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PREVENTION OF HYDROXYL RADICAL-INDUCED ERYTHROCYTE HEMOLYSIS BY PROTEIN THIOLS

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Abstract – A system for studying oxidative hemolysis has been used by controling UV-irradiation and concentration of a novel molecular probe, N,N'-bis(2-hydroperoxy-2-methoxyethyl)-1,4,5,8-naphthalene-tetra-carboxylic-diimide (NP-III), which generates hydroxyl radical upon longer wavelength photoirradiation (> 350 nm). NP-III induces 25-30% of hemolysis at low concentration (50 μM) for 3h-irradiation of UVA. The simultaneous treatment of N-ethylmaleimide (NEM) with NP-III completely hemolyzed erythrocytes under the same conditions as NP-III alone by both decreasing thiol group and increasing lipid peroxidation in erythrocyte membrane. However, thiol-reducing agents prevented the protein-crosslinking and lipid peroxidation on the NEM-synergistic hemolysis by partially scavenging hydroxyl radical and maintaining the thiol group of erythrocyte membrane in the reduced state. In addition, erythrocytes pretreated with 2,2,5,7,8-pentamethyl-6-hydroxychromane (PMC), vitamin E homologue was able to delay and decrease the lipid peroxidation when compared to cells pretreated with both NEM and PMC. We suggest that the presence of reduced thiols in inner membrane protein by GSH can prevent the protein-crosslinking and the lipid peroxidation, and eventually prevent the oxidative hemolysis of erythrocyte.

INTRODUCTION

Reactive oxygen species have been reported to induce serious damages to red blood cell, which results in the cellular aging and hemolysis[1-3]. Carbon-centered radicals, generated from azo compound such as 2,2-azobis(2-aminopropane) dihydrochloride (AAPH) † by thermal decomposition, can induce the peroxidation of erythrocyte membrane and hemolysis[4-6]. It has been reported that free radicals attack the erythrocyte membrane by inducing the oxidation of lipids and proteins, and eventually cause hemolysis[7,8]. Even though the precise mechanism catalyzing oxidation of membrane lipids is not clear, the lipid peroxidation by reactive oxygen species require metal chelates such as iron chelates and iron-ascorbate[9-11].

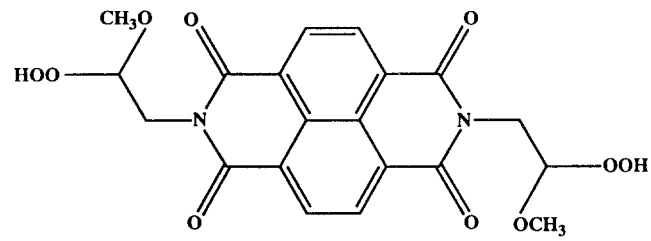

Figure 1. The chemical structure of NP-III.

Reduced glutathione (GSH) plays an important role in the defense against oxidative stress. A few studies have been focusing on the ability of thiol GSH to inhibit lipid peroxidation in microsomal membranes[12-14]. GSH is thought to prevent the lipid peroxidation via the reduction of vitamin E radicals[12,13]. A putative protein factor has been implicated as a GSH-mediator capable of recycling vitamin E radical in microsomal membranes and skin epidermis[12,13,15,16]. However, GSH-mediated free radical reductases has not been identified yet. Protein-thiol group in membrane is also involved in permeabilization[17], GTP-triggered microsomal fusion[18]

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†*Abbreviations* : AAPH, 2,2-azobis(2-aminopropane) dihydrochloride; BCIP, 5-bromo-4-chloro-3-indoylphosphate; DMPO, 5,5-dimethyl-1-pyrroline-N-oxide; DNPH, 2,4-dinitrophenylhydrazine; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid); DTT, dithiothreitol; EPR, electron paramagnetic resonance; mBBr, monobromobimane; NBT, nitroblue tetrazolium; NEM, N-ethylmaleimide; NP-III, N,N'-bis(2-hydroperxy-2-methoxyethyl)-1,4,5,8-naphthalene-tetra-carboxylic-diimide; PBS, phosphate-buffered saline; PMC, 2,2,5,7,8-pentamethyl-6-hydroxychromane; qBBr, monobromotrimethylammioniobimane; TBARS, thiobarbituric acid-reactive substances; TCA, trichloroacetic acid.

and neutrophil adherence to endothelium[19].

In this paper, we have used a novel hydroxyl radical generator, NP-III, upon irradiation with longer wavelength ultraviolet (> 350 nm) (Fig. 1) to study the effect of hydroxyl radical on the oxidation of DNA [20-22]. We also described the protective role of protein thiol groups of ghost membrane on the oxidative hemolysis induced by hydroxyl radicals generated from NP-III.

MATERIALS AND METHODS

Chemicals. 5,5-dimethyl-1-pyrroline-N-oxide (DMPO), 2,4-dinitrophenylhydrazine (DNPH), 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), dithiothreitol (DTT), glutathione (reduced and oxidized), and N-ethylmaleimide (NEM) were purchased from Sigma Chemical Co. (St Louis, MO). Protein molecular mass markers and blockers (non-fat dry milk) were purchased from Bio-Rad, mono-bromobimane (ThiolyteMB, mBBr), and mono-bromotrimethylammioniobimane (ThiolyteMQ, qBBr) were purchased from Calbiochem (La Jolla, CA), mouse monoclonal anti-DNP antibody, anti-mouse IgG (whole molecule) alkaline phosphatase conjugate, and BCIP/NBT (5-bromo-4-chloro-3-indoylphosphate/ nitroblue tetrazolium) pre-mixed solution were purchased from Zymed Laboratories (South San Francisco, CA). 2,2,5,7,8-Pentamethyl-6-hydroxychromane (PMC) was kind gift from Eisai Co. (Tokyo, Japan). N,N'-bis(2-hydroperoxy-2-methoxyethyl)-1,4,5,8-naphthalene-tetra-carboxylic-diimide (NP-III) was synthesized and purified according to the method as described previously[21].

Preparation of erythrocytes. Blood from Sprague-Dawley male rats (250-300 g) by cardiac puncture was collected into heparinized tubes. Erythrocytes were centrifuged for 10 min at 1,000 × g and washed three times with phosphate-buffered saline (PBS; 150 m*M* NaCl, 5 m*M* potassium phosphate, pH 7.4). The supernatant and buffy coat were carefully removed.

Hemolysis procedure. To 200 μL of 50% erythrocyte suspension in PBS was added 100 μL of appropriate concentration of NP-III and/or 100 μL of 50 m*M* DTT, and/or 100 μL of 50 m*M* NEM and finally adjusted to 1 mL of the reaction mixture with PBS. The samples were then irradiated with the Oriel Corporation 68820 apparatus using UVB and UVC cutoff filters (cutoff wavelength, 320 nm) at a distance of 10 cm.

The extent of hemolysis was determined spectrophotometrically as reported previously[4]. The reaction mixtures taken out at defined intervals were centrifuged (15,000 × g for 5 min) and diluted with PBS, and then absorbance was measured at 540 nm. The extent of hemolysis was calculated as the percent of complete hemolysis produced by the addition of 0.1% Triton X-100 in erythrocyte suspension on exposure to NP-III under the same reaction conditions

Preparation of erythrocyte ghosts. Erythrocyte ghosts were prepared from the reaction mixtures by the method of Dodge *et al.*[23] A brief writing for the procedure can be written as follows: At the defined time point, to reaction mixtures was added 40 vol of 5 m*M* potassium phosphate (pH 7.4) and reaction mixtures were centrifuged at 22,000 × g for 20 min and washed three times under the same conditions. The samples were kept at -70 °C before use. The incorporation of PMC into membranes were carried out by the method as described previously[24].

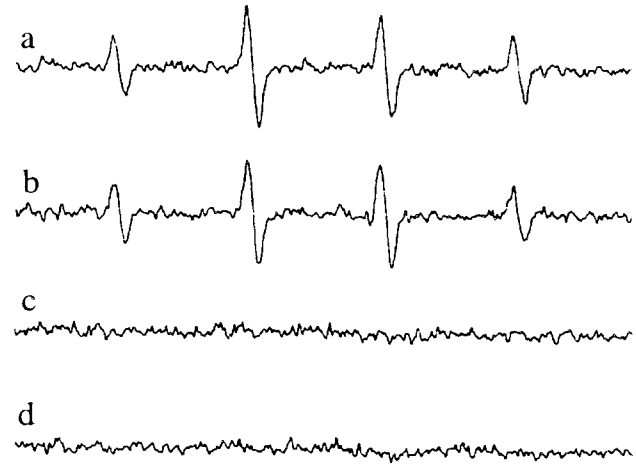

Figure 2. The production of DMPO-OH by NP-III-derived hydroxyl raidical under UVA irradiation.The reaction conditions are described in Materials and Methods. The production of DMPO-OH from the reaction mixture (10 m*M* DMPO + 50(M NP-III) were recorded (a) under UVA irradiation or (c) without irradiation. Spectra in (b) and (d) show the effect of NEM added to (a) and (c), respectively.

Protein content. Protein content of ghosts was measured by Lowry method[25] using BCA protein kit (Pierce) with bovine serum albumin as a standard.

Electrophoresis. SDS-PAGE was carried out with 4% and 12% gels for stacking and resolving, respectively, according to the method of Laemmli[26].

Assay for thiobarbituric acid-reactive substances (TBARS). Lipid peroxidation was assayed with thiobarbituric acid as described by Ohkawa *et al.*[27]. To 1 mL of ghost membrane solution was added 1 mL of 20% TCA solution and 0.8 mL of 0.8% thiobarbituric acid. The final mixture was heated at 100°C for 1 h and cooled immediately on ice. The precipitate was removed by centrifugation and the absorbance of supernatant was measured at 535 nm against a blank that contained all the reagents except ghost membrane proteins. The quantification of TBARS was calculated using a 1,1,3,3-tetramethoxypropane as the standard.

Protein carbonyl assay. Protein carbonyls were measured according to the method of Oliver *et al.*[28]. The ghost proteins of reaction mixtures were divided into two aliquots and each was adjusted to 1 mL of reaction volume and then precipitated with 1 mL of 20% TCA. To the reaction mixture was added 2 mL of 20 μ*M* of DNPH in 2 N HCl and to the other pellet 2 mL of 2 N HCl for 2 h at room temperature. The ghost proteins were precipitated with 2 mL of 20% TCA, washed three times with a 1:1 mixture of ethanol / ethyl acetate and dissolved in 2 mL of 6 *M* guanidine-HCl solution. The absorbance was measured at 360 nm against a HCl-treated ghost proteins. The

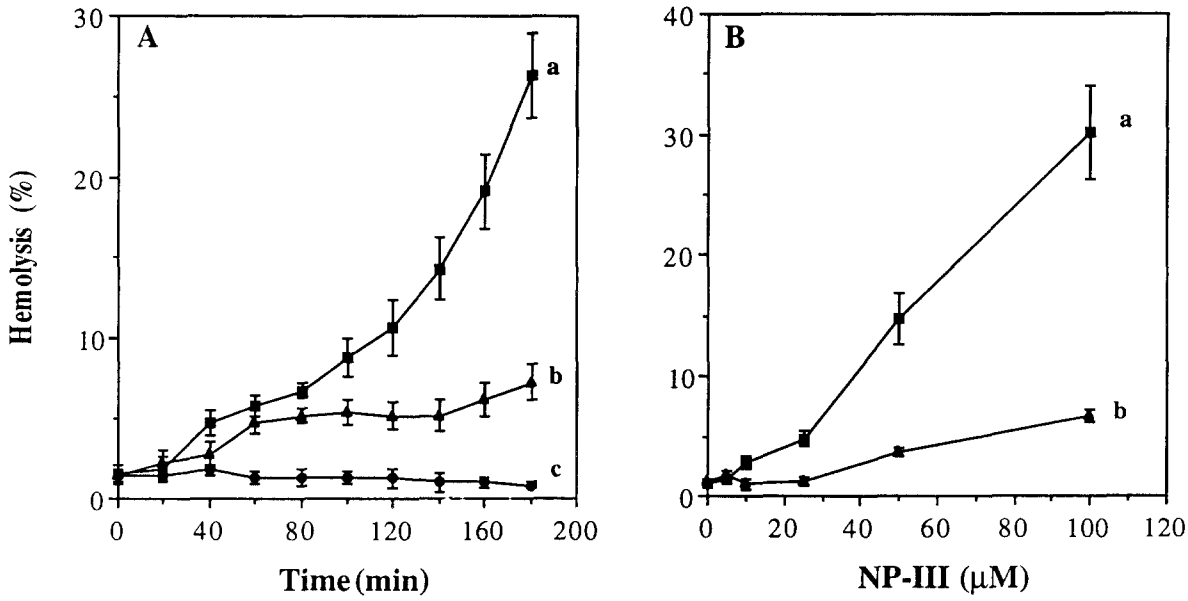

Figure 3. Oxidative hemolysis by hydroxyl radical produced by NP-III upon UVA irradiation. (A) Time-dependent hemolysis. 50 μM of NP-III was used in this experiment. (B) NP-III concentration-dependent hemolysis. Erythrocytes were irradiated for 150 min. (a) NP-III with irradiation, (b) no NP-III with irradiation, (c) NP-III without irradiation. The duplicate samples were experimented three times (n = 3).

content of carbonyls was calculated using a molar extinction coefficient of 21,000 M-1cm-1 for DPNH-derivatives[28].

Western blot for detection of protein carbonyls. For the immuno-detection of protein carbonyls, DNP-conjugated proteins were prepared by following the procedures proposed by Shacter *et al.*[29]. Samples were subjected to 12% SDS-PAGE and electroblotted to nitrocellulose papers[26,30]. Blots were blocked with 2% non-fat milk, incubated with a mouse monoclonal anti-DNP antibody (1:1,000), and sequentially incubated with anti-mouse IgG (whole molecule) alkaline phosphatase conjugate (1:10,000). DNP-conjugated proteins were visualized by BCIP/NBT method.

Thiol determination. For the protein bound SH, the pellet of ghost proteins washed three times with 5 mM potassium phosphate (pH 7.4) was dissolved in 2 mL of 6 M guanidine-HCl and 1 mL of 600 μM DTNB solution was added. The absorbance was measured at 412 nm. The content of sulfhydryl group was calculated using a molar extinction coefficient of 13,600 $M^{-1}cm^{-1}$. For the determination of unbound SH group, proteins were precipitated by the addition of TCA (final concentration, 2%), and then supernatant was used for the detection of unbound SH group. The concentration of unbound SH was measured with the DTNB method as described above.

Spectroscopic study. EPR spectra were recorded at room temperature on a Bruker ER 200 D-SCR spectrometer (X-band). 50 mL of PBS solution containing 10 mM DMPO, 50 mM NP-III and/or 5 mM NEM was taken in a capillary tube and DMPO-OH adduct was recorded in the presence of absence of UVA irradiation. The conditions for obtaining EPR signal is a microwave power of 4 mW, a modulation amplitude of 0.1 mT, a modulation frequency of 100 kHz and a microwave frequency of 9.76 GHz.

RESULTS AND DISCUSSION

Oxidative hemolysis by NP-III

It has been reported in our previous papers that NP-III is a hydroperoxide bearing naphthaldiimide chromophore (Fig. 1) and generates a highly reactive hydroxyl radical upon photoirradiation of UV light at long-wavelength region (> 340 nm) [20-22]. NP-III has been shown to cleave DNA at specific -GG- sites[20,21], and to cause the oxidation of low density lipoprotein (LDL) on irradiation[31]. NP-III was able to produce EPR signal of DMPO-OH within 4 min upon UVA irradiation (Fig. 2a). Based on the chemical property of NP-III, this compound was applied to the study for the oxidative hemolysis of erythrocytes. The increase of hemolysis was dependent on not only time but also NP-III concentration. The presence of 50 μM NP-III upon UV irradiation invoked 25-30% of hemolysis for 3 hr upon UVA irradiation, while neither of UVA light without NP-III nor NP-III without UVA light showed any significant effects on the oxidative hemolysis (Fig. 3).

The targets for hydroxyl radical in erythrocyte membrane were investigated with respect to the oxidation of protein (protein carbonyl, oxidized protein-SH group), and the lipid peroxidation (TBARS). The content of protein-SH group decreased up to 60% of control erythrocytes upon exposure to 50 mM NP-III under UVA irradiation for 200 min (Fig. 4A-a,b). On the other hand, there were no significant changes in TBARS formation in the presence of NP-III upon irradiation (Fig. 4B-a,b). These results indicate that hydroxyl radical itself may not initiate lipid peroxidation. In fact, it has been revealed that metal-chelate can trigger lipid

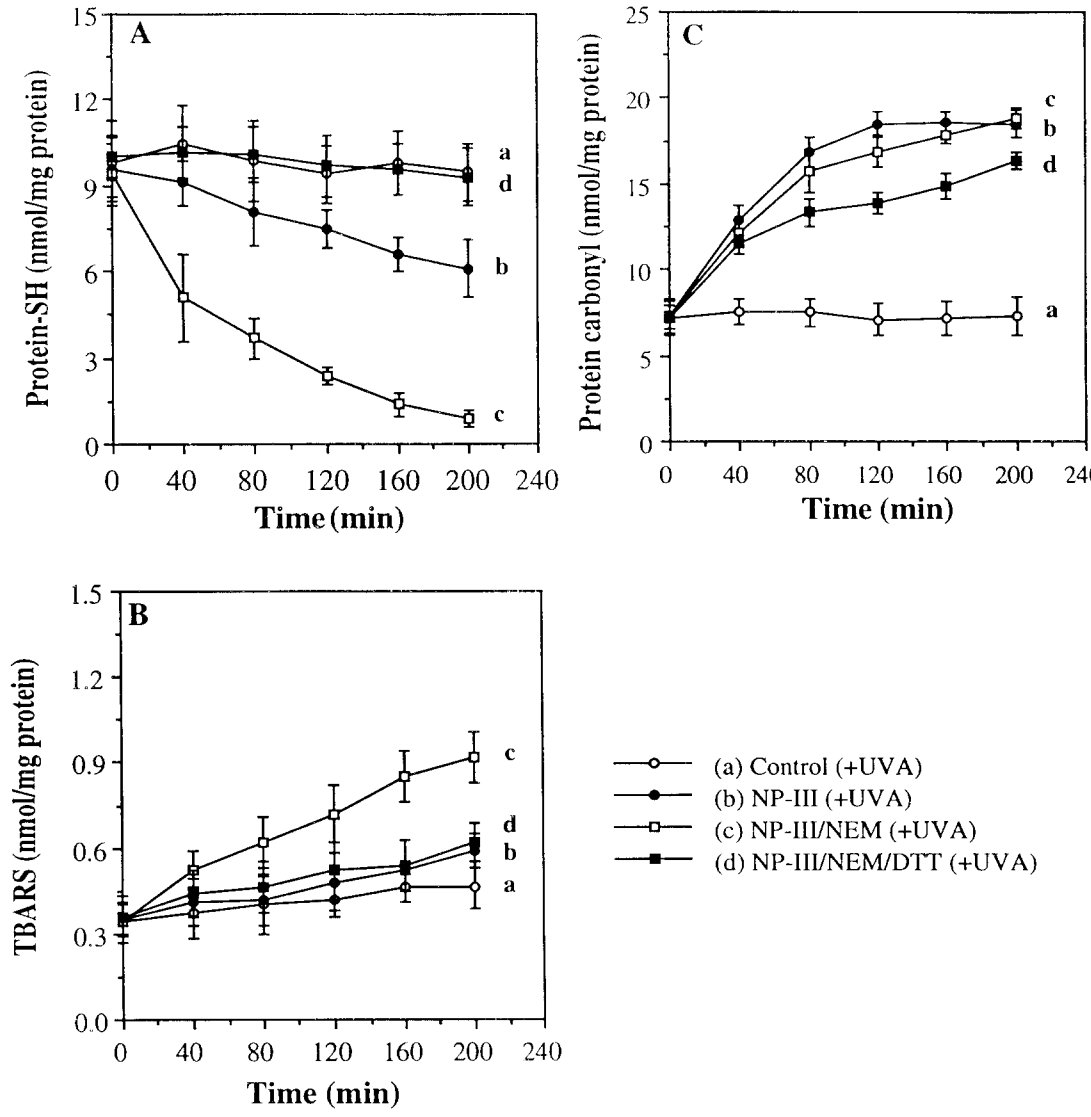

Figure 4. The oxidation of proteins and lipids in erythrocyte ghost membranes during the hemolysis of erythrocyte upon exposure to 50 μM NP-III under UVA irradiation. (A) Protein-bound SH, (B) TBARS, and (C) Protein carbonyl were shown. (a) no NP-III, (b) NP-III, (c) NP-III + 5 mM NEM, and (d) NP-III + 5 mM NEM + 5 mM DTT.

peroxidation in the presence of dioxygen, while reactive oxygen species-superoxide anion radical, hydrogen peroxide, hydroxyl radical- alone are not able to initiate this reaction[9]. The formation of protein carbonyls increased up to approximately 2.5-fold of NP-III non-treated erythrocytes (Fig. 4C-a,b) upon exposure to 50 μM NP-III under UV irradiation for 200 min. It has been reported that reactive oxygen species give rise to the increase of protein carbonyl and the decrease of protein-SH group[32] and that the levels of oxidized protein also show an age-dependent increase in human erythrocyte system[28].

The effect of NEM on the oxidative hemolysis

First of all, it was investigated whether the irreversible modification of protein-SH group with thiol-alkylating reagent, NEM can accelerate the oxidative hemolysis of erythrocyte (Fig. 5). NEM exerts synergistic effect with hydroxyl radicals on accelerating the oxidative hemolysis dependent on both time and NP-III concentration (Fig. 5A, B). The increase of NEM concentration under the same conditions also gave rise to much more rapid hemolysis (data not shown). However, NEM alone was neither able to produce any kind of radicals nor able to synergistically increase the amount of hydroxyl radicals produced by NP-III (Fig. 2b). Therefore, the possibility of NEM as a free radical generator was excluded.

Figure 6 shows SDS-PAGE of erythrocyte ghosts prepared under each reaction conditions. Interestingly, NP-III/NEM oxidative system firstly degraded proteins with high molecular mass such as spectrin and band 3, while low molecular mass proteins such as actin and band 6 were not changed significantly after 1 h irradiation.

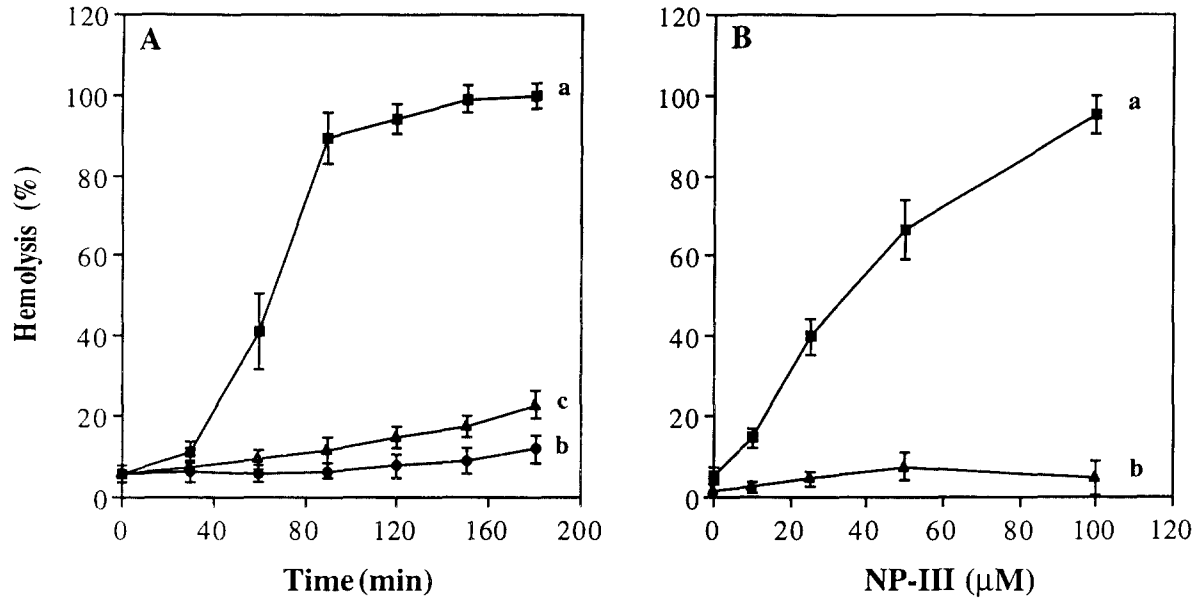

Figure 5. The effect of NEM and DTT on oxidative hemolysis upon exposure to NP-III upon irradiation. (A) Time dependent hemolysis. 50 μM NP-III was used in this experiment. (B) NP-III concentration dependent hemolysis. Erythrocytes were irradiated for 80 min. (a) NEM (5 mM) + NP-III, (b) DTT (5 mM) + NP-III, and (c) NP-III only. The duplicate samples were experimented three times (n = 3).

However, all kinds of ghost proteins were ultimately cross-linked and stagnant in stacking gel and the upper region of running gel after 3 h irradiation. These results indicate that hydroxyl radical may attack spectrin and band 3 predominantly, and then degraded products were cross-linked with each other, and eventually converted to higher molecular mass proteins. The degradation pattern of proteins was not changed on SDS-polyacrylamide gel in the presence or absence of 2-mercaptoethanol, indicating that higher molecular mass proteins were not formed via direct crosslinking between vicinal thiol groups. The protein crosslinking has been observed when ghosts were incubated with AAPH[4] and organic hydroperoxides[33]. On the contrary, the addition of DTT abolished the oxidizable ability of both NP-III and synergistic NP-III/NEM system, probably because DTT can protect the alkylation of protein-SH group by direct reaction of NEM, and DTT also has an ability to scavenge hydroxyl radical. In hydroxyl radical trapping experiment by EPR, 5 mM DTT was able to prevent the production of hydroxyl radical within 4 min. However, after 10 min, hydroxyl radical was also produced even in the presence of DTT (data not shown).

Thiols in membrane on NEM-synergistic hemolysis

The addition of NEM decreased the content of protein-SH to an half of that of control erythrocytes within 40 min (Fig. 4A-c). This result was consistent with the observation that hemolysis rapidly occurred after 40 min (Fig. 5A). In addition, the cooperation of NEM with NP-III resulted in a much more rapid decrease of protein-SH group when compared to the treatment of NP-III alone. However, NEM has been known as a membrane-penetrating reagent, thus has the ability to modify all kind of thiol groups including cytosolic proteins, membrane proteins as well as small thiol molecule. Therefore, we tested additional penetrating and non-penetrating thiol reagents. qBBr, a non-penetrating reagent only modifies some membrane proteins oriented outside, while mBBr, a penetrating reagent modifies both cytosolic and membrane proteins[34]. To investigate the topological importance of thiol groups in membrane, the hemolytic degree of erythrocytes treated with either mBBr or qBBr was estimated (Fig. 7). mBBr could accelerate more rapid hemolysis than qBBr, suggesting that thiol groups in inner membrane proteins or thiol groups inside cells seem to be critical in oxidative hemolysis. Interestingly, mBBr could not invoke such a rapid hemolysis as NEM. This phenomenon may take place due to the difference of chemical characteristics between NEM and mBBr or the presence of NEM-sensitive proteins. So far, a few studies have pointed out the important role of thiol groups in membrane to stabilize as well as to fuse membranes. NEM has been reported to inhibit microsome fusion[18]. NEM-sensitivity in microsomal fusion is associated with not only a homotrimeric ATPase called NEM-sensitive fusion protein (NSF)[35] but also another NEM-sensitive component[36]. The latter component is tightly associated with microsomal membrane and its thiol groups are highly exposed on the membranes. This exposure of thiols has been suggested to play an important role in GTP-triggered fusion[36]. It was also observed that the cooperation of calcium with diimide, an oxidant of vicinal membrane thiol group perturbed the permeabilization of inner mitochondrial membrane due to the cross-linking of protein thiol group[17]. Therefore, it was considered that thiol

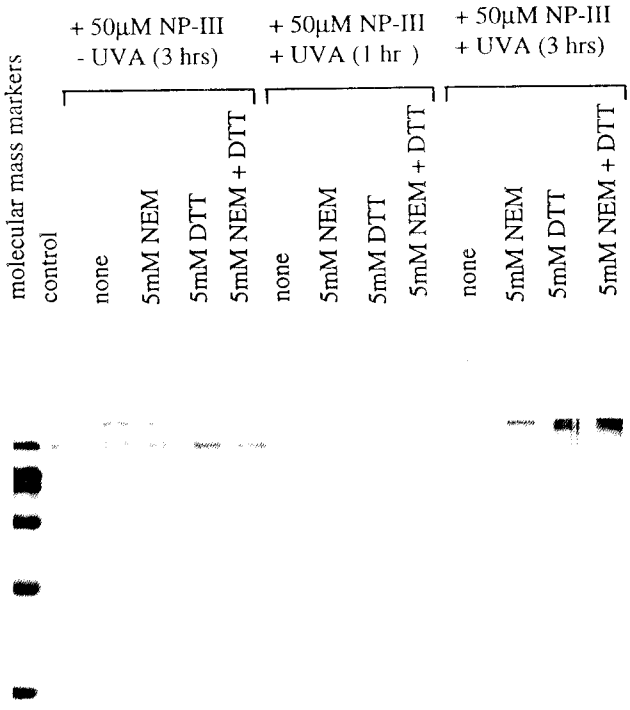

Figure 6. Analysis of erythrocyte membrane proteins on 12% SDS-PAGE. The reaction conditions were described on the top of SDS-polyacrylamide gels. Approximately 60 μg of ghost proteins were loaded onto each lane. The molecular masses were calculated with β-galactosidase (97.4 kDa), serum albumin (66.2 kDa), ovalbumin (45.0 kDa), and carbonic anhydrase (31.0 kDa).

group of membrane may play a role in not only membrane fusion but also membrane breakdown.

Protein carbonyl in membrane on NEM-synergistic hemolysis

The increase of protein carbonyls was not observed when NEM was added to erythrocytes in the presence of NP-III system even though NEM invoked a rapid hemolysis (Fig. 4Cb,c). Namely, the level of protein carbonyl in NEM-treated erythrocyte was nearly equal to or slightly less than those in NEM-non-treated erythrocytes. To confirm the pattern and change in the amount of protein carbonyl, western blot was carried out with monoclonal anti-DNP antibody. The diffused bands representing protein carbonyl were detected in the lanes of stacking gel and the upper region of running gel where samples treated with NP-III and NP-III/NEM were loaded (data not shown). As a control, no bands appeared in samples upon exposure to NP-III and NEM without UVA irradiation within 3 h. Interestingly, protein carbonyls of samples treated with NP-III alone were strongly detected than those treated with NP-III/NEM , this immunoblot result was well consistent with that of the absorbance measurement of DNP-conjugated protein (Fig. 4C). Even though the formation of protein carbonyls was not directly related

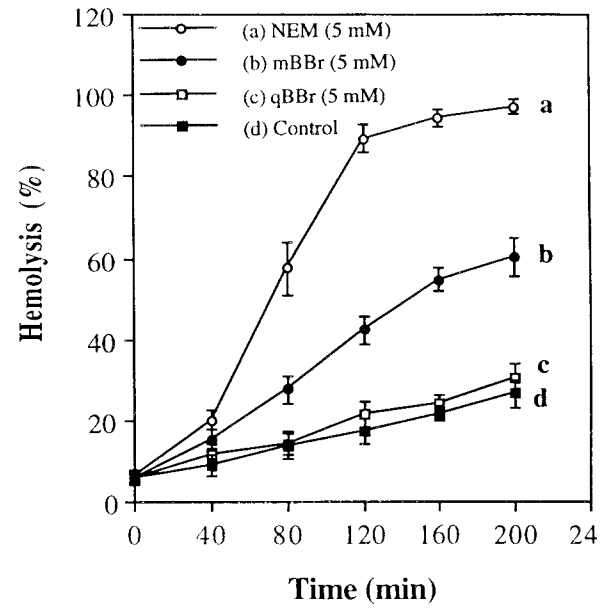

Figure 7. The effect of thiol modification on oxidative hemolysis. To 10% intact erythrocytes were added (a) 5 mM NEM, (b) 5 mM qBBr, (c) 5 mM mBBr, (d) none as a control. The hemolysis was inititated by the treatment of 50 μM NP-III upon UVA irradiation.

with the oxidative hemolysis, the role of protein carbonyl was not able to be excluded in the oxidative damage because it has been suggested that protein carbonyl provides a general mechanism by which proteins are marked for the degradation[37,38]. This suggestion was partially evidenced that neutral/alkaline proteases isolated from rat liver and E. coli exhibited almost complete specificity for the oxidized form of glutamate synthase (GS), not for the native form of GS[39,40].

The Interplay between protein-SH and Vitamin E in Erythrocyte Membrane

The addition of NEM in NP-III system induced a 2-fold increase of TBARS production (Fig. 4C), while NEM alone could neither induce hemolysis nor produce TBARS. Therefore, it was considered that the rapid depletion of glutathione or thiol group in the erythrocyte membrane led to the increase of TBARS. As shown in Fig. 7, one of the reasons why mBBr invoked rapid hemolysis than qBBr in NP-III system is that mBBr may modify reduced glutathione, and eventually disrupt redox balance intracellularly. Actually NEM invoked a rapid decrease of glutathione levels in erythrocyte (data not shown). However, NEM itself did not induce hemolysis. Therefore, to investigate the interplay between GSH-mediated protein-SH and vitamin E in erythrocyte membrane, the TBARS formation and oxidative hemolysis were compared among the cells differently pretreated with NEM and/or PMC (vitamin E homologue), respectively (Table 1). Obviously, the

pretreatment of NEM resulted in the rapid hemolysis and increase of TBARS formation even in PMC-fortified erythrocytes (or ghosts). This finding was consistent with the previous work by Palamanda and Kehrer[14] that lipid peroxidation occurred rapidly and extensively in the microsomes containing fewer thiol groups and vitamin E. Actually, it has been reported that the protein-SH of membrane in the reduced state maintained vitamin E levels in membrane, consequently leading to inhibiting the propagation of the oxidative reactions catalyzing TBARS formation[41,42]. According to Table 1, GSH alone could slightly prevent the TBARS formation and oxidative hemolysis, however, the hydrophilic nature of GSH need inevitably another specific (or random) amphiphilic factor easily accessible to hydrophobic membrane lipid. Therefore, thiol-group in membrane protein seems to be a GSH-mediated protector against lipid peroxidation. GSH-dependent free radical (vitamin E) reductase has been suggested to be as a putative factor in microsomal membrane and skin epidermis[15,16,18,19]. In spite of long-term disputation about a specific GSH-mediated protein, it has not been purified from any kind of membranes so far. In fact the proteins in erythrocyte membrane are well-characterized, so far free radical reductase has not been reported to be isolated from erythrocyte membrane, therefore, we carefully considered that thiols in innermembrane protein, at least some limited protein-SH groups may act as GSH-mediators.

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