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An Increase in Free Radical Production by Means of an Anion Channel Blocker DIDS in Mouse Peritoneal Neutrophils

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ABSTRACT

DIDS (4, 4'-diisothiocyanostilbene-2, 2'-disulfonic acid) has been recognized as an anion channel blocker. In this study, we demonstrated that DIDS significantly enhanced the production of free radicals in mouse peritoneal neutrophils. By means of a luminol-chemiluminescence (LCL) monitoring system, DIDS markedly increased LCL which could be suppressed by SOD, sodium azide (NaN_3), EGTA and BAPTA-AM and only slightly inhibited by staurosporine (STP). Depletion of the endoplasmic reticulum (ER)-Ca^{2+} store by means of thapsigargin (TG) had no effects on DIDS-enhanced LCL, but DIDS significantly increased the amount of intracellular free calcium as monitored by means of fura-2 staining. These results indicate that DIDS may enhance free radical production mediated by Ca^{2+} release from the mitochondria. Both phorbol-12-myristate-13-acetate (PMA) and DIDS can induce increased translocation of p47-*phox* of the neutrophil to the membrane fraction, which is inhibited by STP pretreatment. Since free radical generation could reduce the cytoplasmic pH (pH_i), we further examined whether DIDS was capable of inducing intracellular acidification. The result indicated that DIDS certainly lowered the pH_i, which was also suppressed by pretreatment with either NaN_3 or NaCN, but not by diphenyleneiodonium (DPI). These findings lead us to propose a working hypothesis that DIDS mainly induces superoxide production accompanied by decreasing pH_i mediated through a Ca^{2+}-dependent effect on the mitochondria rather than on NADPH oxidase. Using the lipophilic fluorescent dye $DiOC_6(3)$, we showed that DIDS decreased the transitional mitochondrial membrane potential. NaN_3, but not STP or pyrrolidine dithiocarbamate (PDTC), antagonized DIDS in the course of decreasing the mitochondrial membrane potential. Taken together, all of these findings imply a possible role of anion channels of the mitochondria in modulating free radical production and intracellular acidification of neutrophils through alteration of the mitochondrial transition membrane potential and Ca^{2+}-release.

Key Words: DIDS, free radical, pH_i, mitochondria, membrane potential, neutrophil

I. Introduction

Under stimulation by various stimuli, neutrophils produce various active oxygen species. Superoxide is a short-lived reactive oxygen species (ROS) formed by univalent reduction of molecular oxygen. Activated neutrophils produce substantial quantities of superoxide for microbial killing during phagocytosis (Babior *et al*., 1973; Drath and Karnovsky, 1975). Luminol (5-amino-2, 3-dihydro-1, 4-phthalazinedione) chemiluminescence has been extensively used as a chemiluminigenic probe for assessing $O_2^-\bullet$, H_2O_2, HOCl, $^-OH\bullet$ and $^1O_2^-$ generation by activated phagocytic cells (DeChatelet *et al*., 1982; Hastings *et al*., 1982). However, excessive production of these oxidants may contribute to the generation of tissue injuries, e.g., ischaemic heart failure (Romson *et al*., 1983), respiratory disorders, and atherosclerosis (Quinn *et al*., 1987). The phagocyte respiratory burst, which generates superoxide, is one of the most important mechanisms for killing invasive microbial pathogens. The capacity to generate superoxide anion and other toxic oxygen species depends on the activity of a membrane-bound multi-component oxidase which uses intracellular NADPH as its proximal electron donor (Tauber and Goetzl, 1979; Babior, 1992).

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In resting neutrophils, the components of the NADPH oxidase are segregated into cytosolic and membrane compartments; but during activation, the cytosolic components are translocated to the plasma membrane, where they assemble with membrane-bound components, resulting in an active superoxide-generating system (Chanock *et al*., 1994; Clark *et al*., 1990; Quinn *et al*., 1993). Several lines of evidence support the view that the NADPH-oxidase may consist of a membrane-bound cytochrome *b*-558 and at least three cytosolic proteins (p47-*phox*, p67-*phox*, and Rac-related GTP binding protein ($p21^{rac1}$) (Parkos *et al*., 1987; Volpp *et al*., 1988; Lomax *et al*., 1989; Abo *et al*., 1991). The intracellular signalling route for activation of the oxidase response in neutrophils is well established. NADPH oxidase is activated on binding of extracellular ligands on the phagocyte plasma membrane receptors. Most of the ligands induce intracellular Ca^{2+} mobilization, protein kinase C (PKC) activation, and subsequent translocation of p47-*phox* and p67-*phox* from the cytoplasm to the membrane, eventually leading to NADPH oxidase activation.

On the other hand, mitochondria, other than NADPH oxidase, have been suggested as another cellular source of superoxide anions in neutrophils (Chance *et al*., 1979). It has been documented that direct intervention on mitochondria could lead to superoxide generation and also reduction in the mitochondrial transmembrane potential ($\Delta\Psi m$). $\Delta\Psi m$ is established through the asymmetric distribution of charges between the inner and outer side of the inner mitochondrial membrane, giving rise to a chemical (pH) and electrical potential which is essential for mitochondrial function (Attardi and Schatz, 1988). The inner side of the inner mitochondrial membrane is negatively charged. Consequently, cationic lipophilic fluorochromes, such as 3, 3'-dihexyloxacarbo-cyanine iodide ($DiOC_6(3)$), are distributed to the mitochondrial matrix as a function of the Nernst equation, correlating with $\Delta\Psi m$.

Furthermore, activation of neutrophils is usually accompanied by intracellular acidification. For instance, the production of one mole of superoxide leads to intracellular equimolar proton production (Geinstein and Furuya, 1986; Simchowitz, 1985). However, maintenance of the intracellular pH (pH_i) within a relatively narrow range is crucial for normal cellular function. Thus, intracellular acidification must be opposed if neutrophils are to perform their function of host defense (Geinstein *et al*., 1986).

The impermeant labeling reagent 4, 4'-diisothiocyano-stilbene-2, 2'-disulfonic acid (DIDS), a specific inhibitor of anion transport in red cells, has been shown to protect perfused lung from oxidant injury (Nozil-Grayck *et al*., 1997). This finding apparently correlates with the finding that DIDS inhibits several responses of human neutrophils elicited with immune complexes, the synthetic chemotaxin N-formyl-Met-Leu-Phe (FMLP) and a calcium ionophore A23187 (Terada, 1996).

Thus, in this study, we demonstrated that DIDS not only significantly enhanced the production of free radicals and induced intracellular acidification in the activated mouse peritoneal neutrophils, but also reduced the mitochondrial transmembrane potential. This finding implies the possibility that anion channels of mitochondrial inner membrane may play a role in modulating mitochondrial functions.

II. Materials and Methods

1. Materials

DIDS, luminol, staurosporine (STP), thapsigargin (TG), acetoxymethyl ester of 1, 2-bis(2-aminophenoxy) ethane-N,N,N',N'-tetraacetic acid (BAPTA-AM), sodium azide, diphenyleneiodonium (DPI) and pyrrolidine dithiocarbamate (PDTC) were obtained from the Sigma Chemical Co. (St. Louis, MO, U.S.A.). 2', 7'-bis-(2-carboxyethyl)-5-(and-6) carboxyfluorescein, acetoxymethyl ester (BCECF-AM) and $DiOC_6(3)$ were purchased from Molecular Probe, Inc. (Eugene, OR, U.S.A.). Rabbit polyclonal anti-p47-*phox* was obtained from Transduction Lab. (Lexington, KY, U.S.A.).

2. Preparation of Mouse Activated Peritoneal Neutrophils

Pathogen-free male ICR mice 5 to 6 weeks in age were injected intraperitoneally with 2 ml of sterile 12% (w/v) sodium caseinate in iso-osmotic (0.9%) NaCl. 24 h later, the animals were killed by means of ether asphyxiation, the peritoneal cavity was opened and the peritoneal exudate was collected as previously described by Badwey *et al*. (1983).

3. Effects of Chemicals on Luminol and Lucigenin-Dependent Chemiluminescence

Chemiluminescence amplified by luminol and lucigenin was performed in a luminometer (model 1251, LKB-Wallac, Turku, Finland). The incubation mixture contained 2×10^5 neutrophils (viability > 95% as determined by trypan blue dye exclusion), 50 μM luminol or 100 μM lucigenin, and various chemicals in 200 μl of continuously stirred normal Krebs' buffer. The light output was read as a millivolt response.

4. Measurement of Intracellular Free Calcium Concentration

$[Ca^{2+}]$ was measured according to the procedure of Scharff *et al*. (1988). Briefly, neutrophils (1×10^6 cells

/ml) were incubated for 45 min at 37 ± 0.5˚C with 2.5 μM fura-2-AM in Krebs' solution (145 mM NaCl, 5 mM KCl, 1.2 mM $CaCl_2$, 1.3 mM $MgCl_2$, 1.2 mM NaH_2PO_4, 10 mM glucose, and 20 mM HEPES). The cells were then washed twice by means of centrifugation with Krebs' solution. After a 15-min incubation at 37 ± 0.5˚C to equilibrate them, the cells were centrifugated and resuspended in Krebs' solution. The concentration of the cytoplasmic free Ca^{2+} was determined through fluorescence measurements at 37 ± 0.5˚C with continuous stirring in a SLM-AMINCO 8000 spectrophoto-fluorometer. The monochromator settings were 349 nm for excitation and 510 nm for emission.

5. Preparation of the NADPH Oxidase-rich Membrane Fractions

After treatment of neutrophils (2×10^7 cells/ml) with various stimuli for 20 min at 37 ± 0.5˚C, cells were separated by means of centrifugation (100× g, 5 min) at 4 ± 0.5˚C and were resuspended in ice-cold relaxation buffer. After sonication of the cell suspension, the precipitate was separated by means of centrifugation (85,000× g, 30 min at 4˚C) and resuspended in relaxation buffer. The precipitate thus obtained was used as the NADPH-oxidase enriched membrane fraction and assayed for p47-*phox*.

6. Western Blotting

The membrane fractions obtained from various stimuli were mixed 1:1 with lysis buffer (10 mM Tris-HCl, pH 7.5, 2 mM EDTA, 1 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupetin, 3% (w/v) SDS, 0.1% (v/v) NP-40, 10 mM NaCl and 0.15 mM $MgCl_2$). The mixture was then centrifuged at 20,000× g at 4 ± 0.5˚C for 30 min. The supernatants were then collected, and the protein content was determined using the BCA protein assay reagents (Pierce, Rockford, IL, U.S.A.) with bovine serum albumin (BSA) as standard. Each sample, which contained 50 μg protein, was separated on 8% SDS-polyacrylamide minigels. After electrophoresis, the gels were transferred to a nitrocellulose paper. The loading and transfer of equal amounts of protein in each lane was verified by staining of the protein bands with Amido Black. After extensive washing with distilled water to remove the protein stain, the membrane was incubated with 3% (w/v) BSA in phosphate buffered saline (PBS) containing 0.1% (v/v) Tween-20 to block non-specific immunoglobulins and then immunoblotted with rabbit polyclonal anti-p47-*phox* (Transduction Lab., Lexington, KY, U.S.A.). The primary antibody was detected using anti-rabbit IgG-HRP (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) and the enhanced chemiluminescence (ECL) kit (Amersham, Little Chalfont, Buckinghamshire, England).

7. Measurement and Manipulation of pH*i*

Measurement of pH_i has been described in detail elsewhere. In brief, neutrophils seeded onto a coverslip were loaded with 5 μM BCECF-AM for 10 min at room temperature in HEPES-buffered solution. The cells were then washed with the same solution and excited by alternated 490 and 440 nm wavelength light, using a filter wheel (Cairn Research, Faversham, Kent, U.K.) rotating at 32 Hz. The excitation light was transmitted to the cell under study using a 510 nm dichroic mirror under the microscope nosepiece, and the resulting fluorescence was detected as previously described. The following equation was used to convert the fluorescence ratio into a pH value:

$$pH_i = pK + \log[(R_{max} - R)/(R - R_{min})] + \log(F_{440min}/F_{440max}),$$

where R is the ratio of 530 nm fluorescence at 490 nm excitation and 530 nm fluorescence at 440 nm excitation; R_{max} and R_{min} are the maximum and minimum ratio values, respectively, from the data curve; and pK is the dissociation for the dye, taken as 7.15.

8. Flow Cytometric Analysis of Mitochondrial Trans-membrane Potential ($\Delta\Psi$m)

Flow cytometric analysis was performed on a FACScan (Becton Dickinson, San Jose, CA, U.S.A.) tuned at 488 nm, using the FL1 photomultiplier (bandpass 530 nm, bandwidth 30 nm). For the assessment of $\Delta\Psi$m, after neutrophils were treated with various stimuli for 30 min, cells were incubated for 15 min at 37 ± 0.5˚C in PBS containing 40 nM $DiOC_6(3)$. Thereafter, the cells were washed with PBS for 3 times and then kept on ice until cytofluorimetric analysis was performed within 60 min. In control experiments, cells were labeled after pre-incubation with the uncoupling agent carbonyl cyanide m-chlorophenylhydrazone (CCCP; 50 μM, 37 ± 0.5˚C, 30 min).

9. Statistical Analysis

All data are shown as mean ± S.E., and the Student's *t* test was used to determine if there was a significant difference between the two groups ($p < 0.05$). When multiple means were compared, the significance level ($p < 0.05$) was determined by ANOVA followed by the Student-Newman-Keul's test.

III. Results

1. Production of Free Radical Induced by DIDS

As shown in Fig. 1(a), DIDS at 10 – 100 μM stimulated the luminol-dependent luminescence of mouse peri-

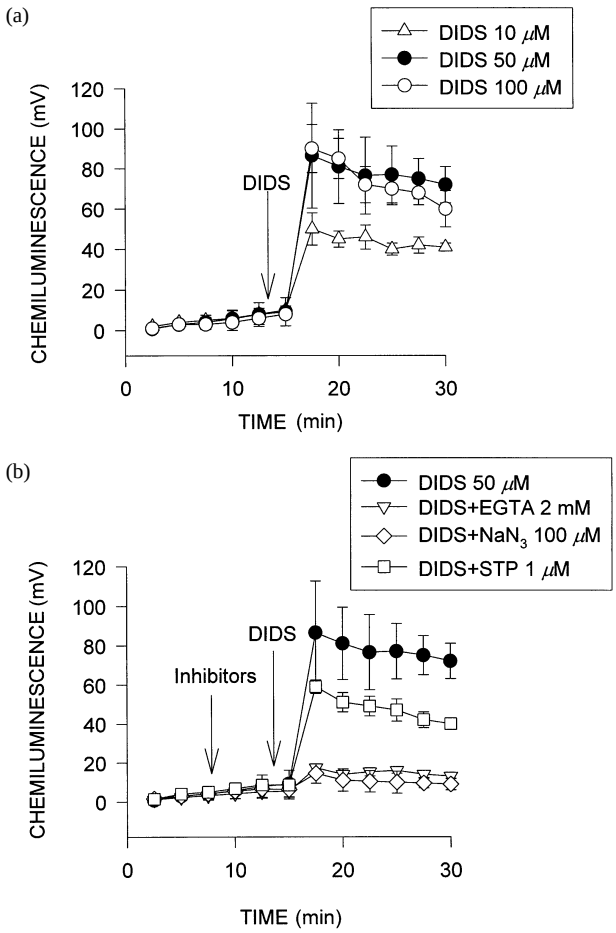

Fig. 1. Effects of NaN_3, EGTA, STP, BAPTA-AM and TG on DIDS-induced luminol-dependent chemiluminescence. (a) DIDS induced concentration-dependent luminol-dependent chemiluminescence production. (b) 2 mM EGTA, 100 μM NaN_3 and 1 μM STP eliminated DIDS and induced luminol-dependent chemiluminescence production, respectively. Data represent means ± S.E. from three different experiments performed in triplicate.

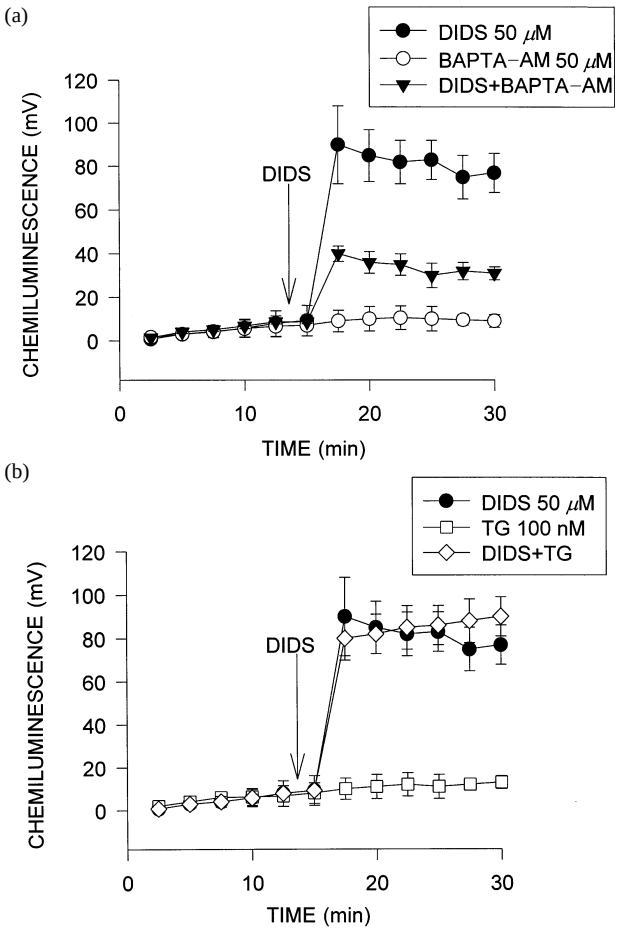

Fig. 2. Effects of BAPTA-AM and TG on DIDS-induced luminol-dependent chemiluminescence. (a) Pretreatment with 50 μM BAPTA-AM for 15 min prior to the addition of 50 μM DIDS. (b) Pretreatment with 100 nM TG for 15 min prior to the addition of DIDS. Data represent means ± S.E. from three different experiments performed in triplicate.

toneal neutrophils in a concentration-dependent manner. EGTA (2 mM, a calcium chelator) and 100 μM NaN_3 (a mitochondrial electron transport chain inhibitor) inhibited the DIDS-induced chemiluminescence of neutrophils by 82 ± 2.5% and 86 ± 1.7%, respectively. Staurosporine (1 μM, a protein kinase C inhibitor) resulted in partial, 32 ± 1.2%, inhibition (Fig. 1(b)). Treatment with superoxide dismutase (SOD, 300 U/ml) depressed the increase of LCL by 70 ± 3.2%. To confirm that the intracellular Ca^{2+} level was involved in the DIDS-induced LCL, we pretreated cells with BAPTA-AM (an intracellular calcium chelator) to block the increase in cytosolic free Ca^{2+}, and under these conditions, the increase of LCL was inhibited (Fig. 2(a)). Investigation of whether the ER-Ca^{2+} store was involved in the DIDS-induced LCL, we pretreated cells with thapsigargin (TG, 100 nM, an ER-Ca^{2+} ATPase inhibitor) to deplete the ER-Ca^{2+} store. The results showed that thapsigargin had no effect on the DIDS-induced LCL (Fig. 2(b)). In order to verify the increased production of superoxide mediated by Ca^{2+} release, we further demonstrated that DIDS significantly increased the lucigenin-dependent chemiluminescence, which was eliminated by 100 μM NaN_3 (Fig. 3). In addition, DIDS significantly increased the intracellular free calcium concentration in neutrophils (Fig. 4).

2. Activation of NADPH Oxidase by DIDS

It has been reported that translocation of p47-*phox* and p67-*phox* is essential for activation of NADPH oxidase, and that continuous translocation is necessary to keep the oxidase in an active state (Chanock *et al*., 1994). To further investigate whether DIDS would activate NADPH oxidase, we detected the translocation of p47-*phox* to the plasma membrane. After 30 min of stimula-

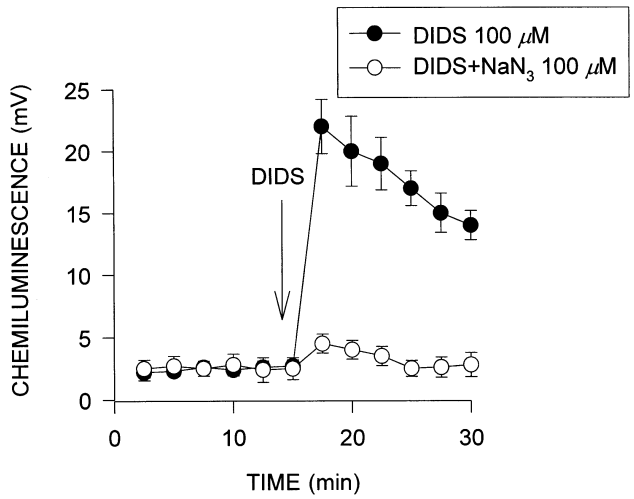

Fig. 3. NaN_3 inhibited DIDS-induced lucigenin-dependent chemiluminescence. DIDS significantly induced lucigenin-dependent chemiluminescence production. NaN_3 (100 μM) could eliminate DIDS while inducing lucigenin-dependent chemiluminescence production. Data represent means ± S.E. from three different experiments performed in triplicate.

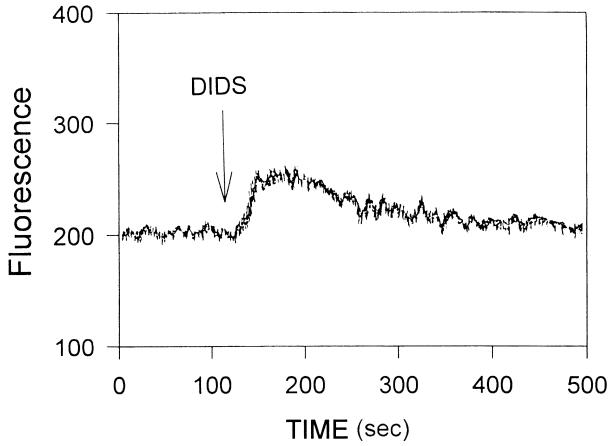

Fig. 4. Intracellular free Ca^{2+} concentration in neutrophils increased by DIDS. The intracellular Ca^{2+} concentration $[Ca^{2+}]_i$ was monitored using the fure-2-AM microfluorometric technique. DIDS (100 μM) increased $[Ca^{2+}]_i$, reaching a maximum after 170 sec and then gradually declining after 500 sec. Similar results were obtained in two separate experiments.

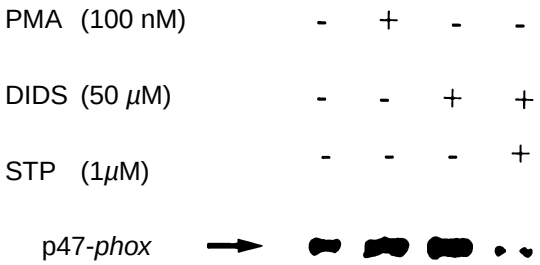

Fig. 5. Effects of DIDS and PMA on translocation of p47-*phox* to plasma membrane fractions. Staurosporine (STP) reduced the DIDS-induced translocation of p47-*phox*. p47-*phox* in plasma membrane fractions prepared from the cells was immunodetected as decribed in Materials and Methods. Similar results were obtained in two separate experiments.

tion, membrane pellets of neutrophils were isolated, and the pellets were analyzed by means of SDS-PAGE, followed by immunoblotting. As shown in Fig. 5, both PMA (phorbol-12-myristate-13-acetate, a protein kinase C activator, 100 nM) and DIDS (50 μM) induced increased translocation of p47-*phox* to membrane fractions (Fig. 5, lanes 2 and 3). Under pretreatment of the neutrophils with 1 μM staurosporine for 10 min, the DIDS-elicited p47-*phox* translocation effect was significantly inhibited (Fig. 5, lane 4).

3. Intracellular Acidification Induced by DIDS

Since it has been suggested that superoxide generation induces cytoplasmic acidification, we tested whether DIDS could induce intracellular acidification. Neutrophils loaded with a pH-sensitive fluorescent probe BCECF-AM were monitored using the fluorescence-ratio technique, and continuous perfusion of the medium was employed to minimize the contribution of probe leaking out from cells. Figure 6(a) shows the effect of perfusion of freshly isolated mouse peritoneal neutrophils with 50 μM DIDS in HEPES-buffered solution at 37 ± 0.5˚C; acidification of pH_i by 0.28 ± 0.04 pH units (n = 6) was observed. To test whether the acidification was due to a chemical reaction between intracellular BCECF and DIDS, BCECF acid was added in the absence of cells; no change in the ratio between the fluorescence intensity at 490 nm and that at 440 nm was seen after the addition of DIDS, showing that the DIDS-induced intracellular acidification was not due to a direct chemical reaction between BCECF and DIDS. The addition of 100 μM diphenyliodonium (DPI, an inhibitor of NADPH oxidase) could not eliminate DIDS-induced acidification (Fig. 6(b), n = 3). In contrast, the addition of either 100 μM NaN_3 or 2 mM NaCN could significantly block DIDS-induced acidification (Fig. 6(c) and (d), n = 3). Pretreatment with NaN_3, an inhibitor of mitochondrial electron transport, could concomitantly antagonize DIDS-induced free radical generation and intracellular acidification.

4. Alteration of Mitochondrial Membrane Potentials Induced by DIDS

Subsequently, we tested whether DIDS could alter the mitochondrial membrane potential when neutrophils generated free radical and acidification. The cationic lipophilic dye $DiOC_6(3)$, a fluorophore, was used to probe the mitochondrial membrane potential, which enabled us to identify the analogous mitochondrial perturbations. Af-

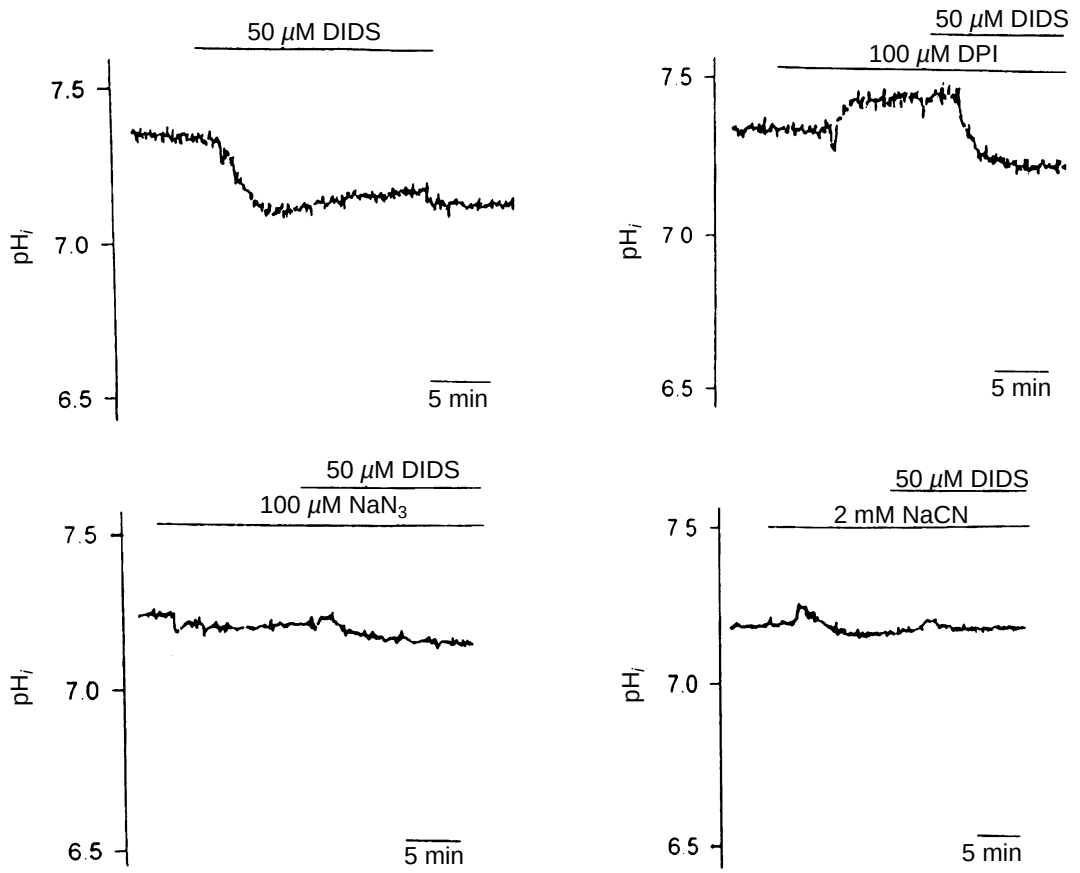

Fig. 6. Effects of DPI, NaN_3 and 2mM NaCN on DIDS-induced acidosis. (a) DIDS (50 μM) induced intracellular acidification. (b) DPI (100 μM) had no inhibitory effect on DIDS-induced intracellular acidification. (c) and (d) 100 μM NaN_3 and 2 mM NaCN inhibited DIDS-induced intracellular acidification, respectively. Similar results were observed in three experiments.

ter the addition of 50 μM DIDS, reduced uptake of $DiOC_6(3)$ was observed in neutrophils, which reached a maximum after 30 minutes of incubation. Similarly, 100 μM NaN_3 could significantly inhibit DIDS-reduced uptake of $DiOC_6(3)$ in neutrophils. In contrast, neither 1 μM staurosporine nor 100 μM PDTC (pyrrolidine dithiocarbamate, an antioxidant) could block DIDS-induced mitochondrial perturbations (Fig. 7).

IV. Discussion

In the present study, we demonstrated that DIDS induced free radical generation, intracellular acidification, reduced mitochondrial membrane potential ($\Delta\Psi m$) and increased intracellular Ca^{2+} concentration in mouse peritoneal neutrophils. DIDS is a stilbene compound that binds covalently and noncovalently to an outward-facing transport site of anion exchange proteins, preventing anion binding (Falke and Chan, 1986). Markert *et al*. (1988) suggested that DIDS causes concentration-related stimulation of oxygen radicals, together with activation of glycolysis in human neutrophils. Terada (1996) also reported that DIDS increased the intracellular superoxide level following reoxygenation in bovine endothelial cells. In contrast, DIDS was found to be capable of blocking superoxide penetration through the anion channels of plasma membranes, thus providing protection against oxidant injury (Nozik-Grayck *et al*., 1997). However, the mechanism of DIDS-mediated stimulation of intracellular superoxide generation still remained to be clarified.

In this study, we used luminol-chemiluminescence (LCL) to detect free radical generation because it offers high sensitivity and instantaneous luminescent reaction, and also because it permits the study of rapid and complex responses, including the generation of $O_2^-\bullet$, H_2O_2, $^-OH\bullet$ and NO (nitric oxide). Furthermore, it was previously shown that weak stimuli, e.g., platelet-activating factor (Poitevin *et al*., 1984), or leukotriene B_4 (Palmblad *et al*., 1984), induced LCL from neutrophils. However, LCL is not specific for superoxide anions but reacts with several products from activated neutrophils, particularly as a result of MPO reactions (DeChatelet *et al*., 1982; Dahlgren and Stendahl, 1983). Therefore, we performed further experiments and found that superoxide dismutase depressed

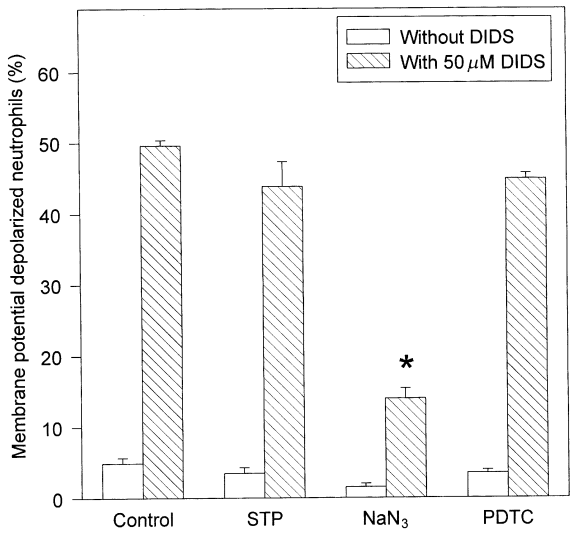

Fig. 7. Effects of inhibitors on DIDS-induced membrane potential depolarization. Cells were incubated with NaN_3 (100 μM), staurosporine (1 μM) or PDTC (100 μM) in the presence or absence 50 μM DIDS for 30 min. Then cells were incubated for 15 min at $37 \pm 0.5^{\circ}C$ in PBS containing 40 nM $DiOC_6(3)$ and analyzed using a flow cytometer. Data represent means ± S.E.

*Significant differences ($p < 0.05$) among treatments were determined by ANOVA as described in Materials and Methods.

the increase of LCL, and that DIDS increased lucigenin-dependent chemiluminescence (specific detection of superoxide, Aniansson *et al*. (1984)). Based on these results, we propose that DIDS mainly induces the production of $O_2^{-}\bullet$ in neutrophils. Further study using water-soluble ascorbate or lipophilic α-tocopherol antioxidants or exogenous free radical scavengers to elucidate the nature of the free radicals generated by DIDS in neutrophils is in progress.

To search for the organelles responsible for DIDS-induced free radical generation, we used the inhibitors of the mitochondrial respiratory chain, NaCN and NaN_3. The results confirmed that DIDS-induced free radicals were inhibited by NaCN and NaN_3, suggesting an important role for mitochondria. To elucidate the machinary underlying free radical generation from mitochondria, we studied the influence of DIDS on the mitochondial transitional membrane potential ($\Delta\Psi$m) and on the change of the intracellular Ca^{2+} concentration $[Ca^{2+}]_i$. Indeed, DIDS disrupted the mitochondrial $\Delta\Psi$m and increased $[Ca^{2+}]_i$. Moreover, not only NaCN and NaN_3, but also EGTA and BAPTA-AM (an intracellular Ca^{2+} chelator) inhibited DIDS-induced free radicals and altered mitochondrial $\Delta\Psi$m. Taking these findings together, we propose that DIDS binds to the mitochondrial membrane, causes a the change of mitochondrial $\Delta\Psi$m, leading to Ca^{2+}-release and then to an increase in free radicals through mainly superoxide generation. The previous reports (Nicholls, 1982; Murphy, 1989) that mitochondrial membrane depolarization enhanced the generation of superoxide anion in a number of cellular preparations also support this contention.

It has been suggested that an increase in superoxide generation is always accompanied by intracellular acidification, which may be derived from NADPH oxidation and /or the activation of the hexose monophosphate shunt (HMS) in normal neutrophils (Masaaki *et al*., 1995). In neutrophils, HMS activation is limited by the availability of mitochondrial $NADP^+$, which can be indicated by the extent of decrease of mitochondrial $\Delta\Psi$m as suggested by Vercesi (1987). In agreement with this notion, we found that DIDS markedly increased cellular acidification, which could also be inhibited by NaCN and NaN_3. These findings again suggest that mitochondria play a role in DIDS-induced cellular acidification due to the excessive production of free radicals. The role NADPH oxidase of neutrophils plays with regard to the effects of DIDS was also studied. Although DIDS increased membrane translocation of p47-*phox*, which could be inhibited by staurosporin (an inhibitor of PKC which can phosphorylate and activate NADPH oxidase, Levy *et al*. (1994)), DIDS-induced free radical generation was only slightly reduced by staurosporin, and DIDS-induced cell acidification could not be inhibited by DPI (an inhibitor of NADPH oxidase, Miesel *et al*. (1995)). All of these results suggest that activated NADPH oxidase may not be the major mechanism in DIDS-induced cellular acidification.

In conclusion, we propose based on the findings obtained in this study that DIDS initially disrupts the mitochondrial membrane potential ($\Delta\Psi$m), causing Ca^{2+} release from the mitochondrial matrix, and then induces superoxide generation and cytoplasmic acidification. This finding implies that anion channels on the mitochondrial inner membrane may play a pivotal role in regulating mitochondrial function.

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DIDS增加小鼠腹腔嗜中性白血球自由基之產生

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摘　要

DIDS 是一種陰離子通道之抑制劑，本研究首先發現DIDS 增加小鼠腹腔嗜中性白血球自由基的產生之作用極強，而此作用可被NaN_3（粒線體呼吸鏈抑制劑），EGTA（鈣螯合劑）和BAPTA-AM（細胞內鈣螯合劑）所抑制，但staurosporine（蛋白激壙C 抑制劑）及thapsigargin（ER Ca^{2+}-ATPase 抑制劑）皆不能抑制。並且DIDS 亦能明顯增加細胞內鈣離子濃度。DIDS 可以使NADPH 之p47-*phox* 轉移至細胞膜上，但staurosporine 卻可以抑制此作用，由這些結果，我們推論DIDS 是經由鈣依靠性途徑促進自由基產生並且主要來自於粒線體而不是NADPH oxidase 。另外，我們發現DIDS 亦會引發細胞內酸化，此作用可被NaN_3及NaCN 所抑制，但diphenyleneiodonium 不能抑制此作用，這些結果證明DIDS 引發自由基產生後導致細胞酸化的作用是經由粒線體所引發。利用$DiOC_6(3)$螢光染劑方法顯示DIDS 能減低粒線體之膜電位，此作用也可被NaN_3所抑制，但staurosporine 和pyrrolidine dithiocarbamate（一種抗自由基劑）依然沒有作用。根據以上數據，我們總結有關DIDS 對小鼠腹腔嗜中性白血球的作用機轉；首先增加自由基之產生，接著引發細胞酸化，並發現粒線體膜電位有降低之現象，這些發現的重點指出粒線體陰離子通道在其膜電位之調控扮演著重要的角色。