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DSM-RX78, a new phosphodiesterase inhibitor, suppresses superoxide anion production in activated human neutrophils and attenuates hemorrhagic shock-induced lung injury in rats

Huang-Ping Yu [a,b], Pei-Wen Hsieh [c], Yi-Ju Chang [c], Pei-Jen Chung [c], Liang-Mou Kuo [d], Tsong-Long Hwang [c,*]

[a] Department of Anesthesiology, Chang Gung Memorial Hospital, Taoyuan 333, Taiwan
[b] College of Medicine, Chang Gung University, Taoyuan 333, Taiwan
[c] Graduate Institute of Natural Products, Chang Gung University, Taoyuan 333, Taiwan
[d] Department of General Surgery, Chang Gung Memorial Hospital at Chia-Yi, Taiwan

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ABSTRACT

Neutrophils are activated following hemorrhagic shock and the accumulation of neutrophils in the lung is associated with lung injury. This research investigated the effects of a semisynthetic 2-benzoylaminobenzoic acid derivative, methyl 2-(2-fluorobenzamido)benzoate (DSM-RX78), on superoxide anion ($O_2^{\bullet-}$) production in formyl-L-methionyl-L-leucyl-L-phenylalanine (FMLP)-activated human neutrophils, and on lung injury in Sprague–Dawley rats subjected to trauma-hemorrhage. DSM-RX78 concentration-dependently inhibited $O_2^{\bullet-}$ production, but not elastase release, in FMLP-activated human neutrophils. DSM-RX78 displayed no superoxide-scavenging ability, and it failed to alter the subcellular NADPH oxidase activity. Significantly, DSM-RX78 increased cAMP formation and protein kinase (PK)A activity in FMLP-activated neutrophils, which occurred through the selective inhibition of cAMP-specific phosphodiesterase (PDE) activity but not an increase in adenylate cyclase function or cGMP-specific PDE activity. These results show that DSM-RX78 is a new inhibitor of cAMP-specific PDE. Moreover, DSM-RX78 reduced FMLP-induced phosphorylation of protein kinase B (Akt), but not calcium mobilization. The inhibitory effects of DSM-RX78 on $O_2^{\bullet-}$ production and Akt phosphorylation were reversed by PKA inhibitors, suggesting that DSM-RX78 regulates $O_2^{\bullet-}$ production of human neutrophils by promoting cAMP/PKA-dependent inhibition of Akt activation. On the other hand, administration of DSM-RX78 significantly attenuated the increase in myeloperoxidase activity and edema in the lung, as well as protein concentrations in bronchoalveolar lavage fluid in rats after trauma-hemorrhagic shock. In summary, these results strongly suggest that DSM-RX78 exerts anti-inflammatory effects, which result from the elevation of cAMP levels and PKA activity through its inhibition of cAMP-specific PDE. Also, our findings show that DSM-RX78 attenuates hemorrhagic shock-induced lung injury in rats.

1. Introduction

Neutrophils play a pivotal role in the defense of the human body against infections. Conversely, there is increasing evidence that overwhelming activation of neutrophils may be a major contributor to tissue damage in inflammatory diseases, such as sepsis [1,2], chronic obstructive pulmonary disease (COPD) [3], acute respiratory distress syndrome (ARDS) [4], and other inflammatory processes [5]. In response to diverse stimuli, activated neutrophils secrete a series of cytotoxins, such as the superoxide anion ($O_2^{\bullet-}$), a precursor of other ROS, granule proteases, and bioactive lipids [6,7]. Therefore, it is crucial to restrain neutrophil functions, such as oxidative burst and degranulation, in physiological conditions

Abbreviations: AC, adenylyl cyclase; Akt, protein kinase B; ARDS, acute respiratory distress syndrome; cAMP, cyclic adenosine 3′,5′-monophosphate; CB, cytochalasin B; COPD, chronic obstructive pulmonary disease; FMLP, formyl-L-methionyl-L-leucyl-L-phenylalanine; GPCR, G protein-coupled receptor; H89, *N*-(2-((*p*-bromocinnamyl)amino)ethyl)-5-isoquinolinesulfonamide; IBMX, 3-isobutyl-1-methylxanthine; KT5720, 9S,10S,12R-2,3,9,10,11,12-hexahydro-10-hydroxy-9-methyl-1-oxo-9,12-epoxy-1H-diindolo(1,2,3-fg:3′,2′,1′-kl)pyrrolo(3,4-i)(1,6)benzodiazocine-10-carboxylic acid hexyl ester; MPO, myeloperoxidase; $O_2^{\bullet-}$, superoxide anion; PDE, phosphodiesterase; PKA, protein kinase A; PKC, protein kinase C; LDH, lactate dehydrogenase; PMA, phorbol myristate acetate; Ro318220, 3-(1-(3-(amidinothio)propyl-1H-indol-3-yl))-3-(1-methyl-1H-indol-3-yl)maleimide; SOD, superoxide dismutase; SPA, scintillation proximity assay; WST-1, 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt.

* Corresponding author at: Graduate Institute of Natural Products, College of Medicine, Chang Gung University, 259 Wen-Hwa 1st Road, Kweishan, Taoyuan 333, Taiwan. Tel.: +886 3 2118506; fax: +886 3 2118506.
E-mail address: htl@mail.cgu.edu.tw (T.-L. Hwang).

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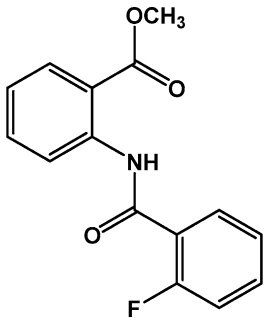

Fig. 1. Chemical structure of methyl 2-(2-fluorobenzamido)benzoate (DSM-RX78).

while potentiating these functions in inflammatory tissues and organs.

Hemorrhagic shock results in excessive production of proinflammatory mediators, which plays a significant role in the development of multiple organ dysfunctions [8,9]. Studies have shown that neutrophils are activated following hemorrhagic shock [10,11], and the subsequent accumulation of neutrophils in the lung is associated with lung injury [8,12]. Furthermore, degranulation of proteolytic enzymes and generation of respiratory burst are recognized as critical neutrophil functions leading to resuscitation-induced lung injury [13,14]. Consequently, regulation of the activity of blood neutrophils is important in controlling lung damage caused by hemorrhagic shock [12,15]. cAMP is an important second messenger with a variety of physiological and pathophysiological manifestations. Elevation of intracellular cAMP levels is believed to suppress the activation of neutrophils [16–18]. Phosphodiesterases (PDEs) are important in regulating intracellular concentrations of cAMP [16,19,20]. Thus, specific PDE inhibitors are currently being investigated as anti-inflammatory drugs because of their suppressive effects on neutrophil functions. The clinical potential of cAMP-elevating agents as inhibitors of neutrophil activities is supported by the suppression of endotoxin-induced acute lung injury in mice by the PDE4 inhibitor, rolipram [21].

In this study, a cellular model in isolated human neutrophils was established to elucidate the anti-inflammatory functions of methyl 2-(2-fluorobenzamido)benzoate (DSM-RX78) (Fig. 1), a semisynthetic 2-benzoylaminobenzoic acid derivative [22]. Based on the screening results, DSM-RX78 showed a potent and selective inhibitory effect on the generation of $O_2^{\bullet-}$ in FMLP-activated human neutrophils. Stimulation of neutrophils leads to increases in their oxygen consumption through the activity of NADPH oxidase which generates $O_2^{\bullet-}$. This phenomenon is the so-called respiratory burst [23]. The mechanisms of action of DSM-RX78 were further investigated in human neutrophils. Our data suggest that DSM-RX78 regulates $O_2^{\bullet-}$ production of human neutrophils by promoting cAMP/protein kinase A (PKA)-dependent inhibition of phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt) activation. Significantly, administration of DSM-RX78 attenuated lung injury in a rodent model of trauma-hemorrhagic shock.

2. Materials and methods

2.1. Reagents

N-(2-((*p*-Bromocinnamyl)amino)ethyl)-5-isoquinolinesulfonamide (H89), 9S,10S,12R-2,3,9,10,11,12-hexahydro-10-hydroxy-9-methyl-1-oxo-9,12-epoxy-1H-diindolo(1,2,3-fg:3′,2′,1′-kl)pyrrolo(3,4-i)(1,6)benzodiazocine-10-carboxylic acid hexyl ester (KT5720), leupeptin, phenylmethylsulfonyl fluoride (PMSF), 3-(1-(3-(amidinothio)propyl-1H-indol-3-yl))-3-(1-methyl-1H-indol-3-yl)maleimide (Ro318220), and rolipram were obtained from Calbiochem (La Jolla, CA). Fluo-3 AM was purchased from Molecular Probes (Eugene, OR). 2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium monosodium salt (WST-1) was purchased from Dojindo Laboratories (Kumamoto, Japan). All other chemicals were obtained from Sigma (St. Louis, MO). When drugs were dissolved in DMSO, the final concentration of DMSO in the cell experiments did not exceed 0.4% and did not affect the parameters measured.

2.2. DSM-RX78

DSM-RX78 was synthesized by our group [22]. The structure of DSM-RX78 was determined by mass and nuclear magnetic resonance (NMR) spectroscopic methods (Fig. 1). DSM-RX78 was a white powder, dissolvable in methanol, with the maximum absorption wavelength locating at about 245 (log ε = 4.41), 263 (sh, log ε = 4.30), and 310 (log ε = 4.02) nm. ^{1}H NMR (400 MHz, $CDCl_3$): δ 11.84 (1H, br.d, J = 7.2 Hz, NH), 8.89 (1H, dd, J = 8.4, 1.2 Hz, Ar–H), 8.07 (2H, m, Ar–H), 7.59 (1H, dd, J = 7.2, 1.2 Hz, Ar–H), 7.51 (1H, m, Ar–H), 7.28 (1H, dd, J = 7.2, 1.2 Hz, Ar–H), 7.20 (1H, m, Ar–H), 7.13 (1H, dd, J = 8.4, 1.2 Hz, Ar–H) and 3.94 (3H, s, COOMe). ^{13}C NMR ($CDCl_3$) δ 168.3 (s, $\underline{C}OOCH_3$), 162.3 (s, $\underline{C}ONH$, J_{C-F} = 3.1 Hz), 160.2 (s, C-2′, J_{C-F} = 249.3 Hz), 141.0 (s, C-2), 134.4 (d, C-4), 133.5 (d, C-4′, J_{C-F} = 8.3 Hz), 130.9 (d, C-6), 131.7 (d, C-6′, J_{C-F} = 2.9 Hz), 124.7 (d, C-5', J_{C-F} = 3.8 Hz), 123.0 (d, C-5), 122.38 (s, C-1′, J_{C-F} = 12.1 Hz), 121.3 (d, C-3), 116.4 (d, C-3′, J_{C-F} = 23.4 Hz), 116.0 (s, C-1) and 52.4 (s, OCH_3). EI-MS m/z: 123 (100), 214 (52) and 273 $[M]^+$. The purity was >97%.

2.3. Preparation of human neutrophils

Blood was taken from healthy human donors (20–32 years old) by venipuncture, using a protocol approved by the Institutional Review Board at Chang Gung Memorial Hospital. Neutrophils were isolated with a standard method of dextran sedimentation prior to centrifugation in a Ficoll Hypaque gradient and the hypotonic lysis of erythrocytes. Purified neutrophils that contained >98% viable cells, as determined by the trypan blue exclusion method, were resuspended in Ca^{2+}-free HBSS buffer at pH 7.4 and maintained at 4 °C before use.

2.4. Neutrophil fractionation

Neutrophils were pretreated with 1 mM PMSF for 30 min at 4 °C, and disrupted in relaxation buffer (100 mM KCl, 3 mM NaCl, 3.5 mM $MgCl_2$, 1 mM ATP, 1 mM EGTA, and 10 mM PIPES; pH 7.3) by sonication. Unbroken cells were removed by centrifugation at 300 × g for 5 min, and the supernatant was then centrifuged at 100,000 × g for 20 min at 4 °C to produce the cytosolic and plasma membrane fractions.

2.5. Measurement of $O_2^{\bullet-}$ generation

The assay of the generation of $O_2^{\bullet-}$ was based on the superoxide dismutase (SOD)-inhibitable reduction of ferricytochrome *c* [24]. In brief, after supplementation with 0.5 mg/ml ferricytochrome *c* and 1 mM Ca^{2+}, neutrophils (6×10^5 cells/ml) were equilibrated at 37 °C for 2 min and incubated with drugs for 5 min. Cells were activated with 100 nM FMLP or 5 nM phorbol myristate acetate (PMA). When FMLP was used as a stimulant, 1 μg/ml cytochalasin B (FMLP/CB) was incubated for 3 min before peptide activation. $O_2^{\bullet-}$ generation by isolated neutrophil fractionation was measured after the addition of 160 μM NADPH to 800 μl of relaxation buffer containing 4×10^6 cell equivalents of membrane extract, 1.2×10^7 cell equivalents of cytosol, 2 μM GTP-γ-S, 0.5 mg/ml ferricytochrome *c*, and 100 μM sodium dodecylsulfate (SDS). To facilitate

the assembly of NADPH oxidase components, all constituents (excluding NADPH) were incubated at 37 °C for 3 min before the addition of NADPH. Drugs were incubated for 2 min before NADPH oxidase assembly. Changes in absorbance with the reduction of ferricytochrome *c* at 550 nm were continuously monitored with a double-beam, six-cell positioner spectrophotometer with constant stirring (Hitachi U-3010, Tokyo, Japan).

2.6. Measurement of elastase release

Degranulation of azurophilic granules was determined by elastase release. Experiments were performed using MeO-Suc-Ala-Ala-Pro-Val-p-nitroanilide as the elastase substrate. Briefly, after supplementation with MeO-Suc-Ala-Ala-Pro-Val-p-nitroanilide (100 μM), neutrophils (6×10^5/ml) were equilibrated at 37 °C for 2 min and incubated with drugs for 5 min. Cells were activated by FMLP (100 nM) in the presence of CB (0.5 μg/ml), and changes in absorbance at 405 nm were continuously monitored for 15 min to determine elastase release. The results are expressed as a percentage of elastase release in the FMLP/CB-activated, drug-free control system.

2.7. Lactate dehydrogenase (LDH) release

LDH release was determined by a commercially available method (Promega, Madison, WI). Cytotoxicity was represented by LDH release in a cell-free medium as a percentage of the total LDH released. The total LDH released was determined by lysing cells with 0.1% Triton X-100 for 30 min at 37 °C.

2.8. $O_2^{\bullet-}$-scavenging activity

The $O_2^{\bullet-}$-scavenging ability of DSM-RX78 was determined using xanthine/xanthine oxidase in a cell-free system, based on a previously described method [25]. After 0.1 mM xanthine was added to the assay buffer (50 mM Tris, pH 7.4, 0.3 mM WST-1, and 0.02 U/ml xanthine oxidase) for 15 min at 30 °C, the absorbance associated with the $O_2^{\bullet-}$-induced WST-1 reduction was measured at 450 nm.

2.9. Determination of cAMP concentrations

cAMP levels were assayed using an enzyme immunoassay kit (Amersham Biosciences, Buckinghamshire, UK). Human neutrophils were incubated with drugs for the indicated time before stimulation with or without FMLP for another 1 min, and the reaction was terminated by adding 0.5% dodecytrimethylammonium bromide. Samples were then centrifuged at $3000 \times g$ for 5 min at 4 °C. The supernatants were used as a source for the cAMP samples. The assay was performed according to the manufacturer's instructions.

2.10. Assay of PKA activity

PKA activity in neutrophils was determined using a non-radioactive protein kinase assay kit (Biosourse, Camarillo, CA). Cells (2×10^7/ml) were incubated with drugs for 5 min at 37 °C. The stimulant, FMLP, was then added, and the reaction mixture was incubated for 1 min. The reaction was stopped on ice, and cells were centrifuged at 4 °C. After removing the supernatants, the pellets were lysed in Omnia cell extraction buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 2 mM EGTA, 30 mM NaF, 10 mM $Na_4O_7P_2$, 0.1 mM Na_3VO_4, 1 mM dithiothreitol, 1% Triton X-100, and 50 mM β-glycerophosphate), then briefly sonicated, and centrifuged at $14{,}000 \times g$ for 30 min at 4 °C. Supernatants were assayed for PKA activity according to the manufacturer's instructions.

2.11. Assay of adenylyl cyclase (AC) and PDE activities

Neutrophils (5×10^7 cells/ml) were sonicated in ice-cold buffer, containing 25 mM Tris–HCl (pH 7.5), 0.25 M sucrose, 2 mM EDTA, 5 mM $MgCl_2$, 10 μM leupeptin, 100 μM PMSF, and 10 μM pepstatin. Unbroken cells were removed by centrifugation at $300 \times g$ for 5 min, and the supernatant was used as a source for the PDE enzyme. For assaying of AC activity, the supernatant was subsequently centrifuged at $100{,}000 \times g$ for 40 min at 4 °C, and the pellet was used as source for the AC enzyme. The reaction mixture (25 mM Tris, pH 7.5, 15 mM $MgCl_2$, 1 mM 3-isobutyl-1-methylxanthine (IBMX), 7.5 mM creatine phosphate, and 3 units creatine phosphokinase) contained 0.5 mM dithiothreitol, 1 mM ATP, and the pellet fraction for assessing AC activity. The reaction was carried out for 20 min at 30 °C and was terminated by boiling for 3 min. cAMP contents were assayed using enzyme immunoassay kits.

PDE activity was analyzed using a tritium scintillation proximity assay (SPA) system, and the assay was performed according to the manufacturer's instructions (Amersham Biosciences). Briefly, assays were performed at 30 °C for 10 min in the presence of 50 mM Tris–HCl (pH 7.5) containing 8.3 mM $MgCl_2$, 1.7 mM EGTA, and 0.3 mg/ml bovine serum albumin. Each assay was performed in a 100-μl reaction volume containing the above buffer, the neutrophil supernatant fraction, and around 0.05 μCi [^{3}H]cAMP. The reaction was terminated by the addition of 50 μl PDE SPA beads (1 mg) suspended in 18 mM zinc sulfate. Assays were performed in 96-well microtiter plates. The reaction mixture was allowed to settle for 1 h before counting in a microtiter plate counter.

2.12. Measurement of $[Ca^{2+}]_i$

Neutrophils were loaded with 2 μM fluo-3 AM at 37 °C for 45 min. After being washed, cells were resuspended in Ca^{2+}-free HBSS to 3×10^6 cells/ml. The change in fluorescence was monitored using a Hitachi F-4500 spectrofluorometer (Tokyo, Japan) in a quartz cuvette with a thermostat (37 °C), while being continuously stirred. The excitation wavelength was 488 nm, and the emission wavelength was 520 nm.

2.13. Immunoblotting analysis of whole-cell lysates

Neutrophils were incubated with drugs for 5 min at 37 °C before being stimulated with FMLP for another 1 min. The reaction was stopped on ice, and cells were centrifuged at 4 °C. After removing the supernatants, the pellets were lysed in 150 μl buffer (20 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1 mM EGTA, 2 mM Na_3VO_4, 1 mM NaF, 1 mM PMSF, 1% dilution of Sigma protease inhibitor cocktails, and 1% Triton X-100). Samples were centrifuged at $14{,}000 \times g$ for 20 min at 4 °C to yield whole-cell lysates. Proteins derived from whole-cell lysates were separated by SDS polyacrylamide gel electrophoresis (PAGE) using 12% polyacrylamide gels and blotted onto nitrocellulose membranes. Immunoblotting was performed using the indicated antibodies and horseradish peroxidase-conjugated secondary anti-rabbit antibodies (Cell Signaling Technology, Beverly, MA). The immunoreactive bands were visualized by an enhanced chemiluminescence system (Amersham Biosciences).

2.14. Trauma-hemorrhagic shock procedure

A rat model of trauma-hemorrhagic shock was used in this study [26]. Thirty-two male Sprague–Dawley rats (275–325 g) obtained from the National Science Council, Taipei, Taiwan were divided into 4 groups of 8 animals each, according to a table of random numbers. They were housed in an air-conditioned room under a reversed light–dark cycle and allowed at least 1 week to

adapt to the environment. Before initiation of the experiment, they were starved overnight but were allowed water *ad libitum*. The rats were anesthetized by isoflurane (Attane, Minrad, Bethlehem, PA) inhalation prior to performing a 5-cm midline laparotomy in the abdomen. The abdomen was closed in layers, and catheters were placed in both femoral arteries and the right femoral vein (polyethylene [PE-50] tubing; Becton Dickinson, Sparks, MD). The wounds were bathed with 1% lidocaine (Elkins-Sinn, Cherry Hill, NJ) throughout the surgical procedure to reduce post-operative pain. Rats were then allowed to awaken and were bled, and the mean blood pressure was maintained at 40 mmHg. This level of hypotension persisted until the animals could not maintain a mean blood pressure of 40 mmHg unless additional fluid in the form of Ringer's lactate was administered. This time was defined as the maximum bleed-out, and the amount of withdrawn blood was noted. Following this, the rats were maintained at a mean blood pressure of 40 mmHg until 40% of the maximum bleed-out volume was returned in the form of Ringer's lactate. The animals were then resuscitated with four times the volume of the shed blood over 60 min with Ringer's lactate. The time required for maximum bleed-out was ~45 min, the volume of the maximum bleed-out was ~60% of the calculated circulating blood volume [27], and the total hemorrhage time was ~90 min. Thirty minutes before the end of the resuscitation period, the rats received DSM-RX78 (1 mg/kg of body weight, intravenously), or an equal volume of the vehicle (~0.2 ml, 10% DMSO, Sigma). The catheters were then removed, the vessels ligated, and the skin incisions closed with sutures. Sham-operated animals underwent the surgical procedure, which included a laparotomy in addition to ligation of the femoral artery and vein, but neither hemorrhage nor resuscitation was carried out. Vehicle or DSM-RX78 was also administered to sham-operated rats after the catheters were put in place. The animals were then returned to their cages and allowed food and water *ad libitum*. The animals were sacrificed 24 h after the end of resuscitation. The current study was approved by the Institutional Animal Care and Use Committee of Chang Gung Memorial Hospital. All animal experiments were performed according to the guidelines of the *Animal Welfare Act* and the *Guide for Care and Use of Laboratory Animals* from the National Institutes of Health, Taiwan.

2.15. Preparation of lung tissue and collection of bronchoalveolar lavage fluid

Twenty-four hours after completing fluid resuscitation or the sham operation, the animals were anesthetized with isoflurane and sacrificed. The chest was opened and the left side of the lung was obtained after clamping the hilum. Excess blood was blotted dry, and the left upper lobe of the lung was measured for tissue water content. Other tissue sections were stored at −80 °C until analyzed. The trachea was then cannulated, and bronchoalveolar lavage fluid was obtained by washing the airways four times with 5 ml of phosphate-buffered saline. The bronchoalveolar lavage fluid was centrifuged at 1200 × *g* for 10 min at 4 °C. The supernatant was collected and stored at −80 °C until analyzed.

2.16. Protein assay in lung lavage

Cell-free bronchoalveolar lavage fluid was evaluated for total protein content (Bio-Rad D_C Protein Assay, Bio-Rad, Hercules, CA).

2.17. Water content assay

In a separate cohort, the left upper lobe of the lung was weighed, and dried for 24 h at 80 °C. The water content of lung tissue was calculated as a wet/dry weight ratio [8].

2.18. Measurement of myeloperoxidase (MPO) activity

MPO activity in whole-lung homogenates was determined as described previously [8,10]. Briefly, equal weights (100 mg wet weight) of lung from various groups were suspended in 1 ml buffer (0.5% hexadecyltrimethylammonium bromide in a 50-mM phosphate buffer; pH 6.0) and sonicated at 30 cycles, twice, for 30 s on ice. Homogenates were cleared by centrifuging at 2000 × *g* and 4 °C, and the supernatants were stored at −80 °C. Protein contents of samples were determined using the Bio-Rad (Hercules, CA) assay kit. Samples were incubated with the substrate, o-dianisidine hydrochloride. This reaction was carried out in a 96-well plate by adding 290 μl of 50 mM phosphate buffer, 3 μl substrate solution (containing 20 g/l o-dianisidine hydrochloride), and 3 μl H_2O_2 (20 mM). The sample (10 μl) was added to each well to start the reaction. Standard MPO (Sigma, St. Louis, MO) was used in parallel to determine the MPO activity in the sample. The reaction was stopped by adding 3 μl sodium azide (30%). The light absorbance at 460 nm was read. MPO activity was determined using a curve obtained from the MPO standard.

2.19. Statistical analysis

Results are expressed as the mean ± S.E.M. Data were analysed using the GraphPad Prism software (GraphPad Software, San Diego, CA). Statistical analysis was performed using Student's *t*-test or one-way analysis of variance (ANOVA) followed by Tukey's multiple-comparison test. A value of $p < 0.05$ was considered statistically significant.

3. Results

3.1. Effects of DSM-RX78 on $O_2^{\bullet-}$ generation and elastase release

Neutrophil respiratory burst and degranulation are important in many inflammatory disorders. The influence of DSM-RX78 on $O_2^{\bullet-}$ generation and elastase release in FMLP/CB-activated human neutrophils was assayed. DSM-RX78 (0.1–10 μM) did not alter basal $O_2^{\bullet-}$ generation under resting conditions, whereas it significantly inhibited $O_2^{\bullet-}$ release in FMLP/CB-treated human neutrophils in a concentration-dependent manner with an IC_{50} value of 0.64 ± 0.10 μM (Fig. 2A). However, PMA (5 nM)-activated $O_2^{\bullet-}$ release by neutrophils was inhibited by Ro318220 (0.1 μM), a non-selective inhibitor of protein kinase C (PKC), but not by DSM-RX78 (Fig. 2C). In addition, DSM-RX78 (0.1, 1, and 10 μM) caused only a slight inhibition of elastase release in FMLP/CB-treated human neutrophils (Fig. 2B). These data suggest that FMLP/CB-mediated elastase release is less sensitive to inhibition by DSM-RX78 than FMLP/CB-induced $O_2^{\bullet-}$ generation. Culturing with DSM-RX78 (up to 30 μM) did not affect cell viability, as assayed by LDH release (data not shown).

To investigate the ability of DSM-RX78 to scavenge $O_2^{\bullet-}$, the effects of DSM-RX78 in a cell-free xanthine/xanthine oxidase system were assayed. DSM-RX78, at concentrations of up to 10 μM, failed to alter WST-1 reduction. SOD was used as the positive control in the xanthine/xanthine oxidase system. Furthermore, DSM-RX78 (10 μM) did not affect the removal of $O_2^{\bullet-}$ by SOD (0.2 U/ml) (Fig. 3A). These data rule out the possibility that the inhibitory effect of DSM-RX78 on $O_2^{\bullet-}$ release occurs through the scavenging of $O_2^{\bullet-}$. To examine whether NADPH oxidase is sensitive to inhibition by DSM-RX78, neutrophil membranes were isolated to assay $O_2^{\bullet-}$ production in a reconstituted system after the addition of NADPH. As shown in Fig. 3B, DSM-RX78 (0.1, 1, and 10 μM) failed to alter the $O_2^{\bullet-}$ generation by SDS-activated NADPH oxidase. Diphenyleneiodonium (10 μM), a well-known NADPH oxidase inhibitor, signifi-

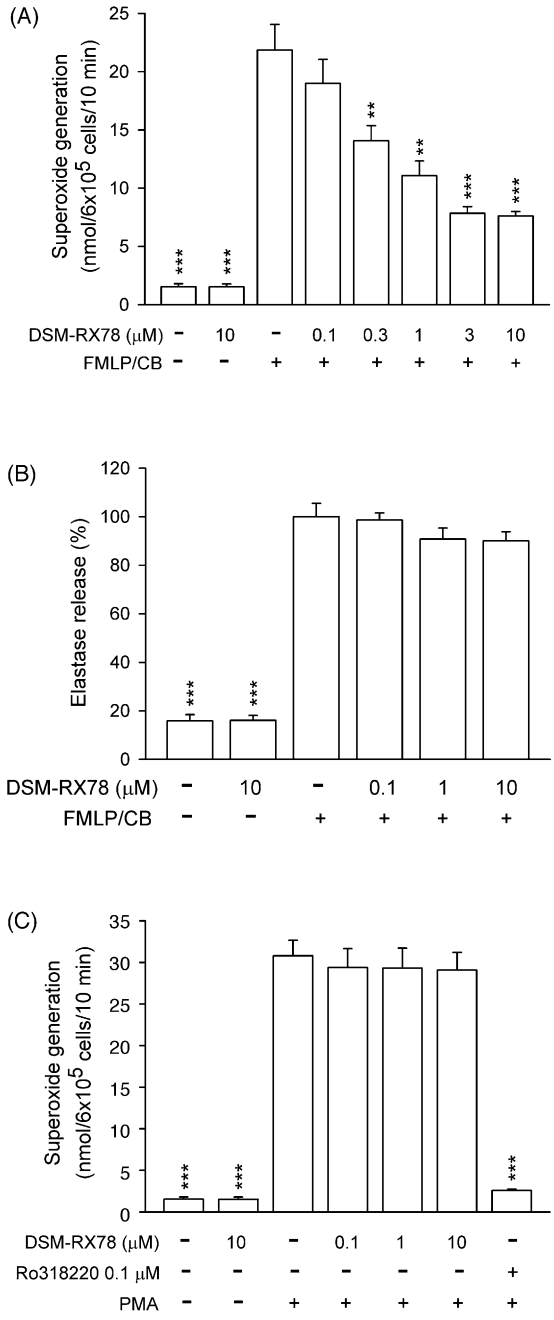

Fig. 2. Effects of DSM-RX78 on $O_2^{\bullet-}$ generation and elastase release in human neutrophils in response to FMLP/CB or PMA. Human neutrophils were incubated with DMSO (control) or DSM-RX78 (0.1–10 μM) for 5 min and then activated by FMLP/CB or PMA. $O_2^{\bullet-}$ generation (A) and elastase release (B) were induced by FMLP/CB. The control value for elastase release was 0.58 ± 0.03 absorbance units/6×10^5 cells/10 min. C, $O_2^{\bullet-}$ generation was induced by PMA. $O_2^{\bullet-}$ generation and elastase release were, respectively, measured using SOD-inhibitable cytochrome *c* reduction and by monitoring *p*-nitroanilide release, as described in Section 2. All data are expressed as the mean ± S.E.M. ($n = 4$ or 7). $^{**}p < 0.01$; $^{***}p < 0.001$ compared to the control.

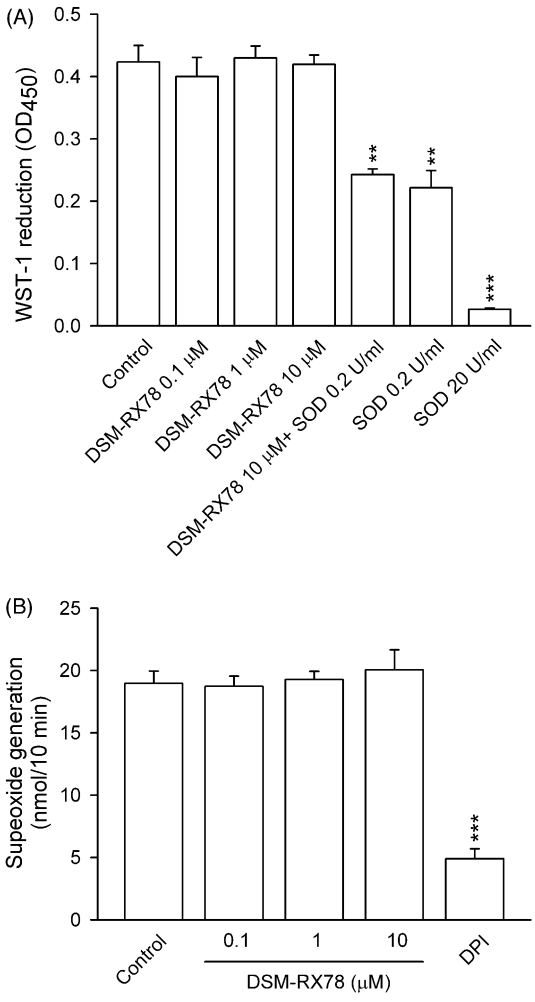

Fig. 3. Effects of DSM-RX78 on $O_2^{\bullet-}$ generation in a cell-free xanthine/xanthine oxidase system and in isolated neutrophil membranes. (A) Reduction of WST-1 was measured spectrophotometrically at 450 in the presence of DSM-RX78 with or without SOD, as described in Section 2. (B) A reactive mixture of the neutrophil cytosolic fraction and membrane fraction was preincubated with DMSO, DSM-RX78 (0.1, 1, and 10 μM), or diphenyleneiodonium (DPI, 10 μM) at 37 °C for 2 min before the addition of SDS (100 μM). The reaction was initiated by adding 160 μM NADPH. $O_2^{\bullet-}$ generation was measured using SOD-inhibitable cytochrome *c* reduction. All data are expressed as the mean ± S.E.M. ($n = 3$). $^{**}p < 0.01$; $^{***}p < 0.001$ compared to the control.

cantly inhibited $O_2^{\bullet-}$ release. These data indicate that DSM-RX78 does not inhibit $O_2^{\bullet-}$ release through the direct inhibition of NADPH oxidase activity.

3.2. Effect of DSM-RX78 on the cAMP pathway

To examine whether the cAMP/PKA pathway is involved in the inhibitory effects of DSM-RX78, the concentrations of cAMP and activities of PKA, AC, and PDE were assayed. DSM-RX78 (1, 3, and 10 μM) caused a slight increase in the cAMP concentration in a concentration-dependent fashion. Notably, DSM-RX78 caused a synergistic increase in FMLP-induced cAMP levels in human neutrophils (Fig. 4A). Furthermore, DSM-RX78 (1, 3, and 10 μM) concentration-dependently increased PKA activity in FMLP-activated human neutrophils (Fig. 4B).

Cellular cAMP concentrations are modulated either by synthesis via AC or by degradation via PDEs. Our data showed that forskolin (30 μM), but not DSM-RX78 (1, 3, and 10 μM), increased the activity of AC (Fig. 5A). On the other hand, DSM-RX78 (1, 3, and 10 μM) inhibited cAMP-specific PDE in a concentration-dependent manner (Fig. 5B). Particularly, DSM-RX78 (1, 3, and 10 μM) was more effective at inhibiting cAMP-specific PDE than cGMP-specific PDE (Fig. 5B and C). Rolipram (1, 3, and 10 μM), a PDE4 inhibitor, and zaprinast (0.1, 1, and 10 μM), a PDE5 inhibitor, were used as positive controls for inhibiting cAMP-specific PDE and cGMP-specific PDE, respectively. IBMX (300 μM), a non-selective PDE inhibitor, almost completely inhibited cAMP- and cGMP-specific PDEs. Moreover, the combination of DSM-RX78 and rolipram did not further inhibit cAMP-specific PDE (Fig. 5B).

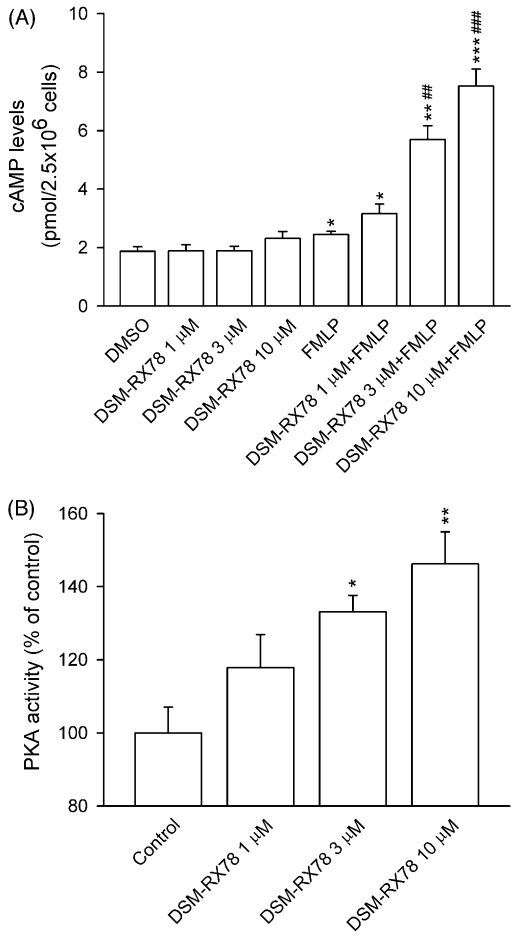

Fig. 4. Effects of DSM-RX78 on cAMP levels and PKA activities. (A) Human neutrophils were incubated with DMSO (control) or DSM-RX78 (1, 3, and 10 μM) for 5 min before stimulation with or without FMLP (0.1 μM) for another 1 min. (B) Cells were incubated with DMSO (control) or DSM-RX78 (1, 3, and 10 μM) in the presence of FMLP (0.1 μM). cAMP levels and PKA activities were measured by enzyme immunoassay kits, as described in Section 2. The control value for PKA activity was 176.00 ± 12.40 RFU/3.3 × 10^5 cells. All data are expressed as the mean ± S.E.M. (n = 3 or 4). $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ compared to the control. $^{\#\#}p < 0.01$; $^{\#\#\#}p < 0.001$ compared to FMLP.

3.3. Effect of DSM-RX78 on FMLP-induced $[Ca^{2+}]_i$ mobilization

FMLP, via a G protein-coupled receptor (GPCR), mobilizes rapid Ca^{2+} release from inositol 1,4,5-triphosphate-sensitive endoplasmic reticulum Ca^{2+} stores. Such Ca^{2+} release depletes endoplasmic reticulum Ca^{2+} stores and subsequently activates extracellular Ca^{2+} influx across the plasma membrane [28]. The magnitude and duration of $[Ca^{2+}]_i$ signal responses to G protein-coupled chemoattractants are obviously important. As shown in Fig. 6, DSM-RX78 (3 and 10 μM) failed to change the $[Ca^{2+}]_i$ mobilization of human neutrophils caused by FMLP.

3.4. PKA mediates the inhibition of FMLP/CB-stimulated $O_2^{\bullet-}$ release by DSM-RX78

To verify whether the inhibitory effects of DSM-RX78 are indeed mediated by cAMP/PKA, pharmacological agents were used to elucidate the mechanisms. The PKA inhibitors, H89 (3 μM) and KT5720 (1 μM), reduced the inhibition of FMLP/CB-stimulated $O_2^{\bullet-}$ formation by DSM-RX78 (1, 3 and 10 μM), PGE_1 (1 μM), and rolipram (1 μM) (Fig. 7). These results suggest that PKA mediates the inhibition of FMLP/CB-activated $O_2^{\bullet-}$ production caused by DSM-RX78.

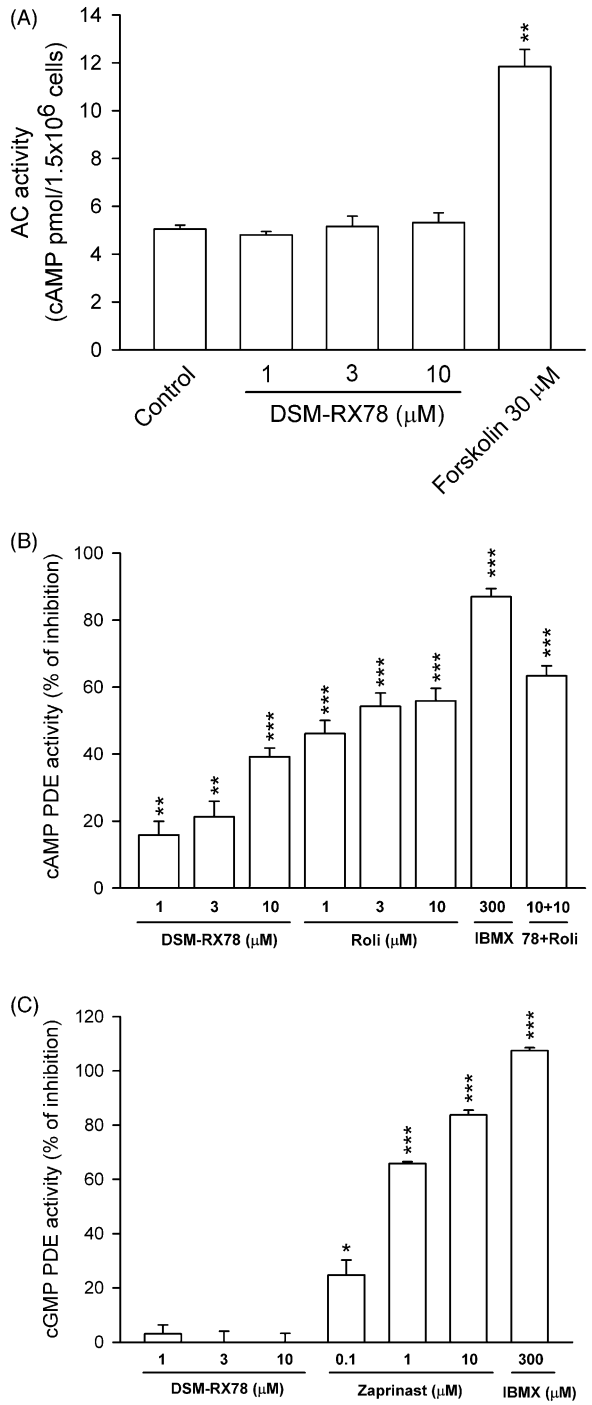

Fig. 5. Concentration-dependent effects of DSM-RX78 on the activities of AC, cAMP-specific PDE, and cGMP-specific PDE. (A) Neutrophil membrane fractions were incubated with DSM-RX78 (1, 3 and 10 μM) and forskolin (30 μM) at 30 °C for 20 min in the presence of 1 mM ATP. cAMP was assayed using enzyme immunoassay kits. Human neutrophil homogenates were incubated with DSM-RX78 (1, 3 and 10 μM), rolipram (Roli, 1, 3 and 10 μM), zaprinst (0.1, 1 and 10 μM), IBMX (300 μM), or DSM-RX78 (10 μM) with Roli (10 μM), and then 0.05 μCi [^{3}H] cAMP (B) or [^{3}H] cGMP (C) was added to the reaction mixture at 30 °C for 10 min. PDE activity was measured as described in Section 2. The control values for cAMP-specific PDE and cGMP-specific PDE activities were 7374.61 ± 393.10 cpm/1 × 10^6 cells and 2619.23 ± 216.52 cpm/3.5 × 10^6 cells, respectively. All data are expressed as the mean ± S.E.M. (n = 3 or 4). $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ compared to the control.

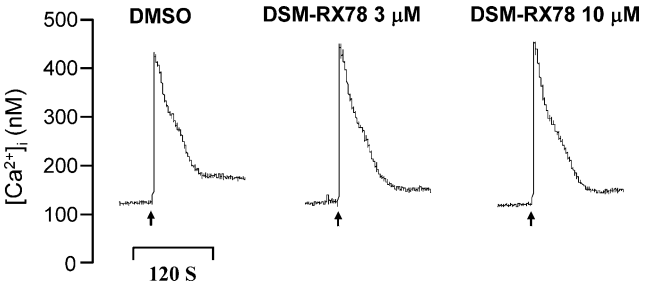

Fig. 6. Typical traces of the effect of DSM-RX78 on Ca^{2+} mobilization in FMLP-activated human neutrophils. Fluo 3-loaded neutrophils were incubated with DSM-RX78 (3 and 10 μM) for 5 min before stimulation with FMLP. Mobilization of Ca^{2+} was determined in real time in a spectrofluorometer, as described in Section 2. Representative traces from one of four experiments are shown.

3.5. Effect of DSM-RX78 on FMLP-induced Akt activation

To test whether other FMLP-mediated downstream signals were affected by DSM-RX78, activation of Akt was assayed using antibodies specific for the phosphorylated, activated form of Akt (Ser 473) as determined by Western blotting. Stimulation of human neutrophils with FMLP resulted in the rapid phosphorylation of Akt. DSM-RX78 (3 μM) reduced phosphorylation of Akt in FMLP-stimulated human neutrophils in a concentration-dependent manner, which was reversed by H89 (3 μM) (Fig. 8).

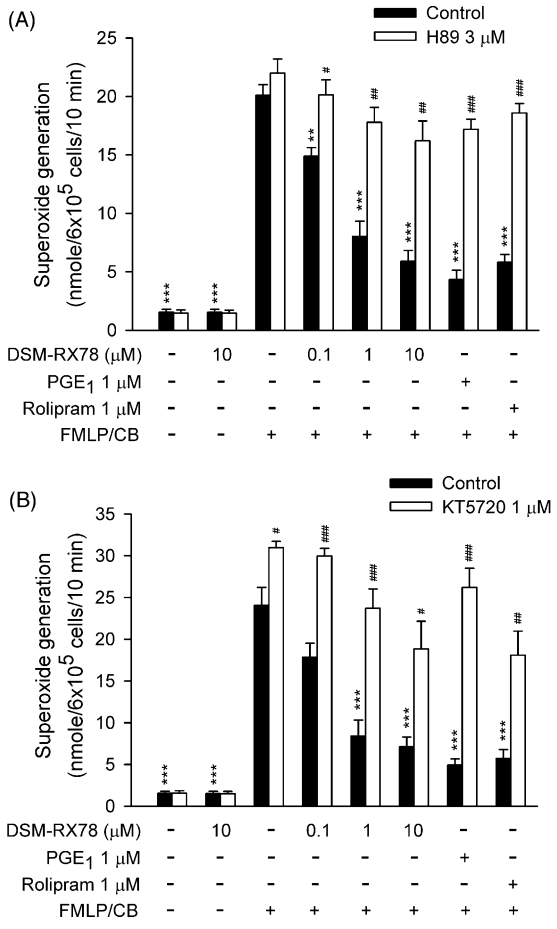

Fig. 7. Effects of PKA on DSM-RX78-caused inhibition of $O_2^{\bullet-}$ generation in human neutrophils. $O_2^{\bullet-}$ generation was induced by FMLP/CB and measured using SOD-inhibitable cytochrome *c* reduction, as described in Section 2. H89 (3 μM) (A) and KT5720 (1 μM) (B) were preincubated for 5 min before the addition of DSM-RX78 (0.1, 1 and 10 μM), PGE_1 (1 μM), or rolipram (1 μM). All data are expressed as the mean ± S.E.M. ($n = 4$ or 5). $^{**}p < 0.01$; $^{***}p < 0.001$ compared to the corresponding DMSO. $^{\#}p < 0.05$; $^{\#\#}p < 0.01$; $^{\#\#\#}p < 0.001$ compared to the corresponding control.

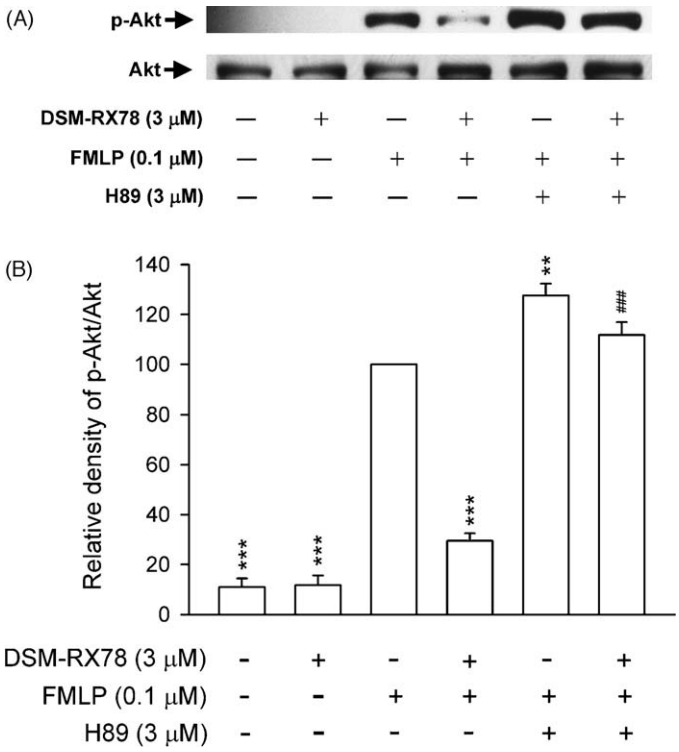

Fig. 8. Effects of DSM-RX78 with or without H89 on the phosphorylation of Akt in human neutrophils. (A) Human neutrophils were incubated with DSM-RX78 (3 μM) for 5 min before stimulation with or without FMLP (0.1 μM) for another 0.5 min. H89 (3 μM) was preincubated for 5 min before the addition of DSM-RX78. Phosphorylation of Akt was analyzed by immunoblotting analysis using an antibody against the phosphorylated form and the total of each protein, as described in Section 2. (B) Bands from A were analyzed by densitometry. Quantitation of the p-Akt/Akt ratio is shown. All data are expressed as the mean ± S.E.M. ($n = 3$). $^{**}p < 0.01$; $^{***}p < 0.001$ compared to FMLP. $^{\#\#\#}p < 0.001$ compared to the corresponding data.

3.6. Effect of DSM-RX78 on lung injury induced by trauma-hemorrhagic shock in rats

There was a significant increase in MPO activity, a marker of neutrophil content, the wet/dry weight ratio in the lung tissue, and total protein content in bronchoalveolar lavage fluid obtained from trauma-hemorrhage rats compared to sham-operated rats (Fig. 9), suggesting that trauma-hemorrhagic shock increases lung injury in rats. In sham-operated animals, no significant differences in the wet/dry weight ratio, MPO activity, or total protein content were found between vehicle- and DSM-RX78-treated groups. Administration of DSM-RX78 (1 mg/kg of body weight) significantly attenuated the increase in the wet/dry weight ratio, MPO activity, and total protein content after trauma-hemorrhagic shock; however, these injury parameters remained higher than in sham-operated animals (Fig. 9).

4. Discussion

There is considerable evidence to suggest that a host's own neutrophil population plays a central role in the development of hemorrhagic shock [10–12], sepsis [1,2], COPD [3], and ARDS [4]. In this study, the effects of DSM-RX78, a semisynthetic 2-benzoylaminobenzoic acid derivative, on respiratory burst in human neutrophils and on lung injury in rats subjected to trauma-hemorrhage were investigated. DSM-RX78 showed potent and selective inhibition of $O_2^{\bullet-}$ generation in FMLP-activated human neutrophils in a concentration-dependent fashion. In addition, our

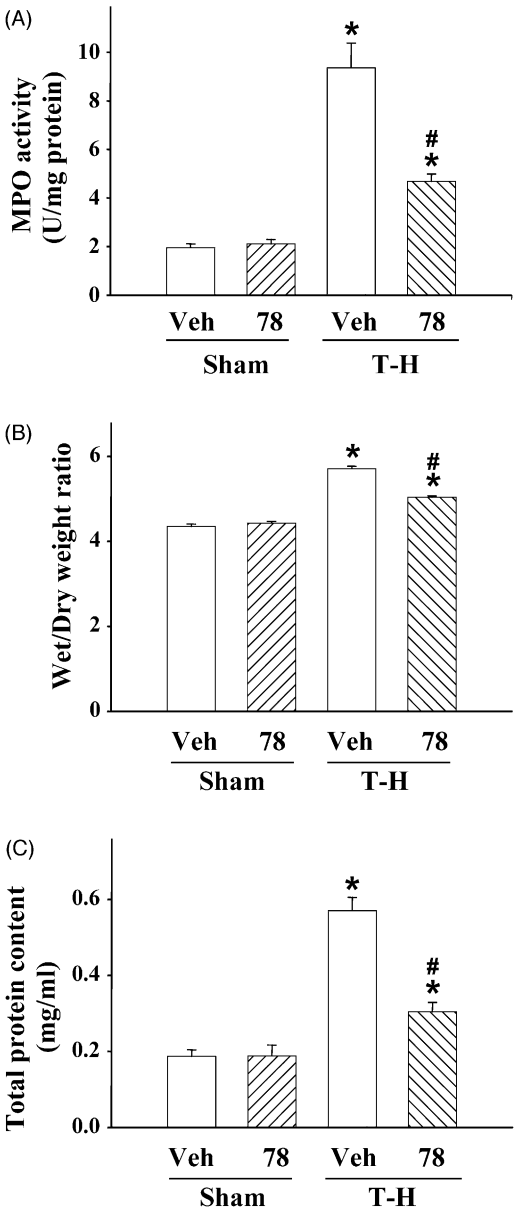

Fig. 9. Effect of DSM-RX78 on lung injury in rats after a sham operation (sham) or trauma-hemorrhage and resuscitation (T-H). Sprague–Dawley rats were treated with DSM-RX78 (1 mg/kg of body weight, intravenously), or an equal volume of the vehicle (~0.2 ml, 10% DMSO). After 24 h, the MPO activity (A) and wet/dry weight ratio (B) in the lung tissue as well as total protein content (C) in bronchoalveolar lavage fluid were assayed, as described in Section 2. Data are shown as mean ± S.E.M. of 8 rats in each group. $^{*}p < 0.05$ compared to sham; $^{\#}p < 0.05$ compared to T-H+Veh.

results showed that DSM-RX78 treatment significantly alleviated hemorrhagic shock-induced lung injury in Sprague–Dawley rats.

The formation of $O_2^{\bullet-}$, a precursor of other reactive oxygen species, by NADPH oxidase is linked to the killing of invading microorganisms, but it can also directly or indirectly cause damage by destroying surrounding tissues [29]. $O_2^{\bullet-}$ production in neutrophils can be inhibited by modulating cellular signaling pathways, but also by directly scavenging $O_2^{\bullet-}$. DSM-RX78 did not scavenge $O_2^{\bullet-}$ in a cell-free xanthine/xanthine oxidase system, indicating that the intracellular signaling pathways are mediated by the action of DSM-RX78. NADPH oxidase is a multicomponent enzyme, including cytosolic and membrane-bound proteins, and remains unassembled in resting cells. Membrane components include a stable, heterodimeric flavocytochrome b_{558} composed of two subunits, gp91^{phox} and p22^{phox}. Cytosolic components include four soluble factors, p67^{phox}, p47^{phox}, p40^{phox}, and Rac, a small G-protein. Upon cell stimulation by soluble inflammatory mediators, such as FMLF, cytosolic components are translocated to the plasma or phagosomal membrane, where NADPH oxidase is assembled [30]. DSM-RX78 inhibited $O_2^{\bullet-}$ generation by intact neutrophils but not by reconstituted NADPH oxidase, suggesting that DSM-RX78 does not inhibit $O_2^{\bullet-}$ release through directly inhibiting of NADPH oxidase activity. The intracellular signaling mechanisms responsible for NADPH oxidase activation in neutrophils are very complex and remain elusive. FMLP activates neutrophils by binding to the GPCR on the membrane. Stimulation of the GPCR induces the Ca^{2+} signal via activation of phospholipase C, which hydrolyzes phosphatidylinositol 4,5-bisphosphate into inositol trisphosphate and diacylglycerol, resulting in an increase in $[Ca^{2+}]_i$ and activation of PKC, respectively. These two second messengers act synergistically in $O_2^{\bullet-}$ production [31]. DSM-RX78 did not change the $[Ca^{2+}]_i$ mobilization of human neutrophils caused by FMLP, suggesting it does not inhibit FMLP-mediated activation of phospholipase C. It is also known that PI3K pathway plays an important role in neutrophil oxidate burst and migration in response to agonists that trigger GPCRs [17,32,33]. Stimulation of human neutrophils by FMLP resulted in the rapid phosphorylation of Akt. DSM-RX78 diminished the FMLP-induced phosphorylation of Akt, suggesting that the suppressive effects of DSM-RX78 on $O_2^{\bullet-}$ generation in FMLP-induced human neutrophils are at least partly mediated by inhibiting of PI3K/Akt activation.

The cyclic nucleotide, cAMP, is an important second messenger with a variety of physiological and pathophysiological manifestations. Recently, we and others showed that elevation of intracellular cAMP levels is able to suppress FMLP-induced $O_2^{\bullet-}$ production in human neutrophils [16,17,34,35]. The contribution of cAMP/PKA in the negative regulation of FMLP-caused human neutrophil $O_2^{\bullet-}$ generation and Akt activation mediated by DSM-RX78 was shown by the observation that the inhibitory effects of DSM-RX78 were reversed by two structurally different PKA inhibitors, H89 and KT5720. In line with these results, the inhibitory effects of PGE_1 and rolipram on $O_2^{\bullet-}$ production were also reversed by PKA inhibitors. In addition, the inhibition of FMLP-induced phosphorylation of Akt by DSM-RX78 was reversed by a PKA inhibitor, suggesting that DSM-RX78 reduces $O_2^{\bullet-}$ production of human neutrophils by promoting cAMP/PKA-dependent inhibition of Akt activation. Several studies established that the addition of chemoattractants to neutrophils leads to a small and temporary increase in the production of cAMP [36–38]. We also noted that FMLP alone was able to induce the weak formation of cAMP. However, the mechanism by which chemoattractants increase cAMP levels in human neutrophils remains to be determined. Interestingly, DSM-RX78 caused a slight increase in cAMP concentrations, whereas it caused a synergistic increase in FMLP-induced cAMP levels in human neutrophils. Cellular cAMP concentrations are modulated either by synthesis via AC or by degradation via cAMP-specific PDEs. Our data showed that DSM-RX78 did not increase AC function, but was able to attenuate cAMP-specific PDE activity. Significantly, DSM-RX78 was more effective at inhibiting cAMP-specific PDE than cGMP-specific PDE, indicating that DSM-RX78 is a selective inhibitor of cAMP-specific PDE. The predominant cAMP-specific PDE in most inflammatory cells including human neutrophils belongs to the PDE4 family [39], and inhibitors of PDE4 are currently being developed clinically as potential anti-inflammatory agents [40]. The combination of DSM-RX78 and rolipram did not further inhibit cAMP-specific PDE, suggesting that DSM-RX78 inhibits the breakdown of cAMP by rolipram-sensitive PDE.

The lung is considered to be a critical organ in the development of delayed organ dysfunction in patients suffering from traumatic

injuries and severe blood loss [41]. Multiple organ failure or dysfunction secondary to a systemic inflammatory response remains the major cause of mortality and morbidity following trauma [42]. The clinical potential of inhibitors of PDE is supported by the suppression of endotoxin-induced acute lung injury in mice by the PDE4 inhibitor, rolipram [21]. Importantly, the anti-inflammatory function of cilomilast, a new-generation PDE4 inhibitor, was confirmed in patients with COPD in whom this agent significantly decreased the numbers of inflammatory cells determined by means of serial bronchial biopsies [43]. Herein, the protective effects of DSM-RX78 in the lung following trauma-hemorrhagic shock were examined. Our study indicates that DSM-RX78 administration decreases neutrophil infiltration in the lung and attenuates lung injury following trauma-hemorrhagic shock. Although the precise mechanism of the salutary effects of DSM-RX78 administration in male Sprague–Dawley rats on organ functions and the contribution of inhibition of PDE activity in reducing organ injuries following trauma-hemorrhagic shock remain unclear, our study provides evidence that inhibition of PDE activity serves as a significant effecter mechanism in reducing lung injury following trauma-hemorrhagic shock. This observation is consistent with a study by Deree et al. that showed pentoxifylline, a non-specific PDE inhibitor, attenuates post-resuscitative lung injury in rats after hemorrhagic shock through modulating neutrophil activities [44]. Multiple observations made in the study suggest that DSM-RX78 suppresses respiratory burst of human neutrophils through an elevation of cAMP levels and PKA activity, and these occur by selective inhibition of cAMP-specific PDE. A similar mechanism of inhibition may be used to control neutrophil-mediated tissue injury. Since DSM-RX78 administration following trauma-hemorrhagic shock decreased lung injury in male Sprague–Dawley rats, this agent might have future potential as a novel adjunct for improving depressed lung function following adverse circulatory conditions.

Acknowledgments

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