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Dietary omega-3 fatty acids attenuate cellular damage after a hippocampal ischemic insult in adult rats☆

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Abstract

The role of omega-3 polyunsaturated fatty acids (3PUFAs) on brain function is increasingly demonstrated. Here, the effect of dietary deprivation of essential 3PUFAs on some parameters related to neuroprotection was investigated. Rats were fed with two different diets: omega-3 diet and omega-3-deprived diet. To assess the influence of 3PUFAs on brain responses to ischemic insult, hippocampal slices were subjected to an oxygen and glucose deprivation (OGD) model of *in vitro* ischemia. The omega-3-deprived group showed higher cell damage and stronger decrease in the [^{3}H]glutamate uptake after OGD. Moreover, omega-3 deprivation influenced antiapoptotic cell response after OGD, affecting GSK-3beta and ERK1/2, but not Akt, phosphorylation. Taken together, these results suggest that 3PUFAs are important for cell protection after ischemia and also seem to play an important role in the activation of antiapoptotic signaling pathways.

Keywords: Omega-3 fatty acids; Hippocampal cell damage; Glutamatergic system; Antiapoptotic; OGD

1. Introduction

Omega-3 (ω3 fatty acids) is a group of essential polyunsaturated fatty acids (PUFAs) that are present in the diet. α-Linolenic acid (18:3 ω3), present in vegetable oils such as linseed and flaxseed oils, is the precursor (in the liver and astrocytes) of eicosapentaenoic acid (EPA 20:5 ω3) and docosahexaenoic acid (DHA 22:6ω3), PUFAs of great relevance to the organism's health. EPA and DHA can also be found in some fatty fishes, such as salmon and tuna. Research over the past 30 years has established that PUFAs are critical for proper infant growth and neurodevelopment. Among the ω3 fatty acids, DHA is one of the most physiologically significant for brain function [1,2].

In the brain, DHA is present in synaptic membrane phospholipids, like phosphatidylethanolamine and phosphatidylserine, and in plasmalogens, compounds that seem to protect cells against oxidative damage [3,4]. The content of DHA in the sn-2 position of phospholipids reaches up to 50% of the total amount of PUFAs in the brain of adult rats [5]. An insufficient dietary supply of ω3 fatty acids during prenatal and postnatal development decreases the levels of DHA in neural tissue with a reciprocal increase of docosapentanoic acid (C22:5ω6) [6], leading to a variety of visual, olfactory, cognitive and behavioral deficits in animal models [7–10].

Many studies were developed to assess the neuroprotective properties of ω3 fatty acids in the central nervous system (CNS). In humans, the decreased levels of DHA were associated with neurodegenerative diseases, such as Alzheimer's disease [11,12]. Deficient dietary intake and low endogenous levels of ω3 fatty acids have been associated with the emergence and prognosis of psychiatric disorders, and many clinical trials have shown that their dietary supplementation was beneficial in patients with depression, bipolar disorder and schizophrenia [13,14]. Although the evidence indicates the beneficial effect of DHA to brain health, underlying mechanisms are not well understood.

Concerning the protective roles of DHA against injuries in animal models, DHA protected rats against excitotoxicity and convulsion [15,16], inhibited epileptiform activity in rat hippocampus [17] and reduced neuronal injury in experimental brain ischemia [18–20]. DHA also reduced β-amyloid cellular damage [7,21–23].

Recently, it was demonstrated that in the onset of brain injury, DHA could be released from the membrane phospholipids by Ca^{2+}-independent phospholipase A_2 and generate the neuroprotectin D1 (NPD1), a docosanoid responsible for the protective effects mediated

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by DHA [24,25]. NPD1 could up-regulate protective protein genes, such as Bcl-2 and Bcl-XL, and down-regulate pro-apoptotic protein genes, such as Bad and Bax [26].

Other proteins were related to neuroprotective features of DHA. Akt signaling, a critical pathway involved in neuronal survival, seems to be activated by DHA [27]. Extracellular signal-regulated kinase (ERK1/2), an enzyme of mitogen-activated protein kinase (MAPK) signaling, involved in cellular proliferation and survival, was also related to protective effects of DHA [28].

Glutamate is the major excitatory neurotransmitter in the CNS involved in various brain parameters, such as learning/memory, brain development and ageing [29–32]. However, besides the essential role of glutamate for normal brain functions, it has been well established that increased amounts of glutamate in the synaptic cleft could lead to neurotoxicity due to overstimulation of glutamate receptors (excitotoxicity). The excitotoxic events are involved in various acute (hypoxia, ischemia, seizure, trauma) and chronic (Parkinson's disease, Alzheimer's disease, Huntington's disease, epilepsy) brain disorders [30,32–34]. The main endogenous process responsible for maintaining the glutamate concentration in the synaptic cleft below the toxic levels is the glutamate uptake, exerted by transporters located mainly in astrocytic cell membranes [30,33–35]. Thus it is reasonable to determine whether the neuroprotective effects of DHA could involve the modulation of glutamate uptake by astrocytes.

The benefit of dietary supplementation with ω3PUFAs is much talked about in the scientific community. Despite that, little is known about the suffering that ω3-deficient animals experience when subjected to an injury. In the light of this knowledge, the objective of the present study was to evaluate in rats the ω3 dietary influence on the toxic effect of *in vitro* ischemic injury in the hippocampus and some of the putative mechanisms involved in these effects.

2. Materials and methods

2.1. Animals and diets

There is no clear consensus in the scientific community about the recommended levels of ω3 fatty acids in the diet; thus many dietetic models are developed to clarify the importance of these fatty acids in an organism's health. Here, in order to evaluate the influence of ω3 fatty acids in the diet and to manage the ω3 fatty acid maternal milk supply, 2 weeks before mating female rats were divided into two groups: the ω3 diet (ω3) and the ω3-deficient diet (D) group, following recommended dietary intakes for essential fatty acids [36]. Wistar female rats were housed in an air-conditioned room (21–22°C) with 12-h dark–light cycle, and food and water were offered *ad libitum*. Both diets were isocaloric, containing 8% total fat and differed only in fatty acid composition (Tables 1 and 2). For the injury experiments, their pups were used ($n=10$ per group; all experiments were performed in triplicate). After weaning, the pups were maintained with the same diet as the dams until the experiments in the adult age (60 days old). All experiments were in agreement with the Committee on Care and Use of Experimental Animal Resources, UFRGS, Brazil.

Table 1
Fatty acid composition of the diet lipids *

	ω3 Diet (%)	D Diet (%)
Saturated		
C16:0	10.9	11.1
C18:0	2.0	2.4
C20:0	0.5	1.3
C22:0	0.1	2.9
C24:0	0.0	1.5
Monounsaturated		
C16:1	0.2	0.2
C18:1	25.4	46.7
C20:1	0.0	1.6
Polyunsaturated		
C18:2ω6	56.6	32.0
C18:3ω3	1.2	0.0
C20:5ω3 **	2.2	0.0
C22:6ω3 **	3.5	0.0

* According to O'Brien [37].
** According to manufacturer information (Naturalis, Brazil).

Table 2
Composition of the diets

	ω3 Diet (%)	D Diet (%)
Casein [a]	22	22
Corn starch	42	42
D-L-Methionin [b]	0, 16	0, 16
Sucrose	21	21
Fibers	2	2
Mineral salt mix [c]	4	4
Vitamin mix [d]	1	1
Peanut oil	0	8
Corn oil	7	0
Fish oil	1	0

Salt and vitamin composition are according to Horwitz [38].
[a] Casein, purity 87% (from Herzog, Porto Alegre, Brazil).
[b] D-L-Methionin (from Merk, Rio de Janeiro, Brazil).
[c] Mineral salt mixture: mg/100 g of diet (from Roche, São Paulo, Brazil): NaCl, 557; KI, 3.2; KH_2PO_4, 1556; $MgSO_4$, 229; $CaCO_3$, 1526; $FeSO_4$–$7H_2O$, 108; $MnSO_4$–H_2O, 16; $ZnSO_4$–$7H_2O$, 2.2; $CuSO_4$–$5H_2O$, 1.9; $CoCl$–$6H_2O$, 0.09.
[d] Vitamin mixture: mg/100 g of diet (from Roche, São Paulo, Brazil): vitamin A (retinyl acetate), 4; vitamin D (cholecalciferol), 0.5; vitamin E (DL-α-tocopheryl acetate), 10; menadione, 0.5; choline, 200; PABA, 10; inositol, 10; niacine (nicotinic acid), 4; pantothenic acid (calcium D-pantothenate), 4; riboflavin, 0.8; thiamin (thiamine hydrochloride), 0.5; piridoxine (pyridoxine hydrochloride), 0.5; folic acid, 0.2; biotin [D-(+)-biotin], 0.04; vitamin B_{12}, 0.003.

2.2. Oxygen and glucose deprivation experiments

After decapitation, hippocampi were immediately isolated and transverse sections (400 μm) were prepared using a McIlwain tissue chopper. Hippocampal slices were divided into two equal sets: control and oxygen glucose deprivation (OGD — *in vitro* brain injury model), placed into separate 24-well culture plates, and preincubated for 30 min in a tissue culture incubator at 37°C with 95% air/5% CO_2 in a modified Krebs–Henseleit solution (preincubation solution, pH 7.4) (in millimolars): 120 NaCl, 2 KCl, 0.5 $CaCl_2$, 26 $NaHCO_3$, 10 $MgSO_4$, 1.18 KH_2PO_4, 11 glucose. After preincubation, the medium in the control plate was replaced with another modified Krebs–Henseleit solution (KHS incubation solution, pH 7.4) (in millimolars): 120 NaCl, 2 KCl, 2 $CaCl_2$, 2.6 $NaHCO_3$, 1.19 $MgSO_4$, 1.18 KH_2PO_4, 11 glucose, and the slices were incubated 60 min in the culture incubator. In the ischemic plate, OGD slices were washed twice with Krebs–Henseleit medium without glucose and incubated for 60 min (OGD period) at 37°C in an anaerobic chamber saturated with N_2, as previously described [39,40]. After incubation, the medium of both plates was removed, supplemented with Krebs–Henseleit solution with glucose and the slices were incubated for 3 h (reoxygenation period) in the culture incubator. Control and OGD sets were used concomitantly with four slices from the same animal in each plate. After reoxygenation, slices were used for determination of cellular damage and viability, glutamate uptake and Western blot analysis.

2.3. Cellular damage and viability

2.3.1. Lactate dehydrogenase assay

Membrane damage was determined by measuring lactate dehydrogenase (LDH) released into the medium [41]. After the reoxygenation period, LDH activity was determined using a kit (Labtest, Minas Gerais, Brazil). Total LDH activity (100%) was evaluated by disrupting the slices by freezing/thawing and homogenization. LDH Activity released into the medium was quantified as a percent of total activity. Results are expressed as a percentage of control.

2.3.2. MTT Colorimetric assay

Slice viability assay was performed by the colorimetric [3(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] (MTT, Sigma) method. After the reoxygenation time, slices were incubated with 0.5 mg/ml of MTT, followed by incubation at 37°C for 45 min. The formazan product generated during the incubation was solubilized in dimethyl sulfoxide and measured at 560 and 630 nm. Only viable slices are able to reduce MTT. Results are expressed as a percentage of control.

2.3.3. Trypan blue incorporation

Membrane permeability was evaluated by trypan blue assay. Briefly, at the end of the recovery time, slices were incubated for 5 min in a solution containing 400 μl of trypsin/EDTA (Gibco) and fetal calf serum at 37°C, gently dissociated by a sequential passage through a Pasteur pipette and allowed to settle during 10 min to remove residual intact tissue. An aliquot of the cell suspension was blended with 1.2% trypan blue solution. After 2 min, cells were counted in a hemocytometer by phase contrast in

an inverted light microscope at ×100 magnification. Each value indicates the percentage trypan blue-labeled cells, counted in four squares of the chamber in four to six separated experiments.

2.3.4. NSE Release assay

Neuron-specific enolase (NSE) released in the medium after the reoxygenation period was used as marker of neuronal damage. NSE was measured using an eletrochemiluminescent assay kit. It consists of a double sandwich assay that uses an anti-NSE antibody bound with ruthenium, which is the luminescent molecule. The reaction and quantification were performed using Elecsys-2010 (Roche Diagnostics Corporation). The assay was carried out in duplicate and the variation coefficient was less than 5%. NSE levels are expressed as nanograms per milliliter [42].

2.4. [^{3}H]Glutamate uptake

2.4.1. Total and Na^+-independent uptake

Glutamate uptake was performed as previously described [43]. After 3 h of reoxygenation, hippocampal slices were preincubated at 33°C for 15 min, followed by the addition of 100 μM [^{3}H]glutamate. Incubation was stopped after 5 min with two ice-cold washes of l ml HBSS, immediately followed by the addition of 0.5N NaOH, which was then kept overnight. Na^+-independent uptake was measured using the same protocol described above, with differences in the temperature (4°C) and medium composition (*N*-methyl-D-glucamine instead of sodium chloride). Results (Na^+-dependent uptake) were considered as the difference between the total uptake and the Na^+-independent uptake. Both uptakes were performed in triplicate. Incorporated radioactivity was measured using a liquid scintillation counter (Wallac 1409). This protocol was used in both ontogeny and OGD experiments.

2.5. Western blotting analysis

For Western blotting analysis of *in vitro* brain injury effects, hippocampal slices were homogenized in 25 mM Hepes (pH 7.4) containing a protease and phosphatase inhibitor cocktail. Samples were normalized to 2 μg protein/μl with a sample buffer (4% sodium dodecylsulfate, 2.1 mM EDTA, 50 mM Tris and 5 % β-mercaptoethanol). Samples (30 μg protein/well) were subjected to electrophoresis and transferred to a nitrocellulose membrane. Membranes were processed as follows: (1) blocking with 5% bovine serum albumin (Sigma) for 2 h; (2) incubation with primary antibody overnight: 1:1000 p-AKT; 1:1000 p-GSK3β; 1:1000 p-ERK1/2 (CellSignal); (3) incubation with horseradish peroxidase-conjugated secondary antibody for rabbit 1:3000 (Amersham Pharmacia Biotech) for 2 h; (4) chemioluminescence (ECL, Amersham Pharmacia Biotech) was detected using X-ray films (Kodak X-Omat, Rochester, NY, USA). The films were scanned and band intensity was analyzed using ImageJ (developed at the US National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/). The loaded protein was always verified by Coomassie blue gel stain.

2.6. Protein determination

Protein concentration was measured by the method of Lowry et al. [44] using bovine serum albumin as standard.

2.7. Statistical analysis

Data are expressed as mean±S.D. One-way ANOVA was used followed by Tukey's *post hoc* test, when significant effects ($P<.05$) were found. Analyses were performed with the SPSS 8.0 software.

3. Results

3.1. Effects of ω3-deficient diet on cellular injury in hippocampal slices subjected to OGD

To compare the hippocampal response of the ω3 and D groups to brain injury, hippocampal slices from both groups were subjected to OGD, a model of *in vitro* ischemia.

Fig. 1 shows the results of cellular responses to OGD. Fig. 1A shows that the D group released more LDH than the ω3 group after 3 h of reoxygenation ($P<.01$). As no difference was observed between both groups after 1 h of recovery, we chose only 3 h of reoxygenation to assess the other parameters of cellular responses.

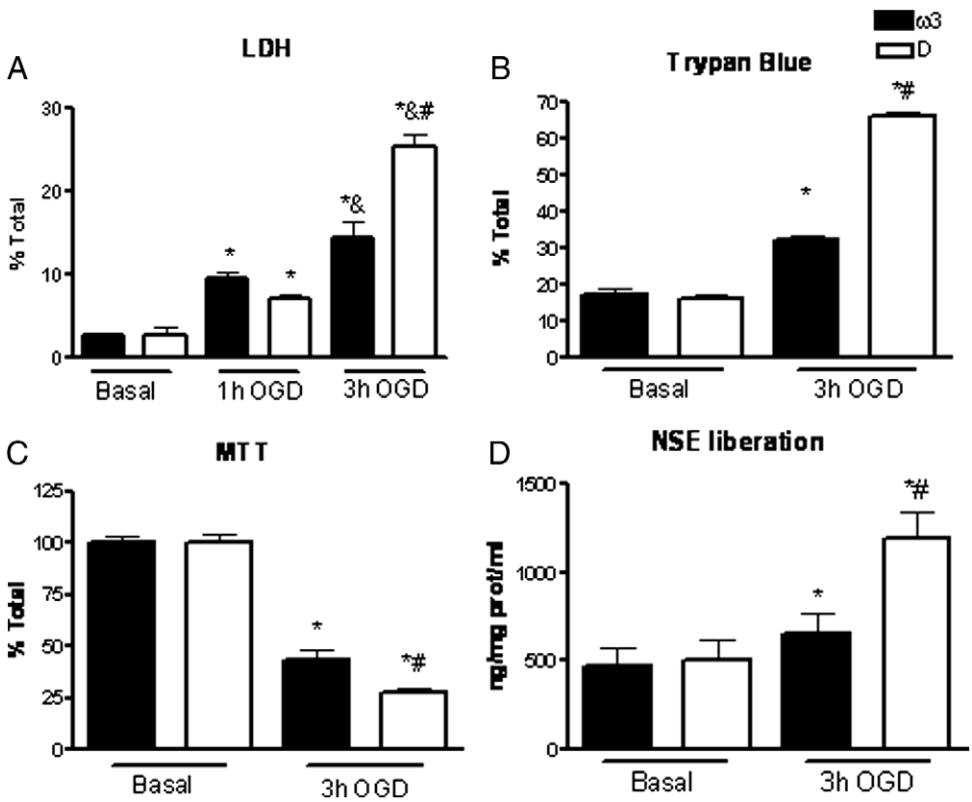

Fig. 1. Effects of ω3 in the diet on cellular viability parameters of hippocampal slices of rats at 60 days of age, subjected to OGD for 60 min. LDH content released in the medium (A) was measured in basal conditions (basal) and after 1 h (1 h OGD) and 3 h (3 h OGD) of reoxygenation. Trypan blue (B), MTT (C) and NSE released in the medium (D) were measured in basal conditions and after 3 h of reoxygenation. ω3, ω3 diet group; D, ω3-deficient diet group. $n=10$ animals per group; experiments in triplicates. Data are expressed as means±S.D. ($^*P<.05$ in relation to its basal control; $^{\&}P<.01$ in relation to 1-h OGD; $^{\#}P<.01$ in relation to ω3 3-h OGD).

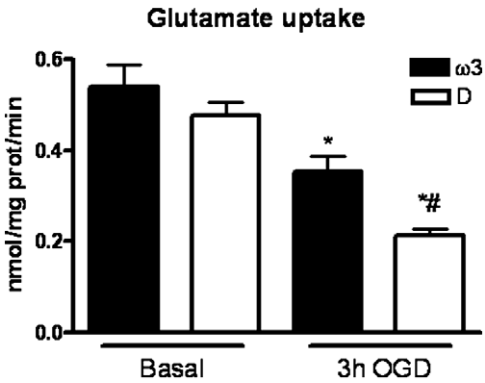

Fig. 2. Effects of ω3 in the diet on [^{3}H]glutamate uptake. [^{3}H]Glutamate uptake was measured after 3 h of reoxygenation in hippocampal slices of rats at 60 days of age subjected to OGD for 60 min. n=10 animals per group; experiments in triplicates. Data are expressed as means±S.D. (*P<.05 in relation to its basal control; #P<.01 in relation to ω3 3-h OGD).

Similarly, for both groups in the trypan blue assay, the D group presented higher membrane injury (P<.05) (Fig. 1B).

MTT Measurement, which assesses mitochondrial viability, showed higher cell death in the D group (Fig. 1C).

To assess specifically neuronal damage by OGD, we measured NSE liberation. Fig. 1D shows that the D group released more NSE after OGD.

3.2. Effects of ω3-deficient diet on [^{3}H]glutamate uptake after OGD

To assess the involvement of astrocytes in hippocampal responses to OGD injury, we measured the glutamate uptake by hippocampal slices. Brain slices maintain the cellular integrity and cell interaction; thus this preparation is necessary to investigate the physiological capacity of astrocytes to keep glutamate concentration in the synaptic cleft below toxic levels, protecting neurons from excitotoxic damage. Fig. 2 shows that OGD decreased glutamate uptake in both groups

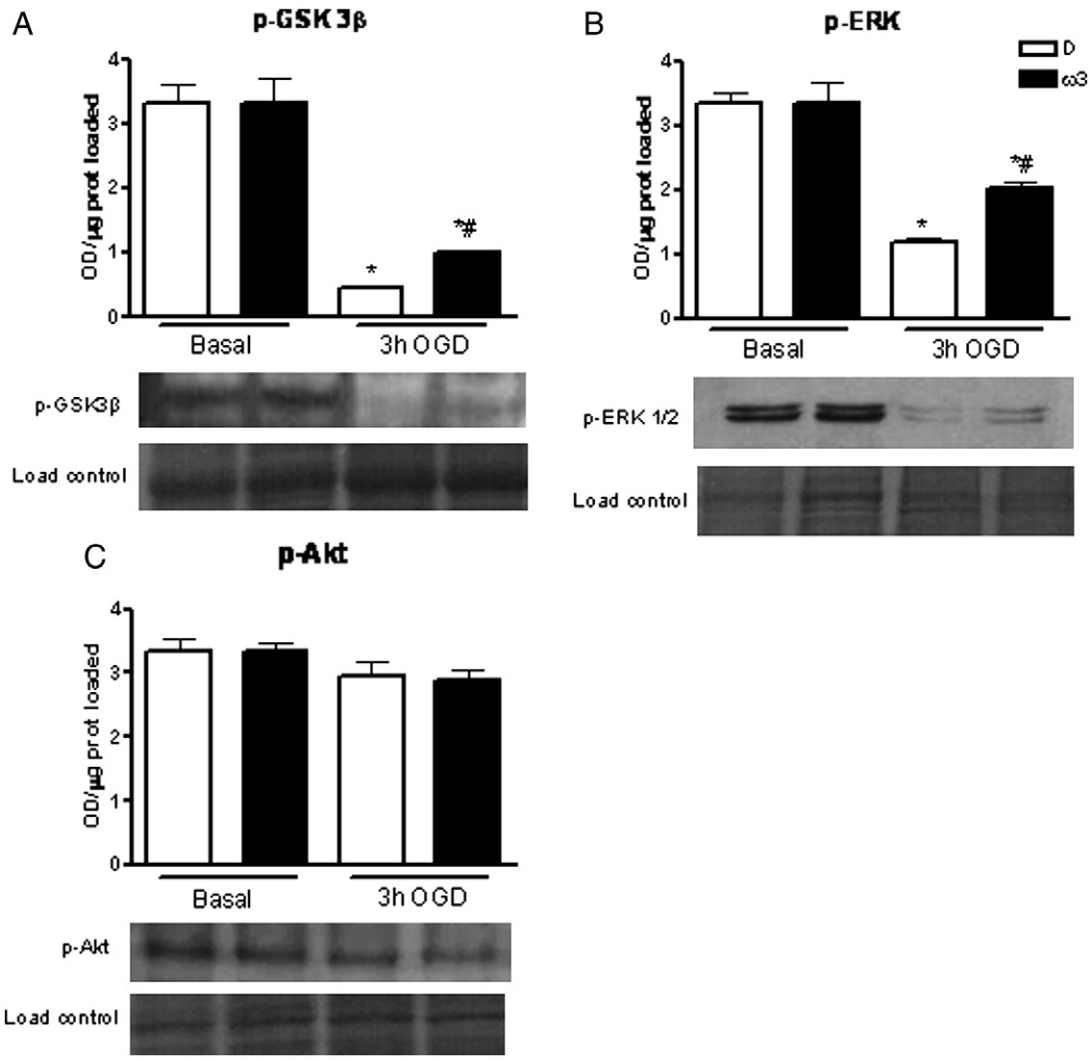

Fig. 3. Influence of ω3 in the diet on proteins involved in apoptotic and antiapoptotic pathway after injury. Evaluation of the phosphorylation state of proteins p-GSK3β (A), p-ERK 1/2 (B) and p-Akt (C), involved in signaling pathways related to apoptosis/antiapoptosis. Phosphorylation was measured in basal conditions (basal) and after 3 h of reoxygenation in hippocampal slices of rats at 60 days of age subjected to OGD for 60 min. The loaded protein was always verified by Coomassie blue gel stain (load control). n=6 animals per group. Data are expressed as means±S.D. of the optical density per microgram of protein loaded in each line (OD/µg protein loaded) (*P<.05 in relation to its basal control; #P<.01 in relation to D 3-h OGD). Representative Western blot images are shown below the respective bars.

($P<.05$). However, again, in the D group this decrease was more pronounced than in the ω3 group.

There was no difference between both groups in basal conditions (not subjected to OGD) in the parameters evaluated in Figs. 1 and 2.

3.3. Influence of ω3-deficient diet on signaling pathways involved in apoptosis after OGD

In this set of experiments, by using Western blot in hippocampal slices subjected to OGD, we investigated the phosphorylation state of proteins involved in apoptotic [glycogen synthase kinase-3β (GSK3β) inactive when phosphorylated] or antiapoptotic (ERK 1/2 and Akt, both active when phosphorylated) signaling pathways (Fig. 3), known to be involved in the protective effect of DHA [21,27,28]. There was a decrease in the phosphorylation state of GSK3β after OGD, which was more accentuated in the D group than in the ω3 group (to 25% and 15% respectively, $P<.01$) (Fig. 3A). Similarly, there was a decrease in the phosphorylation state of ERK1/2 after OGD in both groups, but it was more accentuated in the D group ($P<.01$) (Fig. 3B). Akt phosphorylation was not significantly affected by OGD (Fig. 3C). No parameters, when measured in basal conditions, were affected by the diet.

4. Discussion

In the present study, the ω3-deficient diet in rats caused a lower hippocampal capacity to deal with an ischemic injury.

To assess the influence of ω3 PUFAs on cellular survival, we subjected hippocampal slices of adult rats to OGD and further reoxygenation, and measured the cell viability through various parameters. CNS is particularly sensitive to reactive oxygen species [45], which are generated in excessive amounts during postischemic reoxygenation. Here we have shown that, after reoxygenation, the D group showed an increased susceptibility to hippocampal injury, including neuronal injury specifically (marked with NSE), compared to the ω3 group. Similar results were shown using the same model [19]. They showed a release of DHA during ischemia; both free DHA and the preservation of DHA-containing phospholipids were able to reduce cellular damage.

To determine the putative neurochemical mechanisms for the neuroprotective effects of ω3 PUFAs, we investigated the effect of OGD on glutamate uptake and on apoptotic and antiapoptotic signaling pathways.

Although glutamate plays essential plastic roles in CNS [29–31,46], excessive activity of the glutamatergic system is highly toxic and the glutamate toxicity contributes to brain injury observed in various acute and chronic brain diseases [30,33–36,39–47]. Thus, excessive glutamate has to be removed from the synaptic cleft, and the equilibrium between the physiological/toxic tonus of the glutamatergic system is modulated by the uptake of extracellular glutamate, which is carried out mainly by glutamate transporters located in astrocytic cell membranes surrounding the synaptic cleft [29,30,34,35,48,49].

It has been shown that after OGD, the extracellular glutamate concentration increases [30]. Here, we showed that there was a decrease in the glutamate uptake after OGD, which is in agreement with previous studies [50,51], with the decrease being more pronounced in the D group. Since the basal glutamate uptake was similar in the groups, the lower capacity to take up glutamate could be correlated to the higher cell damage observed in the D group after ischemia. More studies need to be done to elucidate the mechanisms of this parameter.

Akbar et al. [27] showed that DHA promotes neuronal survival via activation of Akt signaling. Akt has direct effects on the apoptotic pathway, by inhibiting proapoptotic proteins, such as Bad, caspase 9 and GSK3β [52]. Moreover, a previous study showed that blocking increased Akt phosphorylation increases subsequent DNA fragmentation [53]. Similarly to Akt signaling, MAPK signaling pathway is required for the antiapoptotic effect and neuronal survival in the brain following ischemic insults, and one study suggests that DHA can modulate ERK/MAPK pathway [28]. Here, we show that ω3 PUFAs consumption was able to partially prevent the increase in apoptotic and the decrease in antiapoptotic signaling pathways, involving GSK3β and ERK1/2 phosphorylation. Despite that, in this study Akt phosphorylation was not affected by both diets. Although it has been previously shown that p-Akt could phosphorylate GSK3β [54], in our study we did not observe it, pointing to the involvement of other mechanism(s) responsible for the increase in the phosphorylation state (inactivation) of GSK3β. DHA can also modulate other proteins involved in apoptosis, such as Bcl-2 and Bcl-xl [22] and caspases [23].

In summary, our data suggest that ω3 PUFAs are relevant for neural cell protection against OGD injury, partially preventing the decrease in the glutamate uptake and partially decreasing the apoptotic response and increasing the cellular capacity to activate antiapoptotic pathways.

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