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Gallic acid selectively induces the necrosis of activated hepatic stellate cells via a calcium-dependent calpain I activation pathway

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ABSTRACT

Aims: The activation of hepatic stellate cells (HSCs) in response to liver injury is critical to the development of liver fibrosis, thus, the blockage of the activation of HSCs is considered as a rational approach for anti-fibrotic treatment. In this report, we investigated the effects and the underlying mechanisms of gallic acid (GA) in interfering with the activation of HSCs.
Main methods: The primary cultured rat HSCs were treated with various doses of GA for different time intervals. The morphology, viability, caspase activity, calcium ion flux, calpain I activity, reactive oxygen species generation and lysosomal functions were then investigated.
Key findings: GA selectively killed HSCs in both dose- and time-dependent manners, while remained no harm to hepatocytes. Besides, caspases were not involved in GA-induced cell death of HSCs. Further results showed that GA toxicity was associated with a rapid burst of reactive oxygen species (ROS) and a subsequent increase of intracellular Ca^{2+} and calpain activity. Addition of calpain I but not calpain II inhibitor rescued HSCs from GA-induced death. In parallel, pretreatment with antioxidants or an intracellular Ca^{2+} chelator eradicated GA responses, implying that GA-mediated cytotoxicity was dependent on its pro-oxidative properties and its effect on Ca^{2+} flux. Furthermore, application of ROS scavengers also reversed Ca^{2+} release and the disruption of lysosomal membranes in GA-treated HSCs.
Significance: These results provide evidence for the first time that GA causes selective HSC death through a Ca^{2+}/calpain I-mediated necrosis cascade, suggesting that GA may represent a potential therapeutic agent to combat liver fibrosis.

Introduction

Most types of chronic liver diseases (including heavy alcohol consumption, drug abuse, autoimmune reactions, nonalcoholic fatty liver disease, and chronic infection with hepatitis virus B or C) can lead to fibrosis of the liver. Liver fibrosis is a characteristic feature of chronic liver injury. It is known as a scarring process that results from an imbalance between synthesis and degradation of extracellular matrix (ECM) proteins. Over time, this process can lead to cirrhosis and serious complications of liver disease, including liver failure and liver cancer (Friedman, 2000).

The activation of hepatic stellate cells (HSCs) is now believed to play an important role in the development of liver fibrosis (Friedman, 2000). In healthy liver, HSCs are quiescent and function in vitamin A storage. However, during liver injury, HSCs become active and undergo profound phenotypic changes from quiescent cells that store retinoid to myofibroblasts capable of secreting ECM; these cells also exhibit de novo expression of α-smooth muscle actin (α-SMA), with the characteristics of a high proliferative rate (Friedman, 2000; Mormone et al., 2011). The expansion of activated HSCs and the accumulation of ECM leading to impaired hepatocellular function and distortion of the normal hepatic architecture constitute the pathological basis of hepatic fibrosis (Friedman, 2000). Therefore, induction of HSC death or suppression of HSC activation is considered as a rational approach for anti-fibrotic treatment.

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Gallic acid (GA) is a phenolic plant secondary metabolite which provides desirable health benefits beyond basic nutrition. Epidemiological evidence suggests that consumption of a diet rich in GA is beneficial to human health, including anti-inflammatory, antimicrobial, antiallergic, and antitumor activity, but the most well-known action of GA is its antioxidant activity. GA exhibits protective effects against CCl_4-induced hepatic damage in rats (Jadon et al., 2007). Furthermore, GA possesses other properties such as hydrogen peroxide production in the presence of certain metals (Kobayashi et al., 2004), the ability to selectively induce apoptosis in tumor cells but not normal cells (Sakaguchi et al., 1998; Inoue et al., 2000) and promotes apoptotic cell death in lung fibroblasts (Chuang et al., 2010) and 3T3-L1 preadipocytes (Hsu et al., 2007). Therefore, GA might act by blocking the initiation of several human diseases. However, despite this wealth of information concerning the role of GA in disease; little has been documented concerning the therapeutic role of GA on liver fibrosis. Therefore, we investigated the toxic effects of GA in primary rat hepatocytes and HSCs. To date, this study is the first report to address GA-induced rapid necrotic cell death in primary culture-activated HSCs, without cytotoxicity to hepatocytes. These results suggest that GA is an active dietary agent that may delay liver fibrosis.

Materials and methods

Chemicals

Gallic acid (GA), acridine orange, *N*-acetyl-Leu-Leu-norleucinal (ALLN), *N*-acetyl-leucyl-leucyl-methioninal (ALLM), 2-aminoethoxydiphenyl borate (2-APB), 1,2-bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid tetrakis(acetoxymethyl ester) (BAPTA-AM), cyclosporine A, dantrolene, ascorbic acid, catalase, collagenase, pronase E, and 4′-6-diamidino-2-phenylindole (DAPI) were purchased from Sigma Co. (St. Louis, MO, USA). Anti-catalase, anti-glutathione peroxidase (GPx), anti-superoxide dismutase (SOD), anti-α-smooth muscle actin (α-SMA), and anti-β-actin antibodies were obtained from Santa Cruz (Santa Cruz, CA, USA). Calpain activity assay kits were obtained from BioVision (Mountain View, CA, USA). Bio-Rad protein assay kit (Bradford method) was purchased from Bio-Rad (Hercules, CA, USA).

HSC and hepatocyte isolation and culture

Primary HSCs were purified using a 2-step pronase E-collagenase perfusion procedure in male Sprague–Dawley rats (weighing 250–300 g). Next, the cells were centrifuged in a 12% Nycodenz (Axis-Shield, Oslo, Norway) two-layer discontinuous density gradient, as described previously (Shu et al., 2004; Weiskirchen and Gressner, 2005). The isolated HSCs were cultured in Dulbecco's modified eagle medium (DMEM) supplemented with 10% heat-inactivated FBS. The quiescent HSCs exhibited vitamin A droplets within the cells (Fig. 1A). After 10–14 days, the cell morphology took on a fibroblastic appearance, and the vitamin A droplets were no longer visible (Fig. 1B). We confirmed the identity of the activated HSCs by assessing their immunoreactivity to α-SMA, a structural protein present in activated fibroblasts (Fig. 1D), but not in quiescent HSCs (Fig. 1C). Next, the purity of the cells was assessed by phase-contrast microscopy (purity > 95%). The activated HSCs used in the experiments described had undergone 4–25 passages. Primary hepatocytes were isolated through collagenase perfusion and were cultured on collagen-coated plates in DMEM supplemented with 2% heat-inactivated FBS as previously described (Alpini et al., 1994). All animal procedures were approved and performed under the guidelines of the Institutional Animal Care and Use Committee of Taichung Veterans General Hospital. The rats were kept in a 12-h light/dark cycle in an open-field cage, air-conditioned at 23–25 °C, 50–60% humidity and commercial basic diet and tap water were provided ad lib.

Cell treatment and the detection of cell death

The cells were cultured in DMEM containing 10% FBS in 12-well plates at a density of 3×10^4 cells/well for 24 h. Next, the cells were incubated with either GA (0–100 μg/mL) or vehicle (DMSO, 0.05% final concentration) for the indicated time periods. In the calpain inhibitor-treated groups, the HSCs were pre-treated with calpain I inhibitor ALLN or calpain II inhibitor ALLM for 24 h. Then, the cells were treated with GA (30 μg/mL). As for the Ca^{2+} chelator-treated groups, HSCs were pre-treated with either EDTA (extracellular Ca^{2+} chelator) or BAPTA-AM (intracellular Ca^{2+} chelator) for 15 min or were pre-treated with dantrolene, 2-APB or cyclosporine A for 2 h in complete DMEM. Next, the HSCs in these treated groups were treated with GA (30 μg/mL). For the antioxidant-treated groups, the HSCs were pre-treated with ascorbic acid or catalase for 2 h. The cells were then treated with GA for indicated time points. Following these procedures, cell death in HSC and primary hepatocytes was measured using the Trypan blue dye exclusion method, whereby cells were counted using a hemocytometer.

Calpain activity assays

The calpain activity was determined using the fluorogenic peptide substrate Ac-LLY-AFC (BioVision Inc., Mountain View, CA, USA) according to the manufacturer's instructions. Briefly, the cell lysates were incubated with calpain substrate. The relative calpain activity was measured by spectrophotometric analysis. We detected the 505-nm fluorescence emitted by the fluorochrome released from the cleavage products. The fluorochrome required excitation by light at 400 nm. Each experiment was repeated at least three times.

Intracellular Ca^{2+} and ROS measurement

To detect the intracellular Ca^{2+} and ROS levels, the HSCs were preloaded with either the redox-sensitive dye DCF-DA (5 μM, Molecular Probes, Eugene, OR, USA) or the Ca^{2+}-sensitive dye fura 2-AM (2 μM, Molecular Probes, Eugene, OR, USA) for 30 min. Then, the cells were washed and stimulated with GA. The changes of intracellular Ca^{2+} concentration were measured for the indicated time period in a multi-well fluorescence plate reader using the 340 nm and 380 nm excitation and 510 nm emission filters. ROS production was measured using a FACSCalibur flow cytometer with Cell Quest software.

Western blot analysis

To examine the different expression levels of catalase, superoxide dismutase, and glutathione peroxidase between HSCs and hepatocytes, western blot analysis was applied. The cells were lysed at 4 °C in a RIPA buffer containing 50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100, 0.25% sodium deoxycholate, 5 mM EDTA (pH 8.0), and 1 mM EGTA. The buffer was also supplemented with protease and phosphatase inhibitors. Following the lysis procedure, cell debris was removed by microcentrifugation, and the supernatants were then quickly frozen. The protein concentration was determined using the Bradford method. Equal amounts of proteins were separated onto SDS-PAGE and then electrophoretically transferred from the gel onto a PVDF membrane (Millipore, Bedford, MA). After blocking, the membrane was treated with specific primary antibodies overnight at 4 °C and was then incubated with a horseradish peroxidase-conjugated secondary antibody for 1 h. The blots were visualized using the ECL-Plus detection kit (PerkinElmer Life Sciences, Inc. Boston, MA, USA).

Assessment of lysosomal membrane integrity

HSCs were treated with either GA (30 μg/mL) or vehicle (DMSO; 0.05% final concentration) for 1 h in the presence or absence of various