Potentiation of Acetaminophen Hepatotoxicity by Doxapram in Mouse Primary Cultured Hepatocytes

Shuuichi Kannō,* Masaaki Ishikawa, Motoaki Takayanagi, Yoshio Takayanagi, and Ken-ichi Sasaki

Department of Pharmacology and Toxicology, Cancer Research Institute, Tohoku Pharmaceutical University, 4-4-1, Komatsushima, Aoba-ku, Sendai 981–8558, Japan. Received October 12, 1999; accepted January 28, 2000

The augmentation by doxapram (DOP) of the reduction in viability and of the apoptosis of cells induced by acetaminophen (AA) was examined in mouse primary cultured hepatocytes. Loss of viability on exposure to AA and/or DOP in cultured hepatocytes was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay and the apoptosis of cultured hepatocytes was detected by nuclear morphologic observation and from a ladder-like DNA fragmentation pattern. The combination of AA (5 μM) and DOP (10, 20, 50 or 100 μM) potentiated the reduction in cell viability and increased the oxidative stress. Hepatocytes exposed for 24 h to AA (5 μM) plus DOP (100 μM), showed atrophy of nuclei, including chromatin condensation and a ladder-like DNA fragmentation pattern, characteristic of apoptosis. Benzyl-oxycarbonyl-Asp-CH$_2$-OC (O)2,6-dichlorobenzene (Z-Asp-CH$_2$-DCB, 50 μM), an inhibitor for caspases, improved the viability and ladder-like DNA fragmentation in cells exposed to DOP (200 or 500 μM) alone or AA (5 μM) plus DOP (100 μM). However, loss of viability on exposure to a high concentration of AA (10 μM) and ladder-like DNA fragmentation were not affected by Z-Asp-CH$_2$-DCB. These results indicated that the synergistic increase in oxidative stress, activation of caspases and DNA fragmentation induced by DOP potentiated the hepatotoxicity of AA.

Key words doxapram; acetaminophen; mouse primary cultured hepatocyte; apoptosis; caspase

Since acetaminophen (AA) is included in cold remedies as an analgesic or antipyretic and frequently used long-term, there are numerous opportunities for its concomitant use with other drugs. Therefore, it is important to evaluate the interaction between AA and other drugs. The mechanisms of hepatotoxicity of AA have attracted considerable interest. In large doses, AA produces centrilobular hepatic necrosis in both humans and experimental animals.1,2 There is general agreement that AA is activated by hepatic mixed-function oxidase enzymes (i.e. cytochrome P-450) to produce N-acetyl-p-benzoquinone imine (NAPQI). Some isomers of the enzyme seem to produce greater quantities of toxic metabolites than others, so their induction could lead to unexpected increases in the toxicity of the drug. Inhibitors of cytochrome P-450 generally decrease toxic metabolite formation and thus might be used to prevent the hepatotoxicity of AA.

Doxapram (DOP) is an agent that produces marked respiratory stimulation and hyperprressor effects with a wider margin of safety than other compounds.3 We have shown that DOP is bound to liver microsomes and a potent inhibitor of mixed-function oxidative metabolism in mice and rats.4–6 Surprisingly, however, we have found that DOP acts synergistically with AA on the mortality and hepatotoxicity in mice.3 Here, we employed mouse primary cultured hepatocytes to elucidate the mechanism by which AA hepatotoxicity is potentiated by DOP.

MATERIALS AND METHODS

Animals and Chemicals Adult male C57BL/6 mice weighing 25—30 g were obtained from Japan SLC (Hamamatsu). Animals were maintained on a 12 h 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 the Guide for the Care and Use of Laboratory Animals published by the Japan Association for the Care of Laboratory Animals. DOP was purchased from Kissel Pharmaceutical Co., Ltd. (Nagano). AA was purchased from Junsei Pharmaceutical Co., Ltd. (Tokyo). Benzyl-oxycarbonyl-Asp-CH$_2$-OC (O)2,6-dichlorobenzene (Z-Asp-CH$_2$-DCB), an inhibitor for caspases, was purchased from Peptide Institute, Inc. (Osaka). Collagenase, insulin, dexamethasone and bisbenzimide H 33258 fluorochrome trihydrochloride (H 33258) were purchased from Wako Pure Chemicals Industries (Osaka). Agarose, fetal bovine serum, Tris–HCl, boric acid, Hanks’ balanced salt solution, penicillin–streptomycin, minimum essential medium (MEM) and Williams’ E medium (WE) were purchased from GIBCO BRL (NY, U.S.A.). All other chemicals were of the highest purity available.

Hepatocyte Culture Hepatocytes were isolated from the mice by a modified version of the two-step collagenase perfusion method of Seglen.8 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, pH 7.2, containing 10 mM HEPES, 0.5 mM EGTA, and 4.0 mM NaHCO$_3$. This was followed by a 15 min perfusion with 37 °C prewarmed Hank’s balanced salt solution at pH 7.5 containing collagenase (0.05%) and buffered with 10 mM HEPES and 4.0 mM NaHCO$_3$. After the second perfusion step, isolated cells were centrifugated (50×g for 1 min, 4 spins) in MEM to remove nonparenchymatous and dead cells. And then the medium was changed from MEM to WE (pH 7.2) containing 5% fetal bovine serum, 10−7 M insulin, 10−4 M dexamethasone, 100 units/ml penicillin and 100 μg/ml streptomycin. This procedure routinely yielded over 90% viability based on trypan blue exclusion testing. Approximately 1×10$^6$/ml parenchymatous cells were plated on 35-mm Falcon collagen coated type I dishes. After an 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 serum-free WE contain-
ing 0.12 µg/ml aprotinin was added and the cells were incubated with AA, DOP and/or an inhibitor.

Cell Viability Cell viability was determined using a modification of the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay. Following exposure of hepatocytes to AA and/or DOP, 50 µl of MTT (10 mg/ml saline) was added, and samples were incubated for 1 h at 37°C. The cells were lysed and solubilized by addition of 500 µl of dimethyl sulfoxide (DMSO) for 2 h at 37°C, and the absorbance of 100 µl aliquots was determined at 590 nm using an Inter-med model NJ-2300 Microplate Reader. Cell viability (%) was calculated relative to control.

Oxidative Stress Oxidative stress was examined using 2′,7′-dichlorofluorescin diacetate (DCFH-DA), which is deacetylated intracellularly to the nonfluorescent compound 2′,7′-dichlorofluorescin which can be oxidized to the highly fluorescent compound 2′,7′-dichlorofluorescin (DCF). This probe measures the overall indicator of oxidative stress. The cells were incubated with drugs and 20 µM DCFH-DA. At the end of each incubation period, the cells were scraped and the fluorescence intensity of the cell suspension was read using a fluorescence spectrophotometer RF-1500 (Shimadzu, Kyoto) with excitation at 488 nm and emission at 525 nm.

Nuclear Morphologic Observation For the study of nuclear morphology, the cells were allowed to attach to 22×22 mm Falcon collagen coated type I coverslips on 35-mm culture dishes. H 33258 (5 µM) was applied to the cells 10 min before the observation. The coverslips were removed from the dishes, washed with PBS and then observed through excitation and emission filters of 360 and 420 nm, respectively, with a fluorescence microscope, Eclipse TE 300 (Nikon, Tokyo).

Agarose Gel Electrophoresis After the medium had been removed, the cells were rinsed twice with PBS. 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% sodium dodecyl sulfate (SDS). Then they were incubated for 3 h with proteinase K (final concentration, 100 µg/ml) at 56°C before being digested overnight with RNase A (final concentration, 10 µg/ml) at 37°C to obtain cell lysate. DNA was extracted from the lysate with phenol:chloroform:isoamyl alcohol (25:24:1) and precipitated by ethanol. DNA samples (10 µg of DNA/lane) were electrophoresed on 1.4% agarose gels containing ethidium bromide (final concentration, 0.16 µg/ml) and visualized by UV fluorescence in order to detect qualitative damage to genomic DNA.

Statistical Analysis Statistical analysis of the results was performed with a one way analysis of variance (ANOVA) followed by Scheffe’s F test using the Statcel (OMS, Tokorozawa) for Apple/Macintosh.

RESULTS

Cell Viability As shown in Fig. 1, neither 5 mM AA nor 200 µM DOP had an effect on the viability of cultured hepato-

---

Fig. 1. Time Course of the AA- and/or DOP-Induced Reduction in Viability of Mouse Primary Cultured Hepatocytes

Cell viability was assessed by MTT assay, as described in Materials and Methods. Values are the mean±S.E. of three replications. *p<0.05 vs. control groups.

---

Fig. 2. AA- and/or DOP-Induced Change in the Oxidative Stress of Mouse Primary Cultured Hepatocytes

Oxidative stress was evaluated from the DCF fluorescence, as described in Materials and Methods. Values are the mean±S.E. of three. Upper panel: Time course of the change in oxidative stress. *p<0.05 vs. control groups. Lower panel: Combination exposure to AA (5 mM) plus DOP (10, 20, 50 or 100 µM) for 6 h. *p<0.05 vs. AA (5 mM) alone treated groups.
tococytes. But a high concentration of AA (10 mM) or DOP (500 μM) produced a reduction in viability. Therefore, we examined the reduction in cell viability of cultured hepatocytes induced by AA (5 mM) in combination with DOP (less than 200 μM). AA (5 mM) plus DOP (10, 20, 50 or 100 μM) dose- and time-dependently potentiated the reduction in cell viability.

**Oxidative Stress** The AA- and/or DOP-induced oxidative stress in primary cultured hepatocytes was assessed by measuring DCF fluorescence (Fig. 2). The AA (5 mM)- or DOP (100 μM)-induced oxidative stress in cultured hepatocytes was little increased in comparison with the control (data on DOP (100 μM) not shown). A high concentration of DOP (500 μM) produced a transient increase in oxidative stress with a peak at 4 h. On treatment with AA (5 mM) plus DOP (100 μM), or a high concentration of AA (10 mM), oxidative stress was increased with a peak at 6 h. Then, we measured oxidative stress in cultured hepatocytes 6 h after simultaneous treatment with AA (5 mM) and DOP (10, 20, 50 or 100 μM). Oxidative stress was dose-dependently and significantly potentiated.

**Morphological Change** There was little change to the nuclei of control hepatocytes during the experimental period (Fig. 3A). And neither AA (5 mM)- nor DOP (200 μM)-exposed hepatocytes differed from the control (Fig. 3B and C). However, on exposure to AA (5 mM) plus DOP (100 μM), or a high concentration of AA (10 mM) or DOP (500 μM), the number of cells exhibiting chromatin condensation increased (Fig. 3D—F).

**DNA Fragmentation** As shown in Fig. 4, the electrophoretograms demonstrated damage to genomic DNA. In the control (Fig. 4, lane 2) and on exposure to AA (5 mM) for
Fig. 4. Exposure to AA and/or DOP for 24 h Induced DNA Fragmentation in Mouse Primary Cultured Hepatocytes

DNA was subjected to electrophoresis on 1.4% agarose gel, as described in Materials and Methods. Each lane contains 10 μg of DNA. Lane 1, marker 123 bp; lane 2, control; lane 3, AA 5 mM alone; lane 4, DOP 200 μM alone; lane 5, AA 5 mM + DOP 100 μM; lane 6, AA 10 mM alone; lane 7, DOP 500 μM alone.

Fig. 5. Effect of Exposure to Caspase Inhibitor for 24 h on the AA- and/or DOP-Induced Reduction in Cell Viability of Mouse Primary Cultured Hepatocytes

Cell viability was assessed by MTT assay, as described in Materials and Methods. Values are the mean ± S.E. of three replications. *p < 0.05 vs. control groups.

Fig. 6. Effect of Exposure to Caspase Inhibitor for 24 h on the AA- and/or DOP-Induced DNA Fragmentation in Mouse Primary Cultured Hepatocytes

DNA was subjected to electrophoresis on 1.4% agarose gel, as described in Materials and Methods. Each lane contains 10 μg of DNA. Lane 1, marker 123 bp; lane 2, control; lane 3, caspase inhibitor 50 μM alone; lane 4, DOP 200 μM + caspase inhibitor 50 μM; lane 5, DOP 500 μM + caspase inhibitor 50 μM; lane 6, AA 10 mM + caspase inhibitor 50 μM; lane 7, AA 5 mM + DOP 100 μM + caspase inhibitor 50 μM.

Similarly, in hepatocytes exposed to DOP (200 or 500 μM) or AA (5 mM) plus DOP (100 μM), DNA fragmentation was prevented by Z-Asp-CH$_2$-DCB. However, Z-Asp-CH$_2$-DCB did not affect the AA (10 mM)-induced DNA fragmentation (Fig. 6).

**DISCUSSION**

Cell death (irreversible loss of vital cellular structure and function) is a fundamental phenomenon among biological organisms. There are two types of cell death, apoptosis and necrosis. Apoptotic cells and bodies are rapidly phagocytosed *in vivo*, so their half-life in organs such as the thymus and liver is very short. The very short half-life of apoptotic cells *in vivo*, and the technical difficulty in recognizing and quantifying these cells, are major reasons why the importance of this mechanism of cell death has been overlooked. In contrast, primary cultured hepatocytes are similar in quality to liver cells, are little affected by factors like phagocytosis, provide excellent experimental reproduction, and so are well suited to investigations of the mechanism of cell death. For these reasons, research on apoptosis is usually carried out using primary cultured hepatocytes. One of the mechanisms of AA-induced hepatotoxicity has been elucidated. In the present study, we have investigated the relation between apoptosis and the mechanism by which DOP potentiates AA hepatotoxicity.

We examined hepatocytes exposed to AA and/or DOP in primary culture for 2 h. Studies have demonstrated a rapid loss of mixed function oxidase activity and decline in the level of cytochrome P-450 in hepatocytes during the early stages of culture. AA hepatotoxicity requires a reactive metabolism involving cytochrome P-450. However, it was confirmed that primary culture for 2 h is not able to recover the function of the liver. Therefore, we extended the culture to 24 h.

Figure 1 shows that not only AA but DOP induces a loss of cell viability in cultured hepatocytes. AA is metabolized by a cytochrome P-450-mediated enzyme system, and the re-
active metabolites (NAPQI, etc.) produce oxidative stress, which causes apoptosis. But the mechanism of cell death induced by DOP is unknown. A high concentration of DOP (500 μm) potentiated both oxidative stress and the characteristic features of apoptosis (chromatin condensation and a ladder-like DNA fragmentation) (Figs. 2–4). Interestingly, exposure to DOP (200 μm) for 24 h produced a ladder-like DNA fragmentation (Fig. 4, lane 4), whereas this concentration had no effect on the cell viability (Fig. 1), oxidative stress (Fig. 2) or morphological change of nuclei (Fig. 3C). The AA (5 mM) plus DOP (10, 20, 50 or 100 μM)-induced reduction in cell viability and oxidative stress were dose-dependently augmented (Figs. 1 and 2). Therefore, we speculated that DOP synergistically promotes oxidative stress and apoptosis, and consequently, potentiates the hepatotoxicity of AA.

In the last few years, enormous progress has been made in the study of the intracellular events that lead to apoptosis.17–19 A key feature of apoptosis appears to be activation of the protease cascade which ultimately leads to the systematic degradation of repair enzymes, structural proteins, and DNA within the cell. This family of proteases is named caspases (cysteine aspartate-specific proteases). Yet, hepatocyte apoptosis involves the processing and activation of caspases.20 One of the most prominent effector caspases is caspase-3, which can directly activate an apoptosis-specific endonuclease.21,22 Selective inhibition of caspases completely prevented apoptosis in rat primary cultured hepatocytes.23 Z-Asp-CH₂-DCB, an extensive inhibitor for caspases, which can preferentially inhibit caspase-3 activity.24,25 Recently, it was reported that AA-induced DNA fragmentation does not require caspase activation,26 but is dependent on Ca²⁺ accumulation in the nucleus.27 Our results indicated that the DOP-induced reduction in cell viability and ladder-like DNA fragmentation are related to caspase activation. Consequently, blocking of caspase protected the cells and prevented ladder-like DNA fragmentation induced by AA (5 mM) plus DOP (100 μM) (Figs. 5 and 6). We conclude that DOP affects the signal transduction pathway for caspase, thereby rendering cells susceptible to apoptosis and potentiating AA hepatotoxicity.

We speculated that DOP would ameliorate the AA-induced hepatotoxicity and mortality, because it is a potent inhibitor of mixed-function oxidative metabolism in mice and rats.4–6 In fact, DOP actually potentiated AA-induced hepatotoxicity and mortality in vivo.7 Our results indicated that the synergistic increase in oxidative stress, activation of caspases and DNA fragmentation, induced by DOP, potentiated the hepatotoxicity of AA. Further study of the mechanisms of DOP-induced cell loss and apoptosis is needed.

REFERENCES