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Coenzyme Q10 protects against statin-induced myotoxicity in zebrafish larvae (*Danio rerio*)

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ABSTRACT

3-Hydroxy-3-methylglutaryl-CoA reductase (HMGCR) is the rate-limiting enzyme of the mevalonic acid pathway and is required for cholesterol biosynthesis and the synthesis of Coenzyme Q10 (CoQ10). Statins inhibit HMGCR, thus inhibiting the downstream products of this pathway including the biosynthesis of decaprenyl-pyrophosphate that is critical for the synthesis of Coenzyme Q10 (CoQ10). We show that zebrafish (*Danio rerio*) larvae treated in tank water with Atorvastatin (ATV; Lipitor) exhibited movement alterations and reduced whole body tissue metabolism. The ATV-inhibition of HMGCR function altered transcript abundance of muscle atrophy markers (atrogen-1, murf) and the mitochondrial biogenesis marker (pgc-1α). Furthermore, ATV-induced reduction in larval response to tactile stimuli was reversed with treatment of CoQ10. Together, the implication of our results contributes to the understanding of the mechanisms of action of the statin-induced damage in this model fish species.

1. Introduction

Atorvastatin (ATV) or Lipitor™, is a 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR) inhibitor, and is one of the best-selling statins for the treatment of hypercholesterolemia (Jackevicius et al., 2012). The increasing prescription rates of these drugs result in their release into aquatic environments. In fact, statin medications are reported at detectable levels in sewage treatment plant (STP) effluents and surface and ground waters in Canada and elsewhere generally at concentrations in the low ng L^{-1} range (Metcalfe et al., 2003; Benotti et al., 2009; Lee et al., 2009; Jelic et al., 2011). A recent review finds high sequence conservation across metazoans (including a number of fish) for HMGCR and in particular the catalytic binding site, implicating statin sensitivity across these species (Santos et al., 2016) thus raising concern of the negative impact statins might have on non-target aquatic species.

Statin drugs or ATV comparatively inhibits HMGC, the rate-limiting enzyme in the mevalonic acid pathway (Santos et al., 2016). In addition to lowering cholesterol, statins reduce the biosynthesis of other lipids including farnesyl prenylated proteins and decaprenyl-PP that ultimately lead to Coenzyme Q10 (CoQ10) (Santos et al., 2016).

CoQ10 is predominantly found in humans, birds, and fish (Battino et al., 1990; Albano et al., 2002; Kyoto Encyclopedia of Genes and Genomes, 2012). Coenzyme Q10 is an essential molecule within the mitochondrial Electron Transport Chain (ETC) where it acts as an electron shuttle for generating the proton gradient for ATP production. Curtailing CoQ10 synthesis therefore could result in oxidative stress and ultimately mitochondrial dysfunction in all body tissues, but in particular those tissues with high energy requirements including cardiac muscle, red oxidative muscle, brain, liver, and kidney (Gold and Cohen, 2001). Studies have shown that statin inhibition of the HMGCR pathway result in muscle damage in humans (Sathasivam and Lecky, 2008), rats (Elhaleem and Elsayed, 2011), mouse muscle cells in vitro (Cao et al., 2009), and zebrafish (*Danio rerio*) larvae (Campos et al., 2016). Elhaleem and Elsayed (2011) reported a correlation between statin-induced muscle damage and diminished CoQ10 levels in rats that was validated by rescuing the damage with CoQ10 treatment.

To further investigate the correlation between statin-induced muscle damage on aquatic species and diminished CoQ10 levels, we utilized zebrafish embryo/larvae as they have similar CoQ10 biosynthetic (Kyoto Encyclopedia of Genes and Genomes, 2012) and mevalonic acid pathways as those in mammals (Thorpe et al., 2004). Their optical clarity in the early stages of development, short lifespan, small size and frequent breeding, make them effective experimental animals for aquatic toxicology experiments.

We chose to use ATV because it is widely used and is detected in the aquatic environment. Here, we exposed zebrafish embryos/larvae to

Abbreviations: ATV, Atorvastatin; EM, embryo medium; HMGCR, 3-hydroxy-3-methylglutaryl-CoA reductase; hpf, hours post-fertilization; PTS, polyoxyethanyl-α-tocopheryl sebacate

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ATV alone, ATV + CoQ10 or ATV + vehicle control and assessed movement alterations, and changes in enzyme and transcript activities. Our results showed that ATV exposure not only increased mortality and the incidence of pericardial edema, but also induced movement alterations, enzymatic (oxidative and glycolytic capacities), and transcript abundance (muscle atrophy markers) changes. The ATV effects on movement and enzyme activities were rescued by the addition of CoQ10, supporting the correlation between myopathies and declining CoQ10 levels. Furthermore, this work validates the use of zebrafish as a model for the role of CoQ10 while contributing to the understanding of the mechanisms of action of statins.

2. Materials and methods

2.1. Chemicals

Atorvastatin (ATV; Pfizer Inc., Connecticut, USA) was dissolved in dimethyl sulfoxide (DMSO; diluted to 10 mg mL^{-1} in sterile/distilled water) and kept at −20 °C until used. Embryos (1–2 hpf) were placed in embryo medium (EM; 5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, 0.33 mM $MgSO_4$, including 0.00001% methylene blue) and treated with various ATV concentrations in embryo medium, with DMSO as control. For rescue experiments, water soluble CoQ10 was utilized. Water soluble-CoQ10 stock formulation contained CoQ10:PTS (50 mg:150 mg in 1 mL of phosphate-buffered saline; 137 mM NaCL, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4) and was a generous gift of Dr. Jagdeep Sandhu, National Research Council (Ottawa, ON, Canada). PTS or polyoxyethanyl-α-tocopheryl sebacate is a vitamin E derived molecule used to produce water soluble CoQ10 and was administrated as vehicle control (Somayajulu-Nitu et al., 2009). All other chemicals were purchased from Sigma–Aldrich Chemical Co. (Oakville, ON, Canada).

2.2. Zebrafish husbandry

Wild-type adult zebrafish (obtained from AQUAlity, Mississauga, ON, Canada) were maintained under a 14:10 light/dark photoperiod and a constant temperature of 28 °C in the University of Ottawa Aquatic Care Facility, and fed a Complete Adult Zebrafish Diet (Zeigler, Garners, PA, USA) daily. Embryos were obtained through natural breeding using a pair-wise trap method in which 2 females and 1 male were placed in 1-L breeding traps (Aquatic Habitat, Apopka, FL, USA). Embryos from several breeding traps were pooled and randomly assigned to 5 cm glass petri dishes containing 35 mL EM and kept at 28 ± 1 °C. All experiments were repeated 4 times and were approved by the University of Ottawa Protocol Review Committee and adhere to the guidelines established by the Canadian Council on Animal Care for the use of animals in research and teaching.

2.3. Drug treatments

The zebrafish early life stage (ELS) test is one of the most popular tools for evaluating the acute effects of aquatic pollutants on fish (Fraysse et al., 2006). Therefore, to establish treatment concentrations, two separate dose-response experiments were carried out to test the acute toxic effects of ATV. The first set of experiments assessed concentrations of ATV verses embryo/larvae mortality using a standard acute 96 hpf (4 days) test to show lethal toxicity endpoint and permit the calculation of an ATV LC_{50} (lethal concentration at which 50% of the embryos died) (Kovirzynych et al., 2013). While conducting the acute lethal toxicity for ATV, it was observed that embryo/larvae that were exposed to ATV concentrations higher than 2 mg L^{-1} displayed a high incidence of pericardial sac edema (data not shown). Consequently, a follow up experiment was conducted assessing concentrations of ATV verses embryo/larvae using a 96 h (4 days) test to show pericardial sac edema as a toxic malformation endpoint and permit the calculation of an ATV LE_{50} (effective concentration at which 50% of the embryos expressed pericardial sac edema). Embryos/larvae for the dose-response experiments were placed in final concentrations that ranged from 0.045 mg L^{-1} (this concentration was chosen because it represents the 1000× environmental concentration; Metcalfe et al., 2003) to 7 mg L^{-1}. The incidence of embryo/larval mortality and pericardial sac edema were assessed daily by inspection using a Leica WILD M10 light dissecting microscope (Leica Microsystems Inc., Concord, ON, Canada) and dead embryos were removed. The 96 h LC_{50} and LE_{50} were calculated by plotting the log-concentration of ATV versus probits of mortality or incidence of pericardial sac edema, respectively (http://ayubmed.edu.pk/JAMC/PAST/21-3/Randhawa.pdf). From the LE_{50} plot, the effective concentration at which 50% of the embryos/larvae expressed pericardial sac edema (probit of 5) was 1 mg L^{-1} or 1.8 μM. All subsequent experiments used a concentration of 0.045 mg L^{-1} as the low dose (or 0.081 μM, representing 1000× environmental concentration), 0.5 mg L^{-1} (0.9 μM) as a mid-dose and 1 mg L^{-1} (1.8 μM) as a very high dose.

A second set of experiments were conducted to understand the role of CoQ10 in ATV-induced myopathy. Within these experiments, first, total displacement and the response to a tactile stimulus in larvae treated with ATV alone or in combination with CoQ10 (rescue experiment) or PTS (vehicle control) were conducted. Following the assessments, larvae from each treatment groups were collected to evaluate possible tissue metabolic and molecular alterations. Twenty five 96 hpf larvae from each treatment group were combined into a 1.5 mL conical centrifuge tube (one sample), immediately frozen in liquid nitrogen and stored at −80 °C until analyzed.

2.4. Response to a tactile stimulus

The zebrafish embryo acquires the ability to respond to tactile stimuli as early as 24 hpf (Kimmel et al., 1995). In order to evaluate the effects of ATV on sensory alterations and the potential role of CoQ10 on these effects, the effect of ATV on larval response to tactile stimulation was examined according to Xi et al. (2010) with slight modifications. In total, 30 larvae per treatment per breeding session were assessed for a total of 4 breeding sessions ($n = 4$). Each larva was placed into a 9 mm glass petri dish and allowed to acclimate for 2 min. Larvae response to a tactile stimulus was assessed by a gentle touch to the tail of the larva using a single tooth brush bristle glued to a 23 gauge needle. In total, two stimuli were applied to each larva. An 'immediate response' was defined as responding to both stimuli, while a 'delayed response' was defined as responding to only one of the two stimuli. Absence of response to both stimuli was defined as 'no response'. Values are expressed as a percentage of the larvae responding.

2.5. Spontaneous displacement

Spontaneous movement can easily be observed during early larval stages (Granato et al., 1996). In order to evaluate the effects of ATV on motor alterations and the potential role of CoQ10 on these effects, total larva displacement were assessed, according to methods described in Xi et al. (2010). Each 96 hpf larva was placed into a 9 mm diameter glass petri dish containing EM and allowed to acclimate for 2 min. In total, four larvae per treatment per breeding session were assessed for a total of 4 breeding sessions ($n = 4$). Live capture of larvae displacement was recorded for 2 min using a high-speed camera (44 mm × 58 mm × 29 mm Grasshopper3, Point Gray Research Inc., Richmond, BC, Canada) powered over a USB 3.1 cable linked to a high performance stereomicroscope (Leica MZ12.5, Meyer Instruments, Houston, TX, USA) using Quick Screen Recorder software (Estrusoft, Kaysville, UT, USA). The recordings were transferred to a computer using a male A micro B cable at 9 frames per second, 3376 × 2704 pixels images using a 5 mm C-Mount Adaptor. Using Window Media Player, videos were analyzed at 2 frames per second by tracking the