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A benzodiazepines derived compound, 4-(3-chlorophenyl)-1,3-dihydronaphtho [2,3-*b*][1,4]diazepin-2-one (ND700C), inhibits fMLP-induced superoxide anion release by activating protein phosphatase 2A in human neutrophils

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ABSTRACT

We studied the mechanism underlying the inhibitory effect of a benzodiazepines derivative, 4-(3-chlorophenyl)-1,3-dihydronaphtho [2,3-*b*][1,4]diazepin-2-one (ND700C), on superoxide anion production induced by formly-methionyl-leucyl-phenylalanine (fMLP) in human neutrophils. ND700C inhibited the fMLP-induced superoxide anion production and cathepsin G release in a concentration-dependent manner with respective IC_{50} values of 5.0 ± 0.5 and 8.7 ± 0.8 μM. In addition, ND700C was found to suppress fMLP-induced intracellular calcium mobilization and the phosphorylation of ERK and Akt. In another study, ND700C was observed to cause a rapid increase in intracellular cAMP level by up to threefold. Furthermore, when H89 was used to inhibit cAMP-dependent protein kinase A (PKA), we discovered that ND700C's suppressive effects on calcium mobilization, phosphorylation, and superoxide anion production were abrogated. ND700C demonstrated additive effect on the PGE_1-induced increase in cAMP. However, this additive effect was not demonstrated with the IBMX-induced rise in cAMP. Our results indicated that ND700C did not directly inhibit the activity of phosphodiesterase 4. In another set of experiments, calyculin A and okadaic acid (both protein phosphatase 2A inhibitors) were found to reverse ND700C's positive effect on cAMP level. This observation suggested the involvement of protein phosphatase 2A in ND700C's cAMP-elevating mechanism. We found that the activity of protein phosphatase 2A was activated by ND700C. Furthermore, protein phosphatase 2A was co-immunoprecipitated with phosphodiesterase 4 after ND700C treatment in human neutrophils. Conclusion: ND700C inhibited fMLP-induced superoxide anion production through a PKA-dependent pathway. ND700C increased cAMP by activating protein phosphatase 2A, which subsequently inhibited phosphodiesterase 4.

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1. Introduction

Neutrophils are a major cellular component of the innate immune response. They constitute 50–60% of the total circulating leukocyte population. In addition, they are the first line of defense against bacterial and fungal assaults, which when compromised, leads to recurrent and severe infections [1,2]. However, a number of pathological conditions such as rheumatoid arthritis, acute respiratory distress syndrome, and ischemia-reperfusion injury, have also been convincingly implicated as the result of neutrophil activation, albeit inappropriately [3,4]. In response to endogenous or exogenous assaults, neutrophils undergo chemotaxis, perform phagocytic functions, release destructive bioactive compounds, and produce reactive oxygen species to defend the host [5–7]. The release of reactive oxygen species formed by NADPH oxidase, so-called a respiratory burst, is a crucial bactericidal mechanism [8].

Chemokines and chemotactic agents such as *N*-formylated peptides (e.g. fMLP) orchestrate neutrophil functions by initiating various signaling cascades [4]. A thorough understanding of the chemoattractant mechanisms may inspire new approaches to achieve a more selective pharmacological intervention. In this study, we employed a small formyl peptide derivative, *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP) as our model chemoattractant for its ability to activate all physiological functions of neutrophils through G-protein-coupled cell surface receptors. The interaction of fMLP with its receptor triggers multiple second messengers by activating PLC [9], PLD and PLA_2. Subsequently, they rapidly stimulate PI3K and activate tyrosine phosphorylation [10]. Increased intracellular levels of cAMP [11] and involvement of kinases, such as PKC [12] and MAPKs [13] have also been demonstrated in this cascade [14].

Protein phosphorylation has been observed in association with the activation of a respiratory burst [15,16]. This led to the hypothesis that kinase and phosphatase, independently or in combination, may control the intensity and duration of a respiratory burst. Supporting evidence of this hypothesis came from the observation that receptor-mediated stimulation of NADPH oxidase is ATP-dependent [17]. In fact, at equimolar concentrations, ATPγS supports respiratory burst more efficiently than ATP. This phenomenon is likely attributed to the fact that thiophosphorylated substrates generated by the hydrolysis of ATPγS are less susceptible to the hyrolysis of cellular phosphatases. At higher concentrations of ATPγS, a spontaneous activation of the respiratory burst was observed. This observation was interpreted as the result of kinases' constitutive action of protein phosphorylation [18]. In contrast, an analogous effect was not observed in the presence of ATP, due to the sustained robust activity of the phosphatase. Yet another supporting evidence came from the observation that okadaic acid, a protein phosphatase 2A inhibitors, also magnified and prolonged the oxidative burst induced by fMLP [19]. These findings together suggest that a respiratory burst may be initiated via two mechanisms: the activation of kinases and the inhibition of phosphatases.

4-(3-Chlorophenyl)-1,3-dihydronaphtho [2,3-*b*][1,4]diazepin-2-one (ND700C) is a novel benzodiazepines belonging to the 1,5-benzodiazpine tricycle. It is known that benzodiazepine exert their better-known anxiolytic, anticonvulsant, and hypnotic effects through interactions with the central-type of benzodiazepine receptors. Interestingly, one line of evidence suggested that interactions with the peripheral-type of benzodiazepine receptors identified in human monocytes, neutrophils and lymphocytes, [20], may play a major role in the regulation of immune functions. Peripheral benzodiazepine receptor ligands have been found to modulate monocyte functions such as cellular chemotaxis [21]. In addition, treatment of mice with Ro5-4864, a diazepam derivative, was shown to markedly reduced macrophage's capacity in producing key mediators of inflammation such as superoxide anion, interleukin-1, TNF-α and interleukin-6 [22]. These data have led us to hypothesize a novel application of peripheral benzodiazepine receptor ligands as anti-inflammatory agents.

We investigated the mechanisms of ND700C's effect on superoxide anion production caused by fMLP and several other agents, such as PMA in human neutrophils. ND700C was found to inhibit fMLP-induced superoxide anion production as well as cathepsin G release via a PKA-dependent pathway. As previous studies have demonstrated in other systems that protein phosphatase 2A increases cAMP level by inhibiting phosphodiesterase activity [23], we set out to test the applicability of this theory to ND700C's effect on neutrophils. We employed okadaic acid and calyculin A, both potent serine-threonine phosphatase inhibitors, to inhibit protein phosphatase 2A. Our results demonstrated that, indeed, ND700C increased cAMP by activating protein phosphatase 2A, which subsequently inhibited phosphodiesterase.

2. Materials and methods

2.1. Materials

ND700C was kindly sponsored by Dr. Chih-Shiang Chang. ND700C was prepared by cyclization of naphthalene-2,3-diamine with 3-(3-chlorophenyl)-3-oxo-propionic acid ethyl ester (3) in xylene under microwave condition (260 W). Procedure: 3-(3-chlorophenyl)-3-oxo-propionic acid ethyl ester (3). To a stirred solution of diethylcarbonate (1.53 g, 12.94 mmol) in dry toluene (30 ml) was added NaH (0.78 g, 19.41 mmol) at reflux for 0.5 h. The mixture was dropwise added 20 ml dry toluene containing 3′-chloroacetophenone (1 g, 6.47 mmol) in period of 30 min. The reacted solution was reflux for another 2 h. After cooling the mixture was added distilled water and neutralized by glacial acetic acid. The organic layer was dried over anhydrous $MgSO_4$ and then concentrated under vacuum to give 3 (yield 95%) as oil. To a stirred solution of compound 3 (1 g, 4.41 mmol) in xylene (30 ml) was added naphthalene-2,3-diamine (0.70 g, 4.41 mmol) at reflux (MW 260W) for 3 h. After cooling the mixture was filtered and washed with acetone to give ND700C ($C_{19}H_{13}ClN_2O$; MW 320.77; yield 75%) as yellow powder (Fig. 1). SOD, PMA, H89, PGE_1, IBMX, pheylymethylsulphonylfluoride (PMSF), bovine serum albumin (BSA), calmodulin, *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP), fura-2 acetoxymethylester (Fura-2/AM), okadaic acid, calyculin A, PD98059, wortmannin, cytochrome *c*, cytochalasin B, *N*-succinyl-Ala-Ala-Pro-Phe *p*-nitroanilide (Suc-AAPF-pNA; a colorimetric substrate for human leukocyte cathepsin G) and

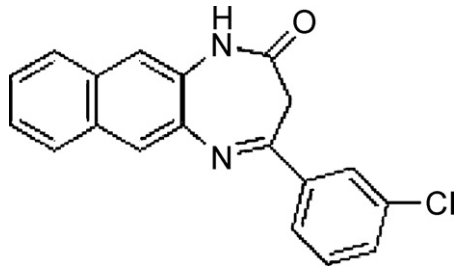

Fig. 1 – Chemical structure of 4-(3-chlorophenyl)-1,3-dihydronaphtho[2,3-*b*][1,4]diazepin-2-one (ND700C).

Hank's buffered saline (HBSS) were purchased from Sigma (St Louis, MO, USA). Phospho-p44/42 MAP kinase (Thr202/Tyr204) antibody (anti-human), p44/42 MAP kinase, phospho-Akt (Ser 473), Akt, phospho-p38 MAP kinase (Thr180/Tyr182) and p38 MAP kinase antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). PP-2A antibody (clone 1D6) and serine/threonine assay kits were purchased from Upstate Technology (Temecula, CA, USA). Rabbit polyclonal phosphodiesterase 4A (phosphos) and phosphodiesterase 4A antibody were purchased from GenTex, Inc. (San Antonio, TX, USA). cAMP ELISA kits were purchased from R&D Systems (Minneapolis, MN, USA).

2.2. Preparation of human neutrophil

Venous blood samples were obtained from healthy volunteers of both gender between the aged 20 and 40 years, using syringes containing heparin (final concentration 20 unit/ml). All protocols were in strict complied with Chang-Gung Memorial Hospital Ethics Committee guidelines. Neutrophils were isolated from blood samples by Ficoll gradient centrifugation then purified by hypotonic lysis of contamination erythrocytes [24]. Briefly, a blood sample was mixed with an equal volume of 3% dextran solution in a 50 ml-centrifuge tube and incubated in an upright position for 20 min at room temperature to allow for the sedimentation of erythrocytes. The leukocyte-rich upper layer was then collected and centrifuged at $250 \times g$ for 15 min at 4 °C. The pellet was then immediately re-suspended in a volume of phosphate buffer saline (PBS) equal to the initial volume of blood. The cell suspension thus obtained was then apportioned into 50-ml centrifuge tubes at 25-ml per tube. Using a pipette, a layer of 10 ml of 1.077 g/ml Ficoll solution was laid beneath the cell suspension. After a final centrifuge ($400 \times g$ for 40 min at 20 °C), the upper (PBS) and lower (Ficoll) layers were removed, leaving only the granulocyte/erythrocyte pellets. To remove the residual erythrocytes, the pellet was re-suspended in 20 ml cold 0.2% NaCl for 30 s. Twenty milliliters cold 1.6% NaCl was then added to restore tonicity. Finally, the remaining neutrophils were pelleted, washed twice with ice-cold PBS and re-suspended in an adequate volume of ice-cold Hank's buffered saline (HBSS). The final preparation thus obtained contained more than 95% neutrophils, as estimated by differentially counting 200 Giemsa stained cells under microscope.

2.3. Superoxide anion measurement

Following isolation, the cells (1×10^6/ml) were re-suspended in HBSS. Superoxide production was determined by measuring cytochrome c reduction [25,26]. One milliliter of cell suspension was placed in a cuvette with HBSS preheated to 37 °C, 80 μM of cytochrome c, and 2.5 μg/ml of cytochalasin B in the presence and absence of SOD (150 U/ml), respectively. The cuvette was then placed in the thermal-controlled chamber of the spectrophotometer (Hitachi UV-3010) and allowed to stabilize at 37 °C After a baseline was established, the cells were stimulated with either 1 μM fMLP or 100 ng/mL PMA. Changes in absorbance readings at 550 nm were measured over a 15-min period. Results were calculated as nanomoles of superoxide produced per million cells over 15 min for total superoxide production. To determine the effect of ND700C on the respiratory burst, the cells were first preincubated at 37 °C with 1, 2, 5, 10, and 20 μM of ND700C, respectively for 3 min in the thermal-controlled chamber. Superoxide anion production, was then quantified again via the aforementioned method. Lastly, to determine the effect of ND700C, neutrophils were pre-incubated with H89 (10 μM) for 10 min at 37 °C before being challenged by ND700C for another 3 min. The cells were then stimulated with fMLP and the superoxide anion production was assayed as previously described.

2.4. Cathepsin G release measurement

Neutrophil (2×10^6 cells/ml) were placed in duplicated into tubes containing different concentration of ND700C for 5 min at room temperature. Neutrophils were stimulated with either fMLP (1 μM) or PMA (100 nM) for 10 min before being centrifuged for 1 min. Duplicate aliquots of the sample (25 μl) were then extracted and placed into the wells of a flat-bottomed 96-well microplate. Tris-buffer (150 μl) was added to each well in addition to 20 μl Suc-AAPF-pNA (1 mM) for cathepsin G activity. After 2 h at room temperature, the colored product was measured spectrophotometrically at 405 nm with a Benchmark Microplate reader (Bio-Rad). To determine if ND700C affected cathepsin G activity, it was added to cellular supernatant from fMLP-treated neutrophils and then incubated with Suc-AAPF-pNA [27].

2.5. Intracellular calcium measurement

The method of Pollock and Rink, with minor modification, was used to measure intracellular calcium [28]. Briefly explained, neutrophils (1×10^6 cells/ml) were incubated with fura-2/AM (2 μM) for 30 min at 37 °C before being centrifuged at $200 \times g$. The resultant pellets were then washed with HBSS and re-suspended in HBSS containing calcium (1 mM). Neutrophils (2×10^6 cells/ml) were treated with ND700C (10 μM) for 3 min before being challenged with fMLP (1 μM). Fluorescence (excitation 340 and 380 nm; emission 500 nm) was measured by Hitachi fluorescence spectrophotometer (model F7000; Tokyo, Japan) at 37 °C At the end of the experiment, the cells were treated with Triton X-100 (0.1%) and EGTA (10 mM) to obtain the maximal and

minimal fluorescence, respectively. Intracellular calcium was calculated as described for fura-2 using the calcium-dye dissociation constant 224 nM [28].

2.6. Western blotting analysis

To analyze the phosphorylation status of ERK, Akt or p38, neutrophils (2×10^6 cells/ml) were incubated with or without ND700C (10 μM) for 3 min at 37 °C and then stimulated with fMLP (1 μM) or PMA (100 nM). The reaction was terminated by placing the cells on ice and subjected them to immediate centrifugation. The pellets were re-suspended in 1× Laemmli sample buffer. After boiling the sample for 10 min, the proteins were stored at −70 °C prior to immunoblotting. The sample was electrophoresed in 8–10% SDS-polyacrylamide gels and transferred electrophoretically onto nitrocellulose. Blots were stained with Ponceau-S (0.2% Ponceau-S 3% TCA, and 3% sulfosalicylic acid) to visualize Mr markers then destained with deionized water followed by TBST (10 mM Tris–HCl (pH 7.5), 100 mM NaCl, and 0.1 % Tween-20). Blots were blocked for 1 h in 5% nonfat milk in TBST. Antibodies were diluted in PBS (pH 7.3) containing 3% BSA and 0.02% sodium azide. Blots were incubated with the appropriate antibody (Phospho-p44/42 MAP kinase, phospho-Akt or Phospho-p38; 1/1000) for 2 h at 25 °C then thoroughly washed (three times, 10 min each) with TBST. Next, blots were incubated for 1 h with an appropriate horseradish peroxidase-conjugated secondary antibody (1/5000) in 5% nonfat milk in TBST, washed thoroughly and examined by enhanced chemiluminescence.

2.7. Cyclic nucleotides analysis

Intracellular cyclic AMP (cAMP) was measured as described in a previous study [29]. Briefly explained, human neutrophils were incubated with DMSO (0.5%; resting), PGE_1 (1 μM), IBMX (200 μM) or ND700C (10 μM) for various durations. The reactions were terminated by the addition of EDTA (10 mM), followed by boiling of the samples for 2 min. The cAMP content was determined by enzyme immunoassay.

2.8. Measurement of phosphodiesterase activity

Phosphodiesterase activity was measured as described by Schudt [30] with modifications. A suspension of cells (10^7 cells/ml) in buffer A (Bis Tris 20 mM, sodium acetate 50 mM, benzamidine 2 mM, EDTA 2 mM, β-mercaptoethanol 5 mM, and PMSF 0.05 mM; pH 6.5) was homogenized and centrifuged. The resultant supernatant was then collected. Cyclic nucleotide phosphodiesterases were assayed following the protocol of Tompson and Strada [31]. The standard incubation mixture contained 40 mM Tris–HCl, 5 mM $MgCl_2$, 3.75 mM β-mercaptoethanol, 1 μM ^{3}H-labelled/unlabelled cyclic nucleotide (~200,000 dpm) in a final volume of 400 μl. When appropriate, phosphodiesterase inhibitors at the concentration tested. The assay was initiated by the addition of 100 μl of the enzyme solution to the standard incubation mixture and allowing the reaction to be carried out for 20 min at 30 °C. Cyclic AMP phosphodiesterase activity was also determined in the presence of Ca^{2+} (10 μM)/calmodulin (1.2 μM) as previously outlined by Cortijo [32]. The content of cyclic AMP was measured as outlined by Cortijo [32]. Cyclic AMP was quantified by use of an enzyme-immunoassay kit (RPN 225; Amersham Life Sciences, UK) following the instructions of the manufacturer. The cyclic AMP content was expressed as pmol/10^6 cells.

2.9. Protein phosphatase 2A activity assay

The protein phosphatase 2A phosphatase assay was performed as stipulated by the manufacturer. Briefly explained, neutrophils were exposed to ND700C at 37 °C for various durations and were lysed in a lysis buffer (1% Nonidet P-40, Tris 50 mM (pH 7.5), 137 mM NaCl, 2 mM NaF, 10 % (v/v) glycerol, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM phenylmethylsuflonyl fluoride, and 1 mM Na_3VO_4). Protein phosphatase 2A was immunoprecipated as described in the ensuing paragraph. The precipitated were washed by centrifugation twice in the lysis buffer and twice in a serine/threonine kinase buffer (100 μM $CaCl_2$ and 50 mM Tris–HCl (pH 7.0)). The pellet was re-suspended in a kinase buffer, and protein phosphatase 2A activity was measured after the addition of phosphopeptide (250 μM). The phosphatase reactions were carried out for 15 min at 30 °C. The release of phosphate from the added phosphopeptide was quantified using a malachite green reagent. In short, a sample (25 μl) of the reaction medium was transferred to a microtiter assay plate, and 100 μl of malachite green reagent was added to each well, after which the plates were incubated for 15 min at 30 °C. Changes in absorbance were measured at 650 nm by a Banchmark microplate reader (Bio-Rad).

2.10. Protein phosphatase 2A immunoprecipitation and phosphorylated phosphodiesterase 4 immunobloting

Neutrophils (10^6/ml) samples were incubated with ND700C (10 μM) for 30 s, 2 and 5 min, respectively. Then, the reactions were terminated with an equal volume of lysis buffer, and the samples were placed on ice for 30 min. The cell lysates were separated from the insoluble material by centrifugation at 13,000 × *g* for 15 min at 4 °C, precleared with 40 μl of protein A-Sepharose, and incubated with polyclonal protein phosphatase 2A for 2 h at 4 °C. It is then incubated with 40 μl of protein A-Sepharose for another 1 h. The immunoprecipitates were washed five times with 1 ml of wash buffer (0.5% Triton X-100, 150 mM NaCl, 10 mM Tris–HCl, pH 7.4), extracted with a Laemmli sample buffer, and boiled for 15 min. Protein phosphatase 2A immunoprecipitates obtained from ND700C-treated cells were separated by electrophoresis in a 10 % SDS-polyacrylamide gel and then electrophoretically transferred to PVDF membranes as described above. The membranes were incubated for 12 h with antibody against the phosphorylated phosphodiesterase 4 before being incubated for 1 h with horseradish peroxidase-conjugated secondary antibody. The bands were identified by enhanced chemiluminescence.

3. Results

3.1. ND700C specifically inhibits fMLP-induced superoxide anion production and cathepsin G release in human neutrophils

The stimulation of human neutrophils with fMLP (1 μM) or PMA (100 nM) induced the production of superoxide anion. ND700C was found to attenuate fMLP-induced superoxide anion in a concentration-dependent manner. Furthermore, it was found that fMLP-induced superoxide anion production was completely inhibited at 10 μM of ND700C. The IC_{50} value for ND700C's inhibitory effect was determined to be 5.0 ± 0.5 μM (Fig. 2A). Conversely, a high concentration of ND700C (10 μM) was found to have no effect on the PMA-induced superoxide anion (Fig. 2B).

The release of cathepsin G was examined to confirm the specificity of ND700C's inhibitory effect on the fMLP-activated human neutrophils. Stimulation of human neutrophils with fMLP (1 μM) or PMA (100 nM) both induced the release of cathepsin G. ND700C attenuated fMLP-induced cathepsin G release in a concentration-dependent manner. The IC_{50} value for the inhibitory action of ND700C was 8.7 ± 0.8 μM (Fig. 2C). In contrast, a high concentration of ND700C (10 μM) demonstrated no affect on the PMA-induced cathepsin G release (Fig. 2D).

3.2. ND700C inhibited fMLP-induced intracellular calcium mobilization

Intracellular calcium mobilization in human neutrophils was induced by fMLP (1 μM) and LTB_4 (100 nM), respectively (Fig. 3A and B). ND700C at 5 and 10 μM concentrations were found to reduce the fMLP-induced intracellular calcium mobilization (Fig. 3A). However, ND700C (10 μM) did not affect the LTB_4 induced intracellular calcium mobilization (Fig. 3B). The increase in calcium concentration was found to be 697.3 ± 14.6 nM for the fMLP-challenged samples and 478.5 ± 67.1 nM for the LTB_4-challenged samples (Fig. 3A; insert illustration). In the presence of ND700C (10 μM), the increases in intracellular calcium induced by fMLP and LTB_4 were reduced to 434.3 ± 56.1 nM (***$P < 0.001$ as compared with fMLP alone) and 451.3 ± 55.7 nM ($P > 0.05$), respectively. Data shown represents the net increase in calcium concentrations after the basal calcium concentrations have been subtracted (Fig. 3A and B; inserted illustration).

3.3. ND700C inhibits fMLP-induced ERK and Akt phosphorylation

In another series of experiments, fMLP (1 μM) and PMA (100 nM) were used to induce ERK and Akt phosphorylations

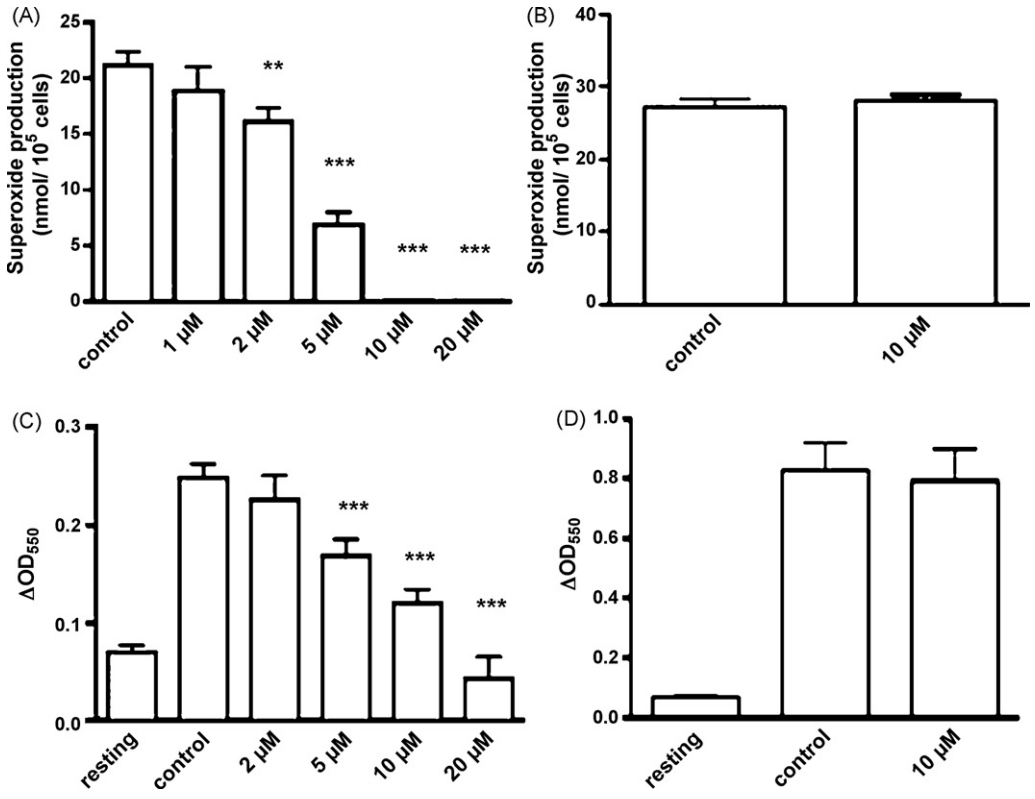

Fig. 2 – Specific effect of ND700C on fMLP-induced superoxide anion production and cathepsin G release in human neutrophils. (A) Concentration response of ND700C on fMLP (1 μM) induced superoxide anion production. (B) PMA (100 nM) induced significant superoxide anion production. ND700C (10 μM) did not affect superoxide anion production by PMA. (C) Concentration response of ND700C on fMLP (1 μM) induced cathepsin G release. (D) ND (10 μM) did not affect cathepsin G release induced by PMA. **$P < 0.01$; ***$P < 0.001$ as compared with control (fMLP).

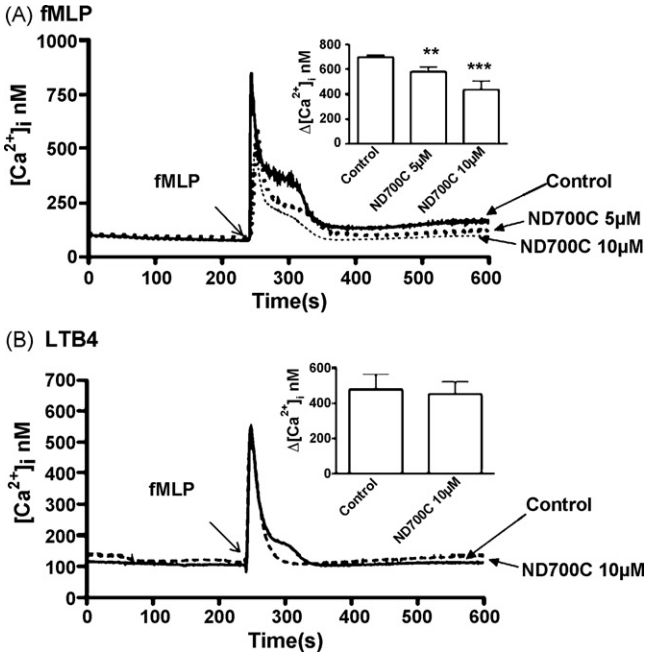

Fig. 3 – Specific effect of ND700C on fMLP-induced intracellular calcium mobilization. (A) fura-2/AM loaded neutrophils were incubated with DMSO or ND700C (5 or 10 μM) for 3 min and stimulated with fMLP (1 μM). (B) Human neutrophils were incubated with DMSO or ND700C (10 μM) for 3 min and stimulated with LTB_4 (100 nM). Insert illustration represent the peak calcium concentration after subtraction of the basal calcium concentration. $^{**}P < 0.01$; $^{***}P < 0.001$ as compared with control (fMLP).

(Fig. 4A and B). ND700C was found to inhibit the fMLP-induced ERK (Fig. 4A, $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ as compared with control) and Akt (Fig. 4B, $^{*}P < 0.05$; $^{***}P < 0.001$ as compared with control) phosphorylations in a concentration-dependent fashion. On the other hand, a high concentration of ND700C (20 μM) exhibited no effect on the PMA-induced ERK and Akt phosphorylations. A positive control was established using PD98059 and wortmannin to inhibit the fMLP- and PMA-induced ERK and Akt phosphorylations (Fig. 4A and B).

3.4. *H89 reversed the inhibitory effect ND700C on fMLP-induced neutrophil activation*

H89 was added 10 min before incubation of with ND700C (10 μM); then fMLP was added after 5 min incubation of ND700C. Superoxide anion, intracellular calcium mobilization and ERK phosphorylation were measured as illustrated in Section 2. As previous stated, ND700C (10 μM) inhibited fMLP-induced superoxide anion (Fig. 5A), intracellular calcium mobilization (Fig. 5B), and ERK phosphorylation (Fig. 5C). However, it was demonstrated that H89 (10 μM) virtually reversed all of these inhibitory effects completely (Fig. 5A–C). In addition, it was found that H89 also reversed ND700C inhibited Akt and p38 phosphorylation (data not shown).

3.5. *ND700C increases cAMP and inhibited by protein phosphatase 2A inhibitors*

ND700C was found to increase cAMP in human neutrophils in a time and concentration-dependent manner (data not shown). Fig. 6A shows that human neutrophils treated with ND700C (10 μM) for various durations. ND700C was found to significantly increase the cAMP level in human neutrophils 2 min after its treatment. In addition, this increase was observed to continue until 10 min after ND700C treatment (Fig. 6A; $^{*}P < 0.05$, $^{***}P < 0.001$ as compared with resting). PGE_1 or IBMX alone was positive control for cAMP levels (Fig. 6B; $^{***}P < 001$ as compared with resting). IBMX caused an additional increase in the PGE_1-induced rise in cAMP level. ND700C was also found to cause an additional increase in the PGE_1-induced rise in cAMP level. However, these additive effects were found to be obliterated when neutrophils were treated simultaneously with ND700C and IBMX (Fig. 6B). Protein phosphatase 2A inhibitors, calyculin A (10 nM) and okadaic acid (5 nM), significantly inhibited cAMP levels induced by ND700C (Fig. 6C and D, $^{**}P < 0.01$ as compared with ND700C alone). Calyculin A or okadaic acid alone failed to affect cAMP level in human neutrophil.

3.6. *ND700C did not directly inhibit cAMP phosphodiesterase activity*

To figure out the mechanism of ND700C increased cAMP level, the effect of ND700C on the cAMP-phosphodiesterase was examined. IBMX (1 mM) significantly inhibited the activity of phosphodiesterase as a positive control (65.7 ± 5.2 % inhibition). Our study showed that ND700C did not inhibit cAMP-phosphodiesterase directly (Fig. 7).

3.7. *ND700C increased the protein phosphatase 2A activity*

To address the possibility that protein phosphatase 2A was activated by ND700C, we measured the phosphatase activity in phosphatase 2A immunoprecipitates after treatment with ND700C. Spectrophotometric analysis revealed a rapid rise in protein phosphatase 2A activity 2 min after the addition of ND700C. This increase continued until 10 min after ND700C stimulation (Fig. 8). This ND700C-induced increase in protein phosphatase 2A activity was abolished by the addition of calyculin A (Fig. 8) and okadaic acid (data not shown).

3.8. *ND700C enhanced the association of protein phosphatase 2A with phosphorylated phosphodiesterase 4*

To address the possibility that protein phosphatase 2A regulated the demonstrated transient phosphodiesterase inhibition effect, we examined the interaction between protein phosphatase 2A and phosphorylated phosphodiesterase 4. Human neutrophils were treated with ND700C (10 μM) for various durations, and the cell lysates were immunoprecipitated with protein phosphatase 2A-antibody. The immunoprecipitant were then subjected to immunoblot analysis using antibody against either phosphorylated phosphodiesterase 4A or phosphodiesterase 4A. Treatment of cells with

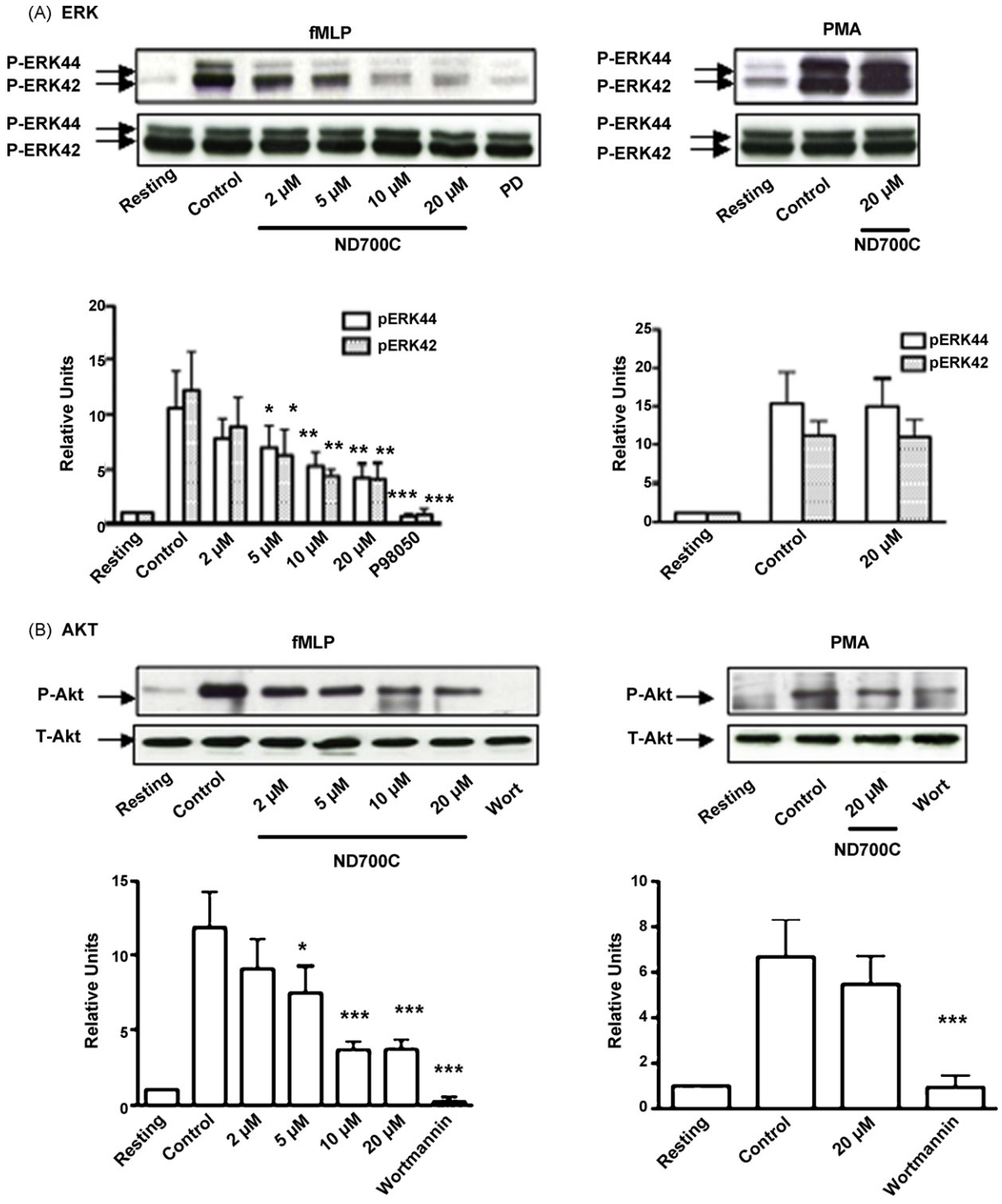

Fig. 4 – Specific effect of ND700C on ERK and Akt phosphorylation induced by fMLP in human neutrophils. Neutrophils were pretreated with DMSO (0.5%, resting and control) or various concentration of ND700C at 37 °C for 3 min and stimulated with fMLP (1 μM) or PMA (100 nM). Reactions were stopped with sample buffer then applied to 10 % gel. (A) P42/44 and (B) Akt were detected with phosphate-P42/44 and phosphate-Akt antibodies, respectively. Data are expressed as mean ± S.E.M. of four independent experiments.*P < 0.05, **P < 0.01, ***P < 0.001 as compared with respective values of cells stimulated with fMLP or PMA. PD (PD98059; 20 μM) and Wort (wortmannin; 100 nM) were the positive control.

ND700C (10 μM; 30 s) significantly demonstrated a rise in the amount of phosphorylated phosphodiesterase 4A in the protein phosphatase 2A immunoprecipitant. This increase was observed to return to baseline after 5 min. This data suggested that ND700C enhanced the association of protein phosphatase 2A with the phosphorylated phosphodiesterase 4 (Fig. 9A and B). Cell lysated was immunoprecipated with protein phosphate 2A-antibody and subsequently immuno-

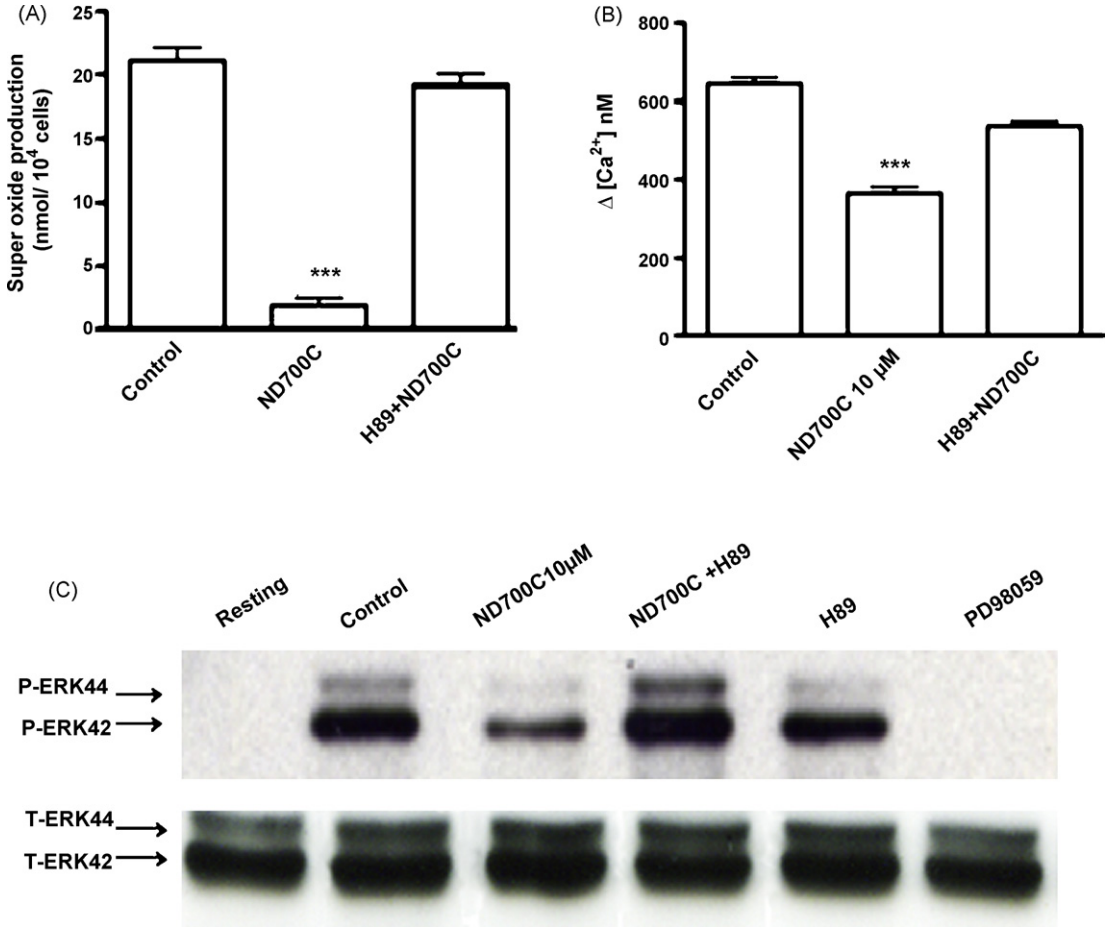

Fig. 5 – H89 reversed the inhibitory effect of ND700C on fMLP-induced human neutrophil activation. Human neutrophils were incubated with H89 (10 μM) 10 min before they were incubated with ND700C (10 μM); then fMLP was added 3 min after incubation with ND700C. (A) Superoxide anion, (B) intracellular calcium and (C) ERK phosphorylation were monitored as indicated in Section 2. ***P < 0.001 as compared with control (fMLP).

blotted with the same antibody to establish an internal control (Fig. 9A panel c).

4. Discussion

This study investigates the inhibitory mechanisms of ND700C on superoxide anion production in human neutrophils. Several chemoattractants, such as fMLP and PMA are using for triggering superoxide anion production. According to our data, ND700C specifically inhibits fMLP-induced superoxide anion production as well as the release of cathepsin G. We further evaluated the mechanism underlying ND700C's effect on fMLP. Cell viability was not affected by high concentrations of ND700C in the present study. Superoxide anion production in human neutrophils was elicited by activating NADPH oxidase with fMLP or PMA. ND700C does not affect either the enzyme activity of NADPH oxidase or its assembly in vitro (data not shown). Moreover, ND700C does not scavenge superoxide anion in a xanthine/xanthine oxidase system (data not shown). This finding suggested that ND700C may inhibit certain steps in the intracellular signaling cascade leading to NADPH oxidase activation.

In order to elucidate the effect of ND700C on fMLP-induced neutrophil activation, intracellular signals induced by fMLP or PMA were evaluated. Intracellular calcium mobilization, ERK, Akt and p38 (data not shown) phosphorylation are all induced by fMLP through fMLP receptor. ND700C inhibits ERK, Akt and p38 (data not shown) phosphorylation induced by fMLP. Conversely, ND700C demonstrated no effect when these proteins phosphorylation were induced by PMA. These observations suggest that ND700C does not affect the signaling induced by PKC. Moreover, in another series of studies, ND700C specifically inhibited the fMLP-induced cathepsin G release confirmed this hypothesis. The specific effects of ND700C on fMLP-induced free radical production, intracellular calcium mobilization, protein phosphorylation (ERK and Akt) and cathepsin G release suggest that ND700C may be a fMLP receptor antagonist. However, we ruled out this possibility by demonstrating the inability of ND700C to inhibit the binding of N-formly-norleucyl-leucy-phenylalanyl-norleucyl-throsyl-lysine-fluorescein to neutrophils (data not shown) [33]. Furthermore, the role of calcium as a primary or secondary messenger in neutrophil activation induced by fMLP has been extensively studied [34,35]. ND700C inhibits the intracellular calcium release and influx induced by fMLP. Even though both

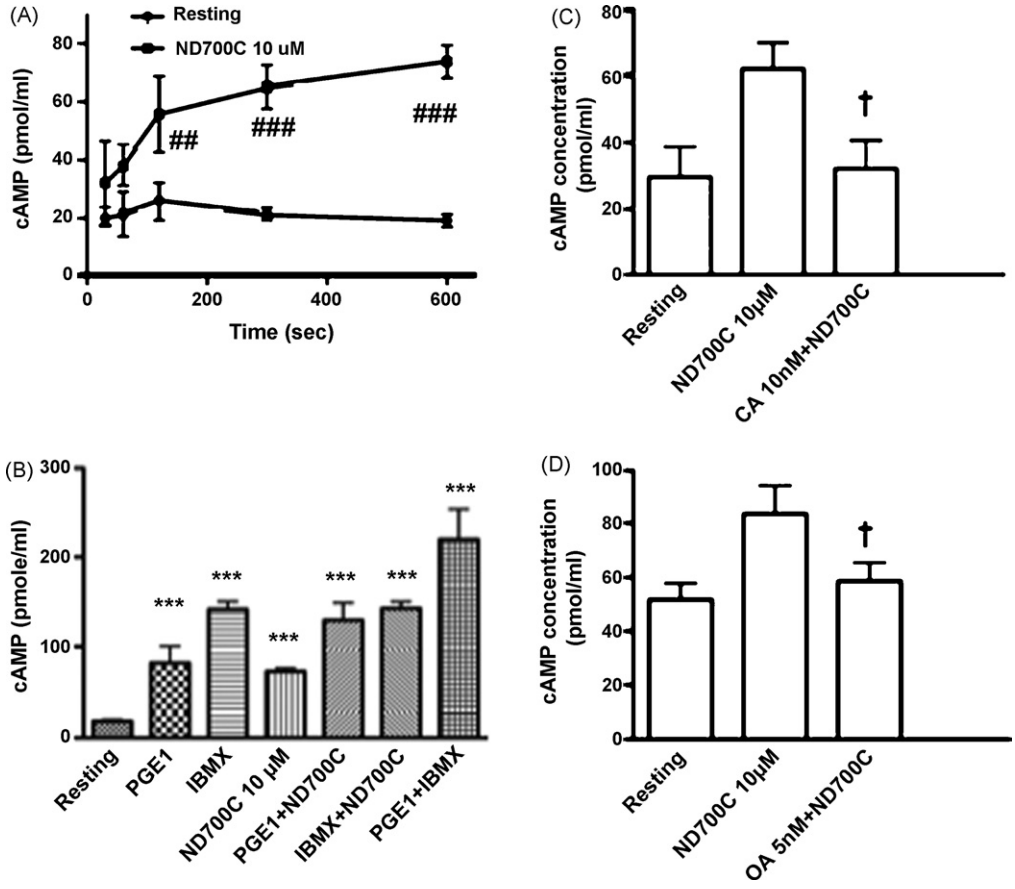

Fig. 6 – ND700C increased cAMP and inhibited by protein phosphatase 2A inhibitors. (A) Neutrophils were pretreated with vehicle or ND700C (10 μM) at 37 °C for indicated times. cAMP was measured with cAMP ELISA kits. $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ as compared with resting. (B) Neutrophils were incubated with PGE_1 (1 μM), IBMX (200 μM), ND700C (10 μM), PGE_1 (1 μM) + ND700C (10 μM), IBMX (200 μM) + ND700C (10 μM), PGE_1 (1 μM) + IBMX (200 μM) for 5 min and cAMP level was measured with cAMP ELISA kits. $^{***}P < 0.001$ as compared with resting. (C) Neutrophils were treated with calyculin A (CA; 10 nM) or (D) okadaic acid (OA; 5 nM) 10 min before ND700C was added. cAMP was measured 5 min after ND700C was added ($n = 5$). $^{\dagger}P < 0.01$ as compared with ND700C 10 μM.

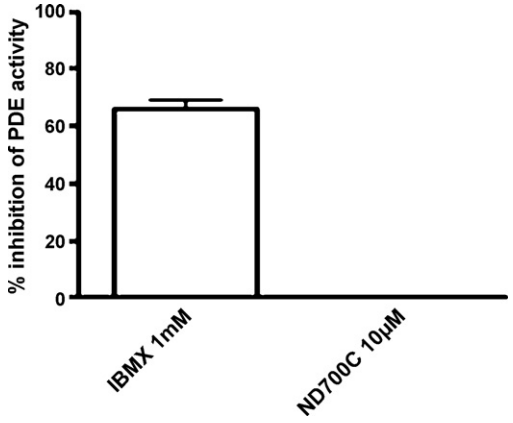

Fig. 7 – ND700C did not abolish phosphodiesterase enzyme activity. Neutrophils were pretreated with IBMX (1 mM) or ND700C (10 μM) for 5 min, the reaction was stopped as indicated in Section 2. Phosphodiesterase activity was determined as show in Section 2.

phases of calcium increase were found to be statistically affected by the addition of ND700C, changes in the sustained phase was found to be pronounced as compared to the initial peak. This observation is analogous to earlier studies that showed an accelerated decline in calcium induced by increased cAMP in human neutrophil [36].

According to previous studies, cAMP-elevating agents inhibited neutrophil adhesion, granule secretion and superoxide anion generation when activated with a variety of agonists including fMLP, LTB_4, but not PMA [37]. cAMP accumulation and PKA activation seem to play important roles in the suppression of neutrophil functional responses. PKA plays an important role in the inhibitory cross talk that leads to the heterologous desensitization of β-adrenergic receptors via direct receptor phosphorylation, which subsequently attenuates the agonist-stimulated GTPase activity [38,39]. In neutrophils, heterologous desensitization of chemoattractant receptors, including fMLP and C5a receptors, has been observed [40]. PKA has been shown to inhibit ERK signaling in a number of cells by interfering with the activation

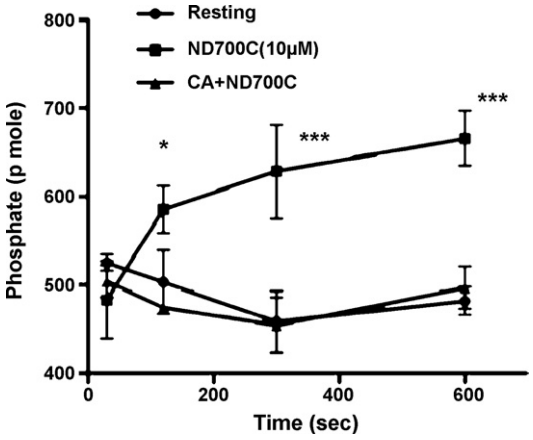

Fig. 8 – Increase protein phosphate 2A activity by ND700C. Human neutrophils (1×10^6/ml) were pretreated with DMSO (0.05%; resting) or ND700C (10 μM) at 37 °C for the indicated periods of time and then immediately lysed. In another set of experiment, calyculin A (10 nM) was added 10 min before ND700C was added, cells were lysated at indicated times. Sample of the lysates were taken for immunoprecipitation with an anti-protein phosphate 2A antibody, and the activity of protein phosphate 2A in the immunoprecipitates was determined by incubation with a phosphopeptide substrate. The amount of phosphate that was released was quantified by recording changes in absorbance.*P < 0.05, ***P < 0.001 as compared with basal.

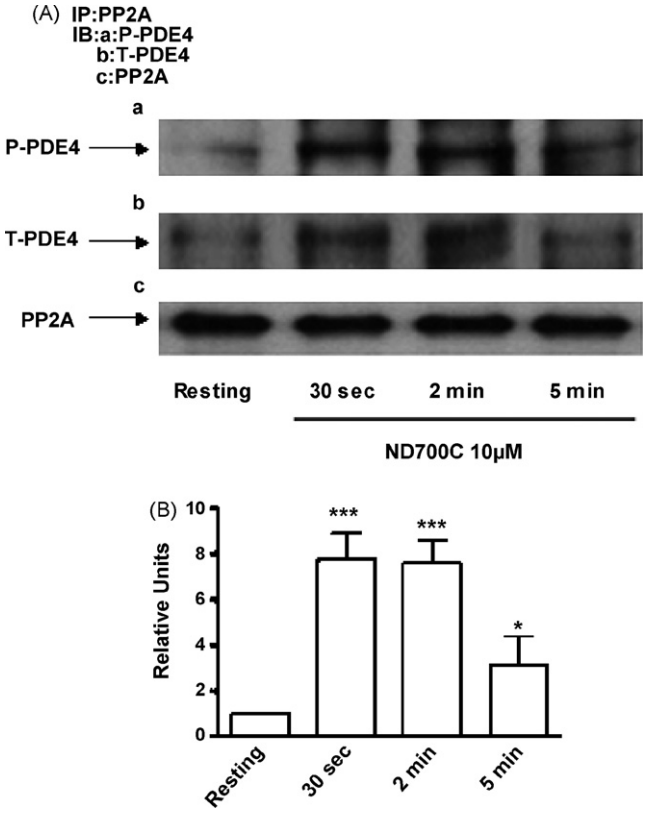

Fig. 9 – ND700C increases in the association of protein phosphate 2A with the phosphorylated phosphodiesterase 4. (A) Neutrophils were incubated with ND700C (10 μM) for various periods as indicated in the figure. (A) The binding of phosphorylated phosphodiesterase 4 (P-PDE4) was monitored by immunoblotting phosphatase 2A immunoprecipitates (IP) with antibodies against phosphorylated phosphodiesterase 4 (IB). (b) The binding of total phosphodiesterase 4 (T-PDE4; phosphorylated and non-phosphorylated) was detected by immunoblotting phosphodiesterase 4 in protein phosphatase 2A's immunoprecipitate. (c) Cell lysates were immunoprecipated with protein phosphatase 2A antibody and immunoblotted with the same antibody as an internal control ($n = 3$). (B) Data shown expressed the relative degree of association between phosphorylated phosphodiesterase 4A and protein phosphate 2A (mean ± SEM of three independent experiments.*P < 0.05, ***P < 0.001 as compared with resting values).

of Raf-1 directly or via Ras [41,42]. In neutrophils, PKA has been shown to affect fMLP-induced phosphoinositide 3-kinase activation, which is a critical event in the signaling steps leading to superoxide formation [43]. In the present study, we found that neutrophils treated with ND700C demonstrated a rapid and dose-dependent rise in cAMP. Our data demonstrate that cAMP/PKA signaling in response to ND700C plays an important role in the suppression of neutrophil function. This is due to the inhibitory action of ND700C on fMLP-induced superoxide anion, protein phosphorylation (ERK, Akt and p38) and intracellular calcium mobilization were reversed when neutrophils were pretreated with H89, a PKA inhibitor. The levels of cAMP are under exquisite control in granulocytes. The only way to inactivate cAMP in cells is through the hydrolysis of cAMP to 5′-cAMP by cAMP phosphodiesterase. The predominant phosphodiesterase in granulocytes belongs to the phosphodiesterase 4 family or cAMP-specific phosphodiesterase [37]. ND700C further increased the PGE_1-induced rise in cAMP, this data indicated that ND700C could inhibit phosphodiesterase 4 activity in human neutrophils. However, ND700C did not inhibit phosphodiesterase 4 activity in cellular lysate assay. Phosphodiesterase 4 can be phosphorylated by PKA [44]. Phosphorylation of a site on the UCR1 (upstream conserved region 1) module causes a conformation change and increase the activity of the catalytic domain [45]. In others studies, protein phosphatase 2A was found responsible for the deactivation of phosphodiesterase 4 in Sertoli cells and gastric smooth muscle cells [46,47]. PKA-mediated phosphorylation [48], and protein phosphatase 2A-mediated dephosphorylation, of phosphodiesterase 4 appear to be the main mechanisms of control of phosphodiesterase 4 activities [46]. Lu's study demonstrated that okadaic acid, a protein phosphatase 2A inhibitor, prolonged the respiratory burst in both intact and permeabilized neutrophils through a process analogous to priming [19]. This event raises that possibility that protein phosphate 2A may play a role in modulation of neutrophil activation. In this study, both okadaic acid and caylculin A, two protein phosphatase 2A inhibitors, demonstrated abilities to significantly reverse ND700C-induced increase in cAMP.

This data indicated that protein phosphate 2A was involved in the regulation of cAMP level in the presence of ND700C. To further elucidate the role of protein phosphate 2A in ND700C-treated neutrophils, we demonstrated that ND700C can activate protein phosphatase 2A. Moreover, ND700C was found to increases the association between protein phosphatase 2A and phosphorylated phosphodiesterase 4. According to this result, we hypothesize that ND700C-activated protein phosphatase 2A may dephosphorylate phosphodiesterase 4, thereby inhibiting its activity. However, the immunoprecipitated phosphorylated phosphodiesterase 4 with protein phosphatase 2A occurs prior to the increase in protein phosphatase 2A activity by ND700C. Further study is needed.

ND700C is a novel benzodiazepines derivative. Little is known about the mechanism through which peripheral benzodiazepine receptor ligands exert their anti-inflammatory action. Peripheral benzodiazepine receptor ligands reduce the macrophage secretion of interleukin-1, interleukin-6 and TNF-α [22]. In paw edema, peripheral benzodiazepine receptor ligands probably have the capacity to inhibit neutrophil migration, which are the main cells present in the mouse paw after the injection of carrageenan [49]. The data obtained in this study suggest that ND700C has anti-inflammatory properties, based on their capacity to inhibit the action of fMLP. This action could be mainly due to the inhibition of cathepsin G release and superoxide anion production induced by fMLP. We hypothesize that ND700C activates protein phosphatase 2A activity, which subsequently dephosphorylates phosphodiesterase 4 and renders it inactive, resulting in an increased cAMP level. In the next study, we will examine if this mechanism is mediated through benzodiazepine receptors. Peripheral benzodiazepine receptor ligands may prove to be of pharmacological interest as a new group of anti-inflammatory agents.

Acknowledgements

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