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Exposure of piglet coronary arterial muscle cells to low alcohol results in elevation of intracellular free Ca^{2+}: relevance to fetal alcohol syndrome

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Abstract

Chronic exposure of cultured piglet neonatal coronary arterial smooth muscle cells to low concentrations of ethanol (46–115 mg/dl) for 7 days resulted in concentration-dependent elevation in intracellular free Ca^{2+} ions ($[Ca^{2+}]_i$); these rises (22–56%) in $[Ca^{2+}]_i$ were not reversible upon short-term exposure to normal, Ca^{2+}-containing physiological salt solution. These findings help to provide a rational basis for why ethanol can result in the well-known fetal alcohol syndrome, including cardiac defects and in-utero death.

Keywords: Ca^{2+}, cytosolic, free; Fetal alcohol syndrome; Digital image analysis

In 1973, a report appeared which described a unique pattern of craniofacial, limb and heart defects, pre- and postnatal growth retardation, and developmental problems in children born to alcoholic women (Jones and Smith, 1973). This was termed the 'fetal alcohol syndrome' (FAS). Over the past 25 years, thousands of these cases have appeared, with large numbers of unexplained deaths in-utero or still-births (Little and Streissguth, 1982; Anonymous, 1993). Ever growing numbers of cases around the globe suggest a rate of 1–3 FAS cases in every 1000 live births, a rate comparable for the incidence of Down's syndrome and spina bifida.

While it is certain that alcohol taken in pregnancy can be teratogenic, and lethal to the fetus, there is no agreement for why alcohol taken during the first trimester of pregnancy produces a very high incidence of cardiac defects (e.g., septal, patent ductus arteriosus, murmurs, tetralogy of Fallot, arrhythmias) and in-utero death (Little and Streissguth, 1982; Anonymous, 1993). Using isolated adult canine, feline, ovine and primate small and large coronary arteries, as well as piglet small and large coronaries, from many regions of the myocardium, we found that alcohol (ethanol) can exert concentration (e.g., from as little as 40 mg/dl) -dependent contractile actions (Altura et al., 1983, 1996a). Using intact, perfused rat working hearts, and ^{31}P-nuclear magnetic resonance spectroscopy, we found that ethanol could produce concentration-dependent, significant and progressive deficits in phosphocreatine and elevation of intracellular inorganic phosphate concomitant with progressive reductions in intracellular pH and loss of intracellular myocardial mitochondrial creatine phosphokinase and lactic acid dehydrogenase (Altura et al., 1996b). We, therefore, tested the hypothesis that in-utero ethanol-induced elevation in fetal-neonatal coronary arterial smooth muscle intracellular free Ca^{2+} ions ($[Ca^{2+}]_i$) might set into motion the alcohol-induced coronary vasospasm resulting in hypoxia and ischemia, facilatating deficits in cardiac and peripheral organ blood flows, the end results being cardiac defects and/or in-utero deaths.

Experiments were carried out on single, cultured primary coronary arterial smooth muscle cells obtained from at least 4–5 different saffan-anesthetized (12 mg/kg i.m., Glaxo or Pittman-Moore) 4–7 day old piglets using digital imaging microscopy with the fluorescent probe fura-2, using modifications of previously established methods

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(Zhang et al., 1992). Prior to enzymatic digestion the arteries were denuded of endothelial cells. By use of monoclonal antibodies for α-actinin and trypan-blue exclusion we found that 96–98% of the cells were pure vascular muscle (Zhang et al., 1992). The cells were cultured in Dulbecco's modified Eagle's medium at 37°C in a humidified atmosphere composed of 95% air/5% CO_2. The cells were exposed to 0, 46, 115 or 460 mg/dl ethanol for 7 days; each day fresh ethanol and media were added. The cells were then loaded with fura-2 (Molecular Probes, Eugene, OR, USA) by incubating them with 2 μM fura-2 acetoxymethyl (AM) ester in the culture media for 60 min under 95% air/5% CO_2. To improve loading efficiency, 0.12% pluronic F-127 (Sigma, St. Louis, MO, USA) was used in the loading media. The labeled cells were washed for 10 min with HEPES buffer solution (in mM: NaCl 118, KCl 4.7, KH_2PO_4 1.2, $MgSO_4$ 1.2, HEPES 5 and glucose 10) containing the respective 0, 46, 115 and 460 mg/dl ethanol. The pH was adjusted to pH 7.4 with NaOH. Measurement of $[Ca^{2+}]_i$ was performed using a TN8500 Fluroplex Image Analyzer (Tracor Northern, Madison, WI, USA). Images of fura-2 fluorescence at 510 nm emissions were obtained with 340 and 380 nm excitation wavelengths using a silicon intensified target (SIT) camera. Fluorescence ratios ($R_{340/380}$) were obtained by dividing the 340 image by the 380 image.

An in-vitro calibration method was used to calculate $[Ca^{2+}]_i$ of single piglet coronary arterial smooth muscle cells employing 0 and 2.54 mM buffered $CaCl_2$ standard solutions plus 10 mM EGTA for the maximum (R_{max}) and minimum (R_{min}) fluorescence ratios of the 340-nm and 380-nm images. $[Ca^{2+}]_i$ was calculated according to the following equation (Grynkiewicz et al., 1985):

$$[Ca^{2+}]_i = K_d \times B \times (R - R_{min})/(R_{max} - R)$$

A K_d of 224 nM was used for the fura-2 complex (Grynkiewicz et al., 1985; Zhang et al., 1992). B is the ratio of fluorescence intensity of fura-2 to the Ca^{2+} bound fura-2 at 380 nM. Particular care was taken to minimize photobleaching of the dye. Experiments were done in total darkness, and exposure to excitation light was less than 2 min in duration in all experiments.

Where appropriate, means ± S.E.M.s were calculated and compared for statistical significance by t-tests and analysis of variance using Scheffe's contrast test for multiple comparisons.

With no ethanol, the basal level of $[Ca^{2+}]_i$ was 104.2 ± 0.65 nM (Table 1), with a heterogeneous distribution of $[Ca^{2+}]_i$. Seven-day exposure to 46 mg/dl ethanol resulted in a 22% rise in cytosolic free Ca^{2+}. Exposure to 115 mg/dl and 460 mg/dl ethanol resulted in a 56% and 58% elevation, respectively in $[Ca^{2+}]_i$.

Reintroduction of, and incubation (for 30 min) with, normal HEPES buffer containing 0-ethanol did not, significantly, alter the gains in cytosolic free Ca^{2+} produced by 7 days of exposure to 46, 115 or 460 mg/dl ethanol.

Table 1
Chronic treatment of piglet coronary arterial smooth muscle with ethanol for 7 days results in elevation of intracellular free Ca^{2+}

Ethanol (mg/dl)	$[Ca^{2+}]_i$ (nM)
0	104.2 ± 0.65
46	126.8 ± 1.93 [a]
115	162.6 ± 0.81 [a]
460	164.7 ± 1.14 [a]

n = 30–40 cells from 4–5 different piglets each. [a] Significantly different from 0-ethanol ($P < 0.01$).

To our knowledge, this is the first demonstration: (1) of use and culture of pure piglet-neonatal, primary cultured coronary arterial smooth muscle cells, and (2) that very low concentrations of chronically applied ethanol (< 125 mg/dl) can result in sigificant, concentration-dependent elevation in $[Ca^{2+}]_i$ in single coronary arterial smooth muscle cells. These new data, thus, show that alcohol is capable of producing by a direct mechanism (in the absence of nervous elements, circulating hormones or blood), significant elevation of cytosolic free Ca^{2+} in neonatal (and probably fetal) coronary smooth muscle cells. Since it is currently thought that only 2-equivalent drinks (i.e., cocktails) or more of alcohol (= 40–80 mg/dl blood alcohol level) per day during the first trimester of pregnancy is needed to induce the sequelae of events termed FAS (Anonymous, 1993), the data shown herein are quite relevant to the etiology of cardiac defects and in-utero deaths. In this context, our data indicate that exposure of coronary muscle cells to less than 50 mg/dl of ethanol for 7 days already results in significant elevation in coronary muscle $[Ca^{2+}]_i$ and most likely coronary vasospasm and reduced myocardial blood flow in the fetus. In view of these observations, it would be important to determine if longer exposure to ethanol will result in coronary muscle cell death, e.g., apoptosis. Progressive Ca overload in fetal coronary muscle cells, due to prolonged maternal ingestion of alcohol would be expected to result in severe ischemia and cell death.

Our findings are consistent with a vasospastic response in fetal coronary arteries leading to coronary vascular occlusion and the well-known cardiac defects observed in FAS. The progressive rise in myocardial $[H^+]$, intracellular inorganic phosphate and lactic acid concomitant with the progressive myocardial loss in phosphocreatine, lactic acid dehydrogenase and creatine phosphokinases, noted recently in intact working hearts, exposed to increasing concentrations of ethanol (Altura et al., 1996b), are consistent with this hypothesis.

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