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Protection by Paeonol on Cytotoxicity of Cultured Rat Hepatocytes Exposed to Br-A23187

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Abstract – The present experiment was performed to investigate the protective effects of paeonol isolated from *Moutan Cortex Radicis* on primary cultured rat hepatocytes exposed to Br-A23187 (Ca^{2+} ionophore). Br-A23187 is frequently used as a model of cell killing as inducing both necrotic and apoptotic cell death. Hepatocytes were isolated by collagenase perfusion from livers of fasted male Sprague Dawley rats and cultured overnight. Cell viability was determined by propidium iodide using fluorocytometry in Krebs-Ringer-HEPES buffer at pH 7.4. In addition, intracellular calcium was measured by excitation at 340 and 380 nm and emission at 505 nm using a luminescence spectrophotometer. Paeonol (20-100 μM) inhibited cell killing induced by 10 μM Br-A23187, in a dose-dependent manner. Paeonol also reduced increased intracellular calcium level when hepatocytes were exposed to Br-A23187. Therefore, the present results suggest that paeonol protects the hepatocytotoxicity induced by Br-A23187, *via* inhibiting the influx of calcium into into rat hepatocytes.

Keywords □ Hepatocytes, Paeonol, Br-A23187, Intracellular [Ca^{2+}], Cytotoxicity.

INTRODUCTION

Moutan Cortex Radicis is a traditional Chinese Herb as the root of *Paeonia suffructicosa* Andrews, which has been commonly used to treat liver diseases in China, Japan and Korea for centuries. It is also traditionally used for the nourishment of blood, activation of circulation, alleviation of pain and regulation of menstruation (Harada and Yamashita, 1969). The scavenging activity of oxygen radicals was observed in this crude drug (Yoshikawa *et al.*, 1992). In addition, various constituents were isolated from *Moutan Cortex Radicis* and have diverse biological activities. The methanol extract of *Moutan Cortex Radicis* showed the effects of reducing urea-nitrogen in rat serum (Shibutani *et al.*, 1981) and vasodilator effects (Goto *et al.*, 1996). Paeonol, a major component of *Moutan Cortex Radicis* has sedative and anti-inflammatory effects (Harada *et al.*, 1969, Harada *et al.*, 1972), inhibits platelet aggregation of rabbit (Shi *et al.*, 1988) and shows protective effects on cultured neonatal rat heart cells, inhibiting Ca^{2+} influx (Tang *et al.*, 1991).

Liver is very often the target organ to the toxic chemicals. Toxicants can induce a variety of toxic effects on different organelles in the cells of liver, exhibiting different types of liver injury. Many models of cell-killing have been developed to understand the cytotoxic mechanisms of toxic chemicals (Nieminen *et al.*, 1992; Qian *et al.*, 1999). Br-A23187, a Ca^{2+} ionophore and a toxic compound to the hepatocytes, has been frequently used as the experimental model experiment of Ca^{2+}-dependent cell killing (Wyllie *et al.*, 1984). The present experiment is designed to investigate the protective effects of paeonol on the cytotoxicity induced by Br-A23187 in cultured rat hepatocytes.

MATERIALS AND METHODS

Isolation of paeonol

The sample (2 kg) was cut into the small size and extracted with methanol in water bath. The methanol extract was evaporated to be dry. The residue (500 g) was suspended with water and partitioned with hexane. The hexane solution was evaporated to dry. The hexane fraction (67 g) was fractionated on silica gel (60-230 mesh) column chromatography using a gradient of hexane-acetone (100:1 → 1:1) afforded three fractions (Fr. 1 ~3). The Fr. 1 afforded crude crystal and it was recrystallized in ethyl acetate to give white crystal (21 g).

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Identification of paeonol

mp 48-50°C, $FeCl_3$ test: positive, UV λ_{max}^{EtOH} nm (log) 314 (3.4), 274 (3.7), 230 (3.6), EI-MS m/z (rel. int.) 166 $[M]^+$ (54), 151 $[M\text{-}CH_3]^+$ (100), 123 $[M\text{-}COCH_3]^+$ (3), ^{1}H-NMR (300 MHz, DMSO-*d6*) δ 7.84 (1H, d, J = 8.7 Hz, H-3),, 6.53 (1H, dd, J = 8.7 Hz and 2.7 Hz, H-4), 6.47 (1H, d, J = 2.7 Hz, H-6), 3.82 (3H, s, OCH_3), 2.56 (3H, s, CH_3). 13C-NMR (75 MHz, DMSO-*d6*) δ 113.7 (C-1), 165.7 (C-2), 100.7 (C-3), 164.1 (C-4), 107.3 (C-5), 133.3 (C-6), 55.7 (OCH_3), 203.1 (C=O), 26.6 (CH_3).

Hepatocyte isolation and culture

Hepatocytes were isolated from overnight fasted male Sprague-Dawley rats (200-250 g) by collagenase perfusion of livers, as described previously (Gores *et al.*, 1988). Cell viability routinely exceeded 90%, as determined by trytophane blue exclusion. Hepatocytes were then cultured in Waymouths MB-7521/1 medium containing 27 mM $NaHCO_3$, 2 mM L-glutamine, 10% fetal calf serum, 100 nM insulin, and 100 nM dexamethasone. For cell viability assay, hepatocytes were plated onto 24-well microtiter plates (Falcon, Lincoln Park, NJ) coated with 0.1% Type 1 rat-tail collagen at a density of 1.5×10^5 cells/well in 1 ml of medium. Hepatocytes were used after overnight (14-16 h) incubation in humidified 5% CO_2, 95% air at 37°C. Experiments exposing cells to Br-A23187 were then carried out in modified Krebs-Ringer-HEPES buffer (KRH) containing 25 mM Na-HEPES, 115 mM NaCl, 5 mM KCl, 1 mM KH_2PO_4, 1.2 mM $MgSO_4$, and 0.5 mM $CaCl_2$ at pH 7.4.

Cell viability assay

Viability of hepatocytes cultured on microtiter plates was monitored by propidium iodide fluorometry using a multi-well fluorescence scanner (FLUOstar 403, BMG LabTechnologies, Durham, NC). Briefly, hepatocytes in 24-well microtiter plates were incubated in KRH containing 30 μM propidium iodide. Fluoroscence from each well was measured using excitation and emission wavelengths of 544 nm (25 nm band pass) and 590 nm (35 mm band pass), respectively. For each experiment, an initial fluororescence measurement (A) was made 20 min after addition of propidium iodide and then at intervals thereafter. Individual experiments were terminated with 375 μM digitonin to permeabilize all cells, and a final fluorescence measurement (B) was obtained 20 min later. The percentage of viable cells (V) was calculated as V = 100(B X)(B A), where X is fluororescence at any given time. Cell killing in this assay corresponds to that assessed by trypan blue nuclear staining (Nieminen *et al.* 1992).

Measurement of intracellular Ca^{+2}

Intracellular calcium concentration was determined by ratio fluorometry as described (Grynkiewick *et al.*, 1985). After hepatocytes were harvested, cells (1.5×10^5 cells) were moved to a spectrophotometer cuvette and were loaded with 10 μM fura-2 AM in KRH buffer. Flouroscence was measured using a Luminescence spectrophotometer (Phonton Technology International) by excitation at 340 and 380 nm, and emission at 505 nm under continuous stirring.

Statistics

All data are expressed as the mean ± SE. The statistical significance was assessed by an analysis of variance (ANOVA). In the case of significant variation, the individual values were compared by Dunnetts test.

RESULTS

Br-A23187-induced cell killing and inhibition by paeonol

Br-A23187 caused dose-dependent cell killing. When hepatocytes in 24-well microtiter plates were exposed to 10 μM Br-A23187 in KRH, loss of viability occurred progressively over 2 hr. Close to half-maximal cell killing occurred after 70 min. Paeonol (20-100 μM) inhibited cell killing induced by Br-A23187 (10 μM), in a dose-dependent manner. However, paeonol itself did not affect cell killing (Fig. 1). However, the pro-

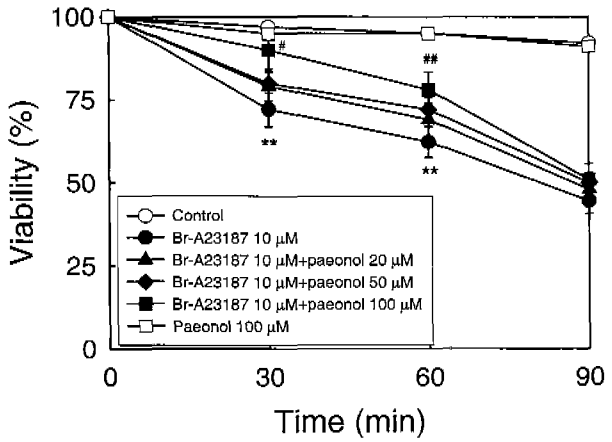

Fig. 1. Protection of Br-A23187-induced cell killing by paeonol. Hepatocytes cultured in 24-well plates were exposed to 10 μM Br-A23187 in KRH. Cell viability was monitored by propidium iodide fluorometry, as described in Materials and Methods. The results were means ± SEM from triplicate experiments with three different cell isolations. Significantly different from vehicle group (**P<0.01) or Br-A23187 group (#P<0.05, ##P<0.01).

tective effects of cells by paeonol were disappeared at 90 min. It seems that this protective effect is no long lasting.

Inhibition of Br-A2187 induced intracellular [Ca^{+2}] by paeonol

10 μM Br-A23187 induced a rapid increase of [Ca^{2+}]. 20-100 μM paeonol reduced increased intracellular [Ca^{2+}] level induced by 10 μM Br-A23187, in a dose-dependent manner (Fig. 2). On the other hand, paeonol itself also decreased the normal calcium level in the cells (Fig. 3). We suggest that paeonol protects against cytotoxicity through decrease of intracellular [Ca^{2+}].

DISCUSSION

Moutan Cortex Radicis has been a commonly used traditional Chinese medicine to treat vascular problems as well as liver diseases such as hepatitis virus infection, liver fibrosis and liver cancer in Asia. Moreover, it is used in combination with other herbs. The methanol extract of Moutan Cortex Radicis inhibited liver fibrosis and damage induced by CCl_4 and D-galactosamine in rat; thus *Paeoniae Radix* has a role in the hepatocyte (Fu, 1992; Qi *et al.*, 1991). The goal of this experiment was to determine whether paeonol, a major component of *Moutan Cortex Radicis*, would inhibit apoptotic and necrotic cell killing induced by Br-A23187. *Moutan Cortex Radicis* has been used in many traditional prescriptions for the treatment of liver disease, and paeonol, a major compound of *Moutan Cortex Radicis* has been shown to be potentially hepatoprotective (Qi *et al.*, 1991). Our results demonstrated that paeonol delays cell killing after exposure of hepatocytes to 10 μM Br-A23187, in a dose dependent manner. Br-A23187 and related Ca^{2+} ionophore are widely used to study Ca^{2+}-dependent cell injury. Toxicity of Ca^{+2} commonly affects the activation of Ca^{2+} dependent degenerative enzymes (McConkey and Orrenius, 1996; Trump and Berezesky, 1995). Br-A23187 increases intracellular and intramitochondrial calcium and induces onset of the mitochondrial permeability transition (MPT) with subsequent acute necrotic cell death (Qian *et al.*, 1999). Here, we employed 10 μM Br-A23187, which causes a slower rate of cell killing. These observations are again consistent with the earlier results of acute cytotoxicity by 10 μM Br-A23187. We found that 100 μM paeonol alone affected neither necrotic nor apoptotic killing of cultured hepatocytes.

In terms of calcium paradox, it has been well known that paeonol reduces intracellular Ca^{+2} level in the heart cells. It was reported that the remarkable inhibition of the Ca^{45} uptake on cultured heart cells by paeonol was associated with the blocking slow calcium channel in the cell membrane (Tang and Shi, 1991). This result indicates that paeonol inhibits myocardial beating rate under normal states. Br-A23187 produces cellular Ca^{+2} overload, which induces uncoupling of mitochondrial oxidative phosphorylation and activation of the oligomycin-sensi-

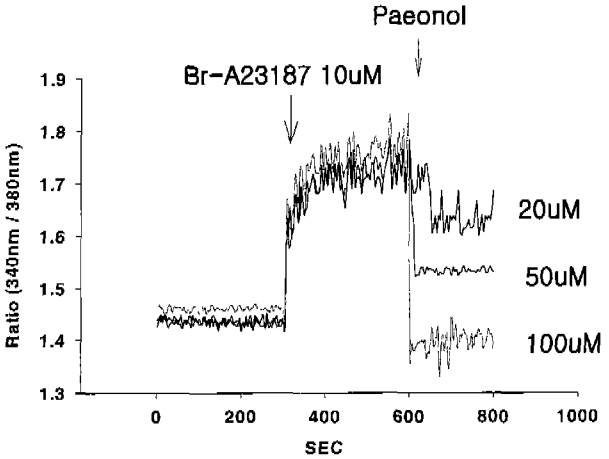

Fig. 2. Inhibition of Br-A23187 induced intracellular [Ca^{2+}] by paeonol. Hepatocytes were harvest and were loaded with fura-2 AM, and the relative ratios were measured as described in Materials and Methods. Cells are incubated for 20 min in KRH prior to adding 10 μM Br-A23187 and 20-100 μM paeonol. Intracellular Ca^{+2} variations are expressed as ratio of Fura-2 fluorescent intensities.

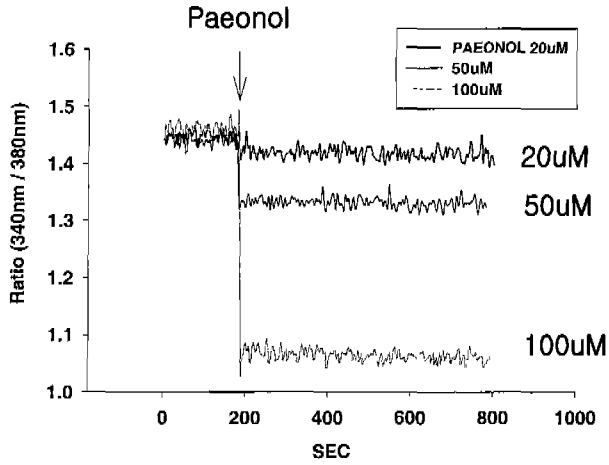

Fig. 3. Effects of paeonol itself on intracellular [Ca^{2+}] on cultured hepatocytes. Hepatocytes were incubated for 20 min in KRH prior to adding 20-100 μM paeonol. Cell death was measured as described in Materials and Methods. Results are mean ± SEM from triplicated experiments with three different cell isolations.

tive mitochondrial ATPase (Kawanish et al., 1991). Therefore, Br-A23187 and other Ca^{+2} ionophores are frequently used as a model of Ca^{+2}-dependent cell killing. Paeonol inhibited hepatocyte killing and reduced intracellular Ca^{+2} level after addition of 10 μM Br-A23187.

Taken together, the data show that paeonol protects hepatocytotoxicity caused by Br-A23187. Since paeonol has been a common and widely used remedy for the treatment of liver disease, the present work raises the possibility that paeonol might subtly protect hepatotoxicity by various hepatotoxicants such as Ca^{+2} ionophores. Further in vivo experiments are needed to elucidate the protective effects of livers.

In conclusion, paeonol could inhibit cell death induced by toxic chemicals. Therefore, paeonol has the potential to beneficially protect the hepatotoxicity by some toxicants.

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