

# Sulfhydryl Modification Induces Calcium Entry through IP<sub>3</sub>-Sensitive Store-Operated Pathway in Activation-Dependent Human Neutrophils

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## Abstract

As the first line of host defense, neutrophils are stimulated by pro-inflammatory cytokines from resting state, facilitating the execution of immunomodulatory functions in activation state. Sulfhydryl modification has a regulatory role in a wide variety of physiological functions through mediation of signaling transductions in various cell types. Recent research suggested that two kinds of sulfhydryl modification, S-nitrosylation by exogenous nitric oxide (NO) and alkylation by N-ethylmaleimide (NEM), could induce calcium entry through a non-store-operated pathway in resting rat neutrophils and DDT<sub>1</sub>MF-2 cells, while in active human neutrophils a different process has been observed by us. In the present work, data showed that NEM induced a sharp rising of cytosolic calcium concentration ( $[Ca^{2+}]_c$ ) without external calcium, followed by a second  $[Ca^{2+}]_c$  increase with readdition of external calcium in phorbol 12-myristate 13-acetate (PMA)-activated human neutrophils. Meanwhile, addition of external calcium did not cause  $[Ca^{2+}]_c$  change of  $Ca^{2+}$ -free PMA-activated neutrophils before application of NEM. These data indicated that NEM could induce believable store-operated calcium entry (SOCE) in PMA-activated neutrophils. Besides, we found that sodium nitroprusside (SNP), a donor of exogenous NO, resulted in believable SOCE in PMA-activated human neutrophils via S-nitrosylation modification. In contrast, NEM and SNP have no effect on  $[Ca^{2+}]_c$  of resting neutrophils which were performed in suspension. Furthermore, 2-Aminoethoxydiphenyl borate, a reliable blocker of SOCE and an inhibitor of inositol 1,4,5-trisphosphate (IP<sub>3</sub>) receptor, evidently abolished SNP and NEM-induced calcium entry at 75  $\mu$ M, while preventing calcium release in a concentration-dependent manner. Considered together, these results demonstrated that NEM and SNP induced calcium entry through an IP<sub>3</sub>-sensitive store-operated pathway of human neutrophils via sulfhydryl modification in a PMA-induced activation-dependent manner.

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## Introduction

As the first line of host defense against invasion of pathogenic microbes, neutrophils play a crucial role in a variety of inflammatory responses [1,2]. In generally, researchers have known that there are two main states for neutrophils: resting and activation. The activation of the human neutrophils leads to a spectrum of responses, including aggregation, stimulation of the respiratory burst and degradation of microbes. During the transition of neutrophils from resting to activation state, the increase of cytosolic calcium concentration ( $[Ca^{2+}]_c$ ), due to the calcium release and calcium entry, has been considered as the major intracellular modification factor [3,4]. As a second messenger,  $[Ca^{2+}]_c$  executes profound effects across a wide range of physiological functions and signaling transductions in neutrophil immunity responses, including the initiation of cytoskeletal changes, degranulation, adhesion, apoptosis, and oxidative burst [5]. In particular, store-operated calcium entry (SOCE), a critical mechanism of  $[Ca^{2+}]_c$  regulation in non-excitable cells [6-8], plays a crucial role in modulating the immune responses of neutrophils [9-11].

Sulfhydryl groups, a key component of proteins, can be modified by different agents through oxidation, reduction, or alkylation. These modifications play a significant role in various signaling transductions though affecting the activity and structure of different kinds of enzymes, receptors, and ion channel proteins [12,13]. For instance, N-ethylmaleimide (NEM), a irreversible sulfhydryl-alkylating agent, has long been used for the chemical modification of sulfhydryl groups of cysteine residues in various cells types [14-16]. More recently, studies have indicated that nitric oxide (NO) conveys a large part of ubiquitous influence via S-nitrosylation of sulfhydryl groups for providing redox-based physiological regulation [17]. S-Nitrosylation of proteins, a modification of cysteine residues by NO [18], first demonstrated by Stamler [19], performs an important regulatory role in cell apoptosis [20], ion channel activation [21], mitochondrial caspases modulation.

For resting rat neutrophils, Wang *et al* demonstrated that alkylating agent NEM and NO donor 5-amino-3-(3,4-dichlorophenyl)-1,2,3,4-oxatriazolium (GEA3162) just stimulated calcium entry through a non-store-operated pathway via direct sulfhydryl modification [22-24]. In addition, Gill *et al* reported that NEM

and membrane-permeant NO donors could activate non-store-operated calcium entry through sulfhydryl modification in DDT<sub>1</sub>MF-2 cells [25–27]. However, in phorbol 12-myristate 13-acetate (PMA)-activated human neutrophils, we have previously shown that NO donor sodium nitroprusside (SNP) induced calcium release from inositol 1,4,5-trisphosphate (IP<sub>3</sub>) receptor-sensitive stores via S-nitrosylation of sulfhydryl groups [28]. These reports indicated that sulfhydryl modification had different effects on [Ca<sup>2+</sup>]<sub>c</sub> of activated neutrophils as compared to resting neutrophils. The present study investigated the calcium entry mechanism induced by the alkylation agent NEM and the exogenous NO donor SNP in PMA-activated human neutrophils. We asked whether the effects of NEM and SNP on [Ca<sup>2+</sup>]<sub>c</sub> were due to the activation of neutrophils. Data showed that NEM or SNP-triggered [Ca<sup>2+</sup>]<sub>c</sub> increase was due to a IP<sub>3</sub> receptor-sensitive calcium release and SOCE via sulfhydryl modification in PMA-induced activation-dependent human neutrophils.

## Results

### TG or FMLP induces SOCE in PMA-activated neutrophils

PMA, a potent protein kinase C (PKC) activator, can activate NADPH oxidase by stimulating PKC and subsequently induce respiratory burst in neutrophils [29,30]. In our experiments, resting human neutrophils were activated by incubation with 2 μM PMA for 5 min at 37°C, which caused adhesion to the glass surface resulting in observable flattening of the cells with differential interference contrast (DIC) microscopy (Fig. 1A and B). To determine whether the mechanism of SOCE was in use after PMA activation, we measured the [Ca<sup>2+</sup>]<sub>c</sub> responses to thapsigargin (TG) [31,32] and N-formyl-methionyl-leucyl-phenylalanine (FMLP) [33,34], two powerful activators of SOCE in neutrophils. Figure 1 showed the effects of TG and FMLP on [Ca<sup>2+</sup>]<sub>c</sub> of PMA-pretreated neutrophils. Addition of 2 μM TG induced a sharp rising of [Ca<sup>2+</sup>]<sub>c</sub> due to calcium store release in Ca<sup>2+</sup>-containing medium, followed by a second extended rising of [Ca<sup>2+</sup>]<sub>c</sub> (Fig. 1C). The latter peak represented extracellular calcium entry and was abolished when the medium was replaced with nominally Ca<sup>2+</sup>-free HBSS as shown in Figure 1C and D. Moreover, readdition of 1 mM external calcium evidently resulted in a repeat of calcium entry (Fig. 1C and D), indicating the familiar response for SOCE. As illustrated in Figure 1G and H, FMLP (2 μM) showed the same effects on [Ca<sup>2+</sup>]<sub>c</sub> in the presence or absence of external calcium in PMA-activated neutrophils. In addition, when 75 μM 2-Aminoethoxydiphenyl borate (2-APB) was added during the entry phase, it rapidly and obviously abolished calcium entry (Fig. 1E). When 75 μM 2-APB was added together with TG, it had little effect on the calcium release but completely inhibited the calcium entry component of [Ca<sup>2+</sup>]<sub>c</sub> in Ca<sup>2+</sup>-containing buffer (Fig. 1F). It is well known that 2-APB is a potent membrane-permeant antagonist of IP<sub>3</sub> receptor [35–38] and a reliable blocker of SOCE [9,38,39]. Taken together, these results clearly indicated that the SOCE mechanism still occurred in PMA-activated neutrophils.

### NEM or SNP induces SOCE via sulfhydryl modification in PMA-activated neutrophils

As shown in Figure 2A and B, NEM (100 μM) or SNP (500 μM) induced a rapid [Ca<sup>2+</sup>]<sub>c</sub> increase followed by a second extended rising of on [Ca<sup>2+</sup>]<sub>c</sub> in PMA-pretreated neutrophils within Ca<sup>2+</sup>-containing HBSS. When cells were pretreated with NEM (500 μM), the effect of SNP on [Ca<sup>2+</sup>]<sub>c</sub> was significantly abolished (gray line in Fig. 2B). Our previous work [28] also demonstrated that application of 4H-8-bromo-1,2,4-oxadiazolo(3,4-d) benz

(b-1,4) oxazin-1-one (NS2028), a specific inhibitor of soluble guanylate cyclase, had no effect on SNP-induced the rising of [Ca<sup>2+</sup>]<sub>c</sub> in PMA-activated neutrophils. These results showed that two kinds of sulfhydryl modification, alkylation by NEM and S-nitrosylation by SNP, could cause obvious [Ca<sup>2+</sup>]<sub>c</sub> increase in PMA-activated neutrophils.

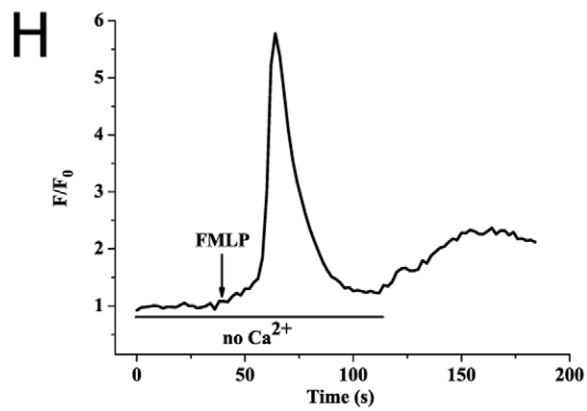
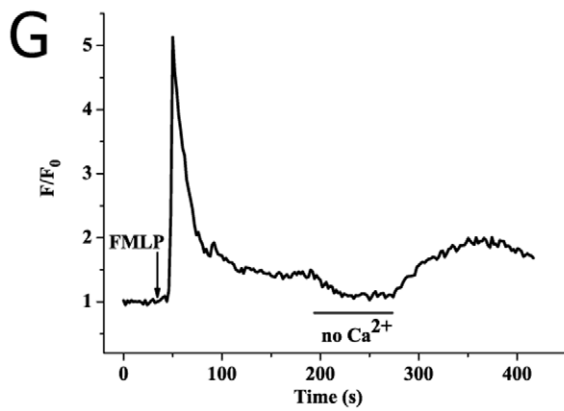
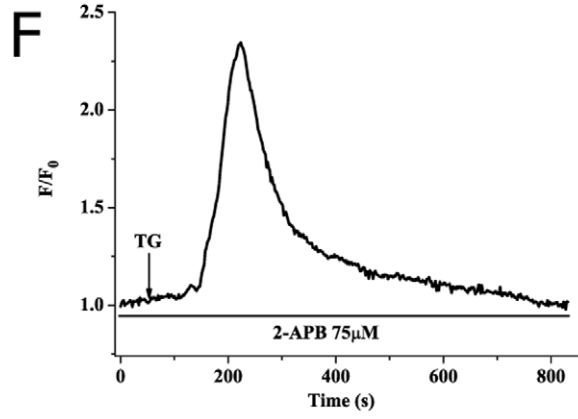
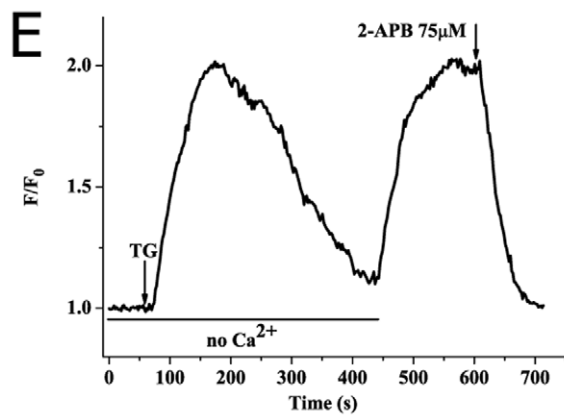
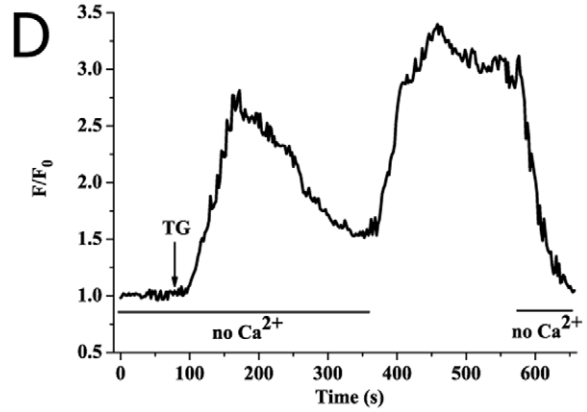
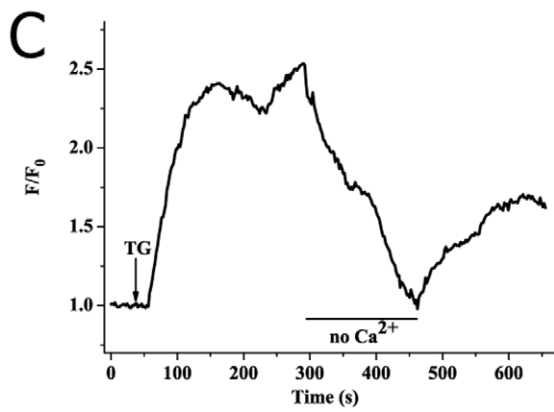
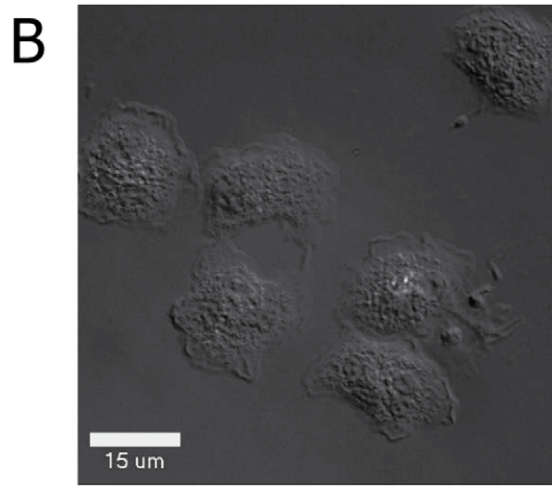
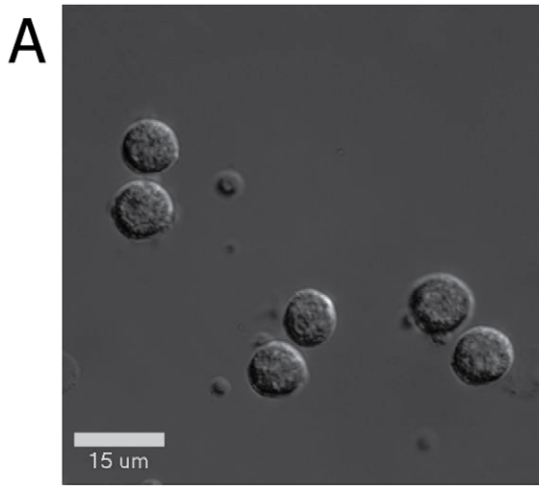
Moreover, in the absence of external Ca<sup>2+</sup>, the increase of [Ca<sup>2+</sup>]<sub>c</sub> was still observed with the latter peak being completely blocked, indicating that calcium stores release and calcium entry were co-existing (Fig. 2C). To further explore the relationship between calcium stores release and calcium entry, [Ca<sup>2+</sup>]<sub>c</sub> were triggered with NEM (100 μM) or SNP (500 μM) in the absence of Ca<sup>2+</sup> followed by readdition of external Ca<sup>2+</sup> in PMA-activated neutrophils. It was found that subsequent addition of external Ca<sup>2+</sup> resulted in a further calcium entry event after stimulation by NEM or SNP, demonstrating calcium entry should be store-depletion-dependent (Fig. 2D and E). Besides, addition of 1 mM external calcium had no effect on [Ca<sup>2+</sup>]<sub>c</sub> of Ca<sup>2+</sup>-free PMA-activated neutrophils before application of stimulation (Fig. 2F). In summary, NEM and SNP could induce believable SOCE via sulfhydryl modification in PMA-activated neutrophils.

### The effect of NEM or SNP on [Ca<sup>2+</sup>]<sub>c</sub> of resting human neutrophils

To further determine whether SOCE induced by NEM and SNP was due to the activation of neutrophils, experiments were performed with resting human neutrophils in suspension (1×10<sup>6</sup>/mL) using spectrofluorometer. It was found that NEM (100 μM) or SNP (500 μM) did not caused [Ca<sup>2+</sup>]<sub>c</sub> increase with Ca<sup>2+</sup>-containing buffer in resting neutrophils as shown in Figure 3A. However, FMLP (2 μM), a classic activator of calcium mobilization in neutrophils [33,34], induced evident rising of [Ca<sup>2+</sup>]<sub>c</sub> in resting neutrophils served as a positive control (Fig. 3B). Considered together, these results suggested that NEM and SNP-induced SOCE via sulfhydryl modification was dependent on activation of neutrophils.

### The effect of 2-APB on NEM or SNP-induced SOCE in PMA-activated neutrophils

To further study these calcium stores release and calcium entry responsible for sulfhydryl modification-induced SOCE in PMA-activated neutrophils, we measured [Ca<sup>2+</sup>]<sub>c</sub> responses to the potent IP<sub>3</sub> receptor [35–38] and SOCE [9,38,39] blocker 2-APB in Ca<sup>2+</sup>-containing medium. It was found that addition of NEM (100 μM) caused no increase in [Ca<sup>2+</sup>]<sub>c</sub> elevation when cells were preincubated with 150 μM 2-APB as shown in Figure 4A (-Δ-curve) and B (maximal value of F/F<sub>0</sub>: 5.062±0.121 for 2-PAB absence vs. 1.121±0.013 for 150 μM 2-APB, n=30 cells, P<0.01). This result indicated that calcium release came from IP<sub>3</sub> receptor-sensitive stores. Meanwhile, application of 75 μM 2-APB prior to NEM stimulation completely blocked the calcium entry (-○- curve in Fig. 4A) but partially inhibited calcium release (maximal value of F/F<sub>0</sub>: 5.062±0.121 for 2-APB absence vs. 3.191±0.103 for 75 μM 2-APB, n=30 cells, P<0.01; Fig. 4B). 2-APB also had the same effect on SNP-induced [Ca<sup>2+</sup>]<sub>c</sub> elevation in PMA-activated neutrophils (data not shown). In addition, addition of 75 μM 2-APB to cells already responding to NEM or SNP resulted in rapid decrease in [Ca<sup>2+</sup>]<sub>c</sub> back to basal levels (Fig. 4C and D). Together, these results demonstrated that 2-APB could totally inhibit calcium entry at 75 μM while blocking calcium release in a concentration-dependent manner, indicating that NEM or SNP induced calcium entry via depletion of IP<sub>3</sub> receptor-sensitive stores.



**Figure 1. SOCE still occurs but is blocked by 2-APB in PMA-activated human neutrophils.** (A) Spherical resting neutrophils visualised by DIC. (B) When PMA was added (2  $\mu$ M for 5 min at 37°C), neutrophils were activated and spread on glass surface thereby flattening their morphology as observed by DIC. (C) After calcium store depletion by a calcium pump blocker TG (2  $\mu$ M), external  $\text{Ca}^{2+}$  (1 mM) was transiently removed 4 min later; then, further addition of 1 mM external  $\text{Ca}^{2+}$  resulted in obvious calcium entry, revealing the putative response for SOCE. (D) 2  $\mu$ M TG was added in the absence of  $\text{Ca}^{2+}$ , followed by readdition of 1 mM external  $\text{Ca}^{2+}$ . (E) After calcium stores depletion by 2  $\mu$ M TG, PMA-activated neutrophils were treated with 75  $\mu$ M 2-APB. (F) 2  $\mu$ M TG was added together with 75  $\mu$ M 2-APB. (G, H) Calcium stores were triggered with G protein-coupled agonist FMLP (2  $\mu$ M) in the presence/absence of calcium followed by removal/readdition of 1 mM external  $\text{Ca}^{2+}$ .  
doi:10.1371/journal.pone.0025262.g001

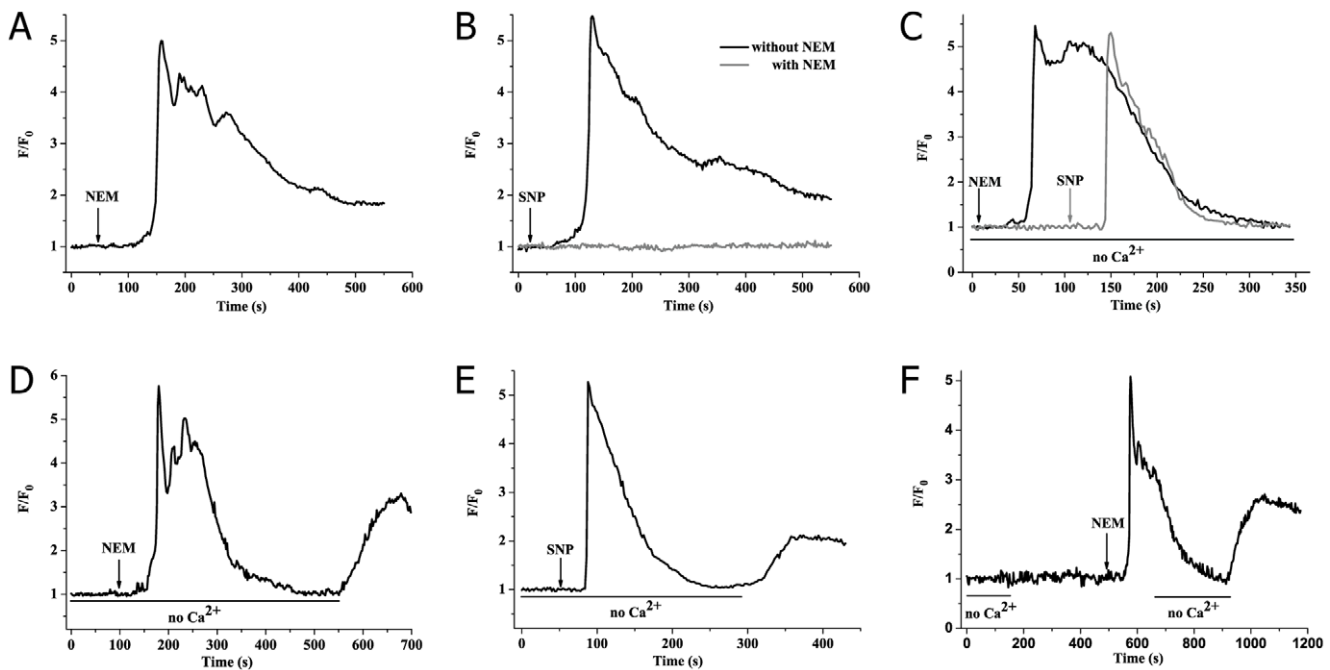
### The effect of FMLP or U73122 on sulfhydryl modification-induced SOCE in PMA-activated neutrophils

The classic G protein-coupled receptors (GPCRs)-G protein-Phospholipase C (PLC)-IP<sub>3</sub> signaling pathway is well-known in the terms of calcium mobilization for neutrophils. FMLP, a classic activator of neutrophils, could result in SOCE on neutrophils through this GPCRs-G protein-PLC-IP<sub>3</sub> signaling pathway [33,34,40]. Therefore, to investigate whether the upstream signaling pathway of sulfhydryl modification-induced SOCE was similar to that of FMLP, PMA-activated neutrophils were stimulated by NEM, SNP and FMLP within  $\text{Ca}^{2+}$ -containing medium in different chronological order. Data showed that FMLP (2  $\mu$ M) had little capacity for inducing NEM (100  $\mu$ M) or SNP (500  $\mu$ M)-pretreated  $[\text{Ca}^{2+}]_c$  change in PMA-activated human neutrophils (Fig. 5A and B). This inhibitory effect proposed that sulfhydryl modification may induce SOCE through a GPCRs-G protein-PLC signaling pathway similar to that of FMLP. On the contrary, addition of NEM or SNP after FMLP activation still caused a rapid rising of  $[\text{Ca}^{2+}]_c$  (Fig. 5C and D). Comparing with results in Figure 5A and B, it indicated that the pathway of GPCRs-G protein-PLC maybe not the only means for sulfhydryl modification-induced calcium mobilization.

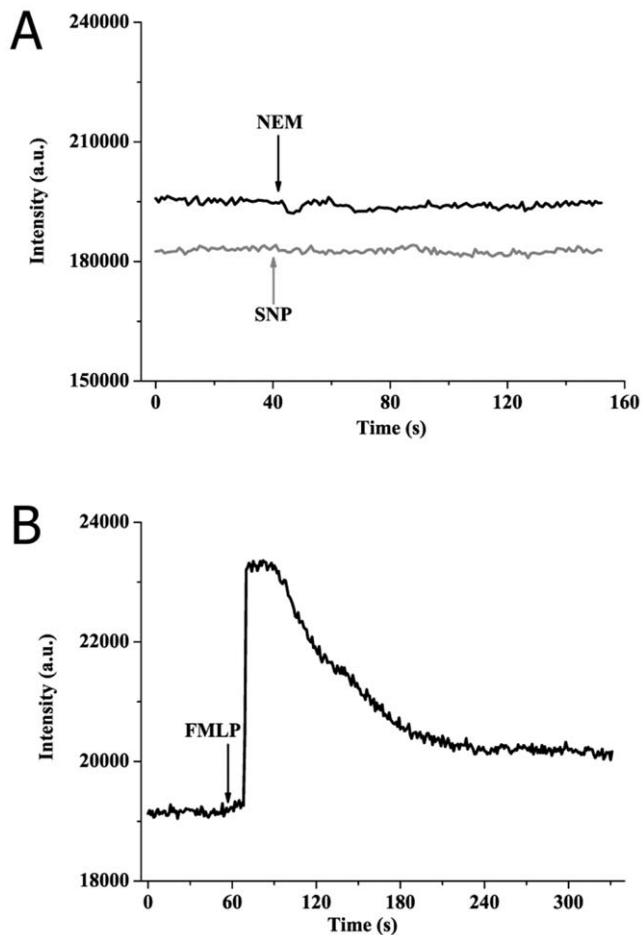
To further study whether sulfhydryl modification-induced SOCE was due to activation of PLC-IP<sub>3</sub>-dependent signaling pathway, PMA-activated neutrophils were stimulated by NEM or SNP in the presence of 1-[6-([(17 $\beta$ )-3-methoxyestra-1,3,5,(10)-trien-17-yl]-amino)-hexyl]-2,5-dione (U73122), a putative inhibitor of PLC [41,42]. We found that NEM (100  $\mu$ M) had no effect on  $[\text{Ca}^{2+}]_c$  elevation in  $\text{Ca}^{2+}$ -containing medium when PMA-activated neutrophils were treated with U73122 (10  $\mu$ M) as summarized in Figure 6A (gray line) and B (maximal value of  $F/F_0$ : 5.062 $\pm$ 0.121 for NEM vs. 1.110 $\pm$ 0.012 for U73122+NEM, n = 30 cells, P<0.01). Meanwhile, the effect of SNP on  $[\text{Ca}^{2+}]_c$  was completely inhibited in  $\text{Ca}^{2+}$ -containing buffer in the presence of U73122 (maximal value of  $F/F_0$ : 4.651 $\pm$ 0.253 for SNP vs. 1.121 $\pm$ 0.012 for U73122+SNP, n = 30 cells, P<0.01; Fig. 6B). Take together, it was suggested that PLC activation may be involved in the signaling transduction mechanism of NEM or SNP-induced SOCE. Besides, we clearly observed that U73122 resulted in morphological contraction of PMA-induced spreading neutrophils (Fig. 6C and D).

### Discussion

There are a variety of agonists can activate resting neutrophils *in vitro*, including PMA, FMLP, leukotriene B<sub>4</sub>, interleukin-8 and



**Figure 2. NEM or SNP results in SOCE via sulfhydryl modification in PMA-activated neutrophils.** (A) Representative tracing of  $[\text{Ca}^{2+}]_c$  elevation induced by 100  $\mu$ M NEM in the presence of external  $\text{Ca}^{2+}$ . (B) Representative tracings of 500  $\mu$ M NEM inhibitory effect on  $[\text{Ca}^{2+}]_c$  change caused by SNP (500  $\mu$ M) within  $\text{Ca}^{2+}$ -containing medium. (C) Typical tracings of  $[\text{Ca}^{2+}]_c$  responses for PMA-activated neutrophils to 100  $\mu$ M NEM or 500  $\mu$ M SNP stimulation in  $\text{Ca}^{2+}$ -free buffer. (D, E),  $[\text{Ca}^{2+}]_c$  of PMA-pretreated neutrophils was triggered with NEM (100  $\mu$ M) within  $\text{Ca}^{2+}$ -free HBSS followed by readdition of 1 mM external  $\text{Ca}^{2+}$ . (F) 1 mM external calcium was added to  $\text{Ca}^{2+}$ -free PMA-activated neutrophils before application of stimulation, followed by addition of 100  $\mu$ M NEM.  
doi:10.1371/journal.pone.0025262.g002



**Figure 3. NEM and SNP have no effect on  $[Ca^{2+}]_c$  of resting human neutrophils.** (A) Representative tracings of  $[Ca^{2+}]_c$  responses to 100  $\mu$ M NEM (black line) and 500  $\mu$ M SNP (gray line). (B) Typical tracing of  $[Ca^{2+}]_c$  change induced by 1  $\mu$ M FMLP. For all experiments, resting human neutrophils were performed in suspension ( $1 \times 10^6$ /mL) within  $Ca^{2+}$ -containing buffer. doi:10.1371/journal.pone.0025262.g003

platelet-activating factor [1,43,44]. PMA, a diacylglycerol (DAG) analog, specifically activates PKC which in turn activates NADPH oxidase, resulting in stimulation of the respiratory burst of neutrophils [29,30]. Although PMA is not a physiological neutrophil activator, it was widely used as a model stimulus to study inflammatory responses of neutrophils in vitro experiments [45–48]. There are two main reasons for us to select PMA as an activator of neutrophils. First, PMA activates neutrophils without an apparent rising of  $[Ca^{2+}]_c$  [29,49,50] and maintains stability of  $[Ca^{2+}]_c$  basal level before stimulation, as shown in all our experiments of  $[Ca^{2+}]_c$  measurement. Second, the activation of the human neutrophils by PMA leads to depolarization of the plasma membrane [29,51] as seen in Figure 1B, as well as inhibiting spontaneous polarization-induced random migration. Both these attributes ease measurement of  $[Ca^{2+}]_c$  in single cell level with fluorescence microscopy. On the contrary, FMLP, a physiological neutrophil activator, not only caused strong calcium mobilization but also induced obvious migration of neutrophils, thereby complicating measurements of  $[Ca^{2+}]_c$  change in single cell level.

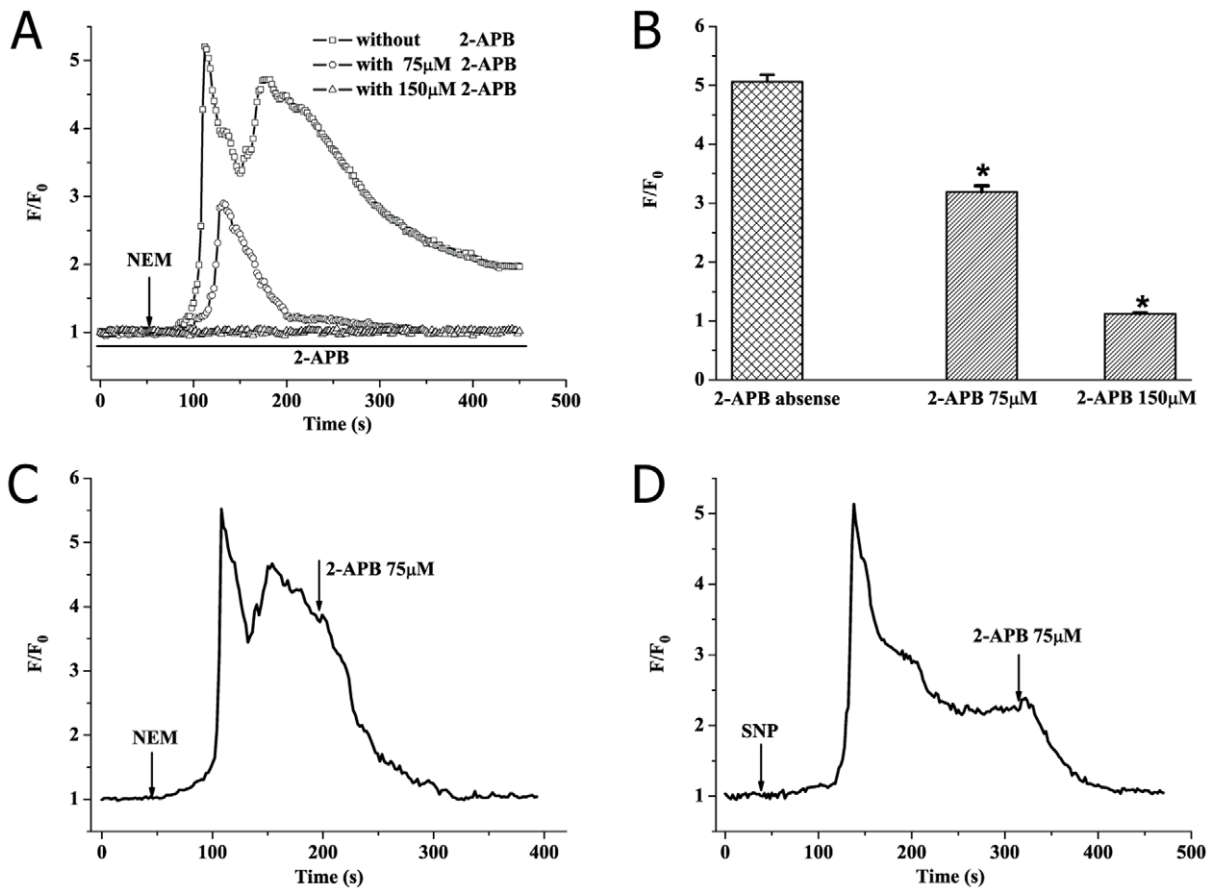
Our studies using TG and FMLP firstly demonstrated that the mechanism of SOCE still exist in PMA-activated neutrophils (Fig. 1). TG, a specific inhibitor of sarcoplasmic/endoplasmic reticulum

$Ca^{2+}$  ATPases [32], causes calcium stores depletion and calcium entry via SOCE pathway in many types of cells including neutrophils [31]. FMLP, a chemotactic tripeptide, is well known as a potent and classic SOCE activator in neutrophils [33,34]. We subsequently found that NEM or SNP induced significant rising of  $[Ca^{2+}]_c$  in PMA-activated neutrophils no matter whether calcium was in the buffer or not (Fig. 2A, 2B, 2C). SNP had no effect on  $[Ca^{2+}]_c$  when PMA-activated neutrophils were pretreated with NEM (Fig. 2B). Readdition of external  $Ca^{2+}$  led to a further calcium entry event after activation of NEM or SNP (Fig. 2D and E), indicating calcium entry should be store-depletion-dependent. Besides, we found that addition of 1 mM external calcium did not induce  $[Ca^{2+}]_c$  increase in  $Ca^{2+}$ -free PMA-activated neutrophils without any stimulation, and subsequently adding NEM (100  $\mu$ M) to achieve an apparent SOCE observed (Fig. 2F). Moreover, we found that NEM or SNP did not induced change of  $[Ca^{2+}]_c$  in resting human neutrophils which were performed in suspension as shown in Figure 3. In conclusion, these results indicated that NEM and SNP could induce a believable response for SOCE via sulfhydryl modification in PMA-induced activation-dependent human neutrophils.

2-APB, a specific inhibitor of IP<sub>3</sub> receptor [35–38], abolished NEM-induced calcium release in a concentration-dependent manner (Fig. 4A and B). 2-APB also had the same effect on SNP-induced  $[Ca^{2+}]_c$  elevation in PMA-activated neutrophils (data not shown). Besides, much research has demonstrated that 2-APB was a reliable blocker of SOCE in recent years [9,38,39]. Gill *et al* reported that 2-APB showed obvious inhibitory effect on SOCE at 75  $\mu$ M [52]. In our experiments, application of 2-APB (75  $\mu$ M) completely inhibited TG, NEM and SNP-induced SOCE in PMA-activated human neutrophils (Fig. 1E, F and Fig. 4). Moreover, we found that ruthenium red, a specific inhibitor of ryanodine receptor-sensitive stores [53], had no effect on NEM or SNP-induced calcium release PMA-activated neutrophils (data not shown). Considered together, these data further indicated that NEM and SNP resulted in calcium entry through IP<sub>3</sub> receptor-sensitive stores-operated pathway in PMA-activated human neutrophils.

In addition, it is easy to find that we use a relatively high an order (2  $\mu$ M) of magnitude higher than usual-PMA-dose to activate neutrophils from the resting state. It should be important to see whether the same phenomenon occurs at a submicromolar concentration. First, we found that 200 nM PMA (30 min at 37°C) could result in neutrophils obvious spreading on glass. Under this condition, NEM (100  $\mu$ M) or SNP (500  $\mu$ M) caused obvious  $[Ca^{2+}]_c$  increase (data not shown). However, further result showed that neutrophils could not spread on glass evidently when neutrophils were preincubated with 200 nM for 5 min at 37°C. Subsequent application of NEM or SNP had no obvious effect on  $[Ca^{2+}]_c$  (data not shown). It could be proposed that the activation ability of PMA on neutrophils were concentration and activation time-dependent. Lower concentration of PMA used, more activation time needed. The degree of neutrophils adhesion on glass substrate may have effect on sulfhydryl modification-induced  $[Ca^{2+}]_c$  change.

Having known this, we sought to offer an explanation as to the upstream signaling pathway of NEM and SNP induced-SOCE. For this aim, we firstly studied the effect of FMLP on NEM or SNP-induced SOCE in PMA-activated neutrophils. It is well known [33,34,42] that FMLP, a classic activator of neutrophils, can cause stimulation of GPCRs on neutrophils, with consequent activation of PLC and generation of IP<sub>3</sub> and DAG by breakdown of membrane phosphatidyl inositol 4,5-bisphosphate. Finally, IP<sub>3</sub> interacts with  $Ca^{2+}$ -mobilizing receptors, resulting in IP<sub>3</sub> receptor-sensitive calcium stores release and SOCE. Our data showed that NEM or SNP blocked calcium signaling induced by subsequent stimulation of FMLP (Fig. 5A and B). In contrast, FMLP failed to



**Figure 4. 2-APB completely blocks SOCE in response to sulfhydryl modification in PMA-activated human neutrophils.** All experiments were performed with cells in  $\text{Ca}^{2+}$ -containing medium. (A) Typical tracings of  $[\text{Ca}^{2+}]_c$  changes of PMA-activated neutrophils induced by 100  $\mu\text{M}$  NEM in the presence of 2-APB at 0  $\mu\text{M}$  ( $\square$ - curve), 75  $\mu\text{M}$  ( $\circ$ - curve), 150  $\mu\text{M}$  ( $\triangle$ - curve), respectively. (B) Statistic data from three independent experiments (means  $\pm$  SEM,  $n = 30$  cells). \* $P < 0.01$ , compared with 2-APB absence. (C, D)  $[\text{Ca}^{2+}]_c$  increase was induced by NEM (100  $\mu\text{M}$ ) or SNP (500  $\mu\text{M}$ ) in PMA-activated neutrophils, followed by addition of 75  $\mu\text{M}$  2-APB at  $t = 197$  or  $t = 314$  s, resulting in return to baseline  $[\text{Ca}^{2+}]_c$ . doi:10.1371/journal.pone.0025262.g004

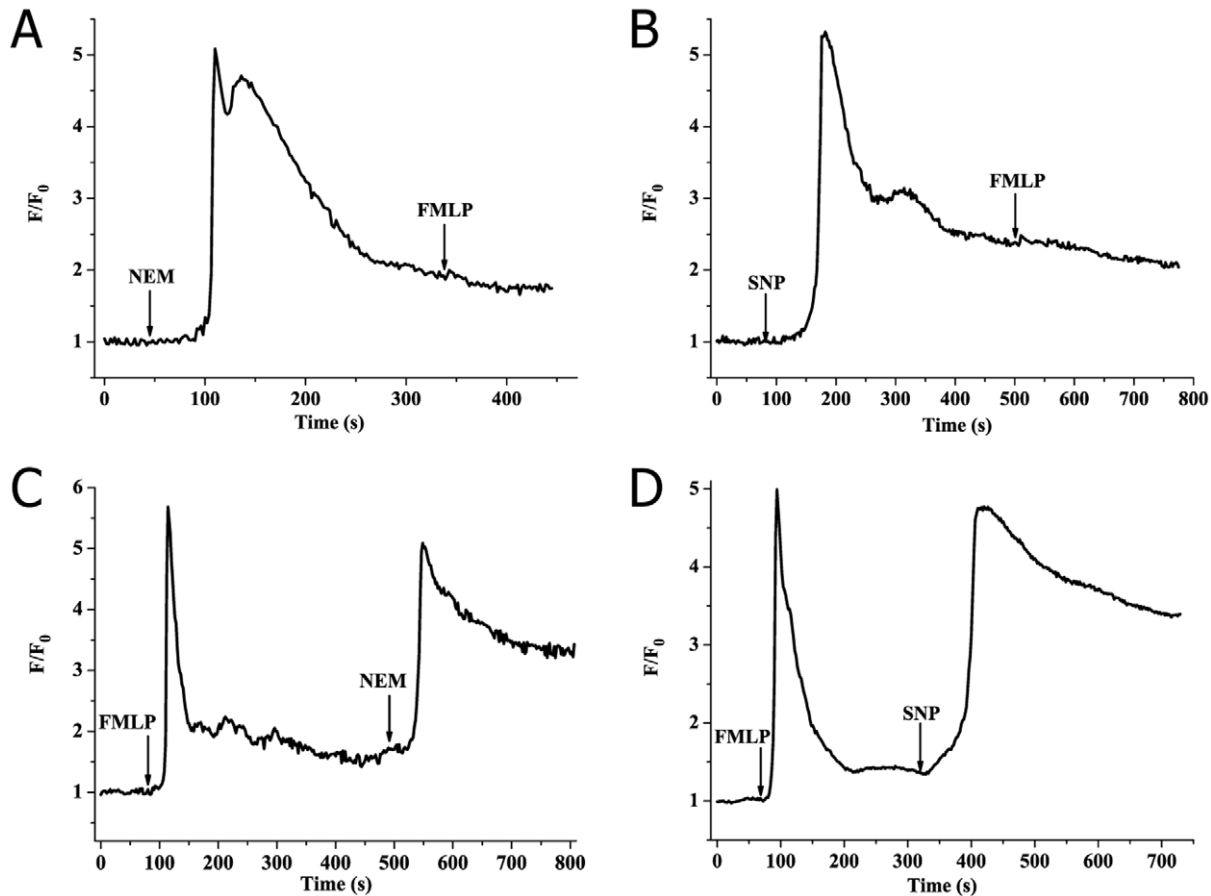
inhibit calcium elevation caused by subsequent activation by NEM or SNP in PMA-activated neutrophils (Fig. 5C and D). Together, these data proposed that NEM or SNP-induced calcium mobilization in PMA-activated neutrophils may involve the same signaling pathway as that of FMLP, namely the GPCRs-G protein-PLC-IP<sub>3</sub> pathway. However, this may not be the only pathway for NEM or SNP-induced SOCE.

Furthermore, it was found that demonstrated that U73122 completely blocked NEM or SNP-induced calcium mobilization in  $\text{Ca}^{2+}$ -containing medium (Fig. 6A and B). U73122 is a putative and specific PLC inhibitor which has become an important tool in establishing the link between PLC activation and intracellular calcium signaling [40,41]. However, we must admit that the inhibitory mechanism of U73122 on NEM or SNP-induced SOCE seems to be problematic because U73122 is a sterol derivative of NEM. Therefore, such data just indicated possible roles for PLC in NEM or SNP-induced calcium responses. In addition, we directly observe an interesting phenomenon that U73122 caused morphological contraction of PMA-induced spreading of neutrophils in glass substrate observed by DIC microscopy (Fig. 6C and D), which is in accordance with observations by Smith *et al* who reported that U73122 could cause a inhibition of PMA-triggered neutrophils adhesion on fibronectin and fetal bovine serum-coated plates [54]. This result also suggested that sulfhydryl modification-induced  $[\text{Ca}^{2+}]_c$

elevation may associate with the degree of neutrophils adhesion on glass substrate similar to that of PMA.

Interestingly, Wang *et al* showed that NO donor GEA3162 and NEM could induce calcium entry through a non-store-operated mechanism via direct sulfhydryl modification in resting rat neutrophils which were performed in suspension [22–24]. Gill *et al* also reported that NO donors and NEM caused non-store-operated calcium entry through sulfhydryl modification in DDT<sub>1</sub>MF-2 cells [25–27]. It is obvious that activation of neutrophils by PMA adherent to a glass substrate give a different result of sulfhydryl modification-induced calcium mobilization compared to resting neutrophils. Other studies reported that neutrophils would give different physiological or psychological responses to stimuli depending on their being in the activation or resting state [44,55]. We analysed that activation of neutrophil respiratory burst may be implicated in the different regulation of  $[\text{Ca}^{2+}]_c$  induced by sulfhydryl modification. To address this hypothesis, diphenylene iodonium (DPI), a putative inhibitor of the respiratory burst-generating NADPH oxidase, was used in the present work. The results provided evidence that DPI had a inhibitory effect on NEM-induced SOCE in PMA-treated neutrophils (data not shown). Our previous report also demonstrated that DPI could block SNP-induced calcium release via S-Nitrosylation [28]. Meanwhile, U73122 completely inhibited NEM and SNP-induced SOCE in PMA-activated neutrophils (Fig. 6A and B). It was reported that





**Figure 5. No further calcium elevation occurs with further addition of FMLP when PMA-activated neutrophils were pretreated NEM or SNP, while addition of NEM or SNP after FMLP stimulation still results in rising of  $[Ca^{2+}]_c$ .** (A, B) Representative tracings of  $[Ca^{2+}]_c$  elevation induced by 2  $\mu$ M FMLP when cells were pretreated NEM (100  $\mu$ M) or SNP (500  $\mu$ M) in PMA-activated neutrophils. (C, D) Typical tracings of  $[Ca^{2+}]_c$  elevation caused by 100  $\mu$ M NEM or 500  $\mu$ M SNP after stimulation of FMLP (2  $\mu$ M). doi:10.1371/journal.pone.0025262.g005

U73122 could inhibit PMA-elicited adhesion-dependent respiratory burst [54]. Moreover, We also found that NEM and SNP did not induce obvious  $[Ca^{2+}]_c$  increase when neutrophils was preincubated with PMA just at submicromolar concentration for little time at 37°C (data not shown). These evidence suggested the activation degree of respiratory burst may contribute to generation of sulfhydryl modification-induced SOCE in neutrophils. However, further study is still needed to elucidate the relationship between activation of respiratory burst-generating NADPH oxidase and sulfhydryl modification.

In summary, we clearly demonstrated that NEM, a thiol-alkylating agent, and SNP, a donor of exogenous NO, induced obvious calcium entry through  $IP_3$ -sensitive store-operated pathway via sulfhydryl modification in PMA-induced activation-dependent human neutrophils. Since SOCE plays a crucial role in neutrophils, this study on investigating sulfhydryl modification-induced SOCE may help contribute towards understanding mechanisms of  $[Ca^{2+}]_c$  regulation surrounding neutrophil inflammatory reactions.

## Materials and Methods

### Ethics Statement

The study was reviewed and approved by the Ethics Committee of Nankai University. All participants gave written informed consent.

### Reagents

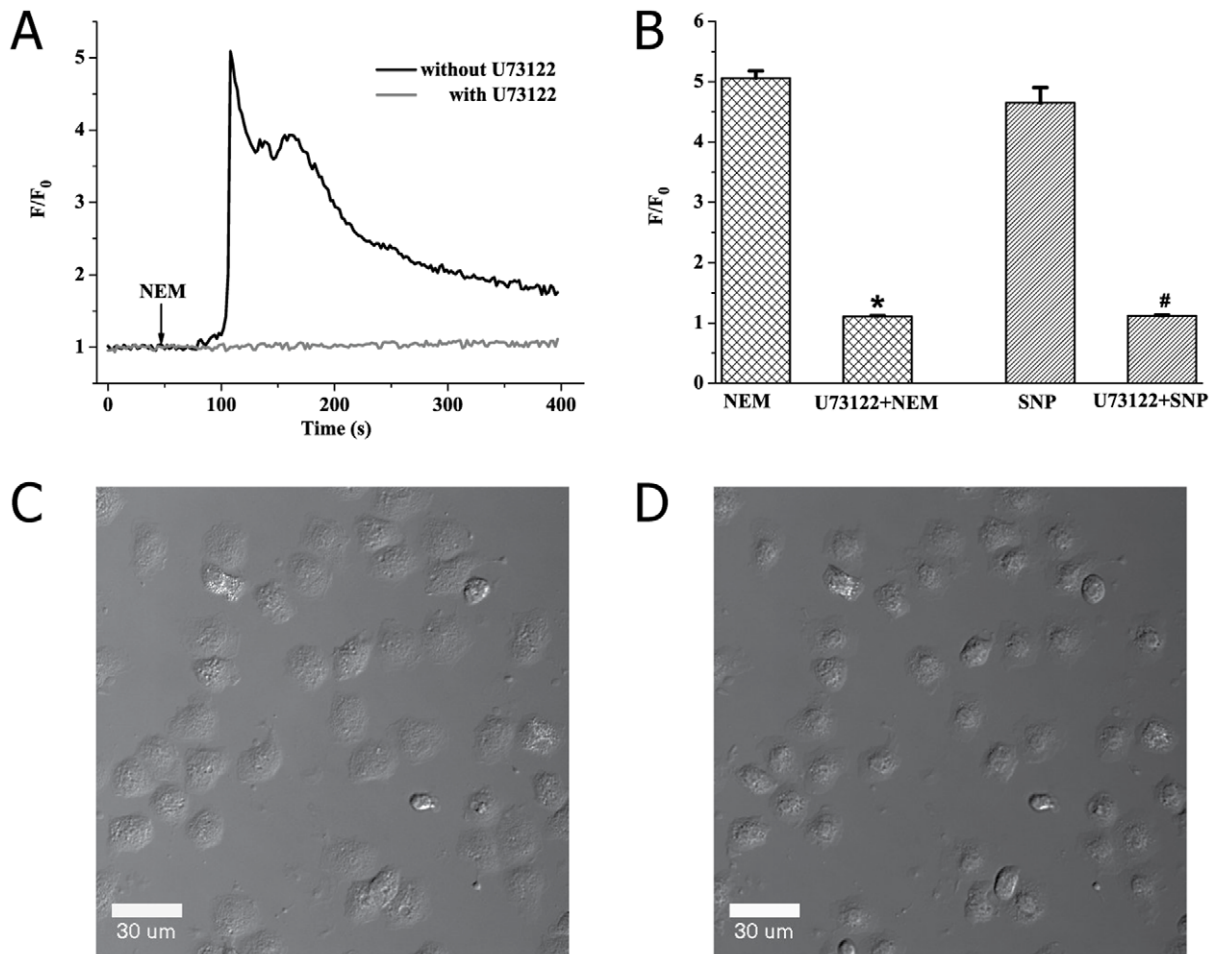
SNP, NEM, PMA, FMLP, 2-APB, TG, U73122, DPI, EGTA, Histopaque 1077 and 1119 solutions, Hoechst 33342, trypan blue were obtained from Sigma–Aldrich (St. Louis, MO, USA). Fluo-3 acetoxymethyl ester (Fluo-3 AM) was from Gibco (Gaithersburg, MD, USA).

### Preparation of human neutrophils

Neutrophils were obtained from the blood of normal healthy adults by step-density gradient centrifugation over Histopaque 1077 and 1119 solutions at 500 $\times$ g for 15 min. The cells were >96% viable as assessed by trypan blue exclusion and had a purity of >95% as demonstrated by Hoechst 33342 nuclear staining. Cells were suspended in HBSS (145 mM NaCl, 5 mM KCl, 1 mM  $CaCl_2$ , 1 mM  $MgCl_2$ , 10 mM HEPES and 10 mM glucose, PH adjusted to 7.4 with NaOH 1 mM) and then kept in an ice bath before use.

### Microscopy

For all imaging experiments, cells in chamber (volume, 1 mL) were observed microscopically at 37°C by using a temperature-controlled stage. Fluorescence was performed with a fluorescent microscope (Axio observer D1, Carl Zeiss, Germany) with a 100 W mercury lamp. A shutter (VS25S2ZM1, Uniblitz, USA) was equipped behind the mercury lamp to control the exposure time and the interval time for fluorescence imaging. Fluorescence



**Figure 6. U73122 completely blocks NEM or SNP-induced SOCE and inhibits PMA-elicited spreading of neutrophils.** (A) representative tracings of  $[Ca^{2+}]_c$  elevation induced by NEM (100  $\mu$ M) in the presence or absence of U73122 (10  $\mu$ M) in  $Ca^{2+}$ -containing buffer. (B) Statistic results are means  $\pm$  S.E.M (n = 30 from three independent experiments). \* $P < 0.01$ , compared with NEM. # $P < 0.01$ , compared with SNP. (C) PMA-activated and adherent neutrophils image was obtained by DIC. (D) The effect of U73122 (10  $\mu$ M) on morphology of PMA-activated neutrophils. doi:10.1371/journal.pone.0025262.g006

signaling was detected by an electron multiplying charge coupled device (EMCCD) (DU-897D-CS0-BV, Andor, U.K.,  $\approx -80^\circ C$ ) which was connected to the left exit side of microscope. The EMCCD was controlled by MetaMorph software version 7.1 (Universal Imaging Corp., USA). DIC and fluorescence images were collected by this microscopy system.

#### Measurement of $[Ca^{2+}]_c$

Human neutrophils were incubated with calcium-sensitive probe Fluo-3 AM 4  $\mu$ M for 30 min at  $37^\circ C$  in HBSS. For single cell calcium imaging,  $[Ca^{2+}]_c$  was measured by the micro-imaging system as shown above. Fluo-3 AM-loaded cells were excited by a mercury lamp with a 485/20 nm excitation filter, and fluorescence was collected by a fluar oil objective with a 510 nm long-pass dichroic mirror and a 540/50 emission filter. Each fluorescence image was acquired for 50 msec with a 2 s interval between frames. The obtained images were quantitatively analyzed for changes of fluorescence intensities within region of interest.  $[Ca^{2+}]_c$  change was represented by relative fluorescence intensity ( $F/F_0$ , intensity after stimulation/basal intensity before stimulation). At least 30 individual cells were selected from three independent experiments, with one characteristic  $[Ca^{2+}]_c$  trace plotted to represent  $>10$  similar traces. Besides,  $Ca^{2+}$ -free HBSS

was prepared by substituting  $MgCl_2$  for  $CaCl_2$  at the same concentration, with 2 mM EGTA. For  $[Ca^{2+}]_c$  measurement in suspension, Fluo-3 AM-loaded neutrophils were suspended in HBSS to  $1 \times 10^6$  cells/mL. Fluorescence was monitored with a spectrofluorometer (FSL920, Edinburgh, U.K.) at 530/30 nm with excitation at 488 nm.

#### Statistical analysis

All data were presented as means  $\pm$  standard error of the mean (S.E.M.). The statistical comparison between groups was carried out using Student's *t*-test.  $P < 0.05$  was considered statistically significant.

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#### Author Contributions

Conceived and designed the experiments: LP XW JX. Performed the experiments: LP XW DZ. Analyzed the data: LP XW DZ. Contributed reagents/materials/analysis tools: XZ IL JX. Wrote the paper: LP NH.



## References

- Nathan C (2006) Neutrophils and immunity: challenges and opportunities. *Nat Rev Immunol* 6: 173–182.
- DeCoursey TE, Cherny VV, Zhou W, Thomas LL (2000) Simultaneous activation of NADPH oxidase-related proton and electron currents in human neutrophils. *Proc Natl Acad Sci USA* 97: 6885–6889.
- Merritt JE, Jacob R, Hallam TJ (1989) Use of manganese to discriminate between calcium influx and mobilization from internal stores in stimulated human neutrophils. *J Biol Chem* 264: 1522–1527.
- Harfi I, Corazza F, D'Hondt S, Sariban E (2005) Differential Calcium Regulation of Proinflammatory Activities in Human Neutrophils Exposed to the Neuropeptide Pituitary Adenylate Cyclase-Activating Protein. *J Immunol* 175: 4091–4102.
- Lew DP (2008) Receptor signalling and intracellular calcium in neutrophil activation. *Eur J Clin Invest* 19: 338–346.
- Venkatachalam K, van Rossum DB, Patterson RL, Ma H, Gill DL (2002) The cellular and molecular basis of store-operated calcium entry. *Nat Cell Biol* 4: E263–E272.
- Parekh AB, Putney JW, Jr. (2005) Store-Operated Calcium Channels. *Physiol Rev* 85: 757–810.
- Putney JW, Jr. (2009) Capacitative calcium entry: from concept to molecules. *Immunol Rev* 231: 10–22.
- Itagaki K, Kannan KB, Livingston DH, Deitch EA, Fekete Z, et al. (2002) Store-Operated Calcium Entry in Human Neutrophils Reflects Multiple Contributions from Independently Regulated Pathways. *J Immunol* 168: 4063–4069.
- Granfeldt D, Samuelsson M, Karlsson A (2002) Capacitative  $Ca^{2+}$  influx and activation of the neutrophil respiratory burst. Different regulation of plasma membrane and granule-localized NADPH-oxidase. *J Leukoc Biol* 71: 611–617.
- Montero M, Garcia-Sancho J, Alvarez J (1993) Transient Inhibition by Chemotactic Peptide of a Store-operated  $Ca^{2+}$  Entry Pathway in Human Neutrophils. *J Biol Chem* 268: 13055–13061.
- Winterbourn CC, Hampton MB (2008) Thiol chemistry and specificity in redox signaling. *Free Radic Biol Med* 45: 549–561.
- Dinkova-Kostova AT, Massiah MA, Bozak RE, Hicks RJ, Talalay P (2001) Potency of Michael reaction acceptors as inducers of enzymes that protect against carcinogenesis depends on their reactivity with sulfhydryl groups. *Proc Natl Acad Sci USA* 98: 3404–3409.
- Smyth DG, Nagamatsu A, Fruton JS (1960) Reactions of N-ethylmaleimide. *J Am Chem Soc* 82: 4600–4604.
- Fryer MW (1992) An N-ethylmaleimide-sensitive G-protein modulates Aplysia  $Ca^{2+}$  channels. *Neurosci Lett* 146: 84–86.
- Gibona J, Tua P, Frazzini V, Sensi SL, Bouron A (2010) The thiol-modifying agent N-ethylmaleimide elevates the cytosolic concentration of free  $Zn^{2+}$  but not of  $Ca^{2+}$  in murine cortical neurons. *Cell Calcium* 48: 37–43.
- Hess DT, Matsumoto A, Kim SO, Marshall HE, Stamler JS (2005) Protein S-nitrosylation: purview and parameters. *Nat Rev Mol Cell Biol* 6: 150–166.
- Stamler JS (1994) Redox signaling: nitrosylation and related target interactions of nitric oxide. *Cell* 78: 931–936.
- Stamler JS, Simon DI, Osborne JA, Mullins ME, Jaraki O, et al. (1992) S-Nitrosylation of proteins with nitric oxide: synthesis and characterization of biologically active compounds. *Proc Natl Acad Sci USA* 89: 444–448.
- Benhar M, Stamler JS (2005) Central role for S-nitrosylation in apoptosis. *Nat Cell Biol* 7: 645–646.
- Xu L, Eu JP, Meissner G, Stamler JS (1998) Activation of the cardiac calcium release channel (ryanodine receptor) by poly-S-nitrosylation. *Science* 279: 234–237.
- Wang J (2003) GEA3162 stimulates  $Ca^{2+}$  entry in neutrophils. *Eur J Pharmacol* 458: 243–249.
- Hsu M, Sun S, Chen Y, Tsai C, Huang L, et al. (2005) Distinct effects of N-ethylmaleimide on formyl peptide- and cyclopiazonic acid-induced  $Ca^{2+}$  signals through thiol modification in neutrophils. *Biochem Pharmacol* 70: 1320–1329.
- Hsu M, Chen Y, Huang L, Tsao L, Kuo S, et al. (2006) GEA3162, a nitric oxide-releasing agent, activates non-store-operated  $Ca^{2+}$  entry and inhibits store-operated  $Ca^{2+}$  entry pathways in neutrophils through thiol oxidation. *Eur J Pharmacol* 535: 43–52.
- Favre CJ, Ufret-Vincenty CA, Stone MR, Ma H, Gill DL (1998)  $Ca^{2+}$  Pool Emptying Stimulates  $Ca^{2+}$  Entry Activated by S-Nitrosylation. *J Biol Chem* 273: 30855–30858.
- Ma H, Favre CJ, Patterson RL, Stone MR, Gill DL (1999)  $Ca^{2+}$  Entry Activated by S-Nitrosylation. *J Biol Chem* 274: 35318–35324.
- van Rossum DB, Patterson RL, Ma H, Gill DL (2000)  $Ca^{2+}$  Entry Mediated by Store Depletion, S-Nitrosylation, and TRP3 Channels. *J Biol Chem* 275: 28562–28568.
- Pan L, Zhang X, Song K, Wu X, Xu J (2008) Exogenous nitric oxide-induced release of calcium from intracellular  $IP_3$  stores via S-nitrosylation in respiratory burst-dependent neutrophils. *Biochem Biophys Res Commun* 377: 1320–1325.
- Tauber AI (1987) Protein kinase C and the activation of the human neutrophil oxidase. *Blood* 69: 711–720.
- Chanock SJ, Benna JE, Smith RM, Babior BM (1994) The respiratory burst oxidase. *J Biol Chem* 269: 24519–24522.
- Foder B, Scharff O, Thastrup O (1989)  $Ca^{2+}$  transients and  $Mn^{2+}$  entry in human neutrophils induced by thapsigargin. *Cell Calcium* 10: 477–490.
- Thastrup O, Cullen PJ, Drobak BK, Hanley MR, Dawson AP (1990) Thapsigargin, a tumor promoter, discharges intracellular  $Ca^{2+}$  stores by specific inhibition of the endoplasmic reticulum  $Ca^{2+}$ -ATPase. *Proc Natl Acad Sci USA* 87: 2466–2470.
- Anderson R, Mahomed AG (1997) Calcium efflux and influx in f-met-leu-phe (MLP)-activated human neutrophils are chronologically distinct events. *Clin Exp Immunol* 110: 132–138.
- Anderson R, Steel HC, Tintinger GR (2005) Inositol 1,4,5-triphosphate-mediated shuttling between intracellular stores and the cytosol contributes to the sustained elevation in cytosolic calcium in FMLP-activated human neutrophils. *Biochem Pharmacol* 69: 1567–1575.
- Maruyama T, Kanaji T, Nakade S, Kanno T, Mikoshiba K (1997) 2-APB, 2-aminoethoxydiphenyl borate, a membrane-penetrable modulator of  $Ins(1,4,5)P_3$ -induced  $Ca^{2+}$  release. *J Biochem* 122: 498–505.
- Ma HT, Patterson RL, van Rossum DB, Birnbaumer L, Mikoshiba K, et al. (2000) Requirement of the inositol trisphosphate receptor for activation of store-operated  $Ca^{2+}$  channels. *Science* 287: 1647–1651.
- Soulsby MD, Wojcikiewicz RJH (2002) 2-Aminoethoxydiphenyl borate inhibits inositol 1,4,5-triphosphate receptor function, ubiquitination and downregulation, but acts with variable characteristics in different cell types. *Cell Calcium* 32: 175–181.
- Peppiatt CM, Collins TJ, Mackenzie L, Conway SJ, Holmes AB, et al. (2003) 2-Aminoethoxydiphenyl borate (2-APB) antagonises inositol 1,4,5-trisphosphate-induced calcium release, inhibits calcium pumps and has a use-dependent and slowly reversible action on store-operated calcium entry channels. *Cell Calcium* 34: 97–108.
- Bootman MD, Collins TJ, Mackenzie L, Llewelyn RH, Berridge MJ, et al. (2002) 2-Aminoethoxydiphenyl borate (2-APB) is a reliable blocker of store-operated  $Ca^{2+}$  entry but an inconsistent inhibitor of  $InsP_3$ -induced  $Ca^{2+}$  release. *FASEB J* 16: 1145–1150.
- Di Virgilio F, Vicentini LM, Treves S, Riz G, Pozzan T (1985) Inositol phosphate formation in fMet-Leu-Phe-stimulated human neutrophils does not require an increase in the cytosolic free  $Ca^{2+}$  concentration. *Biochem J* 229: 361–367.
- Smith RJ, Sam LM, Justen JM, Bundy GL, Bala GA, et al. (1990) Receptor-coupled signal transduction in human polymorphonuclear neutrophils: effects of a novel inhibitor of phospholipase C-dependent processes on cell responsiveness. *J Pharmacol Exp Ther* 253: 688–697.
- Yule DI, Williams JA (1992) U73122 inhibits  $Ca^{2+}$  oscillations in response to cholecystokinin and carbachol but not to JMV-180 in rat pancreatic acinar cells. *J Biol Chem* 267: 13830–13835.
- Witko-Sarsat V, Rieu P, Descamps-Latscha B, Lesavre P, Halbwachs-Mecarelli L (2000) Neutrophils: molecules, functions and pathophysiological aspects. *Lab Invest* 80: 617–653.
- Berton G, Yan SR, Fumagalli L, Lowell CA (1996) Neutrophil activation by adhesion: mechanisms and pathophysiological implications. *Int J Clin Lab Res* 26: 160–177.
- Takei T, Araki A, Watanabe H, Ichinose A, Sendo F (1996) Rapid killing of human neutrophils by the potent activator phorbol 12-myristate 13-acetate (PMA) accompanied by changes different from typical apoptosis or necrosis. *J Leukoc Biol* 59: 229–240.
- Klink M, Jastrzebska K, Bednarska K, Banasik M, Sulowska Z (2009) Effect of nitric oxide donors on NADPH oxidase signaling pathway in human neutrophils in vitro. *Immunobiology* 214: 692–702.
- Petty HR, Worth RG, Kindzelskii AL (2000) Imaging Sustained Dissipative Patterns in the Metabolism of Individual Living Cells. *Phys Rev Lett* 84: 2754–2757.
- Petty HR, Kindzelskii AL (2001) Dissipative metabolic patterns respond during neutrophil transmembrane signaling. *Proc Natl Acad Sci USA* 98: 3145–3149.
- Di Virgilio F, Lew DP, Pozzan T (1984) Protein kinase C activation of physiological processes in human neutrophils at vanishingly small cytosolic  $Ca^{2+}$  levels. *Nature* 310: 691–693.
- Sha'afi RI, White JR, Molski TF, Shefcyk J, Volpi M, et al. (1983) Phorbol 12-myristate 13-acetate activates rabbit neutrophils without an apparent rise in the level of intracellular free calcium. *Biochem Biophys Res Commun* 114: 638–645.
- Seeds MC, Parce JW, Szejda P, Bass DA (1985) Independent stimulation of membrane potential changes and the oxidative metabolic burst in polymorphonuclear leukocytes. *Blood* 65: 233–240.
- Ma H, Venkatachalam K, Parys JB, Gill DL (2002) Modification of Store-operated Channel Coupling and Inositol Trisphosphate Receptor Function by 2-Aminoethoxydiphenyl Borate in DT40 Lymphocytes. *J Biol Chem* 277: 6915–6922.
- Partida-Sánchez S, Cockayne DA, Monard S, Jacobson EL, Oppenheimer N, et al. (2001) Cyclic ADP-ribose production by CD38 regulates intracellular calcium release, extracellular calcium influx and chemotaxis in neutrophils and is required for bacterial clearance in vivo. *Nat Med* 7: 1209–1216.
- Smith RJ, Justen JM, McNab AR, Rosenbloom CL, Steele AN, et al. (1996) U-73122: a potent inhibitor of human polymorphonuclear neutrophil adhesion on biological surfaces and adhesion-related effector functions. *J Pharmacol Exp Ther* 278: 320–329.
- Savage CO, Pottinger BE, Gaskin G, Pusey CD, Pearson JD (1992) Autoantibodies developing to myeloperoxidase and proteinase 3 in systemic vasculitis stimulate neutrophil cytotoxicity toward cultured endothelial cells. *Am J Pathol* 141: 335–342.