ELSEVIER

Alcohol 39 (2006) 169–178

ALCOHOL

# Chronic-alcohol exposure alters IGF1 signaling in H9c2 cells via changes in PKC delta☆

Richard Ila, Michele Solem*

*Department of Pathology, Anatomy and Cell Biology, Thomas Jefferson University, 1020 Locust Street, Philadelphia, PA 19107, USA*

Received 17 May 2006; received in revised form 21 August 2006; accepted 23 August 2006

**Abstract**

Previously, we have demonstrated that chronic-alcohol exposure alters insulin-like growth factor 1 (IGF1) signaling in adult rat heart cells. This report examines the effects of alcohol in vitro on the expression of protein kinase C (PKC) alpha, delta, and epsilon using the embryonic heart cell line, H9c2, and how this may be linked to changes in IGF1 signal transduction. Western blot analyses of H9c2 protein preparations demonstrate that there are significant increases in the total protein levels of PKC delta and epsilon after 4 days exposure to alcohol, and similar increases were found after 2 and 6 days exposure. In addition, there was a significant increase in PKC delta and epsilon in the membranal fractions and a decrease in the cytosolic fractions. No change was found in the expression or activity levels for PKC alpha. Chronic-alcohol exposure (100 mM, 4 days) increased the basal tyrosine kinase activity of the IGF1 receptor (IGF1R), and altered its rate of activation. Chronic-alcohol exposure also reduced the rate of Erk1/Erk2 activation by IGF1. Chronic alcohol blocked the proliferative effects of IGF1 on cell growth and reduced cell viability both in the presence and absence of IGF1, and this alcohol-induced reduction in cell viability was blocked using siRNA to inhibit PKC delta. In addition, a reduction in the amount of myosin light chain 2 was found in the alcohol-exposed cells. In conclusion, chronic alcohol alters PKC delta and epsilon expression and activity, and suppresses the IGF1 signaling pathway in embryonic heart cell culture. Blockage of PKC delta expression using siRNA inhibits the suppressive effects of alcohol on cell viability. 

*Keywords:* Insulin-like growth factor 1 (IGF1); Protein kinase C (PKC); IGF1 receptor (IGF1R); Extracellular-regulated kinase (Erk 44 and 42 kD, also referred to as MAP kinase); Small interfering RNA (siRNA)

## 1. Introduction

Recently, this laboratory has determined that in both adult rat heart cells and in H9c2 embryonic rat heart cells, selective inhibition by PKC alpha blocks the tyrosine kinase activity of the IGF1 receptor (Maniar et al., 2005). However, in cardiomyocytes from adult alcoholic rats, there is loss of IGF1-stimulated PKC alpha activation as well as alterations in the IGF1 receptor activity (Pecherskaya et al., 2002). Since chronic-alcohol exposure is known to alter several different PKC isoenzymes levels of expression and activity, it may be that these proteins interfere with the normal role that PKC alpha plays in the IGF1 signaling pathway. Using H9c2 tissue culture, the following study was designed to determine if PKC plays a role in altering the IGF1 signaling pathway after chronic-alcohol exposure.

Several key studies using transgenic mice have demonstrated the importance of IGF1 in the heart's development and maintenance (DeLaughter et al., 1999; Liu and LeRoith, 1999; Montgomery and Schwartz, 1995). Homozygous IGF1-deficient mice are only half the weight of the wild types, and the vast majority of them die at birth. The hearts as well as other organs, including the liver, kidney, and brain were found to be enlarged in these mice compared to their wild-type littermates (Liu and LeRoith, 1999). In another study, it was found that expression of IGF1 in a transgenic model initially induced physiologic hypertrophy, however, later in life a pathological condition developed characterized by decreased systolic performance and increased interstitial fibrosis (DeLaughter et al., 1999). IGF1 may also serve to inhibit cardiomyocytes from undergoing apoptosis following infarction (Chen et al., 2000; Wang et al., 1998). In primary neonatal cardiomyocyte culture, it was shown that IGF1 protects these cells from

☆ This work was supported by the National Institutes of Health grant (R21AA013306-01A2) to Michelle Solem.

* Corresponding author. Tel.: +1-215-503-2681; fax: +1-215-923-2218.

*E-mail address*: michele.solem@jefferson.edu (M. Solem).

0741-8329/06/$ – see front matter 
doi: 10.1016/j.alcohol.2006.08.006

alcohol-induced cell death (Chen et al., 2000). IGF1 partially suppressed Bax induction, Caspase 3 activation, DNA fragmentation, and increased cardiomyocyte survival.

Many types of PKC isoenzymes have been detected in the heart. In adult rat cardiac tissue, the detection of the classical protein kinase C isoenzyme alpha, a $Ca^{2+}$-requiring isoenzyme, and the novel protein kinase C isoenzymes delta and epsilon, which do not require $Ca^{2+}$, accounted for the majority of PKC activity (Erdbrugger et al., 1997). Alcohol is known to modulate the expression and activity of several of these kinases in the heart and other tissues. Their roles in alcohol-associated heart disease are currently being identified (Chen & Mochly-Rosen, 2001; Chen et al., 2001; Gray et al., 2004; Miyamae et al., 1998; Solem et al., 2000; Zhou et al., 2002). In this report, we examine how chronic-alcohol exposure results in changes in PKC delta and epsilon in embryonic heart cells, and how this may alter the IGF1 signaling pathway. We find that chronic alcohol reduces IGF1R activation as well as downstream Erk1/Erk2 activity. There is also a reduction in cell proliferation and cell viability after serum withdrawal by chronic alcohol. Since we find that there are changes in PKC delta and epsilon, we hypothesize that one or both of these isoenzymes may be involved. By inhibiting PKC delta expression with siRNA, this blocks the alcohol-induced reduction in cell viability and also reduces alcohol's inhibition of IGF1's protective effects. Finally, we measure proteins that are expressed during cardiac cell growth and hypertrophy. We find that IGF1 modestly increases the level of myosin light chain 2, and alcohol reduces the levels of this protein and blocks IGF1's effect. No changes are found in the expression levels of troponin C, beta-actin, or the IGF1 receptor.

## 2. Methods

### 2.1. Materials

Chemicals for buffers were purchased from Sigma Chemicals (St. Louis, MO); bovine serum albumin was purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN); collagenase was purchased from Worthington Biochemicals (Lakewood, NJ). Phenylmethylsulfonyl fluoride (PMSF), leupeptin, aprotinin, and pepstatin were purchased from Sigma Chemicals (St. Louis, MO). IGF1 was purchased from R & D laboratories, (Minneapolis, MN). All cell culture reagents and antibiotics were purchased from Gibco BRL (Gaithersburg, MD). Antibodies to PKC alpha, delta, epsilon, IGF1 receptor (beta subunit), beta-actin, myosin light chain 2, and troponin C were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). CellTiter 96 Aqueous nonradioactive cell proliferation assay was purchased from Promega Corporation (Madison, WI). The siRNA (cat # B-002000-UB-0115, sequence #3) to block PKC delta, the siRNA (cat. # M-04-0103-00) for rat PKC alpha, and the siRNA (cat. # D-087950-01) for PKC epsilon were purchased from Dharmacon RNA Technologies (Lafayette, CO). In addition, the nonspecific control sequence (cat # D-001210-01) was also purchased from Dharmacon. The transfection reagent TransIT-TKO transfection reagent (cat # MIR2154) was purchased from Mirus Corp. (Madison, WI). 100% ethyl alcohol (alcohol) was a gift from the Thomas Jefferson University Pharmacy.

### 2.2. H9c2 cell culture

H9c2 rat embryonic cell culture (ATCC) was grown in DMEM with 10% FBS, streptomycin/penicillin (Gibco BRL, Gaithersburg, MD) at 37°C, 5% $CO_2$ as previously described (Maniar et al., 2005). Ethanol-containing media (100 mM) was changed daily, and the flasks containing the cells exposed to ethanol as well as the paired control flask were capped in order to prevent evaporation of ethanol from the media. This concentration of ethanol is within the range (50–200 mM) that has previously been shown to alter PKC in tissue culture (Coe et al., 1996; Messing et al., 1991). For the experiments, only low passage cells ($<15$) were used.

### 2.3. Western analysis of PKC alpha, delta, and epsilon

Western blotting was performed as previously described (Pecherskaya et al., 2002). Briefly, whole cell lysates from H9c2 cells exposed to 100 mM ethanol (2–6 days) were homogenized in cell lysis buffer (50 mM Tris, pH 7.5, 150 mm NaCl, 1% Triton X-100, 100 μM phenylmethylsulfonyl fluoride, 25 μg/ml leupeptin, pepstatin, aprotinin, 2 mM ethylenediaminetetraacetic acid, 1 mM ethyleneglycotetraacetic acid) (Solem et al., 2000). To isolate the cytosolic and membranal fractions, the detergent was not included in the cell lysis buffer. The cells were homogenized by vortexing for 30 s in an Eppendorf tube and triturated 35 times using a 23-gauge syringe needle. The cell pellet containing the membranal portion was removed by quick centrifugation (10,000 × g for 10 min) and the lysate containing the cytosolic portion was immediately stored at −20°C. Next, the membranal fraction was dissolved in cell lysis buffer containing detergent (1% Triton X-100). Protein concentrations were determined by using the Biorad (Hercules, CA) detergent-compatible protein assay kit. Protein samples (50 μg) were heat denatured in sodium dodecyl sulfate (SDS)-Laemmli sample buffer and loaded onto 10% SDS polyacrylamide gel electrophoresis (PAGE) gels for size separation. Molecular weights were determined by using molecular weight protein markers (Biorad, Hercules CA). The separated proteins were transferred to nitrocellulose and stained with antibody (1:300 dilution for 4 h) to PKC alpha, delta, or epsilon (Santa Cruz Biotechnology, Santa Cruz, CA). The blots were washed (3× at 30 min intervals using TBS containing tween-20 (0.1%)) and stained with anti-rabbit conjugated IgG-HRP secondary antibody (1:3000 dilution for 30 min) and then washed again (3×). These blots were exposed to X-ray film (Fuji