agarose column (8 × 75 mm). The column was washed with three bed volumes of phosphate–3 glycerol–EDTA (0.02 mM) and three bed volumes of phosphate–glycerol–EDTA (0.4 mM). The reductase was eluted from the column with phosphate–glycerol–EDTA (0.02 mM), containing 1 mM 2′-AMP. Fractions containing the reductase activity were concentrated in Amicon microconcentrators and stored at −80° until used.

Assays to determine the activity of the purified cytochrome P450 reductase (cytochrome *c* reduction) contained 80 μM cytochrome *c* and 0.1 mM NADPH in 0.3 M potassium phosphate buffer, pH 7.7. The reduction of cytochrome *c* (25°) was measured by the increase in absorbance at 550 nm. Enzyme activities were calculated from initial rates, using an extinction coefficient of $28.0 \cdot mM^{-1} \cdot cm^{-1}$ [9]. Typical preparations had specific activities of approximately 30 U/mg protein and appeared as a single band on SDS-PAGE.

Typically, 2-mL incubations (37°) consisted of 1.5 mg microsomal protein/mL, in the presence of an NADPH-generating system (NADP, 0.5 μmol; glucose-6-phos-

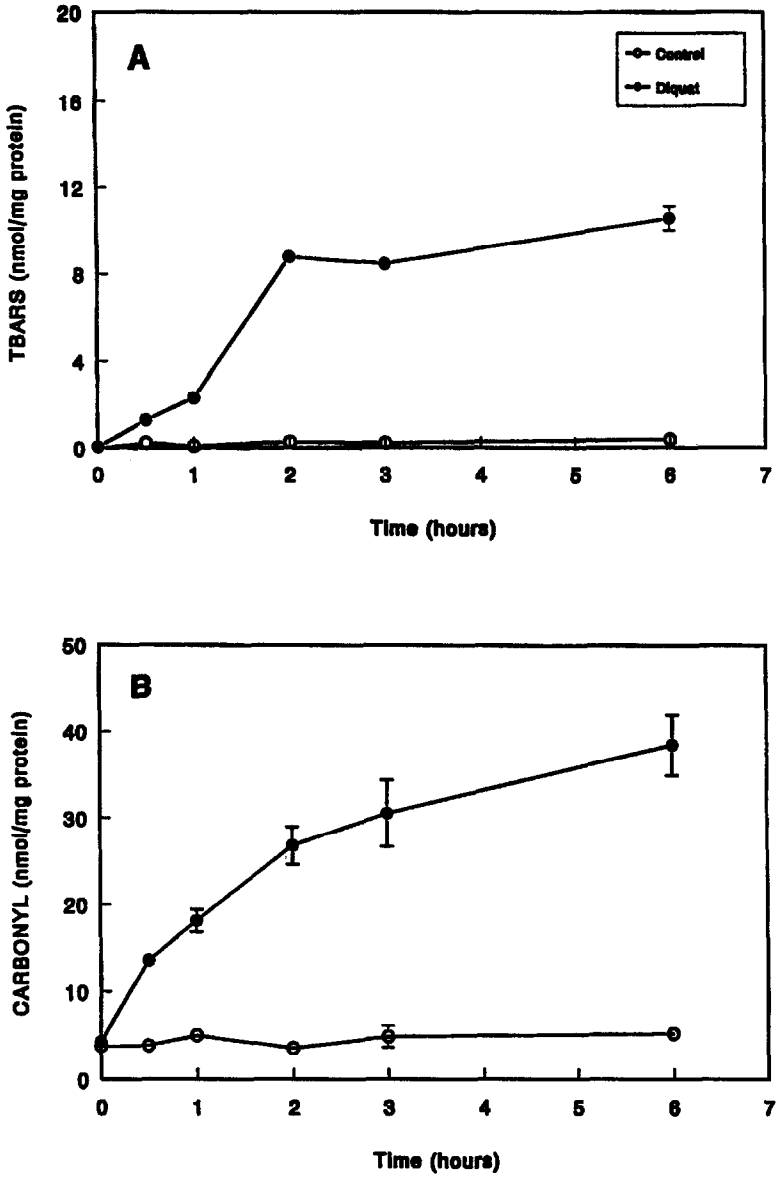


Fig. 2. Time-dependent formation of TBARS (panel A) or protein carbonyl groups (panel B) in rat hepatic microsomes. Data represent the means ± SD of 3 measurements following incubation with diquat (1 mM) at 37° See Materials and Methods for experimental details.

phate dehydrogenase, 1.0 U; $MgCl_2 \cdot 6H_2O$, 10 μmol) in 50 mM Tris-1.15% KCl, pH 7.4. In experiments utilizing purified NADPH cytochrome P450 reductase, 0.6 U was added per 2-mL incubation, and 1.5 mg BSA/mL was substituted for microsomal protein. Where indicated, diquat dibromide (as an aqueous solution) or carbon tetrachloride (neat) was included at final concentrations of 1 mM and 1 μL/mL, respectively. Stock solutions of U-74006F and U-78517G were prepared in methanol and deionized H_2O, respectively. Where indicated, these compounds were added to incubation mixtures to produce a final concentration of 100 μM. Control incubations contained the appropriate vehicle, and concentrations of organic vehicle did not exceed 1% (v/v).

Lipid peroxidation was assayed using TBARS according to the procedure of Buege and Aust [10]. Incubation mixtures were acidified by addition of TCA (10%, w/v; 0.75 mL), and TBA reagent [0.375% (w/v) TBA, 15% (w/v) TCA, 0.25 N HCl; 2 mL] was added. The mixtures were incubated for 15 min at 100°. After cooling, samples were centrifuged, and the absorbance of the supernatant was read at 532 nm (ϵ_{532} = 1.56 × $10^5 \cdot M^{-1} \cdot cm^{-1}$).

PC formation was assessed using an adaptation of the procedure described by Oliver *et al.* [11]. Aliquots of reaction mixtures (as described above) were removed to tubes containing TCA, centrifuged and the supernatants discarded. Pellets were treated with 2 N HCl, or 0.2% DNPH in 2 N HCl, and vortexed at 10-min intervals while incubating at room temperature for 1 hr. Following reprecipitation with additional TCA, pellets were extracted three times with ethanol:ethyl acetate (1:1), dissolved in 6 M guanidine HCl, and centrifuged to remove insoluble debris. The difference spectra of DNPH-

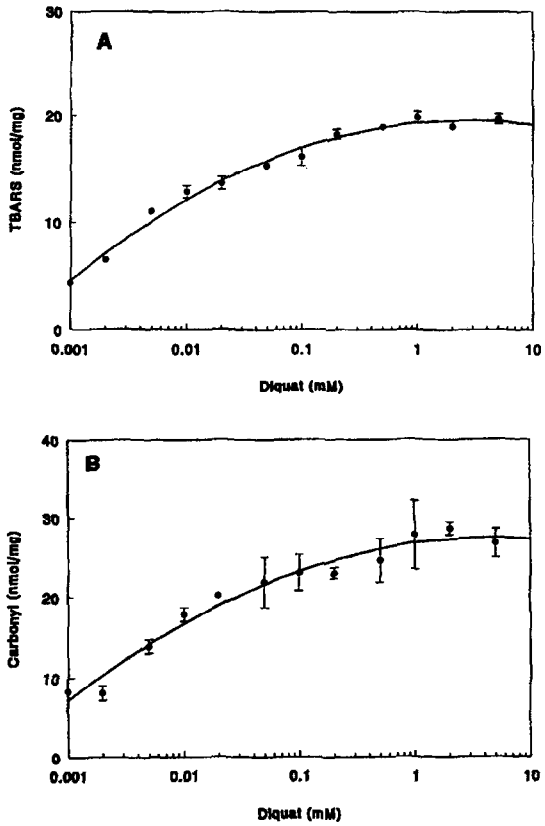


Fig. 3. Concentration-dependent formation of TBARS (panel A) or protein carbonyl groups (panel B) in rat hepatic microsomes. Data represent the means ± SD of 3 measurements following 3-hr incubations at 37°. See Materials and Methods for experimental details.

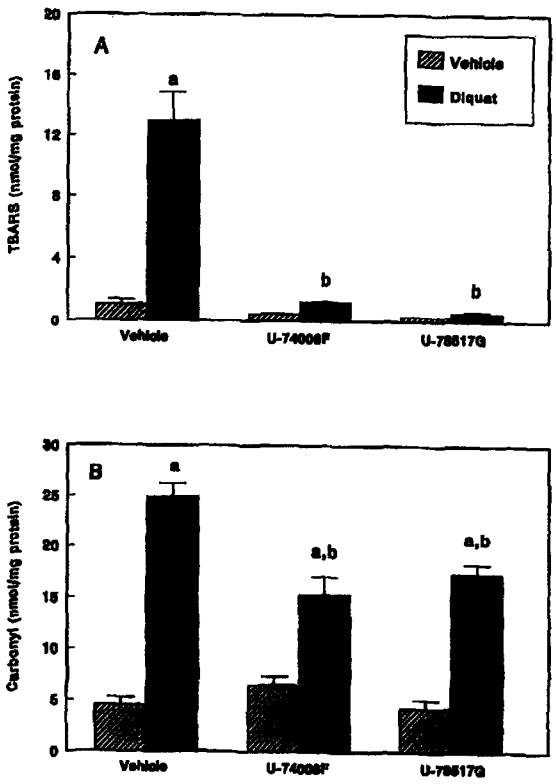


Fig. 4. Effect of antioxidants (U-74006F and U-78517G) on diquat-induced TBARS (panel A) and protein carbonyl formation (panel B) in rat hepatic microsomes. Data represent the means ± SD of 3 measurements following 3-hr incubations at 37°. Diquat concentration, 1 mM; antioxidant concentration, 100 μm. See Materials and Methods for experimental details. Key: (a) significantly different ($P < 0.05$) from vehicle control; and (b) significantly different ($P < 0.05$) from diquat alone.

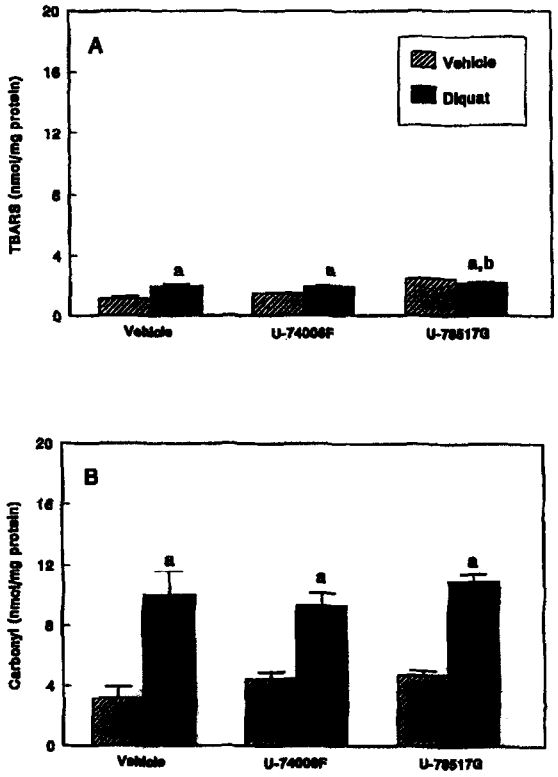


Fig. 5. Effects of antioxidants U-74006F and U-78517G on diquat-induced TBARS (panel A) and protein carbonyl formation (panel B) in a purified cytochrome P450 reductase/BSA incubation system. Data represent the means ± SD of 3 measurements following 1-hr incubations at 37°. Diquat concentration, 1 mM; antioxidant concentration, 100 μM. See Materials and Methods for experimental details. Key: (a) significantly different ($P < 0.05$) from vehicle control; and (b) significantly different ($P < 0.05$) from diquat alone.

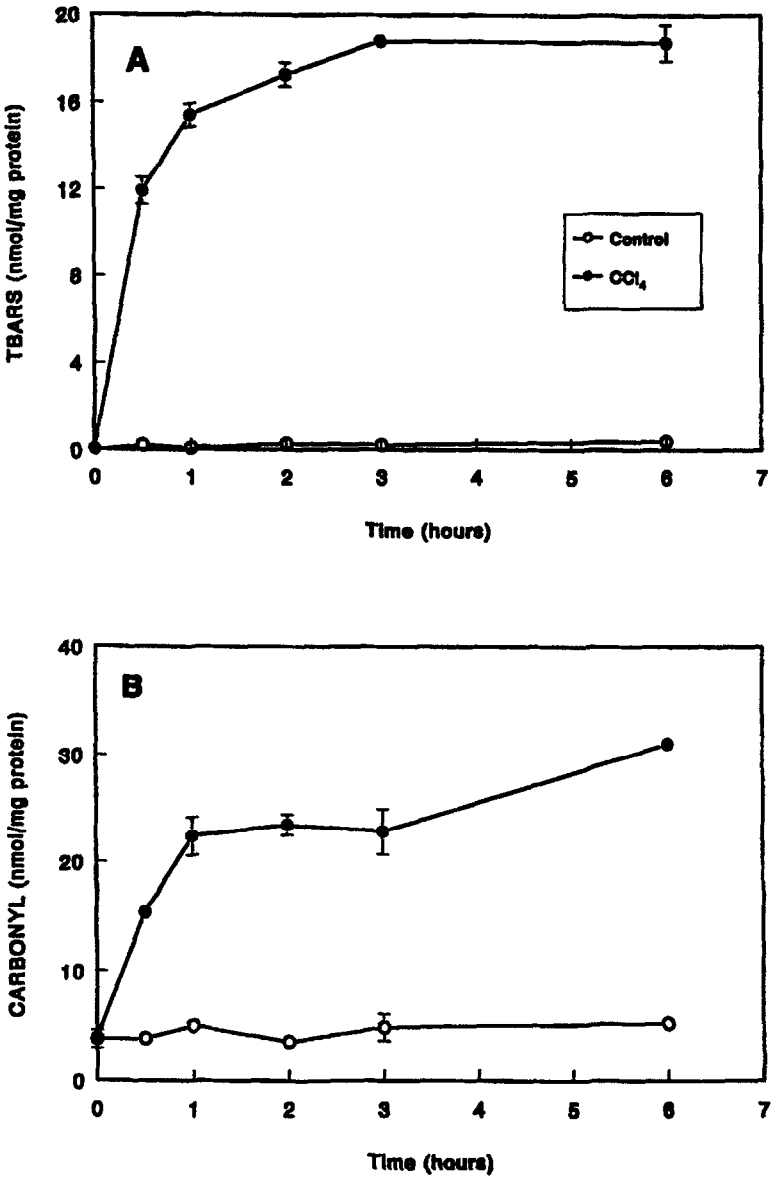


Fig. 6. Time-dependent formation of TBARS (panel A) or protein carbonyl groups (panel B) in incubations of rat hepatic microsomes and CCl_4. Data represent the means ± SD of 3 measurements following 3-hr incubations at 37°. CCl_4 concentration, 1 μL/mL. See Materials and Methods for experimental details.

treated samples versus the control (HCl) samples were determined and carbonyl formation was calculated usingan extinction coefficient of $21.0 \cdot mM^{-1} \cdot cm^{-1}$ at 375 nm.

Data were analyzed by appropriate ANOVA, using SAS® software (Cary, NC). Individual group means were compared using the Bonferroni procedure. Differences were considered significant where $P < 0.05$.

RESULTS

In rat hepatic microsomes, both diquat-dependent TBARS and PC formation were time dependent (Fig. 2). TBARS formation appeared to plateau at approximately 2 hr, while PC formation continued to increase through 6 hr of incubation. Similarly, both phenomena were concentration dependent (Fig. 3), with maxima at approximately 1 mM, which was the concentration used throughout the remainder of the studies. Diquat-dependent TBARS formation in microsomes was completely inhibitable by either U-740006F or U-78517G (Fig. 4A). In contrast, diquat-dependent PC formation was inhibited 47% or less by these compounds (Fig. 4B). When purified cytochrome P450 reductase and BSA were substituted for microsomes, minimal TBARS formation was observed (Fig. 5A). However, protein carbonyl formation remained time dependent (data not shown). Antioxidants did *not* inhibit diquat-dependent PC formation in this system (Fig. 5B).

As with diquat, carbon tetrachloride-induced TBARS and PC formation were time dependent in the microsomal system, although unlike diquat, both plateaued at

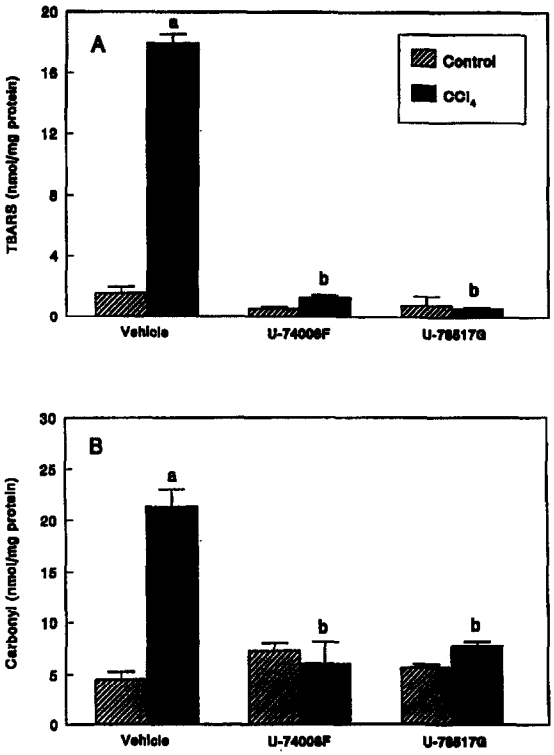


Fig. 7. Effects of antioxidants U-74006F and U-78517G on CCl_4-induced TBARS (panel A) and protein carbonyl formation (panel B) in incubations with rat hepatic microsomes. Data represent the means ± SD of 3 measurements after a 3-hr incubation at 37°. CCl_4 concentration, 1 μL/mL; antioxidant concentration, 100 μM. See Materials and Methods for experimental details. Key: (a) significantly different ($P < 0.05$) from vehicle control; and (b) significantly different ($P < 0.05$) from CCl_4 alone.

approximately the same time in the case of carbon tetrachloride (Fig. 6). Both TBARS and PC formation induced by this compound were completely inhibitable by antioxidants (Fig. 7).

DISCUSSION

Previous studies indicated that diquat-mediated PC formation in rat hepatic microsomes is inhibited by antioxidants [4]. Such inhibition was inconsistent with the mechanism of protein oxidation proposed by Stadtman [6]; (Fig. 1, Mechanism 1), which involves a site-specific reaction of H_2O_2 with a protein–Fe^{2+} complex to generate a carbon-centered radical adjacent to the amine of the complex. This radical dehydrates to a ferrous–imine, which spontaneously hydrolyzes to form a PC with release of NH_3 and ferrous iron. Such a mechanism has been described as "caged" and is reportedly largely insensitive to radical-scavenging agents.

Diquat-mediated TBARS formation was blocked completely by either antioxidant, consistent with a previous report [4]. However, diquat-dependent PC formation was only partially inhibited under identical conditions. This observation suggested that an alternate pathway, or multiple pathways, may be involved in diquat-dependent PC formation. In addition to the mechanism proposed by Stadtman, PC formation may occur secondary to lipid peroxidation [7], secondary to Michael-type addition reaction between hydroxyalkenals and protein sulfhydryl groups or other nucleophiles (Fig. 1, Mechanism 2). Products of that reaction have identical chromophores in the PC assay.

In an effort to determine the contribution of this alternate mechanism to diquat-dependent PC formation, an essentially lipid-free system was devised. This system utilized purified cytochrome P450 reductase instead of microsomes, and included BSA as a target protein. In these incubations, diquat-mediated TBARS was negligible, as expected. These data served as evidence of minimal contamination by lipid in the system. However, PC formation remained time dependent. Antioxidants did *not* inhibit diquat-dependent PC formation in this system, consistent with the metal-catalyzed protein oxidation mechanism (Fig. 1, Mechanism 1).

Several investigators have utilized albumin as a target protein for oxidative damage, using a variety of indicators, including altered molecular weight (aggregation and fragmentation), altered electrical charge, loss of tryptophan, oxidation of sulfhydryl groups, increased susceptibility to enzymic hydrolysis and production of bityrosine [12–19]. PC formation is a common measure of oxidative modification [20]. Data from metal-catalyzed oxidation systems indicate that they are derived from arginine, proline and lysine residues [21] and have indeed been shown to form in albumin after exposure to reactive oxygen species [13,16,19,22–25].

To assess the potential role of lipid peroxidation in PC formation in the microsomal system, similar incubations were conducted in which CCl_4 was substituted for diquat. Unlike diquat, the bioactivation of CCl_4 occurs by reduction to the trichloromethyl free radical without obligatory generation of H_2O_2. Nevertheless, incubations containing rat hepatic microsomes and CCl_4 induced both TBARS and PC formation. Additionally, both phenomena were inhibited completely by U-74006F and U-78517G. Thus, these data are consistent with CCl_4-dependent PC formation secondary to reaction with aldehydic lipid peroxidation products.

Recently Miura *et al.* [22] demonstrated that HO· generated by an ascorbate-EDTA-Fe^{3+} system caused oxidative modification to BSA in the form of carbonyl group formation and that this formation was inhibitable by the radical scavenging agents trolox, mannitol and ethanol. Due to the antioxidant effect, they concluded that PC formation was unlikely due to the site-specific mechanism proposed by Stadtman and suggested that trolox may have competed with molecular oxygen for protein radicals. It was unclear whether the system used by those authors was truly fatty acid free, i.e. the source and purity of BSA were not identified and TBARS formation was not assessed. If that system was *not* lipid free, these results could be explained by the alternate mechanism discussed above (Fig. 1, Mechanism 2). If their system *was* essentially lipid free, the mechanism of diquat-dependent, antioxidant-insensitive PC formation would appear to be different from that induced by iron-EDTA.

Collectively, these data suggest that diquat-mediated PC formation occurs by two pathways. One is lipid independent and antioxidant insensitive, possibly occurring via the site-specific reaction of H_2O_2 with a protein-Fe^{2+} complex. The second pathway is lipid dependent

and antioxidant sensitive. This latter pathway is probably a function of a Michael-type addition reaction occurring between protein sulfhydryl groups or other nucleophiles and aldehydic lipid peroxidation products. Additional studies are required to further elucidate the mechanism of a lipid-independent pathway in other to determine whether the mechanism is, in fact, that proposed by Stadtman [6]. The role(s), if any, of protein modification in diquat-induced hepatotoxicity, including possible differences dependent on the pathway, also requires further study, but must at least be considered as an alternate or supplemental contributor to that toxicity.

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