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ORIGINAL ARTICLE

Recovery of 1-Chloro-2,4-dinitrobenzene Detoxification by N-acetyl-L-cysteine in Glutathione Predepleted Human Erythrocytes

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Abstract: Glutathione is an important thiol-containing compound involved in the detoxification process in erythrocytes. Its thiol group reacts with a variety of xenobiotics in a glutathione S-transferase catalyzed reaction to form conjugates that are effluxed from the erythrocytes by an ATP dependent transport mechanism. A well studied experimental system is the transport of the conjugate of glutathione and 1-chloro-2,4-dinitrobenzene. We investigated whether N-acetyl-L-cysteine protects the free-SH content and restores 1-chloro-2,4-dinitrobenzene detoxification in erythrocytes or replaces glutathione in detoxification process in glutathione predepleted erythrocytes. Our results indicate that N-acetyl-L-cysteine restores the intracellular free-SH content following depletion by 1-chloro-2,4-dinitrobenzene and N-ethylmaleimide. N-acetyl-L-cysteine (10 mM) increased the intraerythrocyte free-SH level to 14 ± 1 µmol/ml erythrocytes in 10 min in erythrocytes treated with N-ethylmaleimide. The control level was 5 ± 0.1 µmol/ml RBC. Results showed that N-acetyl-L-cysteine, in the presence and absence of L-buthioninesulfoximine, significantly recovered the 1-cloro-2,4-dinitrobenzene detoxification process in erythrocytes. The rate of conjugate transport in glutathione predepleted and N-acetyl-L-cysteine treated erythrocytes was 449 ± 38 nmol/ml erythrocytes. In the absence of N-acetyl-L-cysteine the rate of transport was 214 ± 21 nmol/ml erythrocytes which remained similar to the control. Our results suggest that N-acetyl-L-cysteine recovers the dinitrophenyl-glutathione transport and also replaces glutathione in the detoxification of 1-cloro-2,4-dinitrobenzene in glutathione predepleted erythrocytes.

Key Words: GSH, NAC, Erythrocytes, Xenobiotics, Detoxification, Free-SH

Introduction

Glutathione (GSH) is a tripeptide containing a reactive thiol group (1). One of the most important roles of glutathione is to maintain a reduced cellular environment and thus to protect the cells from toxic insults induced by oxidants (2-4). In addition, glutathione is involved in the detoxification of electrophilic xenobiotics (5). In these 2 defense processes of cells, glutathione is mainly converted into oxidized glutathione (GSSG) and glutathione conjugates for the latter. Both of these products are actively transported from the erythrocytes (6-8). The GSH conjugate of 1-chloro-2,4-dinitrobenzene (CDNB), dinitrophenyl-glutathione (Dnp-SG), is actively effluxed from the erythrocytes in an ATP dependent manner (9,10). The effluxed Dnp-SG is then taken up by the kidneys and liver and further metabolized in the mercapturic acid pathway by the sequential action of γ-glutamyltranspeptidase, dipeptidase and N-acetylase (11). It has been demonstrated that the transport of GSSG and xenobiotic conjugates of GSH such as Dnp-SG significantly contributes to intracellular GSH turnover in erythrocytes (6). Depletion of GSH by these mechanisms renders the cells more susceptible to destruction by toxic or oxidant assault. Therefore, the depleted intracellular GSH pool should be recovered to continue viability. N-acetyl-L-cysteine (NAC) has been shown in several experimental systems including erythrocytes to induce the GSH synthesis and increase intracellular GSH levels following depletion by several agents (12-14).

In the present study, we investigated whether NAC restores the CDNB transport from the erythrocytes following depletion of free-thiol (free-SH), which is mainly GSH. Since NAC serves as a precursor for GSH, it would be expected that treatment of GSH predepleted erythrocytes with NAC would regenerate GSH and recover the transport process. We also investigated

whether NAC itself replaces GSH in conjugation with CDNB and is involved in the removal of this xenobiotic from GSH predepleted erythrocytes.

Materials and Methods

Materials

1-Chloro-2,4-dinitrobenzene (CDNB), N-acetyl-L-cysteine (NAC), L-cysteine, L-buthionine [S,R] sulfoximine (BSO) and N-ethylmaleimide (NEM) were obtained from Sigma Chemical Co., USA. 5,5' - Dithiobis (-) (nitrobenzoate) (DTNB) was obtained from Fluka BioChemica, Switzerland.

Preparation of erythrocytes

Heparinized human blood was obtained from a healthy donor. Plasma was separated by centrifugation at 2000 g for 5 min. The plasma and buffy coat were then removed and discarded. The resulting erythrocyte pellet was washed twice with 4 volumes of phosphate buffered-saline (PBS) (9 parts of 0.15 M NaCl and 1 part of 0.1 M potassium phosphate buffer, pH 7.4) and was further used in the experiments at 20% hematocrit (9,11).

Depletion of intracellular free-SH in erythrocytes

Erythrocytes prepared as described above were treated with 0.1, 0.5 and 1 mM NEM containing PBS-glucose and incubated at 37 °C for 30 min. At the end of incubation, erythrocytes were centrifuged and washed. The free-SH groups in washed erythrocytes were then determined as described previously (15). Briefly, 100 µl of NEM treated and washed erythrocytes were lysed in 100 µl of 10% TCA prepared in sodium phosphate-EDTA buffer (0.01 M sodium phosphate/0.005 M EDTA, pH 8.0). The erythrocyte lysates were then centrifuged at 12,000 g for 5 min. At the end of centrifugation, 100 µl of the resulting supernatant was mixed with 2 ml of 0.6 µmol/ml DTNB prepared in sodium phosphate-EDTA buffer. Samples were allowed to stand for 5 min to develop color. The absorbances of the samples were measured at 415 nm and the concentrations of free-SH were calculated by using the mM extinction coefficient of 13.6. The same procedure was followed for the measurement of free-SH following treatment with CDNB.

Free-SH recovery in erythrocytes by NAC

The erythrocytes treated with 1 mM of NEM for 30 min described above were transferred to 10 mM NAC containing PBS-glucose following the centrifugation and the wash steps. Free-SH depleted erythrocytes were incubated in the presence of NAC for 10, 60 and 240 min at 37 °C. The free-SH content of erythrocytes treated in this manner was determined as described above.

Recovery of CDNB transport by NAC in GSH predepleted erythrocytes

The following experiments were designed to investigate whether NAC recovers the CDNB transport or replaces GSH in CDNB detoxification in GSH predepleted erythrocytes. Erythrocyte samples were divided into 5 different groups and marked A, B, C, D and E. In the first stage, A, B, C and D groups in PBS-glucose and the E group erythrocytes were incubated in 10 mM BSO containing PBS-glucose at 37 °C for 2 h. In all of the following stages the E group contained 10 mM BSO in the incubation medium in addition to the others indicated. In the second stage, the free-SH was depleted in C, D and E groups by incubation of erythrocytes with 1 mM NEM in PBS-glucose for 30 min. In the third stage, the A, B, and C group erythrocytes were incubated in PBS-glucose only and the D and E group erythrocytes were incubated in the presence of 10 mM of NAC in PBS-glucose for 1 h to load the erythrocytes with NAC. In the following stage, all groups except the A group were treated with 2 mM CDNB for 20 min and then the rate of Dnp-SG transport was determined in the supernatant spectrophotometrically at 340 nm using the mM extinction coefficient of 9.6 at the end of 2 h (9,11).

Statistical analysis

One-way analysis of variance (ANOVA) and Student-Newman-Keuls multiple comparison tests were applied to process the data statistically. All tests were performed on triplicate samples. Results were expressed as mean ± S.D. $P < 0.05$ values were considered significant.

Results

The results in Figure 1 show that treatment of erythrocytes with 2 mM CDNB depletes the intracellular

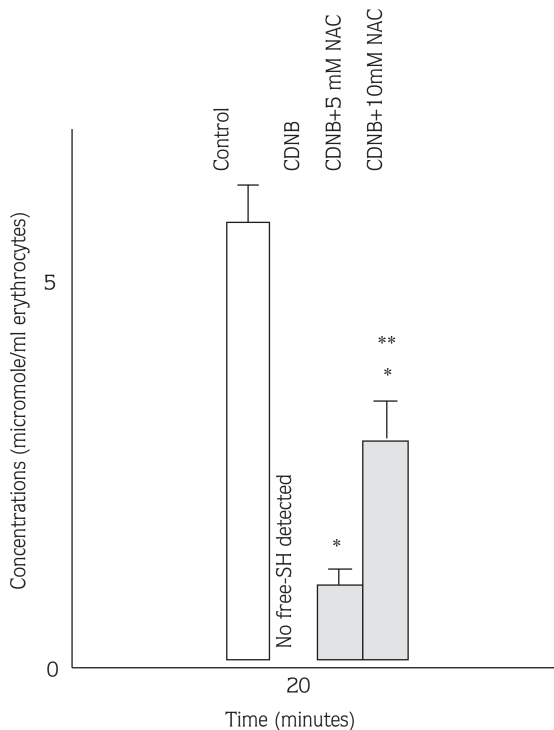

Figure 1. The effect of NAC on free-SH depletion by CDNB. Washed erythrocytes were treated with 2 mM of of CDNB in the presence and absence of 5 and 10 mM of NAC in PBS for 20 minutes. At the end of 20 minutes the free-SH content in erythrocytes were measured. Results are the mean, S.D. of three separate experiments.
*Significantly different from the control.
**Significantly different from CDNB+5 mM NAC group.
$P < 0.05$

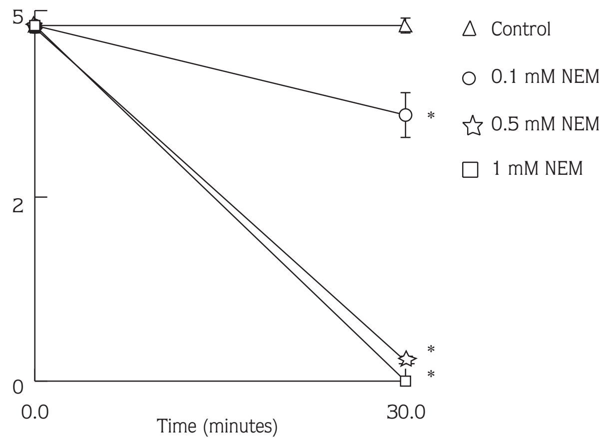

Figure 2. Free-SH Depletion by NEM. Washed erythrocytes were treated with indicated concentrations of NEM for 30 minutes in PBS-glucose at 37 C. At the end of 30 minutes the free-SH content in erythrocytes were measured. Results are the mean, S.D. of three separate experiments.
*Significantly different from the control.
$P < 0.05$.

free-SH content to undetectable levels. However, the presence of NAC in the incubation medium along with CDNB protects against depletion of intracellular free-SH in a dose dependent manner. The intracellular free-SH content remained at 1 ± 0.2 µmol/ml erythrocyte with 5 mM of NAC and at 2.9 ± 0.5 µmol/ml erythrocyte with 10 mM of NAC in CDNB treated erythrocytes. The control level was 5.7 ± 0.5 µmol/ml erythrocyte. In the following experiments we tested whether NAC recovers the Dnp-SG transport or replaces GSH in conjugation reaction with CDNB in GSH predepleted erythrocytes. To deplete the GSH pool, we incubated the erythrocytes with different concentrations of NEM for 30 min to determine the concentration that completely depletes free-SH in our system. As shown in Figure 2, the most effective concentration of NEM was 1 mM and this concentration brought the free-SH content to undetectable levels. Free-SH levels remained at 3.6 ± 0.3 µmol/ml erythrocyte with 0.1 mM of NEM and at 0.3 ± 0.05 µmol/ml erythrocyte with 0.5 mM of NEM. In the following experiment, we established the regeneration of intraerythrocyte free-SH depleted by NEM. As seen in Figure 3, in the presence of NAC intraerythrocyte free-SH content reached a level high above the control level. The increases in free-SH in 10, 60 and 240 min were 14.3 ± 1, 28.5 ± 3.2 and 26.9 ±.1.0 µmol/ml erythrocyte respectively. In the next step, we investigated whether this free-SH pool recovers the Dnp-SG transport or replaces GSH in the detoxification of CDNB. To establish these possibilities, we first depleted GSH by NEM, recovered the free-SH in erythrocytes by NAC in the presence and absence of BSO, treated with CDNB and then measured the transport activity. Figure 4 suggests that NAC both recovers the Dnp-SG transport and replaces GSH in CDNB detoxification in erythrocytes. In the absence of NAC, NEM + CDNB treated erythrocytes (Group C) displayed a transport activity of 214.6 ± 21.5 nmol/ml erythrocyte, which is equal to the control value (235.6 ± 50 nmol/ml erythrocyte). The erythrocytes treated with NAC following NEM exposure (Group D) recovered significant transport activity (449 ± 38 nmol/ml erythrocyte). This recovery may be due to GSH synthesis. However, a recovery in transport activity in the presence of BSO (Group E) is also recorded (544.0 ± 174.6 nmol/ml erythrocyte). The erythrocytes treated only with CDNB (Group B) showed a transport activity 624.6 ± 29.9 nmol/ml erythrocyte.

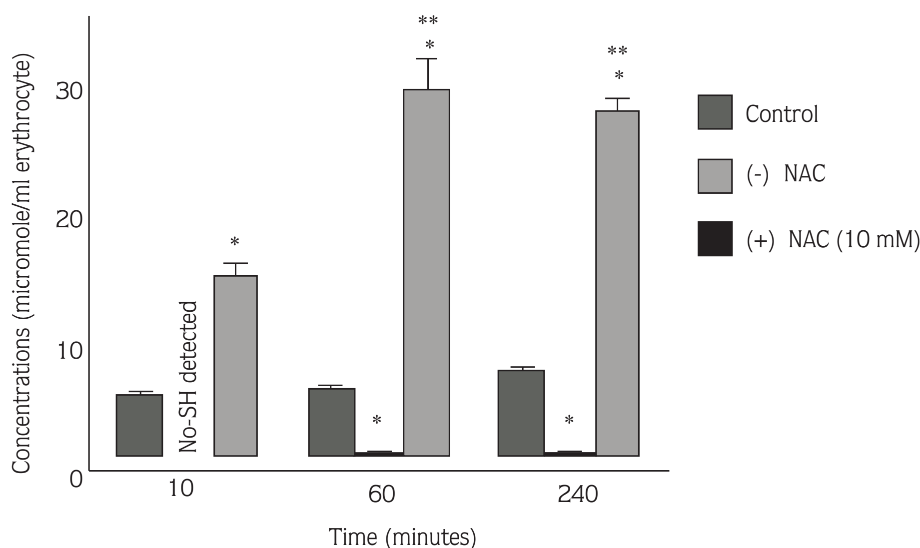

Figure 3. Recovery of Free-SH by NAC. Control erythrocytes received no treatment. (-) NAC group received no NAC following tretament with NEM. (+) NAC group was treated with 10 mM NAC following NEM treatment. Results are the mean, S.D. of three separate experiments.
*Significantly different from the control group.
** Significantly different from the (-) NAC group.
$P < 0.05$

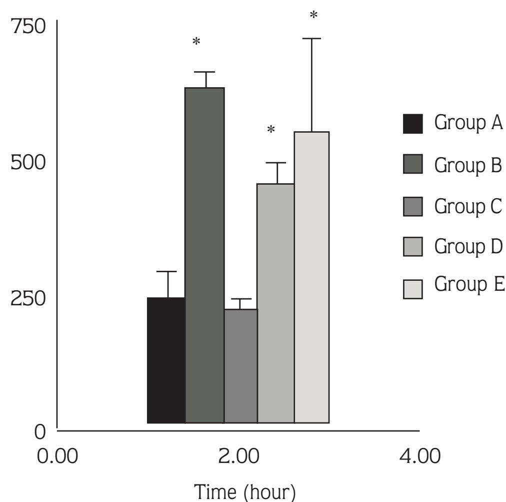

Figure 4. Recovery of CDNB detoxification in GSH predepleted erythrocytes by NAC. (Group A) Control, (Group B) treated only with CDNB, (Group C) GSH was depleted by pretreatment with NEM before CDNB treatment, (Group D) GSH was depleted by pretreatment with NEM and then incubated in the presence of NAC before CDNB treatment, (Group E) GSH was depleted by pretreatment with NEM and then incubated in the presence of BSO + NAC before CDNB treatment. See Materials & Methods for the details. Results are the mean, S.D of three separate experiments.
* Significantly different from the Goup A and C.
$P < 0.05$

Discussion

As our results indicated, NAC protects the erythrocytes from free-SH depletion by CDNB. CDNB is an electrophilic compound and is conjugated with GSH by GST upon entry into the erythrocytes. This conjugation reaction depletes the most important intracellular soluble antioxidant, GSH, and renders the cells more vulnerable to toxic or oxidative insult. Therefore, NAC could be used to alleviate the deleterious effects of GSH depletion by CDNB. CDNB has been known to induce immune stimulation and is also used by some researchers in AIDS therapies (16). In this sense, coadministration of NAC along with CDNB to experimental systems where CDNB is applicable may improve the positive outcome by providing protection against the toxic stress of CDNB. Our results also indicate that NAC recovers the free-SH content of erythrocytes following depletion by NEM. As shown in Figure 3, the recovery is very efficient and the concentrations reach about 2 times the control in 10 min. The data presented also indicate that the uptake of NAC into erythrocytes reaches an equilibrium in 40 min and prolonging the exposure time does not further increase the free-SH level even if NAC is present. In the next step, we investigated whether this free-SH pool regenerated by NAC recovers the Dnp-SG transport or replaces GSH in

the removal of CDNB from erythrocytes. The results displayed in Figure 4 suggest that NAC both recovers the Dnp-SG transport and replaces GSH in CDNB efflux from erythrocytes. In the absence of NAC, NEM + CDNB treated erythrocytes were equal in transport activity to the control. This demonstrates that CDNB is not leaked or diffused out from the erythrocytes but is removed by the presence of NAC. The erythrocytes treated with NAC following NEM exposure recovered significant transport activity. This recovery may be due to GSH synthesis. However, a recovery in transport activity in the presence of BSO was also recorded. Since erythrocytes in this group were pretreated with BSO and exposed to BSO in all the following steps, it cannot be stated that the recovery of CDNB detoxification took place through initial synthesis of GSH and then conjugation with GSH and transport from the erythrocytes. BSO has been shown in several experimental systems to be an effective inhibitor of GSH synthesis and could be used at high concentrations without toxicity (17-19). NAC has been shown to stimulate GSSG reductase (13). In the presence of NAC a rapid conversion of GSSG to GSH could also be expected. Our results suggest that NAC could recover the efflux of CDNB in the presence and absence of BSO. Erythrocytes may possess a GSH independent emergency detoxification mechanism that utilizes moderately low molecular weight soluble-SH containing compounds. Evidence has been presented by other researchers supporting the possibility that NAC may directly react with CDNB (20). It has been shown that hemoglobin-cysteine-DNB adducts can form. These adducts are also detected in enzyme free systems that would be required to catalyze the conjugation of DNB with hemoglobin. In recent studies, it has also been shown that GST may utilize other substrates such as NAC and γ-glutamylcysteine in addition to GSH in conjugation reactions (21,22).

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