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Superoxide Formation and Cytotoxicity of RAW264.7 Macrophages Induced by Nitric Oxide

Hong Lee[1]*, Hyun-Ock Pae[1,2], Chang-Duk Jun[2], Ji Chang Yoo[2], Rae-Kil Park[2] and Hun-Taeg Chung[2]

[1]*Department of chemistry, Wonkwang university, Iksan, Korea.*
[2]*Department of microbiology and immunology, Wonkwang university school of medicine, Iksan, Korea.*
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ABSTRACT *: We have studied cytotoxicity of S-nitroso-N-acetyl- N-DL-penicillamine(SNAP), a Nitric oxide (NO)-releasing compound, in RAW264.7 macrophages. SNAP is cytotoxic to RAW264.7 cells in a concentration-dependent manner. PMA(200 nM) stimulated cells to produce superoxide anion radical($O_2^{\cdot}$) and caused a little loss of RAW264.7 cell viability for 12 hr and diminished the cytotoxicity of SNAP. The mechanism by which PMA can protect cells against NO-mediated cytotoxicity was studied by peroxynitrite-enhanced chemiluminescence method. Observed results suggested that $O_2^{\cdot}$ produced by PMA-stimulated RAW264.7 cells may quench NO released by SNAP and reduce NO, thus attenuating NO-related damages.*

Key Words *: Superoxide, Nitric oxide, Peroxynitrite, Free radical*

I. INTRODUCTION

In the energy-producing processes of our body molecular oxygen is reduced by four electrons to yield two molecules to water as the end products, partially reduced oxygen species or its excited form (singlet oxygen) can be produced(Niki, 1992). Oxygen molecule has two unpaired electrons which have parallel spins. When one of the electrons is excited and inverts its spin, a highly relative singlet oxygen is formed. Univalent reduction of oxygen produces the superoxide anion radical($O_2^{\cdot}$), whereas bivalent reduction generates hydrogen peroxide(H_2O_2) and trivalent reduction generates hydroxyl radical($^{\cdot}OH$)(Niki, 1992). Most of these intermediate forms of oxygen are highly reactive and cytotoxic, but $^{\cdot}OH$ is considered to be the most reactive species. The generation of $^{\cdot}OH$ is accelerated by transition metals, especially iron (Sutton and Winterbourn, 1989).

Nitric oxide(NO) is a unique molecule which can act as either an oxidant or a reductant, depending upon its target molecule. NO can be easily converted to a variety of related molecules such as S-nitrosothiol(RS-NO), nitrosyl-iron(Fe-NO), nitrogen dioxide gas($NO_2^{\cdot}$), peroxynitrite($ONOO^-$), nitrate(NO_2^-) and nitrite(NO_3^-). Some of them are more potent in attacking biological molecules than NO. Particularly, $ONOO^-$ has been as a candidate of actual species for tissue damage due to its potent oxidative power. Simultaneous production of NO and $O_2^{\cdot}$ has been shown to occur in inflammatory conditions(Ischiropoulos, *et al*, 1992). Moreover, the formation of $ONOO^-$ from activated macrophages and human neutrophils is well documented(Ischiropoulos, *et al*, 1992). Beckman and coworkers (Beckman, *et al*, 1990) have suggested that the interaction between NO and $O_2^{\cdot}$ to yield $ONOO^-$ and its conjugate acid, peroxynitrous acid(ONOOH), enhances dramatically the toxicity of either NO or $O_2^{\cdot}$ alone.

In this study, we demonstrated that the formation of $O_2^{\cdot}$ may protect the cytotoxicity of NO toward RAW264.7 macrophages via the formation of $ONOO^-$.

II. MATERIALS AND METHODS

1. Chemicals

*To whom correspondence should be addressed.

S-nitroso-N-acetyl-N-DL-penicillamine(SNAP), superoxide dismutase(SOD), luminol, Hank's balanced salt solution(HBSS) without phenol red, S-nitrosoglutathione(GSNO), methylthiazol tetrazolium bromide(MTT), and phorbol myristate acetate(PMA) were purchased from Sigma chemicals(St. Louis, Mo). All other chemicals and reagents were purchased from commercial sources.

2. Measurement of peroxynitrite.

Peroxynitrite was measured by luminol-dependent chemiluminescence as described in previous papers (Kooy, *et al*, 1994; Deliconstantinos, *et al*, 1996; Radi, *et al*, 1993; Li, *et al*, 1996). All the light(photons) emitted was measured using a Berthold AutoLumat LB9505 luminometer. A stock solution(1 mM) of luminol made in DMSO was diluted in HBSS to final concentration of 10 μM just before use. Luminol was directly injected in the tubes by the luminometer. RAW264.7 macrophages(5×10^6 cells per 1 m*l* of HBSS) were placed in a vial and stimulated with 200 nM PMA in the absence or the presence of SNAP. Light emission was recorded by computer interface and reported as the integrated light emission for a total period of 60 min. Background chemiluminescence was measured in vials containing the reaction mixture without cells. The results were calculated at counts per min(CPM).

3. MTT cytotoxicity assay.

This assay was carried out as described by McQuaid, *et al*(McQuaid, *et al*, 1996). Following incubation with or without SNAP for 12 hr, cells in 6 well plates were washed with 2 m*l* PBS, then 2 m*l* DMEM followed by 200 μ*l* MTT(5 mg/m*l*) were added to each well. After a 3 hr incubation at 37°C, the medium overlying cells was aspirated and cells were solubilized with 1.5 m*l* dimethylsulfoxide(DMSO). The 96 multiwell was then measured on a Dynatech Miroelisa reader at 570 nm or 600 nm. Results were expressed as percentage of control(absorption of control taken to represent 100% viability).

4. Culture of RAW264.7 macrophages.

Cells were cultured in DMEM containing 10% fetal bovine serum. Prior to the experiment, the culture medium was replaced with fresh medium and cells were then exposed to SNAP or PMA.

III. RESULTS AND DISCUSSION

SNAP was well known as NO donor(Ioannidis, *et al*, 1993) and found to cause a significant loss of RAW264.7 cell viability in a concentration-dependent manner(Fig. 1.A). Similar results were also observed by using another NO donor, S-nitrosoglutathione(GSNO), as well(Fig. 1B). Thus, these data indicate that NO derived from these NO donors are indeed cytotoxic to RAW264.7 cells. It should be noted that a significant toxicity was only observed 12hr after treatment of SNAP or GSNO. Therefore, cell viability was determined 12hr after initial exposure to SNAP or GSNO. The toxicity of NO is attributed to NO reaction with iron-containing mitochondrial enzymes and the inhibition of DNA or protein synthesis(Ioannidis, *et al*, 1993). PMA(200nM) caused a little loss of RAW264.7 cell viability as compared with control and clearly diminished the cytotoxicity of SNAP(Fig. 1.A and B). These results raise the possibility that PMA may protect cells against NO-mediated cell death via an unknown mechanism.

$O_2^{\cdot}$ production, a property of both macrophages and neutrophils, is stimulated through the interrelated pathways of calcium mobilization and the activation of protein kinase C. The exposure of macrophages or neutrophils to appropriate stimuli activates a metabolic pathway known as the respiratory burst whose purpose is the production of microbicidal oxidants through the partial reduction of oxygen. The key to this metabolic pathway is the respiratory burst oxidase, a membrane-bound enzyme that catalyzes the one-electron reduction of oxygen to $O_2^{\cdot}$ at the expense of NADPH(Keeling, 1982).

Since PMA, an activator of protein kinase C, stimulates RAW264.7 macrophages to produce $O_2^{\cdot}$, there is a possibility that $O_2^{\cdot}$ produced by PMA-stimulated RAW264.7 macrophages may attenuate the cytotoxicity of NO released by SNAP. To test this possibility, PMA-stimulated RAW264.7 cells

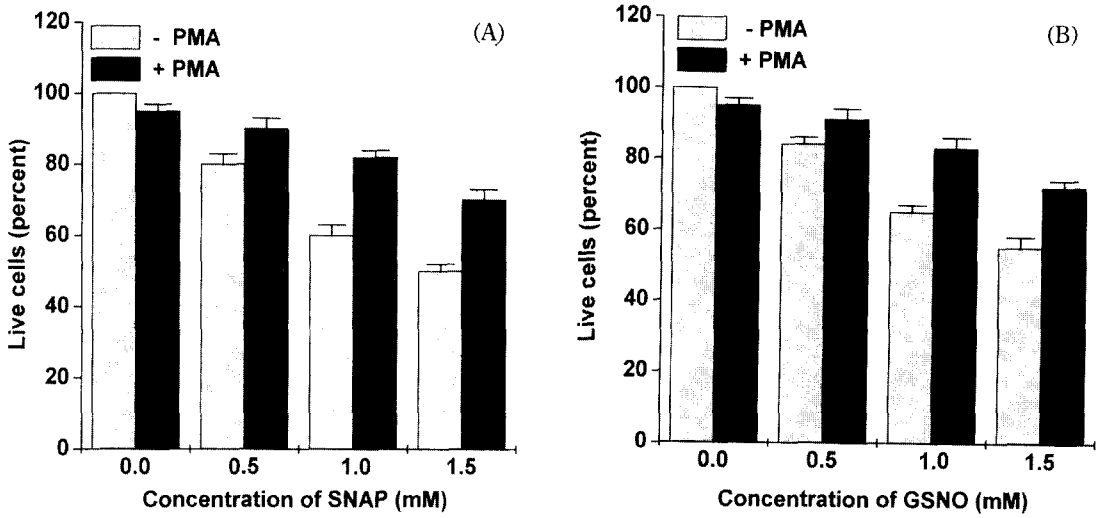

Fig. 1. Effects of PMA on SNAP-mediated cytotoxicity. Cells(10^6/mL) were incubated with or without various concentrations of SNAP(A), GSNO(B), and PMA in 6-well culture plates at 37°C for 12 hr and then MTT assay was carried out. Each value is the mean±SD of triplicate determinations.

were treated with SNAP and survival was assessed in the absence or presence of 200 units/ml SOD that can dismutate $O_2^{\cdot}$ to H_2O_2(Getzoff, *et al*, 1983). As shown in Fig. 2., SOD blocks the protective effect of PMA. This result suggests that $O_2^{\cdot}$ formation may protect NO-mediated cytotoxicity.

It was well studied that $O_2^{\cdot}$ reacts spontaneously with NO to form $ONOO^-$ (Eq.1) (Koppenol, *et al*, 1992). This notion suggests that

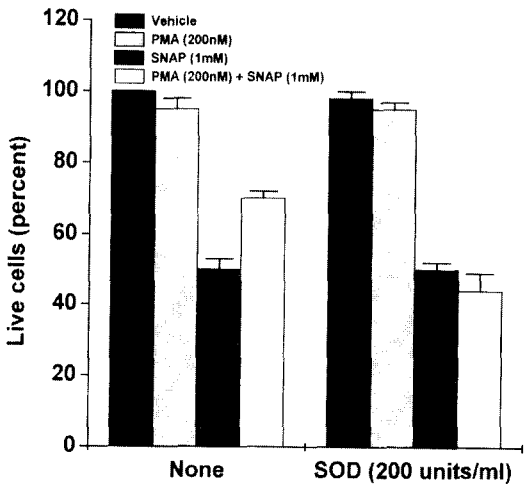

Fig. 2. Effects of SOD on SNAP-mediated cytotoxicity. Cells(10^6/mL) were incubated with or without 1 mM SNAP and 200 nM PMA in 6-well culture plates : 200 units/mL SOD was added immediately before the exposure to SNAP and PMA and then MTT assay was carried out. Each value is the mean±SD of triplicate determinations.

$O_2^{\cdot}$ produced by PMA-stimulated cells may react with NO released by SNAP to form $ONOO^-$. To test this hypothesis, we determined whether $ONOO^-$ can be formed in our experimental conditions. As shown in Fig. 3, NO released by SNAP does not exhibit any detectable luminescence and PMA alone exhibits low luminescence. However, combined treatment of PMA and SNAP resulted in exhibiting strong $ONOO^-$-enhanced chemiluminescence. These results support the above possibility.

Although $ONOO^-$ is cytotoxic more than NO or $O_2^{\cdot}$ alone(Pryor, *et al*, 1984), a low production rate of $ONOO^-$ which is limited by a production rate of $O_2^{\cdot}$ does not contribute to RAW264.7 cell damages. However, $O_2^{\cdot}$ produced by PMA-stimulated RAW264.7 cells may quench NO released by SNAP and reduce NO via the interaction between $O_2^{\cdot}$ and NO, thus attenuating NO-related damages. Moreover, there is an evidence to suggest that $O_2^{\cdot}$ may protect cells and tissue against NO-mediated damages(Brüne, *et al*, 1997). Another possibility(Eq.2-Eq.3) is considered as described below.

$$O^{\cdot} + NO \rightarrow ONOO^- \quad \text{(Eq.1)}$$

$$ONOO^- + NO(\text{excess}) \rightarrow NO_2 + NO_2^- \quad \text{(Eq.2)}$$

$$NO_2 + NO(\text{excess}) \leftrightarrow N_2O_3 \quad \text{(Eq.3)}$$

Under above conditions of limiting $O_2^{\cdot}$ rate and excess NO, excess NO will react with $ONOO^-$ to

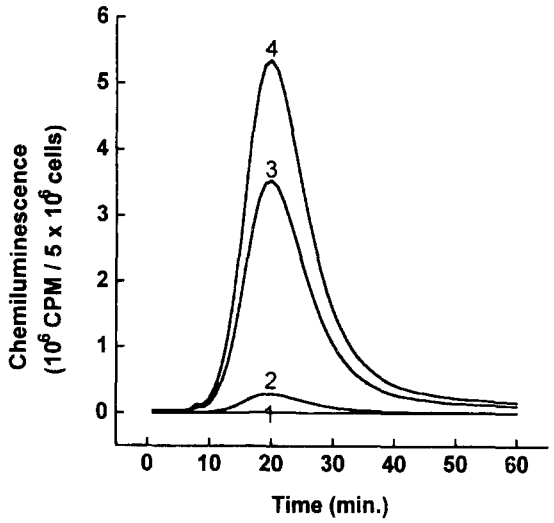

Fig. 3. Typical kinetic curve for peroxynitrite-enhanced chemiluminescence in the presence of 1 mM SNAP(1), 200 nM PMA(2), 1 mM SNAP plus 200 nM PMA(3), 5 mM SNAP plus 200 nM PMA(4).

form nitrogen oxides(e.g. NO_2 , NO_2, N_2O_3) that are not potent oxidants more that ONOO (Miles, *et al*, 1996). This notion may be supported by our observation that at a high concentration of SNAP, ONOO -enhanced chemiluminescence was decreased as shown in Fig. 3.

In conclusion, although ONOO might be formed under our experimental conditions, production of ONOO- under conditions of limiting $O_2^{\cdot}$ rate and excess NO does not contribute to RAW264.7 cell damages. The formation of $O_2^{\cdot}$ by PMA-stimulated cells protects cells against NO-mediated toxicity via the spontaneous interaction between $O_2^{\cdot}$ and NO.

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