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Ion channel modulators mediated alterations in NO-induced free radical generation and neutrophil membrane potential

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Abstract

The present study investigated the effect of various ion (H^+ and K^+) channel modulators on nitric oxide (NO) donors (SNP and SNAP) induced free radical generation and on neutrophil membrane potential. Free radical generation was assessed by DCDHF-DA, using flow cytometry, while membrane potential was measured by a fluorescent dye, DiO-C5-(3). Neutrophil suspension in high potassium containing medium or following addition of NO donors (SNP, SNAP) to the neutrophil suspension led to free radical generation and membrane depolarization. DPI (a dual inhibitor of NADPH-oxidase and NOS), ABAH (MPO inhibitor) and BAPTA-AM (calcium chelator) significantly reduced 80 mM KCl or NO mediated free radical generation. Modulators of large (NS1619), intermediate (Chlorzoxazone) and small conductance (Apamin, chlorzoxazone) calcium activated K^+ channels (TBA), voltage activated K^+ channels (Kv) (4AP, 8Br-cGMP), ATP sensitive K^+ channels (K_{ATP}) (Glybenclamide, pinacidil), Na^+,K^+-ATPase (Ouabain) and Na^+/H^+ exchanger (NHE, Amiloride) altered NO-induced neutrophil free radical generation response and membrane polarity. The results obtained thus suggest an association between rat neutrophil membrane depolarization and NO-dependent free radical generation.

Keywords: *Neutrophils, nitric oxide, membrane potential, ion channel, free radicals*

Introduction

Neutrophils/polymorphonuclear leukocytes (PMNs), the first line of defense, are recruited earliest to the site of inflammation, injury or infection [1]. The major role of PMNs is to internalize and destroy infectious agents by the sequential formation of phagosomes, recruitment of lysosomes, free radical formation and fusion of various types of granules to release proteolytic enzymes as well as anti-microbial peptides, acting in concert to mediate the execution of endocytosed pathogens [1–3]. It has been documented that alterations in ionic current through various ion channels affect immune cell functions [4,5]. K^+ channels also mediate cytotoxic actions of the human neutrophils [2]. Moreover these channels have been documented to regulate alterations in the membrane potential [6]. Various ion channels such as potassium channels, proton pumps, sodium hydrogen exchanger (NHE) and sodium-potassium ATPase have been found on human and mouse neutrophil surface [2,7–11], information regarding the presence of these channels on rat neutrophils is however lacking. Membrane depolarization has been associated with generation of superoxide radicals in the macrophages and neutrophils [12–14]. Previous studies from this lab as well from others have suggested nitric oxide (NO) as an important modulator of free radical generation in rat and human PMNs [15–17]. Since NO is known to modulate various ion channel activities and exert various actions, the present study was undertaken to investigate an association between NO-dependent free

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radical generation and membrane polarity in the rat neutrophils by using various ion channel modulators.

Methods

Isolation of polymorphonuclear leucocytes (PMNs)

PMNs from male SD rats (200–250 g) were isolated from buffy coat diluted with phosphate-buffered saline (PBS) containing tri sodium citrate 15 mM, 0.5% (w/v) bovine serum albumin (BSA) and glucose 10 mM was loaded over 1 ml Histopaque-1077 and centrifuged (1000 *g* at 25°C) for 20 min using the method of Saini et al. [18]. Original density of cells was restored by suspending cells in rat plasma containing 5% (v/v) foetal calf serum for 30 min. PMNs were separated over Histopaque-1083 and -1119 gradients, washed with PBS, counted and suspended in Hanks' balanced salt solution [HBSS; composition (mM): NaCl 138, KCl 2.7, Na_2HPO_4 8.1, KH_2PO_4 1.5, glucose 10, pH 7.2–7.4]. PMNs purity was >90%, as assessed by CD11b/CD45; contaminating cells in the PMN suspension were mostly lymphocytes. NaN_3 (1 mM) was added in the HBSS buffer to rule out the free radical generation from mitochondria [19].

Cell viability

PMNs (1×10^6 cells/ml) were pre-incubated for 5 min with propidium iodide (PI, 5 μg/ml) followed by treatment with Sodium nitroprusside (SNP, 1 μM to 10 mM) or S-nitroso, N-acetyl penicilamine (SNAP, 100 nM to 1 mM) or with various interventions used in the study for 30 min at 37°C to assess their effect on the cell viability. Necrotic cells which varied from 4–10% in the control and most of the treated cells were counted by flow cytometry (FACS Calibur, Becton Dickinson, USA).

DCF response by flow cytometry

PMNs (2×10^6cells/ml) were incubated with vehicle or various interventions such as diphenyleneiodonium chloride (DPI) (10 μM), 4-aminobenzoic acid hydrazide (ABAH, 100 μM), 1,2-bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid (BAPTA-AM, 30 μM), tetrabutyl ammonium phosphate (TBA, 1 mM), paxilline (300 nM), 1,3-dihydro-1-2[2-hydroxy-5-(trifluoromethyl) phenyl]-5-(trifluor omethyl)-2H-benzimidazole-2-one (NS1619, 30 μM), apamin (1 μM), chlozoxazone (500 μM), 4-aminopyr idine (4-AP, 10 mM), 8 bromo cGMP (10 μM), pinacidil (10 μM), ouabain (2 mM), amiloride (1 mM) or valinomycin (1 μM) for 5 min. Cell suspension was subsequently incubated with 2′,7′-dichlorodihydro-fluorescein diacetate (DCDHF-DA, 10 μM), for 5 min and finally with a NO donor, sodium nitroprusside (SNP, 1 μM to 10 mM) or *S*-nitroso-*N*-acetyl-penicillamine (SNAP, 100 nM to 1 mM). Free radical generation in each sample was monitored by flow cytometry by acquiring 10 000 cells and was analysed by Cell Quest program (Becton Dickinson, USA) [16] and the results have been expressed as MSI (mean stimulation index) with respect to the respective controls, which is the ratio of mean fluorescence of the stimulated and unstimulated cells. In all the experiments, mean stimulation index was calculated using respective control from the same experiment.

Membrane potential determination

Membrane potential was determined by using a potential sensitive fluorescent dye 3-3-dipentyloxacarbocyanine, DiO-C5-(3) (0.25 μM) [20]. Cells (2×10^6/ml) were suspended in HBSS (2.7 mM KCl), incubated for 5 min with the dye and basal value of fluorescence was measured (5–10 min); the cells were subsequently stimulated with a NO donor, SNP/SNAP or other interventions to assess change in the fluorescence measured by a fluorimeter (Shimadzu, RF 5000) at the excitation wavelength 490 nm and emission wavelength 506 nm. The value of membrane potential was obtained from a calibration curve by using valinomycin (2 μg/ml) in a cell suspension at various K^+ concentrations (0–140 mM KCl).

Statistical analysis

Results have been expressed as mean ± SEM of at least 3–5 independent experiments. In the experiments comparing only two conditions, statistical analysis was performed using Student's *t*-test and a p-value < 0.05 was considered significant. In all the other experiments, statistical analysis was performed using analysis of variance for repeated measurements. When the *F*-value was significant further pair-wise comparisons were carried out with an adjustment for multiple comparisons using Newman-Keul's analysis. The alpha level of all tests was set at 0.05.

Results

NO-induced free radical generation and membrane polarity alterations

Rat neutrophil suspension in high K^+ (KCl 80 mM) containing medium exhibited a significant augmentation in free radical generation (Figure 1A). DPI (a dual inhibitor of NADPH-oxidase and NOS), ABAH (Myeloperxidase [MPO] inhibitor) and BAPTA-AM (calcium chelator) significantly reduced KCl mediated free radical generation (Figure 1A and C), suggesting a role of enzymatic free radical generation in rat PMNs. NO donors SNP (100 nM–10 mM) or SNAP (100 nM–1 mM) depolarized PMNs (Figure 1B) and also augmented the free radical generation (Figure 1C). Since PMNs exhibited maximal DCF

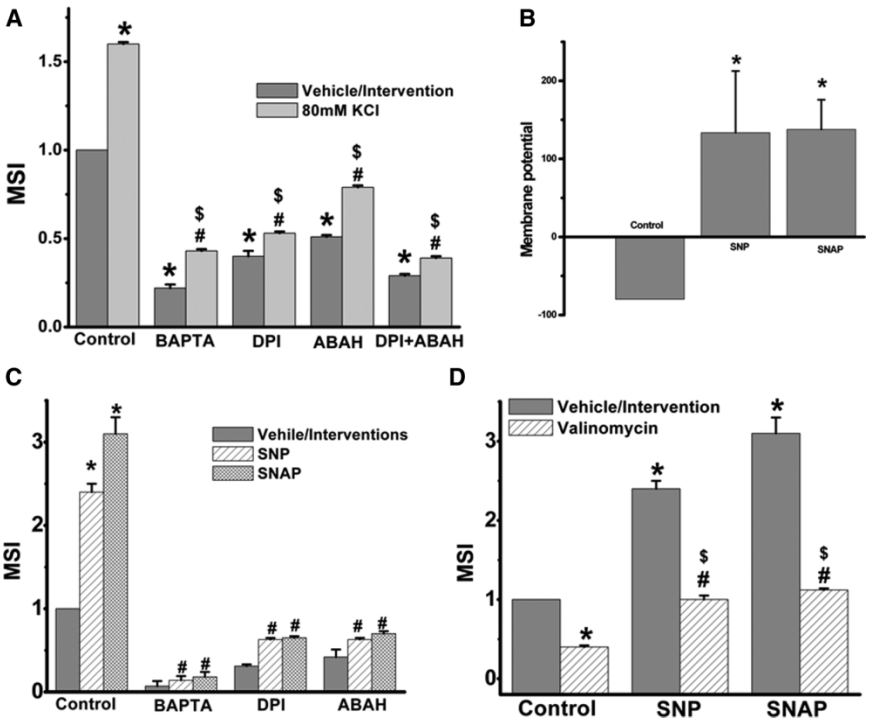

Figure 1. (A) Effect of DPI, ABAH and BAPTA on high extracellular potassium induced free radical generation. The buffers 2.5 mM or 80 mM K^+ were used with DPI (10 μM), ABAH (100 μM) or BAPTA (30 μM) for 30 min at 37°C along with DCF. (B) Effect of SNP and SNAP on membrane potential. The PMNs (1×10^6 cells/ml) preincubated with Di-O-C(5)[3] and valinomycin (4 μM) and were subsequently treated with SNP (100 μM), SNAP (100 μM) and readings were recorded. Effect of DPI (C), ABAH (C) and valinomycin (D) on SNP and SNAP induced PMNs free radical generation. PMNs (1×10^6 cells/ml) were incubated with 10 μM DCF-DA for 5 min at 37°C and were subsequently treated with various interventions. The PMNs incubated with interventions were treated with SNP (100 μM) or SNAP (100 μM) for 30 min; 10 000 from each tube were acquired and analysed by a flow cytometer, control tubes had cells treated with dye but instead of NO donors the same amount of vehicle was used ($^*p < 0.001$ in comparison to the un-stimulated controls, $^{\#}p < 0.001$ in comparison to the stimulated controls PMNs, $^{\$}p < 0.001$ in comparison to the un-stimulated PMNs pre incubated with valinomycin). MSI (mean stimulation index) is the ratio of mean fluorescence of the stimulated and unstimulated cells.

fluorescence following treatment with SNP (100 μM) or SNAP (100 μM) at 30 min, these parameters were kept constant in subsequent studies. Neutrophil treatment with valinomycin significantly reduced SNP or SNAP induced free radical formation (Figure 1D). NO donors did not react with DCF to generate fluorescent adduct and DCF also did not interfere with the NO release (data not shown). Both NO donors in the concentrations used did not affect the cell viability, number of apoptotic and necrotic cells were ~6% in the control and the NO donor-treated cells, which was assessed by PI positive cell population using flow cytometry.

Effect of calcium activated potassium channels

Addition of a non-specific calcium activated potassium channel blocker, tetrabutyl ammonium (TBA) (Figure 2A) or small conductance calcium activated potassium channel (SK) blocker, apamin (Figure 2B), significantly augmented SNP or SNAP induced free radical generation. TBA as well as apamin enhanced free radical generation, even in the absence of SNP or SNAP. Paxilline, an inhibitor of large conductance calcium activated potassium channel (BK), had no effect on SNP or SNAP induced free radical generation. Mean stimulation index (MSI) of the unstimulated PMNs (1 ± 0) was augmented to 2.45 ± 23 following the addition of bacteria in the presence of paxilline. Blocking of BK channels by paxilline had no significant effect on NO-induced free radical generation, which could even be due to the absence of these channels on rat PMNs. NS1619, an opener of BK channels, however, completely abolished SNP or SNAP induced free radical generation (Figure 2D). Chlorzoxazone, opener of small (SK) and intermediate (IK) conductance calcium activated potassium channel augmented basal as well as SNP or SNAP induced free radicals (Figure 2C). Neutrophil membrane was depolarized in the presence of Apamin (60.55 ± 9.0 mV) and chlorzoxazone (24.95 ± 7.9 mV). Moreover these interventions had no adverse effect on the cell viability, as assessed by PI positive cell population using flow cytometry.

Effect of voltage sensitive potassium channel

In human neutrophils, voltage sensitive potassium channels open only above − 60 mv. In rat neutrophils

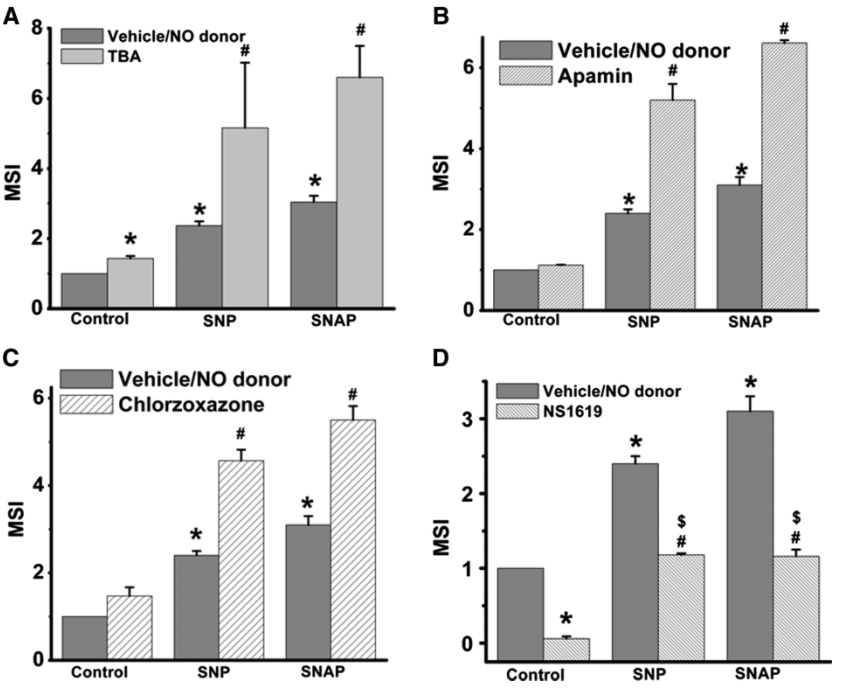

Figure 2. Effect of TBA (1 mM), Apamin (1 μM), NS1619 (30 μM) and chlozoxazone (500 μM) on PMNs. PMNs (1×10^6 cells/ml) were incubated with 10 μM DCF-DA for 5 min at 37°C and were subsequently treated with TBA (A), Apamin (B), chlozoxazone (C) and NS1619 (D) for 5 min at 37°C. The PMNs incubated with interventions were subsequently treated with SNP (100 μM) or SNAP (100 μM) for 30 min and 10 000 from each tube were acquired and analysed by a flow cytometer, control tubes had cells treated with dye but instead of NO donors the same amount of vehicle was used ($^{\star}p < 0.001$ in comparison to the un-stimulated controls, $^{\#}p < 0.001$ in comparison to the stimulated controls PMNs, $^{\$}p < 0.001$ in comparison to the un-stimulated PMNs pre incubated with NS1619). MSI (mean stimulation index) was measured with respect to the respective controls.

we evaluated the effect of 4-Amino pyridine (4-AP), the blocker of voltage sensitive potassium channel, which offered complete inhibition of NO-induced free radical generation (Figure 3A). 8BrcGMP had an opposite effect, as shown in Figure 3B. 4-AP hyperpolarized (-125 ± 10 mV), while 8BrcGMP depolarized (182.56 ± 12 mV) the neutrophil membrane. Cell viability in the presence of 4-Amino pyridine and 8BrcGMP was not affected and remained between 4–7%, as assessed by counting PI positive cell population using flow cytometry.

Effect of ATP sensitive potassium channel, sodium (Na^+)-hydrogen (H^+) exchanger (NHE) and Na^+/K^+ ATPase

Modulation of the ATP sensitive potassium (K_{ATP}) channel is considered important during hypoxia at the site of inflammation, which also exists in the presence of excess NO. We therefore investigated NO-induced free radical generation in the presence of glybenclamide (blocker) or pinacidil (opener-SUR2), both inhibited NO-induced free radical generation (Figures 4A and B). Both interventions used also

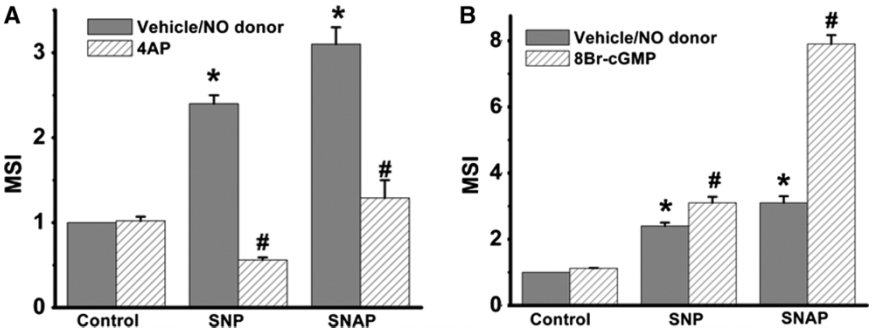

Figure 3. Effect of 4-aminopyridine (10 mM) and 8 bromo cGMP (10 μM) on SNP and SNAP induced free radical generation. PMNs (1×10^6 cells/ml) were incubated with 10 μM DCF-DA for 5 min at 37°C and were subsequently treated with 4AP (A) or 8Br-cGMP (B). The PMNs incubated with interventions were further treated with SNP (100 μM) or SNAP (100 μM) for 30 min and 10 000 from each tube were acquired and analysed by a flow cytometer, control tubes had cells treated with dye but instead of NO donors the same amount of vehicle was used ($^{\star}p < 0.001$ in comparison to the un-stimulated controls, $^{\#}p < 0.001$ in comparison to the stimulated controls PMNs). MSI (mean stimulation index) is the ratio of mean fluorescence of the stimulated and unstimulated or vehicle treated resting cells.

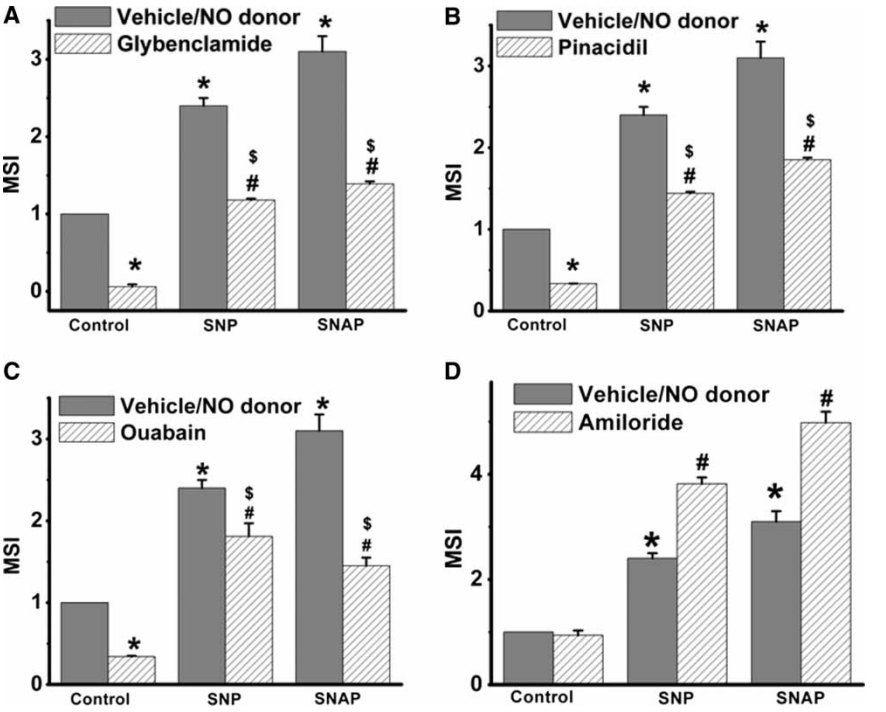

Figure 4. Effect of Glybenclamide (100 μM), pinacidil (10 μM), ouabain (2 mM) and amiloride (1 mM) on SNP, SNAP induced free radical generation. PMNs (1×10^6 cells/ml) were incubated with 10 μM DCF-DA for 5 min at 37°C and were subsequently treated with Glybenclamide (A), pinacidil (B), ouabain (C) and amiloride (D). The PMNs incubated with interventions were then treated with SNP (100 μM) or SNAP (100 μM) for 30 min and 10 000 from each tube were acquired and analysed by a flow cytometer, control tubes had cells treated with dye but instead of NO donors the same amount of vehicle was used ($^{*}p < 0.001$ in comparison to the un-stimulated controls, $^{\#}p < 0.001$ in comparison to the stimulated controls PMNs, $^{\$}p < 0.001$ in comparison to the un-stimulated PMNs pre-incubated with glybenclamide, pinacidil or ouabain). MSI (mean stimulation index) is the ratio of mean fluorescence of the stimulated and unstimulated or vehicle treated cells.

reduced basal DCF fluorescence. Interestingly Glybenclamide and pinacidil hyperpolarized the neutrophil membrane (>−100 mV). Basal DCF response was significantly reduced in the presence of ouabain (Figure 4C), while amiloride had no effect (Figure 4D). Ouabain (Na^+/K^+ ATPase inhibitor) significantly reduced SNP or SNAP induced DCF response (Figure 4C), while inhibition of NHE by amiloride significantly augmented NO-mediated neutrophil free radical generation (Figure 4D) and depolarized neutrophils (-31.56 ± 6 mV). Treatment of PMNs with ATP-sensitive channel modulators did not adversely affect their viability, as measured by counting PI positive cell population, which was not more than 6%.

Discussion

The present study reports NO-mediated free radical formation, although enzymatic, seems to be associated with membrane depolarization and is also modulated by various ion channel modulators. A large amount of NO accumulation as NO metabolite (nitrite ≥ 50 μM), following iNOS induction at the inflammatory site is well recognized in systemic inflammatory response syndrome [21], hypotensive sepsis [22] and nephrotic syndrome [23]. The amount of NO available to neutrophils in the present study is thus similar to the NO concentrations documented in various pathological conditions. It has also been proposed that deoxy haemoglobin, might act as nitrite reductase to enhance NO availability [24].

Neutrophil free radical generation from NADPH-oxidase [25], NOS [26] and MPO [27] is well documented. MPO, a cationic protein packed in the azurophilic granules, with negatively charged sulphated proteoglycan matrix remains inactive in dormant neutrophils. Superoxide dismutates O_2^- to generate hydrogen peroxide (H_2O_2), which is utilized by activated MPO to form hypohalous acid. Segal [2] reported that in the phagocytic vacuole potassium exchanged with MPO in a hypertonic, K^+ rich alkaline environment. Free radical generation due to high extracellular K^+, SNP or SNAP was inhibited by DPI and ABAH, suggesting dependence on enzymatic free radical generation (Figure 1A and C). Generation of free radicals in addition to enzyme activation also seems to be dependent on K^+ [28] and calcium [16]. We also observed that neutrophil membrane depolarization by suspension in high K^+ containing medium exhibited a significant augmentation in free radical generation (Figure 1A). High extracellular K^+ [29–31], SNP or SNAP (Figure 1B)

depolarized neutrophils and augmented free radical generation (Figure 1C). Importance of ionic modulation during inflammatory conditions is mostly associated with neutrophils migration and enhanced NO availability.

Altered Vm is predominantly a result of change in either intracellular K^+ concentrations or K^+ permeability by blockade of K^+ efflux. Blockade of K^+ efflux or increase in K^+ influx depolarize cells. Potassium channels regulate membrane polarity and thus alter the free radical generation as assessed by DCF, which seems to be independent of the electron transport chain [32]. Neutrophil, a major player of innate immune response [2], has a resting membrane potential $\sim -75 \pm 17$ mV, which is primarily maintained by K^+ conductance and to a small extent by sodium/potassium pumps [33,34]. To combat bacterial infection, neutrophils generate superoxide (O_2^-), depolarization in the present study subsequent to addition of NO donors along with basal/enhanced intracellular K^+ seems to be mostly associated with augmented free radical generation.

NO by modulating ion channels alter the responses of various types of cells [35–39]. Opening of SK and IK by chlorzoxazone augmented SNP and SNAP (Figure 2C) induced free radical generation, while treatment with NS1619 (Opener of BK) resulted in the inhibition of free radical generation (Figure 2C and D). Apamin or chlorzoxazone, which depolarized PMNs, augmented free radical generation, while NS1619 hyperpolarized and attenuated the free radical generation from rat neutrophils. The extent of hyperpolarization observed with NS1619 might be due to many factors, besides the effect of this intervention on BK channels alone. Although no direct estimation of potassium has been carried out, blockade of K_{Ca} by TBA or SK by apamin has shown to increase intracellular potassium. Blockade of K_{Ca} by TBA or SK by apamin significantly enhanced NO-dependent free radical generation. In the paxilline-treated neutrophils, however, no effect on SNP or SNAP induced free radical generation was observed; it could be due to the absence of these channels on rat PMNs or the observed effect could also be due to the fact that these channels normally remain closed in resting human neutrophils and are assembled only during phagocytosis [2]. The presence of BK is controversial on human neutrophils. Segal [2] proposed that the electrogenic charge difference generated by the activity of NADPH oxidase is compensated by the BK channel mediated K^+ flux. On the contrary [40], it has been recently reported that BK channels are absent in human and mouse neutrophils and remain to be investigated on rat PMNs. The results therefore needs to be confirmed using molecular studies or by patch clamp studies. Alterations in NO-induced free radical generation in the presence of these K channel modulators however indicated some association with depolarization and hyperpolarization.

K_{ATP} channel, a weak inward rectifier [41,42] open subsequent to ATP depletion [43] and its modulation by NO has been documented [44], which is involved in maintaining the resting membrane potential [42]. Neutrophil migrate to the hypoxic inflammatory site [45], implying the importance of K_{ATP}. Glybenclamide inhibited entry of potassium in the NO-treated neutrophils and reduced SNP or SNAP (Figure 4A) induced free radical generation. Pinacidil (K_{ATP} opener) in the present study hyperpolarized neutrophils and inhibited SNP or SNAP (Figure 4B) induced free radical generation. It has been reported that pinacidil presumably by hyperpolarization reduced the superoxide radical generation in neutrophils [46]. NHE maintains intracellular pH [7], while intracellular acidification results in free radical generation [7]. Na^+/K^+ ATPase, which maintain the resting membrane potential, transport K^+ from inside to outside. In the present study ouabain, an inhibitor of Na^+/K^+ ATPase [47], reduced SNP or SNAP (Figure 4C) induced free radical generation, while amiloride treated neutrophil exhibited augmentation in the neutrophil free radical generation (Figure 4D).

Studies conducted so far to characterize the presence of various channels on mouse and human neutrophils [7–11,13,14] have provided an association between these channels and free radical generation [2,28,40], while similar reports are lacking in rat neutrophils. The present study has explored this possibility in a preliminary manner by using various pharmacophores and suggests the importance of these channels in regulating the NO-dependent free radical generation in rat neutrophils.

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References

[1] Nathan C. Neutrophils and immunity: challenges and opportunities. Nat Rev Immunol 2006;6:173–182.

[2] Segal AW. How neutrophils kill microbes. Annu Rev Immunol 2005;23:197–223.

[3] Babior BM, Lambeth JD, Nauseef W. The neutrophil NADPH oxidase. Arch Biochem Biophys 2002;397:342–344.

[4] Cahalan MD, Wulff H, Chandy KG. Molecular properties and physiological roles of ion channels in the immune system. J Clin Immunol 2001;21:235–252.

[5] Colden-Stanfield M. Clustering of very late antigen-4 integrins modulates K^+ currents to alter Ca2+-mediated monocyte function. Am J Physiol Cell Physiol 2002;283: C990–C1000.

[6] Hatzoglou CH, Gourgoulianis KI, Molyvdas PA. Effects of SNP, ouabain, and amiloride on electrical potential profile of isolated sheep pleura. J Appl Physiol 2001;90:1565–1569.

[7] Nanda A, Grinstein S, Curnutte JT. Abnormal activation of H^+ conductance in NADPH oxidase-defective neutrophils. Proc Natl Acad Sci USA 1993;90:760–764.

[8] Krause KH, Welsh MJ. Voltage-dependent and Ca2(+)-activated ion channels in human neutrophils. J Clin Invest 1990;85:491–498.

[9] Demaurex N, Grinstein S, Jaconi M, Schlegel W, Lew DP, Krause KH. Proton currents in human granulocytes: regulation by membrane potential and intracellular pH. J Physiol 1993;466:329–344.

[10] DeCoursey TE, Cherny VV. Potential, pH, and arachidonate gate hydrogen ion currents in human neutrophils. Biophys J 1993;65:1590–1598.

[11] DeCoursey TE, Grinstein S. Ion channels and carrier in leukocytes. In: Gallin J, Snyderman R, editors.. Inflammation: Basic principles and clinical correlates. 3rd ed. Philadelphia, PA: Lippincott Williams and Wilkins; 1999. p 639–659.

[12] Amatore C, Arbault S, Bouton C, Coffi K, Drapier JC, Ghandour H, Tong Y. Monitoring in real time with a microelectrode the release of reactive oxygen and nitrogen species by a single macrophage stimulated by its membrane mechanical depolarization. Chembiochem 2006;7:653–661.

[13] Henderson LM, Meech RW. Proton conduction through gp91phox. J Gen Physiol 2002;120:759–765.

[14] DeCoursey TE. Interactions between NADPH oxidase and voltage-gated proton channels: why electron transport depends on proton transport. FEBS Lett 2003;555:57–61.

[15] Pieper GM, Clarke GA, Gross GJ. Stimulatory and inhibitory action of nitric oxide donor agents vs. nitrovasodilators on reactive oxygen production by isolated polymorphonuclear leukocytes. J Pharmacol Exp Ther 1994;269:451–456.

[16] Sharma P, Raghavan SA, Saini R, Dikshit M. Ascorbate-mediated enhancement of reactive oxygen species generation from polymorphonuclear leukocytes: modulatory effect of nitric oxide. J Leukoc Biol 2004;75:1070–1078.

[17] Sethi S, Singh MP, Dikshit M. Nitric oxide-mediated augmentation of polymorphonuclear free radical generation after hypoxia-reoxygenation. Blood 1999;93:333–340.

[18] Saini R, Patel S, Saluja R, Sahasrabuddhe AA, Singh MP, Habib S, Bajpai VK, Dikshit M. Nitric oxide synthase localization in the rat neutrophils: immunocytochemical, molecular, and biochemical studies. J Leukoc Biol 2006;79: 519–528.

[19] Camara AK, Riess ML, Kevin LG, Novalija E, Stowe DF. Hypothermia augments reactive oxygen species detected in the guinea pig isolated perfused heart. Am J Physiol Heart Circ Physiol 2004;286:H1289–H1299.

[20] Nanda A, Grinstein S. Protein kinase C activates an H^+ (equivalent) conductance in the plasma membrane of human neutrophils. Proc Natl Acad Sci USA 1991;88:10816–10820.

[21] Spack L, Havens PL, Griffith OW. Measurements of total plasma nitrite and nitrate in pediatric patients with the systemic inflammatory response syndrome. Crit Care Med 1997;25:1071–1078.

[22] Villalpando S, Gopal J, Balasubramanyam A, Bandi VP, Guntupalli K, Jahoor F. *In vivo* arginine production and intravascular nitric oxide synthesis in hypotensive sepsis. Am J Clin Nutr 2006;84:197–203.

[23] Kawashima H, Kashiwagi Y, Watanabe C, Sato S, Nishimata S, Takekuma K, Hoshika A, Watanabe Y. NOx (nitrite/nitrate) in patients with pediatric nephrotic syndrome. Pediatr Nephrol 2007;22:840–843.

[24] Cosby K, Partovi KS, Crawford JH, Patel RP, Reiter CD, Martyr S, Yang BK, Waclawiw MA, Zalos G, Xu X, Huang KT, Shields H, Kim-Shapiro DB, Schechter AN, Cannon RO, 3rd, Gladwin MT. Nitrite reduction to nitric oxide by deoxyhemoglobin vasodilates the human circulation. Nat Med 2003;9:1498–1505.

[25] Babior BM. NADPH oxidase. Curr Opin Immunol 2004;16: 42–47.

[26] Stuehr DJ, Santolini J, Wang ZQ, Wei CC, Adak S. Update on mechanism and catalytic regulation in the NO synthases. J Biol Chem 2004;279:36167–36170.

[27] Klebanoff SJ. Myeloperoxidase: friend and foe. J Leukoc Biol 2005;77:598–625.

[28] Matsumoto T, Takeshige K, Minakami S. Spontaneous induction of superoxide release and degranulation of neutrophils in isotonic potassium medium: the role of intracellular calcium. J Biochem 1986;99:1591–1596.

[29] Bashford CL, Pasternak CA. Plasma membrane potential of neutrophils generated by the Na^+ pump. Biochim Biophys Acta 1985;817:174–180.

[30] Korchak HM, Weissmann G. Changes in membrane potential of human granulocytes antecede the metabolic responses to surface stimulation. Proc Natl Acad Sci USA 1978;75: 3818–3822.

[31] Kawa K. Electrophysiological properties of three types of granulocytes in circulating blood of the newt. J Physiol 1989; 415:211–231.

[32] McArdle F, Pattwell DM, Vasilaki A, McArdle A, Jackson MJ. Intracellular generation of reactive oxygen species by contracting skeletal muscle cells. Free Radic Biol Med 2005;39: 651–657.

[33] Majander A, Wikstrom M. The plasma membrane potential of human neutrophils. Role of ion channels and the sodium/potassium pump. Biochim Biophys Acta 1989;980:139–145.

[34] Sanguinetti MC, Spector PS. Potassium channelopathies. Neuropharmacology 1997;36:755–762.

[35] Pickkers P, Dorresteijn MJ, Bouw MP, van der Hoeven JG, Smits P. *In vivo* evidence for nitric oxide-mediated calcium-activated potassium-channel activation during human endotoxemia. Circulation 2006;114:414–421.

[36] Remillard CV, Yuan JX. Activation of K^+ channels: an essential pathway in programmed cell death. Am J Physiol Lung Cell Mol Physiol 2004;286:L49–L67.

[37] Gao YD, Xiong SD, Xu YJ, Zhang ZX, Liu XS. The effect of nitric oxide on potassium channels of bronchial smooth muscle cells from asthmatic rats]. Zhonghua Jie He He Hu Xi Za Zhi 2003;26:615–618.

[38] Ivancheva C, Rahamimoff R, Radomirov R. Apamin-sensitive nitric oxide- and ATP-mediated motor effects on the guinea pig small intestine. Gen Physiol Biophys 2001;20:97–108.

[39] Kadekaro M, Sua G, Chua R, Lei Y, Li J, Fang L. Nitric oxide upregulates the expression of calcium-dependent potassium channels in the supraoptic nuclei and neural lobe of rats following dehydration. Neurosci Lett 2006;404: 50–55.
[40] DeCoursey TE. Electrophysiology of the phagocyte respiratory burst. Focus on 'Large-conductance calcium-activated potassium channel activity is absent in human and mouse neutrophils and is not required for innate immunity'. Am J Physiol Cell Physiol 2007;293:C30–C32.
[41] Ho K, Nichols CG, Lederer WJ, Lytton J, Vassilev PM, Kanazirska MV, Hebert SC. Cloning and expression of an inwardly rectifying ATP-regulated potassium channel. Nature 1993;362:31–38.
[42] Baukrowitz T, Tucker SJ, Schulte U, Benndorf K, Ruppersberg JP, Fakler B. Inward rectification in KATP channels: a pH switch in the pore. Embo J 1999;18:847–853.
[43] Giblin JP, Cui Y, Clapp LH, Tinker A. Assembly limits the pharmacological complexity of ATP-sensitive potassium channels. J Biol Chem 2002;277:13717–13723.

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[44] Sasaki N, Sato T, Ohler A, O'Rourke B, Marban E. Activation of mitochondrial ATP-dependent potassium channels by nitric oxide. Circulation 2000;101:439–445.
[45] Murdoch C, Muthana M, Lewis CE. Hypoxia regulates macrophage functions in inflammation. J Immunol 2005;175: 6257–6263.
[46] Hu G, Vinten-Johansen J, Salem MR, Zhao ZQ, Crystal GJ. Isoflurane inhibits neutrophil-endothelium interactions in the coronary circulation: lack of a role for adenosine triphosphate-sensitive potassium channels. Anesth Analg 2002;94: 849–856, table of contents.
[47] Simchowitz L, Spilberg I, De Weer P. Sodium and potassium fluxes and membrane potential of human neutrophils: evidence for an electrogenic sodium pump. J Gen Physiol 1982; 79:453–479.