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Aconitine-induced Ca^{2+} overload causes arrhythmia and triggers apoptosis through p38 MAPK signaling pathway in rats

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ABSTRACT

Aconitine is a major bioactive diterpenoid alkaloid with high content derived from herbal aconitum plants. Emerging evidence indicates that voltage-dependent Na^+ channels have pivotal roles in the cardiotoxicity of aconitine. However, no reports are available on the role of Ca^{2+} in aconitine poisoning. In this study, we explored the importance of pathological Ca^{2+} signaling in aconitine poisoning in vitro and in vivo. We found that Ca^{2+} overload lead to accelerated beating rhythm in adult rat ventricular myocytes and caused arrhythmia in conscious freely moving rats. To investigate effects of aconitine on myocardial injury, we performed cytotoxicity assay in neonatal rat ventricular myocytes (NRVMs), as well as measured lactate dehydrogenase level in the culture medium of NRVMs and activities of serum cardiac enzymes in rats. The results showed that aconitine resulted in myocardial injury and reduced NRVMs viability dose-dependently. To confirm the pro-apoptotic effects, we performed flow cytometric detection, cardiac histology, transmission electron microscopy and terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling assay. The results showed that aconitine stimulated apoptosis time-dependently. The expression analysis of Ca^{2+} handling proteins demonstrated that aconitine promoted Ca^{2+} overload through the expression regulation of Ca^{2+} handling proteins. The expression analysis of apoptosis-related proteins revealed that pro-apoptotic protein expression was upregulated, and anti-apoptotic protein BCL-2 expression was downregulated. Furthermore, increased phosphorylation of MAPK family members, especially the P-P38/P38 ratio was found in cardiac tissues. Hence, our results suggest that aconitine significantly aggravates Ca^{2+} overload and causes arrhythmia and finally promotes apoptotic development via phosphorylation of P38 mitogen-activated protein kinase.

Introduction

Aconitum plants have been widely used to treat various diseases, such as shock caused by acute myocardial infarction, coronary heart disease and angina pectoris in China for thousands of years (Liou et al., 2005; Shaheen et al., 2005; Singhuber et al., 2009). Numerous herbal medicines containing aconitum plants as main ingredients have been formulated. However, the high cardiotoxicity of these compounds severely limits their clinical use (Chan, 2012; Kong et al., 2012; Lin et al., 2004). Aconitine, a major bioactive diterpenoid alkaloid derived from aconitum plants, reportedly contributes primarily to the cardiotoxic effects of aconitum plants (Fu et al., 2007; Wada et al., 2005). Previous studies have mainly focused on the cardiotoxic effects of aconitine on voltage-dependent Na^+ channels (Kunze et al., 1985; Wang and Wang, 2003; Wright, 2002). However, little information is available on the role of Ca^{2+} in aconitine poisoning. Therefore, the present study was designed to give a clearer understanding of the importance of defective Ca^{2+} signaling in aconitine poisoning in vitro and in vivo.

It is well known that Ca^{2+} overload has vital roles in the pathogenesis of heart dysfunctions, especially arrhythmia and apoptosis (Lai et al., 2011; Petersen et al., 2005; Rabkin and Kong, 2003; Soni et al., 2011). Many researchers have reported that Ca^{2+} plays an important role in the pathogenesis of arrhythmia and pathological cellular Ca^{2+} overload can lead to an arrhythmogenic state. In other words, arrhythmia is an important event that occurs during aconitine poisoning. In addition, Ca^{2+} overload can be involved in cardiac apoptosis and appears to be a principal mediator of apoptosis. However, the potential role of Ca^{2+} overload in aconitine-induced cardiotoxicity

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remains largely unknown. In our research, we investigated the alterations of Ca^{2+} level in cardiomyocytes induced by aconitine treatment and explored whether Ca^{2+} overload could cause arrhythmia and trigger apoptosis.

Materials and methods

Materials

Aconitine (content ≥ 98%) was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (China). The molecular weight of aconitine is 645.74. Collagenase Type II and Fura-2/AM were purchased from Life Technologies Corporation (Carlsbad, CA, USA). The kits for determining total creatine kinase (CK), aspartate aminotransferase (AST), and LDH (lactate dehydrogenase) were obtained from Biosino Bio-Technology and Science Incorporation (Hong Kong, China). Annexin V/propidiumiodide (PI) apoptosis detection kit was obtained from Life Technologies Corporation (Carlsbad, CA, USA). The terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) assay kit was purchased from Roche Diagnostics (Mannheim, Germany). Primary antibodies against RyR, SERCA, NCX, BCL-2, BAX, P53, caspase-9, caspase-3, ERK, P-ERK, P38 and P-P38 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The anti-rabbit-conjugated horseradish peroxidase antibody was purchased from Zhongshan Goldbridge Biotechnology (Beijing, China). The Bradford protein assay kit was purchased from Pierce Corporation (Rockford, USA) and super-enhanced chemiluminescence detection reagents were purchased from Applygen Technologies (Beijing, China). All of the chemical reagents were obtained from Sigma Chemical Co., Ltd (St. Louis, MO, USA).

Animals and treatments

Eighty adult male Wistar rats (Vital River Laboratories, Beijing, China) weighing 220 to 240 g were used and the procedures were approved by the local animal committee. These rats were kept at standard room temperature (22 ± 2 °C) and relative humidity (60% ± 10%) with a 12 h light/dark cycle. All of the rats were allowed free access to food and water ad libitum during the acclimatization and experimental period. The experiments were performed in accordance with the guidelines of the Experimental Laboratory Animal Committee of Chinese Academy of Medical Sciences and Peking Union Medical College and the principles and guidelines of the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Rats were randomly divided into the following two groups: group A as control and group B as aconitine model. Forty male Wistar rats were designated for each group. Ten rats in each group were implanted with telemetry transmitters for ECG study.

After 1-week of acclimatization, the aconitine model was induced with the protocol described below. Group B orally received 0.146 mg/mL aconitine, diluted in 0.05 N hydrochloric acid, once a day by gavage at 10 mL/kg for 10 consecutive days. Group A was given vehicle instead of aconitine once a day by gavage at 10 mL/kg for 10 consecutive days.

On day 3 or day 6 post-aconitine administration, 10 rats in each group were anesthetized with sodium pentobarbital (50 mg/kg, i.p.), and their hearts were removed rapidly. The left ventricle was excised for hematoxylin-eosin (HE) staining, transmission electron microscopy (TEM) and TUNEL examination. Myocardial homogenates were then prepared for Western blot analysis. On day 10 after the last administration of aconitine, blood was collected from the left ventricle and centrifuged at 800 ×*g* for 10 min to obtain serum, which was kept at −80 °C until analysis. Subsequently, the hearts were removed rapidly. The left ventricle was excised for HE, TEM and TUNEL examination. The myocardial homogenates were prepared for Western blot analysis.

Isolation of adult rat ventricular myocytes and treatments

Individual adult rat ventricular myocytes (ARVMs) were isolated from 13-week-old Wistar rats as described previously (Westfall and Borton, 2003; Westfall et al., 1997). After the rats were anesthetized with ketamine/xylazine (0.1 mL/100 g, i.p.), their hearts were removed and perfused through the aorta cannula with a series of different perfusion solutions at the rate of 6 mL/min. First, the hearts were perfused with Ca^{2+}-containing Tyrode's solution [NaCl, 137; KCl, 5.4; $MgCl_2$, 1.2; HEPES, 10; glucose, 10; and $CaCl_2$, 1.2 (in mM)] for 2 min. Subsequently, the hearts were perfused with Ca^{2+}-free Tyrode's solution containing with the aforementioned components except $CaCl_2$ for 5 min, followed by a 20 min perfusion with Ca^{2+}-free Tyrode's solution containing collagenase Type II (210.00 units/mg). After perfusion, the left ventricles were removed, minced, and filtered through a nylon mesh (300 mm). The filtered myocytes were then washed with Ca^{2+}-containing Tyrode's solution to restore the extracellular Ca^{2+} concentration to 1.2 mM. ARVMs were assayed by trypan blue exclusion assay for viability. Viability was over 80%. Only rod-shaped ARVMs with clear edges were used in this study.

Measurement of beating rhythm, sarcomere shortening, and Ca^{2+} transients in ARVMs

Beating rhythm, sarcomere shortening and Ca^{2+} transients of intact ARVMs at room temperature were assessed simultaneously upon field stimulation (0.5 Hz with 2-ms-duration, 16 V) using a video-based sarcomere contractility and calcium recording module in a SoftEdge MyoCam system (IonOptix Corporation, Milton, MA, USA). Isolated ARVMs were loaded with fura-2/AM (2 μM) for 15 min and washed twice with Ca^{2+}-containing Tyrode's solution after restoration of the extracellular Ca^{2+}. ARVMs were then placed in a Warner chamber mounted on the stage of an inverted microscope (Olympus, IX-70) and superfused with Ca^{2+}-containing Tyrode's solution with aconitine (1 μM), verapamil (10 μM) or verapamil (10 μM) + aconitine (1 μM) at the rate of 1.5 mL/min. Verapamil is a specific L-type Ca^{2+} channel blocker. Beating rhythm, sarcomere shortening and Ca^{2+} transients were recorded in 10 min intervals after the addition of aconitine (1 μM), verapamil (10 μM) or verapamil (10 μM) + aconitine (1 μM). Before measurement, isolated ARVMs were given 10 min for baseline stabilization. Data were recorded and analyzed with IonWizard software (version 6.2.0.59).

Electrocardiography

Electrocardiography (ECG) recordings were taken before aconitine or vehicle treatment and 24 h after each aconitine or vehicle treatment in conscious freely moving rats. Ten rats in each group were implanted with telemetry transmitters (HD-S21) of Data Sciences International (St. Paul, MN, USA) for ECG collection. After the rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p.), HD-S21 transmitters were placed in the abdominal cavity of the rats and fixed to the inner peritoneal wall using silk sutures. All skin incisions were closed using wound clips under sterile conditions. After 2-week surgical recovery, ECG was measured for ten days. When we switched the mode to ON, the transmitters began to sense and transmit data. ECG data were acquired using Dataquest A.R.T. 4.31 software (Data Sciences International). The raw ECG data were then analyzed by DSI's Ponemah Physiology Platform software. This technique facilitated the collection of ECG recordings in the most reliable and efficient method without anesthetizing rats.

Cell culture and aconitine treatment

Primary cultures of NRVMs were prepared as described previously (Gray et al., 1998). NRVMs were isolated from the hearts of 1-day-old