Characterization of Hydrogen Peroxide-Induced Apoptosis in Mouse Primary Cultured Hepatocytes

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The influence of oxidative stress by hydrogen peroxide (H$_2$O$_2$) was examined in mouse primary cultured hepatocytes. A change in morphology was observed in hepatocytes incubated for 30 min in saline A containing H$_2$O$_2$. The percentage of dead cells, as measured by the fluorescence method, was increased in a dose-dependent manner. In addition, a ladder-like DNA fragmentation pattern was detected by agarose gel electrophoresis 1 hr after exposure to 3 mM H$_2$O$_2$. This phenomenon was prolonged for 24 hr. Hydrogen peroxide-induced cell viability reduction and DNA fragmentation were dose-dependently protected by the addition of antioxidants (N-acetylcysteine, l-ascorbic acid), a metal-chelator (1,10-phenanthroline), iron-chelator (deferroxamine) and intracellular calcium ion chelator (quin 2-AM). No influence, however, was detected by endonuclease inhibitors (zinc, aminonucleoside and poly (ADP-ribose) polymerase inhibitors (3-amino benzamide, theophylline). These results following H$_2$O$_2$-induced cell viability reduction suggested that oxidative stress by H$_2$O$_2$ itself or H$_2$O$_2$-derived changes involved in ferrous or intracellular calcium ions resulted in apoptosis in mouse primary cultured hepatocytes. These phenomena are not likely to be associated with endonuclease or poly (ADP-ribose) polymerase.

Key words oxidative stress; hydrogen peroxide (H$_2$O$_2$); primary cultured hepatocyte; apoptosis

Many physiological and environmental toxicants and chemicals (including metabolic poisons and chemotherapeutic drugs) are injurious to cells by mechanisms involving the overproduction of reactive oxygen species. In an effort to define the mechanisms of cell injury by such oxygen intermediates, hydrogen peroxide (H$_2$O$_2$)-induced killing of cultured hepatocytes is a useful model. H$_2$O$_2$ is a relatively stable product of the spontaneously or enzymatically catalyzed dismutation of superoxide anions. It may also participate with superoxide in the iron-catalyzed formation of hydroxyl radicals. The cellular toxicity of H$_2$O$_2$ has been explained as a cascade of biological oxidations resulting in the rapid modification of cytoplasmic constituents, the depletion of intracellular GSH and ATP, a decrease in NAD$^+$ level, an increase in free cytosolic Ca$^{2+}$, and lipid peroxidation. So far, several experimental studies have shown the mechanisms of H$_2$O$_2$-induced apoptosis in cultured hepatocytes. In this experiment, we attempted to clarify the influence of cell viability reduction and to characterize the apoptosis by H$_2$O$_2$ in mouse primary cultured hepatocytes.

MATERIALS AND METHODS

Animals and Chemicals Adult male ddY mice weighing 25—30 g were obtained from Japan SLC (Hamamatsu, Japan). Animals were maintained on a 12 hr light/dark cycle in a humidity- and temperature-controlled facility and allowed free access to food and water during the experiments. All animal studies were performed in compliance with guidelines established in “Guide for the Care and Use of Laboratory Animals” published by the Japan Association of Laboratory Animals. H$_2$O$_2$ was purchased as a 30% stock solution from Santoku Chemical Industries Co., Ltd. (Tokyo, Japan). [Methyl-3H]-thymidine was purchased from Moravek Biochemicals, Inc. (CA, U.S.A.). l-Ascorbic acid (vitamin C; VC), N-acetylcysteine (NAC), 3-amino benzamide (3-AB), bisbenzimide H 33258 fluorescent trihydrochloride (H 33258), ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA-Na$_2$·2H$_2$O), propidium iodide (PI), proteinase K, sodium bicarbonate (NaHCO$_3$), sodium lauryl-sulfate (SDS), theophylline (THO) and 1,10-phenanthroline monohydrate (1,10-PT) were purchased from Nacalai Tesque, Inc. (Kyoto, Japan). Collagenase, insulin, dexamethasone, O,O'-bis (2-aminophenyl) ethylene glycol-N,N',N'',N'''-tetraacetic acid (EGTA) and 2-[4-(2-hydroxyethyl)-1-piperazinyl] ethanesulfonic acid (HEPES) were purchased from Wako Pure Chemicals Industries (Osaka, Japan). Agarose, fetal bovine serum, Tris–HCl, boric acid, Hank’s balanced salt solution, penicillin-streptomycin, minimum essential medium (MEM) and Williams’ E medium (WE) were purchased from Gibco BRL (NY, U.S.A.). Aprotinin, aminonucleoside (ATA), IGEPAL CA-630, deferoxamine mesylate (DFX) and quin 2-AM were purchased from Sigma Chemical Co. (NY, U.S.A.).

Hepatocyte Culture Hepatocytes were isolated from 5-week-old ddY male mice (Japan SLC, Hamamatsu) by a modified version of the two-step collagenase perfusion method of Seglen. Briefly, the liver of a mouse was perfused for 5 min with 37°C prewarmed Ca$^{2+}$- and Mg$^{2+}$- free Hank’s balanced salt solution at pH 7.2 containing 10 mM HEPES, 0.5 mM EGTA, and 40 mM NaHCO$_3$. This was followed by a 15 min perfusion with 37°C prewarmed Hank’s balanced salt solution containing collagenease (0.05%) and buffered with 10 mM HEPES and 4.0 mM NaHCO$_3$ (pH 7.5). After the second perfusion step, isolated cells were centrifuged (50 g for 1 min×4 spins) in MEM to remove nonparenchymal and dead cells. Then, the medium was exchanged from MEM to WE (pH 7.2) containing 5% fetal bovine serum, 10$^{-7}$M insulin, 10$^{-7}$M dexamethasone, 100 units/ml penicillin and 100 µg/ml streptomycin. This procedure routinely yielded 4×10$^9$ cells/ml with greater than 90% viability based on the trypan blue exclusion test. Approximately 1×10$^9$ parenchymatous cells (density was 5×10$^5$ cells/ml) were plated on 35 mm Falcon collagen coated type I dishes. After incubation at 37°C for 2 h in a humidified environment of 5% CO$_2$/95% air, the cultures were rinsed with
warmed phosphate buffered saline (PBS) to remove free cells and debris, and then transferred to serum-free WE containing 0.12 µg/ml aprotinin. After further incubation at 37°C for 24 h, the cultures were used for the following experiments.

**Incubation with H₂O₂ and Other Drugs** The cultures were preincubated for 15 min with various concentrations of other drugs in saline A (0.14 M NaCl, 5 mM KCl, 4 mM NaHCO₃, and 5 mM glucose), then incubated for 30 min with H₂O₂ (final concentration of 0.3, 1, 3 or 9 mM). Then the cultures were rinsed twice with saline A and incubated in drug- and serum-free WE medium.

**Morphology and Fluorescence Dyeing** After the hepatocytes had been exposed to the agents to be tested, they were stained with H 33258 and PI by the modified method of Nishikawa et al.¹⁴ For this procedure, 5 µM H 33258, which stains DNA in both living and dead cells, and 5 µM PI, which stains DNA in dead cells,¹⁵,¹⁶ were applied to the cells for 10 min. After observation by phase-contrast microscopy, the percentage of dead cells was immediately obtained by counting the number of PI-positive cells under a fluorescence microscope, ECLIPSE TE300 (Nikon, Tokyo, Japan), according to {the PI-positive cells (excitation wavelength, 520 nm; emission wavelength, 605 nm)/H 33258- positive cells (excitation wavelength, 360 nm; emission wavelength, 450 nm)} × 100.

**Effect of H₂O₂-Induced Cell Viability Reduction** Cell viability was determined by visual morphological inspection, counting the number of PI-positive cells, measuring the level of ³H-thymidine incorporation, and then by mitochondrial activity assessment by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay. ³H-thymidine at a final concentration of 0.05 µCi/ml was added to cultured hepatocytes 30 min before the measurement time points. The cultured hepatocytes were washed with PBS two times, then the cells were harvested from the dishes by a cell scraper. Samples were emulsified in scintillation fluid and counted in a Beckman LS 6000TA beta scintillation counter. ³H-thymidine incorporation (%) was calculated according to (cpm (test groups)/cpm (control groups)) × 100.

**Agarose Gel Electrophoresis** After removal of the medium and rinsing twice with PBS, the hepatocytes separated from 35 mm dishes were lysed by adding lysis buffer (1% IGEPAL CA-630 in 20 mM EDTA, 50 mM Tris, HCl, pH 7.5) and 1% SDS. They were then incubated for 3 h with proteinase K (final concentration 100 µg/ml) at 56°C, followed by digestion overnight with RNase A (final concentration 10 µg/ml) at 37°C to obtain the cell lysate. DNA was extracted twice from the lysate with equal volumes of phenol-chloroform–isoamyl alcohol (25:24:1), and precipitates with double the volume of ethanol. DNA samples (10 µg of DNA/lane) were electrophoresed on 1.4% agarose gel containing ethidium bromide (final concentration 0.16 µg/ml), and were visualized by UV fluorescence in order to detect qualitative damage to genomic DNA.

**Statistical Analysis** Statistical analysis of the results was performed by one way analysis of variance (ANOVA)

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Fig. 1. Morphological Analysis of Mouse Primary Cultured Hepatocytes
Phase-contrast microscopy photographs of control (A) and incubation with H₂O₂ (3 mM) in saline A for 30 min (B) and cultured for 4 h. Magnification: ×100. Fluorescence microscopy photographs of control (C) and incubation with H₂O₂ (3 mM) in saline A for 30 min (D) cultured for 4 h. Magnification: ×400.
RESULTS

Morphological Changes of Cultured Hepatocytes
Under phase-contrast microscopy, the control hepatocytes showed clear nuclei and nearly no apparent changes during the experimental period (Fig. 1A). On the other hand, following incubation with 3 mM H₂O₂ in saline A for 30 min after 4 h of culture, atrophy of nuclei in the hepatocytes was observed (Fig. 1B). In the fluorescence microscopic examinations, the control hepatocytes alone were dyed H 33258, but not dyed PI (Fig. 1C). H₂O₂ (3 mM)-exposed hepatocytes consisted almost solely of PI-positive cells which showed the accumulation of chromatin in the nuclei or nuclear fragmentation, a feature indicating apoptosis (Fig. 1D).

H₂O₂-Induced Cell Viability Reduction and DNA Fragmentation
As shown in Fig. 2, the H₂O₂ (0.3, 1, 3 or 9 mM)-induced cell viability reduction was evaluated at more than 3 mM H₂O₂ by counting the number of PI-positive cells, and by measuring the level of ³H-thymidine incorporation and mitochondrial activity (MTT assay, data not shown). The dead cells of the control hepatocytes were less than 10% of the initial value 24 h after the operation. The dead cells of the 0.3 mM and 1 mM H₂O₂-exposed hepatocytes slightly increased in comparison with the control. H₂O₂ (3 mM and 9 mM)-exposed hepatocytes markedly increased in the number of dead cells in a dose- and time-dependent manner. As shown in Fig. 3, the electrophoretogram demonstrated time-dependent damage by 3 mM H₂O₂ to genomic DNA. A ladder-like fragmentation pattern, in multiples of 180—200 bp, appeared first after 1 h; as time passed from after 2 h to 24 h, this fragmentation pattern cleared, then disappeared after 48 h, shifting to necrosis. In the control cultures, no such ladder-like fragmentation pattern of DNA was ever observed.

Effect of Antioxidants on H₂O₂-Induced Cell Viability Reduction and DNA Fragmentation
NAC (1, 2, 5 or 10 mM) and VC (0.2, 0.5, 1 or 2 mM), anti-oxidant...
ments in dose-dependent manner (Figs. 4 and 5). However, ATA (5 μM) and Zn²⁺ (100 μM), which are endonuclease inhibitors, and 3-AB (10 μM) and THO (1 μM), which are poly (ADP-ribose) polymerase inhibitors, produced no effect on H₂O₂ (3 mM)-induced DNA fragmentation (Fig. 6). H₂O₂ (3 mM)-induced DNA fragmentation disappeared by the incubation with NAC (10 mM), VC (2 mM), 1,10-PT (25 μM), DFX (300 μM), or quin 2-AM (5 μM) (Fig. 7), whereas no effect was observed with ATA, Zn²⁺, 3-AB and THO (Fig. 8).

**DISCUSSION**

Oxidative stress is a conclusive factor in apoptosis, although it has differential mechanisms in several cell lines.
Monolayer cultured hepatocytes do not divide, thus avoiding any confusion with toxic injury based on the arrest of cell cycle progression. Consequently, these cells are particularly useful for the study of oxidative stress-induced apoptosis. Many experiments of H2O2-induced oxidative stress and apoptosis in primary cultured hepatocytes have been conducted using experimental conditions in which H2O2 and/or other drugs were directly treated with medium containing serum and hormones. The cytotoxic effect of H2O2 in such cases is not observable directly after treatment; it required several hours to become manifest. In our experimental conditions, H2O2 exposed to deficiencies of amino acid, serum and hormones in saline A removed certain unnecessary factors. In the experiments described above we have attempted to study the mechanisms of H2O2-induced cell viability reduction and apoptosis in mouse cultured hepatocytes.

In the cells cultured with more than 3 mM H2O2, a significant difference in viability reduction and apoptosis, i.e. morphological and DNA fragmentation in mouse cultured hepatocytes (Figs. 1, 2 and 3), was shown. In this paper we reported that a higher concentration of H2O2 (3 mM) is required for a significant effect on cell viability, because of different experimental conditions and toxicant assessments compared with other experiments.1 Changes in membrane permeability were found to be late in onset and unreliable as indicators of the toxic action of H2O2.7 In the present study we used the 3H-thymidine incorporation assay as an index of cell viability, since this correlates well with the plating efficiency assay, and is an index of reproductive viability.17,18 Apoptosis in mouse liver has been reported to be mainly induced by cytokines such as TNF,22–24 and through Fas antigen.22,23 In fact, chemically induced differentiation between apoptosis and necrosis is considerably difficult. Our observation of morphological and electrophoretical changes indicated that H2O2-induced cell viability reduction was caused by apoptosis.

A few studies have documented the effects of prolonged culture on antioxidant levels and free radical toxicity in primary cultured or early passage cells. Antioxidants protect cells against a variety of oxidative stress-induced apoptosis. NAC and VC are good examples of antioxidants. Figure 4 illustrates that 3 mM H2O2-induced cell viability reduction was dose-dependently protected by NAC and VC. Moreover, these reagents eliminate the DNA fragmentation caused by 3 mM H2O2 (Fig. 7). It is suggested that in this model, H2O2-induced cell viability reduction and apoptosis were blocked by antioxidants. The molecular mechanisms by which oxidants trigger the cascade leading to cell death are, however, not fully elucidated, but the loss of calcium homeostasis may contribute in a major way to the onset of cytotoxicity. Indeed, a rapid increase in free cytosolic calcium concentration occurred in isolated hepatocytes injured by H2O2, widely used as model compound to induce oxidative stress.24–26 The importance of catalytically active iron in oxidative stress-induced cell killing has been shown in several studies. 1,10-PT,27 quin 2-AM28 and DFX29,30 protected against the oxidative damage induced by H2O2. More importantly, H2O2 is a potential source of one of the most dangerous radicals known, the hydroxyl radical, through the Fenton reaction. The results of Figs. 5 and 7, which involved the use of 1,10-PT and DFX, suggest that an intracellular source of iron seems to be required in order to express the damaging effects of H2O2 on cell and DNA integrity. Namely the 1,10-PT- or DFX-ferrous ion complexes are unable to catalyze a Fenton reaction. Moreover, H2O2 or H2O2-derived radicals may disturb intracellular calcium homeostasis, which results in secondary reactions ultimately leading to DNA fragmentation.

On the other hand, H2O2-induced cell viability reduction and apoptosis could not be blocked by ATA, Zn2+, 3-AB or THO (Figs. 6 and 8). One DNA fragmentation mechanism was prevented by using endonuclease inhibitors such as ATA29 and zinc.30 t-Butyl hydroperoxide-induced cytotoxicity and DNA damage in rat hepatocytes could not be inhibited by these compounds.31 Our data agreed with them. ADP-riboisyltransferase activity was inhibited by 3-AB or THO in cultured hepatocytes.32,33 Inhibition by: ADP-riboisyltransferase prevents that loss of viability by oxidative stress.34,35 In the fibroblasts, the inhibition of poly (ADP-ribose) polymerase protected against the loss of viability occurring with H2O2; on the other hand, the inhibition of this enzyme in hepatocytes had little effect on cell killing by H2O2.36 From the results of this experiment, inhibitors of poly (ADP-ribose) polymerase were shown to have no effect on cell viability reduction or DNA fragmentation of cultured hepatocytes by H2O2. The results indicate that the activity of poly (ADP-ribose) polymerase in response to repairing this damage is not related to cell viability reduction or apoptosis of cultured hepatocytes by H2O2.

In conclusion, these reported results indicate that H2O2-induced cell viability reduction and apoptosis in mouse cultured hepatocytes was protected by antioxidants, iron chelators or intracellular calcium ion chelators, although endonuclease and poly (ADP-ribose) polymerase did not participate. Further examination of the influence of oxidative stress in this model is required.

REFERENCES

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