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CHEMILUMINESCENCE STUDIES ON THE BIOLOGICAL INTERACTION BETWEEN SUPEROXIDE ANION RADICAL AND NITRIC OXIDE PRODUCED BY PHORBOL ESTER-STIMULATED RAW264.7 MACROPHAGES

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Abstract – The rapid and spontaneous interaction between superoxide anion radical and nitric oxide to yield the potent oxidants, peroxynitrite anion and peroxynitrous acid, was investigated in phorbol myristate acetate(PMA)-stimulated RAW264.7 macrophages by means of lucigenin- or luminol-enhanced chemiluminescence method. When RAW264.7 macrophages were stimulated by PMA, peroxynitrite-induced chemiluminescence was clearly observed. To prove observed chemiluminescence due to the reaction between superoxide anion radical and nitric oxide produced by RAW264.7 macrophages, N-nitrosoglutathione (GSNO), a nitric oxide-releasing compound, superoxide dismutase(SOD), an enzyme removing superoxide anion radical by dismutating superoxide anion radical to hydrogen peroxide, and N-acethyl cysteine(NAC), a scarvenging reagent both superoxide anion radical and nitric oxide, were added in the cell system. Peroxynitrite- induced chemiluminescence was increased by exogenous addition of GSNO, whereas observed chemiluminescence was decreased by SOD and NAC. These results suggest that PMA-stimulated RAW264.7 macrophages produce both superoxide anion radical and nitric oxide to form peroxynitrite.

INTRODUCTION

Chemiluminescence has been widely used to detect the production of reactive oxygen species from enzymatic reactions, isolated cell systems, and organ systems[1-3]. To yield light, luminol must undergo a two-electron oxidation and form an unstable endoperoxide. Luminol endoperoxide spontaneously decomposes to an excited-state, 3-aminophthalic acid, which relaxes to the ground state by emitting photons. The kinetics and reaction mechanisms of peroxynitrite-induced luminol-enhanced chemiluminescence have been reported[1]. Peroxynitrite anion or peroxynitrous acid may cause a one-electron oxidation of luminol forming the luminol radical followed by a superoxide-mediated second oxidation forming the endoperoxide[4].

Activated macrophages reduce oxygen to superoxide anion radical($O_2 \cdot^-$) and produce nitric oxide($\cdot NO$)[1-4]. Nitric oxide reacts rapidly with superoxide anion radical yielding the peroxynitrite anion($ONOO^-$), which decays, once protonated, to the very reactive hydroxyl radical($\cdot OH$) and to nitrogen dioxide ($\cdot NO_2$)[1-4]. Formation of $ONOO^-$ has been suggested to dramatically enhance cytotoxicity of either nitric oxide or superoxide anion radical alone[5,6].

To better understand the peroxynitrite formation, we studied the biological reaction between superoxide anion radical and nitric oxide produced by PMA-stimulated RAW264.7 macrophages by means of lucigenin- or luminol-enhanced chemiluminescence methods. Although no quantitative description of the mechanism by which peroxynitrite is formed is available, a knowledge of the interaction between superoxide anion radical and nitric oxide in the biological system is necessary for an understanding of the major cell cytotoxicity. In the ensuing experiments, therefore, our attentions would be forcused on the detection of peroxynitrite formed in RAW264.7 macrophages stimulated with PMA. We would have a discussion on the radicals produced by cells and their reaction.

MATERIALS AND METHODS

Reagents. Lucigenin, luminol, Hank's balanced salt solution without phenol red(HBSS), superoxide dismutate (SOD), S-nitrosoglutathione(GSNO), and phorbol myristate acetate (PMA) were purchased from Sigma Chemical Co. All other chemicals and reagents were from commercial sources.

Measurement of superoxide. PMA-stimulated superoxide production was monitored by measurement of lucigenin-enhanced chemiluminescence at 37°C. The reaction mixture consisted of 10 μM lucigenin in veronal buffered saline(pH 7.4). Chemiluminescence of the mixture was measured by using a six-channel luminometer equipped with a data-analyzing system(Model LB 9505C; Labaratorium Berthold AG, Wildbell, German).

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Measurement of nitric oxide. The secretion of nitric oxide into culture supernant was determined by measuring nitrite as described previously[7].

Measurement of peroxynitrite. Peroxynitrite anion was measured by luminol-enhanced chemiluminescence as described by Radi, *et al.*[1] A stock solution(1m*M*) of luminol made in dimethyl sulfoxide was diluted in HBSS to the final concentration of 10 μM just before use.

Culture of RAW264.7 macrophages. RAW264.7 macrophages were cultured in RPMI containing 10% fetal bovine serum. Prior to the experiment, the culture medium was replaced with fresh medium.

RESULTS

Superoxide anion radical sources

Superoxide anion radical production, a property of both macrophages and neutrophils, is stimulated through the interrelated pathways of calcium mobilization and the activation of protein kinase C[8]. 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 superoxide anion radical at the expense of NADPH[8]. Thus, superoxide anion radical sources can be obtained from agonist-stimulated macrophages.

To yield superoxide anion radical sources, we tested the effect of phorbol myristate acetate(PMA), an activator of protein kinase C, at the concentrations from 50 n*M*(sub-optimal dose) to 250 n*M*(maximal dose) on superoxide production in RAW264.7 macrophages. Cells were stimulated with various concentrations of PMA. Then, superoxide production was monitored by lucigenin-enhanced chemiluminescence method. It was found that PMA increases superoxide production with increase in its concentration up to 250 n*M*(Fig. 1).

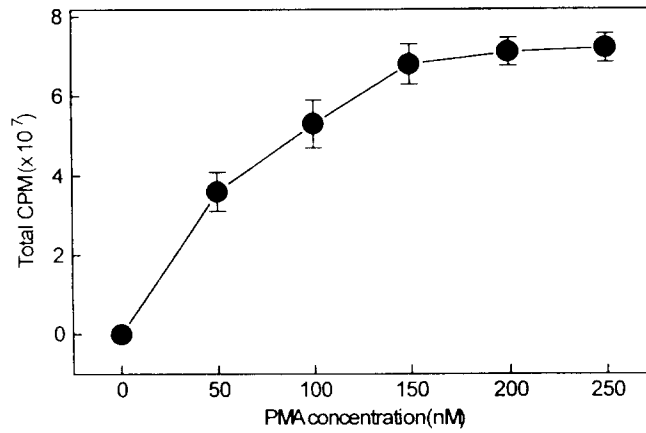

Figure 1. Effect of PMA concentrations on superoxide anion radical production. Cells(2×10^6) were stimulated with PMA at indicated concentrations. Values are the mean ± SD of three experiments.

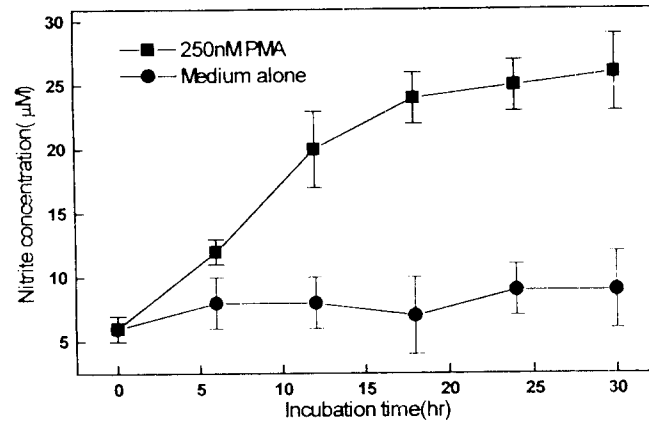

Figure 2. Effects of PMA on the production of nitric oxide in RAW264.7 cells. Cells(2×10^6) were treated with either medium alone or medium containing 250 n*M* PMA for indicated times. Nitric oxide was measured by the method of Griess(nitrite). Results are presented as the mean ± SD of three experiments.

Nitric oxide sources

Macrophages and neutrophils produce not only superoxide anion radical by the respiratory burst oxidase but also nitric oxide by oxidizing L-arginine to citrulline. To determine nitric oxide released by PMA-stimulated RAW264.7 macrophages, cells were stimulated with 250 n*M* PMA. Then, the nitric oxide was determined by the spec-trophotometric measurement. We found that 250 n*M* PMA-stimulated RAW264.7 macrophages released low concentrations of nitric oxide(25 μM for 24 h.) in RAW264.7 macrophages(Fig. 2).

The Interaction between superoxide anion radical and nitric oxide to form peroxynitrite

An important biological reaction of superoxide anion radical may be the one with endothelial-derived nitric oxide[1-4]. 250 n*M* PMA-stimulated RAW264.7 macrophages released low concentrations of nitric oxide, so that peroxynitrite-induced chemiluminescence was not strong. However, Fig. 3 indicates that when RAW264.7 macrophages are stimulated by PMA, detectable amounts of peroxynitrite is formed. To prove this observed chemiluminescence due to the reaction between superoxide anion radical and nitric oxide, we tried to increase the concentration of nitric oxide by adding S-nitrosoglutathione (GSNO), a nitric oxide generator. An exogenous addition of GSNO resulted in enhancing peroxynitrite-induced chemiluminescence. Nitric oxide released by GSNO did not exhibit any detectable luminescence(Fig. 3). Therefore, it is reasonable that oberved chemiluminesence is

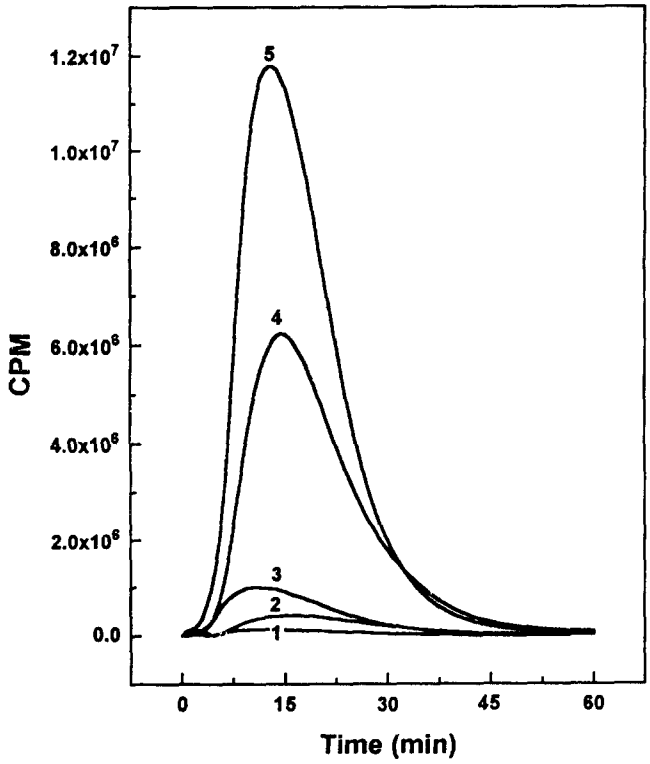

Figure 3. Typical kinetic curves of PMA-induced peroxynitrite formation. 1) 0.01 m*M* GSNO, 2) 50 n*M* PMA alone, 3) 250 n*M* PMA, 4) 50 n*M* PMA in the presence of 0.01 m*M* GSNO, 5) 250 n*M* PMA in the presence of 0.01 m*M* GSNO. RAW264.7 macrophages(2×10^6) were used in these experiments.

due to the reaction between superoxide anion radical and nitric oxide to form peroxynitrite.

To further understand the reaction between superoxide anion radical and nitric oxide, we tried to scavenge superoxide anion radical by using superoxide dismutase (SOD) and both superoxide anion radical and nitric oxide by using N-acetyl cysteine(NAC). As shown in Fig. 4, Chemiluminescence is decreased by SOD and NAC. These results suggestes that observed chemiluminescence resulted from peroxynitrite.

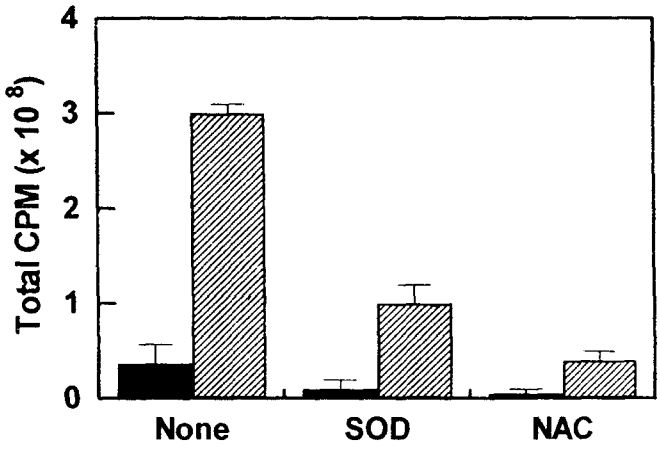

Figure 4. The effects of SOD(200 U/mL) and NAC(0.6 m*M*) on peroxynitrite formation. RAW264.7 macrophages(2×10^6) were stimulated with 250 n*M* PMA in the absence(■) or in the presence(▨) of 0.01 m*M* GSNO. Values are the mean ± SD of three experiments.

DISCUSSION

In the energy-producing processes of our body, molecular oxygen is reduced by four electrons to yield two molecules of water as the end products, partially reduced oxygen species or its excited form(singlet oxygen) can be produced[10]. Oxygen has two unpaired electrons which have parallel spins. When one of the electrons is excited and inverts its spin, a highly reactive singlet oxygen($^1\Sigma_g^+O_2$ or $^1\Delta_gO_2$) is formed. Univalent reduction of oxygen produces the superoxide anion radical, whereas bivalent reduction generates hydrogen peroxide and trivalent reduction generates hydroxyl radical. Most of these active oxygen and related species are highly reactive and cytotoxic, but hydroxyl radical is considered to be the most reactive species[11]. The generation of hydroxyl radical is accelerated by transition metals, especially iron. Considering that nitric oxide has an unpaired electron in its orbital, nitric oxide is thus a free radical, and since nitric oxide undergoes radical-radical reaction with superoxide anion radical, peroxynitrite is not a radical. However, it is a potent oxidant which is spontaneously decomposed to generate hydroxyl radical-like species[11]. It should be noted that oxygen radical species are essential as the immune system to combat pathogens and tumors and free radical reactions are employed in our body for useful purposes such as reactions involving cytochrome P-450 or in the reaction of ribonuleosides[12].

Superoxide anion radical has a limited reactivity toward biological molecules and superoxide anion radical functions as a reducing agent for Fe^{3+} to Fe^{2+} in the Harber-Weiss reaction by promoting the formation of hydroxyl radical in the presence of hydrogen peroxide(H_2O_2). However, this role of superoxide anion radical may be irrelevant since other reducing agents such as ascorbate or glutathione are present in higher concentrations in tissue than superoxide anion radical and these agents can readly reduce Fe^{3+} as compared to superoxide anion radical. Namely, hydroxyl radical can be formed through the reaction between hydrogen peroxide and Fe^{2+} in the absence of superoxide anion radical. Then, there is no logical basis for the effect of SOD on superoxide anion radical-related injury since SOD removes superoxide anion radical by dismutating superoxide anion radical to H_2O_2 i.e., increasing H_2O_2. This is the point which we have been puzzled for a long time. However, if peroxynitrite rather than hydroxyl

radical is the actual cytotoxic molecule, the effect of SOD is readily explained since the removal of superoxide anion radical by SOD simply reduces the production of peroxynitrite and will attenuate the injury.

Several reports[1-7] suggest that the nitric oxide pathway interacts with the respiratory burst of activated inflamatory cells. It was reported that luminol-enhanced chemiluminescence was dependent on the simultaneous production of nitric oxide and superoxide anion radical from agonist-activated macrophages, including Kuffer cells(macrophage-like cells)[1].

In conclusion, we have demonstrated that RAW264.7 macrophages, in response to 250 n*M* PMA, produce both superoxide anion radical and nitric oxide, thereby leading to form detectable concentrations of peroxynitrite.

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