Singlet oxygen from endoperoxide initiates an intracellular reactive oxygen species release in HaCaT keratinocytes

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Singlet oxygen (1O2) is a selective intermediate reactive oxygen species generated naturally in biological systems by light- and non-light mediated processes. Although 1O2 plays an important role in cell signaling and in maintaining homeostasis, it can be toxic due to its ability to diffuse across considerable distances. Several in vitro studies have investigated the pathways by which 1O2 mediates oxidation of biological molecules and potential pathogenesis. However, understanding how singlet oxygen exerts cell injury through the production of subsequent reactive oxygen species remains unexplored. To study this, we used a hydrophobic endoperoxide as a source of 1O2. Endoperoxides are reagents that quantitatively generate singlet oxygen in solution at 35°C by thermal decomposition. Our chemiluminescence and cell viability assay data revealed that 1O2 stimulated a secondary intracellular reactive oxygen species production in a very short time. To determine the source of these reactive oxygen species with endoperoxide exposure, cells were treated with inhibitors targeting NADPH oxidases and platelet activating factor receptors. Our results showed that addition of the platelet activating factor receptor antagonist, Apafant (WEB2086), alleviated cell injury and hydrogen peroxide levels following endoperoxide stimulation. Furthermore, intracellular calcium assay data demonstrated a potential calcium sensitive production of intracellular reactive oxygen species.

Key Words: singlet oxygen, endoperoxide, PAF receptor, HaCaT keratinocytes, intracellular ROS

Singlet oxygen (1O2) is a naturally occurring reactive oxygen species (ROS) that plays important roles in biological systems. Under normal conditions (i.e., oxidative eustress), this ROS functions as a secondary messenger during cell signaling,1) and as an antimicrobial in immune response.2) However, singlet oxygen readily oxidizes several biological molecules, particularly proteins, DNA, and lipids.3,4) Therefore, when its production rises above homeostatic levels, it leads to disease pathogenesis and cell injury (i.e., oxidative distress).5)

Singlet oxygen functions as an intermediate ROS in biological systems, since it can diffuse across considerable distances,6) and processes involving its production readily lead to secondary reactive byproducts such as lipid peroxidation.2,7) Although several in vitro studies have been conducted to understand the functions of singlet oxygen in the cell, none of them have addressed its cell injury pathway via the ROS production cascade. Consequently, the mechanism by which this phenomenon occurs and the specific pathways involved remain unknown.

Photochemical reactions involving photosensitizers are a commonly used source of this ROS. This method, however, is not ideal in exclusively studying the functions of singlet oxygen as it simultaneously produces other ROS.3) However, chemical generation of singlet oxygen in solution by thermal decomposition of endoperoxides (EPs) is a more specific and easily utilized source.5)

EP is a reagent that is widely used in Japan for singlet oxygen absorption capacity assays of antioxidants,9) and previously developed as a useful tool for singlet oxygen stimulation in biological studies.10) Therefore, this study investigated the effects of singlet oxygen from EP as a biological stimulus on the intracellular generation of ROS as well as the possible pathways associated with it.

Materials and Methods

Reagents. EP powder was purchased from Waken Btech, Co., Ltd. (Kyoto, Japan), and the solution was prepared by diluting the powder in 0.1% dimethyl sulfoxide (DMSO; FUJIFILM Wako Pure Chemical Industries, Osaka, Japan). To prepare inactive EP (negative control), 1 mM EP solution was incubated at 37°C with a humidified atmosphere of 5% CO2 (Thermo Fisher Scientific, San Jose, CA) for 24 h. Catalase (CAT; FUJIFILM Wako Pure Chemical Industries), and superoxide dismutase (SOD; Nippon Kayaku Co., Tokyo, Japan) were dissolved in distilled water to a final concentration of 10,000 U/ml, aliquoted, and stored at 4°C. WEB2086 (Sigma-Aldrich, St. Louis, MO) was prepared by dissolving 1 mg in 220 μl of DMSO then 1.98 ml of PBS (−) was added to reach a final concentration of 10 nM (and a DMSO concentration of 10%). Sodium azide (NaN3) was obtained from FUJIFILM Wako Pure Chemical Industries and dissolved in medium to prepare the desired final concentration. 0.143 mg of MnTMPyP (Calbiochem, Merck KGaA, Darmstadt, Germany) was dissolved in medium to a final concentration of 10 μM for all experiments, and the remaining solution was aliquoted and stored at −20°C. Effective concentrations and incubation times for inhibitors, ROS scavengers, et cetera, were decided in consideration of toxicity using cell viability assay.

Cell culture. Human epidermal keratinocytes (HaCaT cells) were provided by Cell Lines Service and used in all experiments carried out in this study. The cells were cultured in Dulbecco’s Modified Eagle’s Medium–high glucose (Sigma-Aldrich) with 10% Fetal Bovine Serum (FBS; Nichirei Biosciences, Tokyo, Japan), and 1% antibiotic antimycotic solution (Sigma-Aldrich). 0.025% Trypsin-EDTA with phenol red (Gibco, Carlsbad, CA)

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was used to passage the cells. Washing was done using 9.6 g of Dulbecco’s phosphate buffer solution (−) [PBS (−); Nissui Pharmaceutical Co., Ltd., Tokyo, Japan] dissolved in 1 L of distilled water and sterilized by autoclaving.

**Cell viability assay.** Cell viability (WST-8) assay is a method used to quantitatively measure viable cells through cellular mitochondrial activity. In a 96 well plate (Greiner Bio-One, Frickenhausen, Germany), the cells were seeded at a concentration of 2.0 × 10^5 cells/ml in 100 μl/well and incubated for 24 h. Following confluence, the culture solution was removed and washed twice with 100 μl PBS (−). PBS (−) was then removed, and stimulation was performed under the desired conditions (Fig. 1). A mixed solution of Cell Counting Kit-8 reagent with water soluble tetrazolium salt (WST-8; Dojindo Chemical Laboratory, Kumamoto, Japan) and D-MEM medium containing 10% FBS (or Zero medium obtained from CSTL, Inc., Miyagi, Japan) was prepared according to the manufacturer, and 100 μl of the solution was added to each well. Blanks were prepared by adding the same solution to wells without cells. The plate was then incubated for 30 min and the absorbance was measured at 450 nm using Varioskan Flash Multimode Reader (Thermo Fisher Scientific).

**Chemiluminescence.** 100 μl of the chemiluminescence (CL) reagents MCLA (TCI, Tokyo, Japan) or L-012 (FUJIFILM Wako Pure Chemicals), 50 μl of ROS scavengers, and 300 μl Krebs-Ringer Buffer (KRB) were added in a number 4 Eiken tube (Eiken Chemical Co., Ltd., Tokyo, Japan) and measurement was initiated for 60 s. 50 μl of EP solution in 0.1% DMSO was then added and measurement was completed for 1,000 s. To measure cellular ROS, HaCaT cells (cultured 48 h prior until confluence) were trypsinized and resuspended in KRB to make a solution with a cellular concentration of 7.0–9.0 × 10^5. For analysis, ROS generation was evaluated by area under the curve starting from 64 s (i.e., total emission following stimulation), and the peak-base value (maximum emission intensity), and represented as a bar graphs.

**Intracellular calcium assay.** HaCaT keratinocytes were seeded at a concentration of 2.0 × 10^5 cells/ml in 100 μl/well in a black 96 well plate (Thermo Fisher Scientific) overnight until confluence. The plate was then washed with PBS (−), and cells were stimulated by incubation with EP concentrations of 0, 50, 100, 500, and 1,000 μM for one hour. Following EP stimulation, the plate was washed again and Calcium Kit II-Fluo 4 (Dojindo) was prepared according to the manufacturer’s instructions, and subsequently added to the cells. Fluorescence intensity was then measured at a wavelength of 485/535 nm in a microplate reader. For groups with DPI pre-treatment, the cells were incubated with 2.5 μM DPI for two minutes and washed prior to 1 mM EP stimulation. For WEB2086 groups, the cells were incubated with 10 μM of the platelet activating factor receptor (PAF-R) antagonist for 10 min and then stimulated with 1 mM EP without washing.

**Hydrogen peroxide assay.** HaCaT keratinocytes were seeded in a black 96 well plate overnight until confluence. The plate was washed using PBS (−), and cells were treated with ROS scavengers, WEB2086, or diphenyleiodonium chloride (DPI) prior to 1 mM EP stimulation similar to previous assays. The same method was used to measure hydrogen peroxide concentration in media only with EP stimulation. Following incubation, Red Hydrogen Peroxide assay kit (Enzo Life Sciences, Farmingdale, NY) was used in accordance to the manufacturer’s instructions, and fluorescence intensity of the standards was measured using a microplate reader at a wavelength of Ex570/ Em590 nm. The standards curve equation was then used to calculate the hydrogen peroxide concentration of the samples.

**Statistical analysis.** Statistical significance between groups was evaluated using Tukey HSD post-hoc test, and one way ANOVA. A p value of less than 0.05 was considered significant. All analyses were performed on IBM SPSS ver. 27 and Microsoft Excel 2016.

**Results**

Endoperoxide releases singlet oxygen in a dose-dependent manner. In order to determine the effective concentration of EP for this study, MCLA CL was employed to measure singlet oxygen release. The data (Fig. 2) shows that singlet oxygen production increased dose-dependently from EP solutions prepared in 0.1% DMSO when measured for 1,000 s.

ROS production from 1 mM endoperoxide is significantly

![Fig. 1. Experimental design for pre-, co-, and post-incubation WST-8 assays with 1 mM EP stimulation. In all three tests, cells were seeded at a concentration of 2 × 10^5 in 96 well plates and incubated for 24 h prior to treatment and stimulation. For treatment, ROS scavengers and the PAF-R antagonist were used at the following concentrations: superoxide dismutase and catalase, 1,000 U/ml; sodium azide and histidine, 1 mM; MnTMPyP and WEB2086, 10 μM.](image-url)
increased in the presence of cells. In both MCLA and L-012 CL analysis data (Fig. 3), ROS production rate was significantly higher in samples containing cells compared to those that did not.

Sustained intracellular superoxide and singlet oxygen production occurred following 1 mM endoperoxide exposure. In MCLA and L-012 CL analysis for detection of superoxide radical and singlet oxygen (Fig. 4), treatment with 10 mM sodium azide (NaN₃), 1,000 U/ml SOD, and 10 µM of the cell-permeable SOD mimic (MnTMPyP) significantly reduced the intracellular ROS production with 1 mM EP stimulation.

One mM EP stimulation lowered cell viability within 1 h. Cell viability assay data for 1-, 3-, 6-, 9-, and 12-h incubation with 1 mM EP solution reveals a significant reduction in viability when compared to the control group (Fig. 5).

ROS scavengers targeting superoxide, singlet oxygen, and hydrogen peroxide improved cell viability. Pre-, co-, and post-incubation cell viability assay data demonstrate significant improvement in viability in groups treated with ROS scavengers; particularly those targeting singlet oxygen, intracellular superoxide, and intracellular hydrogen peroxide with EP stimulation (Fig. 6).

NADPH oxidases (Noxs) are not a major source of intracellular ROS following endoperoxide exposure. Pre-treating cells with 2.5 µM of the Nox inhibitor, DPI, did not cause a significant improvement in cell viability (Fig. 7A) nor did it significantly reduce ROS production with 1 mM EP stimulation (Fig. 7B).

PAF-R pathway could be one of the main sources of ROS following EP stimulation. As stated earlier; in pre-, co-, and post-incubation cell viability assay data, PAF-R antagonist, WEB2086, counteracted the cell injury caused by 1 mM EP (Fig. 6). In addition, hydrogen peroxide assay data (Fig. 8B), demonstrate a significant reduction in hydrogen peroxide concentration when cells were co-incubated with WEB2086 and EP.

EP causes a rise in intracellular calcium levels. This increase, however, was counteracted by blocking Nox and PAF-R. Intracellular calcium assay data revealed a steady, dose-dependent increase in intracellular calcium with EP (Fig. 9A). Pre-treatment with DPI or co-incubation with the PAF-R antagonist, WEB2086, significantly countered the rise in intracellular calcium levels caused by 1 mM EP stimulation (Fig. 9B).

Discussion

In this study, we revealed that singlet oxygen, obtained via thermal decomposition of EPs, initiated an intracellular ROS release, and that this phenomenon may be a major cause of its cell injury pathway.

CL assay was employed to determine an effective concen-
Fig. 4. CL curves and AUC graphs for ROS production with 1 mM EP. (A) MCLA, (B) L-012 in the presence of HaCaT keratinocytes and ROS scavengers. Values are reported as means ± SD (n = 3–4). *p<0.05, **p<0.01.

Fig. 5. Cell viability assay data using WST-8 kit. The cells were incubated with 1 mM EP for 1–12 h prior to reading. Values are reported as means ± SD (n = 4). *p<0.01.

Treatment of the EP. This method can detect ROS via oxidation reactions using specific CL probes or reagents. L-012 (a luminol derivative) and 2-methyl-6-(4-methoxyphenyl)-3,7-dihydroimidazole [1,2-a] pyrazin-3-one (MCLA) probes were selected, according to a previous study that extensively evaluated various CL reagents. This is because of their high sensitivity in detecting different ROS, specifically superoxide radical ($O_2^{-}$) and singlet oxygen ($^{1}O_2$). Our results using MCLA CL revealed a dose-dependent rise in singlet oxygen levels with EP stimulation, and a high detection in 1,000 μM solution (i.e., 1 mM) (Fig. 2). The evaluation was performed for approximately 16 min, with a peak reading at approximately 8 min, followed by a steady decline. This shows that EP releases singlet oxygen for a short time (i.e., less than one hour), and is consistent with previous studies using 1-methylnaphthalene-4-propionate EP, which is hydrophobic and structurally similar to the EP used in this study. 

Skin exposure to several extracellular stimuli such as toxins, ultraviolet radiation, and drugs, generates intracellular ROS in excessive quantities which lead to disease pathogenesis. Interestingly, when samples containing cells were compared to those without in our MCLA and L-012 CL data, ROS detection was significantly higher in the cell group (Fig. 3). These data confirmed that cellular ROS production is possible in response to EP stimulation. Furthermore, CL is a sensitive method for ROS detection, but fails to detect normal intracellular ROS levels. Therefore, the slight increase in the CL reading with cells may indicate an excessive production of ROS. However, this production was not limited to $^{1}O_2$. Our results also showed that EP released $^{1}O_2$, most likely, intracellular SOD mimetic, MnTMPyP (Fig. 4). Although the ROS released seemed to be predominantly singlet oxygen and superoxide, minute amounts of other reactive oxygen species were noted.

To analyze the effect of ROS production on cell function and further confirm the CL findings, the WST-8 kit was used. We found that 1 mM EP, which produced about 5.6 x 10^6 relative light units of singlet oxygen per second (RLU/s) at maximum reading, significantly lowered cell viability after one hour of incubation (Fig. 5). This finding supports the hypothesis that EP at this concentration is toxic to cells. Furthermore, treating the cells with scavengers targeting intracellular superoxide...
Co-incubation

**Fig. 6.** Cell viability assay data using WST-8 kit. (A) HaCaT keratinocytes were pre-treated with the ROS scavengers for 1 h, followed by 1 mM EP incubation for another hour. (B) In co-incubation tests, ROS scavengers were added for 10 min followed by the addition of 1 mM EP solution; the cells were then incubated with both for 1 h. (C) In post-incubation tests, the cells were treated with 1 mM EP for 1 h prior to ROS scavenger treatment for another hour. SOD, superoxide dismutase; CAT, catalase; His, histidine; NaN₃, sodium azide. Values are reported as means ± SD (n = 3–14). **p<0.01 vs inactive EP, *p<0.05, **p<0.01 vs EP.

(MnTMPyP), and singlet oxygen (His and NaN₃) improved viability (Fig. 6A and B), which was consistent with the CL data. Post-incubation data especially upheld the theory that exposure to EP resulted in an intracellular ROS response, as the scavengers and PAF-R inhibitor were still effective even after removal of the stimulant (Fig. 6C). Additionally, when cells were treated with scavengers that exclusively neutralize extracellular superoxide and hydrogen peroxide, cell viability remained unchanged (data not shown). Yet, it is still unclear whether this effect is due to the oxidation of biological molecules or the consequent ROS production caused by singlet oxygen.

Next, it is important to identify the source of intracellular ROS in response to singlet oxygen stimulation. HaCaT keratinocytes express the enzymes, Noxs, particularly Nox-1, 2, 4, and 5 at the mRNA level with different subcellular localizations. These enzymes are a major source of ROS in epidermal cells to maintain homeostasis and may lead to cell injury via excessive ROS production in response to ultraviolet (UV) irradiation. Therefore, the cells were treated with the widely used Nox inhibitor, DPI, prior to EP stimulation to determine whether Nox is involved in this model. Our WST-8 assay data did not show significant improvement in viability when compared to the untreated group (Fig. 7A), which negates Nox as a main source of ROS in this system. To further confirm these findings, ROS production in cells treated with DPI and untreated cells was measured using MCLA CL and the areas under the curve were compared. The data revealed no significant difference in ROS production (Fig. 7B).

HaCaT keratinocytes express functional PAF-Rs and synthesize the potent inflammatory lipid mediator, PAF, and other PAF-R agonists are produced in response to UVRs, and ultimately cause an increase in intracellular ROS levels. Hydrogen peroxide is widely implicated by both inducing PAF synthesis and being intracellularly produced by PAF in immune cells. Accordingly, using the PAF-R antagonist, WEB2086, in our WST-8 assay was effective in improving the cell viability with EP stimulation. These data demonstrate that PAF-R can be a potential source of intracellular ROS production, particularly intracellular hydrogen peroxide, in this system. In order to measure the intracellular concentration of this ROS, an assay kit was used. According to our red hydrogen peroxide assay data in media only, EP released approximately 5 μM extracellular hydrogen peroxide via a normal ROS cascade (Fig. 8A). Hydrogen peroxide at this level, however, is too low to cause oxidative distress, as it requires an extracellular concentration of approximately 10 μM or higher. Furthermore, treating the cells with WEB2086, catalase, sodium azide, and DPI showed significant alleviation of H₂O₂, but this reduction was not clearly represented (Fig. 8B). This is due to the abundance and high turnover rate of catalase in keratinocytes. Therefore, experiments using a catalase inhibitor are required to adequately measure hydrogen peroxide concentration, irrespective of catalase activity. Nonetheless, this does not entirely nullify the action of the ROS scavengers and inhibitors in lowering the hydrogen peroxide levels.

Lastly, intracellular calcium ([Ca²⁺]) plays a critical role in the epidermis for normal physiological functions and initiates ROS production following UV radiation. This rise in ROS levels could be due to the effects of calcium on the cytosolic proteins responsible for Nox activation or via the promotion of the Krebs cycle for ATP synthesis in the mitochondria. Thus, intracellular calcium assay method was employed to determine whether a similar phenomenon occurred in this model. Our data showed a dose-dependent increase in [Ca²⁺] levels following EP stimulation (Fig. 9A). Furthermore, treatment of cells with WEB2086 or DPI notably alleviated [Ca²⁺] levels (Fig. 9B). These results are consistent with those of a previous study in which a dose-dependent increase in calcium and ROS levels was noted with PAF stimulation, and the addition of DPI inhibited this production.

In conclusion, our study highlights the possibility of an indirect sustained release of intracellular ROS in response to singlet oxygen from EP. These ROS are potentially generated through PAF-Rs via a calcium sensitive pathway. Alternatively, WST-8
and CL data disputed Nox involvement in this model with respect to ROS production. However, intracellular calcium and hydrogen peroxide assays revealed a minimal participation of this family of enzymes. Further investigations are required to better understand the roles of Nox and PAF in this system, and to identify which secondary ROS is responsible for this phenomenon. Moreover, we hypothesize that mitochondria are a likely source of continuous intracellular $\text{O}_2^{-}$, $\text{H}_2\text{O}_2$, and $'\text{O}_2$ following EP stimulation. That is because the mitochondria are sensitive to $[\text{Ca}^{2+}]$, and are major producers of ROS in most

Fig. 7. Involvement of Nox in this model. (A) Cell viability assay data using WST-8 kit. In the DPI treated group, cells were incubated with 2.5 µM DPI for 2 min prior to 1 mM EP exposure. Values are reported as means ± SD ($n = 14$). (B) MCLA chemiluminescence curve and AUC graph for ROS production following 1 mM EP stimulation from cells. The same concentration and incubation time was used for DPI pre-treatment. Values are reported as means ± SD ($n = 4–6$).

Fig. 8. Quantification of hydrogen peroxide concentration using red hydrogen peroxide assay kit. (A) ROS scavengers were first added for 10 min to media only (i.e., no cells), followed by 1-h coincubation with 1 mM EP and fluorescence was measured. (B) HaCaT keratinocytes were pre-treated with ROS scavengers for 10 min, followed by coincubation with 1 mM EP solution for 1 h (for DPI group, cells were pre-treated for 2 min only with 2.5 µM DPI instead). Values are reported as means ± SD ($n = 7–12$). *$p<0.05$ vs EP, **$p<0.01$ vs EP.
mammalian cells via the respiratory electron transport chain. Therefore, future experiments are expected to explore the relevance of the mitochondrial pathway as a source for intracellular ROS, and observe this subsequent ROS production using more extensive methods.

**Author Contributions**

HI and YM designed the study. HI supervised the study progress. ME conducted the experiments and analyzed the data. ME wrote the manuscript. HI, YM, and ME reviewed and edited the manuscript before submission.

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