Attenuation of fatty acid-induced apoptosis by low-dose alcohol in neonatal rat cardiomyocytes

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Sparagna, Genevieve C., Chad E. Jones, and Diane L. M. Hickson-Bick. Attenuation of fatty acid-induced apoptosis by low-dose alcohol in neonatal rat cardiomyocytes. Am J Physiol Heart Circ Physiol 287: H2209–H2215, 2004. First published June 24, 2004; doi:10.1152/ajpheart.00247.2004.—Moderate alcohol consumption has been shown to reduce the morbidity and mortality from coronary heart disease. Ethanol elicits its protective effects via mechanisms that include activation of protein kinases linked to growth and survival. Our results in isolated neonatal rat cardiomyocytes demonstrate that repeated short-term, low-dose exposure to ethanol is sufficient to activate the growth and/or survival pathways that involve PKC-ε, Akt, and AMP-activated kinase. In addition, we are able to induce apoptosis in these cardiomyocytes using the saturated fatty acid palmitate. Pretreatment with multiple low-dose ethanol exposures attenuates the apoptotic response to palmitate. This protection is manifested by a reduction in caspase-3-like activity, decreased mitochondrial loss of cytochrome c, and decreased loss of the mitochondrial lipid cardiolipin. We previously reported that incubation of cardiomyocytes with palmitate results in decreased production of reactive oxygen species compared with cells incubated with the nonapoptotic fatty acid oleate. In the present study, we observed an increase in the production of superoxide and the rates of fatty acid oxidation in cardiomyocytes pretreated with ethanol and then exposed to fatty acids. The level of superoxide production in palmitate-treated cells returns to the levels observed in oleate-treated cells after ethanol exposure. Taken together with our observed increase in AMP-activated kinase activity, we propose that ethanol pretreatments stimulate oxidative metabolism and electron transport within cardiomyocytes. We postulate that stimulation of palmitate metabolism may protect cardiomyocytes by preventing accumulation of unsaturated precursor molecules of cardiolipin synthesis. Maintaining cardiolipin levels may be sufficient to prevent the mitochondrial loss of cytochrome c and the downstream activation of caspases.

Palmitate; cardiolipin; reactive oxygen species; AMP-activated kinase

Epidemiological studies have shown that moderate alcohol consumption can reduce morbidity and mortality from coronary heart disease (12, 36, 55). Animal studies support these observations by demonstrating that sustained oral administration of low doses of ethanol provides protection against ischemia-reperfusion injury (60). Similarly, acute exposure of isolated cardiomyocytes and isolated hearts to physiological levels of ethanol also provides protection against ischemic damage (4). The cellular mechanisms by which both chronic and acute alcohol administration elicit these protective responses include the upregulation of protein kinases linked to cardiac growth and survival, namely, PKC-ε (4, 59) and Akt (59). The preconditioning protective response of mouse heart to ischemia-reperfusion injury elicited by repeated alcohol exposure is associated with a translocation (activation) of PKC-ε to a membrane fraction from a cytosolic pool (34).

Cardiac myocytes in adult human hearts undergo apoptosis under a variety of conditions (2, 41, 56). In experimental systems, cardiac apoptosis has been associated with ischemia and reperfusion injury (7, 14), hypertrophy (54), aging (23, 30), vascular wall remodeling (41), and TNF-α exposure (27). Intracellular accumulation of lipid within the heart is also associated with cardiomyopathy and increased cardiomyocyte apoptosis (60). Clinically high levels of circulating fatty acids are seen acutely after myocardial ischemia (35, 40). Palmitate (C16:0) and oleate (C18:1) are major constituents of circulating fatty acids. Palmitate and other long-chain saturated but not unsaturated fatty acids can induce apoptosis in cultured neonatal cardiomyocytes (5, 19, 52). This apoptosis is associated with an increase in caspase-3-like activity, mitochondrial cytochrome c release (52), poly(ADP-ribose) polymerase cleavage, and DNA laddering (51). Incubation with palmitate, compared with the control fatty acid oleate, leads to a decrease in the production of reactive oxygen species (ROS) including superoxide (20), inhibition of the enzyme AMP-activated kinase (AMPK), and decreased fatty acid oxidation (19). Palmitate-induced apoptosis in neonatal cardiomyocytes is also associated with decreased synthesis of the mitochondrial-specific phospholipid cardiolipin, and there is a linear, highly significant correlation between the decrease in this phospholipid and the release of mitochondrial cytochrome c (43).

In this study, we demonstrate that ethanol preconditioning of neonatal cardiac myocytes is effective in attenuating the apoptotic response toward palmitate. Repeated ethanol treatment of these cells leads to increases in activation of PKC-ε, Akt, and AMPK. These observations are concomitant with an attenuation of caspase-3-like activity, decreased mitochondrial cytochrome c release, and loss of cardiolipin after palmitate exposure. Ethanol pretreatment increases the level of superoxide production and fatty acid ß-oxidation by cells exposed to nonesterified fatty acids and thereby increases superoxide production in palmitate-treated cells to levels seen in oleate-treated cells. We suggest that this increase in ROS is a result of increased activation of the electron-transport chain in the presence of increased fatty acid-reducing equivalents. Furthermore, ethanol pretreatment protects cardiomyocytes from palmitate-induced apoptosis by increasing the oxidative metabolism of palmitate, thereby significantly lowering the inhibitory effect of this fatty acid on cardiolipin synthesis, mitochondrial cytochrome c release, and initiation of apoptosis.

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MATERIALS AND METHODS

Primary cell culture. Neonatal rat cardiac myocytes were prepared according to the methods of McMillin et al. (32) using 1- to 2-day-old Sprague-Dawley rat pups. Myocytes were plated in 60-mm dishes (2 × 10^6 cells) and maintained for 48 h in DMEM that contained 0.3 g/l glutamine, 4.5 g/l glucose, and 10% calf serum. All experiments conformed to the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the University of Texas Health Science in Houston animal welfare committee.

Ethanol treatment of cells. Isolated myocytes were treated with 10 mM ethanol in DMEM for 30 min. The media was then removed, and the cells were washed with phosphate-buffered saline. Cells that were to be treated multiple times with ethanol were returned to DMEM plus 10% calf serum and allowed to recover at 37°C overnight before the ethanol treatment was repeated. In each experiment, all cells were maintained in culture for an identical period. The media was also ethanol treatment was repeated. In each experiment, all cells were changed for both experimental and control cells on the same schedule, maintained in culture for an identical period. The media was also ethanol treatment was repeated. In each experiment, all cells were changed for both experimental and control cells on the same schedule, maintained in culture for an identical period. The media was also

Subcellular distribution of PKC-ε. After treatment with ethanol for the required number of times, the subcellular distribution of PKC-ε isoform in cell fractions was determined as described by Goldberg et al. (13). Briefly, ventricular myocyte cultures were washed with phosphate-buffered saline and immediately lysed in 0.4 ml of ice-cold homogenization buffer (20 mM Tris·HCl, pH 7.5, 2 mM EDTA, 2 mM EGTA, 6 mM β-mercaptoethanol, 50 µg/ml aprotinin, 48 µg/ml leupeptin, 5 µg pepstatin A, 1 mM PMSF, 0.1 mM sodium vanadate, and 50 mM NaF), sonicated, and centrifuged at 100,000 g for 1 h. The supernatant was removed (cytosol) and the pellet was resuspended in 0.2 ml of homogenization buffer that contained 1% Triton X-100 to solubilize particulate proteins. After the solution was shaken on ice for 30 min, Triton X-100-insoluble proteins were removed by centrifugation at 10,000 g for 10 min, and the supernatant (which contains virtually all of the membrane-associated PKC isoforms but only about two-thirds of the original cell pellet protein) was saved (particulate fraction). The distribution of PKC-ε in these fractions was determined by immunoblot analysis using a monoclonal antibody specific for this isoform (Transduction Laboratories) at a 1:1,000 dilution.

Western blot analysis of Akt and AMPK. Western blot analysis was used to determine phosphorylated Akt (p-Akt) and phosphorylated AMP kinase (p-AMPK). Cells were lysed, and total cellular extracts were run on 12% SDS-PAGE gels and then electrophoretically transferred to polyvinylidene difluoride membranes. Membranes were blocked using gelatin and were blotted first with the primary antibodies and then by appropriate horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology; Santa Cruz, CA). Signals were detected using the Renaissance Chemiluminescence Reagent Plus Kit (PerkinElmer Life Sciences; Rochester, NY). The p-AMPK antibody (Cell Signaling Technology; Beverly, MA) recognized both the α1- and α2-subunits of AMPK when these subunits are phosphorylated at Thr172. The p-Akt antibody is specific for Ser473 (Cell Signaling Technology). After membranes were probed for p-Akt, they were stripped and reprobed using a primary antibody that recognizes total Akt (Cell Signaling Technology). Protein intensities were quantified by densitometry (52).

Induction of apoptosis in ethanol-treated cells. Cardiac myocytes were repeatedly treated with ethanol. Apoptosis was then induced by incubating the cells in a 2:1 (M/M) palmitate-BSA solution for 16 h (19). Control cells were treated with ethanol as indicated and then incubated in a 2:1 (M/M) olate-BSA solution for 16 h.

Caspase-3-like activity. Caspase-3-like activity was measured by monitoring the cleavage of the fluorescent substrate acetyl-Asp-Glu-Val-Asp (DEVD)-7-amino-4-methylcoumarin (Calbiochem) as described previously (19).

Mitochondrial release of cytochrome c. After treatment with ethanol and fatty acids, cells were washed in PBS, the plasma membrane was permeabilized using digitonin (30 µM), and the supernatant was collected. Lactate dehydrogenase activity was monitored to assure optimal release of cytosolic proteins and the absence of particulate enzyme activity (32). Aliquots of the supernatant were analyzed by Western blot analysis and probed using an antibody to denatured cytochrome c (Pharmeren). Protein intensities were quantified by densitometry (52).

Superoxide production. Cardiomyocyte superoxide content was determined by lucigenin-enhanced luminescence. Cells were treated multiple times with ethanol as described (see Ethanol treatment of cells) in 60-mm plates. After the appropriate incubation periods, cells were washed with PBS, scraped, and resuspended in 350 µl of PBS. Cell extracts (100 µl) were aliquoted in triplicate into a 96-well plate. Lucigenin (100 µM of a 400 µM solution in PBS) was added to the wells, and the chemiluminescence of the samples was measured over a 15-min period in a luminesimeter. Superoxide levels were reported as relative light units (RLU) after background luminescence subtraction and normalization to the protein content of each sample.

Cardiolipin measurements. Cardiolipin was measured in cultured neonatal cardiomyocytes after ethanol treatments using the thin-layer chromatography method previously described by Ostrander et al. (43).

Fatty acid oxidation. Oxidation of [1-14C]palmitate by neonatal cardiomyocytes was determined using procedures previously described in this laboratory (19).

Statistical analysis. Data are represented as means ± SE. Statistical significance was assessed by one-way ANOVA. P values ≤ 0.05 were considered significant.

RESULTS

Chronic alcohol consumption upregulates PKC-ε, which is a signal transduction molecule required for cardioprotective effects of acute ischemic preconditioning (48). Similarly, brief exposure of isolated adult rat cardiomyocytes to low levels of ethanol protects against ischemia and is dependent upon the activation of PKC-ε (4). Likewise in our studies, brief (30 min) exposures of cultured neonatal rat cardiomyocytes to 10 mM ethanol induced activation of PKC-ε (Fig. 1) as assessed by translocation of protein from the soluble to the particulate

![Fig. 1. Effects of repeated exposure to low doses of ethanol on the membrane-bound distribution of PKC-ε in cultured neonatal rat ventricular cardiomyocytes. Cultured myocytes were exposed to repeated low doses of ethanol (see MATERIALS AND METHODS for details). After the last exposure, cell membrane fractions were isolated. Control cells were maintained in culture for an identical period with media being changed at similar intervals to cells treated with ethanol. *P ≤ 0.005, significantly different from control (no ethanol treatments); n = 3 independent experiments.](http://ajpheart.physiology.org/)

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fractions. Multiple cycles of ethanol treatment elicited a reproducible PKC-ε response (Fig. 1). In the mouse heart, chronic consumption of moderate doses of alcohol leads not only to activation of PKC-ε but also to activation of the serine/threonine protein kinase Akt (59). Constitutive activation of Akt has also been shown (8) to protect cardiomyocytes in vivo from reperfusion injury. Western blot analysis of rat neonatal cardiomyocytes after ethanol treatment demonstrates an increase in the content of phosphorylated (activated) Akt within the myocyte. The Akt response to ethanol is ablated by pretreatment with wortmannin, which is a specific inhibitor of phosphatidylinositol 3-kinase (PI3-K), the upstream activator of Akt (Fig. 2A). Maximal Akt activation was sustained after two or more ethanol treatments (Fig. 2B).

Pretreatment of myocytes with multiple ethanol exposures attenuated the apoptotic response of these cells to palmitate.

This is evidenced by the ethanol-induced dose-dependent reduction in caspase-3 activation of palmitate-treated cells (Fig. 3). Mitochondrial release of cytochrome c into the cytoplasm and a concomitant decrease in cardiolipin levels are temporally early events in palmitate-induced apoptosis (43). In the absence of ethanol treatments, incubation of these cells in the presence of the nonapoptotic fatty acid oleate results in cardiolipin levels 142 ± 9% higher than in cells incubated in the presence of palmitate (43). Multiple treatments of myocytes with ethanol resulted in a sequential reduction in the appearance of cytosolic cytochrome c after palmitate incubation (Fig. 4A) and attenuation of the loss of cardiolipin (Fig. 4B). These data imply that ethanol pretreatment protects the cardiomyocyte from palmitate-induced cell death.

Palmitate-induced apoptosis in cardiomyocytes is associated with a decrease in the production of superoxide compared with cells incubated in oleate (20). Repetitive ethanol treatments cause an increase in superoxide production in both oleate- and palmitate-treated cells. Moreover, ethanol treatment increased the superoxide production in palmitate-treated cells to the levels observed in oleate-treated cells (Fig. 5). A small percentage of electrons escape from the electron transport chain, potentially at complexes I and III (18). These electrons are capable of forming ROS including superoxide. Increased β-oxidation rates will lead to increased movement through the electron transport chain and potentially to increased levels of ROS production. Increased superoxide production after ethanol pretreatment is therefore consistent with an increased flux of reducing equivalents through the electron transport chain.

This hypothesis is also supported by our observation that ethanol treatment stimulates the phosphorylation of AMPK by fatty acid incubation (Fig. 6) and a concomitant increase in fatty acid β-oxidation rates (Fig. 7). Activation of AMPK only occurs in the presence of fatty acids (see Fig. 6A), and the...
levels of p-AMPK observed after incubation with fatty acids increased with repeated ethanol treatments (Fig. 6, B and C). AMPK phosphorylates and inactivates acetyl-CoA carboxylase. This in turn reduces the level of malonyl-CoA, which in heart cells is a potent inhibitor of the rate-limiting enzyme for fatty acid oxidation, carnitine palmitoyl transferase I (CPT I). This increase in p-AMPK levels is consistent with the onset of the protective response and increased disposal of palmitate through β-oxidation.

DISCUSSION

In this study, we demonstrated that acute, repetitive exposure to low doses of ethanol when cells are exposed to palmitate-BSA-containing media for 16 h. AMPK phosphorylates and inactivates acetyl-CoA carboxylase. This in turn reduces the level of malonyl-CoA, which in heart cells is a potent inhibitor of the rate-limiting enzyme for fatty acid oxidation, carnitine palmitoyl transferase I (CPT I). This increase in p-AMPK levels is consistent with the onset of the protective response and increased disposal of palmitate through β-oxidation.

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classic ischemic preconditioning in rabbits, i.e., translocation and activation of PKC-ε (28, 46). This isoform-specific translocation and activation of PKC-ε is a critical step in the signaling pathways responsible for short-term and delayed ischemic preconditioning in rabbits and in acute alcohol-induced protection against infarction in rabbit hearts (26). Chronic, moderate alcohol consumption in C57BL/6 mice also leads to translocation of PKC-ε and development of cardioprotection against ischemia (59). This translocation is also associated with activation of Akt, and selective inhibition of PKC-ε translocation prevents increases in Akt kinase activity induced by moderate alcohol consumption (59). We also observed increased Akt activation in our neonatal model. Akt activation has been shown (8) to reduce myocyte apoptosis in models of transient ischemia. In isolated neonatal cardiomyocytes, low amounts of ethanol mimic some of the obligatory signaling pathways activated by ischemic preconditioning.

Induction of apoptosis by saturated fatty acids has been reported in several cell types including cardiomyocytes (5), hematopoetic cells (45), pancreatic β-cells (47), and astrocytes (1). In cardiomyocytes, palmitate induction of apoptosis is associated with increased caspase-3 activity and mitochondrial release of cytochrome c (52). Mitochondrial release of cytochrome c was also demonstrated to be temporally and significantly associated with a decrease in mitochondrial synthesis of cardiolipin (43). In neonatal rat cardiomyocytes, pretreatment with low doses of ethanol attenuates the loss of cardiolipin, mitochondrial loss of cytochrome c, and activation of caspase-3 activity. Ethanol preconditioning therefore prevents apoptosis induced by saturated fatty acids in this cell system. Apoptosis has been reported in a variety of cardiovascular diseases including ischemia-reperfusion (14) and myocardial infarction (22, 41, 42). Multiple studies also indicate that cardiac ischemic preconditioning inhibits both apoptosis and necrosis. The attenuation of apoptosis is mediated by PKC-ε in rat hearts and chick ventricular myocytes (29, 38). Cardiac reperfusion after an ischemic event also exoses myocytes to a high level of circulating nonesterified fatty acids (35, 40). Thus ethanol preconditioning achieved by low-level alcohol consumption may protect heart by attenuating the apoptotic response of myocardium exposed to excess fatty acid. In our experiments, multiple ethanol exposures were required to maximally protect the cells from apoptosis. However, a single exposure to ethanol was sufficient to induce near-maximal PKC-ε translocation. Other investigators have shown that during ischemia-reperfusion, sustained translocation of PKC-ε is not necessary to induce cardioprotection (49, 53). PKC-ε translocation may thus act as a trigger rather than a mediator of cardioprotection (15).

Recent evidence also suggests that AMPK is activated during the late phase of ischemic preconditioning of rabbit hearts (37). Inhibitors of PKC-ε abolished this activation, which indicates an essential role for PKC-ε in phosphorylation of AMPK. Our results in neonatal myocytes support this observation with respect to ethanol preconditioning. In our studies, pretreatment with ethanol stimulated activation of PKC-ε. Activation of AMPK only occurred in the presence of fatty acids, and this response was increased by preexposure to ethanol, which suggests that activation of PKC-ε alone is not sufficient to activate AMPK. Ethanol pretreatment alone does not stimulate phosphorylation of AMPK; rather, these pretreatments appear to “prime” the AMPK response to fatty acids. It seems unlikely that AMPK is directly regulated by PKC-ε, but little is known of the identity and regulation of upstream kinases responsible for AMPK phosphorylation and whether these proteins represent potential targets for PKC-ε. Alternatively, reports suggest that Akt activity can also modulate the phosphorylation of AMPK in heart (25) although Akt activity itself may be regulated by fatty acids (50). The relationships between these kinases and the cellular response to ethanol preconditioning remain to be determined.

Our data demonstrate that ethanol preconditioning increases the level of ROS production by fatty acid-treated cardiomyocytes. We have previously reported that palmitate-treated cardiomyocytes reduced levels of fatty acid oxidation (19) and produced lower levels of ROS compared with cells treated with the nonapoptotic fatty acid oleate (20). In this study, we demonstrated that ethanol preconditioning increases superoxide production in palmitate-incubated cells to the levels observed in oleate-treated cells. In cells, increased electron flow through the electron transport chain, which is caused by increased rates of β-oxidation, leads to concomitant increases in the production of nonpaired electrons. This enhanced rate of nonpaired electron production leads to an increase in ROS synthesis (18, 39). Hence, increased oxidative metabolism increases the net mitochondrial loss of nonpaired electrons from the electron transport chain and can lead to an increase in intracellular ROS. We report an increase in both p-AMPK and fatty acid oxidation rates in cells subjected to ethanol pretreatment before fatty acid exposure. Increased fatty acid oxidation inhibits glucose oxidation (for review, see Ref. 24) and may lead to over reliance of the heart on fatty acids as an energy source. Such a situation has been proposed to perturb the functional recovery of myocardium after ischemia-reperfusion (6, 21). However, in our studies, in the presence of abundant oxygen and without a major glycolytic component to the cellular metabolism, the stimulation of fatty acid oxidation is protective, possibly by its effect on decreasing cardiolipin loss.

Fig. 7. Increased fatty acid oxidation by cells exposed to ethanol. Fatty acid oxidation rates were measured after ethanol exposures as described (see MATERIAL AND METHODS). Control cells were maintained in culture for an identical period with media being changed at similar intervals to cells treated with ethanol. *P < 0.012, significantly different from control; n = 5 independent experiments.
and/or reducing lipid accumulation (lipotoxicity) (33) within myocytes.

Chronic ethanol treatment can lead to proliferation of myocardial peroxisomes (61) and increased mitochondrial volume (31). Ethanol preconditioning may increase peroxisomal capacity for metabolism of long-chain fatty acids to shorter fatty acid chain derivatives that are then substrates for mitochondrial oxidation. Alternatively, activation of mitochondrial ATP-sensitive K⁺ (mKATP) channels, which are putative end effectors of cardioprotection, may play a role. These channels have been closely associated with PKC-ε activation and protection in ischemia-reperfusion preconditioning (9, 11) and ethanol preconditioning in dogs (44) and isolated rabbit and rat hearts (26, 60). The actual mechanism by which opening of these channels protects the heart is presently the topic of much debate. The volume of the mitochondrial matrix has a profound effect on fatty acid oxidation (16, 17), and opening of the mKATP channels is proposed to regulate mitochondrial matrix volume (for review, see Ref. 10). Furthermore, during ischemic preconditioning, opening of the mKATP channels leads to increased mitochondrial production of ROS. This increase triggers gene transcription (3, 58) and is required for protection afforded by ischemic preconditioning (57). In our studies, this protective production of ROS may therefore be the result of activation of the electron transport chain in the presence of increased availability of fatty acid-reducing equivalents.

The mechanism by which ethanol preconditioning prevents cardiolipin loss during palmitate-induced apoptosis is not directly addressed in this study. During palmitate-induced apoptosis, there is a significant increase in the levels of phosphatidic acid and phosphatidylglycerol with predominantly saturated acyl chains (43). These compounds are precursors in the biosynthesis of cardiolipin. The final step in cardiolipin biosynthesis, the conversion of phosphatidylglycerol to cardiolipin, is catalyzed by cardiolipin synthase, and highly unsaturated phosphatidylglycerol is not a good substrate for this enzyme (43). Thus increasing the removal of saturated fatty acids by stimulating oxidation may relieve this inhibition and allow cardiolipin synthesis to proceed, thereby decreasing the mitochondrial loss of cytochrome c and the induction of apoptosis. Little is presently known about the control of cardiolipin synthesis and/or degradation in heart. It remains possible that one or both of these processes are regulated because of alterations in upstream signaling pathways like those modulated by PKC-ε.

In conclusion, we propose that repeated exposure to low levels of ethanol stimulates oxidative metabolism and electron transport, increasing the oxidative metabolism of palmitate, and thereby relieving the inhibition of cardiolipin synthesis that is attributed to accumulation of unsaturated precursor molecules. This in turn protects the mitochondrion from cytochrome c loss and induction of apoptosis. The mechanisms responsible for increased oxidative metabolism are not elucidated, but we implicate upstream regulation of protein kinases including PKC-ε, Akt, and AMPK. These responses may all be downstream effects of mKATP-channel opening.

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REFERENCES


H2214 ETHANOL ATTENUATES PALMITATE-INDUCED APOPTOSIS


