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Cellular mechanisms of inhibition of superoxide anion generation in rat neutrophils by the synthetic isoquinoline DMDI

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Abstract

This study was undertaken to assess the cellular localization of the inhibitory effect of a chemically synthetic isoquinoline compound 1-(3′,4′-dimethoxybenzyl)-6,7-dichloroisoquinoline (DMDI) on the formyl-methionyl-leucyl-phenylalanine (fMLP)-induced respiratory burst in rat neutrophils. The DMDI concentration dependently inhibited the superoxide anion ($O_2^{\bullet -}$) generation and O_2 consumption (IC_{50} 12.2 ± 4.9 and 15.2 ± 8.4 μM, respectively) of neutrophils. DMDI did not scavenge the $O_2^{\bullet -}$ generated during the autoxidation of dihydroxyfumaric acid in a cell-free system. DMDI did not elevate cellular cyclic AMP levels. Inhibition of $O_2^{\bullet -}$ generation by DMDI in neutrophils was not reversed by a cyclic AMP-dependent protein kinase inhibitor, (8*R*,9*S*,11*S*)-(−)-9-hydroxy-9-hexoxycarbonyl-8-methyl-2,3,9,10-tetrahydro-8,11-epoxy-1*H*,8*H*,11*H*-2,7b,11a-triazadibenzo[*a,g*]cycloocta[*cde*]trinden-1-one (KT5720). The DMDI concentration dependently inhibited the late plateau phase but not the initial spike of fMLP-induced $[Ca^{2+}]_i$ changes in the presence of extracellular Ca^{2+}. However, DMDI had no effect on the fMLP-induced $[Ca^{2+}]_i$ changes in the absence of extracellular Ca^{2+}. In addition, DMDI did not affect the fMLP-stimulated phosphatidylinositol 3-kinase (PI3-kinase) activation. DMDI produced a concentration-dependent reduction in the formation of phosphatidic acid and phosphatidylethanol in the presence of ethanol from fMLP-stimulated neutrophils (IC_{50} 13.3 ± 4.0 and 9.4 ± 4.3 μM, respectively). On the basis of the immunoblot analysis of the phosphorylation of the mitogen-activated protein (MAP) kinase, DMDI attenuated the fMLP-stimulated MAP kinase phosphorylation in a similar concentration range. Collectively, these results indicate that the inhibition of the respiratory burst by DMDI in rat neutrophils is mediated through the blockade of phospholipase D and MAP kinase signaling pathways.

Keywords: DMDI (1-(3′,4′-dimethoxybenzyl)-6,7-dichloroisoquinoline); Neutrophil; Respiratory burst; Signal transduction; Phospholipase D; MAP (Mitogen-activated protein) kinase; Ca^{2+} concentration, cellular-free

1. Introduction

Neutrophil activation is associated with the release of large amounts of potent microbicidal reactive O_2 species derived from the superoxide anion ($O_2^{\bullet -}$), and thus, plays a critical role in the host defense against invading microorganisms (Smith and Curnutte, 1991). However, under certain circumstances, the aberrant release of these potentially toxic species can lead to the amplification of inflammation as well as tissue injury. This is probably implicated in the pathogenesis of many diseases (Babior, 2000). To prevent an over-exuberant inflammatory response and to limit the damage to the host, these neutrophil programs should be tightly regulated. Drugs that inhibit the generation of $O_2^{\bullet -}$ from neutrophils are proposed to ameliorate inflammation and tissue damage. The enzyme responsible for $O_2^{\bullet -}$ generation is called NADPH oxidase, which is dormant in resting cells and becomes active upon cell activation. The oxidase consists of membrane cytochrome b_{558} ($p22^{phox}$ and $gp91^{phox}$) and cytosolic components ($p47^{phox}$, $p67^{phox}$ and Rac), which assemble into a functional state upon activation (Segal and Abo, 1993). Recently, it has been suggested that another cytosolic protein, $p40^{phox}$, promotes oxidase activation by increasing the affinity of $p47^{phox}$ for the enzyme (Cross, 2000). Thus, activated neutrophils evoke a respiratory burst in which oxygen uptake from the extracellular medium is increased and a large amount of $O_2^{\bullet -}$ is generated. The signal transduction events pertaining to the respiratory

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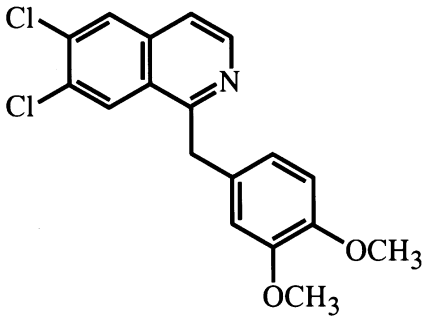

Fig. 1. Chemical structure of DMDI.

burst remain elusive. It has been proposed that the intracellular signals from the formyl-methionyl-leucyl-phenylalanine (fMLP) receptors are mediated by G_i proteins. Phospholipase C is rapidly activated, leading to the hydrolysis of phosphatidylinositol 4,5-bisphosphate to generate inositol trisphosphate and diacylglycerol, resulting in an increase in $[Ca^{2+}]_i$ and in the activation of protein kinase C, respectively (Berridge, 1987). These two second messengers act synergistically in $O_2^{\bullet -}$ generation. Phospholipase D is also activated by fMLP in neutrophils and appears to be functionally linked to $O_2^{\bullet -}$ generation (Bonser et al., 1989). Moreover, activation of phosphatidylinositol 3-kinase (PI3-kinase) and mitogen-activated protein (MAP) kinase by fMLP eventually leads to NADPH oxidase activation (Thelen et al., 1994; Zu et al., 1998).

In a study of the anti-inflammatory activity of chemically synthetic isoquinolines, compound 1-(3′,4′-dimethoxybenzyl)-6,7-dichloroisoquinoline (DMDI) (Fig. 1) was found in preliminary in vitro tests to inhibit the generation of $O_2^{\bullet -}$ from rat neutrophils stimulated by a chemotactic factor. The aims of the present study were to investigate the effect of DMDI on the respiratory burst in rat neutrophils and to elucidate the signaling pathway(s) responsible for the DMDI-operated blockage of the neutrophil response.

2. Materials and methods

2.1. Drugs and chemicals

DMDI was synthesized from 2-benzoyl-6,7-dichloro-1-cyano-1,2-dihydroisoquinoline and 3,4-dimethoxybenzyl chloride by a procedure analogous to that for 1-(4′-methoxybenzyl)-6,7-dichloroisoquinoline (Chen and Liu, 1992) and dissolved in dimethyl sulfoxide (DMSO) to make a 50 mM stock solution. The structure of DMDI (purity >99%) was characterized by the spectral data of infrared, nuclear magnetic resonance and mass spectroscopy. Dextran T-500, cyclic AMP enzyme immunoassay kit, enhanced chemiluminescence reagent and 1-*O*-[^{3}H]octadecyl-*sn*-glycero-3-phosphocholine were purchased from Amersham Pharmacia Biotech (Buckinghamshire, UK). Hanks' balanced salt solution was obtained from Gibco Life Technologies (Gaithersburg, MD, USA). Wortmannin, (8*R*,9*S*,11*S*)-(−)-9-hydroxy-9-hexoxycarbonyl-8-methyl-2,3,9,10-tetrahydro-8,11-epoxy-1*H*,8*H*,11*H*-2,7b,11a-triazadibenzo[*a,g*]cycloocta[*cde*]trinden-1-one (KT5720) and fura-2 acetoxymethyl ester (fura-2 AM) were purchased from Calbiochem-Novabiochem (San Diego, CA, USA). Phosphorus-32 was obtained from NEN Life Science (Boston, MA, USA). Rabbit polyclonal antibodies to phospho-p44/42 MAP kinase, phospho-p38 MAP kinase and p38 MAP kinase were purchased from New England Biolabs (Beverly, MA, USA). Mouse monoclonal pan extracellular signal-regulated (ER) kinase antibody was purchased from BD Transduction Laboratories (Lexington, KY, USA). Polyvinylidene difluoride membrane was from Millipore (Bedford, MA, USA). Other chemicals were purchased from Sigma (St. Louis, MO, USA). The final volume of DMSO in the reaction mixture was $\leq 0.5\%$ (v/v).

2.2. Isolation of neutrophils

Rat (Sprague–Dawley) blood was collected from the abdominal aorta, and the neutrophils were purified by dextran sedimentation, centrifugation through Ficoll–Hypaque and the hypotonic lysis of erythrocytes (Wang et al., 1995). Purified neutrophils containing >95% viable cells were resuspended in Hanks' balanced salt solution (HBSS) containing 10 mM HEPES, pH 7.4, and 4 mM $NaHCO_3$ and kept in an ice bath before use. Cells were used within 4 h after their preparation.

2.3. Measurement of $O_2^{\bullet -}$ generation and O_2 consumption

The generation of $O_2^{\bullet -}$ in neutrophil suspensions (2×10^6 cells) was determined by the superoxide dismutase-inhibitable reduction of ferricytochrome *c* (Wang et al., 1995). For the determination of $O_2^{\bullet -}$ scavenging activity, the $O_2^{\bullet -}$ generated during the autoxidation of dihydroxyfumaric acid (2.5 mM) in a cell-free system was assessed by measuring the reduction of nitroblue tetrazolium (Goldberg and Stern, 1977). Absorbance changes in the reduction of ferricytochrome *c* and nitroblue tetrazolium at 550 and 560 nm, respectively, were monitored continuously in a double-beam spectrophotometer. The O_2 consumption in the neutrophil suspension (2×10^6 cells) was measured continuously with a Clark-type oxygen electrode using a YSI biological oxygen monitor (Ingraham et al., 1982).

2.4. Determination of cellular cyclic AMP levels

Neutrophils were incubated with test drugs for 10 min at 37 °C. The reaction mixture was then added to 0.05 M acetate buffer, pH 6.2, containing 50 μM 3-isobutyl-1-methylxanthine. After boiling for 5 min, the suspension was sonicated and then sedimented. Supernatants were acetylated by the addition of 0.025 volume of triethylamine/acetic anhydride (2:1, v/v). The cyclic AMP content of the aliquots was assayed using an enzyme immunoassay kit.

2.5. $[Ca^{2+}]_i$ measurement

Neutrophils were loaded with 5 μM fura-2 AM (Wang et al., 1995). After washing, the cells were resuspended in HBSS to 5×10^6 cells/ml. Fluorescence was monitored with a double-wavelength fluorescence spectrophotometer at 510 nm with the excitation of 340 and 380 nm in the ratio mode. Calibration of the excitation ratio in terms of $[Ca^{2+}]_i$ was performed (Grynkiewicz et al., 1985).

2.6. PI3-kinase activity assay

Neutrophils were incubated with 150 μCi $^{32}P_i$ in HEPES buffer (30 mM HEPES, pH 7.4, 110 mM NaCl, 10 mM KCl, 1 mM $MgCl_2$ and 10 mM glucose) supplemented with 2 mg/ml of bovine serum albumin at 37 °C for 90 min and were then washed and resuspended in the HEPES buffer to 1×10^7 cells/ml. Cells were incubated with test drugs in the presence of 1 mM $CaCl_2$ for 3 min at 37 °C before stimulation with fMLP. The reactions were terminated by the addition of chloroform/methanol/8% $HClO_4$ (50:100:5, v/v). Lipids in the reaction mixture were extracted, dried and separated on silica gel 60 (Okada et al., 1994) which had been impregnated with 1.2% (w/v) potassium oxalate. The plates were developed with a solvent system containing chloroform/acetone/methanol/acetic acid/water (80:30:26:24:14, v/v), dried and then visualized for [^{32}P]phosphatidylinositol trisphosphate radioactivity with a PhosphorImager (Molecular Dynamics 445 SI) using ImageQuaNT software.

2.7. Phospholipase D activity assay

Neutrophils (4×10^7 cells/ml) were loaded with 10 μCi 1-*O*-[^{3}H]octadecyl-*sn*-glycero-3-phosphocholine in HBSS at 37 °C for 75 min and were then washed. Cells were incubated with test drugs in the presence of 1 mM $CaCl_2$ and 0.5% (v/v) ethanol for 3 min at 37 °C before stimulation with fMLP. Lipids in the reaction mixture were extracted, dried and separated on silica gel 60 (Wang et al., 1997). The plates were developed halfway using a solvent system consisting of hexane/diethyl ether/methanol/acetic acid (90:20:3:2, v/v), dried and developed again to the top using the upper phase of a solvent system consisting of ethylacetate/isooctane/acetic acid/water (110:50:20:100, v/v). The radioactivities of [^{3}H]-phosphatidic acid and [^{3}H]phosphatidylethanol were directly quantified with a PhosphorImager.

2.8. Immunoblot analysis of the phosphorylation of MAP kinase

Cells (2×10^7 cells/ml) were preincubated with test drugs for the indicated time before stimulation with fMLP. The reactions were terminated by the addition of stopping solution (20% (w/v) trichloroacetic acid, 1 mM phenylmethylsulfonyl fluoride, 2 mM *N*-ethylmaleimide, 10 mM NaF, 2 mM Na_3VO_4, 2 mM *p*-nitrophenyl phosphate, 7 μg/ml each of leupeptin and pepstatin). Proteins were resolved by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). The membranes were blocked with 5% (w/v) non-fat dried milk in TBST buffer (10 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.1% Tween 20) and probed with anti-phospho-p44/42 MAP kinase or anti-phospho-p38 MAP kinase antibody. To standardize protein loading in each lane, blots were stripped with buffer containing 62.5 mM Tris–HCl, pH 6.8, 100 mM 2-mercaptoethanol and 2% (w/v) SDS at 50 °C for 30 min. Then, the membranes were washed thoroughly, followed by reprobing with anti-pan ER kinase (p44/42 MAP kinase) or anti-p38 MAP kinase antibody. Immunostains were revealed using the enhanced chemiluminescence reagent. Quantification was by densitometry.

2.9. Statistical analysis

Statistical analyses were performed using the Bonferroni *t*-test method after the analysis of variance. A P value of less than 0.05 was considered significant for all tests. Analysis of the regression line test was used to calculate the IC_{50} values. Data are expressed as means ± S.D.

3. Results

3.1. Effect of DMDI on $O_2^{\bullet -}$ generation

Stimulation of rat neutrophils with 0.3 μM fMLP in the presence of 5 μg/ml of dihydrocytochalasin B induced rapid

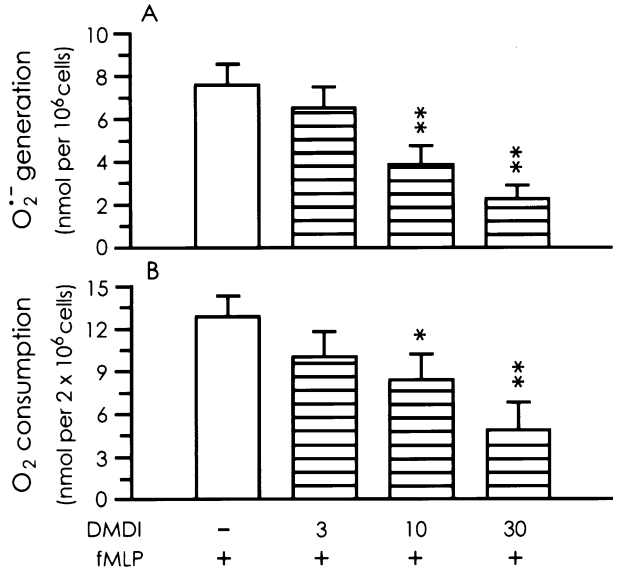

Fig. 2. Effects of DMDI on respiratory burst in rat neutrophils. Cells were preincubated with DMSO (as control) or 3–30 μM DMDI at 37 °C for 3 min (A) in the presence of dihydrocytochalasin B (5 μg/ml) before stimulation with 0.3 μM fMLP for the determination of $O_2^{\bullet -}$ generation for 10 min or (B) in the presence of 1 mM NaN_3 and 5 μg/ml of dihydrocytochalasin B before stimulation with 0.3 μM fMLP for O_2 consumption measurement for 5 min. Values are expressed as means ± S.D. of four to six separate experiments. * $P<0.05$, ** $P<0.01$, as compared with the corresponding control values.

and transient $O_2^{\bullet -}$ generation as assessed by the detection of superoxide dismutase-inhibitable ferricytochrome *c* reduction by spectrophotometry. In the presence of 1 mM NaN_3 and 5 μg/ml of dihydrocytochalasin B, 0.3 μM fMLP induced non-mitochondrial O_2 consumption in neutrophil suspensions. DMDI inhibited fMLP-induced $O_2^{\bullet -}$ generation and O_2 consumption in rat neutrophils in a concentration-dependent manner with IC_{50} values of 12.2 ± 4.9 and 15.2 ± 8.4 μM, respectively (Fig. 2A and B). The viability was ≥90% when the cells were incubated with 100 μM DMDI for 15 min at 37 °C (the cells released 10.2 ± 0.6% lactate dehydrogenase into the medium in comparison with the value of Triton X-100-treated cells). In a cell-free system, to determine $O_2^{\bullet -}$ scavenging activity, DMDI had no effect on $O_2^{\bullet -}$ generation during dihydroxyfumaric acid autoxidation (0.24 ± 0.02 for control vs. 0.23 ± 0.02 ΔA_{560} at 30 μM DMDI, $P>0.05$).

3.2. The role of cyclic AMP signaling pathway on the inhibition of $O_2^{\bullet -}$ generation by DMDI

Treatment of cells with 30 μM DMDI for 10 min at 37 °C did not affect the cellular cyclic AMP levels (Fig. 3A) or the cyclic AMP levels in response to fMLP (data not shown). Elevation of the cellular cyclic AMP level was observed in cells treated with the adenylyl cyclase activator forskolin (10 μM). Next, we examined the involvement of cyclic AMP in the inhibition of $O_2^{\bullet -}$ generation by DMDI. Pre-

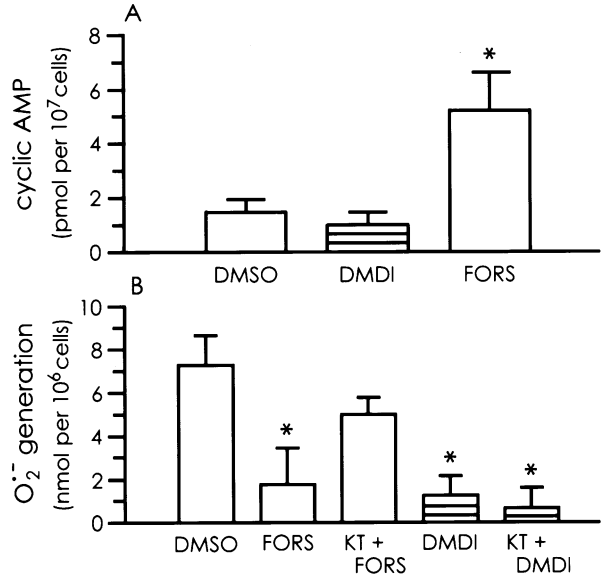

Fig. 3. The role of cyclic AMP on the inhibition of $O_2^{\bullet -}$ generation by DMDI. (A) Cells were incubated with DMSO (as control), 30 μM DMDI or 10 μM forskolin (FORS) for 10 min at 37 °C. The cyclic AMP contents were assayed using an enzyme immunoassay kit. Values are means ± S.D. of 6–10 separate experiments. * $P<0.01$ as compared with the control value. (B) Cells were preincubated with DMSO or 1 μM KT5720 for 10 min at 37 °C, followed by addition of DMSO, 10 μM forskolin or 30 μM DMDI for 3 min in the presence of dihydrocytochalasin B (5 μg/ml) before stimulation with 0.3 μM fMLP for the measurement of $O_2^{\bullet -}$ generation for 10 min. Values are expressed as means ± S.D. of three to six separate experiments. * $P<0.01$, as compared with the control value (first column).

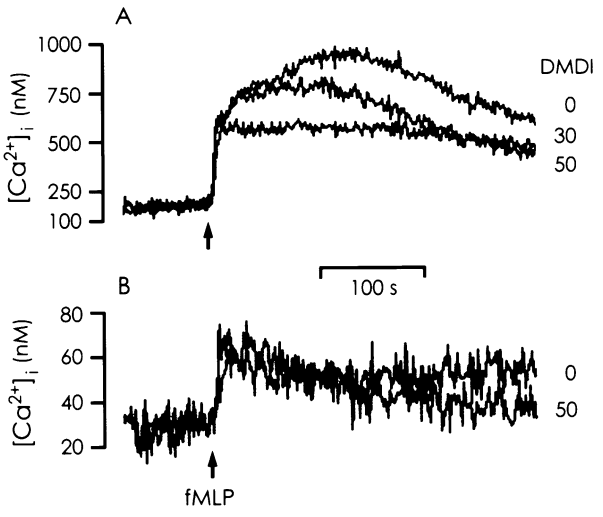

Fig. 4. Effect of DMDI on $[Ca^{2+}]_i$. Fura-2-loaded cells in (A) 1 mM Ca^{2+}-containing or (B) Ca^{2+}-free HBSS were incubated with 30–50 μM DMDI for 3 min at 37 °C, then stimulated (arrow) with 0.3 μM fMLP. Results presented are representative of three independent experiments with similar results.

treatment of cells with 1 μM KT5720, a cyclic AMP-dependent protein kinase inhibitor (Kase et al., 1987), failed to significantly alter the inhibitory effect of 30 μM DMDI (Fig. 3B) but reversed the inhibition of fMLP-induced $O_2^{\bullet -}$ generation by forskolin.

3.3. Effect of DMDI on $[Ca^{2+}]_i$

Addition of fMLP to the fura-2-loaded cells evoked a rapid rising phase followed by a plateau phase of $[Ca^{2+}]_i$ changes in the presence of extracellular Ca^{2+}. DMDI (30 μM) did not affect the rapid rising phase but reduced the plateau phase of the fMLP-induced response (Fig. 4A). Inhibition of the plateau phase was more pronounced at 50 μM DMDI. In the absence of extracellular Ca^{2+}, fMLP induced a small spike of $[Ca^{2+}]_i$ change. This response was not modified by 50 μM DMDI (Fig. 4B). The fMLP-induced $[Ca^{2+}]_i$ changes were abolished by 1 μM 1-[6-[[(17β)-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1*H*-pyrrole-2,5-dione (U73122), a phospholipase C inhibitor (Smith et al., 1990), both in the presence and absence of extracellular Ca^{2+} (data not shown).

3.4. Effect of DMDI on PI3-kinase activation

The addition of 1 μM fMLP to $^{32}P_i$-labeled cell suspension in the presence of dihydrocytochalasin B (5 μg/ml) at 37 °C resulted in an about 2.8-fold increase in radioactivity in phosphatidylinositol trisphosphate (12 143 ± 2925 vs. 35 353 ± 11 618 counts, $P<0.01$) as measured indirectly by a PhosphorImager. Cells were then pretreated with wortmannin, a PI3-kinase inhibitor (Powis et al., 1994), or DMDI for 3 min. The former greatly attenuated (93.1 ± 7.9% inhibition at 3 μM wortmannin), while the later had little effect

on the formation of phosphatidylinositol trisphosphate by fMLP (32 850 ± 8411 and 27 285 ± 7806 counts at 30 and 50 μM DMDI, respectively, $P>0.05$).

3.5. Effect of DMDI on phospholipase D activation

Phospholipase D catalyses the hydrolysis of phosphatidylcholine to produce phosphatidic acid and choline (Billah et al., 1989). In the presence of ethanol, phosphatidic acid yields phosphatidylethanol via a transphosphatidylation reaction. The addition of 1 μM fMLP to a 1-*O*-[^{3}H]octadecyl-*sn*-glycero-3-phosphocholine-loaded cell suspension in the presence of dihydrocytochalasin B (5 μg/ml) for 0.5 min significantly increased the formation of phosphatidic acid and phosphatidylethanol (about 4.7- and 16.2-fold, respectively). This effect was abolished by 100 μM genistein, a general tyrosine kinase inhibitor (Akiyama et al., 1987) and was attenuated by DMDI in a concentration-dependent manner with an IC_{50} value of 13.3 ± 4.0 and 9.4 ± 4.3 μM, respectively (Fig. 5).

3.6. Effect of DMDI on the phosphorylation of MAP kinase

Stimulation of rat neutrophils with fMLP resulted in a rapid phosphorylation of ER kinase and p38 MAP kinase. The ability of fMLP to stimulate the phosphorylation of MAP kinase was diminished by DMDI in a concentration-dependent manner. Significant inhibition was observed at concentrations of DMDI ≥ 10 μM. However, DMDI inhibited ER kinase phosphorylation to a greater extent (IC_{50} value of 14.3 ± 4.5 μM) than it inhibited the p38 MAP kinase phosphorylation (Fig. 6A and B). The phosphorylation of both ER kinase and p38 MAP kinase was inhibited by 50 μM genistein (data not shown). DMDI (100 μM) alone had no significant effect on the phosphorylation of ER kinase and p38 MAP kinase.

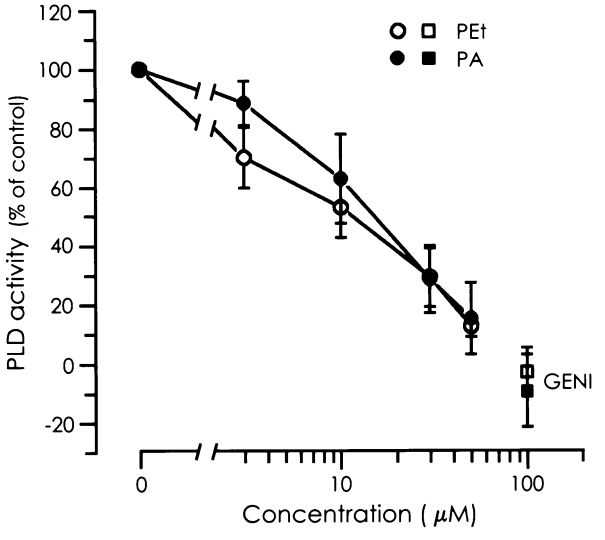

Fig. 5. Effect of DMDI on phospholipase D (PLD) activity. 1-*O*-[^{3}H]-Octadecyl-*sn*-glycero-3-phosphocholine-loaded cells were incubated with DMSO (as control), 3–50 μM DMDI or 100 μM genistein (GENI) in the presence of 0.5% ethanol for 3 min in the presence of dihydrocytochalasin B (5 μg/ml) at 37 °C before the addition of 1 μM fMLP for 0.5 min. Lipids in the reaction mixture were extracted and separated. The radioactivities of phosphatidic acid (PA) and phosphatidylethanol (PEt) were counted. Results were calculated as the % response of the control values (3422.7 ± 456.7 and 2856.6 ± 362.8 counts, respectively). Values are expressed as means ± S.D. of four to six separate experiments.

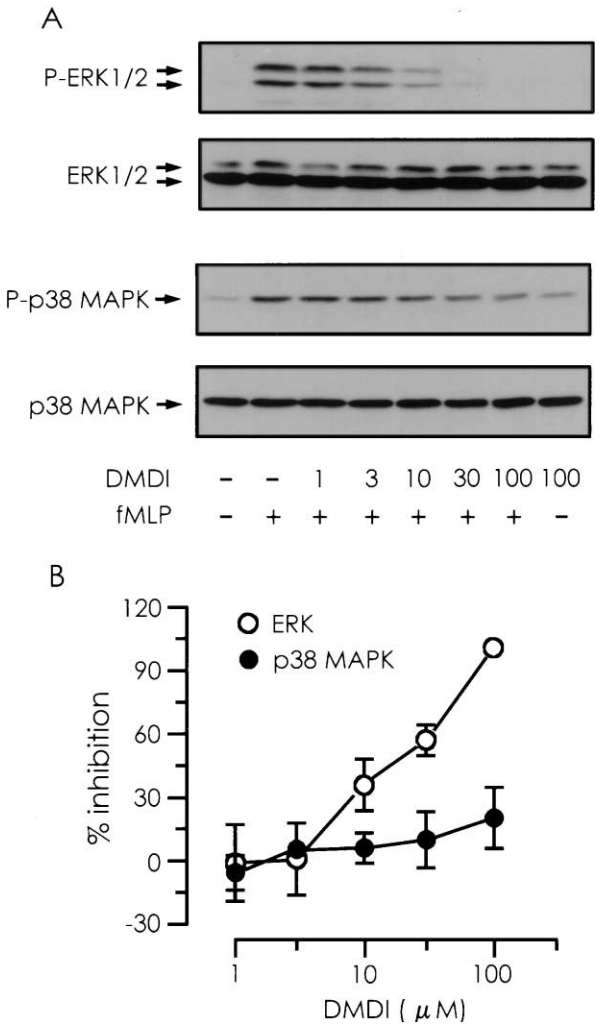

Fig. 6. Effect of DMDI on fMLP-stimulated MAP kinase phosphorylation. Cells were preincubated with DMSO or 1–100 μM DMDI for 3 min in the presence of dihydrocytochalasin B (5 μg/ml) at 37 °C before the addition of 0.1 μM fMLP for 1 min. Phosphorylation of ER kinase and p38 MAP kinase was detected by immunoblot analysis using anti-phospho-p44/42 MAP kinase or anti-phospho-p38 MAP kinase antibodies, respectively. The blots above were then stripped and reprobed with anti-pan ER kinase and anti-p38 MAP kinase antibodies, respectively. (A) represents the results of Western blot analysis and (B) shows means ± S.D. of % inhibition of MAP kinase phosphorylation in three to four independent experiments.

4. Discussion

The results showing that DMDI inhibited fMLP-induced O_2 consumption and $O_2^{\bullet -}$ generation in a parallel fashion,

together with the negligible effect of DMDI on $O_2^{\bullet -}$ generation during dihydroxyfumaric acid autoxidation, preclude that DMDI has $O_2^{\bullet -}$-scavenging activity. The inhibition of the respiratory burst by DMDI was not attributable to a cytotoxic effect because cell viability remained relatively unchanged at effective concentrations of DMDI. It is plausible that the inhibition of the respiratory burst by DMDI is mediated through an interaction with certain signal transduction steps that follow the fMLP receptor activation.

Some isoquinoline compounds have been reported to act as cyclic AMP-elevating agents (Ruppert and Weithmann, 1982; Nourshargh and Hoult, 1986). It is well recognized that the elevation of cellular cyclic AMP levels inhibits fMLP-induced responses. Cyclic AMP acts by cyclic AMP-dependent protein kinase in many cellular systems. However, the role of cyclic AMP in the inhibition of the respiratory burst by DMDI can be eliminated because cellular cyclic AMP levels were not increased by DMDI treatment. This hypothesis is further confirmed by the observation that the inhibition of $O_2^{\bullet -}$ generation by DMDI is not reversed by KT5720, a cyclic AMP-dependent protein kinase inhibitor (Kase et al., 1987) under conditions which significantly reverse the inhibition induced by the adenylyl cyclase activator forskolin.

The fMLP-induced respiratory burst is a Ca^{2+}-dependent process. The $[Ca^{2+}]_i$ elevation by fMLP is composed of a rapid rising phase, supported primarily by the inositol trisphosphate-induced release of Ca^{2+} from intracellular stores, followed by a plateau phase, which is sustained by the Ca^{2+} entry from the extracellular medium (Meldolesi et al., 1991). In the presence of extracellular Ca^{2+}, DMDI reduces the plateau but not the initial rising phase. In addition, the Ca^{2+} spike in the absence of extracellular Ca^{2+} was unaffected by DMDI. These results indicate that DMDI failed to alter the phospholipase C/inositol trisphosphate signaling pathway but attenuated extracellular Ca^{2+} entry. It has been reported that cytochrome *P*450, protein kinase C and tyrosine kinase are responsible for the regulation of Ca^{2+} entry in neutrophils (Montero et al., 1993, 1994). The mechanism of the DMDI-induced inhibition of Ca^{2+} entry awaits further investigation. These inhibitory effects occur at concentrations of DMDI higher than the IC_{50} value for the inhibition of $O_2^{\bullet -}$ generation, which suggests that the inhibition of $[Ca^{2+}]_i$ alone might play a certain but not a prominent role in the inhibition of the fMLP-induced respiratory burst by DMDI.

Neutrophil activation by chemoattractants is associated with the generation of polyphosphorylated phosphoinositides through the stimulation of the PI3-kinase (Stephens et al., 1994). PI3-kinase phosphorylates phosphatidylinositols at the D3 position, thereby converting phosphatidylinositol 4,5-bisphosphate to phosphatidylinositol 3,4,5-trisphosphate, which is a critical component of the signaling pathway leading to NADPH oxidase activation by fMLP (Okada et al., 1994). Neutrophils contain two classes of PI3-kinase: the classical p85/p110α heterodimer and a novel G-protein $\beta\gamma$ subunit-regulated PI3-kinase, p110γ (Stephens et al., 1994). A recent study indicated that the stimulation of neutrophils by fMLP is associated with the activation of p110γ (Naccache et al., 2000). Unlike the PI3-kinase inhibitor wortmannin (Powis et al., 1994), DMDI did not alter fMLP-induced PI3-kinase activation as assessed by counting the radioactivity of the [^{32}P]phosphatidylinositol trisphosphate product, which suggests that PI3-kinase signaling pathways are not involved in the inhibition of the respiratory burst by DMDI.

Two mammalian phospholipase D isoenzymes, phospholipase D1 (phospholipase D1a and phospholipase D1b) and phospholipase D2, have been cloned and the splice variants have been described (Exton, 1998). Phospholipase D1 is under an elaborate control, whereas phospholipase D2 exhibits high basal activity and appears less subject to control (Steed et al., 1998). The phospholipase D1b form is the predominant phospholipase D1 isoform expressed in the rat tissue (Katayama et al., 1998). The phospholipase D signaling pathway regulates neutrophil function through phosphatidic acid, and probably, its metabolite, diradylglycerol, as second messengers. Phosphatidic acid may activate a novel protein kinase that induces $p47^{phox}$ phosphorylation and NADPH oxidase activation (Waite et al., 1997). DMDI inhibited fMLP-induced phospholipase D activation in rat neutrophils in a concentration-dependent manner. This loss of phospholipase D activity was coincident with the inability of fMLP to stimulate $O_2^{\bullet -}$ generation, suggesting a crucial role for the phospholipase D signaling pathway in the inhibition of the respiratory burst by DMDI. In neutrophils, phospholipase D activation by fMLP has been shown to be Ca^{2+}- and tyrosine kinase-dependent but independent of protein kinase C (Planat et al., 1996). It has been reported that phospholipase D1 is stimulated in vitro in a direct and synergistic manner by protein kinase C-α, ADP-ribosylation factor and Rho family members (Zhang et al., 1999) and that the translocation to the membrane of protein kinase C, ADP-ribosylation factor and RhoA is required for phospholipase D activity in vivo (Fensome et al., 1998). Whether or not DMDI inhibits tyrosine kinase activity and/or the membrane translocation of phospholipase D-activating factors in rat neutrophils stimulated with fMLP needs further investigation.

Three distinct mammalian MAP kinases have been identified, including ER kinase, p38 MAP kinase and *c-Jun* N-terminal kinase, each having different physiological roles. The upstream signaling cascade that phosphorylates and activities MAP kinase via phosphorylation of both tyrosine and threonine residues (Derijard et al., 1995). It has been reported that both ER kinase and p38 MAP kinase phosphorylate $p47^{phox}$ in vitro (El Benna et al., 1996). Our previous reports have demonstrated that exposure of rat neutrophils to fMLP rapidly induces the phosphorylation of ER kinase and p38 MAP kinase (Chang and Wang, 1999, 2000). The present study demonstrates that the phosphor-

ylation of ER kinase was inhibited by DMDI with a similar IC_{50} value to that for the inhibition of the respiratory burst.

In conclusion, the DMDI concentration dependently inhibits fMLP-induced $O_2^{\bullet -}$ generation and O_2 consumption in rat neutrophils. These effects are not attributed to the scavenging of generated $O_2^{\bullet -}$, elevation of cellular cyclic AMP or suppression of PI3-kinase activity but are attributed to the blockade of phospholipase D and MAP kinase activation.

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