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Modification of cell vulnerability to oxidative stress by N-(3-oxododecanoyl)-L-homoserine-lactone, a quorum sensing molecule, in rat thymocytes

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Highlights

· N-(3-oxododecanoyl)-L-homoserine-lactone (ODHL) potentiates H_2O_2 cytotoxicity.

· ODHL does not potentiate the cytotoxicity of calcium ionophore A23187.

· ODHL further augments H_2O_2-induced elevation of intracellular Zn^{2+} level.

· ODHL greatly attenuates H_2O_2-induced increase in intracellular Ca^{2+} level.

· Zn^{2+} contributes to ODHL-induced potentiation of H_2O_2 cytotoxicity.

Abstract

N-(3-oxododecanoyl)-L-homoserine-lactone (ODHL), a quorum sensing molecule, affects intracellular Zn^{2+} concentration ($[Zn^{2+}]$i) and cellular levels of nonprotein thiols ([NPT]i) of rat thymic lymphocytes, both of which are assumed to affect cell vulnerability to oxidative stress. Therefore, it is interesting to examine the effects of ODHL on the cells under oxidative stress. ODHL augmented the cytotoxicity of H_2O_2, but not calcium ionophore A23187. ODHL potentiated the H_2O_2-induced elevation of $[Zn^{2+}]$i, wherein, it greatly attenuated the H_2O_2-induced increase in intracellular Ca^{2+} concentration. ODHL did not affect [NPT]i in the presence of H_2O_2. Therefore, we conclude that the elevation of $[Zn^{2+}]$i is involved in the ODHL-induced potentiation of H_2O_2 cytotoxicity. Our findings suggest that ODHL modifies cell vulnerability to oxidative stress in host cells.

Keywords: lymphocytes; 3-oxododecanoyl-homoserine-lactone; hydrogen peroxide; zinc

Introduction

N-(3-oxododecanoyl)-L-homoserine-lactone (ODHL) is known as a quorum sensing molecule in Gram-negative bacteria [1,2]. QS molecules induce synchronized biological events through cell-to-cell communication in bacteria [3.4] and the cellular activities of host cells [5.6]. In our previous study using rat thymic lymphocytes as cell models [7], the treatment of rat lymphocytes with ODHL at low micromolar concentrations increased the cellular content of nonprotein thiols ([NPT]i), intracellular Zn^{2+} levels ([Zn^{2+}]i), and the cellular content of superoxide anions ([O_2^-]i). An increase in [NPT]i protects the cells against oxidative stress [8]. Elevation of [Zn^{2+}]i induces reciprocal actions; the increase in [NPT]i [9] and the augmentation of cytotoxicity of hydrogen peroxide (H_2O_2), a metabolite of O_2^- [10]. Thus, it is suggested that ODHL can modify the redox status in host cells [7]. Some compounds, which elicit oxidative stress, elevate [NPT]i at low concentrations and reduce it at high concentrations [11,12]. Zn^{2+} is involved in the elevation of [NPT]i by the compound at low concentrations. Furthermore, the removal of intracellular Zn^{2+} potentiates the cytotoxicity of A23187, a calcium ionophore [13]. There are complicated relations between [Zn^{2+}]i, [NPT]i, and the concentrations of compounds that increase [Zn^{2+}]i and/or induces oxidative stress. Therefore, it is interesting to examine the effects of ODHL on the cells suffering from oxidative stress because of the following reasons: ODHL may affect (increase or decrease) cell vulnerability to oxidative stress. Moreover, during bacterial infection, oxidative stress is implicated in the development of inflammation [14]. It is also reported that ODHL induces anti-inflammatory molecules in human immune cells [15]. In addition, H_2O_2 is a central redox-signaling molecule in physiological oxidative stress [16]. Therefore, this study provides interesting insights into the interaction between QS and host cells.

2. Materials and methods

2.1. Chemicals

ODHL was obtained from Sigma-Aldrich (St. Louis, Missouri, USA). Specific reagents and fluorescent probes used to photochemically estimate the changes in biological parameters of rat thymocytes are described in Table 1. Other chemical reagents were products of Wako Pure Chemicals (Osaka, Japan).

(Table 1 near here)

2.2. Cell preparation

The university committee for animal experiments approved this study (T29-52). Thymuses were isolated from Wistar male rats (8–12 weeks), which were intraperitoneally anesthetized with thiopental (50 mg/kg). Isolated thymuses were immersed in ice-cold Tyrode's solution. Tyrode's solution was prepared with NaCl (150 mM), KCl (5 mM), $CaCl_2$ (2 mM), $MgCl_2$ (1 mM), 2-[4-(2-Hydroxyethyl)-1-piperazinyl] ethanesulfonic acid (5 mM) with the appropriate amount of 2–3 mM NaOH for pH adjustment at 7.3–7.4. The thymuses immersed in ice-cold Tyrode's solution were sliced with a razor. Sliced preparations were mechanically dispersed in the Tyrode's solution to disperse thymic lymphocytes (thymocytes) for preparing cell suspensions. The solution containing dissociated thymocytes was passed through a mesh (50 μm in pore diameter) and then incubated at a bath temperature of 36–37°C for 50–60 min until further use. The density was 5–7 $\times 10^5$ cells/ml in the cell suspension.

2.3. Experimental procedures and cytometric measurements

All experiments were performed at 36–37ºC. ODHL was dissolved in dimethyl sulfoxide (DMSO). The ODHL solution (3–30 mM ODHL in DMSO) was added to the cell suspension to achieve the final ODHL concentrations (3–30 μM).

To photochemically measure the change in $[Zn^{2+}]i$ or $[Ca^{2+}]i$, the cells were pretreated with 1 μM FluoZin-3-AM [17] or 1 μM Fluo-3-AM [18] for 60 min at least before the experiments. FluoZin-3 or Fluo-3 fluorescence was detected only from living cells with intact membranes by a flow cytometer (CytoACE-150, JASCO, Tokyo, Japan). Dead cells and the cells with

deteriorated membranes were stained with water soluble propidium iodide and the cells with propidium fluorescence were neglected from the measurement.

The cells were treated with 500 nM 5-CMF-DA for 20 min before measuring the 5-CMF fluorescence. The fluorescence measurement allows measuring the changes in [NPT]i in this preparation [19]. The fluorescence of 5-CMF attains a steady intensity at 20–30 min after the start of the treatment of cells with 5-CMF-DA [19]. Notably, the cells were further incubated with the test agent for 20–30 min in the experiments using 5-CMF-DA.

2.4. Statistical analysis

Statistical analysis using Tukey's multivariate test was performed. P-values of less than 0.05 were statistically considered significant. Numerical results (columns and bars in figures) indicate the mean and standard deviation of four samples, respectively. The experiment was carried out two or three times.

3. Results

3.1. Augmentation of H_2O_2 cytotoxicity by ODHL

As shown in the cytograms (Figure 1), the treatment of cells with 300 μM H_2O_2 for 4 h increased the population of cells with propidium fluorescence (presumably dead cells), while this was not the case with 30 μM ODHL. Thus, ODHL itself was not cytotoxic at the concentration of 30 μM. However, the simultaneous application with 30 μM ODHL and 300 μM H_2O_2 further increased the population of cells exhibiting propidium fluorescence (Figure 1), indicating the increase in cell lethality. It is likely that ODHL potentiated the cytotoxicity of H_2O_2. The threshold concentration of ODHL to further increase the percentage population of dead cells (cell lethality) in the simultaneous presence of 300 μM H_2O_2 was 10 μM (Figure 2). The further potentiation of H_2O_2 cytotoxicity by 30 μM ODHL was statistically significant ($P < 0.01$). Results concerning the concentration-dependent change of H_2O_2 cytotoxicity by ODHL were summarized in Figure 2.

(Figures 1 and 2 near here)

3.2. Effect of ODHL on the cells treated with A23187

Oxidative stress induced by H_2O_2 elevates $[Ca^{2+}]i$, which promotes cell death [20-22]. Calcium ionophore A23187 causes Ca^{2+} overload, which induces cell death in the presence of a sufficient extracellular Ca^{2+} [23,24]. Therefore, to determine whether ODHL potentiates the Ca^{2+}-dependent cell death, the effect of ODHL on the cells simultaneously treated with 100 nM A23187 was examined. ODHL at 30 μM did not significantly increase the population of dead cells in the simultaneous presence of A23187 (Figure 3). Thus, it is unlikely that Ca^{2+} is involved in the ODHL-potentiation of H_2O_2 cytotoxicity.

(Figure 3 near here)

3.3. Effects of ODHL on $[Zn^{2+}]i$ and $[Ca^{2+}]i$ increased by H_2O_2

Excessive increases in $[Zn^{2+}]i$ and $[Ca^{2+}]i$ are considered to be cytotoxic [21,22,25,26]. Changes in the intensity of FluoZin-3 and Fluo-3 fluorescence by 300 μM H_2O_2 were examined in the absence and simultaneous presence of 30 μM ODHL. As shown in Figure 4, the incubation with 30 μM ODHL alone for 1 h increased the intensity of FluoZin-3 fluorescence as a parameter of $[Zn^{2+}]i$. This increase was statistically significant. ODHL at 30 μM caused further augmentation of FluoZin-3 fluorescence in the simultaneous presence of H_2O_2 (Figure 4). Thus, it is likely that ODHL augments the effect of H_2O_2 to elevate $[Zn^{2+}]i$.

The treatment of cells with H_2O_2 significantly increases the $[Zn^{2+}]i$ [10]. The estimation of $[Ca^{2+}]i$ by Fluo-3 fluorescence is not possible owing to excessive increase in $[Zn^{2+}]i$ because the affinity of Zn^{2+} to Fluo-3 is much higher than that of Ca^{2+} [18]. Therefore, the effects of ODHL, H_2O_2, and their combination on Fluo-3 fluorescence as a parameter of $[Ca^{2+}]i$ were examined in the presence of 10 μM TPEN, a chelator of intracellular Zn^{2+}. TPEN does not affect the A23187-induced augmentation of Fluo-3 fluorescence [27]. TPEN is necessary for this experiment because Zn^{2+} augments Fluo-3 fluorescence [28]. The incubation with 30 μM ODHL alone for 1 h did not significantly increase the intensity of Fluo-3 fluorescence under the condition that intracellular

Zn^{2+} was chelated by TPEN. As shown in Figure 5, H_2O_2 still induced significant increase in the intensity of Fluo-3 fluorescence in the presence of TPEN. However, ODHL greatly attenuated the H_2O_2-induced augmentation of Fluo-3 fluorescence (Figure 5). ODHL is assumed to attenuate the H_2O_2-induced elevation of $[Ca^{2+}]$i.

(Figures 4 and 5 near here)

3.4. Effect of ODHL on [NPT]i increased by $ZnCl_2$

The [NPT]i is linked to the vulnerability of cells to oxidative stress [29]. The treatment of cells with ODHL increases the [NPT]i in part *via* a Zn^{2+}-dependent mechanism [7]. The $ZnCl_2$-induced elevation of $[Zn^{2+}]$i also increases the [NPT]i [9]. The [NPT]i of rat thymocytes is significantly reduced in the presence of H_2O_2 [19] and H_2O_2 causes excessive increase in $[Zn^{2+}]$i in the presence of $ZnCl_2$ [10]. Because the ODHL-induced changes of [NPT]i in respective presence of $ZnCl_2$ and H_2O_2 were not predictable, the changes in 5-CMF fluorescence by 30 μM ODHL were examined in respective presence of 3 μM $ZnCl_2$ and 300 μM H_2O_2 were examined. The application of micromolar $ZnCl_2$ (10–30 μM) greatly increased [NPT]i [9]. However, in the presence of H_2O_2, $ZnCl_2$ at 30–100 μM exerted cytotoxic action [10]. $ZnCl_2$ at 1 μM or more increased $[Zn^{2+}]$i [9]. Therefore, 3 μM $ZnCl_2$ was chosen for the study. ODHL at 30 μM elevated $[Zn^{2+}]$i in a manner being largely dependent on extracellular Zn^{2+} [7]. The combination of 3 μM $ZnCl_2$ and 30 μM ODHL was expected to further increase intracellular Zn^{2+} levels. As shown in Figure 6, ODHL did not significantly affect the changes in 5-CMF fluorescence by $ZnCl_2$ and H_2O_2. It is unlikely that the change in [NPT]i by ODHL is involved in the ODHL-induced augmentation of H_2O_2 cytotoxicity.

(Figure 6 near here)

3.5. Possible contribution of Zn^{2+}

Zinc is considered to be protective against oxidative stress [30]. However, in rat thymocytes, $ZnCl_2$ at concentrations of 30 μM or more potentiates the cytotoxicity of 3 mM H_2O_2 [10]. ODHL increases the $[Zn^{2+}]$i [7] (also shown in Figure 4). To understand whether Zn^{2+} contributes to the

augmentation of H_2O_2 cytotoxicity, the effects of 30 μM $ZnCl_2$ and 10 μM TPEN on the cytotoxicity of 300 μM H_2O_2 were examined. The simultaneous incubation of cells with H_2O_2 and $ZnCl_2$ for 4 h further increased the population of dead cells (Figure 7). However, in the case of H_2O_2 and TPEN, the cytotoxicity of 300 μM H_2O_2 was significantly attenuated.

(Figure 7 near here)

4. Discussion

4.1. Involvement of Zn^{2+}

Ca^{2+} is known to contribute to the cell death induced by oxidative stress [21,22]. The present study confirms that Zn^{2+} also contributes to the cell death caused by H_2O_2-induced oxidative stress in rat thymocytes (Figure 7). ODHL at 30 μM itself did not change cell lethality (Figures 1 and 2). However, it further increased the population of dead cells in the presence of H_2O_2 (Figures 1 and 2). It is likely that Zn^{2+} is involved in this ODHL-induced phenomenon because ODHL augmented the H_2O_2-induced increase in $[Zn^{2+}]$i, but attenuated the same in $[Ca^{2+}]$i (Figures 4 and 5), and because ODHL did not affect the A23187-induced increase in the population of dead cells (Figure 3). Thus, Ca^{2+} is neglected in this case although ODHL increases intracellular Ca^{2+} release in some cells [31]. Zn^{2+} strengthens the cytotoxicity of H_2O_2, and TPEN, a chelator of intracellular Zn^{2+}, weakens its cytotoxicity (Figure 7). TPEN augments the cytotoxicity of A23187, a calcium ionophore [13]. These results indicate that Zn^{2+} is involved more profoundly than Ca^{2+} in this study. Zn^{2+} complicatedly modifies the cytotoxicity of chemicals. For example, the combination of $ZnCl_2$ and clotrimazole induced potent cytotoxic action on rat thymocytes with "bell-shape" dose–response relation [32]. Therefore, it is also considered that ODHL may modify some drug actions under in vivo conditions.

The further elevation of $[Zn^{2+}]$i by ODHL presumably potentiates the cytotoxicity of H_2O_2 under present *in vitro* conditions. It might also be argued that ODHL exerts additive oxidative stress because the treatment of rat thymocytes with ODHL at 10–100 μM increased BES-SO

fluorescence, an indicator of superoxide anions in cells [7]. However, this is unlikely because ODHL did not significantly affect [NPT]i in the presence of H_2O_2 (Figure 6), suggesting that oxidative stress induced by 300 μM H_2O_2 masks that induced by 30 μM ODHL.

4.2. Attenuation of H_2O_2-induced increase in $[Ca^{2+}]i$ by ODHL

It may be interesting that the increase in $[Ca^{2+}]i$ by 300 μM H_2O_2 in the presence of 30 μM ODHL was less than that in absence of ODHL (Figure 5) although ODHL potentiated the cytotoxicity of H_2O_2 that was estimated with the changes in cell lethality (Figure 2). In this preparation, dithiothreitol, which protects [NPT]i, greatly reduces the H_2O_2-induced increase in $[Ca^{2+}]i$ [20]. ODHL at 30 μM increased the [NPT]i in absence of H_2O_2 [7]. However, the effect of ODHL on the [NPT]i is negligible in presence of H_2O_2 (Figure 6). The increase in $[Ca^{2+}]i$ by H_2O_2 is due to the increases in membrane Ca^{2+} permeability and intracellular Ca^{2+} release from intracellular calcium stores [20]. ODHL mobilizes Ca^{2+} from endoplasmic reticulum of murine embryonic fibroblast cells [33]. Therefore, ODHL may reduce the H_2O_2-induced increase in membrane Ca^{2+} permeability.

4.3. Possible mechanism and implication

Incubation of cells with H_2O_2 at concentrations ranging from 100 μM to 1 mM causes big transition from intact living cells to annexin V-positive living cells [34]. In the presence of H_2O_2 the percentage population of annexin V-positive living cells is more than 80%. Therefore, ODHL presumably accelerates the transition from annexin V-positive cells to dead cells. Annexin V-positive status indicates that the cells are at an early stage of apoptosis [35]. Thus, ODHL may promote cell death in living cells at early stage of apoptosis. It is likely because ODHL accelerates the process of apoptosis in neutrophils and macrophages [5].

ODHL is a quorum sensing molecule, which is a signal mediator used by bacteria to synchronize their biological events [36]. ODHL also possesses many direct actions on host cells [5,37]. Of them, this molecule induces inflammation in host cells [38]. Oxidative stress is

involved in the inflammation process [39,40]. Therefore, these observations hint towards the possibility that ODHL also exacerbates inflammation in host cells.

Some papers concerning toxicological roles of Zn^{2+} (possible contribution of Zn^{2+} to the cytotoxicity of chemicals) in rat thymocytes were published from our laboratory [9-13]. Therefore, rat thymocytes were chosen in this study because it is on the extension of experimental evidences accumulated. It is important to confirm our results in other cells including human cell lines. In addition, ODHL is reported to induce anti-inflammatory molecules in human immune cells [15]. This anti-inflammatory molecules may be important to interpret the effect of ODHL on the cells treated with H_2O_2. Such experiments will be done in future studies.

5. Conclusion

ODHL is considered to increase cell vulnerability to H_2O_2-induced oxidative stress in mammalian cells. This study provides additional hints into the interaction between quorum sensing molecules and host cells.

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Figure legends

Figure 1. Changes in cytograms (forward scatter versus propidium fluorescence) by ODHL, H_2O_2, and their combination. Each cytogram was constructed with 2000 cells. Effects were examined at 180 min after the start of drug application. Dotted line under cytogram and arrow indicate the population of cells exhibiting propidium fluorescence, presumably dead cells.

Figure 2. Changes in cell lethality (percentage population of cells exhibiting propidium fluorescence) by ODHL, H_2O_2, and their combination. H_2O_2 (as shown with filled column) and ODHL (respective concentrations shown in horizontal axis) were simultaneously applied to the cells. The column and bar indicate the mean and standard deviation of four samples, respectively. Asterisks (**) show the significant difference ($P < 0.01$) between the control group (left side column) and respective test groups. Pounds (##) also indicate the significant difference ($P < 0.01$) between the groups of cells treated with H_2O_2 alone and with the combination of H_2O_2 and ODHL.

Figure 3. Cell lethality of cells treated with A23187 alone and with a combination of A23187 and ODHL (middle pair). A23187 and ODHL were simultaneously applied to the cells. The column and bar indicate the mean and standard deviation of four samples, respectively. Asterisks (**) show the significant difference ($P < 0.01$) between the control group (left side column) and respective test groups. Pounds (##) also indicate the significant difference ($P < 0.01$) between the group of cells treated with H_2O_2 alone, and with the combination of H_2O_2 and ODHL (right pair). This result confirms that of Figure 2.

Figure 4. Changes in the intensity of FluoZin-3 fluorescence by ODHL in the absence (left pair) and presence (right pair) of H_2O_2. H_2O_2 and ODHL were simultaneously applied to the cells. Asterisks (**) show the significant difference ($P < 0.01$) between the control group (left side

column) and respective test groups. Pounds (##) also indicate the significant difference ($P < 0.01$) between the group of cells treated with H_2O_2 alone, and with the combination of H_2O_2 and ODHL.

Figure 5. Changes in the intensity of Fluo-3 fluorescence by ODHL in the absence (left pair) and presence (right) of H_2O_2. H_2O_2 and ODHL were simultaneously applied to the cells. Asterisks (**) show the significant difference ($P < 0.01$) between the control group (left side column) and the respective test groups. Pounds (##) also indicate the significant difference ($P < 0.01$) between the group of cells treated with H_2O_2 alone, and with the combination of H_2O_2 and ODHL.

Figure 6. Changes in the intensity of 5-CMF fluorescence by ODHL in the absence (left pair) and presence of $ZnCl_2$ (middle pair) or H_2O_2 (right pair). ODHL was simultaneously applied to the cells with $ZnCl_2$ or H_2O_2. Asterisks (**) show the significant difference ($P < 0.01$) between the control group (left side column) and the respective test groups. NS indicates no significant difference between the groups of cells treated with $ZnCl_2$ alone, and with the combination of $ZnCl_2$ and ODHL.

Figure 7. Changes in the cell lethality by H_2O_2 in the presence of $ZnCl_2$ or TPEN. Asterisks (**) show the significant difference ($P < 0.01$) between the control group (left side column) and the respective test groups. Pounds (##) also indicate the significant difference ($P < 0.01$) between the group of cells treated with H_2O_2 alone, and with the combination with $ZnCl_2$ or TPEN.

Table 1. Reagents and fluorescent probes used in this study

A. Chemical Reagents

Chemical Name	Manufacturer
N-(3-oxododecanoyl)-L-homoserine-lactone (ODHL)	Sigma Aldrich (St. Louis, Missouri, USA)
A23187 calcium salt	Sigma Aldrich
N,N,N',N'-Tetrakis(2-pyridylmethyl)ethylenediamine (TPEN)	Dojin Chemical (Kumamoto, Japan)

B. Fluorescent Probes

Chemical Name	Manufacturer	Excitation	Emission
Propidium Iodide	Invitrogen (Eugene, Oregon, USA)	448 nm	600 ± 20 nm
5-Chloromethylfluorescein Diacetate (5-CMF-DA)	Invitrogen	448 nm	530 ± 20 nm
FluoZin-3-AM	Invitrogen	448 nm	530 ± 20 nm
Fluo-3-AM	Dojin Chemical	448 nm	530 ± 20 nm

Figure 1

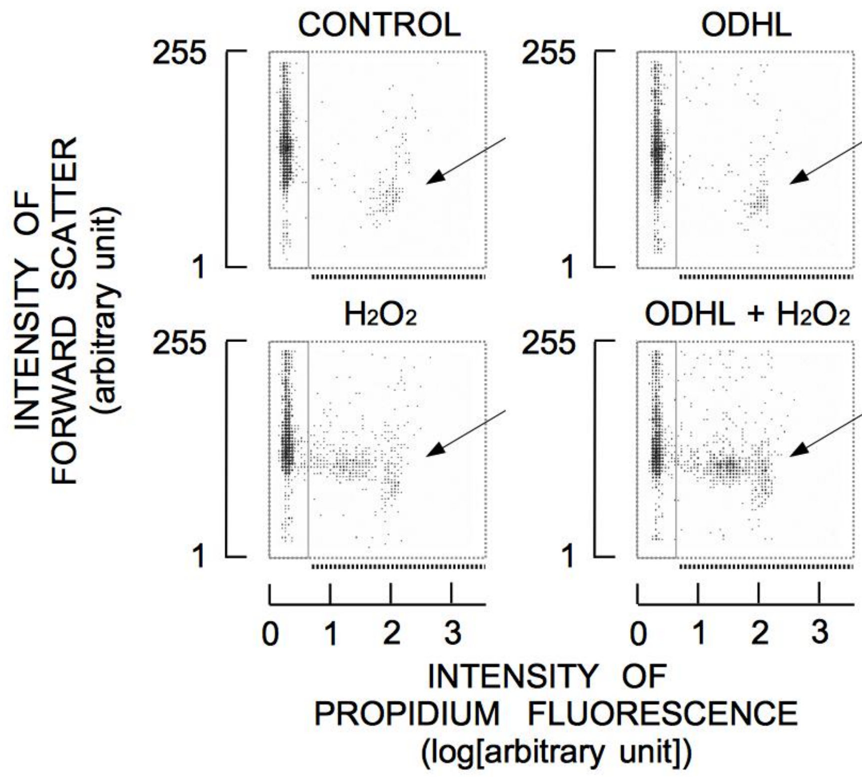


Figure 2

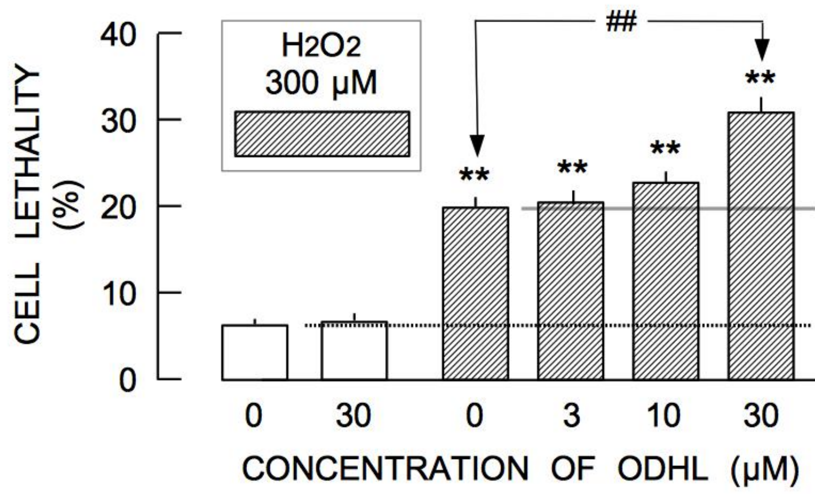


Figure 3

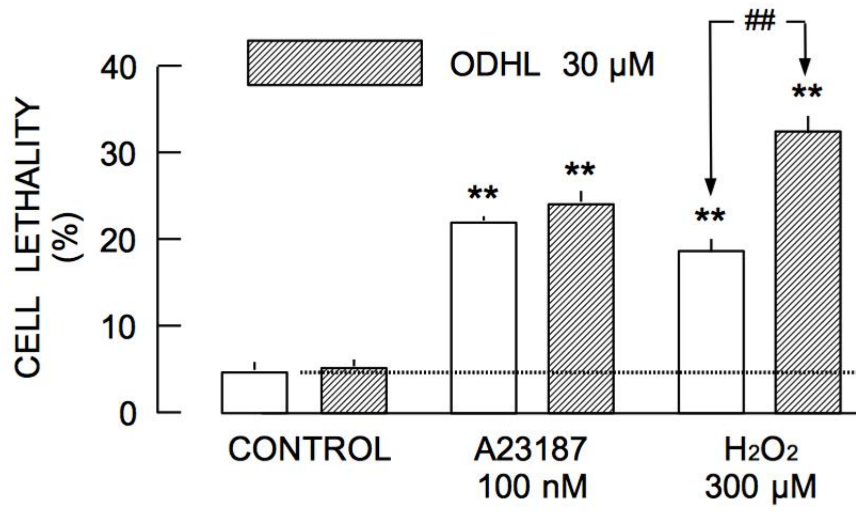
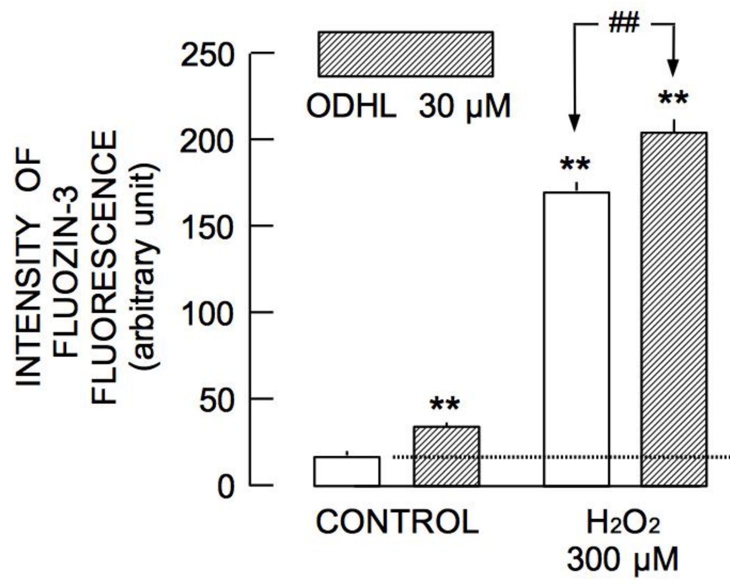


Figure 4



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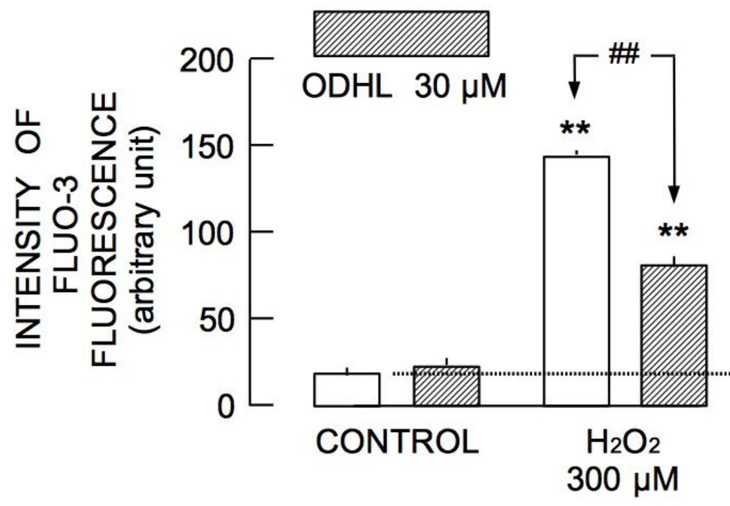

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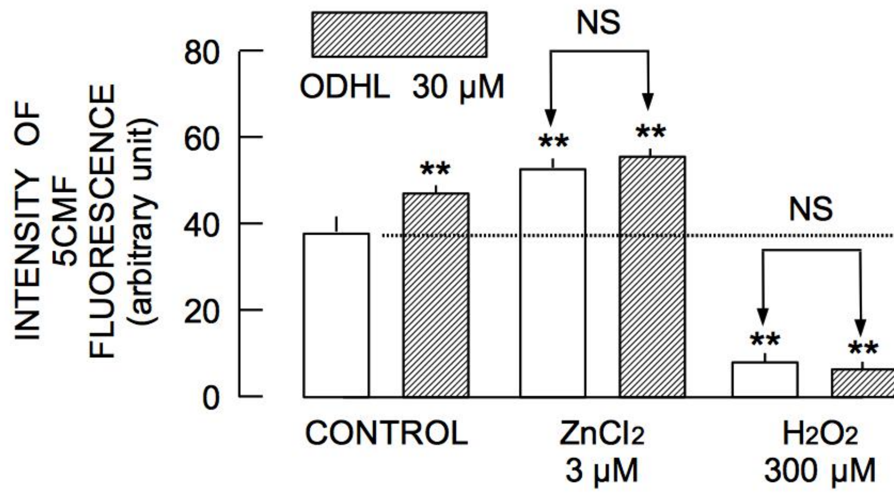

FIGURE 7

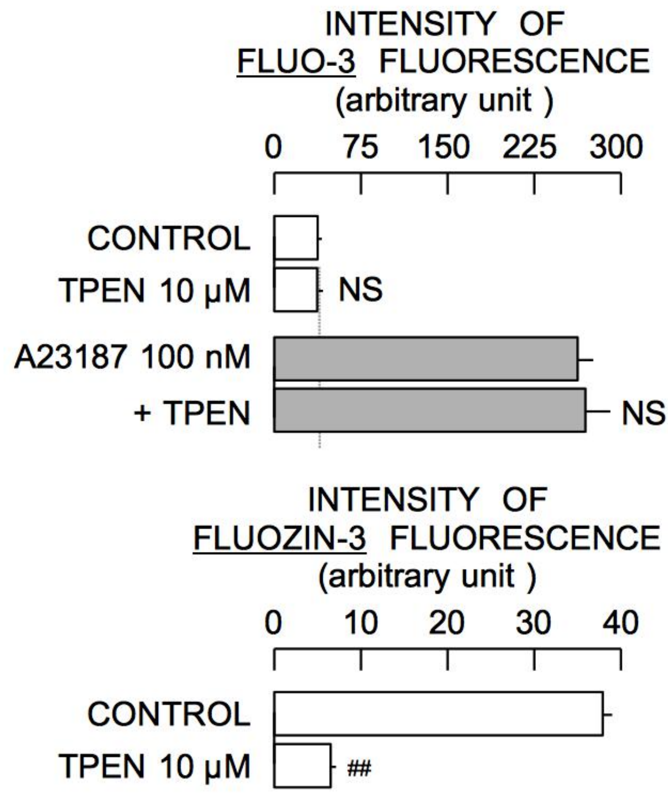

FIGURE 8