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Palmitic acid increases apoptosis of neural stem cells *via* activating c-Jun N-terminal kinase

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Abstract Elevated plasma free fatty acid (FFA) level is common in many pathological conditions, including neurological disorders, and their deleterious effects on various cells have been well documented. However, it remains to be investigated whether elevated FFAs would have a direct effect on neural stem cells (NSCs). Here, we reported that palmitic acid (PA) impaired cell viability and increased apoptosis of NSCs significantly in a dose- and time-dependent manner. Increased protein levels of Bax and cleaved caspase 3 coupled with decreased expression of Bcl-2 were also observed in NSCs with increasing dose or time of PA treatment, whereas caspase 3 expression remained relatively unaltered. In parallel to this, the expression of phospho-c-Jun N-terminal kinase (p-JNK) in NSCs challenged with PA was increased significantly; however, JNK expression appeared stable. Remarkably, JNK inhibitor effectively reduced the apoptosis of NSCs induced by PA. The expression of phospho-p38 (p-p38), p38,

Abbreviations: BSA, bovine serum albumin; DAPI, 4′, 6-diamidino- 2-phenylindole; E13.5, embryonic day 13.5; ERK1/2, extracellular regulated protein kinase 1/2; FFAs, free fatty acids; GFAP, glial fibrillary acidic protein; JNK, c-Jun N-terminal kinase; MAP2, microtubule-associated protein 2; MAPKs, mitogen-activated protein kinases; MTT, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide; NSCs, neural stem cells; PA, palmitic acid; p-ERK1/2, phospho-extracellular regulated protein kinase 1/2; p-JNK, phospho-c-Jun N-terminal kinase; p-p38, phospho-p38; SA, stearic acid; TUNEL, terminal deoxynucleotidyttransferase-mediated dUTP nick ending labeling.

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phospho-extracellular regulated protein kinases 1/2 (p-EKR1/2) and EKR1/2 in NSCs was not affected by PA treatment. In consideration of the above, it is suggested that elevated plasma FFA level may induce apoptosis of NSCs *in vivo*, and that this might be one of possible underlying mechanisms for the cognitive disturbance in neurological disorders.

Introduction

Free fatty acids (FFAs), including saturated and unsaturated FFAs, are the major source of lipid energy in human body and released mainly from adipose tissue by lipolysis of triglycerides (Miles and Nelson, 2007). FFAs circulating in the plasma are largely bound to the albumin, with a concentration of ~0.1 to 1.0 mM. Lipotoxicity follows when non-adipose cells are exposed to chronic elevation of FFAs, which can result from disturbed balance between release and clearance of FFAs, *e.g.*, on a high fat diet or under other metabolic conditions (Mittendorfer, 2011; Boden, 2008). Besides fatty acids trafficking alterations, high level of FFAs may also be a consequence of membrane phospholipid degradation caused by activated phospholipases in many pathological conditions, such as trauma, hypoxia and stroke (Farooqui and Horrocks, 1998). It has been reported that FFAs accumulated acutely after traumatic brain injury, with the concentration of palmitic acid (PA) in the brain increasing from ~60 to 180 μM and stearic acid (SA) from ~50 to 350 μM (Lipton, 1999). Moreover, elevated plasma FFA level can inhibit insulin's anti-lipolytic action which further increases the release of FFAs into circulation (Boden, 2008).

Pathological accumulation of saturated FFAs in the human body can pose serious threat to normal cellular homeostasis, which has been suggested to be involved in the development of many chronic diseases, such as obese, diabetes and cardiovascular diseases (Gomez-Lechon et al., 2007; Saunders et al., 2008). Importantly, brain could uptake FFAs from plasma through the blood–brain barrier (Wang et al., 1994). In other words, elevated FFA level can exacerbate cellular damage directly on brain, leading to different extent of cognitive decline and brain abnormalities (Adibhatla and Hatcher, 2008). In addition, excessive saturated FFAs could freely penetrate placental barrier, thereby interfering with the development and growth of embryos, including the development of brain (Yu et al., 2009; Elahi et al., 2009; Tozuka et al., 2009). These data suggest that FFAs can readily enter and exert their destructive effects on the brain.

There is growing evidence indicating that the deleterious effects of saturated FFAs on the brain have been associated with alterations of its cells. Cell death was obvious when nerve growth factor-differentiated PC12 cells were exposed to FFAs, such as SA and PA (Ulloth et al., 2003; Almaguel et al., 2009). FFAs exerted Alzheimer-like pathological effects on neurons through their actions on primary rat cortical astroglia (Patil et al., 2007). Neural stem cells (NSCs), residing in both fetal and adult brain, have been suggested to differentiate into various kinds of neurons and astrocytes and play important roles in the development and injuries repair of the brain (Temple, 2001; Alvarez-Buylla et al., 2002; Kim, 2004). Many factors have been shown to exert their effects on NSCs, influencing their proliferation, apoptosis and differentiation (Gao and Gao, 2007; Anthony et al., 2008). However, there is an apparent lack of information about the effects of saturated FFAs on NSCs. In view of this, it is desirable to explore the effects of elevated saturated FFAs on NSCs, as this might offer a possible explanation for the cognitive alterations and brain abnormalities of neurological disorders due to elevation of FFAs. In the present study, we have employed suspension culture to obtain mouse NSCs *in vitro* in the form of neurospheres. We next investigated the impact of PA, the most abundant saturated fatty acid in the diet or in the plasma, on NSCs *in vitro* with an aim to gain a further understanding of the underlying molecular mechanism of its effects on the stem cells.

Materials and methods

All experiments were carried out in accordance with "the National Institute of Health Guide for the Care and Use of Laboratory Animals revised 1996", which is also adopted by the Laboratory Animal Center, Shandong University. All efforts were made to minimize the pain and suffering of mice during all the procedures.

Primary culture of embryonic NSCs

Embryos of Kung Ming mouse (purchased from Laboratory Animal Center, Shandong University) at E13.5 were collected by Cesarean section of pregnant mice, and then the telencephalons of the embryos were isolated and mechanically disrupted into single cells by repeated pipetting in a serum free DMEM/F12 (1:1) medium (Invitrogen, Carlsbad, CA, USA) for three times. After being filtered, cell number was counted and cells were seeded at 5×10^5 cells/ml in DMEM/F12 (1:1) medium supplemented with 2% B27 (Gibco, Gaithersburg, MD, USA), 20 ng/ml basic fibroblast growth factor (R&D, Minneapolis, MN, USA), 100 U/ml penicillin (Sigma-Aldrich, St. Louis, MO, USA) and 100 μg/ml streptomycin (Sigma-Aldrich) in a humidified atmosphere of 5% CO_2 and 95% air at 37 °C. After 5 days of culture, neuropheres were harvested by centrifugation, dissociated using trypsin and EDTA (Sigma-Aldrich), and re-seeded for following experiments.

Characterization of NSCs

For examination of NSC specific marker, neurospheres were seeded on coverslips and cultured for 24 h before fixation. For proliferation of NSCs, dissociated NSCs were seeded on coverslips and cultured for 3 days and supplemented with BrdU 4 h before fixed. To examine the differentiation of NSCs, dissociated NSCs were seeded on coverslips, added with differentiation medium (containing 2% fetal bovine serum without growth factors) and cultured for 5 days. After fixation, cells on coverslips were incubated with mouse anti-Nestin monoclonal antibody (1:500; Millipore, Billerica, MA, USA), mouse anti-BrdU monoclonal antibody (1:1000;