Original Article
Docosahexaenoic acid protects neuronal and vascular of retinal ganglion cells from retinal ischemia and reperfusion injury in rats

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Abstract: Studies have shown that docosahexaenoic acid can dilate blood vessels, regulate blood pressure and improve tissue perfusion to play a role in the cardiovascular system protection. In the present study, we examined the effect of docosahexaenoic acid prevents neuronal and vascular of retinal ganglion cells after retinal ischemia and reperfusion (RI/R) injury in rats and to investigate underlying mechanisms of the drug effects. RI/R injury model was established by elevating the intraocular pressure for 60 min. RI/R injury rats were treated with docosahexaenoic acid for 100 mg/kg. Pre-treatment using docosahexaenoic acid in diets significantly inhibited retinal I/R induced capillary degeneration. Next, docosahexaenoic acid pre-treatment inhibited NF-κB activation, STAT5 and MCP protein expressions after RI/R injury in rats. Moreover, docosahexaenoic acid administered 2 days after the injury also showed caspase-3 activation, the protein expression of MAPK, Bcl-2 and Bax expression in rats with retinal RI/R injury. Overall, our findings suggest that docosahexaenoic acid protects retinal neurons and vascular RI/R injury in rats. The beneficial effects of docosahexaenoic acid on neurovascular degeneration may occur through anti-inflammation and anti-apoptosis in RI/R injury rats.

Keywords: Docosahexaenoic acid, retinal ischemia and reperfusion, neuronal, vascular

Introduction

In retinal ischemia reperfusion (RIR) injury, a series of metabolic and structural changes occur in the retinal tissue, resulting in the damage to the retinal nerve tissue, thus causing permanent vision loss [1]. RIR injury can be seen in diabetic retinopathy, glaucoma, central retinal (leg) vein occlusion, ischemic optic neuropathy and retinopathy of premature children [2]. It is generally considered that the measure for saving retinal ischemia is to restore retinal blood reperfusion, and a large number of experiments show that early reperfusion is very useful for the recovery of retinal function [3]. However, with the deepening of research, many researchers have observed significant retinal dysfunction does not occurs after a certain period of ischemia, but after the realization of reperfusion, there is significant dysfunction, and even irreversible damage to structure and function can occur [4]. Retinal damage occurs after ischemia and the injury has been aggravated rather than abated after the realization of blood reperfusion [5].

Docosahexaenoic acid and eicosapentaenoic acid belong to polyunsaturated fatty acid (PUFA), which are difficult to synthesize in human body, and need essential fatty acids provided by food [6]. They are closely related to the body's physiological function, which can maintain the normal function and growth of brain, retina and so on, with the effects such as platelet aggregation inhibition, anti-thrombosis, lipid regulation, immunity improvement, intelligence promotion and others, and can inhibit inflammation, some cancers and diabetes effectively [7-9]. Now they are widely used in all kinds of health care products, milk, dairy products and various bakery products [10].
Materials and methods

Adult male Wistar rats (230-280 g) were obtained from the Linyi People's Hospital Laboratory, and housed in standard environmental conditions of light (a 12/12 h light/dark cycle), temperature (22 ± 2°C), 50 ± 10% humidity with free access to water and food. Briefly, an infusion line of normal saline was attached using a 27-gauge needle from the anterior chamber cannulated. Briefly, a pressure infuser (Infusurg, Ethox Corp., Buffalo, NY) was used to measure pressure of the eye. After ischemia 60 min, the needle was withdrawn, and reflow of the retinal circulation was immediately reflowed.

Grouping

Adult male Wistar rats randomly allocated into three groups: (1) sham group, (2) retinal ischemia-reperfusion group (Model group) and (3) docosahexaenoic acid group. In sham group, Wistar rats were received injection of normal saline (0.1 ml/100 g). In Model group, retinal ischemia-reperfusion rats were received injection of normal saline (0.1 ml/100 g). In docosahexaenoic acid group, retinal ischemia-reperfusion rats were received injection of 100 mg/kg body weight of docosahexaenoic acid for 90 days [11].

Isolation of retinal vasculature and quantitation of degenerate capillaries

Eyes were fixed with 10% neutral buffered formalin, retinas were isolated and steeped using water overnight, and then incubated with 3% Difco crude trypsin (Sangong Biotech, Shanghai, China) at 37°C for 2 h. Non-vascular cells were washed with normal saline from the vasculature in rat. Then, the vasculature was peeled and periodic acid-Schiff and hematoxylin (PASH) was used to stain. The vasculature stained was putted into the middle under 200 × magnification. ImagePlus 6.0 software (Media Cybernetics, Bethesda, Maryland) was used to quantitate and identify acellular (degenerated) capillaries using capillary-sized vessel tubes (.30% diameter of regular capillary).

Examination of NF-κB activation and oxidative stress

Blood samples were extracted and serum samples centrifuged at 3000 rpm for 25 min. Nuclear factor-kappaB (NF-κB), methane dicarboxylic aldehyde (MDA) and superoxide dismutase (SOD) activations were measured by Luminex xMAP technology (Bioplex-200 system; Bio-Rad Laboratories, USA).

Examination of caspase-3 activation

Retinas tissue samples were isolated, homogenated with RIPA buffer (Beyotime Biotech, China) and centrifuged at 12,000 g for 10 minutes at 4°C. Protein concentration was determined with the BCA protein assay (Beyotime Biotech). The lysis buffer prior and equivalent protein were blended and used to analyze caspase-3 activation for 37°C for 30 min. Then miscible liquids were incubated with Ac-DEVD-pNA (2 mM) at 37°C for 4 h.

Western blot analysis of MMP-9, HO-1 and Nrf-2

Retinas tissue samples were isolated, homogenated with RIPA buffer (Beyotime Biotech, China) and centrifuged at 12,000 g for 10 minutes at 4°C. Protein concentration was determined with the BCA protein assay (Beyotime Biotech). Equivalent protein was separated by SDS-PAGE and transferred to PVDF membrane (Millipore). The membranes were then stained with 5% non-fat milk to Tris-buffered saline. After blocked, the membranes were incubated with anti-MMP-9 (1:5000 dilution, Beyotime Biotech), anti-HO-1 (1:2000 dilution, Beyotime Biotech), anti-Nrf-2 (1:2000 dilution, Beyotime Biotech) and β-actin (1:5000 dilution, Beyotime Biotech) overnight at 4°C. All blots were washed with respective horseradish peroxidase coupled secondary antibodies (1:5000, Bio-Rad). After washing, protein bands detected by the antibodies were visualized by ECL reagent followed by exposure on X-OMAT film.

Statistical analysis

Data are expressed as mean ± standard error of the mean (SEM) and analyzed by the non-
parametric Kruskal-Wallis test followed by the Mann-Whitney test. Differences were considered significant when $P<0.05$.

**Results**

**Docosahexaenoic acid inhibited retinal I/R induced capillary degeneration**

The chemical structure of docosahexaenoic acid (Sigma-Aldrich Co. LLC, USA) was indicated in Figure 1. Retinal I/R effectively induced capillary degeneration in rat, compared to sham group (Figure 2). But, docosahexaenoic acid effectively reduced the retinal I/R-induced capillary degeneration, compared to retinal I/R model group (Figure 2).

**Docosahexaenoic acid inhibited the NF-κB activation after retinal I/R injury**

We investigated whether docosahexaenoic acid inhibits the NF-κB activation after retinal I/R injury. As shown in Figure 3, retinal I/R availably induced the NF-κB activation in rat, compared to sham group. However, treatment with docosahexaenoic acid availably inhibited the retinal I/R-induced NF-κB activation in rat, compared to retinal I/R model group (Figure 3).

**Docosahexaenoic acid inhibited oxidative stress after retinal I/R injury**

We investigated whether docosahexaenoic acid inhibits the MDA and SOD activations after retinal I/R injury. The MDA activity was observably increased and the SOD activity was observably decreased by retinal I/R, compared to sham group (Figure 4). Nevertheless, pretreatment with docosahexaenoic acid markedly inhibited the retinal I/R-induced MDA activity and inhibited SOD activity (Figure 4).

**Docosahexaenoic acid inhibited caspase-3 after retinal I/R injury**

To explore anti-apoptotic effect of docosahexaenoic acid on retinal I/R injury, we measured caspase-3 activity. The retinal I/R-induced caspase-3 activity was observed in retinal I/R rat, compared to sham group (Figure
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Moreover, administrate of docosahexaenoic acid signally weakened the retinal I/R-induced caspase-3 activity, compared to retinal I/R model group (Figure 5).

**Docosahexaenoic acid induced MMP-9 after retinal I/R injury**

To search anti-protective effect of docosahexaenoic acid on retinal I/R injury, we measured MMP-9 protein expression in retinal I/R rat. Compared to sham group, the MMP-9 protein expression was significantly suppressed in retinal I/R injury rat (Figure 6). Figure 6 showed that supplement with docosahexaenoic acid significantly promoted the MMP-9 protein expression in retinal I/R rats (Figure 6).

**Docosahexaenoic acid induced HO-1 after retinal I/R injury**

To search anti-protective effect of docosahexaenoic acid on retinal I/R injury, we measured HO-1 protein expression in retinal I/R rat. As shown in Figure 7, retinal I/R injury significantly reduced in rat, compared to sham group. As shown in Figure 7, docosahexaenoic acid significantly enhanced the decrease HO-1 protein expression in retinal I/R rats (Figure 7).

**Docosahexaenoic acid induced Nrf-2 after retinal I/R injury**

In order to test the protective effect of docosahexaenoic acid on retinal I/R injury, we measured Nrf-2 protein expression in retinal I/R rat. The down-regulated of Nrf-2 protein expression was significantly decreased in retinal I/R rats, compared to sham group (Figure 8). Specially, docosahexaenoic acid could significantly activate the down-regulated of Nrf-2 protein expression in retinal I/R rats (Figure 8).

**Discussion**

RIR is associated with the development of many eye diseases, such as retinal vascular...
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Figure 6. Docosahexaenoic acid induced MMP-9 after retinal I/R injury. Docosahexaenoic acid induced MMP-9 protein expression using Western blot analysis (A) and statistical analysis of MMP-9 protein expression (B) after retinal I/R injury. Sham, sham group; Model, retinal I/R model group; Treated, docosahexaenoic acid treated group. **P<0.05 versus sham group; ***P<0.05 versus sham group.

Figure 7. Docosahexaenoic acid induced HO-1 after retinal I/R injury. Docosahexaenoic acid induced HO-1 protein expression using Western blot analysis (A) and statistical analysis of HO-1 protein expression (B) after retinal I/R injury. Sham, sham group; Model, retinal I/R model group; Treated, docosahexaenoic acid treated group. **P<0.05 versus sham group; ***P<0.05 versus sham group.

Figure 8. Docosahexaenoic acid induced Nrf-2 after retinal I/R injury. Docosahexaenoic acid induced Nrf-2 protein expression using Western blot analysis (A) and statistical analysis of Nrf-2 protein expression (B) after retinal I/R injury. Sham, sham group; Model, retinal I/R model group; Treated, docosahexaenoic acid treated group. **P<0.05 versus sham group; ***P<0.05 versus sham group.

occlusive disease, neovascular age-related macular degeneration, proliferative diabetic retinopathy and glaucoma, all of which can lead to severe visual impairment and even RIR mechanisms including oxidative stress, inflammation and immune response, apoptosis and necrosis neuronal and glial cell activation [2, 5, 12]. Most pathological changes above are interrelated, and such a link forms vicious cycle in ischemia to increase the damage and lead to poor prognosis [13]. For example, the metabolites of oxidative stress can promote nerve cell death [14]. In the OGD injury model, the applications NF-κB selective blocker and NADPH oxidase inhibitors in vitro can reduce the loss of ganglion cells [15]. Many studies on obstructive disease show oxygen plays an important role in ischemia model, so the treatment of anti-oxida-
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tive stress is particularly important for inhibiting oxidative stress development and protecting the retinal cells [16]. Oxidative stress refers to the imbalance between the formed reactive oxygen species and the scavenging capacity of antioxidant endogenous substances for reactive oxygen species, and excessive reactive oxygen species are important cytotoxin for pigment cells apoptosis [17]. Most of apoptotic cells are located in the inner layer of retina, such as nuclear layer, inner plexiform layer and ganglion cell layer [18]. In this study, we found that docosahexaenoic acid significantly reduced the retinal I/R-induced capillary degeneration, NF-κB activation and oxidative stress in retinal I/R rats through anti-apoptotic-mediating caspase-3 activity. Hong et al. reported that docosahexaenoic acid therapy alleviates focal cerebral ischemia through suppression of inflammation in rats [19]. Kielar et al. demonstrated that docosahexaenoic acid meliorates acute renal ischemic failure through inflammation in mice [8]. Yang et al. indicated the effect of docosahexaenoic acid suppressed hippocampal neurons inflammatory through involvement of PI3K/AKT/nuclear factor-κB, oxidative stress and decreasing caspase-3 and caspase-9 expression [7]. These results suggested that the effects of docosahexaenoic acid are neuronal protective effect for retinal I/R rats.

Matrix metalloproteinase (MMPS) is a zinc-dependent endogenous peptide enzyme [20]. Matrix metalloproteinase, especially MMP-9, can break the connection between cells and matrix, thus undermining the integrity of cerebral blood flow barrier and leading to neuronal cell death [20]. It is found that MMP-9 level is elevated in the patients with ischemic or hemorrhagic stroke blood equipment, which further supports the effect of MMP-9 in the development of stroke [21]. The integrity of extracellular matrix is essential for neuronal homeostasis. Elevated MMP-9 and reduced number of survival ganglion cells can be found in the injuries of many animal models including RIR and glaucoma [20, 22]. Therefore, ischemia can lead to elevated level of MMP-9, and thus MMP-9 promotes the ganglion cell damage. In the present study, docosahexaenoic acid significantly activated the MMP-9 protein expression in retinal I/R rats. Perez et al. suggested that docosahexaenoic acid alters pregnant through increasing of MMP-2 and MMP-9 in rat [9]. Velten et al. reported that docosahexaenoic acid attenuates inflammation and enhances pulmonary function through MMP-9 in a newborn mouse model of perinatal inflammation [23].

Currently there are three known isoforms of HO [24]. HO-1 is induced by oxidation stress or hypoxemia isoforms [24]. HO-2 is a basic isoform expressed under the condition of dynamic equilibrium [25]. HO-1 and HO-2 are ubiquitously expressed with catalytic activity [25]. A third isoform, HO-3 has no catalytic activity but is regarded with oxygen sensitivity. HO-1 can generate CO, iron, and biliverdin by degrading heme, to realize potential indirect antioxidant effect [26]. Furthermore, these intermediary products play an important role in cell metabolism, oxidative stress alleviation and other aspects [27]. The generation of HO-1 and its metabolites by induction can achieve protective effect for many diseases, such as sepsis, wart disease, endogenous toxic shock, organ transplant rejection, type 2 diabetes and obesity [28]. NF-E2-related factor-2 (Nrf-2) is an anti-oxidation protection cell transcription factor [29]. When oxidation damage occurs, HO-1 expression level has also been increased as a downstream factor Nrf-2 [29]. After light stimulation on photoreceptor cells, overexpression of HO-1 can protect them from secondary damage [30]. Studies in the eyes of human retinal epithelial cells have shown that HO-1 is reduced with age. Related protective agent can fight against oxidative stress and reduce the generation of reactive oxygen species by increasing the expression level of HO-1 [28]. After RIR, the increase of HO-1 expression level is considered as endogenous self-protection mechanism [28]. This explains why HO-1 is a representative antioxidant cell protection medium, and explains the effect against the oxidation stress injury caused by ischemia [30]. Our results suggest docosahexaenoic acid significantly induced HO-1 and Nrf-2 protein expression in retinal I/R injury. Furthermore, Kusunoki et al. indicated that docosahexaenoic acid has an anti-oxidant effect via the Nrf-2/HO-1 pathway in 3T3-L1 adipocytes [10]. Wang et al. reported that docosahexaenoic acid induced HO-1 expression in human cancer cells [6].

In this study, we demonstrate that docosahexaenoic acid results in a partial, but significant, prevention of neuronal and vascular of
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retinal ganglion cells after retinal I/R injury in rats. Docosahexaenoic acid inhibits inflammation, oxidative stress and anti-apoptotic that are activated by retinal I/R injury, raising a possibility that MMP-9 and Nrf-2/HO-1 pathways might contribute to the inhibition of retinal neurovascular damage by the drug.

Disclosure of conflict of interest

None.

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