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Nitrite attenuated peroxynitrite and hypochlorite generation in activated neutrophils

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ABSTRACT

Oxidative stress is usually considered as an important factor to the pathogenesis of various diseases. Peroxynitrite ($ONOO^-$) and hypochlorite (OCl^-) are formed in immune cells as a part of the innate host defense system, but excessive reactive oxygen species generation can cause progressive inflammation and tissue damage. It has been proven that through mediating nitric oxide (NO) homeostasis, inorganic nitrite (NO_2^-) shows organ-protective effects on oxidative stress and inflammation. However, the effects of NO_2^- on the function of immune cells were still not clear. The potential role of NO_2^- in modulating $ONOO^-$ and OCl^- generation in neutrophil cells was investigated in this study. As an immune cell activator, lipopolysaccharide (LPS) increased both $ONOO^-$ and OCl^- production in neutrophils, which was significantly attenuated by NO_2^-. NO_2^- reduced superoxide (O_2^-) generation via a NO-dependent mechanism and increased NO formation in activated neutrophils, suggesting a crucial role of O_2^- in NO_2^--mediated reduction of $ONOO^-$. Moreover, the reduced effect of NO_2^- on OCl^- production was attributed to that NO_2^- reduced H_2O_2 production in activated neutrophils without influencing the release of myeloperoxidase (MPO), thus limiting OCl^- production by MPO/H_2O_2 system. Therefore, NO_2^- attenuates $ONOO^-$ and OCl^- formation in activated neutrophils, opening a new direction to modulate the inflammatory response.

1. Introduction

Overproduction of reactive oxygen species (ROS) is of major importance in the development and progression of various diseases (Lu et al., 2010; Pacher et al., 2007; Valko et al., 2007). Immune cells like neutrophils and macrophages, are the major sources of ROS, in particular peroxynitrite ($ONOO^-$) and hypochlorite (OCl^-) (Pacher et al., 2007; van der Veen et al., 2009). Myeloperoxidase (MPO) is the only human enzyme known to generate reactive OCl^-, and it utilizes Cl^- as substrate to form OCl^- in the presence of H_2O_2 (van der Veen et al., 2009). Both $ONOO^-$ and OCl^- are effective in the innate host defense system and crucial for immune cell function. On the other hand, excessive production of $ONOO^-$ and OCl^- can become detrimental and lead to progressive inflammation and severe tissue injuries (Pacher et al., 2007; van der Veen et al., 2009).

Nitrite (NO_2^-) is one of the major end products of nitric oxide (NO) metabolism, closely reflecting nitric oxide synthases (NOS) activity, and from the daily consumption (Lu et al., 2014a; Whiteman et al., 2002). NO_2^- has recently emerged as an endogenous signaling molecule with potential therapeutic implications for cardiovascular disease (Kevil et al., 2011; Lundberg et al., 2009). It is now clear that as a NO donor, NO_2^- can act as a promising therapeutic agent to provide cytoprotective effects in vitro, and protect blood and organs against ischemia-reperfusion injuries (Bryan et al., 2007; Kevil et al., 2011; Lundberg et al., 2009). Dietary NO_2^- restores NO homeostasis and is cardioprotective in NOS-deficient mice (Bryan et al., 2008). The reduction of NO_2^- to NO can be achieved by a variety of enzymes in vivo, including heme-containing proteins and xanthine oxidase (Kevil et al., 2011; Lundberg et al., 2009).

Abbreviations: DETA, diethylenetriamine/nitric oxide adduct; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; MPO, myeloperoxidase; NADPH oxidase, nicotinamide adenine dinucleotide phosphate oxidase; NAME, N_ω-Nitro-L-arginine methyl ester hydrochloride; NO, nitric oxide; NO_2^-, nitrite; NOS, nitric oxide synthase; O_2^-, superoxide; OCl^-, hypochlorite; $ONOO^-$, peroxynitrite; PTIO, carboxy-PTIO potassium salt; ROS, reactive oxygen species

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It has been found that endogenous levels of NO_2^- in human blood and tissues may reach the micromolar range (Hensley et al., 1998; Sureda et al., 2004, 2006), and significantly lower NO_2^- levels are reported in the brains of Alzheimer's disease (AD) patients (~2.7 μM in healthy brain, ~1.7 μM in AD) (Hensley et al., 1998). The decrease in plasma NO_2^- levels reflects the endothelial dysfunction in humans and is correlated with increasing cardiovascular risk load (Kleinbongard et al., 2006). Moreover, NO_2^- treatment also protects against lipopolysaccharide (LPS)-induced tissue injuries in rats as well as morbidity and mortality in mice (Cauwels et al., 2009; Hamburger et al., 2013). Recently, inorganic NO_2^- attenuates NADPH oxidase-derived superoxide (O_2^-) generation in LPS-activated mice peritoneal macrophages or human monocytes (Yang et al., 2015). Taken together, these studies demonstrate therapeutic effects of NO_2^- on disorders characterized by oxidative stress and inflammation. It has been proven that this beneficial effect is mainly attributed to the attenuation of oxidative stress via enhanced NO generation (Kevil et al., 2011; Lundberg et al., 2009; Yang et al., 2015). Although immune cells are one of the most important sources of ROS, it still remains unclear if NO_2^- can directly modulate the formation of $ONOO^-$ and OCl^- in immune cells.

2. Materials and methods

2.1. Neutrophils isolation

Human neutrophils were isolated by a procedure utilizing Histopaque (Kagan et al., 2010; Lu et al., 2014b; Vlasova et al., 2012). Heparinized venous blood was collected from healthy volunteers. The leukocyte-rich supernatant was harvested and subjected to centrifugation as described in the manual. The neutrophil-rich supernatant was carefully aspirated and washed twice with PBS, thus obtained cells were suspended and cultured in RPMI.

2.2. Substances used in the experiments

The following substances were purchased from Sigma and used in the experiments: lipopolysaccharide (LPS) from *Escherichia coli*, sodium nitrite ($NaNO_2$, 10 μM), diethylenetriamine/nitric oxide adduct (DETA, 0.5 mM), carboxy-PTIO potassium salt (PTIO, 20 μM), N_ω-Nitro-L-arginine methyl ester hydrochloride (NAME, 1 mM), allopurinol (40 nM). All solvents and other reagents were the highest purity commercially available. $NaNO_2$ and DETA were administered simultaneously with LPS, whereas all other treatments were administered 20 min prior to LPS. Cells were then treated for 12 h (Lu et al., 2014a; Whiteman et al., 2002).

2.3. Assessment of ROS generation in neutrophils

The generation of $ONOO^-$ was measured using hydroxyphenyl fluorescein following the manufacture's protocol (Yang et al., 2015). 4-Amino-5-methylamino-2′, 7′-difluorescein was used for fluorescence detection of NO production in neutrophils (Yang et al., 2015). O_2^- and H_2O_2 production in neutrophils were measured by an SOD-inhibitable cytochrome c reduction assay and Amplex Red assay, respectively (Kagan et al., 2010; Lu et al., 2014b; Vlasova et al., 2012). Levels of MPO and OCl^- in cells were determined by ELISA kit and 5-thio-2-nitrobenzoic acid, respectively (Kagan et al., 2010; Lu et al., 2014b; Vlasova et al., 2012).

2.4. Western blot analysis

For detection of specific protein, equal amounts of proteins were subjected to SDS-PAGE. After electrophoresis, the proteins were transferred to a nitrocellulose membrane and then immunoblotted with antibody against NADPH oxidase or iNOS (Sigma). The antibody was detected using a secondary antibody conjugated to horseradish peroxidase. Chemiluminescence was used to identify specific proteins according to the ECL system (Pierce).

Xanthine oxidase activity and expression was determined with a commercial kit and Western blotting, respectively (Montenegro et al., 2011).

2.5. Statistical analysis

All of the experiments were performed at least three times. The results were reported as the means ± S.D. of at least triplicate determinations. One-way ANOVA was used for statistical analyses, and $P < 0.05$ was considered significant.

3. Results

3.1. NO_2^- decreased $ONOO^-$ and OCl^- generation in LPS-activated neutrophils

First, we explored whether the presence of NO_2^- influenced the biological action of LPS in human blood neutrophils. Consistent with previous studies that $ONOO^-$ and OCl^- were generated upon activation of neutrophils (Lu et al., 2014b; Pacher et al., 2007; van der Veen et al., 2009), both $ONOO^-$ and OCl^- generation from LPS-activated neutrophils were significantly higher compared to nonactivated cells (Fig. 1A and B). Compared with control, treatment with NO_2^- in 10 μM concentration in nonactivated cells slightly increased $ONOO^-$ production and decreased OCl^- generation without statistical significance. Moreover, simultaneous administration of NO_2^- and LPS markedly reduced $ONOO^-$ and OCl^- formation compared with LPS alone. These results demonstrated that NO_2^- could significantly decrease $ONOO^-$ and OCl^- production in LPS-activated neutrophils. In subsequent experiments, we used the concentration of NO_2^- at 10 μM.

3.2. NO_2^- reduced O_2^- production in activated neutrophils

The highly reactive $ONOO^-$ is usually formed as a result of O_2^- interacting with NO (Pacher et al., 2007). Then, we investigated if NO_2^--mediated reduction in $ONOO^-$ formation was dependent on O_2^- and NO generation. Neutrophils stimulated with LPS showed significantly higher O_2^- generation compared to nonactivated cells (Fig. 1C). NO_2^- treatment significantly reduced O_2^- production in LPS-activated cells. Similar to the effect of NO_2^-, the classical NO donor DETA also attenuated O_2^- production during LPS activation, suggesting that the effects of NO_2^- on O_2^- generation were linked to NO generation.

Further, a NO scavenger (PTIO) or a NOS inhibitor (NAME) was administered to investigate the effects of NO on O_2^- production. PTIO alone increased O_2^- level in nonactivated cells, suggesting that scavenging of NO was favor for O_2^- formation. Simultaneous LPS and PTIO treatment also elevated O_2^- formation compared with that of PTIO group (Fig. 1D). In the presence of PTIO, the NO_2^--mediated reduction in O_2^- generation was abolished during LPS activation, and subsequently simultaneous LPS and NO_2^- treatment showed no difference compared with cells treated with LPS. NAME (NOS inhibitor) significantly elevated O_2^- generation in both control and LPS-activated cells. However, in the presence of NAME, simultaneous LPS and NO_2^- treatment showed lower O_2^- level than cells treated with LPS (Fig. 1E). Thus, simultaneous treatment with NAME did not abolish the NO_2^--mediated reduction in O_2^- generation during activation with LPS, implying that the effects of NO_2^- on O_2^- generation was independent on NOS. Taken