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Opposing mechanism of extracellular signal-regulated kinases on hydrogen peroxide-induced apoptosis in PC12 cells

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Abstract

The effects of two different doses of hydrogen peroxide (H_2O_2), a principal source of reactive oxygen species (ROS), upon the apoptotic response in pheochromocytoma12 (PC12) cells were examined by comparing the activities of the extracellular signal-regulated kinases (ERKs) and caspase-3. In our current study, we report that a 150 μM concentration of H_2O_2 decreases cell viability within 24 hours. Moreover, PD98059 (PD), a specific inhibitor of the ERKs-mediated signaling pathway, prevents this cell death although viable cells were found to decrease upon treatment with 50 μM H_2O_2 in the presence of PD. The caspase-3 activity, measured using a fluorogenic substrate, was lower in the presence of PD and 150 μM H_2O_2 than in the absence of this inhibitor, but this situation was reversed in the same experiments using 50 μM H_2O_2. These data indicate that the ERKs accelerate the apoptotic response through a caspase-3-dependent mechanism at levels of 150 μM H_2O_2, but not at a 50 μM dose of this compound. Our current experiments thus indicate that the ERKs-mediated signaling pathway regulates apoptosis via caspase-3 only at higher doses of H_2O_2 in PC12 cells.

Introduction

Reactive oxygen species (ROS) have a close association with many cell functions and also maintain the homeostasis of cells. On the other hand, ROS are also found to contribute to a number of chronic cell disorders[1-3]. Moreover, oxidative stress involving ROS can also be observed during aging[4-6]. Hydrogen peroxide (H_2O_2) is a primary source of functional ROS, and plays a number of pivotal roles in the cell. For instance, H_2O_2 is produced during both receptor-mediated signal transduction[7] and in the progression of neuronal death, such as Alzheimer's disease[8], Parkinson's disease[9], and ischemia[10)11]. Although these phenomena involve H_2O_2, the underlying intracellular mechanisms are characterized by a complicated crosstalk between redundant and additive signaling pathways. Thus, a simple explanation of the survival action and chronic action of H_2O_2 is needed, based upon an overview of the related molecules[12].

It is known that H_2O_2 activates caspase-3, of the cysteine protease family, during the process of apoptosis. Caspase-3 is activated by caspase-9 and promotes DNA fragmentation by activating a caspase-activated DNase (CAD) to induce apoptosis[13-15]. The caspase-mediated apoptotic cascade is also fundamentally involved in H_2O_2-induced apoptosis[16]. Extracellular signal-regulated kinases (ERKs) are also major molecules in the receptor-mediated or H_2O_2-mediated signal transduction pathway[17-19]. It is well-known that ERKs are activated by mitogen-activated protein kinase kinase1 (MAPKK1 or MEK1), which is an up-stream kinase of ERKs, in the mentioned signal transduction pathway. The physiological role of the ERKs is om-

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nifarious, however, and few explanations have been found for their differences. Interestingly, it has been reported that the ERKs phosphorylate caspase-9 and inactivate the caspase-mediated apoptotic cascade[20]. Thus, it is suggested that the caspase-mediated apoptotic cascade and the ERKs-mediated signal transduction pathways are closely associated with a chronic state of cellular disorder.

To investigate the crucial role for ERKs in H_2O_2-mediated signal transduction pathway involved in some disorders, we analyzed the relation between cell viability and the activity of ERKs in the presence or absence of PD98059 (PD), which is an inhibitor of an upstream regulator of the ERKs, MEK1 in H_2O_2-treated pheochromocytoma12 (PC12) cells. In our current study, we have found a possible correlation between the concentration of H_2O_2, the activity of the ERKs and the progression of cell death. We also find that PD increases the rate of cell death by exposure to 50 μM H_2O_2, but causes a reduction in the loss of cell viability at doses of 150 and 250 μM H_2O_2. Interestingly, PD increases the activity of caspase-3 at 50 μM H_2O_2 and decreases it at a 150 μM dose, suggesting that MEK1 controls cell viability by sensing a high concentration of H_2O_2. Thus, we conclude that ROS-mediated neuronal death is at least in part controlled by MEK1 and that this activity leads to cell death via the activation of caspase-3.

Materials and methods

1. Reagents

PD98059 (PD) was purchased from Calbiochem (U.S.A.). Caspase substrate (acetyl-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin (Ac-DEVD-MCA)) was obtained from the Peptide Institute (Japan). Antibodies against phospho-ERKs (pERKs) and ERKs were purchased from Cell Signaling Technology Inc (U.S.A.).

2. Cell line

The cell line used in this study was established and designated as PC12 by Drs. L.A. Greene and A.S. Tischler (Harvard Medical School, U.S.A.) from a solid adrenal tumor passaged subcutaneously in New England Deaconess Hospital strain white rats. The PC12 cells were generously provided by Dr. Hatanaka (Osaka University, Japan).

3. Cell culture

PC12 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% (v/v) fetal bovine serum, 5% (v/v) heat-inactivated horse serum and 0.1% (v/v) penicillin-streptomycin solution (Gibco BRL, U.S.A.). For the measurement of cell viability and for fluorescence microscopy analysis, naive PC12 cells were seeded onto either collagen-coated 96-well plates or 8-well chamber slides at 2×10^5 cells/cm^2. The following day, the medium was changed to serum-free DMEM, and hydrogen peroxide, or hydrogen peroxide plus PD was added as required, then 24 hours after the addition of these reagents, cell viability was measured or cells were stained with 1 μg/mL Hoechst 33258.

4. Measurement of cell viability

Viable cells were quantified by use of the AlamarBlue™ (alamarblue) (BioSource International Inc., U.S.A.) assay which measures mitochondrial activity in a similar manner to the MTT assay. Measurements were then made as described in our previous study[21]. Briefly, cells were seeded into 96-well plates and the medium was changed in order to assay at the appropriate time. The medium was then changed to serum-free DMEM containing 10% (v/v) alamarblue solution and the cells were incubated for 3 hours. The intensity of the fluorescence was detected with a spectrofluorometer (Fluoroskan Ascent, L-5210420, Dainippon-pharm, Japan) at 560 nm excitation and 595 nm emission. The cell viability was defined as $\{$(test sample count)-(blank count)/(untreated control count)-(blank count)$\}\times100$.

5. Immunoblot analysis

PC12 cells were seeded in 6-well plates and the medium was replenished with serum-free DMEM overnight to reduce the level of phosphorylation in the cells, or with serum-free DMEM supplemented with inhibitors as necessary. Then, the cells were lysed with buffer containing 50 mM Tris-HCl (pH 7.8), 150 mM NaCl, 1% sodium dodecylsulfate (SDS), 1 mM ethylenediamine tetraacetic acid (EDTA), 1 mM phenymethylsulfonyl fluoride (PMSF), 2 μg/mL of aprotinin and 1 mM Na_3VO_4. The supernatant was used for the analysis after the elimination of debris by centrifugation. The lysates (20 μg of protein per lane from PC12 cells) were then subjected to sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in Laemmli buffer, and blotted onto a polyvinylidene difluoride (PVDF) membrane using a semi-dry blotter (AE-6677, Atto, Japan). The primary antibody was used at a dilution of 1 : 1,000, after blocking treatment with Block-Ace (Dainippon-pharm, Japan), and the resulting horse radish peroxidase-conjugated signal was enhanced using an avidin-biotin complex (ABC) kit following the manufacturer's instructions (Vecta Stain kit, Vector Laboratories, U.S.A.). The bands were detected by enhanced chemiluminescence (ImmunoStar Reagent, WAKO, Japan) and visualized with a light-capture system (AE6962N, Atto, Japan). The band intensities were measured with a CS analyzer (version 1.03b, Atto, Japan).

6. Measurement of the activity of caspase-3

The activity of caspase-3 was measured using a fluorogenic peptide as described previously[22]. Briefly, PC12

cells were collected and lysed in buffer containing 10 mM Hepes-KOH (pH 7.4), 2 mM EDTA and 1 mM PMSF. Once centrifuged, one volume of supernatant was mixed with 2×ICE buffer containing 20 mM Hepes-KOH (pH 7.4), 20% glycerol (v/v), 2 mM PMSF, 4 mM dithiothreitol and 50 μM Ac-DEVD-MCA, and incubated for 1 hour at 37°C. The linearization of the activity response was tested for at least two hours. After the addition of 200 μl of distilled water, fluorescence was detected with a spectrofluorometer (Fluoroskan Ascent, L-5210420, Dainippon-pharm, Japan). Then, the values were shown as fold increase.

7. Statistical evaluation

All data were expressed as the means±SEM. Either Student's t-test or one-way analysis of variance (ANOVA) was used to compare the means between groups. A p value of less than 0.05 was considered to indicate a statistically significant difference.

Results

1. PD98059 accelerates cell death at a low dose of H_2O_2 but prevents cell death at higher concentrations of H_2O_2

To elucidate the characteristics of H_2O_2-mediated death in PC12 cells, we analyzed features of the extracellular signal-regulated kinases (ERKs)-mediated cell death using PD98059 (PD). PD is a specific inhibitor of the ERKs-mediated signaling pathway. As shown in Fig. 1A, H_2O_2 promotes cell death in PC12 cells within 24 hours at all concentrations. Interestingly, PD was found to cause accelerated cell death at 50 μM of H_2O_2 but PD inhibited cell death at both the 150 and 250 μM doses. To assess this intriguing effect further, we measured the activity of the ERKs using phosphorylation specific antibodies against these factors in the presence of H_2O_2 or H_2O_2 plus PD. As shown in Fig. 1B, the maximal levels of these kinases were observed after 15 minutes in the presence of 150 μM H_2O_2 and a lesser effect was observed at 50 μM (Fig. 1C). PD treatment completely decreased the activity of the ERKs to basal levels after 15 minutes in the presence of either 50 or 150 μM of H_2O_2 (Fig. 1C, D). Then, to investigate whether the cell death is apoptosis or not, the corresponding morphological features of the cells were observed following Hoechst 33258 staining, and we found that 150 μM H_2O_2 produced more apoptotic cells than 150 μM H_2O_2 plus PD (Fig. 2E, F), whereas the opposite was true at 50 μM of H_2O_2 (Fig. 2B, C). These results suggest that the balance between the cell death promoting and preventive effects of PD depends, at least in part, on the intracellular activity of ERKs.

2. The activity of caspase-3 is controlled by the PD inhibitor

The caspase family of proteins promotes apoptosis

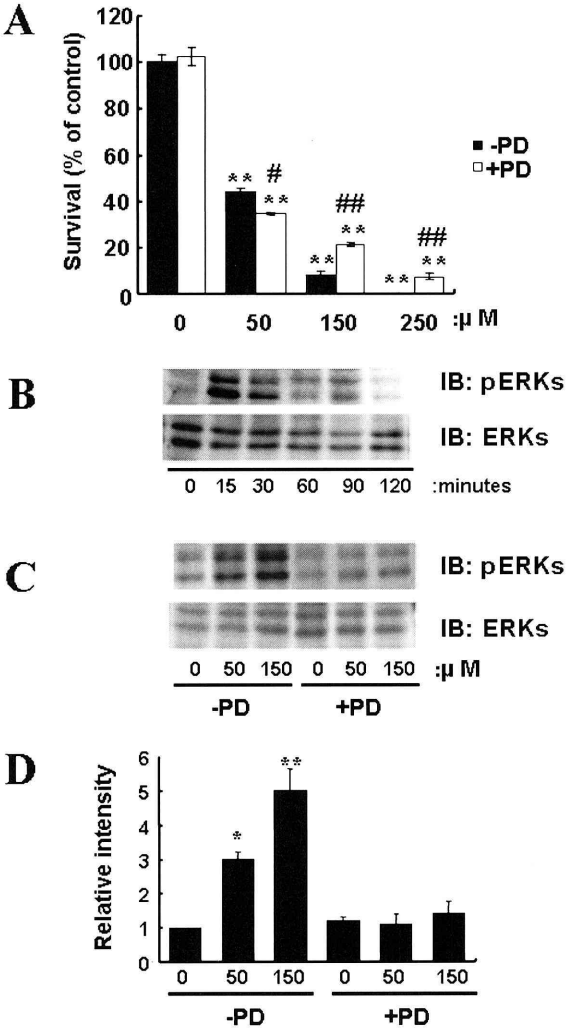

Fig. 1 (A) 25 μM PD was added to the serum-free DMEM for 24 hours in the presence of the indicated concentrations of H_2O_2. The values shown are the means±SEM (n=4). The +PD and −PD groups (**P<0.01), 0 μM and 50 μM of PD (# P<0.05), 150 and 250 μM of PD (## P<0.01). (B) The H_2O_2-treated samples were collected at specific points (minutes) and subjected to SDS-PAGE. The specific bands (phosphorylated ERKs: upper panel, unphosphorylated ERKs: lower panel) were visualized by immunoblotting (IB) using a light-capture system. The activity of the ERKs was estimated as shown using obtained intensity of the band as described in Materials and methods. (C, D) Different doses (50 or 150 μM) of H_2O_2 were added to PC12 cells for 15 minutes in the presence or absence of PD, and the samples were treated for IB as described above. Then, the specific phosphorylated or unphosphorylated bands were obtained and the intensity of the bands of pERKs in Fig. 1C, which was normalized by ERKs, was measured with a CS analyzer. The data (means±SEM) represent three independent experiments (*P<0.05, **P<0.01).

induced by H_2O_2[16]. Hence, we examined whether the activity of caspase-3 was altered by PD in the H_2O_2-induced apoptotic response in PC12 cells. Thus, we added 50 or 150 μM H_2O_2 to PC12 cells in the presence

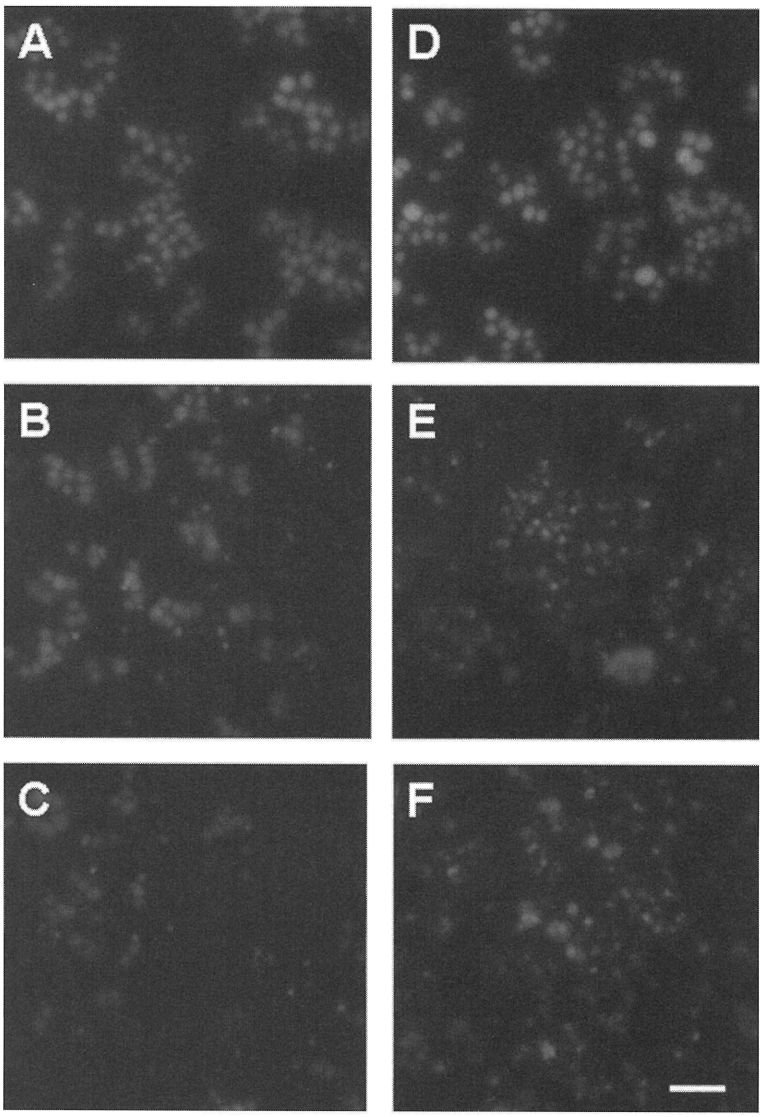

Fig. 2 Condensed chromatins were detected by Hoechst 33258 staining as described in Materials and methods. Cells that were untreated (A), 50 μM H_2O_2-treated (B), 50 μM H_2O_2-treated in the presence of PD (C), PD-treated (D), 150 μM H_2O_2-treated (E), or 150 μM H_2O_2-treated in the presence of PD (F) are shown. Apoptotic condensed chromatins were observed and images were captured using fluorescence microscopy. Bar denotes 20 μm.

or absence of PD for indicated time and measured the activity using collected lysates as described in Materials and methods. As shown in Fig. 3, the activity of caspase-3, which was indicated as fold increase, was increased by 50 μM H_2O_2, but to a lesser extent than by 150 μM H_2O_2. However, these effects were reversed by PD exposure. These data strongly suggest that the MEK1-ERKs pathways regulate the activity of caspase-3 and can induce apoptosis.

Discussion

We present evidence that the level of ERKs activity induced by ROS alters cell viability. Caspase-3 is activated by different stimuli via ROS stimulation[23)24)], leading to apoptosis and possibly disease[25)26)]. ROS have now been found to be present during ischemia and ischemia/reperfusion injury in previous reports[10)11)], and in the thronboembolic infarction system in rats in our experiments (manuscript in preparation). We also demonstrate that the effects of H_2O_2 upon cell viability are altered by different doses, and we therefore speculate that the control of the impact of intracellular ROS is quite difficult and would prove to be very problematic when attempting to apply it to new therapeutic strategies. We also show that the effects of PD are altered by different doses of H_2O_2. The underlying mechanism of this is not yet clear, but we do think that phosphatases may have a crucial role in this regard as earlier reports have shown that they are regulated in part by ROS[27)28)]. Moreover, we consider that functional change of ERKs by effective dose of ROS may be existed to activate caspase-3 although the activity of ERKs is increased

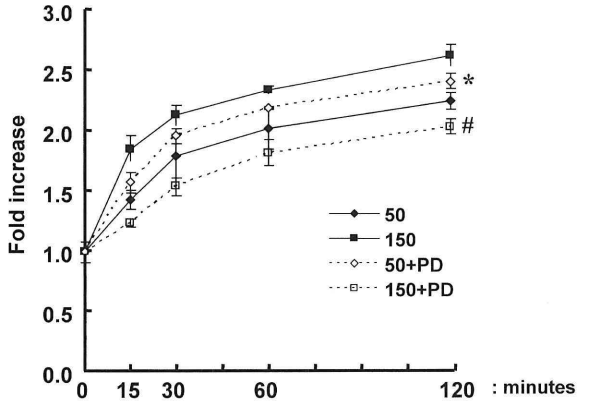

Fig. 3 After the treatment of 50 or 150 μM H_2O_2 in the presence or absence of PD, lysates were collected with lysis buffer to measure the activity of caspase-3 as described in Materials and methods. The activity of caspase-3 was measured using specific fluorogenic substrates (50 μM Ac-DEVD-MCA). The activity was denoted as fold-increase. The values shown are the means±SEM ($n=4$) and statistical analysis was carried out using the Student's t-test. The data were then compared between cells treated with 50 μM H_2O_2 and 50 μM H_2O_2 plus PD (*$P<0.05$), and with 150 μM H_2O_2 and 150 μM H_2O_2 plus PD (# $P<0.05$).

dose-dependently. Both hyper- and hypo-activation of ERKs may lead to activation of caspase-3. Thus, we think that we could speculate the effective and specific intracellular dose in this study. Cheng et al. have reported a significant reduction in the loss of cell viability following the intracerebroventricular administration of a caspase inhibitor[29]. The authors reported that this inhibitor is neuroprotective, even during the short period after administration and following the onset of reperfusion. Moreover, edaravone (3-methyl-1-phenyl-2-pyrazolin-5-one), which is a potent scavenger of free radicals, has been shown to prevent hypoxia/ischemia in mice showing a correlation between neuronal cell death and the caspase-12 activity[30]. Taken together, these findings suggest that there may be a specific cell death-inducing signal pathway that involves caspases and ERKs in hypoxia/ischemia. Kulich et al. have reported that the sustained activation of ERKs leads to Parkinson's disease[31]. In addition, apoptosis signal-regulating kinase1 (ASK1), a mammalian mitogen-activated protein kinase kinase kinase (MAPKKK), is a ROS-sensitive mediator of apoptosis[32][33]. The effect of ASK1 leads to the activation of c-Jun N-terminal kinase (JNK) and p38 mitogen-activated protein kinase (MAPK), which are stress activated proteins and belong to the MAPK superfamily[33][34]. But we have not yet further examined its effects. Allan et al. have also reported that the ERKs inhibit the activation of caspase-9[20], suggesting that it should be considered whether ERKs contribute to anti-apoptotic signaling pathway. It must therefore be clarified what condition is critical for pro-apoptotic or anti-apoptotic signaling pathways and how this family of proteins does participate in pro-apoptotic or anti-apoptotic signaling pathways.

Although we have focused on caspase-3 in our current study, caspase-9 also exists in the seriate cascade[35]. In a recent report, caspase-12 was shown to be involved in the ischemic brains of rats[36] and in hypoxia/ischemia in mice[30]. It has been found as the result of an in vitro study that the seriate cascade comprises caspase-3, -9 and -12[37][38]. This cascade leads to endoplasmic reticulum (ER) stress-mediated apoptosis. ASK1 is also involved in ER stress-mediated apoptosis via a different mechanism from ROS[39]. In the ER stress-mediated apoptotic response, glucose-regulated protein 78 or 94 (GRP78 or GRP94), which are chaperones for misfolded proteins, are up-regulated. This endogenous mechanism for protecting against ER stress is therefore very noteworthy in the context of these phenomena. We consider that further study correlated with ER stress is necessary for total interpretation of the ROS-mediated cytotoxicity and its preventive mechanism via ERKs.

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過酸化水素刺激によって惹起されたアポトーシス進行過程における ERKs を介した細胞生存率の制御機構

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【要旨】 アポトーシス進行の基礎過程において、ミトコンドリアの機能障害が引き起こされることが知られている。また、その際には、過酸化水素をはじめとする活性酸素種 (ROS) が、細胞内で産生されることも知られている。一方で、上皮成長因子 (EGF) 受容体を介した細胞内でのリン酸化を介したシグナル伝達経路においても過酸化水素が、EGF の機能発現に重要な役割を有していることも報告されている。本報では、PC12 細胞に対して、過酸化水素をそれぞれ、50、150、250 μM 添加したときに、濃度依存的にアポトーシスが誘導されたことを示す。その際に、extracellular signal-regulated kinases (ERKs) を活性化するリン酸化酵素である mitogen-activated protein kinase kinase 1 (MAPKK1 または MEK1) の特異的阻害剤である PD98059 (PD) を添加すると、50 μM の過酸化水素の存在下では、アポトーシスを促進し、150、250 μM の過酸化水素存在下では、アポトーシスを有意に抑制したことを報告する。この実験系において、アポトーシスを実行する caspase-3 の活性を測定したところ、50 μM の過酸化水素の存在下では、PD 未添加時と比較すると、添加時の活性が有意に高かった。150 μM の過酸化水素存在下では、逆に、PD 未添加時の活性が有意に高かった。以上から、PC12 細胞内の過酸化水素の濃度依存的に ERKs の活性化が調節され、その結果、caspase-3 を介するアポトーシスによる細胞死の程度を調節する機構の存在が明らかになった。

〈キーワード〉 活性酸素種、ERK、カスパーゼ、アポトーシス、PC12 細胞