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Report

Implication of NF-κB Activation on Ozone-Induced HO-1 Activation

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The controlled and moderate oxidative stress such as ozone induces both inflammatory and anti-inflammatory response. This balance is important for homeostasis of living organisms. Furthermore, it has been shown that this conflict response is mainly regulated by two transcriptional factors, nuclear transcriptional factor κB (NF-κB) and nuclear factor erythroid 2-related factor 2 (Nrf2). NF-κB is involved in inflammatory responses by regulating expression of cyclooxygenase-2 (COX-2) and various inflammatory cytokines while Nrf2 is involved in anti-inflammatory responses by controlling expression of numerous antioxidant enzymes such as heme oxygenase-1 (HO-1). We here demonstrate the molecular mechanisms of the crosstalk between NF-κB and Nrf2 activation during the moderate oxidative stress induced by ozone. We first confirmed the activation of NF-κB and Nrf2 signaling during the moderate oxidative stress in HeLa cells. Induction of NF-κB-mediated *COX-2* mRNA expression was observed at the early phase after stimulation (30-60 min after ozone treatment). However, induction of *HO-1* mRNA expression was observed at the late phase of stimulation (6 h after stimulation). To reveal the crosstalk between NF-κB and Nrf2, we tested whether reduction of NF-κB expression affects ozone-induced Nrf2 activation by knocking down of NF-κB in HeLa cells. Importantly, the *HO-1* induction by ozone was remarkably decreased by a reduction in NF-κB expression. These results suggest that the moderate oxidative stress by ozone initially induces NF-κB activation, and this NF-κB activation is required for *HO-1* induction at the late phase of the moderate stress.

Key words ozone therapy, oxidative stress, NF-κB signaling, Nrf2 signaling

INTRODUCTION

The process from inflammation to wound healing is an important step in maintaining homeostasis in the body, which is strictly regulated by the balance of inflammatory/anti-inflammatory signals.

NF-κB is a key mediator of inflammatory response, that is a family of transcription factors consisting of RelA (p65), RelB, c-rel, p50 and p52.[1)] Pro-inflammatory cytokines such as Tumor necrosis factor alpha (TNF-α), interleukin 1 beta (IL-1β) and bacterial lipopolysaccharide (LPS) are well known as NF-κB activator. Binding of these cytokines to those receptors induces phosphorylation of IκBα, a negative regulator of NF-κB, followed by ubiquitination and proteasomal degradation of IκBα and releasing NF-κB subunits, such as p65/p50, which then translocate to the nucleus.[2)] In nucleus, NF-κB activates transcription of various pro-inflammatory genes, such as cyclooxygenase-2 (COX-2),[3,4)] inducible nitric oxide synthase (iNOS)[5-8)] and NADPH oxidase-2 (NOX2)[9)] that are enzymes related to reactive oxygen species (ROS) production, resulting in induction of inflammation and oxidative stress. Further, some studies have shown that ROS, in particular, hydrogen peroxide (H_2O_2), can activate NF-κB signaling in various cell types.[10-12)]

On the other hand, Nrf2 is known as a key transcription factor mediating anti-oxidant/anti-inflammatory response contributing cell homoeostasis in response to oxidative stress.[13,14)] Nrf2 is expressed in a wide variety of tissues, but its amount of protein is kept low through Kelch-like erythroid cell-derived protein with CNC homology-associated protein 1 (Keap1) dependent ubiquitination-proteasomal degradation under basal condition.[15)] Oxidative stress causes structural change of Keap1 and release Nrf2. Then Nrf2 translocates to the nucleus and regulates the expression of its target genes related to drug metabolism and disposition, antioxidant defense, such as heme oxygenase 1 (HO-1) and NAD(P)H quinone oxidoreductase-1 (NQO-1).[16-18)]

The balance of Nrf2 and NF-κB pathways is important for the physiological homeostasis of cellular redox status under oxidative stress. Severe oxidative stress induces excessive activation of NF-κB signaling, resulting in chronic inflammation and tissue injury via the production of COX-2 and inflammatory cytokines. On the other hand, mild oxidative stress induces Nrf2 activation and anti-oxidative enzymes that not

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only protect cells from oxidation and inflammation but contribute to recover various tissue injury.

Ozone (O_3) has been widely known as strong oxidant and is toxic for pulmonary system and skin during prolonged exposure.[19-26] However, scientific evidence proved that the effects of ozone exposure are dose-dependent: high dosages stimulate severe oxidative stress resulting in inflammatory response and tissue injury, whereas low doses of ozone induce a moderate oxidative eustress activating antioxidant pathways.[27-37] These properties make ozone a useful medical tool, which can be used as either a disinfectant or an adjuvant agent in the therapy of numerous diseases.[32,38-40]

This paradox of ozone effect should be explained by the balance of NF-κB and Nrf2 signaling. Actually, many studies have been reported about the cross-talk between NF-κB and Nrf2, that Nrf2 plays a critical role in counteracting NF-κB driven inflammatory response.[41-43] However, its molecular mechanism is still unclear.

Here, we demonstrated the molecular mechanisms of the crosstalk between NF-κB and Nrf2 activation during the moderate oxidative stress induced by ozone.

MATERIALS AND METHODS

Reagents and Cells N-Acetyl-L-cysteine (NAC) was purchased from Wako (Tokyo, Japan). HeLa cells were cultured in DMEM (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% FBS (Sigma-Aldrich, St. Louis, MO, USA) at 37°C in a humidified 5% CO_2, 95% air atmosphere.

Knockdown For knockdown of NF-κB (p65) expression in HeLa cells, we used the small interfering RNAs (siRNAs) targeting human NF-κB, detailed as follows: siNF-κB#1, 5′-UUACGUUUCUCCUCAAUCCGG-3′; siNF-κB#2, 5′-CUUGUCGGUGCACAUCAGC-3′. Control siRNA was obtained from Qiagen (nonsilencing; catalog 1022076, Venlo, The Netherlands). HeLa cells were plated on 24-well plates at 2×10^4 cells/well and incubated with a siRNA–Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) mixture at 37°C for 4 h, followed by the addition of fresh medium containing 10% FBS. To confirm the knockdown of NF-κB (p65) in HeLa cells, an aliquot of total cell lysates was analyzed by immunoblotting using anti-p65 (Santa Cruz, CA, USA) or anti-Actin antibody (Santa Cruz, CA, USA). Western blotting assays were performed as described previously.[44]

Culture Condition and Ozone Treatment Ozone was generated from medical-grade oxygen by a streamer discharge type ozone generator (Model TK20, Otec. Lab., Tokyo, Japan), which allows the gas flow rate and ozone concentration (0.0~40.0 μg/mL) to be controlled in real time by photometric determination. Produced ozone/oxygen mixture was taken from the syringe port of the generator with a sterile syringe and then injected into cell culture medium with dose of 30 μg O_3 /mL. This ozonated medium was prepared immediately before use. Cells were cultured in 24-well plates with 500 μL of DMEM containing 10% FBS. For ozone treatment, the culture medium was replaced with ozonated medium prepared as above.

RNA Isolation and Quantitative Real-Time PCR (qPCR) Cells were harvested, and total RNAs were prepared using IsoGen (Nippon Gene, Tokyo, Japan) and used in RT-PCR. RT-PCR was performed using an RT-PCR high-Plus-Kit (TOYOBO, Tokyo, Japan). Quantitative real-time PCR analyses of *COX-2* and *HO-1*, as well as the control *beta-Actin* mRNA transcripts, were carried out using the assay-on-demand gene-specific fluorescently labeled TaqMan MGB probe in an ABI Prism 7000 sequence detection system (Applied Biosystems, Foster City, CA, USA).[44] The significance of differences between group means was determined by Student's t-test.

Measurement of Intracellular ROS Intracellular ROS was detected with ROS assay Kit - Highly Sensitive dichlorodihydro-fluorescein diacetate (DCFH-DA) (Dojindo, Tokyo, Japan), according to manufacture's standard protocol. The fluorescence was measured after 30 min of incubation with 10 μM DCFH-DA using excitation and emission wavelengths of 488 and 515 nm by Zeiss LSM 510 laser scanning microscope with an Apochromat x63/1.4 oil immersion objective and x4 zoom.

RESULTS

To confirm activation of NF-κB and Nrf2 signaling induced by ozone, we measured time-dependent mRNA expression of *COX-2* and *HO-1* as a major target of each signaling in HeLa cells before and after cultured with ozonated medium. As a result, *COX-2* was induced in early phase (0.5-1 h ozone treatment), while *HO-1* mRNA expression increased after 6 h (Fig. 1A, B). This result indicated that NF-κB signaling was activated by ozone treatment, prior to Nrf2. To investigate the crosstalk between NF-κB and Nrf2 during ozone treatment, we tested the effect of NF-κB (p65) knockdown in HeLa cells by using siRNA. We measured *HO-1* mRNA expression induced by ozone treatment in WT or NF-κB knockdown HeLa cells. As a result, we observed *HO-1* induction by ozone treatment was dramatically decreased by NF-κB knockdown. This result suggested that NF-κB activation by ozone at early phase was important for *HO-1* induction at late phase (Fig. 1C).

It has been well known that activation of NF-κB signaling followed by induction of various inflammatory molecules (such as COX-2) causes ROS production and oxidative stress. To confirm the ROS production after ozone treatment, we treated HeLa cells with DCFH-DA and monitored the fluorescence of oxidized DCFH-DA in HeLa cells after ozone treatment. As a result, we observed the ROS production by ozone treatment at early phase (Fig. 2A). To investigate the effect of ROS production on *HO-1* induction by ozone treatment at late phase, we used NAC as ROS inhibitor. We confirmed that NAC treatment blocked ROS induction by ozone treatment in HeLa cells (Fig. 2A). Further, we measured the mRNA expression of *COX-2* and *HO-1* induced by ozone treatment with or without ROS inhibitors. Interestingly, *HO-1* induction was completely blocked by ROS inhibitor, while *COX-2* induction was not affected (Fig. 2B, C). These results suggested that ROS production by ozone treatment via NF-κB signaling is an essential step for anti-oxidant response, such as *HO-1* induction (Fig. 3).

Conclusion Here we demonstrated that the rapid NF-κB activation followed by ROS production is necessary for Nrf2-target gene, *HO-1* induction by ozonated medium treatment (Fig. 1C and Fig. 2). However, it is still unknown how ozonated medium activates NF-κB signaling. In major autohemotherapy (MAH), that is representative ozone therapy, peripheral blood from the patient is mixed with ozone gas, and then reinfused into the patients. Injected ozone gas is immediately dissolved in blood and reacts with unsaturated fatty acid

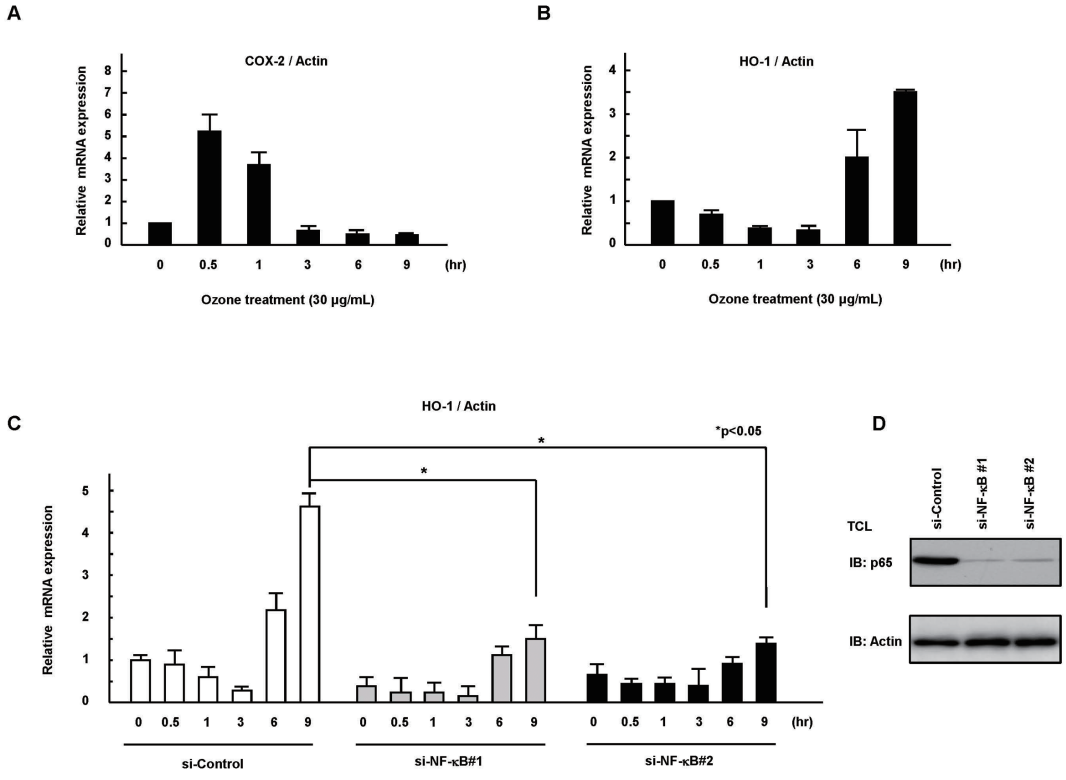

Fig. 1. Ozone Treatment Activates NF-κB Signaling, Prior to *HO-1* Induction

A,B, HeLa cells were cultured with ozonated medium (30 μg O_3/mL) for indicated period in a 24-well plate, and then mRNA expression of *COX-2*/*HO-1* were measured by qPCR. Results are representative of three independent experiments, and the error bars represent the SD. C, HeLa cells in a 24-well plate were transfected with human NF-κB (p65) or control siRNAs (20 pmol). At 24 h after transfection, cells were cultured with ozonated medium (30 μg O_3/mL) for indicated period, and then mRNA expression of *HO-1* was measured by qPCR. Results are representative of three independent experiments, and the error bars represent the SD. D, To confirm the knockdown of NF-κB (p65) in HeLa cells, an aliquot of total cell lysates was analyzed by immunoblotting using anti-p65 or anti-Actin antibody.

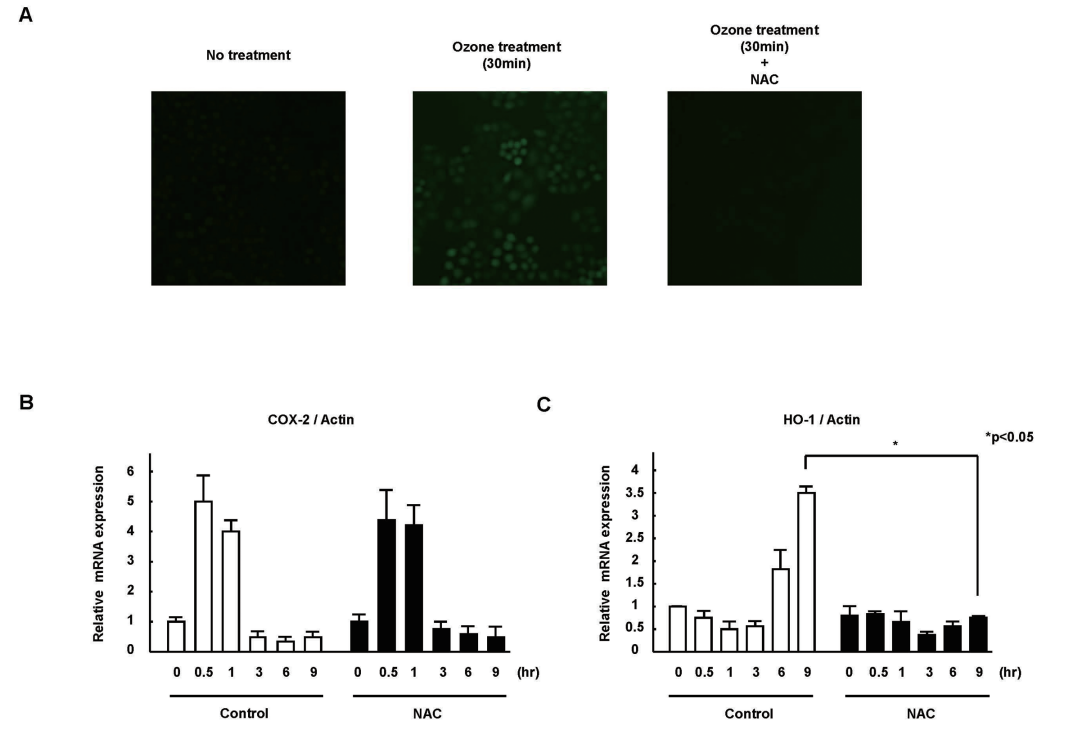

Fig. 2. ROS Production via NF-κB Signaling is Critical for *HO-1* Induction by Ozone Treatment

A, HeLa cells in a 6-well plate were cultured with ozonated medium and 3 mM NAC (ROS inhibitor) for 30 min, and then cells were incubated with 10 μM DCFH-DA for 30 min. The ROS production was observed by confocal microscope as fluorescence intensity (green) of oxidated DCFH. Results are representative of three independent experiments. B-E, HeLa cells were cultured with ozonated medium and NAC for indicated period in a 24-well plate, and then mRNA expression of *COX-2*/*HO-1* were measured by qPCR. Results are representative of three independent experiments, and the error bars represent the SD.

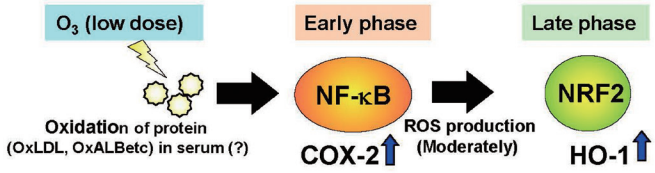

Fig. 3. Schematic Model of NF-κB and Nrf2 Activation for *HO-1* Induction

leading to the generation of second messengers such as H_2O_2, 4-hydroxynonenal (4HNE) and other lipid oxidation products (LOPs). It has been reported that these second messengers are the key factor for Nrf2 and NF-κB activation resulting in antioxidant reaction.[34,36] From these findings, it can be predicted that NF-κB signaling is activated not by ozone gas directly, but by some second messengers in ozonated medium (Fig. 3). To identify the mediator for NF-κB activation in ozonated medium, we have examined the necessity of FBS and low-density lipoprotein (LDL) receptor for NF-κB activation. However we are still not getting the direct evidence indicating which components are the novel mediator of NF-κB activation during ozone treatment from our preliminary data (data not shown). Further study will be needed to understand the detailed mechanism of NF-κB and Nrf2 activation by ozonated medium, that will be useful to improve clinical efficacy of ozone therapy.

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Conflict of interest The authors declare no conflict of interest.

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