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Effects of Krill-derived phospholipid-enriched n − 3 fatty acids on Ca^{2+} regulation system in cerebral arteries from ovariectomized rats

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ABSTRACT

Aims: To investigate the effects of n − 3 polyunsaturated fatty acids on cerebral circulation, ovariectomized (OVX) rats were administered with phospholipids in krill oil (KPL) or triglycerides in fish oil (FTG); effects on the Ca^{2+} regulating system in their basilar artery (BA) were then analyzed.
Main methods: The rats were divided into 4 groups: control, OVX, OVX given KPL (OVXP), and OVX given FTG (OVXT) orally, daily for 2 weeks. Time dependent relaxation (TDR) of contractile response to 5HT in BA was determined myographically, Na^+/Ca^{2+} exchanger (NCX) 1 mRNA expression was determined by real time PCR, and nucleotides were analyzed by HPLC.
Key findings: The level of TDR in OVX that was significantly lower in the control was inhibited by L-NAME and indomethacin; TEA inhibited TDR totally in the control but only partly in OVXP and OVXT. Relaxation induced by the addition of 5 mM KCl to the BA pre-contracted with 5-HT was inhibited by TEA in the controls, OVXP and OVXT, but not in OVX. Overexpression of NCX1 mRNA in the BA from OVX was significantly inhibited by FTG. The ratio of ADP/ATP in cerebral arteries from OVX was significantly inhibited by KPL and FTG. Levels of triglyceride and arachidonic acid in the plasma of OVX increased, but were significantly inhibited by KPL and FTG.
Significance: Ovarian dysfunction affects Ca^{2+} activated-, ATP-sensitive-K^+ channels and NCX1, which play crucial roles in the autoregulation of cerebral blood flow. Also, KPL may become as good a supplement as FTG for postmenopausal women.

Introduction

Cerebrovascular dysfunction is more common in postmenopausal than premenopausal women, suggesting the vascular protective effects of estrogen (Serock et al., 2008; Stefanick, 2010). The actions of estrogen on the brain include increased cerebral blood flow (Duckles and Krause, 2007), promotion of endothelium-dependent relaxation by increasing nitric oxide, prostacyclin, and hyperpolarizing factor, and inhibition of the mechanisms of vascular smooth muscle contraction including intracellular Ca^{2+}, protein kinase C (Reslan and Khalil, 2012) and lipid profile (Pines et al., 2002). Because changes in cerebrovascular functions contribute to the pathogenesis of stroke (Li et al., 2011), estrogen presents a potential treatment for cognitive decline in women with dementia syndromes, such as Alzheimer's disease (Sano et al., 2008). A better understanding on the action of estrogen on cerebrovascular function holds promise for the development of new therapeutic entities that could be useful in preventing or treating a wide variety of cerebrovascular diseases. Controversy surrounding the use of hormone therapy for cardiovascular and neuronal health has contributed to the decline in its post-menopausal use (Kelly et al., 2005; Speroff, 2010). Many women now use alternative therapies for postmenopausal health including dietary soy and isoflavone supplements instead of, or in addition to, traditional hormone therapy (Newton et al., 2002; Kurzer, 2003). Moreover, n − 3 polyunsaturated fatty acids (PUFAs) such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) have been suggested as being involved in the development and maintenance of learning memory performance (Gamoh et al., 1999; Hashimoto et al., 2009a, 2011; Dyall et al., 2010; Boucher et al., 2011). The n − 3 PUFAs ameliorated endothelial dysfunction in diabetic rats (Matsumoto et al., 2009), and led to attenuation of the contractile responses of isolated resistance arteries (MacLeod et al., 1994).

Fish oil typically contains n − 3PUFAs in the form of triglycerides (TG) or as fatty acid ethyl esters (Harris et al., 1988). Fish intake and ingestion of EPA, DHA and in some cases alpha-linolenic acid (ALA) have been associated with reduced risk of cardiovascular events and death

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(Erkkilä et al., 2006). Antarctic krill (*Euphausia superba*) is a shrimp-like crustacean rich in both EPA and DHA (Gamoh et al., 2011). Knowledge of any interaction between n − 3 PUFAs and estrogen would provide better strategies for the development and maintenance of brain circulation and learning memory. The contractile response of the intact basilar artery (BA) to 5-HT comprises a phasic contraction followed by a time-dependent relaxation (TDR) (Enkhjargal et al., 2010). The endothelium-dependent TDR includes nitric oxide (NO)- and cyclooxygenase-independent components, which is related to K^+ channel pathways and Na^+ pump activity in BA (Enkhjargal et al., 2010). The effects of estrogen deficiency on TDR in cerebral circulation is not well understood, particularly with regard to the roles of K^+ channels and the Na^+/Ca^{2+} exchanger (NCX). The aim of this study was to determine whether a combined treatment with EPA and DHA, as provided by phospholipids in krill oil (KPL) or by triglycerides in fish oil (FTG), has a beneficial effect on the Ca^{2+} regulating system in BA isolated from ovariectomized (OVX) rats.

Materials and methods

Animals and diet

All rats were handled and killed in accordance with the procedures outlined in the Guidelines for Animal Experimentation of Shimane University, as compiled from the Guidelines for the Animal Experimentation of Japanese Association for Laboratory of Animal Science. Wistar rats purchased from CLEA Japan (Osaka, Japan) were housed in a room under the following controlled environmental conditions: 23 ± 2 °C, 50 ± 10% relative humidity, 12/12 h light/dark cycle, 13–15 cycles of air exchange/h, and given food and water ad libitum. The animals were provided with a fish-oil-deficient diet (F-1 TM; Funabashi Farm, Funabashi, Japan). Inbred second-generation female rats fed the same F-1 diet were used in the study (Gamoh et al., 2011).

KPL obtained from Nippon Suisan Co. Ltd. (Tokyo, Japan) was weighed and emulsified daily in twice its volume with sterilized water. The fatty acid composition of the KPL emulsion was as described previously (Gamoh et al., 2011). The rats were divided into 4 groups: Sham (Control), ovariectomized (OVX), OVX treated with KPL (182 mg EPA + 118 mg DHA; OVXP), and OVX treated with FTG (203 mg EPA + 97 mg DHA; OVXT). Control and OVX rats received sterilized water only. All the rats were provided with F-1 TM.

Blood sample preparation

The rats were anesthetized with diethyl ether and exsanguinated from the inferior vena cava with heparinized syringes. Blood samples were collected into polyethylene tubes and centrifuged for 20 min at 3000 rpm at 4 °C to separate the platelet-poor plasma, as previously described (Hashimoto et al., 2009a).

Fatty acid composition of plasma and fat tissue

Analysis of fatty acid levels in plasma was carried out with a modified one-step reaction as reported by Lepage and Roy (1986) and described previously (Hashimoto et al., 1999). Gas chromatograph separation was done on a Model 5890 I1 gas chromatograph (Hewlett-Packard, Avondale, PA, USA) equipped with a flame ionization detector and an automatic sampler (Model 7673).

Basilar artery ring preparation and organ bath setup

The rats were anesthetized with diethyl ether, the whole brain quickly removed, and the basilar artery (BA) isolated from the brain was gently cleaned of any connective tissue in Krebs–Henseleit buffer (KHB), containing 118 mM NaCl, 4.5 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.5 mM $MgSO_4$, 25 mM $NaHCO_3$, and 11 mM glucose, pH 7.4, and bubbled with 95% O_2/5% CO_2, then cut into 2 ring preparations (each 2.5–3.0 mm long) under a dissecting microscope. The cleaned preparations were placed in an organ chamber (UFER, Medical Kishimoto, Japan) containing KHB at 37 ± 0.5 °C. Two fine tungsten wires (Ø 50 μm) were then passed through the lumen of the basilar artery, with one end of each wire connected to an isometric transducer (T7-8-240, ORIENTEC, Tokyo, Japan) and the other attached to the holder; the isometric tension was then recorded on a polygraph (RECTIGRAPH-8 K, San-ei, Tokyo, Japan). The experiments were monitored by a computer-based analysis system in Mac-Lab and Chart 4.1 software (AD Instruments, Inc., Colorado Springs, Co, USA) as described previously (Enkhjargal et al., 2010).

Quantitative RT-PCR for NCX1 and TREK-1

Total mRNA from the rat basilar artery was prepared using an RNeasy kit (QIAGEN) according to the manufacturer's instructions. RNA (0.5 μg) was reverse transcribed to cDNA by QuantiTect® Reverse Transcription (QIAGEN), and real-time PCR reactions were carried out with the use of QuantiTect®S YBR® Green PCR (QIAGEN) on a 7500 Fast Real-Time PCR System (Applied Biosystems). Gene specific primer sets were purchased from Sigma Genosys. GAPDH housekeeping genes were used as internal controls to normalize mRNA expression. The primers used were NCX1 forward: 5′-CTCACCATTATTCGAAGAGG-3′, reverse: 5′-CCAGGTTTGAAGATCACAGT-3′ (Iwamoto et al., 2007). TREK-1 forward: 5′-CCCCTCTTTGGTTTTCTACT-3′, reverse: 5′-CGAGAT GATACGAATCTTGG-3′; and GAPDH forward: 5′-AACGACCCCTTCATTG AC-3′, reverse: 5′-TCCACGACATACTCAGCAC-3′ (Blondeau et al., 2007).

Analysis of nucleotides in cerebral vascular tissue

Mixed cerebral arteries were homogenized with citrate buffer and centrifuged for 15 min at 3000 rpm at 4 °C. The supernatant was processed for the determination of ATP, ADP, AMP and adenosine by high performance liquid chromatography (HPLC) with fluorescence detection (Hashimoto et al., 1999).

Drugs

Nω-nitro-L-arginine methyl ester hydrochloride (L-NAME), 5-Hydroxytryptamine (5-HT), and tetraethylammonium chloride (TEA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The drugs dissolved in distilled water were stocked as 1–10 mM aliquots. All chemicals and materials of the highest grade available commercially were used at the following final concentrations: 5-HT (100 nM); TEA (1 mM), a non-selective Ca^{2+}-activated K^+ channel (K_{Ca}) blocker; L-NAME (100 μM), a non-selective NO synthase inhibitor; indomethacin (10 μM), a non-selective cyclooxygenase inhibitor.

Statistical analysis

Results are expressed as means ± S.E.M. All parameters were analyzed by one-way ANOVA with Bonferroni multiple comparison student t tests. $P < 0.05$ was considered statistically significant.

Results

Characteristics of experimental rats

Body and uterine weight characteristics of the 4 groups of rats are shown in Table 1. The mean body weight of the rats stood at 137.7 ± 4.7 g and was not significantly different among the 4 groups before ovariectomy. Two weeks after ovariectomy, however, it differed between the control and the OVX, OVXP, OVXT rats. Two weeks after the treatment of OVX rats with either KPL or FTG, their body weight