Effects of intermittent fasting on age-related changes on Na,K-ATPase activity and oxidative status induced by lipopolysaccharide in rat hippocampus

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A B S T R A C T
Chronic neuroinflammation is a common characteristic of neurodegenerative diseases, and lipopolysaccharide (LPS) signaling is linked to glutamate-nitric oxide-Na,K-ATPase isoforms pathway in central nervous system (CNS) and also causes neuroinflammation. Intermittent fasting (IF) induces adaptive responses in the brain that can suppress inflammation, but the age-related effect of IF on LPS modulatory influence on nitric oxide-Na,K-ATPase isoforms is unknown. This work compared the effects of LPS on the activity of α2,3 Na,K-ATPase, nitric oxide synthase gene expression and/or activity, cyclic guanosine monophosphate, 3-nitrotyrosine-containing proteins, and levels of thiobarbituric acid–reactive substances in CNS of young and older rats submitted to the IF protocol for 30 days. LPS induced an age-related effect in neuronal nitric oxide synthase activity, cyclic guanosine monophosphate, and levels of thiobarbituric acid–reactive substances in rat hippocampus that was linked to changes in α2,3-Na,K-ATPase activity, 3-nitrotyrosine proteins, and inducible nitric oxide synthase gene expression. IF induced adaptive cellular stress-response signaling pathways reverting LPS effects in rat hippocampus of young and older rats. The results suggest that IF in both ages would reduce the risk for deficits on brain function and neurodegenerative disorders linked to inflammatory response in the CNS.

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1. Introduction

Aging has been associated with an increasing neuroinflammatory process (Richwine et al., 2005; Ye and Johnson, 1999), and systemic inflammation may be associated with several pathologies, such as, coronary heart disease, diabetes, multiple sclerosis, Alzheimer’s disease, and Parkinson’s disease (Fito et al., 2007). However, anti-inflammatory therapies have shown mixed and discouraging results (Imbimbo et al., 2010; In t’ Veld et al., 2001; Stewart et al., 1997; Vlad et al., 2008).

Dietary strategies visibly influence inflammation, as related through both observational studies and controlled feeding trials in which subjects had limited food consumption (Giugliano et al., 2006; Harvie et al., 2011; Johnson et al., 2007; Mozaffarian et al., 2009). The most prominent dietary factor that affects the risk of many different chronic diseases is energy intake. Thus, the excessive calorie intake increases the risk while reducing energy intake by controlled caloric restriction or intermittent fasting (IF) increases lifespan and protects various tissues against diseases. Hormesis is a dose-response phenomenon characterized by a low dose stimulation and a high dose inhibition. Cumulating misfolded proteins in response to proteotoxic environmental stress conditions triggers the cellular stress response. Nutritional antioxidants are able to activate vitagenes, such as heme oxygenase, Hsp70, thioredoxin reductase, and sirtuins, which represent an integrated system for hormetic cellular stress tolerance. Thus, at least part of the effect of IF could be mediated by hormetic mechanisms involved in the activation of vitagenes resulting in enhanced defense against energy and stress resistance homeostasis disruption with consequent impact on longevity processes (Calabrese et al., 2012; Cornelius et al., 2013).

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Two distinct dietary restriction regimens have been shown to promote an extensive array of health benefits. Both restricting the amount of food intake in caloric restriction, or the frequency of feeding in IF can significantly increase life span, reduce free radicals production, and attenuate age-related diseases and impairment in cognitive and motor functions (Masoro, 2006; Mattson, 2005; Mattson and Wan, 2005).

Na,K-ATPase is a transmembrane spanning enzyme consisting of 3 different subunits, α (catalytic), β, and γ. This enzyme also can act as a signal transducer and an nuclear factor (NF)-κB activator by interacting with neighboring membrane proteins and organized cytosolic cascades of signaling proteins (Aperia, 2007; Kawamoto et al., 2012). The activity of Na,K-ATPase has been shown to be modulated by glutamate by N-methyl-d-aspartate (NMDA) receptor–nitric oxide (NO) production, leading to cyclic GMP-PKG activation (Munhoz et al., 2005; Scavone et al., 2005). It has been shown that inflammatory stimulus in the brain increases excitatory glutamatergic transmission, especially at NMDA receptor, leading to NO modulation by glutamate by NF-κB activation (Glezar et al., 2003). In previous studies, we showed an age-related decrease in α2,3-Na,K-ATPase activity in rat cerebellum that is not linked to changes in the amount of protein (α1,3,2-Na,K-ATPase) but result of changes on the cyclic GMP–PKG pathway (Kawamoto et al., 2008b). Aging reduction in Na,K-ATPase activity results in increase in reactive oxygen species that could be linked to neurodegenerative processes (Mattson and Liu, 2002). The present study investigated the effect of IF for 30 days (every other day feeding) on the systemic inflammation induced by lipopolysaccharide (LPS), a highly conserved component of the wall of Gram-negative bacteria, on the activity of isoforms α1 and α2,3-Na,K-ATPase, inducible nitric oxide synthase (iNOS) gene expression, and/or neuronal NOS activity, cyclic guanosine monophosphate (cGMP), levels of thiobarbituric acid–reactive substances (TBARS), and protein nitrosylation in hippocampus of young and older rats.

2. Methods

2.1. Animal and tissue preparation and viability test

Four-, 12-, and 24-month-old male Wistar rats (Biomedical Sciences Institute, University of São Paulo) were kept under 12-hour light/dark cycle (lights on at 7:00 AM) and allowed free access to food and water. Animals from these 3 groups were treated with LPS dissolved in sterile saline (1 mg/kg, intravenous [i.v.] bolus) or sterile saline and euthanized 2 hours after the administration. Four- and 24-month-old animals were also submitted to IF (every feeding every other day) or control diet for 30 days followed by LPS or saline and euthanized 2 hours after the administration. All animals were euthanized by decapitation (between 9:00 and 11:00 AM) following procedure approved by the Biomedical College of Animal Experimentation. All procedures were also approved by the Ethical Committee for Animal Research of the Biomedical Sciences Institute of the University of São Paulo. The brain was immediately removed and immersed in cold phosphate-buffered saline. The hippocampus were rapidly dissected, quickly immersed in liquid nitrogen, and stored at ~80°C for later use.

2.2. Chemicals and kits

Routine reagents and LPS from Escherichia coli O111:B4 were purchased from Sigma Chemical (St. Louis, MO, USA); the Bio-Rad protein assay kit was purchased from Bio-Rad (Hercules, CA, USA). Cyclic GMP Enzyme Immunoassay kits were purchased from Enzo Life Sciences Inc. (Farmingdale, NY, USA). The antibody against 3-nitrotyrosine (3-NT) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). All solutions were prepared immediately before use.

2.3. TBARS determination in rat hippocampus

Thiobarbituric acid reacts with products of lipid peroxidation, mainly malondialdehyde, producing a colored compound. Lipid peroxidation was determined through the production of TBARS, as previously described (Kawamoto et al., 2013). Hippocampi tissues were homogenized in saline buffer and precipitated proteins were removed by centrifugation at 12,000g for 10 minutes. The supernatant was mixed with thiobarbituric acid (1% in NaOH 50 mm) and HCl 25%. The samples were then heated in a boiling water bath for 10 minutes and, after cooling, were extracted with 1.5 mL of butanol. The mixture was centrifuged at 12,000g for 10 minutes and the absorbance of the supernatant was determined (Freitas et al., 2001).

2.4. Measurement of cyclic GMP levels and platelets and brain samples

Cyclic GMP levels in the hippocampus were determined in triplicate by using direct, competitive enzyme immunoassay kits (Stressgen; Enzo Life Sciences Inc.). Hippocampi and cerebella were homogenized in 10 volumes of 0.1 M HCl and centrifuged at 2500g for 20 minutes at room temperature. The supernatant was acetylated to improve signal detection and samples were run in duplicate. The concentration cyclic GMP is expressed as pmol/mL.

2.5. Measurement of NOS activity

For NOS activity assay, the tissue samples were homogenized in ice-cold 0.32 M sucrose/20 mM HEPES buffer (pH 7.4) containing 1 mM dithiothreitol in an ice bath for 1 minute using a Teflon homogenizer. Each homogenate was centrifuged at 10,000g for 30 minutes at 4°C. The supernatant was passed through a Dowex AG 50 Wx–8 (Na+ form) column to remove the endogenous arginine. The arginine-free eluent was used to assay the NOS activity. NOS activity in hippocampus slices was determined by the enzymatic conversion of [3H]-arginine to [3H]-citrulline as described by McKee et al. (1994) with some modifications. To summarize, the NOS assay reaction medium of 200 μL contained 100 mM HEPES, pH 7.4; 1 mM nicotinamide adenine dinucleotide phosphate; flavin mononucleotide, flavin adenine dinucleotide, tetrahydrobiopterin 0.45 mM CaCl2; 80 units of calmodulin, 100 μM L-arginine, and 1 μM L-[2,3-3H]-arginine (0.5 μCi), or with no addition of CaCl2 and calmodulin (in the presence of 0.425 mM EDTA); and 100 μL of hippocampus cytosolic protein (0.2 μg/μL). The reaction mixture was incubated for 30 minutes at 37°C and stopped by the addition of stop buffer containing 20 mM HEPES at pH 5.5. The entire reaction mixture was passed through a column packed with Na+ form of Dowex AG 50 Wx-8 resin. The flow through fraction containing [3H]-citrulline was counted for radioactivity using a Beckman 6000 liquid scintillation counter. The NOS activity was expressed as picomoles [3H]-citrulline per milligram protein per minute. Samples of rat cerebellum were analyzed simultaneously as a positive control. Inhibition of the enzyme was evaluated in all tissues using N-nitro-L-arginine methyl ester hydrochloride (10−6 to 10−4 M). The biochemical characterization of hippocampal NOS showed that spontaneous activity of NOS in the cytosol of hippocampus tissue was greatly reduced when nicotinamide adenine dinucleotide phosphate or Ca2+/calmodulin was omitted from the incubations medium for the conversion of L-arginine to L-citrulline. The biochemical characterization data of the constitutive NOS isoform in the hippocampus suggest that NOS isoform seems to be similar to...
the neuronal type enzyme (NOS I) found in cerebellum. Our previous data also found a similar result in rat dorsal periaqueductal gray compared with cerebellum tissue (Chiavegatto et al., 1998).

2.6. Reverse transcription polymerase chain reaction (RT-PCR) determination of iNOS mRNA levels

The effect of IF on LPS-modulated gene expression in the hippocampus of rats was measured. Total RNA was isolated with Trizol reagent (Invitrogen, Carlsbad, CA, USA) from hippocampus of 4- and 24-month old animals according to the instructions of the manufacturer. Semi-quantitative RT-PCR amplification was performed using the ThermoScript RT kit (Invitrogen) according to the instructions of the manufacturer. The primer sequences were iNos (GenBank access number 0126113.6–651 bp) 5’-GTGCTAATGCAGAAGCTCATA-3’ (sense) and 5’-CCAAATCTGGTGTGCACAC-3’ (antisense). The PCR conditions consisted of 5 minutes at 94°C, 33 cycles of 94°C for 45 seconds, 63°C for 45 seconds, and 72°C for 1 minute and 30 seconds and a final extension at 72°C for 10 minutes. Glyceraldehyde-3-phosphate dehydrogenase (Gapdh; GenBank accession number 0170083.3–264 bp) also was amplified as an internal PCR control using the following primers: 5’-GGGGAAGCTTGTTGAGATGG-3’ (sense) and 5’-GCCAGTGATGCTGACAGTG-3’ (antisense). In this case, the temperature cycling conditions were as follows: 5 minutes at 94°C, 20 cycles of 94°C for 45 seconds, 63°C for 45 seconds, and 72°C for 1 minute and 30 seconds and a final extension at 72°C for 10 minutes. Gel electrophoresis of the PCR product was performed using an ethidium bromide-containing agarose gel (2%), and resulting bands were visualized under ultraviolet light. The photographs were captured by photo documentation system DP-001-FDC (VilberLourmat, France), and the optical density of the bands was determined using NIH ImageJ software (http://rsb.info.nih.gov/ij). Results were expressed in relation to the intensity of Gapdh mRNA levels.

2.7. Immunoblotting

Electrophoresis was performed using a 10% polyacrylamide gel. In brief, the proteins present in the hippocampus extract (15 μg) were size separated in 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (90 V). The proteins were blotted onto a nitrocellulose membrane (Bio-Rad) and incubated with specific antibody against 3-NT (sc-2731; Santa Cruz Biotechnology). The Ponceau method of immunoblotting was used to ensure equal protein loading. Proteins recognized by antibodies were revealed by the electrochemiluminescence technique, following the instructions of the manufacturer (Amersham, Buckinghamshire, England). The membranes were exposed to x-ray film. To quantify the immunoblots, we used the NIH ImageJ software. Several exposure times were analyzed to ensure the linearity of the band intensities. β-actin antibody (sc-1616; Santa Cruz Biotechnology) was used as an internal control for the experiments. Results were expressed in relation to the intensity of β-actin band.

2.8. Measurement of Na,K-ATPase activity

Na,K-ATPase activity was determined by assaying inorganic phosphate released from ATP hydrolysis. This inorganic compound forms a complex with molybdate, which can be read spectrophotometrically at 700 nm (Esmann, 1988). For this colorimetric assay BioRad Bradford, 1976). Na,K-ATPase activity was tested by adding 10 μg of the particulate fraction (in 40 μL of buffer) to 360 μL of buffer containing: 3 mM ATP, 120 mM NaCl, 2 mM KCl, 3 mM MgCl2, and 30 mM histidine (pH 7.2), with or without ouabain (OUA) (3 μM or 3 mM). After 20 minutes of incubation at 37°C Na,K-ATPase activity was measured. The reaction was terminated by the addition of a quenching solution (0.6 mL) containing 1 N H2SO4 and 0.5% ammonium molybdate. Formation of a phosphomolybdate complex was determined spectrophotometrically at 700 nm (Esmann, 1988). The total ATPase, Mg-ATPase, α1- and α2,3-Na,K-ATPase activities were linearly related up to 20 minutes. In rodents, the α2,3-Na,K-ATPase isoform is a thousand times less sensitive to the cardiac glycoside than the α2,3-Na,K-ATPase activity as measured as the difference between ouabain-untreated and ouabain-treated samples. The high-affinity α2,3-isofrom fraction was calculated by subtracting the activity obtained with 3 μM ouabain from total-ATPase activity. To determine the low affinity fraction (α1 subunit-associated Na,K-ATPase activity), the values obtained in the presence of 3 μM OUA were subtracted from those obtained in the presence of 3 mM OUA. The Na,K-ATPase activity was expressed as nmol/mg protein × min.

2.9. Statistical analysis

Results are expressed as mean ± SEM of the indicated number of experiments. Statistical comparisons for age-related changes in inducible or neuronal NOS activity, TBARS, cyclic GMP, iNos mRNA levels, and Na,K-ATPase activity were performed by one-way analysis of variance (ANOVA), followed by the Newman-Keuls test. Statistical analysis for age-related effects of LPS-induced changes in NOS, TBARS, cyclic GMP, RT-PCR, and Na,K-ATPase assays at 4, 12-, and 24-month-old animals were performed by two-way ANOVA (control × LPS) with ages (4-, 12-, and 24-month-old animals) and Bonferroni post hoc tests when significant main effects or interactions were detected. Statistical comparisons for age-related changes induced by IF in the presence or absence of LPS in TBARS, cyclic GMP, iNos mRNA levels, and Na,K-ATPase activities in 4- and 24-month-old animals were performed by one-way ANOVA, followed by the Newman-Keuls test. Statistical comparisons for age-related changes induced by IF in the presence or absence of LPS in TBARS, cyclic GMP, 3-NT protein levels, as well as iNos mRNA levels, and Na,K-ATPase activities in 4- and 24-month-old animals were performed by two-way ANOVA (IF × IF+LPS) with ages (4- and 24-month-old animals) and Bonferroni post hoc tests when significant main effects or interactions were detected. All analyses were performed using a Prism 6 software package (GraphPad Software, San Diego, CA, USA).

3. Results

3.1. Age-related changes in cyclic GMP, TBARS, and α2,3-Na,K-ATPase activity induced by LPS in rat hippocampus

Aging induces a progressive decrease in hippocampal total-ATPase activity, which is caused by a reduction in α2,3-Na,K-ATPase activity, whereas α1-Na,K-ATPase and Mg-ATPase activities are not changed (Fig. 1A–D). The α2,3-Na,K-ATPase activity at 12 and 24 months is reduced to 27.2% and 61.1% of the values detected at 4 months, respectively (Fig. 1D). Therefore, a progressive significant reduction in α2,3-Na,K-ATPase activity between 4 and 12, as well as 12 and 24 months, was observed. LPS caused a decrease of the total-Na,K-ATPase activity 2 hours after i.v. injection. The effect was specific to α2,3-Na,K-ATPase...
activity, because $\alpha_1$-Na,K-ATPase and Mg-ATPase activities are not changed (Fig. 1A–D). The $\alpha_{2,3}$-Na,K-ATPase activity at 4 and 12 months was reduced to 30.6% and 18.7% in LPS-treated animals compared with the values detected at 4 and 12 months, respectively (Fig. 1D). Two-way ANOVA revealed a significant main effect of age and LPS treatment at 4 and 12 but not at 24 months. The effect seems to be common to other brain areas since it was also observed in cerebellum (Supplementary Fig. 1A–D).

Aging induces a progressive increase of calcium-dependent NOS activity in hippocampus (Kawamoto et al., 2013). The neuronal NOS activity, because $\alpha_1$-Na,K-ATPase and Mg-ATPase activities are not changed (Fig. 1A–D). The $\alpha_{2,3}$-Na,K-ATPase activity at 4 and 12 months was reduced to 30.6% and 18.7% in LPS-treated animals compared with the values detected at 4 and 12 months, respectively (Fig. 1D). Two-way ANOVA revealed a significant main effect of age and LPS treatment at 4 and 12 but not at 24 months. The effect seems to be common to other brain areas since it was also observed in cerebellum (Supplementary Fig. 1A–D).

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activity at 12 and 24 months is increased to 126.8% and 179.2% of the values detected at 4 months, respectively. In addition, the enzyme activity at 24 months is also increased (141.2%) compared with the activity obtained in hippocampus from 12-month-old animals (Fig. 2A). The Ca\(^{2+}\)-independent form of NOS also showed an age-related effect because it represents <2% of total NOS activity in the hippocampus from 4-month-old animals, <5% at 12, and near 14% at 24 months (data not shown).

Samples from rat hippocampus also showed that cyclic GMP levels decreased 38.7% at 12 months and 62.9% at 24 months compared with the basal values measured at 4-month-old animals (Fig. 2B). TBARS determinations showed that aging is linked to progressive increase in products of lipid peroxidation in hippocampus. The TBARS levels at 12 and 24 months are increased to 104.9% and 250.8% of the values detected at 4 months in hippocampus. The TBARS levels at 24 months are also increased (171.2%) when compared to values obtained from samples of 12-month-old animals (Fig. 2C).

LPS treatment increased constitutive NOS activity without change inducible NOS (iNOS) in hippocampus at 4-, 12-, and 24-month-old animals (Fig. 2A). These results confirmed previous data that showed an increase in iNOS gene expression after LPS 1 mg/kg, i.v., 2 hours, but not in iNOS protein or activity (Munhoz et al., 2006). This effect could be attributable to the time course used in the present study since it was based on the peak of NF-κB activation, and not in the protein expression (Glezer et al., 2003). Other studies in our laboratory also have confirmed that the peak of transcription factor activation is not always linked to protein expression (Kawamoto et al., 2008a). In addition, LPS treatment induced a decrease in cyclic GMP levels at 4- and 12-month-old animals but not at 24 months compared with the respective age control groups (Fig. 2B). In contrast, TBARS activity measured in hippocampus homogenates was increased after LPS in 4-, 12-, and 24-month-old animals compared with the respective age control groups (Fig. 2C). Two-way ANOVA revealed a significant effect of age and LPS treatment to both cyclic GMP (4 and 12 months) and TBARS (4, 12, and 24 months) levels (Fig. 2A–C).

3.2. IF reverts LPS-induced changes in cyclic GMP and TBARS levels and Na,K-ATPase activity in rat hippocampus

The experimental schedule design of IF and LPS administration is shown in Supplementary Fig. 2. On the basis of similar LPS effect on 4- and 12-month-old animals, we decided to evaluate the age-related effects of IF on LPS modulatory influence on NO-Na,K-ATPase isoforms only in 4- and 24-month-old animals. Four-
24-month-old rats in IF during 30 days were challenged by i.v. LPS or vehicle injection 24 hours after the last day of schedule with food.

The animals of both groups (4 and 24 months) were weighed before and after IF. The results showed that there is a progressive age-related increase in the body weight of the animals in the control groups (Supplementary Fig. 3). However, rats at 4 months showed less increase in the body mass after 30 days of IF (Δ = 39.0 ± 1.2 g) compared with the gain of weight obtained by the control group (Δ = 60.0 ± 3.0 g). In contrast, although 24-month-old animals lost weight after 30 days of experiment, the reduction in the animal body weight was greater in IF (Δ = −90.2 ± 1.0 g) compared with the control group (Δ = −50.9 ± 2.3 g) (Supplementary Fig. 3).

IF by itself in both 4- and 24-month-old animals induces an increase in total-ATPase activity, which is linked to 3,2-Na,K-ATPase activity, whereas 3,1-Na,K-ATPase and Mg-ATPase activity are not changed (Fig. 3A–D). In addition, IF was able to prevent an LPS-induced decrease of the total-Na,K-ATPase activity 2 hours after LPS i.v. injection in 4-month-old animals (Fig. 3A). The effect was specific to 3,2,1-Na,K-ATPase activity, because 3,1-Na,K-ATPase and Mg-ATPase activities are not changed (Fig. 3B–D). Two-way ANOVA analysis revealed a significant main effect of age (4 vs. 24 months) and IF treatment. The effect seems to be common to other brain areas because it was also observed in cerebellum (Supplementary Fig. 4A–D).

Changes in cyclic GMP content showed that IF by itself does not cause an increase in this second messenger in hippocampus at 4-month-old animal whereas at 24 months IF induced an increase of 50.2% compared with the control group (Fig. 4). Samples from rat hippocampus exposed to IF schedule and challenged with LPS also showed that IF reverts LPS-induced decrease in this second messenger at 4 months and increases 40.6% of cyclic GMP levels at 24 months compared with the levels observed in LPS group. Two-way ANOVA analysis revealed a significant main effect of age (4 vs. 24 months) and IF treatment (Fig. 4).

3.3. IF also induced a decrease in TBARS, 3-NT, and iNOS mRNA levels in 4- and 24-month-old animals compared with respective control or LPS groups

The TBARS levels in IF group was decreased to 35.4% in 4-month-old animals compared with the values detected in hippocampus of the rats that received LPS but not submitted to IF. Two-way ANOVA revealed a significant main effect of age (4 vs. 24 months) and IF treatment (Fig. 5).

By the same token, although the 3-NT protein level is not changed in the IF group at 4 months of age, it decreases 32.5% in 24-month-old animals compared with the levels detected at the respective control group. In addition, IF induced a decrease of 36.3% and 52% in 4-month-old and 24-month-old animals compared with the values detected in hippocampus of the rats that received LPS but not submitted to IF. Two-way ANOVA revealed a significant main effect of age (4 vs. 24 months) and IF treatment (Fig. 6).

The age-related effect of IF on iNos gene expression in the presence or absence of LPS was then evaluated by RT-PCR assay. Densitometric analysis showed that LPS induced an increase of iNos mRNA levels in both 4- and 24-month-old animals (Fig. 7). Although IF does not change iNos mRNA levels at both ages studied compared with control group, it was able to partially revert the increase of iNos mRNA levels induced by LPS in both 4- and 24-month-old animals. Two-way ANOVA revealed a significant main effect of age (4 vs. 24 months) and IF treatment (Fig. 7).
In contrast, there have been no studies to evaluate the effects of IF on neuroinflammatory processes in NO-cyclic GMP-Na,K-ATPase signaling in young and older rats.

In the first part of this study we investigate the age-related effects induced by LPS on cyclic GMP, TBARS, and iNOS mRNA levels and α1 and α2,3-Na,K-ATPase activities in rat hippocampus and cerebellum because this pathway can mediate protective action in the central nervous system (CNS). The results confirmed that aging induces a progressive decrease in hippocampal and cerebellar total-ATPase activity, which is attributable to a reduction in α2,3-Na,K-ATPase activity, whereas α1-Na,K-ATPase and Mg-ATPase activities are not changed (Scavone et al., 2005). In addition, the present study also has reinforced that aging is linked to a progressive decrease in cyclic GMP and increase in TBARS in the hippocampus (Kawamoto et al., 2013). The reduction of the Na,K-ATPase activity can cause an increase in the intracellular calcium concentration (Xiao et al., 2002). It is widely accepted that an increase in intracellular calcium above certain limits is excitotoxic (Mattson, 1998), and that the reduction in neuron membrane potential, which occurs during aging, is also associated with decrease in Na,K-ATPase (Tanaka and Ando, 1990). Glutamate stimulates Na,K-ATPase activity resulting in a subsequent improvement in Nai/Cai− exchange (Gloor, 1997). However, glutamate positive modulation of cerebellar α2,3-Na,K-ATPase is decreased by aging and this process is linked to a defective PKG signaling pathway (Scavone et al., 2005). In fact, glutamate signaling is essential for hippocampus-dependent spatial working memory (Reisel et al., 2002). Evidence suggests that age-related changes in the regulation of synaptic function by protein kinase and phosphatase activity result in parallel changes in the induction and expression of synaptic plasticity during aging as well as the phosphorylation patterns in long-term potentiation and/or depression (Norris et al., 1998).

Energy deficiency and dysfunction of the Na,K-ATPase are common consequences of many pathological insults in the CNS. Glutamate through NMDA-NOS or alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid-CO-cyclic GMP pathways has been shown to stimulate α2,3- Na,K-ATPase activity in the CNS.
(Munhoz et al., 2005; Nathanson et al., 1995), and NMDA signaling cascade receptor activation can mediate an adaptive, neuroprotective response to amyloid peptide (Aβ) (Kawamoto et al., 2008a). Studies in vitro also have shown that PKG activation and PP-1 inhibition is linked to a glutamate-NMDA-neuronal NOS and cyclic GMP levels cascade acting on 2,3- Na,K-ATPase activity in the CNS (McKee et al., 1994; Munhoz et al., 2005; Nathanson et al., 1995; Scavone et al., 2000).

Increased glutamate levels have been shown after LPS administration (Huang et al., 2008), which can activate NF-κB at least partially due to NMDA-NOS cascade in different brain areas (Glezer et al., 2003). We tested age-related changes in cyclic GMP, TBARS, and 2,3- Na,K-ATPase activity induced by LPS in rat hippocampus. The systemic injection of LPS induced a decrease of cyclic GMP levels and 2,3- Na,K-ATPase activity at both 4 and 12 but not at 24 months compared with control groups in 2 different brain areas (hippocampus and cerebellum). In addition, TBARS levels measured in hippocampus homogenates were increased after LPS treatment in all 3 ages studied when compared with respective control groups. During the aging process, mitochondrial oxidative phosphorylation becomes less efficient, and this probably contributes to an increase in the production of free radicals. The increase in O$_2^-$ production would lead to an accumulation of intracellular calcium and activation of calcium-dependent enzymes, like NOS, which is responsible for the formation of NO$. NO$-mediated neurotoxicity is engendered, at least in part, by reaction with O$_2^-$, apparently leading to formation of ONOO$. and not by NO$ alone. However, if there is NO$ formation, the NMDA receptor will be inhibited and a neuroprotective effect will appear. Therefore, NO behavior as a free radical or as an antioxidant agent will be dependent on O$_2^-$ levels. If the concentration of this anion is high, NO will lead to lipid peroxidation; otherwise, NO will have an antioxidant behavior (Wink et al., 1996). By the same token, LPS-induced NOS activation and increase in free radical production leading to a decrease in cyclic GMP levels and 2,3- Na,K-ATPase activity. In fact, our previous data showed an age-related increase in NOS activity in frontal cortex, hippocampus and in platelets of rats (Harman, 1995; Kawamoto et al., 2013).

Most of the neurodegenerative conditions are associated with a chronic inflammation. Although there is controversy whether inflammation is causative or a consequence of the disease process, it is now clear that it can greatly influence its pathogenesis (Floyd, 1999; Teunissen et al., 2003). Even though aging and neurodegeneration share the same basic mechanisms, it is difficult to establish the limits between these 2 processes; there are mounting evidences that neurodegeneration might be an extension of the normal aging process, which might increase the susceptibility for neurotoxic events (Smith et al., 1991; Swerdlow, 2007).

The present study showed for the first time that IF by itself or in the presence of inflammatory stimulus revert the decrease in 2,3- Na,K-ATPase activity (hippocampus and cerebellum) and cyclic GMP levels as well as the increase in TBARS levels (hippocampus) at 4- and 24-month-old animals induced by LPS in rats. Increased levels of NO can result in reactive nitrogen species that can cause protein modifications resulting in protein-resident 3-NT. These nitrated proteins may have altered functions resulting in detrimental effects on cell viability (Butterfield et al., 2011). In agreement with TBARS results, 3-NT protein levels analysis showed that IF reverts the increase in 3-NT proteins induced by LPS in hippocampus of 4- and 24-month-old animals.

In addition, this study is the first evidence that IF can act during aging process since we showed that IF induces adaptive response restoring the glutamate modulatory action on 2,3- Na,K-ATPase at 24-month-old animals. In fact, several studies have shown that IF can protect neurons against degeneration in animal models of injury that involve local inflammatory processes (stroke and seizures) and a model of Alzheimer’s disease (Arunagam et al., 2010; Bruce-Keller et al., 1999; Halagappa et al., 2007).

Our previous study showed that LPS and IF activate nuclear RELA translocation and NF-κB binding activity with a similar pattern in NF-κB nuclear extracts (Vasconcelos et al., 2014). In addition, we showed that LPS response induced an increase in iNOS proinflammatory gene within 2 hours in rat hippocampus at least partially due to NMDA and neuronal NO$ activation (Glezer et al., 2003; Munhoz et al., 2006). However, the present data indicated that IF induced different changes in the expression of NF-κB target genes involved in synaptic plasticity and cell survival linked to a cell specific NF-κB activation compared with LPS, because IF is mediating a shift in LPS-induced proinflammatory gene expression towards an anti-inflammatory signaling cascade. There is evidence supporting a dual role of NF-κB in neurodegenerative diseases in the CNS; activation of NF-κB in neurons promotes their survival, whereas activation in glial and immune cells mediates pathological inflammatory processes (Camandola and Mattson, 2007). Confirming this hypothesis, we found that levels of multiple markers of neuroinflammation induced by LPS via NF-κB activation were reduced in rats on the IF diet compared with those on the control diet, including iNOS and other proinflammatory cytokines in 4-month-old animals (Vasconcelos et al., 2014).

Here we present new evidence that IF inhibits LPS-induced production of proinflammatory iNOS not only at 4- but also in 24-month-old animals. These effects of IF are indicative of decreased activation of inflammatory cascades linked to NF-κB mediating proinflammatory response (Ghosh and Karin 2002; Kawai and Akira 2010). In fact, it is known that high concentrations of NO, unlike low concentrations (Calabrese et al., 2007; Contestabile et al., 2003), may promote excitotoxicity, which may contribute to cognitive impairment (Calabrese et al., 2007; Guix et al., 2005; Moncada and Bolanos, 2006; Zanelli et al., 2006). Our results showed that IF not only reduced the production of oxidative stress, but it was able to mitigate the induction of iNOS expression by LPS at 4 months and during the aging process.

5. Conclusion

Taken together, the present work suggests that IF can be a good strategy to induce an age-related health benefit and by doing it IF can postpone several pathologies in the CNS.

Disclosure statement

The authors have no actual or potential conflicts of interest.

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