Free Rad. Res. Comms., Vol. 16, No. 2, pp. 111-122
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PEROXYL RADICAL-MEDIATED HEMOLYSIS: ROLE OF LIPID, PROTEIN AND SULFHYDRYL OXIDATION

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(Received October 28, 1991; in revised form November 12, 1991)

The objective of this study was to define the relationship between peroxyl radical-mediated cytotoxicity and lipid, protein and sulfhydryl oxidation using human erythrocytes as the target mammalian cell. We found that incubation of human erythrocytes with the peroxyl radical generator 2,2′ azobis (2-amidinopropane) hydrochloride (AAPH) resulted in a time and dose-dependent increase in hemolysis such that at 50 mM AAPH maximum hemolysis was achieved at 120 min. Hemolysis was inhibited by hypoxia and by the addition of certain water soluble free radical scavengers such as 5-aminosalicylic acid (5-ASA), 4-ASA, N-acetyl-5-ASA and dimethyl thiourea. Peroxyl radical-mediated hemolysis did not appear to involve significant peroxidation of erythrocyte lipids nor did they enhance protein oxidation at times preceding hemolysis. Peroxyl radicals did however, significantly reduce by approximately 80% the intracellular levels of GSH and inhibit by approximately 90% erythrocyte Ca^{2+}-Mg^{2+} ATPase activity at times preceding the hemolytic event. Our data as well as others suggest that extracellular oxidants promote the oxidation of intracellular compounds by interacting with certain redox active membrane components. Depletion of intracellular GSH stores using diamide did not result in hemolysis suggesting that oxidation of GSH alone does not promote hemolysis. Taken together, our data suggest that neither GSH oxidation, lipid peroxidation nor protein oxidation alone can account for peroxyl radical-mediated hemolysis. It remains to be determined whether free radical-mediated inactivation of Ca^{2+}-Mg^{2+} ATPase is an important mechanism in this process.

KEY WORDS: Azo compounds, erythrocyte, 5-Aminosalicylic acid, antioxidants, glutathione, ATPase.

INTRODUCTION

Reactive oxygen metabolites have been implicated in a variety of pathophysiological conditions including atherosclerosis, post-ischemic tissue injury, arthritis, cancer, Parkinson's disease and certain autoimmune diseases.[1-3] In addition, oxy radicals have been suggested to play an important role in mediating the tissue injury associated with the metabolism of certain drugs and xenobiotics.[3] Although the mechanisms by which reactive metabolites of oxygen injure cells and tissue remain rather speculative, many investigators have suggested that oxidation of membrane associated poly-unsaturated fatty acids (PUFA) represent a major pathway by which free radicals mediate their cytotoxic effect.[1,3] However, many of these studies have used as their target cell, purified micellar lipids devoid of important membrane and cytosolic

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components such as protein and sulfhydryl-containing compounds. More recent studies suggest that protein and sulfydryl-containing compounds may represent important targets for oxidative tissue injury.[4–6] Therefore, the objective of this study was to define the relationship between free radical-mediated cytotoxicity and lipid, protein and sulfhydryl oxidation using human erythrocytes as a target cell. Because most free radical generators require the addition of potentially interfering cofactors and transition metals we chose to use the thermolabile peroxyl radical generator, 2,2′azobis-2-amidinopropane-dihydrochloride (AAPH), as our source of free radicals.[7,8]Data presented in this study demonstrate that peroxyl radicals hemolyze human erythrocytes in a time and dose-dependent manner. Only GSH oxidation and Ca^{2+}–Mg^{2+} ATPase inactivation occurred at times preceding hemolysis whereas neither lipid nor protein oxidation appeared to play a role in this process. The data are discussed in relation to mechanisms by which free radicals injure cells and tissues.

MATERIALS AND METHODS

Chemicals and Biochemicals

Sulfasalazine, sulfapyridine, brain extract (Folch fraction III, 85% phosphatidylserine), dimethylsulfoxide, 2,4 dinitrophenylhydrazine, butylated hydroxytoluene, 5,5′dithionitrobenzoic acid, sodium azide, thiobarbituric acid, Fiske & Subbarow reducer, guanidine, Tris (hydroxymethyl) aminomethane (Tris) hydrochloride and human serum albumin (fatty acid free) were purchased from Sigma Chemicals Co. (St. Louis, MO). Hydrogen peroxide was from Fischer Scientific. Dimethyl thiourea was from Aldrich Chemicals Corp. Inc. (Milwaukee, WI). 5-aminosalicylic acid, N-acetyl-5-aminosalicylic acid and 4-aminosalicylic acid were provided by Dr. Thomas Berglindh, KABi-Pharmacia (Uppsala, Sweden). 2,2′azobis (2 amidinopropane) hydrochloride was obtained from Polysciences Inc. (Warrington, PA). Sodium chromate (^{51}Cr) was obtained from Dupont NEN products (Boston, MA). Saponin was supplied by Calbiochem (La Jolla, CA).

Methods

Erythrocyte preparation. Blood was collected from human volunteers by venepuncture. The erythrocytes were separated from leukocytes and platelets by dextran sedimentation and the erythrocyte pellet was washed by centrifugation three times with phosphate buffered saline (PBS). The cells (2×10^9 cells/ml) were then incubated with 100 μCi sodium chromate for 1 h at 37°C. The cells were then washed three times with PBS and resuspended to 2×10^9 cells/ml with Dulbeccos phosphate-buffered saline (DPBS). For the hemolysis experiments, erythrocytes (2×10^8 cells/ml) were incubated with varying concentrations of AAPH in DPBS for varying lengths of time at 37°C with occasional swirling. AAPH is a water soluble free radical initiator that decomposes unimolecularly without requirement for enzymes or biotransformation to yield nitrogen and carbon-centered radicals.[7,8] These carbon-centered radicals react rapidly with molecular oxygen to yield peroxyl radicals. The rate of radical formation is proportional to the concentration of AAPH. At 15 min intervals aliquots of cell suspension were removed, centrifuged and the supernatant and pellet counted for the presence of ^{51}Cr to determine the extent of hemolysis. For some experiments oxygen

was purged from the buffer by bubbling the DPBS with nitrogen gas for 30 min on ice. The solution was then capped and allowed to return to room temperature. This solution was used to suspend the erythrocytes as well as used for the experiments.

Lipid peroxidation Lipid peroxidation was quantified by measuring the formation of thiobarbituric acid reactive substances (TBARS).[9,10] Briefly, AAPH (50 mM) was incubated in DPBS containing either erythrocytes (2×10^8 cells/ml) or brain extract phospholipid (0.7 mM phospholipid phosphate) for varying lengths of time at 37°C. For some experiments erythrocytes were pretreated with 1 mM sodium azide for 10 min at 37°C to inhibit endogenous catalase activity and then 10 mM H_2O_2 was added to induce lipid peroxidation.[9] At 15 min intervals aliquots (0.5 ml) of either the cell suspension or lipid were removed from each tube and added to 1 ml of a solution containing 15% (w/v) trichloroacetic acid, 0.375% (w/v) thiobarbituric acid and 0.25 N HCl. Protein precipitate was removed by centrifugation. To prevent spurious lipid peroxidation during subsequent steps, 0.02% butylated hydroxytoluene was added prior to the heating step. Each mixture was then heated in a boiling water bath for 15 min, the tubes were allowed to cool and then centrifuged at 8000 × g for 5 min to remove precipitant. The absorbance of each sample was determined at 532 nm.

Protein oxidation. Protein oxidation was quantified using the interaction between 2,4 dinitrophenylhydrazine (2,4 DNP) and the carbonyls generated from the peroxyl radical-mediated oxidation of proteins.[5,6] This reaction yields a chomophore that absorbs strongly at 380 nm. Briefly, AAPH (50 mM) was incubated with human serum albumin (1 mg/ml; HSA), intact or hemolyzed erythrocytes (2×10^8 cells/ml) in DPBS for varying lengths of time at 37°C. For those experiments using HSA, 0.5 ml aliquots were removed and the protein precipitated by the addition of 10% TCA. Protein was collected by centrifugation and resuspended in 0.5 ml of 10 mM 2,4 DNP in 2 M HCl. These samples were incubated for 1 hr at 25°C with occasional mixing. Protein was then precipitated by the addition of 10% TCA, collected by centrifugation and the pellet washed three times with 1 ml of an ethanol : ethyl acetate (1 : 1) solution to remove any unreacted 2,4 DNP. The protein precipitate was solubilized in 1 ml of 6 M guanidine (pH 2.3) and the absorbance determined at 380 nm. The carbonyl content was calculated assuming a molar extinction coefficient of 22,000.[5] For those experiments involving erythrocytes, an aliquot of intact or hemolyzed cells (containing approximately 1 mg/ml protein) was removed and added to 10 volumes of acidified acetone (100 volumes acetone : 3 volumes HCl) to remove the heme.[6] The apoprotein precipitate was washed three times with 20% TCA, the precipitate dissolved in 2,4 DNP in HCl and treated exactly as described above. The protein content of the erythrocyte suspension was determined by the modified Lowry protein assay.[11]

Reduced gluthathione (GSH) determination. Acid soluable thiol content (GSH)[12,13] was quantified by using a minor modification of the method of Harlan *et al.*[4] Following incubation of erythrocytes (2×10^8 cells/ml) with 50 mM AAPH, 5 ml aliquots of cells (1×10^9 cells) were removed, pelleted by centrifugation and lysed by the addition of 0.6 ml water. Protein was then precipitated by the addition of 10% TCA and removed by centrifugation. The supernatant was neutralized by the addition of a small aliquot of 10 N NaOH and incubated with 0.13 M Tris (pH 8.2) containing 0.16 mM DTNB for 10 min at 37°C. The absorbance of each sample was determined at 412 nm. GSH content was calculated assuming a molar extinction coefficient of 13,600 at 412 nm.

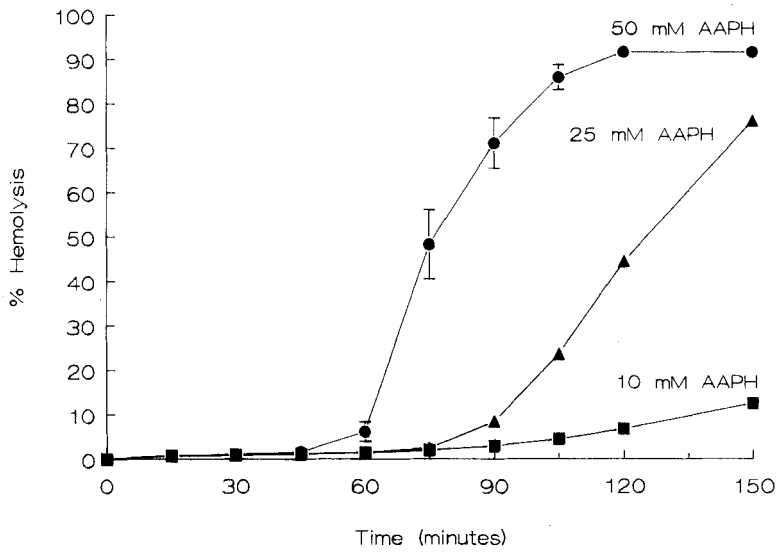

FIGURE 1 Peroxyl radical-induced hemolysis of human erythrocytes. Human erythrocytes (2×10^8 cells/ml) were incubated in DPBS with 10 mM AAPH, 25 mM AAPH or 50 mM AAPH at 37°C for varying lengths of time. Each data point represents the mean ± SEM for duplicate determinations from at least 3 different donors for the experiments using 50 mM AAPH. Each data point represents the mean for the duplicate determinations from 2 different donors for the experiments using 10 and 25 mM AAPH.

Ca^{2+}-Mg^{2+} ATPase activity. Erythrocytes (2×10^8 cell/ml) were incubated with 50 mM AAPH in DPBS for varying lengths of time (0–60 min) at 37°C. Aliquots of the cell suspension were removed, washed three times with 0.172 M Tris (pH 7.6) and suspended to 1×10^9 cells/ml in the Tris buffer. The cells were then lysed by the addition of isotonic saponin (0.1 mg/ml).[14] The assay mixture contained 0.1 ml lysate, 0.2 ml of the Tris-HCl buffer and 0.7 ml of a reaction mixture containing 30 mM imidazole, 100 mM KCl, 4.5 mM $MgCl_2$, 0.3 mM ouabain, 4.5 mM ATP and either 0.025 mM $CaCl_2$ or 0.1 mM EGTA.[15] Following precipitation of protein with 5% TCA, inorganic phosphorous in the supernatant was quantified using the method of Fiske-Subbarow.[16] Calcium ATPase activity was calculated by subtracting the Pi content of the EGTA-containing samples (Mg^{2+} ATPase) from that of the calcium-containing sample (Ca^{2+}-Mg^{2+} ATPase). Results are expressed as μmole Pi liberated/60 min/10^9 cells.

RESULTS

Incubation of human erythrocytes with AAPH resulted in a time and dose dependent increase in hemolysis (Figure 1). Using a 50 mM AAPH significant hemolysis did not occur until 60–75 min and maximum hemolysis (90–95%) was achieved at 120 min of incubation at 37°C. AAPH appears to require oxygen for maximal hemolysis. Figure 2 shows a significant reduction in extent of hemolysis in a hypoxic environment. Furthermore, addition of certain water soluble antioxidants such as 5-aminosalicylate (5-ASA), 4-aminosalicylate (4-ASA) or N-acetyl-5-aminosalicylate (N-5-ASA)

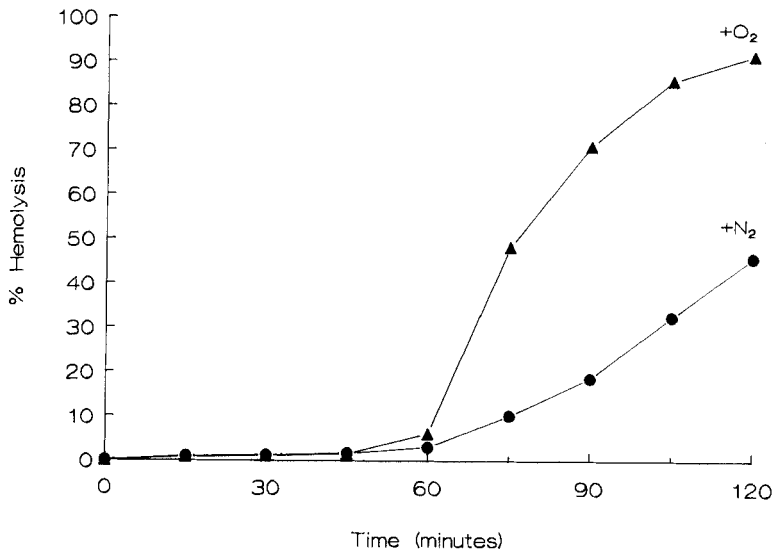

FIGURE 2 Effects of hypoxia on AAPH-induced hemolysis. Erythrocytes (2×10^8 cells/ml) were incubated in DPBS equilibrated with ambient oxygen or buffer that had been purged with nitrogen gas.

significantly inhibited AAPH-induced hemolysis (Figure 3). Sulfasalazine and sulfapyridine were either ineffective or only minimally effective at inhibiting peroxyl radical-mediated hemolysis (Figure 3). In addition to 5-ASA, another free radical scavenger dimethyl thiourea (DMTU) also exhibited an inhibitory effect on hemolysis whereas dimethylsulfoxide (DMSO) did not inhibit peroxyl radical-induced hemolysis at concentrations up to 50 mM (Figure 4).

Figure 5 demonstrates that AAPH promotes extensive peroxidation of phospholipid as measured by increases in TBARS formation. However, incubation of AAPH with erythrocytes did not increase TBARS formation (Figure 6). The fact that H_2O_2 produced extensive lipid peroxidation using the same numbers of erythrocytes as in the AAPH experiments suggests that erythrocyte lipids were not limiting (Figure 6). In an analogous series of experiments we found that incubation of human serum albumin (1 mg/ml) with AAPH produced significant increases in oxidized protein as measured by increased carbonyl formation (Figure 7). Addition of AAPH to a suspension of intact erythrocytes resulted in no significant protein oxidation whereas AAPH did induce protein oxidation (albeit at reduced amounts) using hemolyzed erythrocytes (Figure 7). Again, the fact 2×10^8 cells/ml contained adequate amounts of protein (5–6 mg/ml) suggested that protein was not limiting. We found that incubation of erythrocytes with AAPH decreased GSH content by approximately 80% prior to the onset of hemolysis (Figure 8). Finally, AAPH inhibited the enzyme Ca^{2+}–Mg^{2+} ATPase in a time and dose dependent such that virtually all activity was lost at times preceding hemolysis (60 min; Figure 9).

DISCUSSION

Many investigators have suggested that much of the injury associated with the

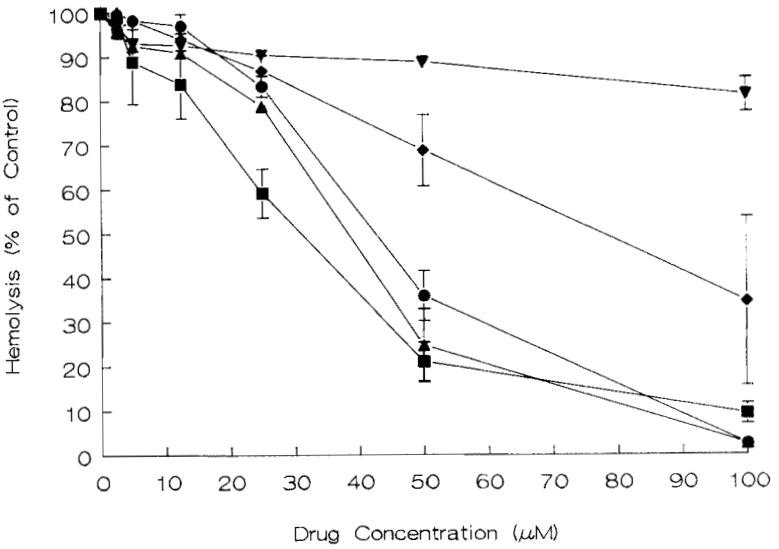

FIGURE 3 Effect of sulfasalazine and its metabolites on AAPH-induced hemolysis. Erythrocytes (2×10^8 cells/ml) were incubated with 50 mM AAPH in the absence or presence of anti-inflammatory drugs for 120 min at 37°C. (●) 5-ASA, (▲) 4-ASA, (■) N-acetyl-5-ASA, (◆) sulfapyridine and (▼) sulfasalazine. Each data point represents the mean ± SEM or duplicate determinations from at least 3 different donors.

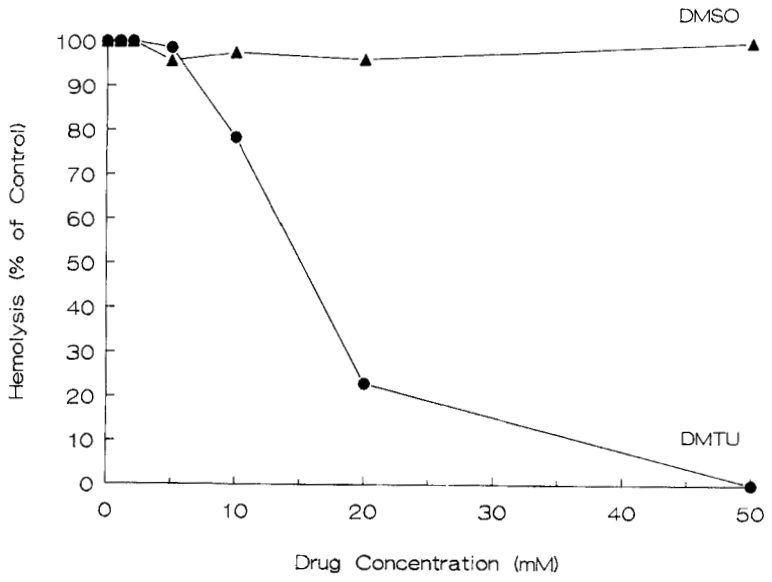

FIGURE 4 Effect of DMTU and DMSO on AAPH-induced hemolysis. Erythrocytes (2×10^8 cells/ml) were incubated with 50 mM AAPH in the absence or presence of varying concentrations of DMTU or DMSO for 120 min at 37°C. Each data point represents the mean for duplicate determinations from 2 different donors.

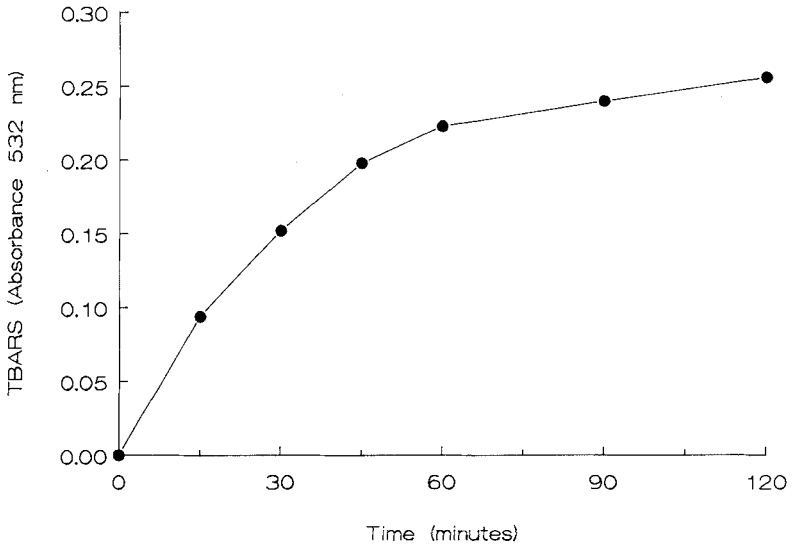

FIGURE 5 Peroxyl radical-induced peroxidation of phospholipid. Brain extract (0.7 mM; 85% phospholipid phosphate) was incubated with 50 mM AAPH in DPBS for varying lengths of time at 37°C. Thiobarbituric reactive substances (TBARS) were quantified by measuring the absorbance at 532 nm. Each data point represents the mean from triplicate determinations and did not vary by more than ±7%.

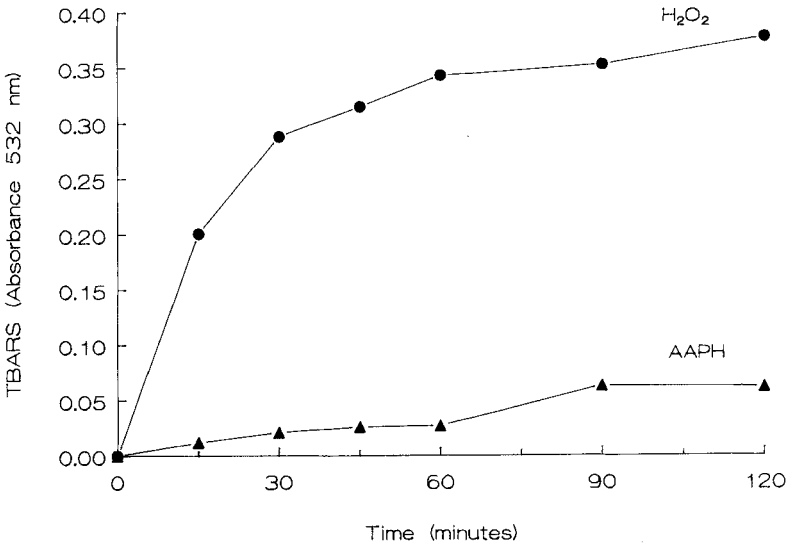

FIGURE 6 Peroxidation of erythrocyte lipids by AAPH and H_2O_2. Erythrocytes (2×10^8 cells/ml) were incubated with 50 mM AAPH or 10 mM H_2O_2 in DPBS at 37°C. For the H_2O_2 experiments, erythrocytes were treated with 1 mM sodium azide to inhibit endogenous catalase activity. Each data point represents the mean for duplicate determinations from 2 different donors.

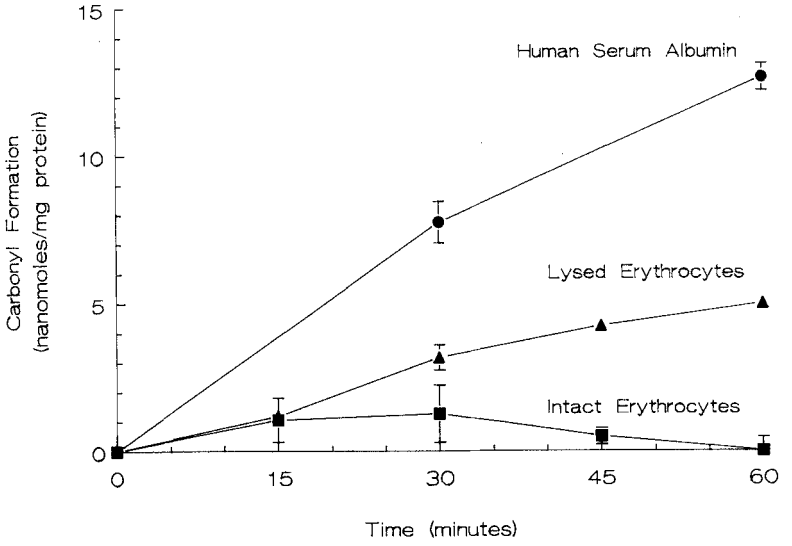

FIGURE 7 Peroxyl radical-induced protein oxidation. Intact Erythrocytes (10^9 cells/ml), hemolyzed erythrocytes or human serum albumin (HSA; 1 mg/ml) was incubated with 50 mM AAPH in DPBS for varying times at 37°C. Aliquots of intact hemolyzed erythrocytes containing approximately 1 mg/ml protein were used to assess protein oxidation. Protein oxidation was assessed by measuring carbonyl formation. Each data point represents the mean ± SEM for duplicate samples from 3 different donors. The background levels of carbonyls were 8.38 ± 0.46 nanomoles/mg (erythrocytes) and 4.2 ± 0.62 nanomoles/mg (HSA).

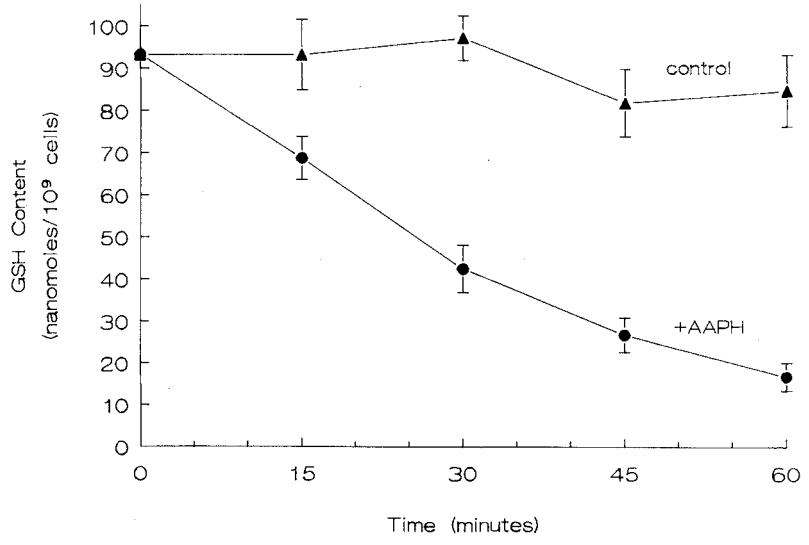

FIGURE 8 Peroxyl radical-mediated oxidation of erythrocyte GSH. Erythrocytes (2×10^8 cells/ml) were incubated with 50 mM AAPH in DPBS for varying lengths of time at 37°C. Each data point represents the means ± SEM for duplicate determinations from at least 3 different donors.

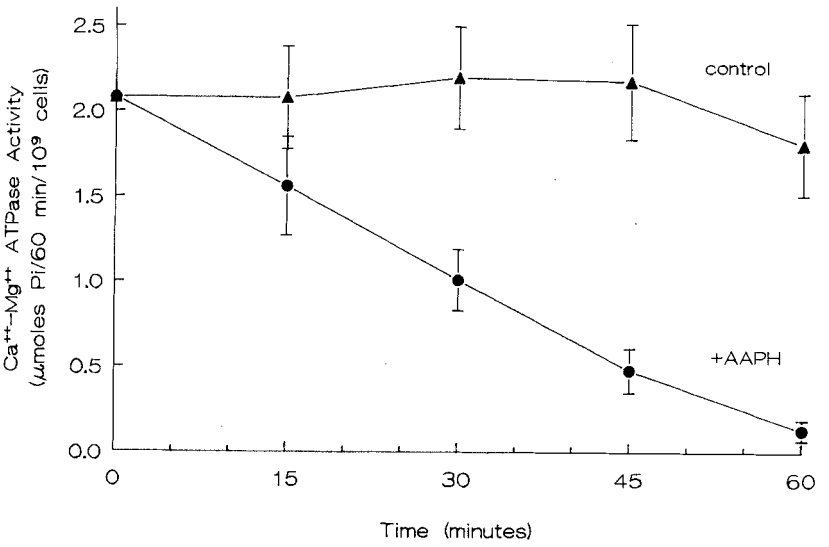

FIGURE 9 Peroxyl radical-mediated inhibition of erythrocyte Ca^{2+}–Mg^{2+} ATPase activity. Erythrocytes (2×10^8 cells/ml) were incubated with 50 mM AAPH in DPBS for varying lengths of time at 37°C. Each data point represents the mean for duplicate determinations from at least 3 different donors.

overproduction of reactive oxygen species, such as O_2^- and/or H_2O_2 is due to metal-catalyzed production of the highly reactive $OH^\cdot$ via superoxide-dependent (or independent) Fenton chemistry:

$$O_2^- + Fe^{+3} \rightarrow O_2 + Fe^{+2}$$

$$H_2O_2 + Fe^{+2} \rightarrow OH^- + OH^\cdot + Fe^{+3}$$

The interaction between $OH^\cdot$ and certain carbohydrates, proteins, nucleotide bases and lipids is known to produce peroxyl radicals as intermediates.[17,18] Indeed, peroxyl radical intermediates have been demonstrated to increase damage to biomolecules and are known to contribute significantly to the propagation of the free radical reactions involving polyunsaturated fatty acids (PUFAs).[3] Because peroxyl radicals are less reactive than $OH^\cdot$ and thus have "extended" half lives of seconds instead of nanoseconds they may be expected to react with cellular targets "distant" to their site of formation.[19] In this way the cytotoxic potential of $OH^\cdot$ may be transferred via the intermediate formation of peroxyl radicals. Thus it is of interest to define the relationship between peroxyl radical-mediated cytotoxicity and lipid, protein and sulfhydryl oxidation using erythrocytes as the target cell.

Data obtained in this study suggest that AAPH promotes hemolysis of human erythrocytes in a time and dose-dependent manner (Figure 1). Inhibition of AAPH-mediated hemolysis using hypoxic buffers suggests that an oxygen-derived species such as peroxyl radical is responsible for the injurious effects of AAPH (Figure 2). The fact that we observed any hemolysis in a "hypoxic" buffer most probably represents either the inadvertent introduction of oxygen upon repeated sampling or toxicity induced by carbon-centered radicals accumulated during hypoxia. These observations are in agreement with those from Niki *et al.*,[8] who showed similar effects with rabbit erythrocytes. Furthermore, we found that certain water soluble free radical scavengers

were able to inhibit AAPH-induced hemolysis (Figures 3 and 4). We have previously demonstrated that certain aryl amines such as 5-ASA and 4-ASA possess potent peroxyl radical scavenging activity.[20] Indeed, we show in this study their ability to inhibit AAPH-mediated hemolysis (Figure 3). It is of interest to note that 5-ASA is the pharmacologically active moiety of sulfasalazine, a compound which has been shown to be very effective in attenuating the mucosal inflammation and injury associated with ulcerative colitis. Recent work suggests that this disease may involve free radical-mediated processes.[20,21] In addition we demonstrated that DMTU but not DMSO (both $OH^{\cdot}$ scavengers) was very effective at inhibiting AAPH-mediated hemolysis suggesting that peroxyl radicals and not $OH^{\cdot}$ were responsible for AAPH-induced hemolysis (Figure 4). The dependence upon O_2 for maximal hemolysis coupled to the fact that certain water soluble antioxidants inhibit AAPH-mediated hemolysis suggest that peroxyl radicals are the damaging species as reported by Niki and coworkers.[7,8]

$$A\text{–}N{=}NA \rightarrow N_2 + 2A^{\cdot}$$

$$A^{\cdot} + O_2 \rightarrow AOO^{\cdot}$$

One possible mechanism that has been proposed for free radical-mediated hemolysis is peroxidation of erythrocyte membrane lipids, thereby disrupting its integrity.[7,8,22–27] In the present study we demonstrated that the peroxyl radicals generated from the decomposition of AAPH are capable of peroxidizing phospholipid (Figure 5). However, AAPH did not induce significant lipid peroxidation of human erythrocytes (Figure 6). Since H_2O_2 did promote extensive peroxidation of azide-treated cells we conclude that lipid substrate was not limiting (Figure 6). These data suggested that the peroxyl radicals were either unable to gain access to the PUFAs localized on the cytosolic face of the erythrocyte membrane or were scavenged by endogenous antioxidants. The latter possibility appears to be the case since we did not observe any significant lipid peroxidation even when the erythrocytes were hemolyzed prior to adding AAPH (data not shown). This is not surprising since it is well known that erythrocytes contain relatively high concentrations of nonenzymatic antioxidants. For example, Miki *et al.*[22] observed that in rat erythrocytes α-tocopherol is an important antioxidant against peroxyl radical-mediated hemolysis. Furthermore, Takenaka *et al.*[23] observed that thiols exert a sparing effect on membrane tocopherol and thus provide an additional line of defense against free radical-mediated injury to sulfhydryls.

Yamamoto *et al.*[24] have shown that erythrocyte ghosts are oxidized to peroxyl radicals and that the proteins as well as the polyunsaturated fatty acids of membrane lipids are oxidized to give high molecular weight proteins. They suggested that the protein oxidation was in the form of thiol oxidation to yield disulfide bonds and possibly polymerization of protein. Our data demonstrate very little protein oxidation of erythrocytes subjected to AAPH as measured by increases in carbonyl formation (Figure 7). If however, the cells were hemolyzed prior to adding the peroxyl radical generator we observed a significant increase in protein oxidation although not as great as with comparable amounts of HSA (1 mg/ml). These data suggest a lack of accessibility of AAPH with intact cells (Figure 7). The fact that protein oxidation was only 30% of that produced with similar amounts of HSA suggests that endogenous free radical scavengers are also effective at inhibiting this reaction.

Another mechanism by which oxidants are thought to mediate cellular injury is via oxidation of important sulfhydryl components.[4] Erythrocytes contain relatively high concentrations of GSH which many investigators feel is important to protect cells

from oxidative insult.[4] We found that the extracellular administration of a peroxyl radical generator resulted in the loss of approximately 80–85% of the intracellular GSH content at times preceding hemolysis (Figure 8). Although these data suggested that GSH oxidation may be an important mechanism by which AAPH promotes hemolysis, we were unable to observe any significant hemolysis when erythrocytes were depleted of GSH using diamide and incubated for 120 min (data not shown). These data are similar to those of Eaton and coworkers who observed that diminished glutathione levels per se do not predispose to erythrocyte oxidant injury.[28] Assuming that AAPH is only minimally accessible to the intracellular compartment in the first 30–60 min it raises the interesting possibility that an extracellular free radical may promote the oxidation of intracellular compounds. It is not inconceivable that this type of reaction could be initiated by the interaction between extracellular free radicals and certain redox-active membrane components located on the outer aspect of the cell membrane resulting in the oxidation of intracellular GSH. There is support for this concept in work by Reglinski *et al.*[29] in which they demonstrated that oxidative stress on the exofascial surface of the erythrocyte membrane resulted in oxidation of the reduced glutatione pool within the erythrocyte in the absence of cytolysis.

Sulfhydryl groups are important components of many enzymes. One such erythrocyte membrane enzyme is Ca^{2+}-Mg^{2+} ATPase. This enzyme is responsible for maintaining the ionic balances by actively pumping Ca^{2+} to the exterior and preventing it's intracellular accumulation.[30] Ca^{2+} plays an important role in mediating several essential intracellular processes including activation of a variety of enzymes, excitation contraction coupling and exocytosis. Inhibition of this enzyme activity by peroxyl radicals prior to the onset of hemolysis may lead to alterations in the intracellular osmotic balance resulting in water movement and cell swelling (Figure 9). Alternatively, accumulation of intracellular Ca^{2+} may also lead to activation of intracellular, Ca-dependent proteases resulting in uncontrolled proteolysis.[31] The lack of a specific inhibitor that will inhibit this enzyme in intact cells make it difficult to assess the relative role of Ca^{2+}-ATPase in peroxyl radical-mediated hemolysis.[32]

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Accepted by Prof. B. Halliwell