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Critical roles of Rho-associated kinase in membrane blebbing and mitochondrial pathway of apoptosis caused by 1-butanol

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ABSTRACT

Alcohols are widely used as industrial solvents and chemical intermediates but can cause serious damage to human health. Nevertheless, few studies have addressed the molecular mechanisms underlying the cytotoxicity of industrial alcohols, with the notable exception of ethanol. The goal of our current study is to elucidate the molecular mechanism of cytotoxicity caused by primary alcohols containing longer carbon chains than ethanol. We find that 1-butanol induces morphological changes in H9c2 cardiomyoblastoma including nuclear condensation and membrane blebbing, both of which are features of apoptotic response. Moreover, a decrease in the mitochondrial membrane potential, the cytosolic release of cytochrome *c*, and the activation of caspase 9 and 3 was observed, thus revealing the activation of the mitochondrial apoptotic pathway by 1-butanol. The addition of Y-27632, a specific inhibitor of Rho-associated kinase (ROCK), suppressed the membrane blebbing and mitochondrial apoptotic pathway. In comparison z-VAD-fmk, a pan-caspase inhibitor, did not inhibit membrane blebbing but did prevent cell death following exposure to 1-butanol. These results indicate that mitochondrial pathway of apoptosis and membrane blebbing are parallel phenomena that occur downstream of ROCK. This kinase thus plays an essential role in 1-butanol cytotoxicity and subsequent cell death in H9c2 cells.

1. Introduction

Alcohols are important chemical intermediates, solvents, and plasticizers used in industrial processes. It has long been assumed that due to their bifurcate nature with both hydrophilic (hydroxyl) and hydrophobic (hydrocarbon) groups within their structures, alcohols will readily dissolve in extra- and intra-cellular fluids and interact with plasma membranes *in vivo* (Strubelt et al., 1999). These physicochemical properties of alcohols are considered to underlie their behavior as anesthetics and strong correlations have been observed between the hydrophobic properties of alcohols and their ability to disturb plasma membrane structures in brain synaptosomes and liver epithelial cells *in vitro* (Lyon et al., 1981; McKarns et al., 1997). As a primary component of alcoholic beverages, ethanol has been extensively studied in the field of toxicology (Forney and Harger, 1969). Chronic ingestion of high doses of ethanol can result in liver cirrhosis, hepatitis, and alcoholic cardiomyopathy (Zima and Kalousova, 2005; Beier and McClain, 2010; Zhang et al., 2004). Direct exposure of cardiomyocytes to ethanol induces the mobilization of Ca^{2+}, and subsequent activation of the mitochondrial apoptotic pathway (Hajnoczky et al., 2005). Studies of the harmful effects of other alcohols are severely lacking however, despite their prevalence in industrialized countries.

Apoptosis is a type of cell death involved in development, differentiation, and in the pathogenesis of multicellular organisms (Kroemer et al., 2009). Apoptotic cell death is characterized by morphological and biochemical features that include nuclear condensation, the digestion of chromosomes into nucleosome units, and plasma membrane blebbing (Kroemer et al., 2009). Although also observed in some forms of necrotic cell death, plasma membrane blebbing is mainly observed in apoptotic cell death and is executed through the activation of Rho-associated coiled-coil domain containing protein kinase (Rho-associated kinase; ROCK) (Barros et al., 2003; Charras and Paluch, 2008). ROCK is activated either by binding of the GTP-bound form of RhoA, by caspase 3-dependent cleavage, or by the binding of several lipids including arachidonic acid (Riento and Ridley, 2003). Membrane blebbing is a phenomenon whereby spherical protrusions from the plasma membrane form and then disappear, and is thought to be driven by hyperactivation of the

Abbreviations: ROCK, Rho-associated coiled-coil protein kinase; z-VAD-fmk, N-benzyloxycarbonyl-Val-Ala-Asp(O-Me) fluoromethyl ketone; MLC, myosin light chain; COX IV, cytochrome *c* oxidase IV; GFP, green fluorescent protein; PI, propidium iodide.

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actin-myosin system, which leads to a strong contractile force and results in unequal intracellular pressure (Charras et al., 2005, 2008; Tinevez et al., 2009). In regions where the membrane-cytoskeleton attachment is weakened, blebs are expanded. The activation of ROCK also leads to the initiation of the mitochondrial apoptotic pathway, which is executed through sequential activations of caspase 9 and 3 (Del Re et al., 2007). Although the true role of membrane blebbing and whether it is a protective mechanism in cells is still controversial, this phenomenon has been observed during the exposure of cardiomyocytes (Guan et al., 2004) and astrocytes (Minambres et al., 2006) to ethanol.

The purpose of our present study was to determine whether the established correlation between the hydrophobicity of alcohols and their efficacy as toxicants is also observed in cardiac cells *in vitro*. We further investigated the mechanism of cell death caused by 1-butanol, a representative alcohol containing a longer carbon chain than ethanol. We found from our analysis that 1-butanol induces blebbing of the plasma membrane as well as activation of the mitochondrial apoptotic machinery in cardiac cells, both of which are dependent on ROCK activity.

2. Materials and methods

2.1. Cell culture and antibodies

H9c2 rat cardiomyoblastoma cells were cultured at 37 °C in a humidified atmosphere containing 5% CO_2 in DMEM supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. One day prior to the experiments, the cells were trypsinized and replated on a new dish. In the experiments using inhibitors, the caspase inhibitor z-VAD-fmk (Calbiochem, San Diego, CA, USA) and ROCK inhibitor Y-27632 (Wako Pure Chemical, Japan) were added to the medium 1 h before exposure to 1-butanol at final concentrations of 10 and 20 μM, respectively. Anti-cleaved caspase 3 and 9 antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). Anti-actin antibody was obtained from Sigma–Aldrich (St. Louis, MO, USA). Anti-phospho MYL9 and -ROCK-1 antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Anti-cytochrome *c* and -COX IV antibodies were purchased from Pharmingen (San Diego, CA, USA) and Molecular Probes (Leiden, The Netherlands), respectively.

2.2. Measurements of cell viability, apoptotic, and necrotic cells

Cell viability was assessed by assaying the cellular dehydrogenase activity with Cell Counting Kit-8 (CCK-8; Dojindo, Kumamoto, Japan) in accordance with the manufacturer's instructions. This assay is a modified version of MTT assay using a highly water-soluble tetrazolium salt. Briefly, 100 μl aliquots of cell suspension were dispensed into 96-well plates and cultured for 24 h. The cells were exposed to alcohols for the indicated times, and then treated with a 10 μl solution of CCK-8. After incubation for 15 min, the absorbance at 450 nm was measured using a microplate reader. In addition to the evaluation of cell viability by CCK-8 assay, we morphologically evaluated apoptotic as well as necrotic cell death by staining cells with propidium iodide (PI) and Hoechst33342. Briefly, cells were cultured on 3.5 cm diameter dishes for 24 h and treated with 1-butanol. After incubation for 10 min with PI (1 μg/ml) and Hoechst 33342 (1 μg/ml), the cells were observed under a fluorescence microscope (BZ-8100; Keyence, Osaka, Japan).

2.3. Western blot analysis

Adherent cells were scraped, collected together with floating cells by centrifugation, and then subjected to ultrasonic wave disruption (5 × 5 s; Sonifier 150, Branson, Danbury, CT, USA) in STE buffer (0.32 M sucrose, 10 mM Tris–HCl, pH 7.4, 5 mM EDTA, 50 mM NaF, 2 mM Na_3VO_4) on ice. Equal amounts of protein (10–20 μg) were resolved by SDS–PAGE, according to Laemmli (1970), and transferred to a PVDF membrane. After blocking with TBS-Tween (150 mM NaCl, 10 mM Tris–HCl, pH 7.4, 0.05% Tween 20) containing 3% skim milk, the membrane was incubated overnight at 4 °C with the specific antibodies. After washing with TBS-Tween, the membrane was incubated for an hour at room temperature with peroxidase-conjugated anti-rabbit, anti-mouse or anti-goat antibodies as required (Promega, Madison, WI, USA). Signals were detected using Western Lightning Chemiluminescence Reagent Plus Kit (Perkin Elmer Life Science, Boston, MA, USA), and signal intensities were quantified using an image analyzer (CS analyzer; Atto, Tokyo, Japan).

2.4. Measurement of mitochondrial membrane potential

Mitochondrial membrane potentials were detected using the JC-1 cationic dye (Molecular Probes). Briefly, cells were plated on 96-well (black with clear flat bottom) plate and cultured for 1–2 days. The cells were incubated in Hank's balanced salts solution (HBSS) containing 2 μg/ml JC-1 for 10 min, and then exposed to 1-butanol. . Fluorescence was measured first at 450 nm (green) and then at 570 nm (red) using a microplate reader. Mitochondrial depolarization is judged by the decrease in the ratio of red/green fluorescence intensity.

2.5. Cytosolic release of cytochrome c

Cytosolic and mitochondrial fractions were separated as described previously (Zucchini-Pascal et al., 2009). Briefly, cells were collected from 10 cm diameter dishes, washed with ice-cold PBS, and resuspended in ice-cold mitochondria isolation buffer (20 mM Hepes–KOH, pH 7.5, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM sodium EDTA, 1 mM DTT, 0.1 mM PMSF, 10 μg/ml pepstatin A and 10 μg/ml leupeptin). Cells were disrupted by syringing seven times with a 26-gauge needle and then centrifuged at 14,000*g* for 15 min at 4 °C. The resultant supernatant (cytosolic fraction) and the pellet (mitochondrial fraction) were used for Western blot analysis.

2.6. Statistical analysis

All data were analyzed using the Tukey–Kramer statistical method or Student's *t* test and expressed as the mean ± S.D. of at least three independent experiments. *P* values of <0.05 were considered to be statistically significant.

3. Results

3.1. Cytotoxicity by normal primary alcohols on H9c2 cardiomyoblastoma cells

We first examined whether the extent of the cytotoxic effects of alcohols upon cardiomyocytes correlates with their carbon chain length. A series of normal primary alcohols from methanol (C1) to 1-pentanol (C5) were used for this purpose. H9c2 rat cardiomyoblastoma cells were exposed to a fixed concentration (150 mM) of each of these alcohols for 6 h and the cytotoxicity was measured by the decrease in viability. Cell viabilities were assessed as total cellular dehydrogenase activities, which were measured as reduction of a tetrazolium salt (CCK-8 assay). As shown in Fig. 1A, greater cytotoxic effects were observed for alcohols containing longer carbon chains. Since 1-butanol (C4) caused a substantial decrease in viability (~90%), we selected this alcohol for further examination.