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Mechanistic studies on ketamine-induced mitochondrial toxicity in zebrafish embryos

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ABSTRACT

Ketamine, a phencyclidine derivative, is an antagonist of the Ca^{2+}-permeable *N*-methyl-D-aspartate (NMDA)-type glutamate receptors. It is a pediatric anesthetic and has been implicated in developmental neurotoxicity. Ketamine has also been shown to deplete ATP in mammalian cells. Our previous studies showed that acetyl L-carnitine (ALCAR) prevented ketamine-induced cardiotoxicity and neurotoxicity in zebrafish embryos. Based on our finding that ALCAR's protective effect was blunted by oligomycin A, an inhibitor of ATP synthase, we further investigated the effects of ketamine and ALCAR on ATP levels, mitochondria and ATP synthase in zebrafish embryos. The results demonstrated that ketamine reduced ATP levels in the embryos but not in the presence of ALCAR. Ketamine reduced total mitochondrial protein levels and mitochondrial potential, which were prevented with ALCAR co-treatment. To determine the cause of ketamine-induced ATP deficiency, we explored the status of ATP synthase. The results showed that a subunit of ATP synthase, *atp5α1*, was transcriptionally down-regulated by ketamine, but not in the presence of ALCAR, although ketamine caused a significant upregulation in another ATP synthase subunit, *atp5β* and total ATP synthase protein levels. Most of the ATP generated by heart mitochondria are utilized for its contraction and relaxation. Ketamine-treated embryos showed abnormal heart structure, which was abolished with ALCAR co-treatment. This study offers evidence for a potential mechanism by which ketamine could cause ATP deficiency mediated by mitochondrial dysfunction.

1. Introduction

The pediatric general anesthetic ketamine is a noncompetitive antagonist of the *N*-methyl-D-aspartic acid (NMDA) receptor (Kohrs and Durieux, 1998). Although recognized as a dissociative anesthetic (Corssen et al., 1968), ketamine also has analgesic and amnesic effects and possesses fast-acting anti-depressant properties (Aroni et al., 2009; Berman et al., 2000; Niesters and Dahan, 2012; Rowland, 2005). Ketamine is a Schedule III controlled substance in the USA (Morgan et al., 2010). Lately, several studies have shown ketamine's adverse effects on mitochondrial function. Ketamine decreased mitochondrial membrane potential in neural stem cells leading to apoptosis (Bai et al., 2013). In primary neurons, ketamine induced neurotoxicity by increased nitrotyrosine formation (Wang et al., 2008). In human lymphocytes and hepatocytes, ketamine caused mitochondrial dysfunction (Braun et al., 2010; Lee et al., 2009). Additionally, in various regions of rat brains, ketamine at subanesthetic doses altered mitochondrial respiratory chain activity (de Oliveira et al., 2011). Ketamine led to mitochondrial dysfunction in neurons derived from human induced pluripotent stem cell (Ito et al., 2015) and acute ketamine administration has been shown to impair mitochondrial function in rat brains (Venancio et al., 2015). Ketamine suppresses ATP biosynthesis in HepG2 cells (Chang et al., 2009). However, the molecular mechanism of how ketamine causes mitochondrial dysfunction and suppresses ATP production is not known.

There are growing concerns that long-term ketamine abuse harms the heart and increases the risk of sudden death (Weiner et al., 2000). Ketamine is known to induce serious side effects including cardiac deregulation (Craven, 2007) by altering the sympathomimetic effects (White and Ryan, 1996) through excessive production of catecholamines. Increased production of catecholamines results in oxidative damage to the heart (Haskova et al., 2011). Ketamine reduced heart rates in newborn human infants (Saarenmaa et al., 2001) as well as pregnant and infant non-human primates (Hotchkiss et al., 2007). Ketamine-induced cardiotoxicity in rats and its prevention by antioxidants (disulfiram, metyrosine and metoprolol) have been reported (Ahiskalioglu et al., 2015; Cetin et al., 2015) suggesting that these drugs may prove useful in clinical practice for any potential development of cardiotoxicity that may occur due to ketamine anesthesia. In both rats and rabbits, chronic ketamine treatment caused significant ventricular

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myocardial apoptosis and fibrosis that not only altered the electrophysiological properties of the heart but also increased its susceptibility to malignant arrhythmia leading to sudden cardiac death (Li et al., 2012). Metoprolol prevented such ketamine-induced cardiotoxicity, opening a potential new therapeutic avenue (Li et al., 2012).

Acetyl L-carnitine (ALCAR) is an L-lysine derivative and belongs to the family of carnitines, a group of naturally occurring compounds that is essential for β-oxidation of fatty acids in mitochondria during ATP generation (Bieber, 1988). It plays a major role in the transport of long chain fatty acids into mitochondria that enter the β-oxidation cycle (Bohles et al., 1994). ALCAR effectively prevents mitochondrial injury resulting from oxidative damage (Palacios et al., 2011). Carnitines have neuroprotective effects on conditions associated with mitochondrial dysfunction, oxidative stress and possibly in neurodegenerative disorders (Beals et al., 2003). During early developmental stages, co-administration of ALCAR and ketamine significantly diminished reactive oxygen species (ROS) generation and provided significant protection of neurons from ketamine-induced neurodegeneration in rats (Liu et al., 2013). Additionally, ALCAR protected neurons from inhalation anesthetic-induced neurotoxicity in rat cortical neurons (Zou et al., 2008). In ischemic swine hearts perfused in the presence of free fatty acids, ALCAR preserved mechanical function (Liedtke and Nellis, 1979). In rats, ALCAR ameliorated ketamine-induced behavioral alterations and body weight deficits (Boctor et al., 2008).

The zebrafish (*Danio rerio*) embryo has become an important experimental model for studying various diseases, protein functions, drug toxicities, and various physiological responses (Kanungo et al., 2014; Vogel, 2000). Accordingly, the therapeutic and toxic responses for both cardiac and non-cardiac drugs can be assessed by simply immersing the embryos in water with the test drug (Kanungo et al., 2012; Langheinrich et al., 2003; Milan et al., 2003). We have previously shown in zebrafish embryos that consistent with its effects on the nervous system and heart function in mammals (Hotchkiss et al., 2007; Irifune et al., 1997; Irifune et al., 1991; Kim et al., 2006; Zausig et al., 2009), ketamine adversely affects the motor neurons (Kanungo et al., 2013), serotonergic and dopamine systems (Robinson et al., 2016; Robinson et al., 2015) as well as heart rate (Kanungo et al., 2012). Additionally, we have shown that ALCAR prevents ketamine's adverse effects on the heart rate (Guo et al., 2017; Kanungo et al., 2012; Robinson et al., 2016; Robinson et al., 2015). Our studies also suggested that ALCAR could function through modulating ATP synthase (Guo et al., 2017).

ATP synthase is a multi-subunit enzyme (Cross and Muller, 2004) and its dysregulation adversely affects mitochondrial respiration. Mitochondrial dysfunction leads to the development of cardiomyopathy (Walters et al., 2012). Since cardiac contraction and relaxation processes are dependent on energy in the form of ATP produced by the mitochondria, mitochondrial dysfunction results in developmental defects of the heart (Sinatra, 2009). Additionally, myocardial ATP depletion is a major factor in heart failure (Gupta et al., 2012; Sinatra, 2009). Ketamine has been shown to cause an undesirable strain on the myocardium of patients (Spotoft et al., 1979) and we have previously reported on ketamine's adverse effects on heart rate in the absence of ALCAR (Kanungo et al., 2012). Here, as a follow up to our earlier studies, we investigated ketamine's effects on the components of the bioenergetic system and whether such effects could be prevented by ALCAR. This study was undertaken primarily to understand the molecular mechanism(s) of ketamine's effects on the heart and investigate potential preventive measures for unintended adverse effects during ketamine anesthesia or abuse.

2. Materials and methods

2.1. Animals

Adult wild type (WT) zebrafish (*Danio rerio*, AB strain) were obtained from the Zebrafish International Resource Center (www.zirc.org) (Eugene, OR, USA). The fish were kept in fish tanks (Aquatic Habitats, FL, USA) at the NCTR/FDA zebrafish facility containing buffered water (pH 7.5) at 28.5 °C and were fed daily live brine shrimp and Zeigler dried flake food (Zeiglers, Gardeners, PA, USA). Each 3-liter tank housed eight adult males or females. Handling and maintenance of zebrafish were in compliance with the NIH Guide for the Care and Use of Laboratory Animals and were approved by the NCTR/FDA IACUC. The day–night cycle was maintained at 14:10 h. For in-system breeding, crosses of males and females were set up the previous day in the tanks with partitions that were taken off the following morning at the time of light onset at 7:30 AM to stimulate spawning and fertilization. Fertilized eggs were collected from the bottom of the tank, placed in Petri dishes, washed thoroughly with buffered egg water [reverse osmosis water containing 60 mg sea salt (Crystal Sea®, Aquatic Eco-systems, Inc., Apopka, FL, USA) per liter of water (pH 7.5)] and then allowed to develop in an incubator at 28.5 °C for later use.

2.2. Reagents

Ketamine hydrochloride was purchased from Vedco, Inc. (St. Joseph, MO, USA). Acetyl L-carnitine (ALCAR) and other reagents were purchased from Sigma (St. Louis, MO, USA) unless stated otherwise. ALCAR stock (1 M) solutions were made fresh with buffered egg water.

2.3. Treatment of zebrafish embryos with ketamine and acetyl L-carnitine (ALCAR)

For treatment with ketamine and ALCAR, 28 h post-fertilization (hpf) embryos were used since our previous studies showed that these embryos as whole organisms respond to ketamine anesthesia (Cuevas et al., 2013; Kanungo et al., 2012) and ketamine exposure at the gastrula stage leads to non-specific effects displayed during later development (Robinson et al., 2017). For each experiment that analyzed all the endpoints, eggs laid at the same time from several females were pooled. For each treatment, 50 embryos were placed in individual 60 mm Petri dishes containing 35 ml buffered egg water and a total of 450 embryos (nine Petri dishes) were used for all the exposures in each experiment. The experiments were repeated three times. Ketamine (2.0 mM) treatment continued for 20 h (static exposures) in the presence or absence of ALCAR (1.0 mM). In our previous studies, these specific concentrations of ketamine and ALCAR for the specific time of exposure were found to be effective in inducing detectable toxicities and their amelioration, respectively (Kanungo et al., 2012; Robinson et al., 2017). Previously, we have shown that the amount of ketamine accumulation (~ 8 μM) in the zebrafish embryos/larvae treated with 2 mM is closer to the lower range of human anesthetic range (plasma concentration) (Trickler et al., 2013). Untreated control groups were examined in parallel.

2.4. Measurement of ATP concentration

Zebrafish embryo ATP content was determined using ATP colorimetric assay kit (Biovision, Milpitas, CA, USA). Briefly, for each sample,10 embryos (out of the 50 embryos in each Petri dish) were homogenized in 200 μl ATP assay buffer and the homogenates were centrifuged at 15,000 × *g* for 2 min to pellet insoluble materials. Supernatants were collected and 50 μl supernatant was added to a 96-well plate. ATP reaction mix (50 μl) was added to the samples in each well. After mixing by shaking gently, the 96-well plate was incubated at room temperature for 30 min, protected from light. The ATP levels were detected at 570 nm using a Synergy MX microplate reader (BioTek, Winooski, VT, USA). ATP levels in the embryo extracts were quantified using an ATP standard curve generated to calculate the amount of ATP and were normalized to the protein concentration of the samples. Protein concentrations were determined using a bicinchoninic