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Short communication

Palmitate induces nitric oxide production and inflammatory cytokine expression in zebrafish

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ABSTRACT

Inflammation markers in zebrafish embryos reflect a toxic response that is common to other animal models and humans. Free fatty acids (FFAs) are known to cause damage in various tissues by inducing inflammation. In this study, we investigated whether a FFA (palmitate) induces inflammation in zebrafish embryos. Nitrous oxide (NO) production and cyclooxygenase-2 (COX-2) mRNA expression were increased in palmitate-treated zebrafish embryos in a dose-dependent manner. mRNA expression of pro-inflammatory cytokines, interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF- α), were also increased. Additionally, the mRNA expression of p65 nuclear factor-kB and I-kB-α were significantly increased after palmitate-treatment. Increased reactive oxygen species (ROS) expression was observed in palmitate-treated zebrafish embryos as well as pericardial edema. Additionally, mRNA expression of pro-inflammatory cytokines were increased in zebrafish liver and pancreas fed with palmitate-contained diet. Taken together, these results indicated that palmitate increases pro-inflammatory mediators in zebrafish embryos, suggesting that zebrafish could be an alternative animal model for inflammatory disease including diabetes.

1. Introduction

Mild inflammation by activation of the innate immune system is known to play a role in the pathogenesis of type 2 diabetes (T2D) [1], thus it can be regarded as a chronic inflammatory disease. In addition, obesity is closely associated with peripheral as well as hepatic insulin resistance [2] and, along with low grade inflammation, is characterized by elevation of proinflammatory cytokines in blood and tissues [3]. Inflammation in obesity contributes to the development of T2D.

Plasma free fatty acids (FFAs) such as palmitate are usually elevated in obesity because they are released by the enlarged adipose tissue mass (8). Several studies have shown that palmitate exposure results in various physiopathologies, including impairment of insulin transport in adipose microvascular endothelial cells [4], activation of NF-kB transcription and increased proinflammatory cytokine production in 3T3-L1 adipocytes [5], and alteration of neuropeptide Y expression in hypothalamic appetite-stimulating neurons in mice [6].

The functional innate immune system is established in zebrafish at 48 h post-fertilization with many of the same immune cells, cytokines and chemokines found in humans. Furthermore, in the last decade, the zebrafish has emerged as an alternative *in vivo* model for diseases including diabetes [7] and as a tool for drug screening. These characteristics suggest that the zebrafish would be an excellent model for the study of inflammatory pathologies during T2D. However, whether palmitate induces a similar pattern of inflammation in the zebrafish as it does in mammals has not been studied. Therefore, in the present study, we investigated the effects of palmitate in zebrafish embryos as a possible model for inflammatory T2D.

2. Materials and methods

2.1. Zebrafish maintenance

Wild-type and transgenic expressing enhanced green fluorescent protein under the control of the insulin promoter *Tg*(ins-egfp) zebrafish embryos were obtained from Korean Zebrafish Organogenesis Mutant Bank. At 3 days post-fertilization (dpf), embryos were arrayed in 12-well plates for experiments. Zebrafish embryo procedures used in the present study were conducted according to the guidelines established by the Gachon University Ethics Review Committee for Animal Experiments.

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Table 1
Primer sequences.

Gene name	Sequence 5′-3′	
IL-1β	Forward	5` - TCAAACCCCAATCCACAGAG- 3′
	Reverse	5` - TCACTTCACGCTCTTGGATG- 3′
TNF-α	Forward	5` - AGAAGGAGAGTTGCCTTTACCGCT- 3′
	Reverse	5` - AACACCCTCCATACACCCGACTTT- 3′
p65	Forward	5` - TCCCTGGAGAGAAGAGCAAC- 3′
	Reverse	5` - CAGTCTTTTCCCACCAGCTC - 3′
I-kBα	Forward	5` - TTTCGGAGGAGATGGAGAGA - 3′
	Reverse	5` - CTGTTCAGGTACGGGTCGTT - 3′
COX-2	Forward	5` - AGCCCTACTCATCCTTTGAGG - 3′
	Reverse	5` - TCAACCTTGTCTACGTGACCATA - 3′
β-actin	Forward	5` - AATCTTGCGGTATCCACGAGACCA - 3′
	Reverse	5` - TCTCCTTCTGCATCCTGTCAGCAA - 3′

2.2. Palmitate preparation

A stock solution of palmitic acid (Sigma, St. Louis, MO) was prepared by conjugating palmitic acid with fatty acid-free BSA (Sigma, St. Louis, MO) as reported previously [8]. In brief, palmitic acid was dissolved in pre-heated 0.1N NaOH at 60 °C and diluted 1:10 in pre-warmed 12% BSA solution to give a final concentration of 10 mM. Control media contained 0.1N NaOH and BSA without palmitate.

2.3. Preparation of diets

For feeding experiment, Mini-bit flake (Aquaplus, Ltd., Korea) was ground and mixed with 0.03%, 0.06%, and 0.12% of palmitate, respectively. Control diet was used as Mini-bit flake. One month old 10 zebrafish were randomly divided into four groups in individual tank. The diets were fed to zebrafish at twice a day. Two weeks later, zebrafish were anaesthetized using 2-phenoxy ethanol (Sigma, St. Louis, MO) and liver and pancreas were isolated and used for the experiment.

2.4. Treatment of zebrafish embryos with palmitate

At 3 dpf, embryos (n = 25) were transferred to 12-well plates and maintained in 1 ml of embryo media (0.003% sea salt, 0.0075% calcium sulfate) Embryos were incubated in the presence of palmitate (0, 0.1, 0.5, 1, 2, or 5 mM) for 24 or 55 h, thereafter the embryos were rinsed in embryo media and anaesthetized using 2-phenoxy ethanol before experiments.

2.5. Measurement of heart rates

The heart rates of both atrium and ventricle were measured as previously reported [9] at the end of palmitate treatment. Counting and recording of atrial and ventricular contractions were performed for 3 min under a microscope (SZX7, Olympus, Japan), and results were presented as the average heart rate per min.

2.6. Estimation of intracellular reactive oxygen species (ROS) and nitric oxide (NO)

Intracellular ROS production using dichloro-dihydro-fluorescein diacetate (DCFH-DA) (Invitrogen, Carlsbad, CA) as a fluorescent probe and NO production using 3-amino, 4-aminomethyl-2′,7′-difluoro-fluorescein diacetate (DFAM-DA; Sigma, St. Louis, MO) as a probe on zebrafish were examined as previously described [10]. Briefly, the zebrafish embryos were incubated with 10 μM of DCFH-DA or 5 μM DAFMDA for 10 min after treatment of palmitate. The fluorescence image was observed using a fluorescence microscope (MVX10, Olympus, Japan). To quantitatively evaluate the fluorescent images, the RGB image was analyzed by ImageJ software (http://rsb.info.nih.gov/ij) and the mean value was used to obtain the bar graph.

2.7. qRT-PCR

Total RNA was extracted from zebrafish embryos, adult zebrafish liver and pancreas using RNAiso plus (Takara Bio Inc., Japan), and cDNA was prepared using a PrimeScriptTM cDNA synthesis kit (Takara Bio Inc., Japan) according to the manufacturer's instructions. cDNA samples were analyzed by the SYBR® Premix Taq™, ROX plus (Takara Bio Inc., Japan) on Bio-Rad cyclers (Hercules, CA). Gene expression was normalized to the endogenous housekeeping control gene, β-actin, which was not influenced by palmitate. Relative expression was calculated for each gene using the Δ Δ CT (where CT is the threshold cycle) method. The primer sequences used are listed in Table 1.

2.8. Statistical analysis

All measurements were made in triplicate and all data are represented as mean ± S.E. The results were subjected to an analysis of variance using the Tukey test to analyze the differences among treatments. Values of $p < 0.05$ were considered significant.

3. Results

3.1. Palmitate induced toxicity in zebrafish embryos

First, to determine palmitate toxicity in zebrafish embryos, 0.5, 1, 2, or 5 mM of palmitate was used. As expected, palmitate showed toxicity in a dose-dependent manner (Fig. 1A). When the zebrafish embryos were treated with 2 or 5 mM of palmitate, embryos did not survive to the end of experiment (24 h incubation). Treatment with 0.5 and 1 mM of palmitate did not affect survival (**data not shown**), however pericardial edema was observed in embryos after long-term exposure (55 h

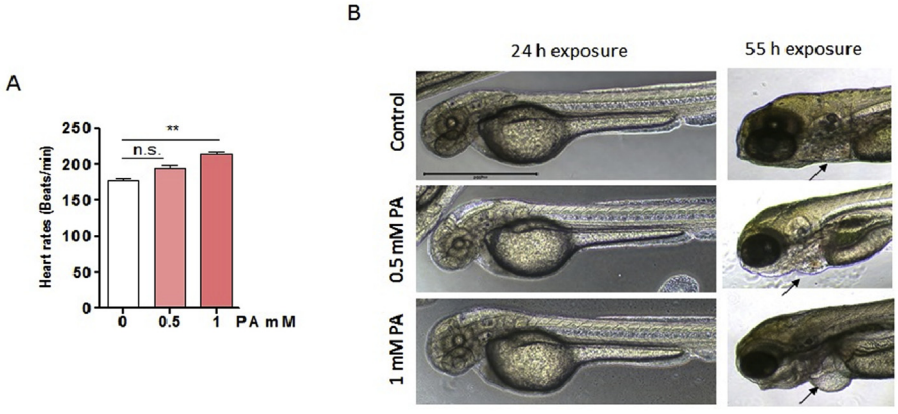

Fig. 1. Palmitate induced toxicity in zebrafish embryos. At 3 days post-fertilization, zebrafish embryos were incubated with palmitate. A. Zebrafish embryos were incubated with the indicated concentrations of palmitate for 24 h. The heart rates were measured at 4 days post-fertilization, the number of heart beats in 3 min was counted, and the results are expressed as the beats/min. B. Zebrafish embryos were incubated with the indicated concentrations of palmitate for 24 h or 55 h. Phase contrast images of zebrafish embryos. Arrow indicates pericardia. PA: palmitic acid. Scale bar: 500 μm n = 20–27 embryos. $^{**}p < 0.01$, n.s. indicates no significance.