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U-101033E (2,4-Diaminopyrrolopyrimidine), a Potent Inhibitor of Membrane Lipid Peroxidation as Assessed by the Production of 4-Hydroxynonenal, Malondialdehyde, and 4-Hydroxynonenal–Protein Adducts

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ABSTRACT. 4-Hydroxy-2-nonenal (4-HNE) and malondialdehyde (MDA) are major lipid peroxidation products generated by free radical attack on membranes and appear to contribute to the cytotoxic effects of oxidative stress by a mechanism involving adduct formation with cellular proteins. In the present studies, we investigated the relationship between lipid peroxidation and eventual inactivation of plasma membrane proteins using a model system consisting of purified red blood cell membranes and Fe^{2+}/EDTA. Using this system, we also analyzed the ability of a novel antioxidant, U-101033E (2,4-diaminopyrrolopyrimidine), to inhibit lipid peroxidation and associated protein damage. Our results demonstrated that significant levels of MDA and 4-HNE are generated in this model system, and that both aldehydes are capable of cross-linking membrane proteins. In addition, we used a monoclonal antibody to demonstrate the presence of 4-HNE–protein adducts in this system. The generation of 4-HNE–protein adducts closely paralleled the time course of lipid peroxidation and membrane protein cross-linking, suggesting that 4-HNE may contribute to membrane protein cross-linking. Analysis of U-101033E in this system showed that this antioxidant inhibited lipid peroxidation, prevented the appearance of 4-HNE–protein adducts, and strongly reduced membrane protein cross-linking, with an EC_{50} of 0.5 μM. We also show that these antioxidant effects were not due to the scavenging of superoxide anion. Thus, these studies demonstrate the potential usefulness of U-101033E for treating certain disease processes where lipid peroxidation plays a role in disease pathogenesis. BIOCHEM PHARMACOL **56**;10:1371–1379, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. 4-hydroxy-2-nonenal; lipid peroxidation; antioxidant; protein cross-linking; free radicals; oxidant stress

Oxidative stress occurs in living organisms when the production of ROS§ exceeds the ability to prevent their accumulation [1, 2]. In this regard, oxidative stress has been implicated in over 100 diseases [2, 3]. One major consequence of oxidative stress is the initiation and propagation of lipid peroxidation [4–6], a process that may lead to disruption of ionic gradients and eventual cell death [7]. In the past decade, considerable attention has focused on the identification and characterization of reactive aldehydes produced as breakdown products of lipid peroxidation in biological membranes [8, 9]. Previous studies have shown that two major aldehyde species, MDA and 4-HNE, are formed during the lipid peroxidation process [8, 9]. These aldehydes are thought to contribute to subsequent cellular injury due to their high reactivity with membrane proteins [9]. For example, 4-HNE has been shown to react with lysine and histidine residues on target proteins [9, 10], and 4-HNE–protein conjugates have been identified in modified low-density lipoproteins [11–13], in tissues from patients with Parkinson's disease [14] and Alzheimer's disease [15], and in the phagosomes of human neutrophils [16]. In addition, elevated levels of MDA have been correlated with the aging process [17] and with autoimmune diseases [18] in humans, and increased levels of MDA and MDA-modified proteins also have been observed in tissues from aged rats [19, 20].

Because of the role of ROS in the etiology of a number of diseases, considerable efforts have been directed towards the discovery of effective antioxidant compounds that can impede the lipid peroxidation process [6, 21]. The 21-aminosteroids and the pyrrolopyrimidines are a novel series of antioxidants that have shown promising activity in brain injury and ischemic models [22–27]. The efficacy of such compounds is derived primarily from their ability to prevent

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§ *Abbreviations:* 4-HNE, 4-hydroxy-2-nonenal; MDA, malondialdehyde; RBC, red blood cell; ROS, reactive oxygen species; SOD, superoxide dismutase; and TBARS, thiobarbituric acid reactive substances.

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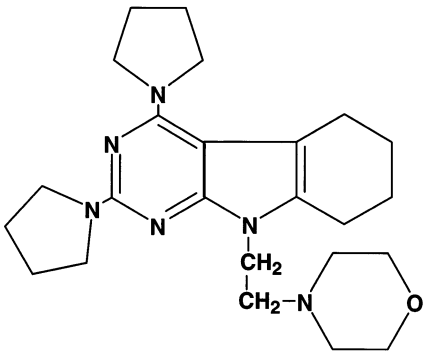

U-101033E

FIG. 1. Chemical structure of the pyrrolopyrimidine U-101033E.

lipid peroxidation, and, therefore, the accumulation of toxic by-products produced during the lipid peroxidation process.

The purpose of the present study was 2-fold. First, we sought to determine if the production of 4-HNE leads to protein–adduct formation in a model system of lipid peroxidation using RBC membranes and Fe^{2+}/EDTA [28, 29]. Second, using one member in the pyrrolopyrimidine series, U-101033E (2,4-diaminopyrrolopyrimidine), we tested whether this compound could prevent lipid peroxidation and accumulation of toxic aldehydes, including MDA and 4-HNE, as well as subsequent membrane protein cross-linking and aldehyde–protein adduct formation.

MATERIALS AND METHODS

Materials

All chemicals used were of the highest purity grade available. Imidazole, histidine, ferrous sulfate, 1,1,3,3-tetraethoxypropane, xanthine, and BSA were purchased from the Sigma Chemical Co. Alkaline phosphatase-conjugated goat anti-mouse IgG and an alkaline phosphatase substrate kit were from Bio-Rad. Protran (nitrocellulose transfer and immobilization membrane) was from Schleicher & Schuell. Lipid peroxidation assay kits (No. 437634) and bovine milk xanthine oxidase were purchased from Calbiochem. 4-HNE (≥98% pure) was purchased from Cayman Chemical. MDA was prepared by the acid hydrolysis of 1,1,3,3-tetramethoxypropane at 45° for 1 hr as described by Esterbauer *et al.* [9]. U-101033E was a gift from Dr. Edward Hall (Pharmacia/Upjohn Co.). For reference, the structure of U-101033E is shown in Fig. 1.

Preparation of 4-HNE–BSA Adducts

4-HNE (2 mM final concentration) was incubated with 1 mg/mL of BSA for 6 hr at 25° to generate 4-HNE–BSA adducts, following the procedure of Waeg *et al.* [30].

Preparation and Peroxidation of RBC Membranes

Isolated plasma membranes from human RBCs were prepared as previously described [28]. Membrane protein content was determined by the bicinchoninic acid method [31], using BSA as a standard. The membranes were stored on ice in the refrigerator until used.

Peroxidation of RBC membranes was performed as previously described [28] with the following modification: lipid peroxidation was initiated by the addition of Fe^{2+}/EDTA in a ratio of 2:1 (final concentrations of 200 and 100 μM, respectively). U-101033E was dissolved in DMSO at a concentration of 10 mM and stored at −20°. On the day of the experiment, the stock solution was serially diluted in DMSO to achieve the desired drug concentration. For controls in experiments where U-101033E was tested (i.e. samples labeled 0 μM U-101033E), an equal volume of DMSO was added, which served as a vehicle control. The presence of 1% DMSO had no effect on any of the parameters monitored (data not shown), and as demonstrated previously [29].

Measurement of Lipid Peroxidation

MDA and 4-HNE were quantified by the spectrophotometric procedure of Smith *et al.* [32], using a kit from Calbiochem. Briefly, 200 μg RBC membranes were incubated with 15.4 M methanesulfonic acid at 45° for 40 min (allows detection of both MDA and 4-HNE) or with 12 N HCl at 45° for 60 min (allows detection of MDA only, even in the presence of 4-HNE). The sample absorbance was then measured at 586 nm on a Cary 3E spectrophotometer (Varian Instruments), and aldehyde concentrations were calculated using apparent molar extinction coefficients determined from standard curves of known concentrations of either MDA or 4-HNE. Standard curves were generated using the same procedure as described above except known concentrations of MDA (1,1,3,3-tetramethoxypropane), 4-HNE, and a mixture of both aldehydes at equal concentrations were analyzed. Then 4-HNE concentrations were calculated by subtraction of the MDA concentration from the total MDA plus 4-HNE concentration. For this assay, the lower limit of measurable MDA or 4-HNE in a sample was ~0.5 μM.

Generation of Superoxide Anion by a Xanthine/Xanthine Oxidase System

Superoxide anion (O_2^-) was generated and detected using a modification of the microassay method of Laight *et al.* [33]. Briefly, we used an assay mixture consisting of 10 mU/mL of xanthine oxidase, 0.5 mM xanthine, 100 μM ferricytochrome *c*, 125 mM NaCl, and 50 mM HEPES, pH 7.4, to make a total volume of 200 μL in a 96-well microtiter plate. The rate of O_2^- production was then monitored at 25° by following the reduction of cytochrome *c* at 550 nm using a Molecular Devices THERMOmax microplate reader. Sim-

ilar assays containing 75 U/mL SOD were performed to demonstrate specificity of the assay for detecting O_2^-. To analyze the effect of U-101033E, we included 1 μM U-101033E or a similar volume of DMSO (vehicle control) in the reaction mixture. The results are presented as V_{max} rates, calculated using $\varepsilon = 21\ mM^{-1}\ cm^{-1}$ for cytochrome *c*, and expressed as micromoles O_2^- per minute per milligram of protein.

Electrophoresis and Western Blotting

SDS–PAGE was performed as previously described [28, 29] using a 7.5% polyacrylamide separating gel and a 4% polyacrylamide stacking gel for resolving proteins, and the gels were stained with Coomassie brilliant blue.

Western blot analysis was performed as described previously [34]. Transfers were blotted with primary antibody for 3 hr, followed by alkaline phosphatase-conjugated goat anti-mouse IgG secondary antibody (1:1000 dilution) for 1 hr. All incubations were performed at room temperature, and blots were developed using an alkaline phosphatase development kit. Pre-stained SDS–PAGE molecular weight standards were used on all gels (Gibco BRL).

RESULTS

Antibody Specificity

In these studies, we utilized a monoclonal antibody previously described by Waeg *et al.* [30] to analyze for the presence of 4-HNE–protein adducts. This antibody (HNE-1g4h7) was characterized previously using ELISA and was shown to primarily recognize 4-HNE bound to histidine residues; however, there was also some cross-reactivity with 4-HNE–lysine and 4-HNE–cysteine adducts [30]. In addition, this antibody is specific for 4-HNE–protein adducts and does not react by ELISA with BSA adducts of 4-hydroxyhexenal, 2-nonenal, nonanal, or MDA [30]. To test the specificity of this antibody in western blotting, we first prepared 4-HNE–BSA adducts and examined the ability of the antibody to recognize this 4-HNE–protein adduct on western blots. As shown in Fig. 2, HNE-1g4h7 specifically recognized 4-HNE–BSA adducts but not BSA alone. Therefore, this antibody is specific for 4-HNE–protein adducts and would be anticipated to recognize such adducts on various proteins.

Time Course of Iron-Mediated Lipid Peroxidation, 4-HNE–Protein Adduct Formation, and Membrane Protein Cross-Linking

MDA and 4-HNE are toxic aldehydes produced from the peroxidation of polyunsaturated fatty acids, and, thus, serve as a reliable index of lipid peroxidation [9, 35]. The classical determination of MDA by the TBARS method is reportedly associated with numerous pitfalls, including poor specificity and reproducibility [36]. Therefore, we chose to measure MDA and 4-HNE using a colorimetric assay that

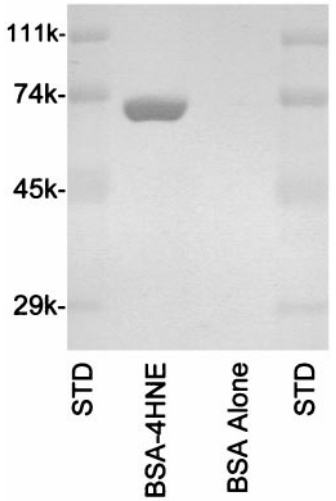

FIG. 2. Specificity of monoclonal antibody HNE-1g4h7 for 4-HNE–protein adducts. BSA conjugated with 4-HNE or BSA alone (2 μg/lane) were separated by SDS–PAGE and western blotted, as described under Materials and Methods, using monoclonal antibody HNE-1g4h7. Prestained molecular weight markers are shown on each side of the blot (STD). The data are representative of three independent experiments.

allows specific quantification of both of these aldehydes [32]. As shown in Fig. 3, incubation of RBC membranes with Fe^{2+}/EDTA resulted in a time-dependent formation of both MDA and 4-HNE. Aldehyde formation appeared to increase linearly for the first 2 hr, and then leveled off after 4 hr with maximal values of 43.0 ± 0.36 μM MDA and 16.4 ± 3.36 μM 4-HNE (mean ± SD, N = 3). Surprisingly, the values obtained for MDA using this assay were

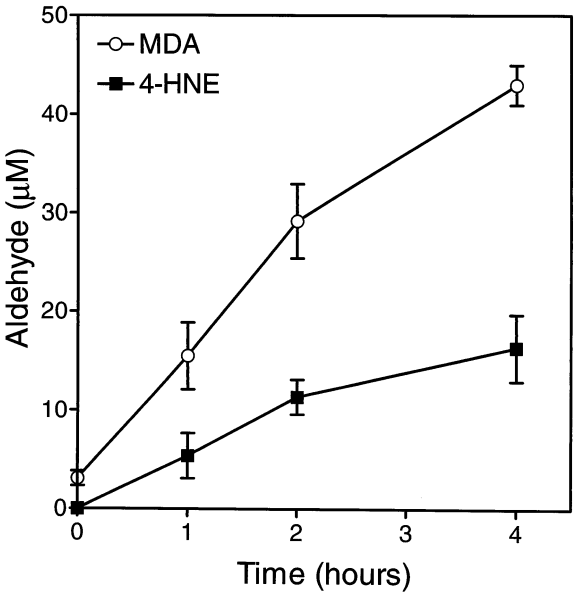

FIG. 3. Time course of iron-catalyzed aldehyde formation in RBC membranes. MDA or 4-HNE levels were determined following incubation of RBC membranes with Fe^{2+}/EDTA for various time periods, as described under Materials and Methods. The data are representative of three independent experiments (±SD).

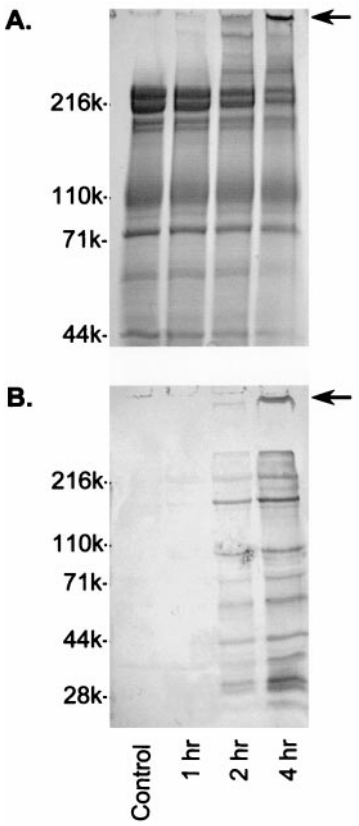

FIG. 4. SDS–PAGE and western blot analysis of RBC membranes treated for various times with Fe^{2+}/EDTA. Following the incubation times indicated, RBC membrane proteins were analyzed by SDS–PAGE (panel A) and western blotting with monoclonal antibody HNE-1g4h7 (panel B), as described under Materials and Methods. Sample lanes are identical for both panels. Arrows to the right of panels A and B indicate the presence of high molecular weight protein complexes at the entrance of the stacking gel. The data are representative of at least three independent experiments.

very similar to those obtained in previous studies [28, 29] using the classical TBARS method. Therefore, the TBARS assay, though often maligned, appears to be a useful index of MDA formation and lipid peroxidation *in vitro*, at least in our hands.

As previously demonstrated [28, 29], incubation of RBC membranes with Fe^{2+}/EDTA resulted in non-specific cross-linking of RBC membrane proteins and the appearance of a high molecular weight proteinaceous complex in a time-dependent manner (Fig. 4A). The appearance of this high molecular weight complex was of sufficient molecular weight that it barely entered the stacking gel (Fig. 4A, see arrow). Also, compared with control membranes, Fe^{2+}/EDTA promoted a general loss of all the RBC membrane proteins, indicating a relative lack of selectivity of this treatment for any one RBC membrane protein.

Based on our previous studies [28, 29], it was hypothesized that one potential mechanism for Fe^{2+}/EDTA-mediated damage and inactivation of certain RBC membrane proteins, such as the Ca^{2+} pump ATPase, is through cross-linking by reactive aldehydes produced following the initiation of lipid peroxidation. To test this hypothesis directly, we incubated RBC membranes with various concentrations of 4-HNE and MDA and analyzed for the presence of high molecular weight protein complexes. As shown in Fig. 5, protein cross-linking occurred when RBC membranes were incubated with either 4-HNE or MDA at concentrations that were similar to those generated by treatment of membranes with Fe^{2+}/EDTA (see Fig. 3). Furthermore, our results show that MDA is able to cause a significantly higher level of protein cross-linking than 4-HNE in this system. This would be expected since the dialdehyde MDA has two reactive aldehyde moieties. The addition of both 4-HNE and MDA also resulted in significant protein cross-linking; however, the level of cross-linking appeared similar to that induced by MDA alone. Thus, our results suggest that although MDA plays a major role in protein cross-linking, 4-HNE also contributes to protein cross-linking observed during membrane lipid peroxidation.

To determine whether the observed protein cross-linking following incubation of RBC membranes with Fe^{2+}/EDTA could be due to reactive aldehydes, we monitored for the presence of 4-HNE–protein adducts by western blot analysis. Figure 4B depicts the results of such an experiment, with representative lanes being identical to those presented in Fig. 4A. No detectable 4-HNE–protein adduct formation occurred in the absence of Fe^{2+}/EDTA, as indicated by the absence of any immunoreactivity to the antibody in the control lane. After 1 hr of incubation with Fe^{2+}/EDTA, only faint immunoreactivity was observed (Fig. 4B, lane marked 1 hr), which correlated with the low production of

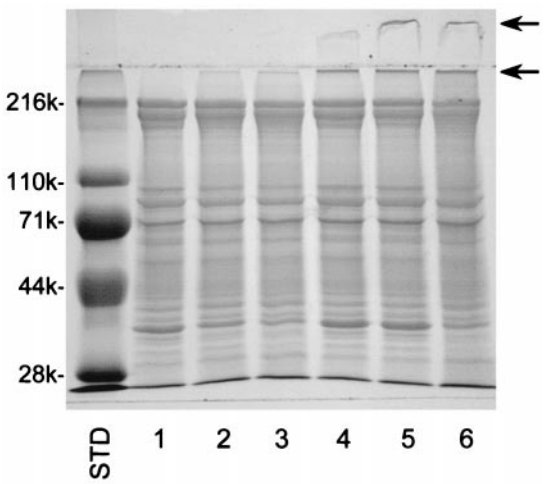

FIG. 5. Analysis of RBC membranes treated with 4-HNE and MDA. Control, untreated RBC membranes (lane 1) and membranes incubated for 2 hr at 37° with 25 μM 4-HNE (lane 2), 50 μM 4-HNE (lane 3), 25 μM MDA (lane 4), 50 μM MDA (lane 5), or 50 μM 4-HNE + 50 μM MDA (lane 6) were analyzed by SDS–PAGE, as described under Materials and Methods. Prestained molecular weight markers are shown on the left (STD). Arrows indicate the presence of high molecular weight protein complexes at the entrance of the stacking gel. The data are representative of two independent experiments.

4-HNE (Fig. 3), and the low level of RBC membrane protein cross-linking (Fig. 4A) observed at this time point. These data support the idea that with little or no lipid peroxidation, minimal 4-HNE–protein adduct formation and membrane protein cross-linking occur. However, at the 2- and 4-hr time points where there were significant levels of 4-HNE (Fig. 3) and protein cross-linking (Fig. 4A), we detected significant levels of 4-HNE–protein adduct formation (Fig. 4B). Indeed, in all regards, the appearance of 4-HNE–protein adducts paralleled the time course of both lipid peroxidation and membrane protein cross-linking. Several other features of this protein–adduct formation closely paralleled membrane protein cross-linking. For example, as shown in Fig. 4B, the action of 4-HNE was nonspecific in that numerous immunoreactive bands were visible following western blot analysis, indicating that 4-HNE is able to react with most RBC membrane proteins in promoting protein–adduct formation. In addition, a very strong signal corresponding to 4-HNE–protein adduct immunoreactivity was observed at the 4-hr time point near the top of the stacking gel (Fig. 4B, see arrow). This observation supports the conclusion that the presence of the high molecular weight complex in Fig. 4A is a direct result of protein–adduct formation mediated, at least in part, by lipid peroxidation by-products such as reactive aldehydes (4-HNE and MDA).

Effects of a Novel Antioxidant on Lipid Peroxidation, 4-HNE–Protein Adduct Formation, and Membrane Protein Cross-Linking

We next examined the ability of a novel pyrrolopyrimidine antioxidant, U-101033E, to prevent iron-mediated lipid peroxidation in RBC membranes and found that U-101033E was very effective in preventing lipid peroxidation in RBC membranes incubated with Fe^{2+}/EDTA. As shown in Fig. 6, aldehyde formation, measured either as MDA or 4-HNE, was inhibited completely by U-101033E in a concentration-dependent manner with an apparent IC_{50} of approximately 0.5 μM. It should be noted that DMSO, the solvent used, had no effect on aldehyde formation in similar assays (data not shown).

We also analyzed what effect inhibition of lipid peroxidation by U-101033E had on subsequent membrane protein cross-linking and the formation of aldehyde–protein adducts. As shown in Fig. 7, both membrane protein cross-linking and the formation of 4-HNE–protein adducts were reduced significantly in a concentration-dependent manner in samples treated with U-101033E. Even at concentrations as low as 0.3 μM U-101033E, there was a significant decrease in 4-HNE–protein adduct immunoreactivity (Fig. 7B), and a decrease in the formation of the high molecular weight complex in the stacking gel (Fig. 7A). At 1 μM U-101033E, essentially no membrane protein cross-linking or 4-HNE–adduct formation was observed. This effect was not due to U-101033E interacting directly with and scavenging aldehyde, however, as we found that membrane protein cross-linking induced by direct treatment of RBC membranes with 4-HNE or MDA was not blocked by treatment with U-101033E (Fig. 8).

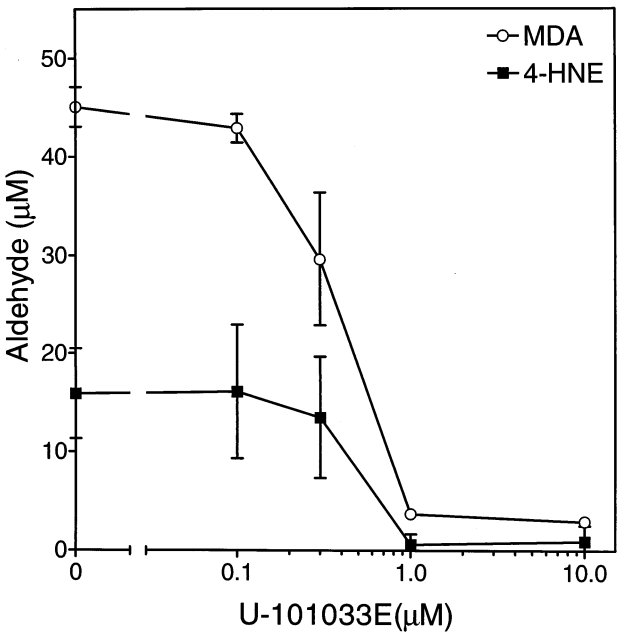

FIG. 6. Inhibition of lipid peroxidation by U-101033E. RBC membranes were incubated for 4 hr in the presence of Fe^{2+}/EDTA and increasing concentrations of the antioxidant U-101033E. Following incubation, aldehyde levels were determined as described under Materials and Methods. Experiments conducted with 0 μM U-101033E contained an equal volume of DMSO alone, which served as the solvent control. Values are the means ± SD of three separate experiments.

In previous studies, Rohn *et al.* [28] found that SOD inhibited lipid peroxidation induced by Fe^{2+}/EDTA, presumably by scavenging superoxide anion (O_2^-) generated by this system. Therefore, we analyzed whether U-101033E might similarly be scavenging O_2^-, thereby blocking subsequent lipid peroxidation. For these studies, we utilized an O_2^--generating system consisting of xanthine and xanthine oxidase. As shown in Fig. 9, addition of 1 μM U-101033E to this system had no significant effect on SOD-inhibitable O_2^- production. Thus, U-101033E does not appear to be a direct scavenger of O_2^-. Rather, the antioxidant U-101033E most likely interferes with the propagation of lipid peroxidation via a chain-breaking mechanism.

DISCUSSION

The reactive aldehydes 4-HNE and MDA are major lipid peroxidation by-products generated from free radical attack on membranes [37], and their formation has been shown to correlate well with lipid peroxidation *in vivo* [8, 9, 38]. The production of 4-HNE is thought to contribute significantly to subsequent cell injury due to its high reactivity with important membrane proteins [8, 9]. In this regard, 4-HNE has been demonstrated to accumulate during ischemia–reperfusion injury of rat small intestine [39] or myocardium

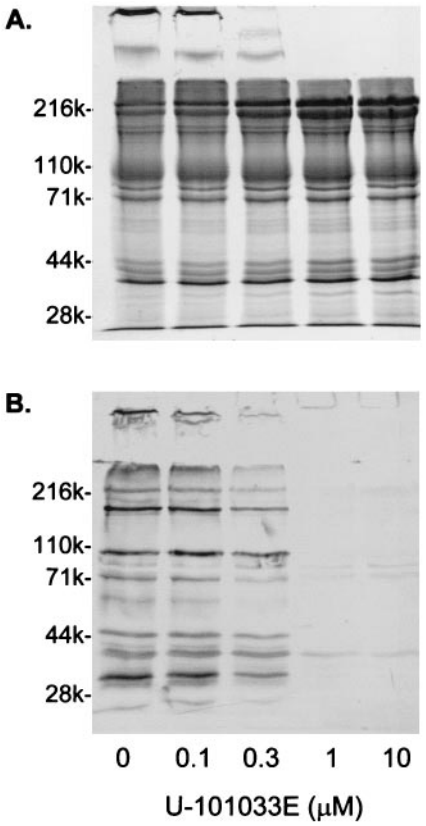

FIG. 7. Inhibition of iron-mediated membrane protein cross-linking and 4-HNE–protein adduct formation by U-101033E. RBC membranes were preincubated for 4 hr in the presence of Fe^{2+}/EDTA and increasing concentrations of U-101033E. Lanes marked 0 μM contained an equal volume of DMSO alone, which served as the solvent control. Following incubation, RBC membrane proteins were analyzed by SDS–PAGE (panel A) and western blotting with monoclonal antibody HNE-1g4h7 (panel B), as described under Materials and Methods. Sample lanes for panels A and B are identical, and the data are representative of at least three independent experiments.

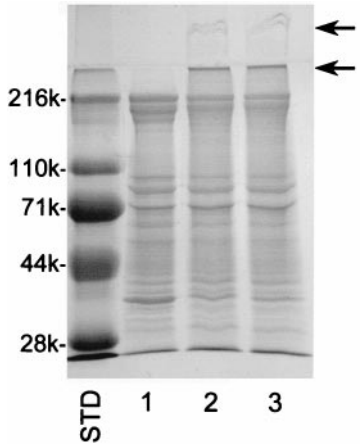

FIG. 8. Effect of U-101033E on protein cross-linking in RBC membranes treated with 4-HNE and MDA. Control, untreated RBC membranes (lane 1) and membranes incubated for 2 hr at 37° with 50 μM 4-HNE + 50 μM MDA and 10 μL DMSO (vehicle control) (lane 2) or 50 μM 4-HNE + 50 μM MDA and 1 μM U-101033E (lane 3) were analyzed by SDS–PAGE, as described under Materials and Methods. Prestained molecular weight markers are shown on the left (STD). Arrows indicate the presence of high molecular weight protein complexes at the entrance of the stacking gel. The data are representative of two independent experiments.

[40], induce cell death [41, 42], damage neurons [43], cause modification of low-density lipoproteins [44], exhibit chemotactic activity towards neutrophils *in vivo* [45, 46], and inhibit a variety of enzymes, such as glyceraldehyde-3-phosphate dehydrogenase [47] and Na^+/K^+-pump ATPase [48]. MDA has also been shown to play an important role in tissue injury associated with lipid peroxidation [36], and increased MDA levels have been observed during aging [19] as well as in a number of disease processes, including perinatal hypoxia [49], lipoprotein oxidation [50], and oxidant-mediated hepatocyte injury [51, 52].

One of the goals of the present study was to establish a direct relationship between the initiation of lipid peroxidation and eventual inactivation of plasma membrane proteins using a model system consisting of purified RBC membranes and Fe^{2+}/EDTA. Using this system, we would also be able to test the ability of novel antioxidant drugs to inhibit lipid peroxidation and associated tissue damage. Our results demonstrate that significant levels of MDA and 4-HNE were generated in this model system, and that both aldehydes were capable of cross-linking RBC membrane proteins. In addition, we used a monoclonal antibody that recognized 4-HNE–protein adducts [30], to show unequivocally that iron-catalyzed lipid peroxidation results in the formation of 4-HNE–protein adducts and cross-linked protein material that contains 4-HNE modifications. Thus, our results support a strong correlation between the occurrence of lipid peroxidation, the production of 4-HNE–protein adducts, and membrane protein cross-linking. In addition,

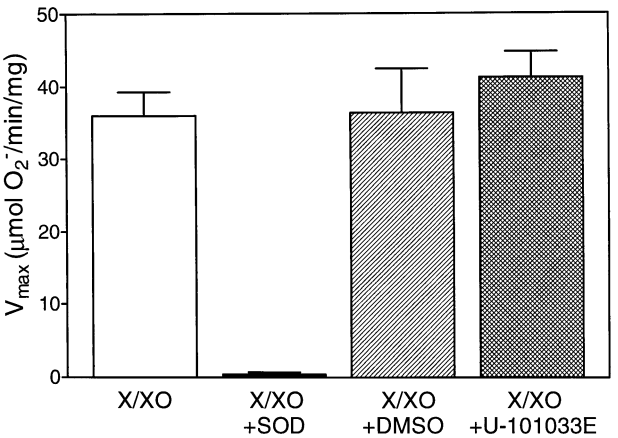

FIG. 9. Analysis of the ability of U-101033E to scavenge superoxide anion in a xanthine/xanthine oxidase system. O_2^- was generated using a xanthine/xanthine oxidase (X/XO) system and detected by monitoring the reduction of cytochrome *c* at 550 nm, as described under Materials and Methods. As indicated, the effect of adding U-101033E to this system was determined (X/XO + U-101033E). Also included are controls for specificity (X/XO + SOD and X/XO + DMSO). Data represent the means ± SEM of three independent experiments.

our studies suggest that MDA, another toxic aldehyde produced in our model system, also plays a significant role in mediating membrane protein cross-linking by causing intermolecular cross-link formation via its two reactive aldehyde moieties [38]. The ability of 4-HNE to cross-link proteins has been well documented in a number of systems. For example, Uchida and Stadtman [47] showed that 4-HNE cross-links glyceraldehyde-3-phosphate dehydrogenase, resulting in loss of enzyme activity. Friguet *et al.* [53, 54] showed that 4-HNE cross-links glucose-6-phosphate dehydrogenase, making this enzyme resistant to proteolysis by the multicatalytic protease/proteosome. Finally, Blanc *et al.* [55] recently found that 4-HNE cross-links the glutamate transporter (GLT-1) in cortical astrocytes, causing impaired glutamate transport and neuronal cytotoxicity. According to Uchida and Stadtman [47], 4-HNE-mediated protein cross-linking could arise from secondary reactions of carbonyl groups from 4-HNE-derived Michael adducts of cysteine, histidine, and lysine with the amino groups of lysine residues on the same or neighboring proteins, resulting in inter- and intramolecular cross-links.

The usefulness of antibodies against reactive aldehyde–protein adducts has been demonstrated in a number of previous studies. For example, Palinski *et al.* [12] used anti-4-HNE–adduct antibodies to demonstrate the presence of 4-HNE–protein conjugates in oxidized low density lipoprotein (LDL). Their results supported the conclusion that LDL undergoes oxidation *in vivo*, an important step in the development of atherosclerosis. More recent studies have shown immunohistochemical evidence of 4-HNE–protein adducts in patients with Parkinson's disease and Alzheimer's disease [14, 15], supporting the idea that oxidative stress contributes to the cell death associated with these diseases. Antibodies recognizing MDA–protein adducts have also been useful in exploring the role of MDA–protein adduct formation disease processes. For example, Greilberger and Jurgens [56] recently found that accumulation of MDA–protein adducts in oxidized high density lipoprotein changes its immunological properties and its binding affinity for collagen. Accumulation of MDA–protein adducts has also been shown in renal tissues of diabetics [57, 58] and in hepatic tissue from patients with chronic liver disease [59]. Finally Mooradian *et al.* [19] showed that MDA–protein adducts accumulate in certain tissues in aging rats. Clearly, these studies, along with the present study, demonstrate that the use of aldehyde–protein adducts as markers of lipid peroxidation provides a powerful tool to link free radicals with protein alteration and damage in tissues subjected to oxidative stress.

Due to the role of ROS in the etiology of CNS injury, efforts have been undertaken towards the discovery of effective antioxidant compounds that exhibit neuroprotective activity following CNS injury. The 21-aminosteroids are one class of compounds that have shown promising activity in experimental models of CNS ischemia–reperfusion injury (for a review, see Ref. 60). The most extensively studied 21-aminosteroid from this series of compounds is tirilazad mesylate, an antioxidant currently in phase III clinical trials for head and spinal cord injury, stroke, and subarachnoid hemorrhage [61]. Recently, a new group of antioxidants, the pyrrolopyrimidines, have been developed based on the structure of the 21-aminosteroids. These compounds possess significantly increased efficacy over the 21-aminosteroids in protecting cultured neurons against free radical-mediated injury [24, 27]. For example, U-101033E, the compound used in our studies, was shown to protect spinal cord and hippocampal neurons during focal cerebral ischemia, to reduce infarct size in a cerebral artery occlusion model, and to exhibit superior brain penetration as compared with tirilazad [27]. In addition, these newer compounds appear to exhibit a greater potency than tirilazad in protecting cultured fetal mouse spinal neurons from iron-induced lipid peroxidation [27].

In the present studies, we examined the ability of U-101033E to act as an antioxidant in further detail, using a model system of lipid peroxidation. We found that U-101033E was a very effective inhibitor of iron-induced lipid peroxidation. In this regard, U-101033E prevented the production of the noxious aldehydes MDA and 4-HNE with an IC_{50} of approximately 0.5 μM. In addition, U-101033E prevented iron-mediated membrane protein cross-linking, presumably by blocking the formation of 4-HNE and MDA and subsequent aldehyde–protein adduct formation. Based on previous studies [28, 29], U-101033E appears to be ~27 times more potent than tirilazad in preventing iron-induced lipid peroxidation. The protective action of U-101033E most likely results from its ability to act as a lipid peroxidation chain-breaking antioxidant similar to vitamin E [38], and our studies clearly show that U-101033E does not scavenge O_2^-, which is an obligatory intermediate in iron-induced lipid peroxidation in this model system [28]. The ability of U-101033E to act as an effective antioxidant is based on its structure (see Fig. 1). In contrast to the 21-aminosteroids, the pyrrolopyrimidines lack the highly lipophilic steroid moiety, which may serve to lessen the high affinity for, and retention in, lipid bilayers [28]. Thus, by possessing a more amphipathic structure, it would be predicted that U-101033E would be localized to a large extent near the water/lipid interface of cell membranes, an ideal location for a compound to act as an inhibitor of lipid peroxidation.

In conclusion, we have demonstrated a direct link between the onset of lipid peroxidation and the eventual inactivation of certain membrane-bound proteins. This link appears to operate, at least in part, through adduct formation and protein cross-linking by toxic aldehydes such as 4-HNE and MDA. This idea is based upon the results demonstrating that 4-HNE and MDA are able to react with and cause membrane protein cross-linking. Indeed, we found an excellent correlation among the lipid peroxidation process, the appearance of 4-HNE–protein adducts, and membrane protein cross-linking. Finally, we demonstrated that U-101033E is a potent inhibitor of lipid peroxidation and able to prevent completely the formation

of toxic aldehydes, thereby inhibiting subsequent protein adduct formation and cross-linking caused by these aldehydes. This ability of U-101033E to act as an efficacious antioxidant may be of potential usefulness where ROS are known to play a central role in the pathogenesis of certain diseases.

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