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Apolipoprotein-J prevention of fetal cardiac myoblast apoptosis induced by ethanol

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Abstract

Over-consumption of ethanol (EtOH) represents a major health problem. This study was to test the cytotoxicity of EtOH in cardiac stem cells or myoblasts, and the potential protective effect of apolipoprotein-J (ApoJ), a stress-responding, chaperone-like protein in high-density lipoprotein, on EtOH-injured cardiac myoblasts. In culture, EtOH-exposed canine fetal myoblasts underwent apoptosis in a concentration- and time-dependent manner. Expression ApoJ by cDNA transfection markedly reduced EtOH-induced apoptosis in the cells. ApoJ expression also restored partially the mitochondrial membrane potential and prevented the release of cytochrome-*c* from mitochondria into cytoplasma. Thus, ApoJ serves as a cytoprotective protein that protects cardiac stem cells against EtOH cytotoxicity.

Keywords: Apoptosis; Stem cells; Ethanol; Cytochrome-*c*; Mitochondria; Lipoprotein; Heart

Alcohol abuse is one of the main factors that lead to hospital admissions in the USA. In a complex process, ethanol (EtOH)-induced toxicity causes widespread cell damage [1,2] and death via, either apoptosis, or necrosis [3,4]. The cytotoxic actions of EtOH are complicated, and perhaps mediated, in part, through oxidative stress and altered calcium homeostasis [5]. As a storehouse for intracellular calcium, a source of reactive oxygen species, and a sensor of oxidative stress [6,7], mitochondria play a key role in regulation of apoptosis under a variety of pathological conditions, including ischemia, hypoxia, and myocardial infarction [8–10].

The electrochemical potential across mitochondrial membrane, $\Delta\Psi$, is known to be highly sensitive to apoptotic stimulation and results from the asymmetric distribution of protons and other ions across membranes. As an index of mitochondrial function in living cells, $\Delta\Psi$ can be measured with an indicator dye, e.g., rhodamine 123 (Rh123), which fluoresces in direct proportion to $\Delta\Psi$ [11]. Decreased $\Delta\Psi$ occurs in cells undergoing apoptosis induced by oxidative agents, such as oxidized low density lipoprotein (oxLDL) [12] and hydrogen peroxide [13]. Injured mitochondria can release cytochrome-*c* into the cytoplasm cells treated with proapoptotic stimuli [14,15]. On entry into the cytosol, cytochrome-*c* activates the apoptosome containing the caspase-activating proteins Apaf-1 and caspase-9, and, subsequently, induces apoptosis [16]. EtOH exposure can induce apoptosis of human neural stem cells. The mechanisms by which EtOH induces myogenic stem cell apoptosis are not well defined; therefore, no therapeutic approach is available for preventing EtOH-induced stem cell toxicity.

Apolipoprotein-J (ApoJ) is a multifunctional glycoprotein widely existing in tissues and body fluids. ApoJ-deficient mice exhibit an increased susceptibility to autoimmune myocarditis [17]. Increased expression of ApoJ confers resistance to apoptosis induced by heat shock and oxidative stress [18,19]. As a component of high-den-

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sity lipoprotein (HDL) [20,21], ApoJ protects endothelial cells against apoptosis by inhibiting the dissipation of mitochondrial potential and the release of cytochrome-*c* into the cytoplasm [22]. This study examined the effects of ApoJ on EtOH-induced mitochondrial dysfunction, the release of cytochrome-*c*, and apoptosis in canine fetal myoblasts.

Materials and methods

Fetal myoblast cell cultures. Canine myoblast cell cultures were prepared from fetal hearts. Briefly, myoblasts were separated by digesting fetal heart tissues in 10 ml of 0.25% trypsin on a shaking water bath for 5 min at 37 °C. After dissociation, cells were counted in a hemacytometer and plated in 25 cm^2 tissue culture flask (Becton–Dickinson) at a density of 10^6 cells/flask. IMDM (Gibco) supplemented with 15% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin was used for culture. The cells were incubated at 5% CO_2/95% room air for 10–14 days before use.

ApoJ cDNA transfection. Human ApoJ cDNA (hApoJ, Accession No. BC19588) was purchased from ResGen IMAGE consortium cDNA clones (IMAGE:4915444). Briefly, cDNA coding for human ApoJ was isolated from pCMV-sport 6 plasmids by using *Hin*dIII and *Eco*RV. The 1.7 kb fragment containing the full length human ApoJ cDNA was ligated into the mammalian expression vector pcDNA3 (Invitrogen) and transfected into canine fetal myoblast cells by using Lipofectamine2000 (Invitrogen). Transfected cells were divided 48 h later and maintained in complete medium containing 300 μg/ml G418. After attaining confluent growth, the cells were subjected to experimental assays.

Determination of ApoJ protein expression by Western blot analysis. ApoJ-transfected and mock myoblast cells were lysed in 50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 1% Igepal CA-630 (NP-40), 0.5% sodium deoxycholate, 1% sodium dodecyl sulfate, and a mixture of protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, and 1 μg/ml leupeptin). Protein extracts were prepared by sonicating the lysed cells 3 times for 5 s. Cell debris was removed by centrifugation at 13,000 rpm for 20 min at 4 °C. An equal volume of 2× SDS reducing gel-loading buffer was then added, and the samples were boiled for 5 min. The protein concentration in the cell extracts was quantified via a spectrophotometer with the use of the BCA protein assay kit (Pierce) before adding the loading buffer. Proteins were fractionated in a gradient SDS-PAGE (4–10%) and transferred to PVDF membrane. Polyclonal antibody against ApoJ was obtained from Santa Cruz Biotechnology. Immunopositive bands were visualized by enhanced chemiluminescence (ECL, Amersham). Blots were stripped and probed again with monoclonal anti-β-actin antibody (Sigma) as a control for equal loading.

Quantification of fragmented DNA in cell death by ELISA. DNA fragmentation in cell death was analyzed using a cell death detection ELISA kit (Roche) according to the manufacturer's instructions. Briefly, canine fetal myoblast cells were exposed to 4 different concentrations of EtOH (25, 50, 75, and 100 mM) for three different time periods (16, 24, and 48 h) to determine the effect of EtOH on apoptosis. After EtOH exposure, cells were lysed in 100 μl of lysis buffer and centrifuged for 10 min at 1500 rpm. Triplicate 20 μl samples of supernatant were placed into the streptavidin-coated microtest plates for analysis. DNA fragmentation was quantified by measuring absorbance at 405 nm with a reference wavelength at 492 nm.

Determination of mitochondrial $\Delta\Psi$. To determine the effects of EtOH on $\Delta\Psi$, fetal myoblast cells were exposed to EtOH at the doses up to 100 mM for 3 different time periods (4, 8, and 16 h). After the experimental treatment, the cells were washed with PBS (pH 7.2, 1 ml) 3 times, then incubated for 15 min with 5 μM Rh123 (Molecular Probes) in 1 ml of PBS. After 15 min the cells were washed 3 times with PBS, and 1 ml of PBS was then added to the plates. Cells were removed from the culture plates with a scraper and placed into cytometer tubes. The indicator fluorescence was measured (excitation/emission wavelengths 480/530 nm) with a fluorescence plate reader. The emission values were expressed as the mean fluorescent peak height of the samples normalized to a percentage of the control value.

Cytochrome-c measurement. The cytosolic level of cytochrome-*c* was determined by using an ELISA kit (R&D Systems, Minneapolis, MN, USA). Briefly, after EtOH exposure, ApoJ transfected and non-transfected fetal myoblast cell cultures were washed twice with PBS, and cell membrane permeabilized in PBS with 0.5% Triton X-100. The cell lysates were centrifuged at 14,000 rpm for 15 min at 4 °C. Cytosolic proteins were determined using the Bradford assay, and the same amounts of proteins from each sample loaded into 96-well microplates with immobilized anti-cytochrome-*c*. After incubation for 2 h at room temperature, the plates were washed 3 times with washing buffer. A substrate solution containing tetramethylbenzidine and hydrogen peroxide was added to each well, and the plates were incubated for 30 min at room temperature. Then, a stop solution containing hydrochloric acid was added to terminate the reaction. Absorbance was determined by using a microplate reader at 450/575 nm dual wavelengths. The data were expressed as the mean optical density of the samples normalized to a percentage of the control value.

Statistics. Data are presented as means ± SEM. Statistical analysis was performed by using ANOVA or Student's *t* test when appropriate. A p value <0.05 was considered significant. All experiments were performed at least 3 times.

Results

EtOH exposure induces apoptotic cell death in canine fetal myoblasts

In culture, canine fetal myoblasts exposed to EtOH underwent apoptotic death in a manner dependent upon incubation time and concentrations of EtOH. Analysis of histone-associated DNA fragments showed that incubation of cells with EtOH caused a concentration and time-dependent DNA fragmentation and reduction in cell viability in the cultures during the 48 h interval (Fig. 1). Also, significant cell death occurred in cultures exposed to EtOH at 100 mM for 48 h. The untreated myoblasts showed little changes in DNA fragmentation and morphology when cultured under the same conditions except for EtOH treatment.

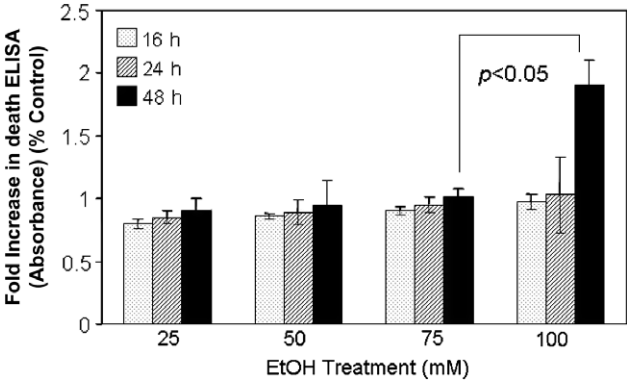

Fig. 1. EtOH-induced fetal myoblast cell apoptosis. The non-transfected fetal myoblast cells were exposed to the specified concentration of EtOH for 16, 24, and 48 h, and cells were assessed for apoptosis by analyzing histone-associated DNA fragments.

ApoJ expression confers resistance to EtOH-induced apoptosis of canine fetal myoblasts

To determine whether ApoJ exerts a protective effect on cardiovascular cells against a toxic agent, such as EtOH, ApoJ expression was induced in canine fetal myoblasts by stable cDNA transfection. Western blot analysis with anti-human ApoJ antibodies confirmed a marked increase in ApoJ expression in the transfected myoblasts but not mock transfected cells (Fig. 2A). To determine the effect of ApoJ in EtOH-induced apoptosis, the cells overexpressing ApoJ were treated with 100 mM EtOH. The death ELISA showed that cells with stable expression of ApoJ had significantly lower levels of histone-associated DNA fragments than the mock control cells when exposed to 100 mM EtOH for 48 h and than non-transfected cells (Fig. 2B).

ApoJ expression partially restores the mitochondrial membrane potential ($\Delta\Psi$) in canine myoblasts exposed to EtOH

Mitochondrial dysfunction characterizes apoptosis induced by cytotoxic substances. Analysis of the mitochondrial membrane's potential $\Delta\Psi$ was performed as a functional measurement of mitochondrial in the fetal myoblasts. Canine fetal myoblasts exposed to EtOH showed a concentration and time-dependent manner decrease in $\Delta\Psi$ (Fig. 3A). At the exposure cytotoxic dose (100 mM), EtOH decreased $\Delta\Psi$ after 16 h, but not after 4 h and 8 h exposure (Fig. 3B). ApoJ expression by transduction with cDNA protected fetal myoblast cells against the cytotoxicity of EtOH on $\Delta\Psi$; the EtOH—induced decrease in $\Delta\Psi$ after 16 h was significantly less in ApoJ—transfected cells than in non-transfected stem cells (Fig. 3B).

ApoJ expression reduces the release of cytochrome-c from mitochondria into the cytoplasma

Concurrently with the changes in the mitochondrial membrane protential, mitochondrial cytochrome-*c* may be released into the cytoplasma due to a partial leakage of mitochondrial membrane triggered by cytotoxic agents. Canine fetal myoblasts exposed to 100 mM EtOH showed increased cytosolic levels of cytochrome-*c* after 24 h of

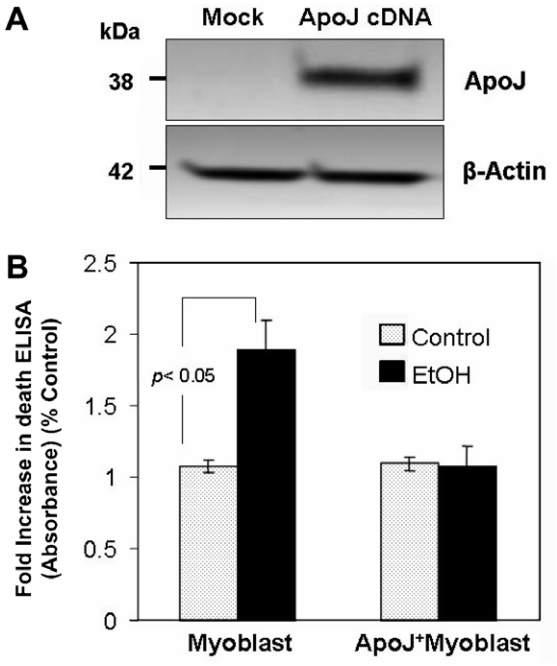

Fig. 2. ApoJ expression transduced by cDNA transfection prevents EtOH-induced apoptosis in fetal myoblast cells. (A) Immunoblot analysis showing ApoJ expression in fetal myoblasts transfected with or without ApoJ. Total cellular proteins extracted from fetal myoblasts and from fetal myoblasts transfected with ApoJ were subjected to 4–15% SDS–PAGE, then transferred to polyvinylidene fluoride membranes, blotted with anti-ApoJ and anti-β-actin antibody, and detected by enhanced chemiluminescence. Anti-β-actin antibody served as a control for equal loading. (B) Apoptosis of ApoJ-transfected or mock control canine myoblasts exposed to EtOH. Both fetal myoblasts transfected with or without ApoJ were exposed to 100 mM EtOH for 48 h, and apoptosis was analyzed for histone-associated DNA fragments.

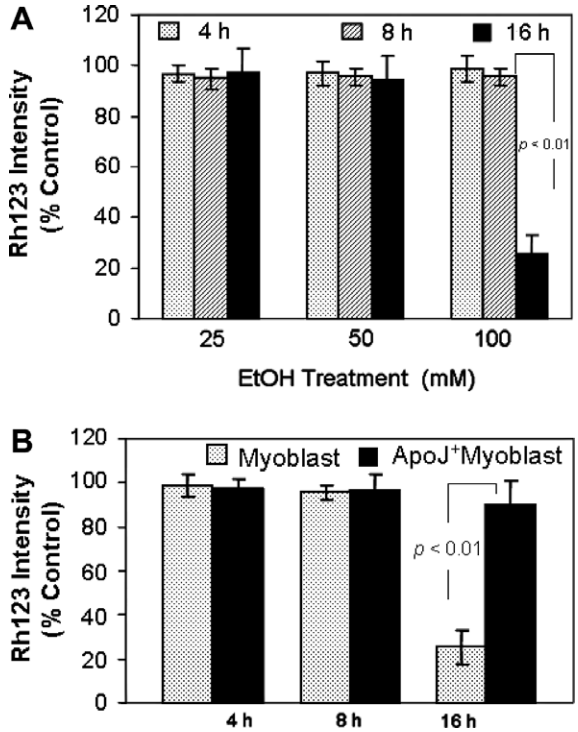

Fig. 3. ApoJ expression restores the mitochondrial membrane potential in fetal myoblasts exposed to EtOH. (A) Effect of EtOH on mitochondrial membrane potential in fetal myoblast cells. The non-transfected fetal myoblast cells were exposed to 25, 50, and 100 mM EtOH for 4, 8, and 16 h, then loaded with 1 μM rhodamine 123 (Rh123). After washing, the cells were analyzed for fluorescence at 480/530 nm (excitation/emission) in a fluorescence plate reader. (B) Effects of ApoJ on EtOH-induced mitochondrial dysfunction. Both fetal myoblasts transfected with or without ApoJ were exposed to 100 mM EtOH for 4, 8, and 16 h, then loaded with 1 μM rhodamine 123 (Rh123). After washing, the cells were analyzed for fluorescence at 480/530 nm (excitation/emission) in a fluorescence plate reader.

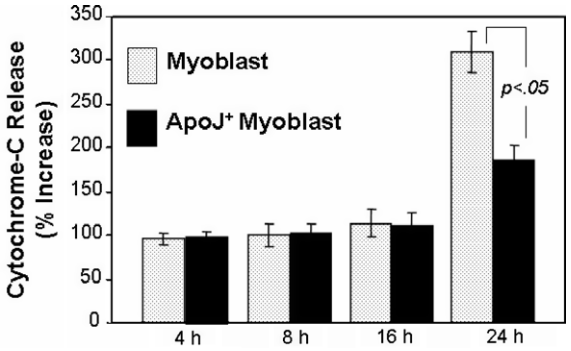

Fig. 4. Effects of EtOH on cytochrome-*c* release in ApoJ-transfected or mock control fetal myoblasts. Both fetal myoblasts transfected with or without ApoJ were exposed to 100 mM EtOH for 4, 8, 16, and 24 h, and the cytosolic fractions were obtained by centrifugation. Cytochrome-*c* levels were assayed by ELISA, with absorbance determined using a microplate reader at 450/575 nm (excitation/emission) dual wavelengths. The results were expressed as a percentage of the untreated control ± SEM of at least three plates per group in three replicate experiments.

exposure but not after 4, 8, and 16 h (Fig. 4). Expression of ApoJ by cDNA transfection attenuated this EtOH-induced increase in the cytosolic levels of cytochrome-*c* as shown by the significant smaller change in EtOH—treated transfected cells as compared with non-transfected cells (Fig. 4).

Discussion

This study provides the first experimental evidence showing that ApoJ attenuates EtOH-induced apoptosis in fetal myoblast cells. Furthermore, the studies of mitochondrial function demonstrate that protection against cytotoxic doses of EtOH conferred by ApoJ may be mediated, at least partially, through stabilization of $\Delta\Psi$ and the retention of mitochondrial cytochrome-*c*. Similarly, others have reported that ApoJ attenuates apoptosis induced by heat shock and oxidative stress [18]. ApoJ is a heat shock protein-like stress-responding protein, and a component of HDL, known to be cytoprotective. Our findings appear to be consistent with recent reports on the anti-apoptotic effects of ApoJ in non-stem cell cells. Others have shown that HDL inhibits apoptosis induced by ox-LDL in vascular cells [23,24], prevents apoptosis of endothelial progenitor cells through the inhibition of caspase-3 activity [25], and blocks apoptosis of endothelial cells by inhibiting dissipation of mitochondrial potential and release of cytochrome-*c* into the cytoplasm [22].

Our observation that exposure to EtOH for 16 h decreased $\Delta\Psi$ without affecting cytochrome-*c* release (measured 24 h after exposure to EtOH) or toxicity (measured 48 h after exposure to EtOH), suggests that the EtOH-induced decrease in $\Delta\Psi$ by itself is not sufficient to induce toxicity. However, the finding that EtOH affects $\Delta\Psi$ only at cytotoxic concentrations (100 mM) and not at non-cytotoxic concentrations (25 and 50 mM) indicates that EtOH-induced mitochondrial dysfunction contributes to the cytotoxicity towards myoblasts.

Our observation shows that the EtOH-induced decrease in $\Delta\Psi$ (16 h) occurred before the release of cytochrome-*c* (24 h), suggesting that EtOH might have attacked the mitochondria and increased the permeability of its membrane. This EtOH-induced cytochrome-*c* release may be a consequence of the opening of the mitochondrial permeability transition (MPT) channel. The finding is consistent with other reports showing that impaired mitochondria can release cytochrome-*c* into the cytoplasm, where it can bind to Apaf-1 and activate the caspase networks that induce apoptosis [26–28]. Furthermore, the finding that only cytotoxic concentrations of EtOH could induce cytochrome-*c* release and the fact that cytochrome-*c* release (24 h) occurred before cytotoxicity was induced (48 h) indicates that EtOH-induced cytotoxicity requires the release of cytochrome-*c* from mitochondria. This is consistent with previous findings that cytochrome-*c* release is necessary for apoptosis in many cell types [14,29,30]. However, the experiments that directly examine the requirement for cytochrome-*c* in apoptosis must await the generation of conditional null mutants of cytochrome-*c* that eliminate the death-promoting activity but maintain the essential function of cytochrome-*c* in oxidative phosphorylation [15].

In conclusion, our findings show that ApoJ inhibits EtOH-induced apoptosis in fetal myoblast cells by reducing mitochondrial dysfunction. Specifically, the protective mechanism of ApoJ involves preserving the mitochondrial membrane potential and maintaining the mitochondrial retention of cytochrome-*c*. These results may be therapeutically useful in preventing EtOH-induced stem cell toxicity.

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