Original Article

Participation of metabolic activation of 2,4,6-trinitrotoluene to 4-hydroxylamino-2,6-dinitrotoluene in hematotoxicity

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ABSTRACT — Exposure to 2,4,6-trinitrotoluene (TNT) causes methemoglobin (metHb) formation, hemolysis and negative heme balance *in vivo*, but the mechanistic details are poorly understood. In the present study, we examined the participation of metabolic activation in TNT-mediated hematotoxicity. Exposure of rats with TNT (300 mg/kg, i.p.) for 4 days resulted in a decrease of hematocrit value coupled to an increase in metHb formation. The red blood cells treated with 4-hydroxylamino-2,6-dinitrotoluene (HADNT), a metabolite of TNT, underwent readily hemolysis *in vitro*, whereas such a phenomenon was not seen with TNT. Consistent with this, HADNT is active toward metHb formation and the decrease in thiol content of the globin moiety compared with TNT and its metabolites 4-amino-2,6-dinitrotoluene (ADNT) and 4-acetylamino-2,6-dinitrotoluene (AADNT). Furthermore, interaction of purified rat oxyhemoglobin (oxyHb) with HADNT, but not TNT, ADNT, and AADNT, caused a concentration-dependent production of H₂O₂ and ferrylhemoglobin (ferrylHb) which is a highly oxidizing state formed by reaction of oxyHb with H₂O₂. Notably, hemein was released during interaction of oxyHb with HADNT. Taken together, these findings suggest that HADNT is an active metabolite that mediates TNT-induced hematotoxicity via formation of prooxidants such as H₂O₂ and ferrylHb.

Key words: 2,4,6-Trinitrotoluene, Metabolic activation, Hemoglobin, Hematotoxicity, Hydrogen peroxide

INTRODUCTION

Nitroaromatics are important precursors for the synthesis of a wide variety of industrial chemicals such as munitions, pesticides, herbicides, and dyes. Their widespread use has led to environmental contamination, and nitroaromatics are recognized as priority pollutants, due to their toxicity (Kovacic and Somanathan, 2014). 2,4,6-Trinitrotoluene (TNT) has been used as a military explosive. Acute and chronic exposures of workers to TNT have been shown to cause toxicity in the liver, the hematopoietic system and the eye (Härkönen *et al.*, 1983; Hathaway, 1977; Sabbioni *et al.*, 2005). Hematotoxic action of TNT is manifested as hemolysis, destruction of hemoglobin (Hb) in erythrocytes, formation of methemoglobin (metHb), and/or formation of sulfhemoglobin (sulfHb) (Crawford, 1954; Djerassi and Vitany, 1975; Levine *et al.*, 1984). Tenhunen *et al.* (1984) and Savolainen *et al.* (1985) reported that exposure of rats or humans to TNT resulted in the reduction of the enzyme

activity responsible for synthesis of delta-aminolevulinic acid which is an intermediate for heme synthesis, but increased the heme oxygenase activity. Although these findings suggest that TNT is associated with negative heme balance, no mechanistic details to clarify these observations have been reported.

The metabolism of TNT has been extensively studied because it is recognized that most nitroaromatic compounds require reductive activation (Biaglow *et al.*, 1986; Kappus, 1986) to exhibit hematotoxic actions. With experimental animals, it was found that TNT is primarily metabolized by a reductive pathway to form the hydroxylamines, which are further reduced to the respective amines or diamino derivatives (Channon *et al.*, 1944). These metabolites were also identified in urine of munition workers (Ahlborg *et al.*, 1988; Woollen *et al.*, 1986). Leung *et al.* (1995) showed that microsomal enzymes are capable of biotransforming 4-hydroxyamino-2,6-dinitrotoluene (HADNT) and 4-amino-2,6-dinitrotoluene (ADNT) from TNT. Recently, we have iden-

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tified NADPH-cytochrome P450 reductase (P450R) from rat liver as an enzyme responsible for catalyzing the reduction of TNT to HADNT, leading to release of nitrite via chemical interaction of HADNT and TNT (Shinkai *et al.* unpublished observation). We also reported that ADNT is further metabolized to 4-acetylamino-2,6-dinitrotoluene (AADNT) by N-acetyltransferase in cytosolic enzymes from rat liver in the presence of acetyl CoA (Li *et al.*, 1998).

With respect to hematologic toxicity, redox processes of Hb have been linked to the toxic effects of drugs and to the accelerated aging of red blood cells (Beutler, 1969). In erythrocytes, Hb is protected against oxidative damage by reactive oxygen species; however, under certain conditions Hb is susceptible to oxidative damage associated with elevation of hydrogen peroxide (H_2O_2) (Hebbel and Eaton, 1989; Schacter, 1986). Reaction with H_2O_2 can transform oxyhemoglobin (oxyHb) and metHb into a higher oxidation state, ferrylhemoglobin (ferrylHb) (Kanas and Acker, 2010). Although toxic actions of TNT have been extensively studied, the role of metabolic activation in TNT-induced hematotoxicity involved in oxidative stress is poorly understood. In the present study, we explored the participation of TNT metabolites in TNT-mediated hematotoxic action *in vivo* and *in vitro*.

MATERIALS AND METHODS

Materials

Ferriprotophyrin IX (hemin), hemoglobin (from human erythrocytes) and catalase were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA). Sodium azide and 5,5'-dithiobis (2-nitrobenzoic acid) were obtained from Nacalai Tesque (Kyoto, Japan). DEAE-Sephadex A-50 and Sephadex G-100 were purchased from Pharmacia LKB Biotechnology (Uppsala, Sweden). All other chemicals used were of the highest grade available. TNT was synthesized from 2,4-dinitrotoluene by the method of MacGookin *et al.* (1940). HADNT and ADNT were synthesized by reduction of TNT under hydrogen sulfide gas and separated by silica gel column chromatography with different ratios of benzene to ethyl acetate as a solvent. Finally, HADNT and ADNT were recrystallized with 1,1,1-trichloroethane and ethanol, respectively. 4-Acetylamino-2,6-dinitrotoluene (AADNT) was synthesized from ADNT with acetic anhydride. These synthetic compounds showed > 95% purity. The chemical structures of TNT, HADNT, ADNT and AADNT characterized by CI-MS analysis, NMR, and IR spectroscopy were as follows. TNT: m/z 228 (MH^+); $^1\text{H-NMR}$ (ppm) 2.58 (3H, s), 9.02 (2H, s); IR (KBr, cm^{-1}) 1,540 ($-\text{NO}_2$),

1,353 ($-\text{NO}_2$); HADNT: m/z 214 (MH^+); $^1\text{H-NMR}$ (ppm) 2.30 (3H, s), 7.51 (2H, s), 9.00 (1H, br s), 9.14 (1H, br s); IR (KBr, cm^{-1}) 3,348 ($-\text{OH}$), 3,291 ($-\text{NH}$), 1,535 ($-\text{NO}_2$), 1,342 ($-\text{NO}_2$); ADNT: m/z 198 (MH^+); $^1\text{H-NMR}$ (ppm) 2.23 (3H, s), 6.16 (2H, s), 7.30 (2H, s); IR (KBr, cm^{-1}) 3,482 ($-\text{NH}_2$), 3,382 ($-\text{NH}_2$), 1,535 ($-\text{NO}_2$), 1,357 ($-\text{NO}_2$); AADNT: m/z 240 (MH^+); $^1\text{H-NMR}$ (ppm) 2.11 (3H, s), 2.40 (2H, s), 8.38 (2H, s), 10.68 (1H, s); IR (KBr, cm^{-1}) 1,683 ($-\text{C=O}$), 1,540 ($-\text{NO}_2$), 1,356 ($-\text{NO}_2$). IR spectroscopy was carried out with a JASCO FT/IR-7300 instrument, NMR-spectra were recorded with a JNM-EX270, and mass spectra with a Shimadzu QP-1000 instrument.

Treatment of animals

Male Fischer 344 (F344) rats (7 weeks, Clea Japan, Inc., Tokyo, Japan) were used. The animals were housed in an air-conditioned room (25°C) with a 12 hr light/dark cycle. They were allowed free access to water and food. The animals were orally administered TNT (300 mg/kg/day, suspended in corn oil) for 4 days. At 1 hr after final injection of TNT, the animals were sacrificed and the blood was collected. Control animals received the same treatment without exposure to TNT. The hematocrit (Ht) value was measured by using microcapillary tube centrifugation.

Hemolysis of erythrocytes by TNT and its metabolites

Freshly drawn rat blood was centrifuged at 500 g for 10 min at 4°C. After removal of plasma and white buffy coat, the cells were washed three times with 0.9% NaCl. Cell suspensions were prepared at 20% in phosphate-buffered saline (10 mM potassium phosphate (pH 7.4)-139 mM NaCl). The final suspensions (10%) were incubated with TNT, ADNT, HADNT and AADNT with the indicated concentrations at 37°C for 60 min in a shaking water bath. For the controls, a similar incubation mixture without TNT and its metabolites or acetone was used. Percent hemolysis was determined as $100 \times A / 10A_{100\%}$, where A is the absorbance of the peak of Soret band of hemoglobin present in the supernatant of the red blood cell suspension after centrifugation, and $A_{100\%}$ is the absorbance at the peak of the Soret band present in the entire red blood cell suspension after 10-fold dilution with distilled water.

Preparation of oxyHb and metHb

OxyHb was purified from the hemolysate as described by Winterbourn (Winterbourn, 1990). Erythrocytes were collected from male F344 rats and washed three times with 2 volumes 0.9% NaCl. The erythrocytes were lysed by addition of 2 volumes of distilled water and then the hemolysate was centrifuged at 29,000 g for 40 min to

remove cell membranes, white blood cells and platelets. Supernatants obtained (94 mL) were dialyzed overnight against 5,000 mL of 5 mM Tris-HCl (pH 8.6) at 4°C. The dialyzed sample was concentrated to 107 mL using an Amicon ultrafiltration system with a YM-10 membrane. The concentrated sample was applied on a DEAE Sephadex A-50 column (23 x 5 cm, i.d.), which had been equilibrated with 5 mM Tris-HCl buffer (pH 8.6) at a flow rate of 120 mL/hr. The column was washed with the same buffer until the eluate was clear. OxyHb was eluted with 10 mM Tris-HCl buffer (pH 7.1)-40 mM NaCl. Fractions (397 mL) containing oxyHb were combined and concentrated to 49 mL. The concentrated sample was applied on a Sephadex G-100 column (65 x 5 cm, i.d.), which had been equilibrated with 50 mM potassium phosphate (pH 7.0)-0.15 M KCl. OxyHb fractions were combined and dialyzed against 3,000 mL of 50 mM potassium phosphate (pH 7.0) buffer. Resulting samples showed two bands based on alpha- and beta-chains of Hb with molecular weights of 14.5 kDa and 16 kDa, respectively, on SDS-PAGE and were free of catalase and superoxide dismutase. The final preparation of oxyHb was mixed at a sodium dithionite/Hb molar ratio of 1.2 for 15 hr at 4°C to reduce the ferrous form completely because most of the oxyHb was converted to metHb during purification. The mixture was passed through an Econo-Pack 10DG column (Bio-Rad) to remove unreacted dithionite. To prepare metHb, oxyHb was mixed with potassium ferricyanide at a 2-fold excess for 15 hr at 4°C. Unreacted potassium ferricyanide was removed by the same method as described above for preparation of oxyHb. Concentrations of oxyHb and metHb were determined by the method of Evelyn and Malloy (Evelyn and Malloy, 1938).

Formation of prooxidants

H₂O₂ generation was determined by the method of Hildebrandt and Roots (Hildebraunt and Roots, 1975). Incubation mixture (0.2 mL) consisted of 10 µM oxyHb, indicated concentrations of TNT or its metabolites, 0.2 mM sodium azide and 20 mM potassium phosphate buffer (pH 7.0). The reaction mixture was preincubated with sodium azide, and then reaction was initiated by addition of TNT or its metabolites. After incubations were carried out at 25°C for 30 min, reactions were terminated by addition of perchloric acid (final 2.5%). Then the mixtures were centrifuged at 15,000 g for 10 min. The each supernatant (0.2 mL) was transferred to test tubes to which were added 10 mM ferrous ammonium sulfate (0.24 mL) and 2.5 M potassium thiocyanate (0.12 mL). The formation of Fe(SCN)₃ was measured at 480 nm.

FerrylHb formation was determined according to the

method of Giuliv and Davies (1990). Optical absorption spectra were recorded in a Shimadzu UV-1600 spectrophotometer (Kyoto, Japan). OxyHb (80 µM) was incubated with indicated concentrations of TNT or its metabolites and 20 mM potassium phosphate buffer (pH 7.0) in a total volume of 0.2 mL at 25°C. The formation of ferrylHb was calculated with the absorption coefficients as reported by Yoshida *et al.* (1994). FerrylHb was also detected by its reaction with sodium sulfide to convert into sulfHb (Giulivi and Davies, 1990). Briefly, sodium sulfide (2 mM) was added to the reaction mixture and sulfHb formation was determined at 620 nm after incubation for 15 min.

Sulfhydryl content

Concentrations of the sulfhydryl groups in oxyHb were determined as reported previously (Kumagai *et al.*, 1990). The incubation mixture (0.2 mL) consisted of 10 µM oxyHb, 25 µM TNT or its metabolites and 10 mM potassium phosphate buffer (pH 7.0). After the reaction at 25°C for 30 min, each aliquot (0.1 mL) of the reaction mixture was mixed with 0.3 mL of 0.2 M Tris-HCl (pH 8.2)-20 mM EDTA, 20 µL of 10 mM 5,5'-dithiobis(2-nitrobenzoic acid), and 0.15 mL of 5% SDS. After mixing, the absorbance at 412 nm was measured against a blank reagent to determine the total sulfhydryls, using an extinction coefficient of 13.6 mM⁻¹cm⁻¹.

Detection of hemin

Release of hemin was determined by the method of Liu *et al.* (1988). OxyHb (25 µM) was incubated with TNT or its metabolites in 20 mM potassium phosphate buffer (pH 7.0) at 25°C for the indicated times. After the reaction, NaCl was added to the reaction mixtures to make a final concentration of 1.5 M. Aliquots (1 mL) were applied on a Dowex AG 1-X8 column (1 mL volume), which had been equilibrated with 10 mM potassium phosphate buffer (pH 7.8)-1.5 M NaCl. The column was extensively washed with 20 mL of the same buffer to remove oxyHb. Hemin was eluted with 2 mL of 5% SDS. The concentration of hemin was determined spectrophotometrically at 385 nm using an extinction coefficient of 58.4 mM⁻¹cm⁻¹.

HPLC analysis

For measurement of HADNT, Shimadzu HPLC system was used. After the addition of 2.5% TCA, an aliquot (50 µL) was applied to a YMC packed column AM-type (250 x 4.6 mm i.d., 5 µm particle size, Kyoto, Japan) at a flow rate of 1 mL/min. HADNT was detected at 255 nm and water/acetonitrile (1:1, v/v) was used as the mobile phase.

Statistical analysis

Statistical significance was assessed with Student's t-test, and $p < 0.05$ was considered significant.

RESULTS

Hematotoxicity caused by TNT exposure *in vivo*

We first examined the effect of TNT on hematotoxicity *in vivo*. Exposure of rats to TNT (300 mg/kg/day) by oral administration for 4 days resulted in a significant decrease in the Ht value and increase in metHb formation at 1 hr after the final administration (Fig. 1), suggesting that TNT exhibits hematotoxicity and decreases red blood cells *in vivo*.

Hematotoxic effects of TNT and its metabolites *in vitro*

We described the chemical structure of TNT and its metabolites we examined in this study (Fig. 2A). To compare the hematotoxicity among these compounds, we performed *in vitro* experiments. As shown in Fig. 2B, incubation of HADNT with the erythrocytes at 37°C for 60 min caused a significant elevated hemolysis in a concentration-dependent manner. Little appreciable hemolysis was seen when the erythrocytes incubated with TNT at concentration 0.5 mM (Fig. 2B), suggesting that TNT-mediated hematotoxicity observed *in vivo* may require a reductive transformation to HADNT. Consistent with this notion, HADNT significantly stimulated metHb formation as well, whereas TNT, ADNT and AADNT did not (Fig. 2C). Formation of metHb following incubation of HADNT with oxyHb increased with HADNT concentrations up to 0.5 mM (data not shown). It has been

reported that hydroxylamine and its derivatives are reactive towards oxyHb to form metHb and H_2O_2 (Spooren and Evelo, 2000). If H_2O_2 is produced during incubation of HADNT with oxyHb, sulphydryl contents would be decreased because of its oxidation. As expected, although the reaction of either TNT or ADNT reduced the thiol content of oxyHb significantly, HADNT was the most effective agent for decrease in the SH groups (Fig. 2D); the consumption of oxyHb sulphydryls showed a concentration-dependent reaction up to 50 μM (data not shown). These results support our hypothesis that metabolic activation of TNT to HADNT is involved in the hematotoxic actions of TNT *in vivo*.

Generation of H_2O_2 during interaction of oxyHb

Consistent with the results of thiol content, incubation of HADNT with oxyHb (25°C for 10 min) resulted in a production of H_2O_2 in a concentration-dependent fashion (10 μM HADNT, 14.5 μM ; 100 μM HADNT, 21.2 μM ; 1000 μM HADNT, 32.3 μM). However, there was minimal generation of H_2O_2 by reactions with TNT, ADNT and AADNT as shown in Table 1. The produced H_2O_2 was completely abolished by addition of catalase. Under these conditions, no superoxide or thiyl radical was seen using ESR spectrometry (data not shown).

Since H_2O_2 , generated within red blood cells, can undergo a variety of reactions, one of which is with oxyHb to form ferryl heme species (Giulivi and Davies, 1990), formation of ferrylHb by reaction of oxyHb (80 μM) with different concentrations of HADNT (25°C, 10 min) was examined (Fig. 3A). FerrylHb was produced in a concentration-dependent manner and reached a plateau level at 0.5 mM. When the concentration of HADNT was fixed at 1 mM, ferrylHb formation increased to 5 min and the steady-state level was maintained during the reaction up to 15 min (data not shown). In contrast, negligible formation of ferrylHb during incubation of oxyHb with TNT, ADNT or AADNT was detected under these conditions (data not shown). To confirm whether ferrylHb was formed as an intermediate in the reaction of oxyHb with HADNT, sodium sulfide (2 mM) was added to the incubation mixture, because sodium sulfide has been used to trap the ferryl oxidation state of hemoglobin (Giulivi and Davies, 1990). As expected, the appearance of a new peak at 622 nm, corresponding to the formation of sulfHb (Berzofsky *et al.*, 1971), accompanied with a decreased absorbance maximum at 543 nm and 575 nm, which corresponds to oxyHb, was observed (Fig. 3B).

Fig. 1. TNT causes the decrease in Ht value and the increase in metHb formation *in vivo*. TNT (300 mg/kg) was administered orally to rats for 4 days and then sacrificed 1 hr after injection. Each data is the mean $\pm$ S.D. of three to four animals. **, $P < 0.01$ compared with corn oil.

Interaction of hemoglobin with TNT metabolites

Fig. 2. Effects of TNT and its metabolites on hematotoxic actions *in vitro*. **A.** Chemical structures of TNT and its metabolites. **B.** Effects of TNT and HADNT on hemolysis in rat erythrocytes. Hemolysis of 10% suspensions of erythrocytes was determined after incubation for 1 hr at 37°C in the presence of TNT or HADNT. Measurement of hemolysis was performed as described in Materials and Methods. Each point is the mean $\pm$ S.D. of three determinations. **C.** MethHb formation following incubation of purified oxyHb with TNT and its metabolites *in vitro*. OxyHb (0.1 mM) was incubated with TNT and its metabolites (0.2 mM) in 10 mM KPi (pH 7.0) at 25°C for 30 min, and then methHb was measured. Each value is the mean $\pm$ S.D. of four determinations. **D.** Decrease in thiol groups of Hb following incubation of purified oxyHb with TNT and its metabolites *in vitro*. OxyHb (10 μ M) was incubated with TNT and its metabolites (25 μ M) in 10 mM KPi (pH 7.0) at 25°C for 30 min, and then the sulfhydryl content was measured. Each value is the mean $\pm$ S.D. of four determinations. *, $P < 0.05$; **, $P < 0.01$ compared with control. Acetone (with rat erythrocytes) or dimethyl sulfoxide (with oxyHb) was used as control.

Table 1. Generation of hydrogen peroxide during incubation of oxyHb with TNT and its metabolites.

Compounds	H ₂ O ₂ (μ M)
TNT	0.6 $\pm$ 1.0
HADNT	32.3 $\pm$ 2.8
ADNT	1.2 $\pm$ 0.5
AADNT	n.d.

OxyHb (10 μ M) was incubated with 1 mM of TNT or its metabolites at 25°C for 10 min in 20 mM KPi (pH 7.0)-0.2 mM NaN₃. Each data is the mean $\pm$ S.D. of three determinations. Each compound was dissolved in dimethyl sulfoxide and H₂O₂ formation was estimated by deducing H₂O₂ formed with dimethyl sulfoxide (5.2 μ M). n.d., not detectable.

Heme release during interaction of Hb with HADNT

From the results so far, it is suggested that HADNT is a reactive metabolite to produce H₂O₂ in the presence of Hb species, resulting in ferrylHb formation. Since it has been shown that H₂O₂ is able to release the heme moiety from hemoprotein (Balla *et al.*, 1993; Karuzina *et al.*, 1999), we monitored heme release during the reaction of oxyHb (25 μ M) with HADNT (Fig. 4). As expected,

HADNT (0.1 or 1 mM) stimulated heme release from oxyHb in time- and concentration-dependent manners. By incubation with 1 mM HADNT for 60 min, the rate of release of heme from added oxyHb was 3.2% of the oxyHb added. However, a minimal release of heme was detected by reaction with TNT at a concentration of 1 mM. When ADNT or AADNT was used instead of TNT, a similar result was also obtained (data not shown). With respect to metabolic rate of TNT, when TNT (1 mM) was incubated with NADPH (1 mM) and P450R (4.2 μ g/mL) in 300 mM KPi (pH 7.7)-0.1 mM EDTA at 37°C for 30 min, more than 0.1 mM of HADNT was produced (data not shown). This also supports the notion that metabolic transformation to HADNT can be a cause of TNT-mediated hematotoxicity.

DISCUSSION

The present study indicates that hematotoxicity of TNT requires reductive activation corresponding to formation of the 4-electron reduction metabolite HADNT. Oral administration of rats to TNT *in vivo* resulted in a significant methHb formation and decrease in the Ht val-

Fig. 3. FerrylHb formation (A) and the spectra of sulfHb formed (B) during the reaction of oxyHb with HADNT. A. OxyHb (80 μM) was incubated with the indicated concentrations of HADNT in 20 mM KPi (pH 7.0) at 25°C for 10 min. Each value is the mean ± S.D. of four determinations. B. OxyHb (80 μM) was pre-incubated with sodium sulfide (2 mM) at 25°C for 15 min, and then incubated with HADNT (1 mM) at 25°C for 15 min to convert the ferryl species into the sulfheme derivative. 1, 0 min; 2, 0.5 min; 3, 15 min.

ue (Fig. 1) but exposure of purified oxyHb or packed red blood cells to TNT did not mediate these hematotoxic actions. In contrast, incubation of oxyHb with HADNT, but not ADNT and AADNT, caused oxidation of metHb from oxyHb and hemolysis of red blood cells (Fig. 2). Under these conditions, only HADNT produced H_2O_2 and ferrylHb during reaction with oxyHb (Fig. 3). These findings suggest that HADNT, which can undergo reaction between the hydroxylamine group and the heme iron, is a major metabolite involved in metHb formation and hemolysis caused by TNT exposure *in vivo*. We speculate that metabolic transformation of TNT mainly occurs in the liver and that the expression levels of enzymes responsible for catalyzing reduction of TNT to HADNT such as P450R are quite low in the erythrocytes.

It has been reported that hydroxylamine analogs are reactive towards oxyHb to form metHb (Harrison and Jollow, 1986; Stolze *et al.*, 1996; Reilly *et al.*, 1999; Spooren and Evelo, 2000). In general, hydroxylamine and its derivatives are capable of forming metHb in the erythrocytes, together with a production of H_2O_2 during interaction with oxyHb (Spooren and Evelo, 2000). In addition, Kawanishi and Caughey (1985) reported that human oxyHb reacts with aquopentacyanoferrate (II) to yield H_2O_2 -metHb via a formation of metHb-O-O-Fe(III)(CN)₅³⁻. Based on these concepts, it is likely that interaction of oxyHb with HADNT resulted in formations of metHb- H_2O_2 and HADNT radical species; then, H_2O_2 was released from the heme moiety because the affinity of H_2O_2 to ferric form of heme was quite negligible. Formed H_2O_2 itself can also oxidize oxyHb to metHb (Kanas and Acker, 2010). In this case, however, a potent oxidizing

agent ferrylHb, which is believed to mediate the peroxidation of lipids, proteins and nucleic acids (Newman *et al.*, 1991; Everse and Hsia, 1997) was formed as a transient intermediate as shown in Fig. 3A. This reactive species of Hb was further confirmed as sulfHb in the presence of sodium sulfide (Fig. 3B).

It was reported that rat Hb is a hetero-tetramer consisting of 2 alpha-chains with 3 cysteine residues and 2 beta-chains with 2 cysteine residues (Chua *et al.*, 1975; Satoh *et al.*, 1987) but 6 thiol groups of 10 cysteine residues are active (Rossi *et al.*, 1997). In the present study, reaction of oxyHb with HADNT resulted in a marked oxidation

Fig. 4. Release of hemin during interaction of oxyHb with TNT or HADNT. OxyHb (25 μM) was incubated with TNT (1 mM) or HADNT (0.1 or 1 mM) in 20 mM potassium phosphate buffer (pH 7.0) at 25°C. After the reaction, NaCl was added to the reaction mixtures to make a final concentration of 1.5 M and then the hemin released was determined. Each point is the average of two determinations.

of globin sulfhydryls. In our preliminary study, SDS-PAGE in the absence of 2-mercaptoethanol revealed that the decreased thiol groups of oxyHb by HADNT were accompanied by cross-linking of Hb subunits through disulfide formation, thereby forming unusual polymer with high molecular weight presumably via intra-/inter-molecular S-S binding (Song Li, unpublished observation). Davies *et al.* (Davies and Delsignore, 1987; Davies *et al.*, 1987) reported that reactive oxygen species could be capable of oxidative modification of amino acids such as cysteine, thereby causing conformational alterations into protein molecules. The production of reactive oxygen species, including H_2O_2 and ferrylHb has been reported to be associated with extensive formation of disulfide-linked Hb adducts on red blood cell membrane skeletal proteins and disulfide-linked Hb polymers (Kiese, 1966; Grossman *et al.*, 1992). Thus, a reasonable explanation is that H_2O_2 and/or ferrylHb generated by the interaction of oxyHb with HADNT potentially participates in the disulfide formation of the globin moiety, resulting in Hb aggregation.

The heme in metHb is more likely to dissociate from the pocket in the protein than the reduced heme in oxyHb that is tightly bound to the globin (Umbreit, 2007), thereby increasing release of heme. Since heme is known to be a potent hemolytic agent that nonspecifically impairs lipid-protein and protein-protein interactions in human erythrocytes (Kirschner-Zilber *et al.*, 1982), we determined the release of heme during the reaction of oxyHb with TNT or its metabolites. As expected, HADNT but not TNT, ADNT, and AADNT induced release of heme from oxyHb in time- and concentration-dependent manners (Fig. 4). To our knowledge, this is the first report to demonstrate dissociation of heme moiety from the Hb molecule caused by an active metabolite of TNT. Early studies on heme biosynthesis in erythroid tissue have indicated that heme itself inhibits the production of delta-aminolevulinic acid, a precursor for heme, both *in vivo* and *in vitro* (Karibian and London, 1965; Gallo, 1967). On the other hand, heme induces heme oxygenase, the rate-limiting enzyme for heme degradation (Tenhunen *et al.*, 1970). These findings indicate that if heme is released from the Hb molecule by TNT exposure, biosynthesis and decomposition of heme would be down-regulated and up-regulated, respectively, leading to a negative heme balance. Thus, the present findings suggest that TNT-mediated negative heme balance is, at least in part, due to release of heme during interaction of oxyHb with HADNT derived from TNT.

In summary, the present findings suggest that prooxidants such as H_2O_2 and ferrylHb generated during interaction of oxyHb with HADNT formed from TNT play

important roles in the metHb formation and decrease in the Ht value caused by TNT exposure *in vivo*. We also found that HADNT causes oxidation of globin sulfhydryls and heme release; as a consequence, aggregation of Hb and hemolysis appears to occur. We speculate that TNT-induced decrease in the Ht value is due to an increased removal of the damaged erythrocytes caused by HADNT in the spleen because Jollow *et al.* (1995) reported that the N-hydroxy metabolites of dapsone induce the premature sequestration of the damaged cells by the spleen.

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Conflict of interest---- The authors declare that there is no conflict of interest.

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