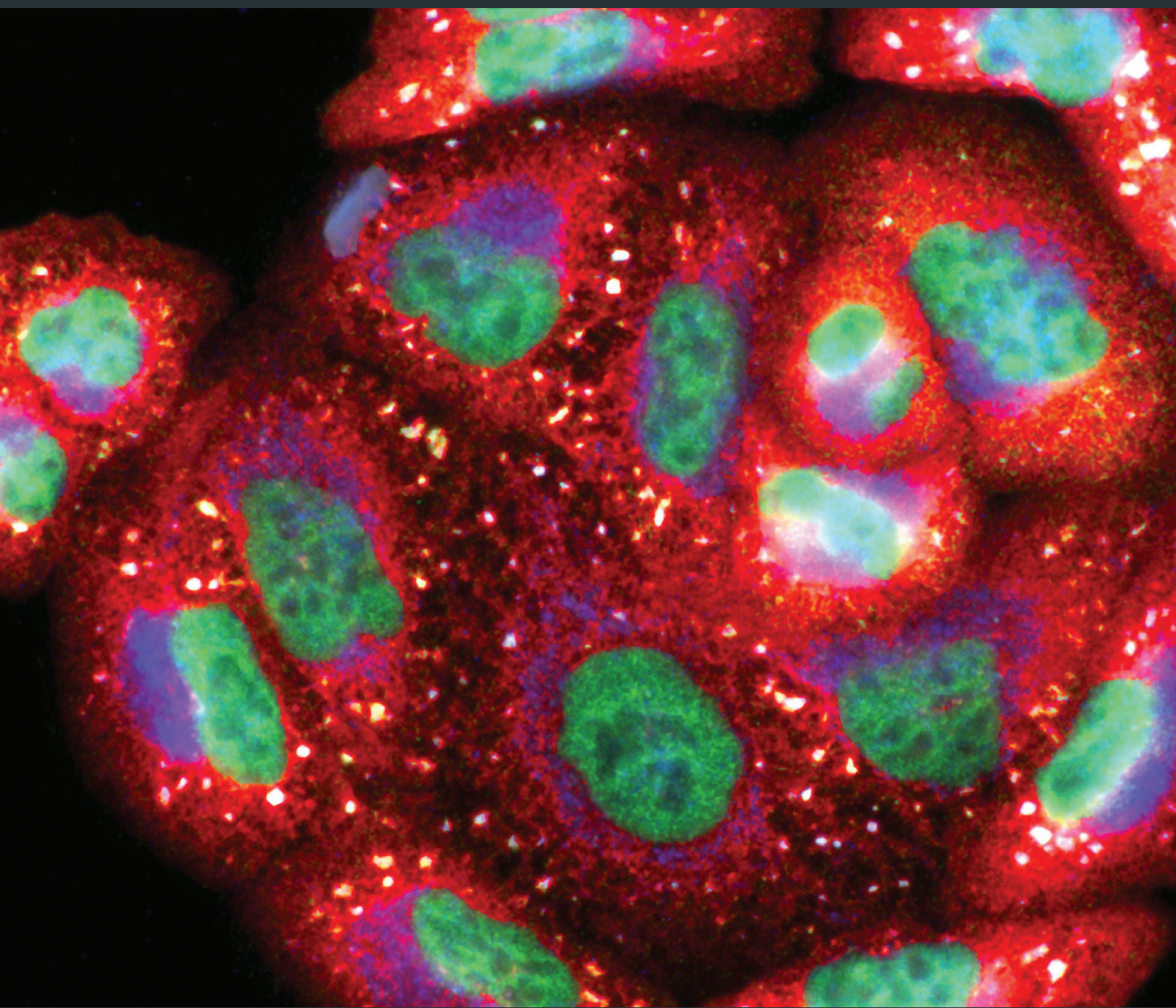
Oxidative Medicine and Cellular Longevity

Oxidative Stress: Neuropathy, Excitability, and Neurodegeneration

Lead Guest Editor: Sebastien Talbot
Guest Editors: Alexandra S. Latini, Réjean Couture, and Maria M. Campos



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Editorial Board

Contents

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Editorial

Oxidative Stress: Neuropathy, Excitability, and Neurodegeneration

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The energy produced from the aerobic cellular metabolism generates free radicals. The imbalance between the production of these radicals and their elimination via antioxidant defenses leads to oxidative stress. The toxicity of these stressors contributes to protein and DNA injury, inflammation, tissue damage, and subsequent cellular apoptosis. Of note, neurons are especially susceptible to reactive oxygen species- (ROS-) mediated damages. Thus, they have a very high demand in energy, a large number of mitochondria, a limited capability to regulate glucose uptake, and weak antioxidant defenses. Alongside environmental and genetic factors, oxidative stress contributes to the onset of various pathologies such as amyotrophic lateral sclerosis, Alzheimer's disease, and Parkinson's disease, as well as diabetic-induced neuropathy and retinopathy. Thus, reduced antioxidant defenses, or the overproduction or incorporation of free radicals from the environment, can lead to neurodegeneration. This special issue on "Oxidative Stress: Neuropathy, Excitability, and Neurodegeneration" aimed at addressing the physiology, the mechanisms, and the impact of oxidative stress in neuron excitability, neurodegeneration, retinopathy, and aging. Overall, this topic compiles seven quality scientific manuscripts including three research articles and four critical reviews, which provide comprehensive evidence demonstrating the therapeutic significance of endogenous and exogenous antioxidants in the control of neurodegenerative pathologies.

Retinal detachment and blindness are increasingly more common and, while photocoagulation and anti-VEGF therapy help control the onset of the disease, these pathologies remain largely intractable. To identify novel therapeutic targets for the treatment of retinopathy of prematurity, diabetic retinopathy, and age-related macular degeneration, Chemtob and colleagues provide a state-of-the-art review addressing the role of oxidative stress and inflammatory processes in ischemic retinopathies. More specifically, they assess how microvascular change-induced ischemia promotes abnormal neovascularization and how antioxidants and anti-inflammatory agents emerged as novel therapeutic targets to reverse these ischemic retinopathies.

Kynurenic acid, one of the tryptophan metabolites, is increased in the presence of free radicals and inflammatory cytokines and was shown to have neuroactive and redox properties. Through its action on NMDA and nicotinic receptors, kynurenic acid is thought to modulate the release of neurotransmitters, and it was shown to regulate inflammation in the context of aging, neurodegeneration, and psychiatric disorders. In this issue, Chavez and colleagues reviewed various alternative mechanisms through which kynurenic acid is produced, exploring the possible involvement of the

D-amino acid oxidase, indole-3-pyruvic acid, and myeloperoxidase pathways, and how such paths may be exploited to prevent neurodegeneration.

The central nervous system possesses various pathways designed to control and protect the host from the excessive production of oxidative stress, one of which is the hydrogen sulfide pathway. Impairment in hydrogen sulfide production was posited to be an early trigger for Alzheimer's, Parkinson's, and Huntington's diseases. The review article by Jung and colleagues discusses the antioxidant roles of hydrogen sulfide; how cystathionine β-synthase, cystathionine γ-lyase, cysteine aminotransferase, and 3-mercaptopyruvate sulfurtransferase enzymatic activities control its production; and how neuroinflammation might be controlled through its antioxidant capability. Among the others, hydrogen sulfide helps maintain CNS homeostasis by shielding neurons against hypoxic injury, preventing hypochlorous acid-mediated oxidative damage, enhancing glutathione generation, and repressing mitochondrial oxidative stress.

The review of Kim and colleagues explores how glutathione S-transferase superfamily of enzymes mediated cellular detoxification of oxidative damage. The omega subfamily (GSTO) is the most recently identified member of this class and appears to be expressed in a wide range of organisms including bacteria, insects, yeasts, mammals, and plants. GSTOs play an essential role in reducing the oxidative stress caused by stressors and cellular processes. Genetic polymorphisms in human GSTO1 and GSTO2 genes have been implicated in neurodegenerative diseases such as Alzheimer's and Parkinson's, and the levels of GSTO2 expression were described to be reduced in patients affected by these pathologies. Altogether, by regulating the MAPK signaling pathway, GstO1 gene helps to prevent neurotoxicity.

The uncoupling proteins (UCPs) are anion-carrier proteins found in the inner membrane of the mitochondria and are involved in the reduction of the transmembrane proton gradient. In this research paper, Antônio Silva Jr and colleagues explored whether UCP2 can protect neurons from epilepsy-induced damage. The authors demonstrated that the UCP2 mRNA expression was transiently increased in the brain post status epilepticus. UCP2 antisense oligonucleotides significantly reduced the transcript expression of UCP2 in SE-silent phase, preventing the expression of p-AKT and antiapoptotic Bcl-2 mRNA. UCP2 silencing also increases oxidative stress levels (reflected by augmented protein carbonyl levels and the activity of the antioxidant enzymes superoxide dismutase, and catalase), the liberation of proinflammatory markers, and enhanced cell apoptosis. Overall, these data revealed that UPC2 might inhibit apoptotic factors and oxidative stress in the pilocarpine model of epilepsy, and, in doing so, reduced neurodegeneration.

Given that polyphenols help to prevent neurodegeneration, Eckert and colleagues assessed how highly purified olive secoiridoids might abolish the cognitive decline typically observed in aged mice. They found that a polyphenol treatment prevented brain ATP reduction, as well as the transcript expression of NADH reductase, cytochrome c oxidase, and citrate synthase. Overall, a diet rich in purified olive polyphenols appears to protect from the decline in spatial working memory and motor coordination observed in aging. Such protective phenomena appear to be mediated by the increase in central ATP levels.

The colostrum is rich in nutrients crucial for newborn development. The latter also contains bioactive peptides, which can help to control the oxidative damage observed in neurodegenerative diseases. In this research manuscript, the group of Prof. Zabłocka presents a novel method to isolate NP-POL nonapeptide from the milk colostrum. Next, by inhibiting ROS overproduction, they found that this peptide protects PC12 cells from 6-hydroxydopamine-induced neurotoxicity. Overall, these results suggest that the NP-POL nonapeptide may help prevent ROS-triggered neurodegeneration.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this article.

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Paula Juliana Seadi Pereira
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Research Article

Effects of Long-Term Treatment with a Blend of Highly Purified Olive Secoiridoids on Cognition and Brain ATP Levels in Aged NMRI Mice

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Aging represents a major risk factor for developing neurodegenerative diseases such as Alzheimer's disease (AD). As components of the Mediterranean diet, olive polyphenols may play a crucial role in the prevention of AD. Since mitochondrial dysfunction acts as a final pathway in both brain aging and AD, respectively, the effects of a mixture of highly purified olive secoiridoids were tested on cognition and ATP levels in a commonly used mouse model for brain aging. Over 6 months, female NMRI mice (12 months of age) were fed with a blend containing highly purified olive secoiridoids (POS) including oleuropein, hydroxytyrosol and oleurosid standardized for 50 mg oleuropein/kg diet (equivalent to 13.75 mg POS/kg b.w.) or the study diet without POS as control. Mice aged 3 months served as young controls. Behavioral tests showed deficits in cognition in aged mice. Levels of ATP and mRNA levels of NADH-reductase, cytochrome-c-oxidase, and citrate synthase were significantly reduced in the brains of aged mice indicating mitochondrial dysfunction. Moreover, gene expression of Sirt1, CREB, Gap43, and GPx-1 was significantly reduced in the brain tissue of aged mice. POS-fed mice showed improved spatial working memory. Furthermore, POS restored brain ATP levels in aged mice which were significantly increased. Our results show that a diet rich in purified olive polyphenols has positive long-term effects on cognition and energy metabolism in the brain of aged mice.

1. Introduction

Aging represents one of the major risk factors for developing neurodegenerative diseases such as Alzheimer's disease (AD). Currently, five million Americans are suffering from dementia, and by 2050, there will be one new case every 66 seconds [1]. The multifactorial pathology makes it difficult to develop feasible therapies, and current approved drugs attenuate symptoms but do not cure the disease. Research into AD also had several failures in terms of developing disease-modifying therapies [2]. Since AD starts many years before the first symptoms occur, new scientific approaches focus on early stages, which are discussed to be important in aging and the onset of AD. In this sense, there is growing interest in dietary patterns, stimulation of the brain, and physical activity as potential modifiable risk factors [3–6]. It has been shown that adherence to a Mediterranean diet (MedDiet) pattern significantly reduces the risk of AD [7, 8], which has been confirmed and recently summarized in reviews and meta-analysis [9–11].

One important component of the MedDiet is a high consumption of extra virgin olive oils (EVOO) [12], and a combination of MedDiet and EVOO seems to better improve cognitive function including improved performance in visual and verbal memory domains [13–15].

EVOO contains several different polyphenols [12] including secoiridoid derivatives, phenolic alcohols, and lignans as well as flavonoids which seem to have neuroprotective properties on the aging process [16–18]. Hydroxytyrosol and oleuropein are two of the main antioxidative compounds

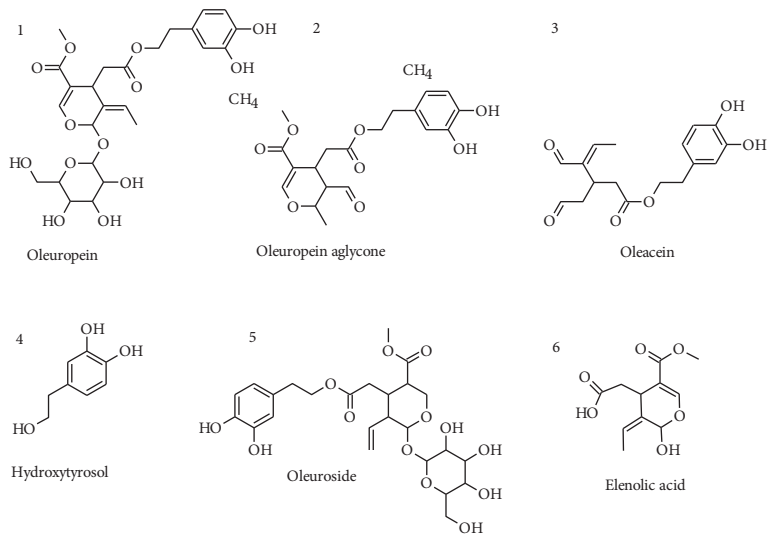

Figure 1: Secoiridoid derivatives (1–5) and their degradation products are the predominant phenolic compounds present in EVOO and in the tested POS. Secoiridoids are characterized by the presence in their molecules of elenolic acid (6) or its derivatives.

present in olives [19–22] providing neuroprotection [22–26]. Thus, olive polyphenols are proposed as new promising agents to combat aging-associated neurodegeneration [27].

Using a recently developed technology, olive polyphenols were isolated and highly purified from olive leaves that represent a rich source of bioactive ingredients [28]. We investigated the effects of a mixture of six highly purified secoiridoid polyphenols (Figure 1) on cognition and brain energy metabolism in aged NMRI mice. Expression of genes involved in longevity, mitochondrial biogenesis and function, synaptic plasticity, and antioxidative properties was determined to elaborate molecular mechanisms. Effects on ATP levels were confirmed in neuronal SH-SY5Y cells.

2. Materials and Methods

2.1. Cell Culture. SH-SY5Y cells were cultured in Dulbecco's Modified Eagle Medium supplemented with 10% heat-inactivated fetal calf serum, 0.3 mg/mL hygromycin, 60 units/mL penicillin, 60 μg/mL streptomycin, 4500 mg/L D-glucose, MEM Vitamin solution, MEM Nonessential Amino Acids, and 1 mM sodium pyruvate at 37°C in a humidified incubator containing 5% CO_2.

Two days prior to incubation, cells were seeded into 96-well plates (20,000 cells/well). Cells were incubated with the respective POS concentrations (0.001 nM–10 μM dissolved in DMEM) for 24 h (basal ATP levels) or preincubated with POS for 1 h and insulted with rotenone (25 μM) for 24 h (ATP levels after insult). The emitted light is linear to the ATP (Lonza, Switzerland) concentration and was measured with a VICTOR™ X2 Multilabel Plate Reader (Perkin Elmer).

2.2. Animals and Treatment. Female NMRI (Navar Medical Research Institute) mice, a commonly used aging model, were purchased from Charles River (Sulzbach, Germany) and were housed according to the German guidelines for animal care with access to water and food ad libitum. Mice were maintained on a 12 h light/dark cycle until they reached the age of 12 months. Young (3 months old) NMRI mice served as the control group. Mice were fed with a well-proven C1000 standardized diet (Altromin, Lage, Germany), C1000 containing vitamin A (2500 IU/kg), vitamin E (20 mg/kg), and selenium (150 mcg/kg) [29, 30]. The verum group received the identical diet supplemented with a blend of highly purified secoiridoid polyphenols for 6 months. Based on the average food consumption, a daily intake of 13.75 mg POS/kg b.w. was calculated. Feeding studies in mice reporting biological effects applied olive polyphenols in a dose range of 1 to 10 mg/kg b.w. [26, 31–33]. The estimated daily POS dose (13.75 mg/kg b.w.) in the current study equals a single human dose of approximately 1.1 mg/kg b.w/day [34]. The feeding period of the young control mice started 3 months later than the feeding period of aged mice to ensure that both of them end at the same time point. Behavioral testing was performed before the starting points and at the end of the feeding period. On the basis of behavioral testing at the beginning, mice were divided into 2 groups of the same performance level. Mice were killed by cervical dislocation and decapitation. Brains were quickly dissected on ice after the removal of the cerebellum, the brain stem, and the olfactory bulb. All experiments were carried out by individuals with appropriate training and experience according to the requirements of the Federation of European Laboratory Animal Science Associations and the European Communities Council Directive (Directive 2010/63/EU). Experiments were approved by the regional authority (Regierungspraesidium Darmstadt; #V54–19 c 20/15–FU/1062).

2.3. Composition of the POS. The POS was provided from N-Zyme Biotec GmbH, Darmstadt, Germany. The composition is listed in Table 1.

2.4. Passive Avoidance Test. The test was conducted using a passive avoidance step-through system (cat. no. 40533/mice Ugo Basile, Gemonio, Italy) and a protocol similar to the

Table 1: Composition of the highly purified secoiridoid-rich extract (POS), manufactured by N-Zyme Biotec GmbH, Darmstadt, Germany.

Secoiridoid derivative	Content (%)
Oleuropein aglycone	36.7
Hydroxytyrosol	24.7
Oleacein	14.6
Elenolic acid derivatives	10.5
Oleuropein	7.2
Oleurosid	6.2

protocol published by Shiga et al. [35]. On the first day of the experiment, the mouse was put into the light chamber (light intensity of 1350 lux). After 30 s, the door toward the dark chamber was opened, and time till entering the dark chamber was measured. In the dark chamber, the mouse received an electric shock (0.5 mA, 1 s duration). The test was stopped if the mouse did not enter the dark chamber after 180 s. The test was repeated after 24 h with the door opening towards the dark chamber after only 5 s. Again, time till entering the dark chamber was recorded. This time no electric shock was applied after crossing the door. The test was stopped after 300 s.

2.5. One-Trial Y-Maze Test. One-trial Y-Maze test was conducted using a custom-made Y-Maze (material: polyvinyl chloride, length of arms: 36 cm, height of arms: 7 cm, width of arms: 5 cm, and angle between arms: 120°). At the beginning of the test, the mouse was put into one of the three arms of the Y-Maze and the sequence of the entries was recorded for 5 min. Spontaneous alternation was determined using the formula (number of alternations/number of entries)/2 [36].

2.6. Preparation of Dissociated Brain Cells for the Measurement of the Mitochondrial Membrane Potential and Determination of ATP Level. One hemisphere was used to prepare dissociated brain cells (DBCs) for ex vivo studies according to the method of [30]. DBCs were resuspended in 4.5 mL DMEM without supplements. For the measurement of ATP levels, DBCs were seeded in 50 μL aliquots into a 96-well plate. Cells were incubated for 3 h in a humidified incubator (5% CO_2). Respectively, 6 wells were incubated for 3 h with sodium nitroprusside (0.5 mM for ATP measurement) in DMEM. The remaining cell suspension was reserved for protein determination (stored at −80°C).

2.7. Protein Quantification. Protein content was determined using Pierce™ Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA). Instructions were followed as given by the manufacturer.

2.8. Glutathione Peroxidase Activity. Glutathione peroxidase activity was determined using a commercially available assay kit (kit number: ab102530; Abcam Plc., Cambridge, UK). 50 mg of frozen brain tissue was used as described in the manufacturer's manual. Glutathione peroxidase generates GSSG from GSH during H_2O_2 reduction, and the generated GSSG is reduced back to GSH by glutathione reductase during consumption of NADPH. The reduction of NADPH is proportional to glutathione peroxidase activity; thus, it can be measured calorimetrically at 340 nm.

2.9. Citrate Synthase Activity. Citrate synthase activity was determined photometrically in isolated brain mitochondria as recently described in Hagl et al. [37].

2.10. Isolation of Brain Mitochondria and Measurement of Complex I and IV Activities. Half a brain hemisphere (the frontal part) was used to isolate brain mitochondria. The protocol is described in Hagl et al. [37]. The pellet obtained from the last centrifugation step was dissolved in 250 μL MIRO5. 80 μL of the resulting cell suspension was injected into the Oxygraph 2k-chamber. A complex protocol (elaborated by Prof. Dr. Erich Gnaiger) was used to investigate the function of the respiratory chain complexes. The capacity of the oxidative phosphorylation (OXPHOS) was determined using complex I-related substrates pyruvate (5 mM) and malate (2 mM) and ADP (2 mM) followed by the addition of succinate (10 mM). Mitochondrial integrity was measured by the addition of cyctochrome c (10 μM). Oligomycin (2 μg/mL) was added to determine leak respiration (leak (omy)), and afterwards, uncoupling was achieved by carbonyl cyanide p-(trifluoromethoxy) phenyl-hydrazone (FCCP, injected stepwise up to 1–1.5 μM). Complex II respiration was measured after the addition of rotenone (0.5 μM). Complex III inhibition was achieved by the addition of antimycin A (2.5 μM) and was subtracted from all respiratory parameters. COX activity was measured after ROX determination by applying 0.5 mM tetramethylphenylenediamine (TMPD) as an artificial substrate of complex IV and 2 mM ascorbate to keep TMPD in the reduced state. Autoxidation rate was determined after the addition of sodium azide (>100 mM), and COX respiration was additionally corrected for autoxidation.

2.11. Gene Expression Analysis by Quantitative Real-Time PCR (qRT-PCR). Total RNA was isolated using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions using ~20 mg RNAlater stabilized samples (Qiagen, Hilden, Germany). RNA was quantified measuring the absorbance at 260 and 280 nm using the NanoDrop™ 2000c spectrometer (Thermo Fisher Scientific, Waltham, MA, USA). RNA purity was assessed using the ratio of absorbance 260/280 and 260/230. To remove residual genomic DNA, samples were treated with a TURBO DNA-free™ kit according to the manufacturer's instructions (Thermo Fisher Scientific, Waltham, MA, USA). Complementary DNA was synthesized from 250 ng total RNA using the iScript cDNA Synthesis Kit (Bio-Rad, Munich, Germany) according to the manufacturer's instructions and was stored at −80°C. qRT-PCR was conducted using a CfX 96 Connect™ system (Bio-Rad, Munich, Germany). Oligonucleotide primer sequences, primer concentrations, and product sizes are listed in Table 2. All primers were received from Biomol. cDNA for qRT-PCR was diluted 1 : 10 with RNase-free water (Qiagen, Hilden, Germany), and all samples were performed in

Table 2: Oligonucleotide primer sequences, product sizes, and primer concentrations for quantitative real-time PCR. bp: base pairs; conc: concentration.

Primer	Sequence	Manufacturer	Product size (bp)	Conc. (μM)
AMPK (beta subunit)	5′-agtatcacggtggttgctgt-3′ 5′-caaatactgtgcctgcctct-3′	Biomol Hamburg, Germany	190	0.1
B2M	5′-ggcctgtatgctatccagaa-3′ 5′-gaaagaccagtccttgctga-3′	Biomol, Hamburg, Germany	198	0.4
CI	5′acctgtaaggaccgagaga-3′ 5′-gcaccacaaacacatcaaaa-3′	Biomol, Hamburg, Germany	227	0.1
CIV	5′-ctgttccattcgctgctatt-3′ 5′-gcgaacagcactagcaaaat-3′	Biomol, Hamburg, Germany	217	0.1
CS	5′-aacaagccagacattgatgc-3′ 5′-atgaggtcctgctttgtcct-3′	Biomol, Hamburg, Germany	184	0.1
GAP43	5′ agggagatggctctgctact-3′ 5′ gaggacggggagttatcagt-3′	Biomol Hamburg, Germany	190	0.15
GPx-1	5′-gtccagcgtgtatgccttct-3′ 5′-ctcctggtgtccgaactgat-3′	Biomol, Hamburg, Germany	217	0.1
PGK1	5′-gcagattgtttggaatggtc-3′ 5′-tgctcacatggctgacttta-3′	Biomol, Hamburg, Germany	185	0.4
Sirt1	5′-gtgagaaaatgctggcctaa-3′ 5′-ctgccacaggaactagagga-3′	Biomol, Hamburg, Germany	161	1
SOD2	5′-acagcgcatactctgtgtga-3′ 5′-gggggaacaactcaactttt-3′	Biomol, Hamburg, Germany	183	0.1
Synaptophysin 1	5′-tttgtggttgttgagttcct-3′ 5′-gcatttcctccccaaagtat-3′	Biomol, Hamburg, Germany	204	0.1

triplicate. PCR cycling conditions were an initial denaturation at 95°C for 3 min, followed by 45 cycles of 95°C for 10 s, 58°C for 45 s, and 72°C for 29 s. Gene expression was analyzed using the $-(2\Delta\Delta C_q)$ method using BioRad CfX manager software and was normalized to the expression levels of beta 2 microglobulin (B2M) and phosphoglycerate kinase 1 (PGK1).

2.12. Statistics. Unless otherwise stated, values are presented as mean ± standard error of the mean (SEM). Statistical analyses were performed by applying one-way ANOVA with Bonferroni's multiple comparison posttest (Prism 7.0 GraphPad Software, San Diego, CA, USA). Statistical significance was defined for p values of <0.05.

3. Results

Female NMRI mice were fed over 6 months with a standardized pelleted diet (aged control) or diet supplemented with a blend of highly purified secoiridoid polyphenols (13.75 mg POS/kg b.w.) (aged + POS) for 6 months. Young control mice (young control) received a standardized pelleted diet for 3 months. There was no significant difference in body weight and life span between the aged control and the aged intervention group mice. At the end of the feeding period, cognitive function and brain mitochondrial function were assessed.

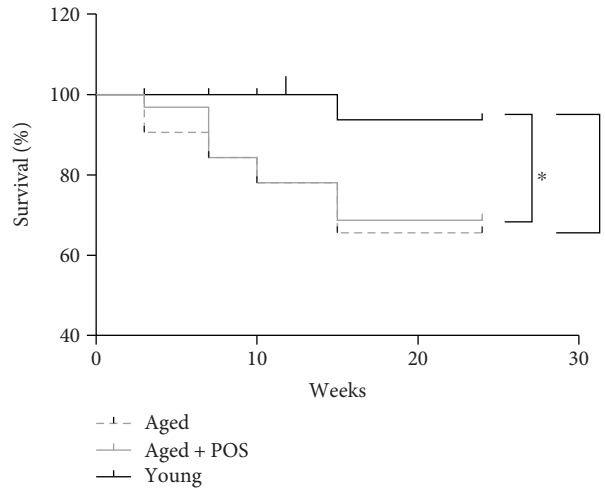

Figure 2: Survival rates of NMRI mice after feeding with or without POS. Aged (12 months old) mice were fed with a standardized pelleted diet (aged) or pelleted diet containing POS (13.75 mg POS/kg b.w., aged + POS) for 6 months. As further control, young mice (3 months old, starting point of the analysis: 12 weeks) were fed with a pelleted standard diet for 3 months (young); $n = 15 - 27$; mean without SEM; log-rank (Mantel-cox) test; $^*P < 0.05$.

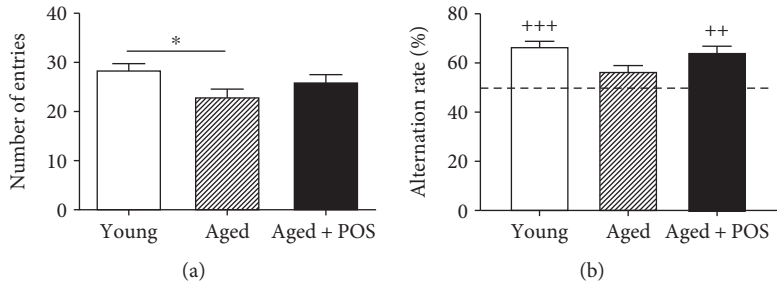

Figure 3: Y-Maze spontaneous alternation of young, aged, and POS-treated mice (13.75 mg/kg b.w.) during a 5 min period time of testing. Number of entries (a) and alternation rate (b); $n = 16$ mean ± SEM, one-way ANOVA with Bonferroni posttest; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. Alternation rate (c) was compared to a theoretical value of 50% using a univariate t-test with $^{++}P < 0.01$ and $^{+++}P < 0.001$.

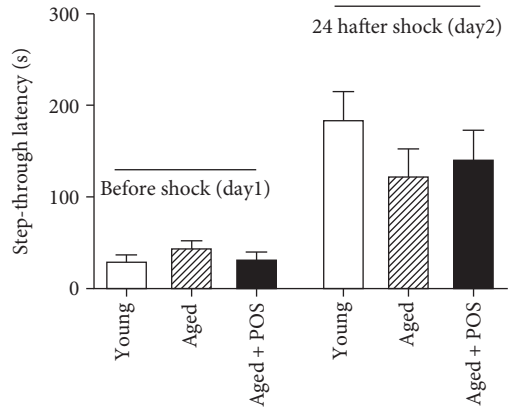

Figure 4: Passive avoidance test with young, aged, and long-term POS-treated mice. On day one, mice receive an electric shock (0.5 mA) and time is recorded when the mouse needs to enter into the dark chamber; 24 h after the first testing period, the test is repeated and time is recorded when the mouse needs to reenter the dark chamber; $n = 16$; mean ± SEM.

3.1. Survival. Survival rates of young and aged control mice were 93 and 66% ($P < 0.05$), while the survival rate of aged mice administrated with POS was 69% ($P < 0.05$). Thus, POS treatment did not increase the survival rate over a 6-month feeding period (Figure 2).

3.1.1. Behavioral Testing. In the Y-Maze test, aged control mice showed a significantly decreased alternation rate (56.7 ± 2%) and number of entries (23 ± 1.5) during a 5 min testing phase compared to young controls (Figure 3(b)). POS administration for 6 months significantly increased the alternation rate (64 ± 2%) and slightly but not significantly increased the number of entries (26 ± 1) (Figure 3(a)).

On day one, aged control mice showed a slightly but not significant longer latency time to enter into the dark chamber (45 ± 7 s) compared to young control animals (31 ± 6 s) in the passive avoidance test. POS-treated mice showed almost the same latency time as young mice (33 ± 7 s). On day two, aged mice showed a numerically shorter time to reenter the dark chamber (123 ± 29 s) compared to young control mice (185 ± 29 s). POS treatment for 6 months led to a slightly but not significant increased step-through latency time (142 ± 31 s) (Figure 4).

3.1.2. Effect of Long-Term POS Treatment on Brain ATP Levels. Basal ATP levels were measured in dissociated brain cells (DBCs) of young, aged, and POS-treated mice. Aged control mice showed significantly lower ATP level (1.41 ± 0.05 nmol/mg protein) in contrast to young animals (1.75 ± 0.01 nmol/mg protein) which were restored after long-term treatment with POS (1.74 ± 0.1 nmol/mg protein; Figure 5(a)). Furthermore, DBCs were incubated for 3 h with sodium nitroprusside (SNP) to examine the resistance against nitrosative stress. However, no differences were detected after SNP incubation between young (74.4 ± 2.2%), aged (74.7 ± 1.3%), and POS-treated aged mice (81.3 ± 2.2%). Long-term POS treatment resulted in a slight increase of ATP concentrations after SNP insult which did not reach a level of significance (Figure 5(b)). To confirm the effects of POS on ATP levels in vitro, SH-SY5Y cells were incubated with different concentrations of POS. A POS concentration of already 0.1 nM significantly enhanced basal ATP levels (Figure 6(a)). At this concentration, SH-SY5Y cells were also protected from nitrosative stress induced by SNP (Figure 6(b) and Table 3).

Expression of genes involved in longevity, mitochondrial biogenesis and function, synaptic plasticity, and antioxidative properties was determined in young, aged, and POS-treated mice to elaborate molecular mechanisms. All considered genes showed significantly decreased mRNA levels after aging with the exception of SOD2. Long-term POS treatment did not show any significant effects on mRNA expression levels between aged and POS-treated mice (Table 4).

3.2. Activities of Complex I, Complex IV, GPx-1, and CS. In comparison to young control animals, activities of the respiratory chain complexes I and IV and CS activity were unaffected during the aging process and after long-term POS treatment in isolated brain mitochondria. Furthermore, we measured the activities of the antioxidative enzyme GPx-1 in total brain homogenate. The activity of GPx-1 was numerically but not significantly reduced in aged control animals compared to young mice (Table 5).

4. Discussion

In the current study, the effects of long-term feeding of a blend with highly purified olive secoiridoids on cognition

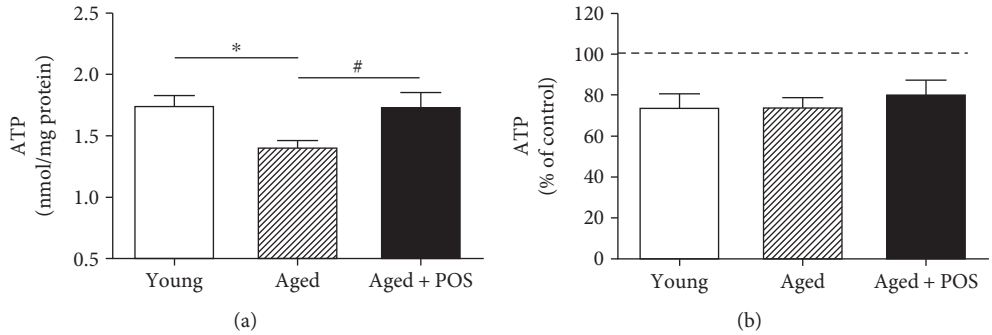

Figure 5: Basal ATP level (a) and ATP concentrations after insult with sodium nitroprusside (SNP, 3 h, 0.1 mM) (b) of dissociated brain cells (DBCs) from young, aged, and POS-treated mice; basal ATP concentrations served as control for normalization in (b); $n = 10$; mean ± SEM; one-way ANOVA with Bonferroni posttest; $^{*}P < 0.05$ vs. young; $^{\#}$one-way ANOVA aged vs. aged + POS with $^{\#}P < 0.05$.

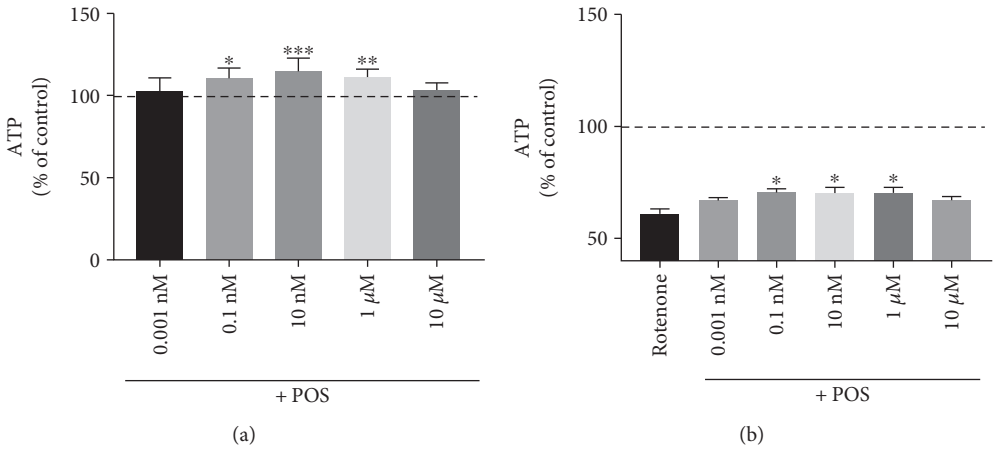

Figure 6: Basal ATP level (a) and ATP concentrations after incubation for 24 h in SY5Y-Mock cells with POS of different concentrations (0.001 nM–10 μM) and insult with rotenone 250 nm (b) in SH-SY5Y control cells; basal ATP concentrations served as control, $n = 7$, mean ± SEM, one-way ANOVA with Bonferroni posttest; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

Table 3: Basal ATP level and ATP concentrations after incubation for 24 h in SY5Y-Mock cells with POS of different concentrations (0.001 nM–10 μM) and after insult with rotenone (250 nM) in SH-SY5Y-Mock cells; basal ATP concentrations served as control, $n = 7$, mean ± SEM, one-way ANOVA with Bonferroni posttest; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

Correlated values	ATP (% of control)	ATP after insult (% of control)
0.001 nM vs. control	102.6 ± 2.9	66.9 ± 1.2
0.1 nM vs. control	110.6* ± 2.0	70.6* ± 1.5
10 nM vs. control	114.9*** ± 3.3	70.3* ± 2.56.1
1 μM vs. control	111.3** ± 1.8	70.4* ± 2.4
10 μM vs. control	103.4 ± 1.7	67.1 ± 1.6

and brain ATP levels were tested in aged female NMRI mice. This strain represents a well-established model for aging studies [30, 38–40]. The results show that a diet rich in purified olive polyphenols has positive long-term effects on cognition and energy metabolism in the brain of aged mice.

4.1. Cognitive Performance in Aged and POS-Treated NMRI Mice. Aged NMRI mice showed deficits in spatial working memory and mobility which is in agreement with earlier studies [30, 38, 41]. Our findings indicated beneficial effects of POS on spatial learning memory and mobility. In agreement with our findings, administration of olive polyphenols has been associated with the improvement of cognitive functions [26, 42, 43].

Pitozzi et al. investigated the effects of long-term dietary administration of EVOO rich in polyphenols in aged C57Bl/6J mice [43]. Comparable to our study, mice were fed from middle age to senescence (total polyphenol dose/day of 6 mg/kg b.w.), and results showed improved contextual memory and prevention of the age-related impairment in motor coordination [43]. EVOO containing different concentrations of polyphenols (e.g., tyrosol, hydroxytyrosol, verbascoside, and oleuropein di-aldehyde) induced similar beneficial effects at a comparable dose as pure oleuropein [42]. Synaptophysin 1 (Syp1) and growth-associated protein 43 (GAP43) are involved in neuronal plasticity and cognition [44, 45]. However, mice lacking SYP1 show significantly reduced learning behavior [46], and enriched environment has been reported to have positive effects on SYP1 brain

Table 4: Relative normalized mRNA expression levels in brain homogenate from aged and aged POS-treated mice determined using quantitative real-time PCR in comparison to young control animals; mRNA expression of young control mice is 100%; $n = 9$; mean ± SEM with one-way ANOVA and Bonferroni posttest with $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; results are normalized to the mRNA expression levels of beta 2 microglobulin (B2M) and phosphoglycerate kinase 1 (PGK1).

	Aged	Aged + POS
AMP-activated protein kinase (beta subunit)	66.19* ± 6.00	72.63 ± 8.38
cAMP response binding protein 1 (CREB1)	64.27** ± 4.29	63.28** ± 5.94
Citrate synthase (CS)	66.75* ± 4.92	61.35*** ± 9.16
Complex I (CI)	75.16* ± 3.34	67.53** ± 7.60
Complex IV (CIV)	58.89** ± 5.80	60.97** ± 8.00
Glutathione peroxidase 1 (GPx-1)	67.65* ± 8.76	58.78** ± 8.18
Growth-associated protein 43 (GAP43)	63.43* ± 9.23	56.73** ± 8.13
Sirtuin 1 (Sirt1)	74.71* ± 4.65	75.36** ± 5.99
Superoxide dismutase 2 (SOD2)	97.23 ± 5.26	88.46 ± 10.39
Synaptophysin 1 (SYP1)	83.23 ± 6.08	72.26* ± 10.43

levels [47]. GAP43 is a nervous tissue-specific protein and is mainly involved in development and axonal remodelling in adult brains [48]. Recently, we have reported that mRNA levels of those two proteins were significantly decreased in the brains of aged NMRI mice [49], which is confirmed by our recent data. These findings indicate less synaptic plasticity and remodelling in the brains of aged NMRI mice which might be responsible for age-related cognitive decline in memory and motor performance [50, 51].

4.2. Brain ATP Levels in Aged and POS-Treated NMRI Mice. The high-energy compound ATP is the key energy source in eukaryotic cells, which is mainly generated in the mitochondria by oxidative phosphorylation (OXPHOS). The mammalian OXPHOS system comprises five large complexes (including NADH oxidoreductase, succinate reductase, cytochrome c oxidoreductase, cytochrome c reductase, and ATP synthase) at the inner mitochondrial membrane [52]. In DBCs isolated from the brains of aged NMRI mice, significantly lower ATP level was determined compared to that of young controls. This finding is in agreement with earlier reports relating lower ATP levels to an impairment of CI and CIV of the OXPHOS system [30, 49, 53, 54]. Accordingly, our current data show significantly decreased expression levels of CI and CIV which also have been reported recently [30]. Long-term treatment with POS significantly improved ATP levels in the DBC of aged NMRI mice, an effect that has not been reported yet for olive polyphenols *in vivo*. POS also improved ATP levels in neuronal SH-SY5Y cells. Recent studies indicate that a mixture of 6 polyphenols (tannic acid, resveratrol, quercetin, rutin, gallic acid, and morin) is able to increase ATP levels during age-related hearing loss [55] in female rats and in the brains of a transgenic mouse model of AD [56]. In a previous study, we showed that short-term administration of hydroxytyrosol-rich olive mill waste water extract (HTRE) to NMRI mice significantly enhanced the mitochondrial membrane potential in DBC isolated from treated mice [23]. In the same study, DBCs were treated with HTRE *in vitro*, and a concentration-dependent improvement of the MMP was detected. In this study, ATP levels were not determined. However, the MMP is the driving force for complex V of the mitochondrial respiration chain (CV; F0/F1-ATPase) to generate ATP [52]. In a following study, HTRE was tested in PC12 cells and HTRE or purified hydroxytyrosol (HT) neither improved MMP nor ATP levels, indicating a different mode of action for POS and HTRE. However, both HTRE and HT protected MMP and ATP levels in PC12 cells from nitrosative stress in a concentration-dependent manner [57]. In the current study, we only detected a numerical increase of ATP levels after SNP insult in DBCs isolated from the brains of POS-treated mice. This result also indicates that POS did not provide antioxidative properties in our current study. Accordingly, POS did not improve mRNA levels and enzyme activity of GPx-1, which is involved in the endogenous response against oxidative stress in the central nervous system [58]. Sirt1 and AMPK are important players in mitochondrial biogenesis since they activate peroxisome proliferator receptor gamma coactivator 1-α (PGC1-α) [59]. PGC1α itself is activated by deacetylation via sirtuins (SIRT) and phosphorylation via AMP-activated protein kinases (AMPK). Furthermore, phosphorylated cAMP response element-binding protein (CREB) can induce gene expression of PGC1α. PGC1α facilitates the expression of transcription factors nuclear respiratory factor 1 (Nrf1) and mitochondrial transcription factor A (Tfam) which in turn induces mitochondrial biogenesis. POS treatment did not influence the expression levels of Sirt1 and AMPK. Additionally, we measured citrate synthase activity in isolated mitochondria which is a marker for the determination of mitochondrial content [60]. Significant lower citrate synthase (CS) mRNA expression was determined in the brains of aged and aged + POS-fed NMRI mice whereas CS in isolated mitochondria was unaffected (Table 5) indicating that other molecular mechanisms were responsible for the improvement of cognition. Possibly, the increased ATP levels and the resulting improvement of cognition are a consequence of an enhanced glycolysis which supports important functions such as neuroprotection and dramatically decreases with age [61]. Typically, glucose-6-phosphatase converts glucose into pyruvate which generates two molecules of ATP. Thus, future studies should determine the levels of glucose, lactate, glucose-6-phosphatase, and pyruvate to confirm this hypothesis.

5. Conclusion

Long-term feeding of a blend containing highly purified secoiridoid polyphenols (POS) provided beneficial effects on spatial working memory and motor coordination which were probably mediated by the increased ATP brain levels.

Table 5: Activities of the respiratory chain complexes I and IV in isolated mitochondria of young, aged, and aged mice fed with POS determined using an Oxygraph-2k; $n = 10$, mean ± SEM. GPx-1 was measured in brain homogenate using a calorimetric kit; $n = 6 \pm$ SEM. CS activity was measured in isolated mitochondria; $n = 10 \pm$ SEM.

Correlated values	CI activity [(pmol/s*IU CS)]	CIV activity [(pmol/s*IU CS)]	GPx-1 activity (mU/mL)	CS activity (IU/mg protein)
Young vs. aged	1545 ± 101 vs. 1625 ± 80	5487 ± 157 vs. 5673 ± 191	1139 ± 50 vs. 1032 ± 48	927 ± 108 vs. 898 ± 104
Aged vs. aged + POS	1625 ± 80 vs. 1587 ± 108	5673 ± 191 vs. 5806 ± 145	1032 ± 48 vs. 1116 ± 60	898 ± 104 vs. 843 ± 118

Therefore, POS might represent a suitable nutraceutical for age-related cognitive decline.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that they have no conflict of interest.

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Supplementary Materials

The supplementary materials include 11 tables showing the complete statistical results including *F* value, degrees of freedom, and *P* value. Supplementary Table 3a: complete statistical results including *F* value, degrees of freedom, and *P* value of number of entries in Y-Maze spontaneous alternation test of young, aged, and POS-treated mice. Supplementary Table 3b: complete statistical results including *F* value, degrees of freedom, and *P* value of alternation rate in Y-Maze spontaneous alternation test of young, aged, and POS-treated mice. Supplementary Table 4: complete statistical results including *F* value, degrees of freedom, and *P* value of number of entries in Passive Avoidance test of young, aged, and POS-treated mice. Supplementary Table 5a: complete statistical results including *F* value, degrees of freedom, and *P* value of basal ATP level in DBCs of young, aged, and POS-treated mice. Supplementary Table 5b: complete statistical results including *F* value, degrees of freedom, and *P* value of ATP concentration in DBCs of young, aged, and POS-treated mice after insult with sodium nitroprusside. Supplementary Table 6a: complete statistical results including *F* value, degrees of freedom, and *P* value of basal ATP concentrations in SY5Y-Mock cells after incubation with POS. Supplementary Table 6b: complete statistical results including *F* value, degrees of freedom, and *P* value of ATP concentration in SY5Y-Mock cells after insult with rotenone. Supplementary Table 7a: complete statistical results including *F* value, degrees of freedom, and *P* value of complex I activity in isolated brain mitochondria of young, aged, and POS-treated mice. Supplementary Table 7b: complete statistical results including *F* value, degrees of freedom, and *P* value of complex IV activity in isolated brain mitochondria of young, aged, and POS-treated mice. Supplementary Table 7c: complete statistical results including *F* value, degrees of freedom, and *P* value of GPx-1 activity in brain homogenate of young, aged, and POS-treated mice. Supplementary Table 7d: complete statistical results including *F* value, degrees of freedom, and *P* value of citrate synthase activity in isolated brain mitochondria of young, aged, and POS-treated mice. *(Supplementary Materials)*

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Research Article

Protective Role of UCP2 in Oxidative Stress and Apoptosis during the Silent Phase of an Experimental Model of Epilepsy Induced by Pilocarpine

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Neuroprotection is a desirable process in many neurological disorders, yet complex mechanisms involved in this field are not completely understood. The pilocarpine epilepsy model causes potent, seizure-induced excitotoxicity cell death and mitochondria impairment. The present study is aimed at investigating the role of UCP2, a ROS negative regulator, in the neuroprotection after cholinergic insult. Our data demonstrated that UCP2 expression was augmented in the rat hippocampus 3 days after *status epilepticus* (SE), reaching a peak on the fifth day, then returning to basal levels. Concomitantly, phospho-AKT expression levels were higher in the hippocampus during the early silent phase (5 days after SE). Additionally, it was demonstrated that the blockade of UCP2 by antisense oligonucleotides (ASO) in SE rats successfully diminished both UCP2 mRNA and protein contents. SE ASO rats presented increased mitochondrial proapoptotic factor expression, caspase-3 activity, inflammatory cytokine expression, and ROS formation. Moreover, ASO treatment diminished p-AKT expression and antioxidant enzyme activities after pilocarpine insult. In conclusion, the present results highlight the neuroprotective actions of UCP2, acting in the inhibition of apoptotic factors and oxidative stress, to increase neuron survival after SE onset.

1. Introduction

Mitochondria are energy-producing organelles widely involved in cell homeostasis maintenance and have been described as a potential site for the intricate events that result in pathological disorders and cell death. The uncoupling proteins (UCPs) are anion-carrier proteins found in the inner membrane of the mitochondria and are involved in diminishing the transmembrane proton gradient [1]. This activity reduces the drive for ROS production and consequently decreases cell death [2, 3]. Thus far, five UCP isoforms have been described based on their sequence homology with UCP1 and their distinct functions [4].

UCP2 is one member of this family and is widely expressed in neurons and immune cells [5–7]. Increased UCP2 expression was observed in immune and nonimmune cells during pathological states such as atherosclerosis [8], type I diabetes [9], infections [10], cerebral ischemia [11], and experimental autoimmune encephalomyelitis (EAE) [12]. Several pathophysiological conditions might generate stimuli that can lead to increased UCP2 expression, resulting in a neuroprotection process. UCP2 mitigates reactive oxygen

species (ROS) production, therefore protecting these cells from the damage of oxidative stress [2, 5, 13]. UCP2 has been implicated in intracellular calcium regulation, ATP production, synaptic transmission, neuronal plasticity, and apoptosis [5, 6, 14, 15].

Inflammatory signaling activation is an important mechanism leading to augmented ROS production and, consequently, to incremental cell death in several cell types, including neurons [16–18]. In experimental epilepsy, after the insult that generates seizures, a potent inflammatory state and significant neuronal death can be observed [19–21]. UCP2 is considered as an important neuroprotective element in many inflammatory and degenerative states of the central nervous system [22], especially for its ability to decrease reactive oxygen species [12].

For more than a decade, our group has been dedicated in studying the role of inflammation in the pathophysiology of epilepsy, which is supported by the findings that interleukins and vasoactive peptide system (kallikrein-kinin and renin-angiotensin) components have distinct actions protecting or worsening seizure activity in both mesial temporal lobe epilepsy (MTLE) patients and experimental animal models [23–26]. The acute administration of high-dose of pilocarpine in rats is an experimental model that has revealed alterations that are comparable to those in human TLE (for review, see [27]). Besides that the mechanism of pilocarpine-induced neurotoxic seizures is well established, it is presently hypothesized that excitotoxicity inflicted by *status epilepticus* (SE) induced by pilocarpine results in pathological increases in neuronal lesions in response to excessive ROS production [23, 24, 26].

To our knowledge, few studies show a relation between seizure activity and UCP2 expression in epilepsy or its implication in apoptosis activation [28–31]. In the present study, we tested the hypothesis that UCP2 can act as an endogenous protective factor against epilepsy-induced damage, using antisense oligonucleotide (ASO) administration, in an experimental pilocarpine model.

2. Methods

2.1. Experimental Groups. We used thirty male Wistar rats (200–250 g) that were randomized into 3 experimental groups. The experimental protocol was approved by the Committee on the Ethics of Animal Experiments of Universidade Nove de Julho (0034/2012). Rats were anesthetized with intraperitoneal (ip) sodium pentobarbital (50 mg/kg), and then the animals received a single dose of pilocarpine (350 mg/kg, ip). To prevent peripheral cholinergic effects, scopolamine methyl nitrate was injected subcutaneously at a dose of 1 mg/kg, 30 min before pilocarpine administration. A group of animals ($n = 5$) was killed 5 h after *status epilepticus* onset (5 h SE, the acute group). Another group ($n = 5$) was killed during the seizure-free period (5 days after SE onset, the silent group), and the last set ($n = 5$) was killed 60 days after SE induction (period of spontaneous recurrent seizures, the chronic group). Saline-treated animals ($n = 15$) were killed 5 h, 5 days, or 60 days after saline and scopolamine methyl nitrate injections and were used as control (control groups). Seizures were observed and scored using the Racine scale [32] for 4 h and then rats received a 4 mg/kg dose of diazepam to terminate SE. To verify UCP2 expression in a time course protocol through the three phases of the pilocarpine model, we used 25 Wistar male rats that were anesthetized and randomly killed at 1, 5, and 24 hours and 3, 5, 7, 45, and 90 days after pilocarpine administration.

All investigation followed the university guidelines for the use of animals in experimental studies, and all efforts were made to minimize suffering, conformed to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH publication number 85–23, revised 1996). The animals were kept on a 12 : 12 h artificial light : dark cycle with rodent chow and water provided ad libitum. All samples were used to perform mRNA-, protein-, or ROS-related compounds quantification.

2.2. Immunohistochemistry. SE rats (5 hours (5H), 5 days (5D), sixty days (60D), and their "control") were anesthetized with a lethal sodium dose of pentobarbital and subjected to transcardiac perfusion with a solution of paraformaldehyde 1% (pH 7.4, 15 mL/rat, infusion rate 15 mL/minute) followed by a solution of paraformaldehyde 4% (pH 7.4, 150 mL/rat, infusion rate 15 mL/minute). After perfusion, the brain was carefully detached from the skull, fixed in paraformaldehyde 4% for 48 hours, and immersed in a solution of sucrose 30% for cryoprotection for 48 hours. Forty-micrometer-thick coronal slices were obtained using a cryostat (HM 505E Micromeria, Zeiss) and stored in 0.1 M phosphate buffer (pH 7.4). The slices were collected throughout the hippocampus and stored in 0.1 M phosphate buffer. The slices were mounted on gelatin-coated slides for immunohistochemistry with p-AKT antibody (Santa Cruz, 1 : 200). Briefly, free-floating slices were treated with hydrogen peroxide 1% for 10 minutes, washed with phosphate-buffered saline (PBS) (pH 7.4), and then treated with Triton X-100 0.4% for 30 minutes. Slices were washed with PBS, preincubated with albumin 10% for 2 hours, and incubated with primary antibody overnight at 4°C. The slices were washed and then incubated at room temperature with appropriate secondary antibodies (1 : 200, biotinylated immunoglobulin G, Calbiochem) for 2 hours. Sections were washed and incubated in avidin-biotin-peroxidase complex (ABC Kit, Vector) for 90 minutes, then washed with Tris-HCl (pH 7.6), and finally developed with diaminobenzidine (DAB) (1 tablet/15 mL of Tris-HCl). Next, slices were washed in PBS and mounted on histological slides. Analysis and documentation of results were performed using a Leica FW 4500 B microscope (Wetzlar, Germany). The degree of staining was provisionally graded by the following criteria: 1+, low staining; 2+, moderate staining; and 3+, intense staining detected by light microscopy ×100 augmentation (10x objective).

2.3. Quantitative mRNA Expression. Thawed hippocampi were homogenized in 1 mL of TRIzol reagent (Gibco BRL, Gaithersburg, MD), and total RNA was isolated according to the manufacturer's instructions. One microgram of total RNA was used for cDNA synthesis and real-time PCR gene expression analysis. Firstly, DNase I (Invitrogen) treatment

at a concentration of 1 unit/μg RNA in the presence of 20 mM Tris-HCl, pH 8.4, containing 2 mM $MgCl_2$ for 15 min at 37°C, followed by incubation at 95°C for 5 min was performed to remove DNA contamination. Reverse transcription (RT) was carried out in a 20 μL reaction in the presence of 50 mM Tris-HCl, pH 8.3, 3 mM $MgCl_2$, 10 mM dithiothreitol, 0.5 mM dNTPs, and 50 ng of random primers with 200 units of Moloney murine leukemia virus reverse transcriptase (Invitrogen). This reaction was performed as follows: 20°C for 10 min, 42°C for 45 min, and 95°C for 5 min. cDNA was at that point amplified by real-time PCR on the 7500 Sequence Detection System (ABI Prism, Applied Biosystems, Foster City, CA) using the SYBR Green core reaction kit (Applied Biosystems). Polymerase enzyme was heat activated for 10 min at 95°C, 40 cycles of 15 sec at 95°C, and 1 min at 60°C amplified the transcript, and data was collected at each cycle. Experiments were performed in triplicates for each data point. Target gene mRNA expression was quantified as a relative value compared with an internal reference, GAPDH, whose expression was believed not to change between the varying experimental conditions. Rat primers used for mRNA quantification were UCP2 (GenBank accession number NM_019354.2) forward 5′-CCACAGCCACCGTGAAGTT-3′ and reverse 5′-CGGACTTTGGCGGTGTCTA-3′; Bcl2-associated death promoter (Bax) (GenBank accession number NM_017059.2) forward 5′-ACTCCCCCCGAGAGGTCTT-3′ and reverse 5′-AGTTGAAGTTGCCATCAGCAAA-3′; Bcl-2 (GenBank accession number NM_016993.1) forward 5′-GCTACGAGTGGGATACTGG-3′ and reverse 5′-GTGTGCAGATGCCGGTTCA-3′; TNF-α (GenBank accession number X66539) forward 5′-AAATGGGCTCCCTCTATCAGTTC-3′ and reverse 5′-TCTGCTTGGTGGTTTGCTACGAC-3′; interleukin-1b (GenBank accession number M98820), forward 5′-CACCTCTCAAGCAGAGCACAG-3′ and reverse 5′-GGGTTCCATGGTGAAGTCAAC-3′; and interleukin-6 (GenBank accession number E02522) forward 5′-TCCTACCCCAACTTCCAATGCTC-3′ and reverse 5′-TTGGATGGTCTTGGTCCTTAGCC-3′. GAPDH primers were forward 5′-TGCACCACCAACTGCTTAGC-3′ and reverse 5′-GCCCCACGGCCATCA-3′ (GenBank accession number NM_017008). A second pair of a housekeeping gene (18S rRNA, GenBank accession number Rn18s) was used to validate the results. One microliter of RT reaction was used for real-time PCR. Quantitative values for target gene and GAPDH mRNA transcription were obtained from the threshold cycle number, where the intensification in the signal associated with an exponential growth of PCR products begins to be detected. Melting curves were generated at the end of every run to confirm product uniformity. The relative target gene expression level was normalized based on GAPDH expression as an endogenous RNA control. ΔC_t values of the samples were determined by subtracting the average C_t value of target gene mRNA from the average C_t value of the internal control (GAPDH). The $2^{-\Delta\Delta Ct}$ parameter was used to express the relative expression data.

2.4. UCP2 Silencing. Antisense oligonucleotide (ASO) protocol was performed using 24 Wistar male cannula-implanted rats. Briefly, rats were anesthetized with intraperitoneal injections of ketamine and xylazine (35 mg/kg and 5 mg/kg, resp.), shaved, and placed in a stereotaxic frame. Then the animal eyes were protected and hydrated with Ocry-gel. A mid-line scalp incision was performed, and the skull was exposed and cleaned of blood and periost. Subsequently, a cannula (gauge 23) was implanted unilaterally at the hippocampus according the coordinates of Paxinos et al. (AP: 3.5 mm behind the bregma, lateral: 3.1 mm, and vertical: 4.5 mm from the cerebral cortex [33]. Two screws were positioned in the skull, and each cannula was affixed into place with dental cement poured around the outer cannula and screws. A stainless steel bar extending just beyond the tip of the cannula was inserted and left in place until inoculation. All rats received ~5 mL of 0.9% saline via ip injection to rehydrate and aid in recovery from surgery. Rats were allowed 7 days postsurgical recovery before any additional experimental procedures. Pilocarpine administration was performed as previously described ($n = 22$). Five animals that received pilocarpine did not enter SE. A group that underwent *status epilepticus* (SE 5d group) was set with 7 rats, and the other 10 SE rats were subjected to ASO administration (SE ASO 5d group). Oligonucleotides were designed according to the UCP2 sequence deposited at the NIH-NCBI (NM 011671). The sequences of antisense oligodeoxynucleotides (ASO) (Invitrogen, Carlsbad, USA) were as follows: sense 5′-TGC ATT GCA GAT CTC A-3′ and antisense 5′-TGA GAT CTG CAA TGC A-3′. The effectiveness of these oligonucleotides in inhibiting UCP2 expression has been previously shown [7, 34]. Antisense oligonucleotides were dissolved in artificial cerebrospinal fluid (aCSF) immediately before administration. Microinjection (500 pmol), at a volume of 100 nL, into the bilateral hippocampal CA3 subfield was performed daily starting on the day before pilocarpine administration until 5 days post-SE. Rats that received bilateral microinjection of the same amount of aCSF ($n = 13$, being 7 pilocarpine-treated and 6 control rats) served as vehicle controls. The animals were killed 6 days after first AOS microinjection. The treatment with the sense oligonucleotides produced no variance in the expression of UCP2 as compared to control (data not shown).

2.5. Enzyme-Linked Immunosorbent Assay (ELISA). UCP2, active caspase-3, phospho-AKT, IL-1β, and IL-6 concentrations of the rat hippocampi from control, SE, and SE ASO were quantified by ELISA. Tissues were excised and immediately frozen at −80°C. For protein extraction, the tissues were sonicated on ice in tissue extraction reagent (Invitrogen) containing protease inhibitor cocktail (Roche, Indianapolis, IN). After centrifugation at 12,000 xg at 4°C for 20 min, the supernatant was assayed for uncoupling protein 2 (UCP2) (Rat Mitochondrial uncoupling protein 2 ELISA kit, Cusabio®, Wuhan, China), caspase-3 activity (Caspase-3/CPP32 colorimetric assay kit, Biovision, Milpitas, CA), phospho-AKT (AKT-pS473 ELISA kit, Abcam, Cambridge, UK), IL-1β, and IL-6 (rat IL-1β or rat IL-6 Quantikine; R&D Systems, Abingdon, United Kingdom).

2.6. Determination of Oxidative Stress Parameters. Oxidative stress parameters were evaluated within the hippocampus homogenates of control, SE-treated, and SE ASO rats. Superoxide dismutase (SOD) activity was measured using a colorimetric commercial kit (Cayman Chemical Co., Ann Arbor, MI, USA) based on inhibition of NADH oxidation, in which superoxide radicals were generated by xanthine oxidase and hypoxanthine and detected at 450 nm using tetrazolium salt. The specific activity is represented as units per milligram of protein. Catalase activity (CAT) was measured spectrophotometrically by a commercial kit (Sigma-Aldrich, St Louis MO, USA), based on the measurement of the decomposition of H_2O_2. One CAT unit is defined as 1 mol of hydrogen peroxide consumed per minute, and the specific activity is reported as units per milligram of protein. MDA levels in tissue were measured spectrophotometrically as described by [35], using a commercial kit (Sigma-Aldrich, St Louis MO, USA) based on the reaction of malondialdehyde (MDA) with thiobarbituric acid (TBA), which produces a colorimetric product. MDA accumulation is indicative of the extent of cell membrane lipid peroxidation and was represented as nmol/mg protein. For the quantitative measurement of protein carbonyl content, we used a commercial kit (Sigma-Aldrich, St Louis MO, USA) based on the derivatization of protein carbonyl groups, which leads to the formation of stable dinitrophenyl (DNP) hydrazone adducts. The carbonyl content was calculated based on the molar extinction coefficient of DNPH and the results were expressed in nmol/mg protein.

2.7. Statistical Analysis. Data were analyzed with GraphPad Prism software 6.0 (La Jolla, CA, USA). The Shapiro–Wilk and Levene tests were used to verify normality and error variances, respectively. Two-way analysis of variance (ANOVA) complemented by Tukey's test was used to detect differences between three groups in samples with normal distribution. Unless indicated otherwise, the biochemical and molecular biology experiments were performed in triplicate. A p value ≤ 0.05 was considered significant. Values are expressed as means ± standard error of mean (SEM).

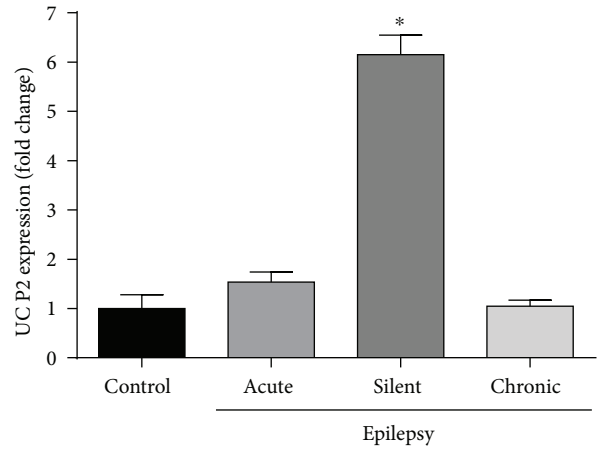

Figure 1: Quantitative real-time PCR for UCP2 mRNA after SE. An increased gene expression of UCP2 was found in the silent phase of the pilocarpine epilepsy model. $^*p < 0.001$ according to two-way ANOVA followed by Tukey's post hoc tests. Values are means ± SEM.

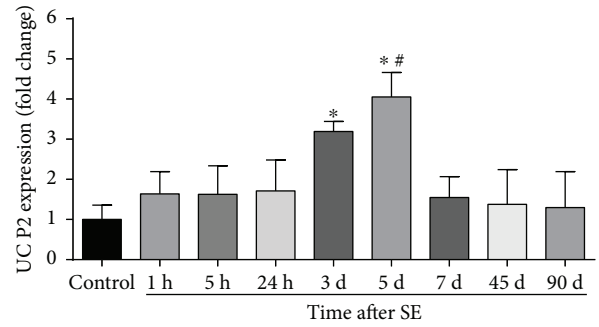

Figure 2: Time course expression of UCP2 mRNA after SE. UCP2 expression increases after SE in the early silent phase of pilocarpine-induced epilepsy. $^*p < 0.001$ versus control, 1 h, 5 h, 24 h, 7 d, 45 d, and 90 d; $^\#p < 0.05$ versus 3 d according to two-way ANOVA followed by Tukey's post hoc tests. Values are means ± SEM.

3. Results

We hypothesized that UCP2 as a neuroprotector may counterbalance the neuronal damage provoked by SE induced by pilocarpine in the experimental epilepsy model. Analyzing the hippocampus of SE rats, we noticed unaltered expression of UCP2 mRNA in the acute phase (0.80 ± 0.22) relative to vehicle-treated controls (0.52 ± 0.19). However, UCP2 mRNA expression observed in the silent phase was about 4-fold higher than that observed in the acute phase (3.26 ± 0.28). The UCP2 mRNA content in the chronic phase (0.53 ± 0.11, Figure 1) returned to basal levels and was similar to that of the control group. Both GAPDH and 18S rRNA showed similar quantification results in all real-time PCR experiments (data not shown). Therefore, we analyzed the time course expression of UCP2 mRNA during the all phases of this experimental epilepsy model. We noticed that, three days after pilocarpine injection, there was a significant upregulation of UCP2 expression (1.47 ± 0.14, Figure 2) when compared to that of control. Augmented UCP2 mRNA expression reached a peak 5 days after SE onset (3.10 ± 0.31), to further decline to baseline levels after this period. Concomitantly, we detected by immunohistochemistry robust expression of phosphorylated AKT (p-AKT), a survival cell marker, at the silent phase (5 days after pilocarpine administration) when compared to those of the control and any other experimental group (Table 1 and Figure 3).

Treatment with UCP2 antisense oligonucleotides (ASO) has shown to effectively inhibit UCP2 generation [34]. We used the same oligonucleotides to analyze the epileptogenesis onset with UCP2 subtraction. In fact, UCP2 silencing successfully decreased UCP2 mRNA expression 5 days after SE (0.51 ± 0.21, Figure 4(a)) compared to those of the control and SE groups (0.83 ± 0.16 and 3.36 ± 0.32, resp.). ASO treatment was well tolerated and no mortality was observed in the experimental group. Rats that received UCP2 antisense oligonucleotide administration presented 100% onset of SE

Table 1: Immunoreactivity for phospho-AKT in the CA1, CA3, and dentate gyrus in an epilepsy model induced by pilocarpine administration.

Hippocampal formation	Control (saline)	Acute (5H)	Silent (5D)	Chronic (60D)
CA1	+	+	+++	++
CA3	++	+	+++	+
Dentate gyrus	+	+	+++	++

Staining of phospho-AKT was scored by two independent observers as follows: +: low; ++: moderate; +++: high intensity based on photomicrographs presented in Figure 3. Control: saline-treated rats; acute: 5 hours after *status epilepticus* (SE); silent: 5 days after SE; chronic: 60 days after SE.

(i.e., all animals treated with ASO followed by pilocarpine administration developed *status epilepticus*, $n = 10$). In contrast, animals that received only pilocarpine were less prone to enter *status epilepticus* (7 from 12 rats). UCP2 protein quantification presented diminished values after in the SE ASO group (0.91 ± 0.21 pg/mL) compared to the SE (3.55 ± 0.21 pg/mL) and control groups (2.38 ± 0.21 pg/mL, Figure 4(a)). Furthermore, we quantified the p-AKT expression after ASO administration. SE ASO rats presented a diminished p-AKT expression (0.71 ± 0.21 ng/g, Figure 4(c)) in comparison to SE and control rats (1.17 ± 0.11 ng/g and 1.82 ± 0.34 ng/g, resp.).

We then analyzed the expression of inflammatory mediators within the hippocampus samples of control, SE, and SE ASO rats. We observed an augmentation of TNF-α (1.33 ± 0.05), IL-1β (2.89 ± 0.41), and IL-6 mRNA (2.48 ± 0.29) 5 days after SE onset when compared to that of control (0.90 ± 0.03, 1.40 ± 0.47, and 1.22 ± 0.39, resp.; Figure 5(a)). Nevertheless, after ASO treatment, the hippocampi of SE ASO rats presented nearly 2-fold higher expression of these proinflammatory mediators (2.49 ± 0.21, 4.13 ± 0.12, and 4.48 ± 0.47) compared to those of SE rats. Augmentation of IL-1β and IL-6 detected in mRNA quantification was corroborated using ELISA detection method to determine protein expression. Besides the increased expression of IL-1β and IL-6 in SE rats (4.01 ± 0.19 pg/mL and 2.26 ± 0.19 pg/mL, resp.), the SE ASO group showed significant and strong expression of these proinflammatory markers (5.37 ± 0.29 pg/mL and 3.19 ± 0.19 pg/mL) compared to any other experimental group (control IL-1β: 2.35 ± 0.13 pg/mL and IL-6: 1.23 ± 0.05 pg/mL).

Then we analyzed the mRNA expression of apoptotic factors in the presence or absence of ASO treatment in the SE rat hippocampi in the silent phase. SE onset induced not only a reduction in antiapoptotic bcl2 mRNA expression (0.72 ± 0.21) but also a robust mRNA expression of bcl2-associated death promoter (bad) (3.58 ± 0.44), suggesting an activation of apoptosis when compared to that of control (1.95 ± 0.26 and 1.69 ± 0.32, resp.). Then we observed a strong diminished expression of bcl2 mRNA after 5 days of SE onset and ASO treatment (0.26 ± 0.05) compared to that of the SE group. The bad mRNA expression increased with SE ASO treatment (6.75 ± 0.53) compared to that of any other group (Figure 6(a)). This higher decrease in bcl2 : bad mRNA ratio suggests a more pronounced apoptotic cell death related with UCP2 silencing in the silent phase of the pilocarpine-induced epilepsy model (Figure 6(b)). To investigate that ASO treatment altered survival by increasing apoptosis, we analyzed the hippocampi for the level of active caspase-3. As shown in Figure 6(c), caspase-3 activity at day 5 was significantly higher in the hippocampus of the SE ASO group (4.35 ± 0.36 ng/mL) compared to the control (1.23 ± 0.18 ng/mL) and SE groups (1.73 ± 0.21 ng/mL), indicating augmentation of apoptosis after ASO administration.

Lipid peroxidation was quantified by measuring MDA production. MDA levels were found to have increased in the hippocampus of rats that were subjected to pilocarpine administration (SE group, 0.91 ± 0.07 nmol/mg) compared to control (0.56 ± 0.15 nmol/mg). ASO treatment markedly increased the levels of MDA in the hippocampus of pilocarpine-treated rats (1.39 ± 0.11 nmol/mg, Figure 7(a)). In SE rats, the levels of carbonyl protein were found to be elevated within the hippocampus (0.92 ± 0.11 nmol/mg) compared to those of control (0.47 ± 0.23 nmol/mg). The ASO treatment increased the levels of carbonyl protein in the hippocampus of rats injected with pilocarpine (1.42 ± 0.09 nmol/mg, Figure 7(b)).

To assess the efficacy of ASO treatment on the intracellular antioxidant system, we measured ROS-related enzyme activities. The SOD enzyme activity was unaltered in the hippocampus of the SE group (0.90 ± 0.17 U/mg) compared to control (0.81 ± 0.11 U/mg). However, ASO treatment significantly increased SOD activity of SE rats (2.48 ± 0.34 U/mg; Figure 7(c)). We demonstrated that there was an increase in the levels of catalase activity (3.62 ± 0.16 U/mg) in the hippocampus after SE compared to control (1.82 ± 0.11 U/mg). It must be noted that treatment with ASO resulted in augmented CAT activity in the hippocampus (4.79 ± 0.23 U/mg, Figure 7(d)).

4. Discussion

In the present study, we demonstrated that rats subjected to the animal model of epilepsy induced by pilocarpine had increased expression of UCP2 in the hippocampus during the early silent phase (between 3–5 days after SE). ASO treatment successfully diminish UCP2 mRNA and protein expression in this period after SE. In addition, the brains of SE rats injected with ASO showed an increase in oxidative stress, marked by damage caused by lipid peroxidation, higher levels of protein carbonyl, and increase in the activity levels of antioxidant enzymes, SOD, and catalase. Moreover, when SE rats received ASO, we observed increase of proinflammatory marker expression and enhanced apoptosis.

Our group has reported the participation of several molecules with effects on the inflammatory process that are gradually taking an important role in epilepsy and its potential treatment [23, 25]. In order to establish mechanisms of epileptogenesis, we have observed numerous cellular and molecular changes that, with time and increasing understanding, broaden the list of new molecules that may contribute to the pathological state of the disease. While

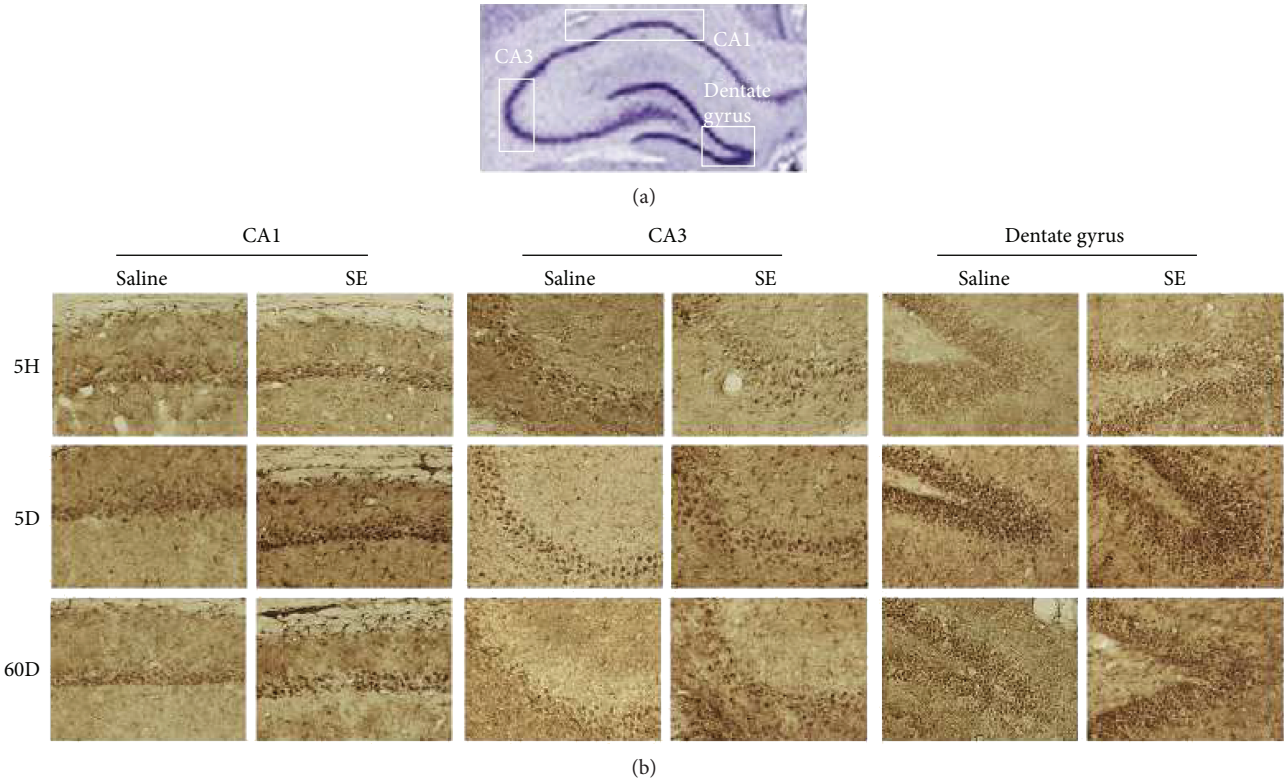

Figure 3: Phospho-AKT expression in the hippocampus CA1, CA3, and dentate gyrus regions of the acute (5 hours after SE (5H)), silent (5 days after SE (5D)), and chronic (60 days after SE (60D)) groups. (a) Rat hippocampal formation with Nissl staining highlighting the CA1, CA3, and dentate gyrus areas. (b) Photomicrographs of the CA1, CA3, and dentate gyrus regions of the rat hippocampus after SE processed for phospho-AKT (p-AKT) immunohistochemistry and respective controls. Pilocarpine-treated animals at the 5th day after SE (silent phase) showed higher expression of p-AKT in comparison to any other group. ×100 augmentation (10x objective).

UCP2-based neuroprotection has been widely reported in many species [11, 36–44], its participation in hippocampal epilepsy needs further investigation. To this end, we observed increased expression of UCP2 after pilocarpine administration in the silent phase, reaching a peak on the fifth day after *status epilepticus* onset. This is corroborated by groups who analyzed kainic acid-induced epilepsy in rodents [43, 45, 46].

UCP2 gene expression during the silent phase suggests that this molecule might be required during epileptogenesis in neuronal cells. The silent phase of the pilocarpine-induced model is characterized by no differentiated phenotype but many metabolic alterations. We observed an increase in p-AKT in the same period in which UCP2 expression is incremented. The relation between UCP2 and AKT has already been described [11, 47], although its relevance to epilepsy remains unclear. The kinase AKT, also known as protein kinase B, is a serine/threonine-specific protein kinase that has a central role in the signaling pathways that regulate metabolism and cellular transformation. AKT can regulate cell growth positively and apoptosis negatively by activating a series of different downstream signaling molecules [48, 49]. In epilepsy, AKT phosphorylation is very welcome because they positively influence neuronal survival by reducing the cell damage observed after insult. While it does not have kinase activity, UCP2 has a close interaction with cell survival factors based on recent reports [11]. Derdák et al. [47], using a UCP2 knockout mouse model, provided the first *in vivo* evidence for a link between UCP2 and cancer, when transgenic mice showed an imbalance between epithelial cell proliferation and apoptosis.

The silencing of UCP2 gene expression is a molecular protocol targeting the mRNA of this molecule, limiting its availability by annealing between complementary nucleotide sequences. This model has previously been used with interesting results. For example, De Souza et al. [34] demonstrated that the silencing of UCP2 ameliorates the hyperglycemic syndrome in two distinct animal models of obesity and diabetes. In 2008, Degasperi et al. [7] reported that inhibiting UCP2 expression increases TNF-α-induced expression of markers of ROS accumulation and apoptosis. The authors postulated that induction of UCP2 expression in the rat hypothalamus could be considered an endogenous protective mechanism that might minimize the harmful effects of potent inflammatory stimuli. Our observation that rats that underwent UCP2 antisense treatment were more susceptible to *status epilepticus* might be an evidence of neuroprotection elicited by UCP2.

SE induced by pilocarpine may promote oxidative stress and could be reflected in direct activation of antioxidant enzymes. Under healthy conditions, a balance between the production of ROS and their destruction by antioxidant

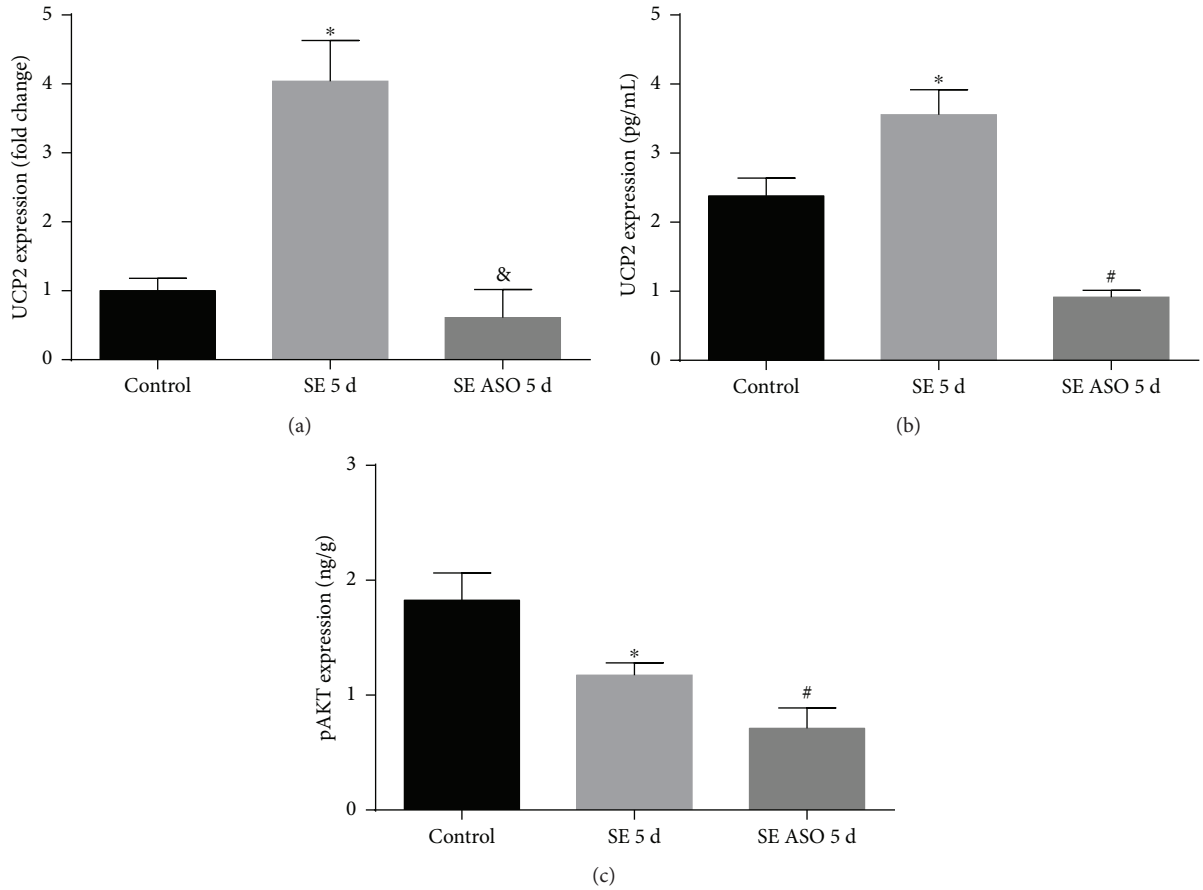

Figure 4: Expression of hippocampal UCP2 and p-AKT in the presence or absence of antisense oligonucleotide (ASO) treatment in SE rats. (a) Treatment with ASO diminished UCP2 mRNA expression in rats 5 days after SE onset. $^{*}p < 0.001$ versus control and 5 d ep ASO; $^{\&}p < 0.001$ versus 5 d ep; (b) UCP2 protein expression presented high levels 5 days after SE. ASO treatment successfully decreased UCP2 protein expression 5 days after SE. $^{*}p < 0.001$ versus control; $^{\#}p < 0.001$ versus control and SE 5 d; (c) p-AKT expression diminished after ASO treatment. $^{*}p < 0.001$ versus control; $^{\#}p < 0.001$ versus control and SE 5 d according to two-way ANOVA followed by Tukey's post hoc tests. Values are means ± SEM.

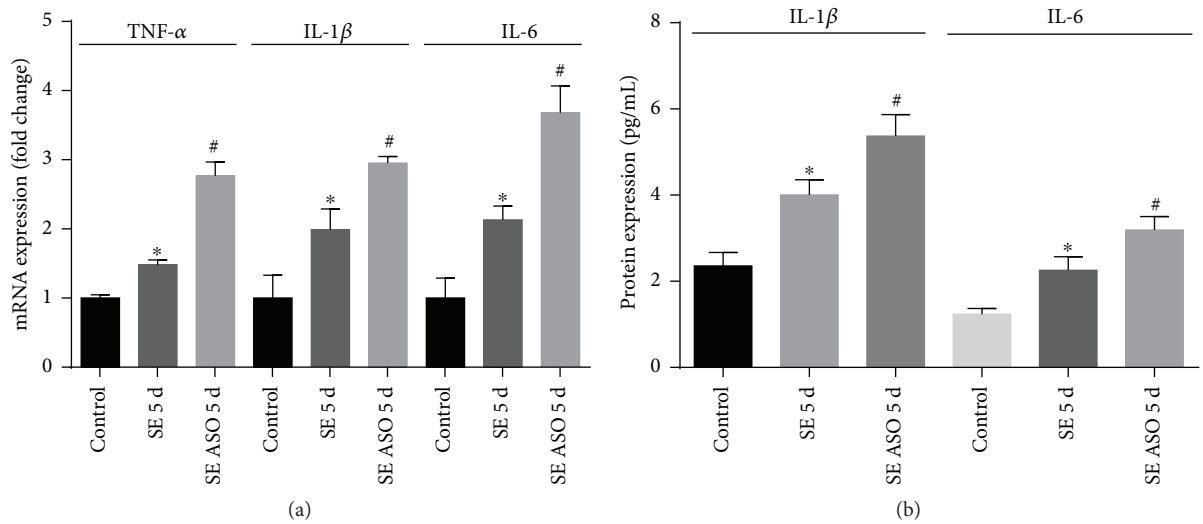

Figure 5: Modulation of expression of proinflammatory markers within the hippocampi with or without ASO treatment. (a) SE onset increased inflammatory marker (TNF-α, IL-1β, and IL-6) mRNA expression, while ASO treatment resulted in higher mRNA expression of these markers. (b) SE onset and ASO treatment lead to increment of IL-1β and IL-6 protein content. $^{*}p < 0.001$ versus control; $^{\#}p < 0.001$ versus control and SE 5 d according to two-way ANOVA followed by Tukey's post hoc tests. Values are means ± SEM.

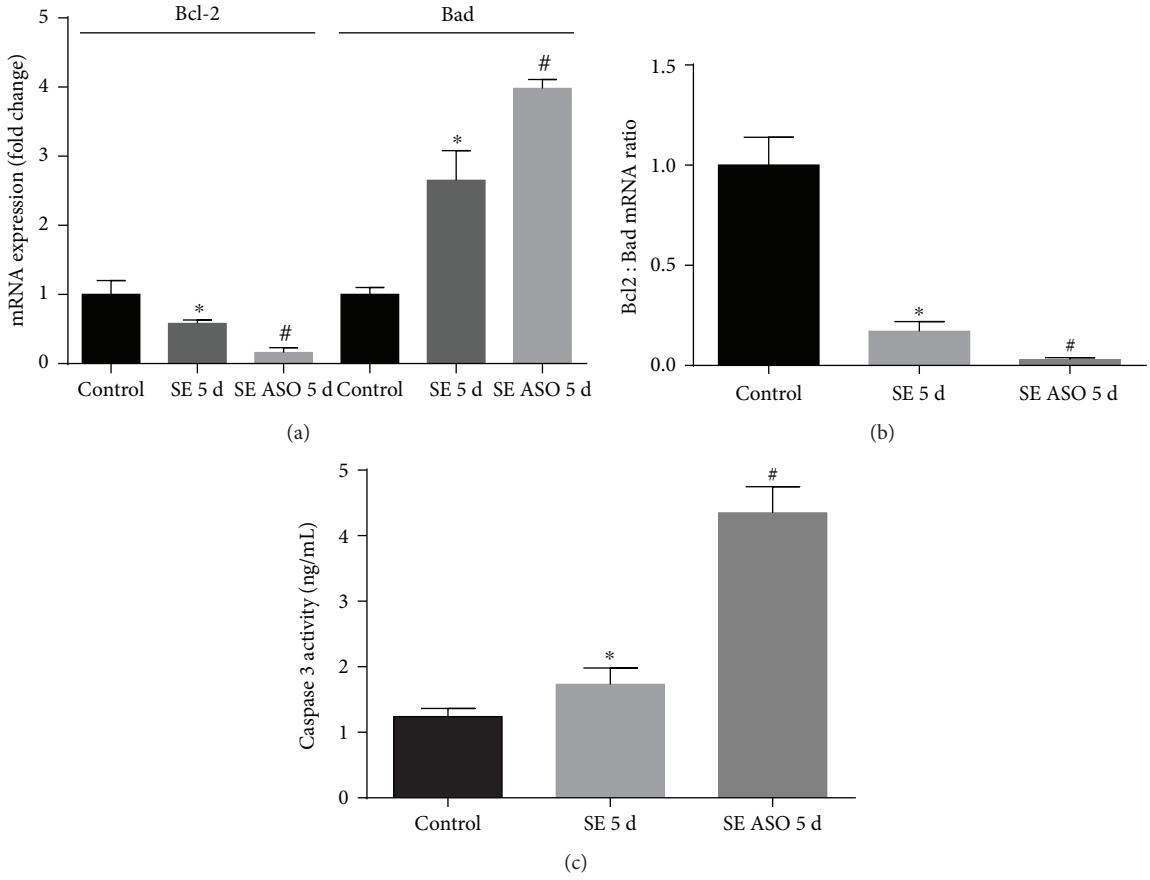

Figure 6: Apoptosis modulation in the presence or absence of ASO treatment in SE rats. (a) SE onset diminished bcl-2 mRNA expression, while increasing proapoptotic factor bad mRNA expression. ASO treatment increased bad expression in SE rats, with low levels of antiapoptotic factor bcl2 mRNA expression. (b) Bcl2 : bad ratio, indicating that SE followed by ASO treatment stimulated apoptosis. (c) Caspase-3 activity is increased 5 days after SE, while a marked activity augmentation was observed when SE rats were submitted to ASO treatment. $^{*}p < 0.001$ versus control; $^{\#}p < 0.001$ versus control and SE 5 d according to two-way ANOVA followed by Tukey's post hoc tests. Values are means ± SEM.

systems is found. Nevertheless, this balance can be altered either by increased ROS production or by a decrease in cellular antioxidant systems. Pilocarpine-induced seizures produce several changes in variables related to the generation and elimination of oxygen free radicals in adult rats [50]. SOD enzyme reaction results in H_2O_2 and water from dismutation of the superoxide (O_2^-) radical, and catalase converts H_2O_2 to oxygen and water [51]. Tejada et al. [52] reported that an increase in enzyme antioxidant activities was observed after SE, indicating that neurons try to counteract excessive SE-induced ROS. However, we observed an unaltered SOD activity after SE onset. SOD activity is related to mechanisms involved in the initiation and/or propagation of seizures induced by pilocarpine. This data is corroborated by another study showing unaltered SOD activity 24 h after pilocarpine treatment, suggesting that SOD activity only changes during the initiation of seizures [53].

Cao et al. [54] reported that splenocytes from UCP2 knockout mice were more susceptible to pathogen activation-induced apoptosis and that the high level of ROS in UCP2 KO mice might be the cause of the apoptotic susceptibility. In our study, when UCP2 was inhibited, the activities of antioxidant enzymes CAT and SOD were significantly increased. These results are in accordance with [7, 34] that suggested that the damaging effect of reduced UCP2 expression was linked to augmented ROS formation. Also, we showed that ASO treatment resulted in MDA and protein carbonyl augmentation in SE rats. Oxidative stress induces cell apoptosis when the endogenous antioxidant factors were decreased [7, 55]. Our results suggest that SE onset induces apoptosis in the rats' hippocampi by decreasing the antioxidant enzyme activities. The augmentation of these endogenous antioxidant systems after ASO treatment may protect the hippocampi against oxidative stress induced by pilocarpine.

Bengzon et al. [56] reported that changes in the expression and activity of cell death regulatory proteins, such as members of the Bcl2 and caspase families, occur in regions vulnerable to cell degeneration. This suggests an involvement of these factors in apoptosis following seizures. Chen et al. [57] observed that kainic acid administration led to marked neuronal apoptosis in the hippocampus, accompanied by increased levels of Bax, activated caspase-3, and decreased

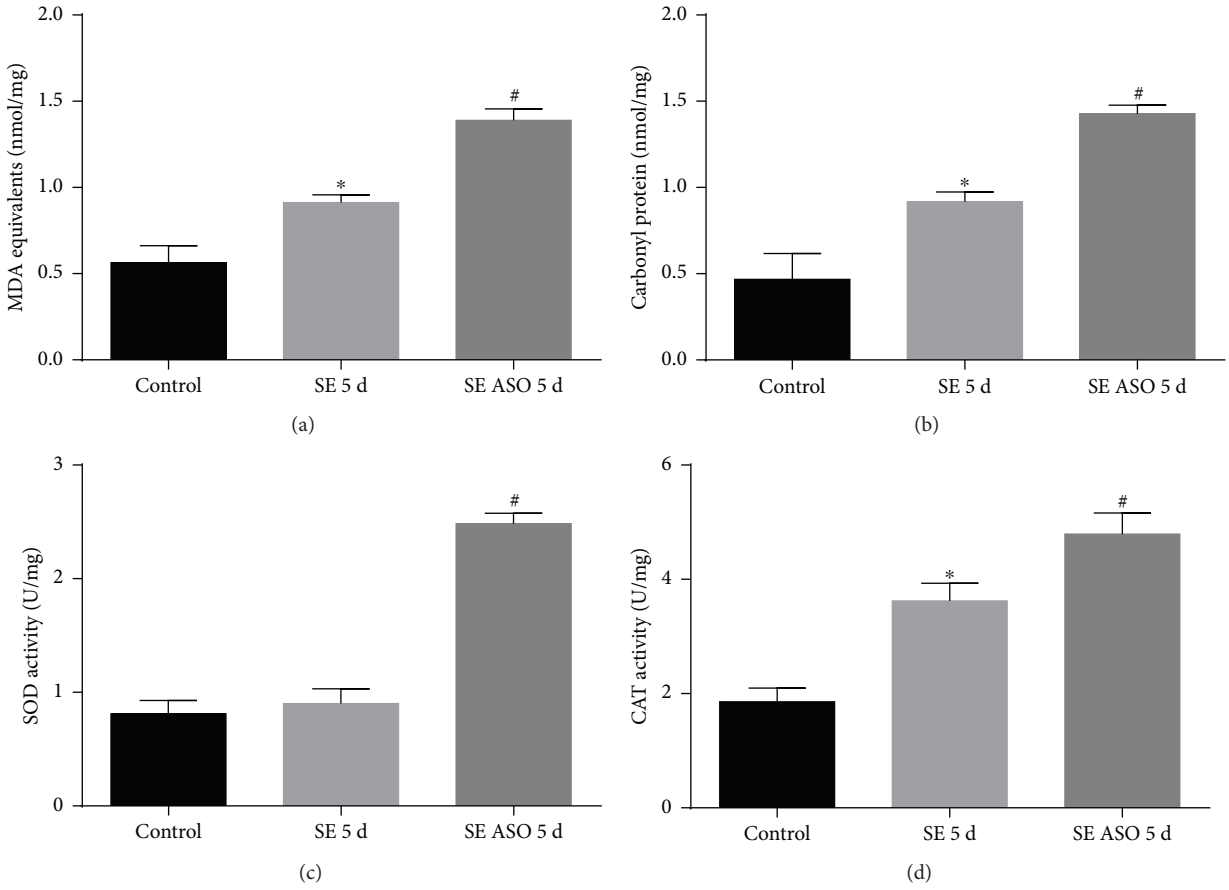

Figure 7: Oxidative stress within the SE rat hippocampi with and without ASO treatment. (a) MDA levels, (b) carbonyl protein, (c) SOD activity, and (d) catalase activity. $^{*}p < 0.001$ versus control; $^{\#}p < 0.001$ versus control and SE 5 d according to two-way ANOVA followed by Tukey's post hoc tests. Values are means ± SEM.

levels of Bcl2. In our study, bcl2 mRNA was decreased relative to bad expression after pilocarpine-induced epilepsy. Reduction of UCP2 expression results in even greater modulation of both factors, indicating more pronounced activation of apoptotic cell death when ASO are administrated, data that is corroborated by Degasperi et al. [7]. While increased caspase-3 activity was observed after SE onset, a higher augmentation was observed after SE and ASO treatment. We can then infer that neuronal cells likely had a higher susceptibility to cell death after SE. However, after ASO, this susceptibility increased. This data along with the bcl2 : bad ratio after antisense treatment highlight the neuroprotective role of UCP2. Together, these evidences demonstrate a mechanism for a protective cell defense against the insult, in accordance with several other studies [4, 44, 58].

Although the neuroprotective role of UCP2 may be dependent on the activation of several other molecules, its inhibition had been showed a decrease in the activated form of AKT, which in turn prevented AKT from exerting its catalytic activity and subsequently activating other cell survival-related molecules [59, 60]. In fact, in SE ASO rats, we observed a diminished expression of p-AKT, corroborating these findings. UCP2 is a neuroprotective factor both through its direct effects in decreasing mitochondrial ROS and through a change in the spectrum of secreted cytokines towards a more anti-inflammatory spectrum [61]. Indeed, when we reduced UCP2 expression, the proinflammatory marker expression was increased. Our data corroborate a study using neurotoxin MPTP-treated UCP2-deficient mice that also showed an enhanced expression of proinflammatory cytokines such as tumor necrosis factor-α and interleukin 1β [62].

5. Conclusion

Our data suggest that UCP2 expression can modulate apoptotic responses and ROS formation to alleviate cellular damage in the hippocampus generated by an excessive neuronal insult as long-lasting SE induced by pilocarpine. Although further studies are needed to identify the molecular pathways of epileptogenesis, our results contribute to the increasing knowledge about the activity of the UCP2-survival-apoptosis axis in epilepsy.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this article.

Authors' Contributions

Marina Rascio Henriques Dutra and Regiane dos Santos Feliciano contributed equally to this work.

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Research Article

Isolation and Characterization of NP-POL Nonapeptide for Possible Therapeutic Use in Parkinson's Disease

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Colostrum and milk are the initial mammalian nourishment and rich reservoir of essential nutrients for newborn development. Bioactive peptides isolated from natural sources, such as colostrum, serve as endogenous regulators and can be used as alternative therapeutic agents in the treatment of neurodegenerative diseases. One example is the previously unknown NP-POL nonapeptide isolated from Colostrinin. In the present study, we investigated a method of NP-POL nonapeptide isolation using Bio-Gel P2 molecular sieve chromatography. We showed the protective effect of NP-POL on 6-hydroxydopamine- (6-OHDA-) induced neurotoxicity using rat adrenal pheochromocytoma (PC12 Tet On) cells. Treatment of PC12 cells with NP-POL nonapeptide reduced 6-OHDA-induced apoptosis and caused transient phosphorylation of extracellular signal-regulated kinases (ERK 1/2), which were shown to promote cell survival. NP-POL nonapeptide also protected neuronal cells against oxidative injury induced by 6-OHDA. These results showed a potential use of NP-POL in the therapy of Parkinson's disease.

1. Introduction

Parkinson's disease (PD) is considered the second most common neurodegenerative disease after Alzheimer's disease, involving 0.3% of industrialized country populations, with a prevalence rising with age from 1% in people over 60 years of age to 4% in those over 80 [1, 2]. PD results from the progressive loss of dopaminergic neurons in the parts of the brain that control muscle movement—the basal ganglia and the extrapyramidal area. Pathological indicators of PD are cytoplasmic inclusions—Lewy bodies and massive atrophy of dopaminergic neurons in substantia nigra pars compacta. Clinically, PD is characterized by motor symptoms (such as bradykinesia, hypokinesia, cogwheel rigidity, resting tremor, and postural instability), sleep disorders, hyposomia, anxiety, and depression [3]. Although the etiology of Parkinson's disease is still not completely clear, some causes have been found, including neuroinflammation, genetic mutation of genes, and mitochondrial and proteasomal dysfunction, as well as α-synuclein aggregation [1, 2].

Several studies have reported that the overproduction of free oxygen radicals and an impaired antioxidative defense system are initial steps in PD [4, 5]. Oxidative stress is a product of an imbalance between oxidative and antioxidative

systems in cells, generating increased levels of free oxygen and nitrogen radicals, leading to impairment in proteins, lipids, and DNA, as well as mitochondrial dysfunction [6, 7]. High levels of redox active metals, decreased activity of antioxidant enzymes, and reduced level of glutathione play a pivotal role in the etiology of PD [8, 9].

Reactive oxygen species (ROS) are also generated in dopaminergic neurons during enzymatic degradation of dopamine by monoamine oxidase, as well as during nonenzymatic dopamine autoxidation to neuromelanin [10, 11]. 6-Hydroxydopamine (6-OHDA) detected in both rat and human brains after long-term L-3,4-dihydroxyphenylalanine (L-DOPA) administration has also been proposed as a neurotoxin in the pathogenesis of PD [12, 13]. 6-OHDA acts via inducing ROS overproduction and energy depletion [13–15]. The toxic effect of 6-OHDA results from the overproduction of ROS through three pathways: extracellular autooxidation, intracellular metabolism by monoamine oxidase, and direct inhibition of the mitochondrial respiratory chain. The increase in the level of ROS by 6-OHDA leads to a decrease in cellular antioxidant enzymes and, subsequently, neuronal apoptosis [16].

The treatment of PD has not changed substantially in the past 30 years, with the key role of dopamine replacement therapy, including L-3,4-dihydroxyphenylalanine (L-DOPA) and dopamine agonists, supported by the use of peripheral decarboxylase inhibitors, catechol-O-methyl transferase inhibitors, and monoamine oxidase-B (MAO-B) inhibitors [17]. The current treatments do not prevent the continuing loss of dopamine neurons, and eventually treatment-related side effects result in severe disability.

One viable alternative to the drugs used in treating PD can be natural products—nutraceuticals or functional foods—commonly used for preventing or attenuating the process of aging. The strong therapeutic potential of bioactive compounds obtained from natural products in age-related disorders such as Parkinson's disease is associated with their multidirectional action. They are more available and considerably safer to use and can promote prosurvival signals and act as antioxidants [18, 19]. These substances can act as agonists for dopaminergic neurons, improve cognitive function, promote mitochondrial function, inhibit ROS generation, and also possess immunomodulatory activity [20, 21]. An example of such a compound is the previously unknown nonapeptide NP-POL isolated as a component of the proline-rich polypeptide complex (PRP, also known as Colostrinin).

PRP is a complex of low-molecular weight peptides ranging from 500 Da to 3000 Da, first isolated from ovine colostrum, and also present in human, bovine, and caprine colostrum [22]. It is one of the many important constituents of colostrum, the first mammalian nourishment, which may stimulate the neonate immune system and play a regulatory role in newborn development, next to immunoglobulins, cytokines, and lymphokines. It is active both *in vivo* and *in vitro* and is not cytotoxic even at 1.25 g/kg body weight.

Because of its multicomponent character, PRP shows pleiotropic activity. It has immunoregulatory properties, regulating both humoral and cellular immune responses. It modulates the innate immune response, including phagocytosis and the balance between oxidants and antioxidants, thus regulating redox-sensitive cellular signaling [23–25]. Additionally, PRP can affect learning, memory, and lifespan and possesses neuroprotective activity [23, 26, 27]. The activity of PRP suggests a potential therapeutic use in the case of diseases associated with changes in innate immunity, for example, Alzheimer's disease [23]. It has also been suggested that PRP has potential in treating other neurodegenerative diseases, such as multiple sclerosis, Parkinson's disease, and amyotrophic lateral sclerosis.

The present study shows a method of isolation and purification of a previously unknown PRP constituent, NP-POL nonapeptide. In addition, we used the PC12 Tet On cell line to investigate the protective effect of NP-POL in 6-OHDA-induced oxidative stress. Our study provides new evidence that NP-POL may protect PC12 cells against 6-OHDA cytotoxicity through a neuroprotective and antioxidant activity. Our results indicate a potential use of NP-POL in the therapy of Parkinson's disease.

2. Materials and Methods

2.1. Reagents. High-glucose Dulbecco's modified Eagle's medium (DMEM) and phosphate-buffered saline (pH 7.4) (PBS) were sourced from the Laboratory of General Chemistry of the Institute of Immunology and Experimental Therapy, PAS (Poland). L-glutamine, antibiotics (penicillin/streptomycin mixture), donor horse serum, and fetal bovine serum (FBS) were produced by BioWest (Nuaille, France). Stabilized hydrogen peroxide 30%, 2,7-dichlorofluorescein diacetate (DCFH), 1,1-diphenyl-2-picrylhydrazyl (DPPH), ferrozine, Trolox, 3-(4,5-dimethylthiazol-2-yl)-2-5-diphenyltetrazolium bromide (MTT), 2′,7′-dichlorofluorescein diacetate (DCFH-DA), and Tween 20 were from Sigma (St. Louis, MO, USA). Reagents for SDS-PAGE were from Bio-Rad (California, USA). 2.5S NGF (from mouse submaxillary glands) and BDNF Emax ImmunoAssay System were from Promega (Madison, USA). Page Ruler™ Plus Prestained Protein Ladder (10 kDa–250 kDa) was obtained from Thermo Scientific (Waltham, MA, USA). 6-Hydroxydopamine (6-OHDA) was provided by Tocris Bioscience (Bristol, UK). Rabbit anti-ERK/anti-phosphoERK monoclonal antibodies and alkaline phosphatase-conjugated anti-rabbit IgG antibodies were from Cell Signaling Technology (MA, USA). 5-Bromo-4-chloro-3-indolyl phosphate disodium salt (BCIP) and nitroblue tetrazolium (NBT) were from Carl Roth GmbH (Karlsruhe, Germany).

Synthetic NP-POL peptide used to determine biological activity was obtained by chemical synthesis at Lipopharm (Gdańsk, Poland).

2.2. Isolation of NP-POL Nonapeptide from Colostrinin

2.2.1. Isolation of Colostrinin. Sheep and bovine colostrum were obtained from sheep farms in Szczenyrz and Grywałd and the farm of the Wrocław University of Environmental and Life Sciences, Poland, respectively. Colostrum was collected from sheep and cow up to 24 hours after delivery.

Colostrinin (CLN) was separated from colostrum using the classical method according to Janusz et al. [22]. Also, a two-step purification method based on the alcohol/salt extraction/precipitation procedure according to Kruzel et al. [28] was used. This method involves the extraction of peptides with 60% methanol directly from raw colostrum (MOHS sample) or after conditioning with EDTA/$CaCl_2$ (ECa sample) and precipitation with 50% ammonium sulfate (Figure 1). A consistent pool of essentially IgG-free polypeptides is obtained at a high yield with these protocols.

2.2.2. Separation of NP-POL Nonapeptide from CLN, MOHS, and ECa Samples. 100 mg of Colostrinin or 10 mg of MOHS or 100 mg of ECa sample were dissolved in 5 ml, 1 ml, and 5 ml of 50 mM EDTA, respectively, and applied onto the Bio-Gel P2 molecular sieve beads equilibrated with the same solution. One ml fractions are collected and the protein profile plotted upon the protein concentration measured in each fraction.

2.2.3. RP-HPLC. RP-HPLC analysis was carried out on a Nucleosil 100 C-18 column (particle size 10 μm, 250 mm × 8 mm). The peptides of peak "c" eluted from Bio-Gel P2 were dissolved in 0.1% TFA and applied to a column equilibrated with 20% ACN in 0.1% TFA. The proteins were eluted with a linear gradient of ACN from 20% to 100% in 0.1% TFA (*v*/*v*) at a flow rate of 1 ml/min in 60 min and detected at 220 nm.

2.2.4. SDS-PAGE. SDS/polyacrylamide slab gels were prepared under reducing conditions according to Schägger and von Jagow [29]. The gel slabs were cast 24 h ahead of electrophoresis and stained with Coomassie Brilliant Blue R-250 for proteins.

2.2.5. Amino Acid Sequence Analysis. The amino acid sequence of the peptide separated from fraction 2 obtained after RP-HPLC analysis was made to undergo the Edman degradation method [30].

2.3. Cell Cultures. Whole blood samples from healthy donors were kindly provided by the Station of Blood Donation, 4th Military Hospital, Wrocław, Poland. Samples were collected into syringes containing 10 U/ml of heparin. Within 2 h of collection, the blood was diluted 10-fold with RPMI 1640 medium supplemented with 100 units/ml penicillin, 100 mg/ml streptomycin, and 0.5 mg/ml L-glutamine. Whole blood samples were used for the determination of cytokine level.

PC12 Tet On (ATCC) rat pheochromocytoma cells used as a model of neuronal cells were kindly provided by Professor Janusz Matuszyk (Institute of Immunology and Experimental Therapy, PAS, Wrocław). The cells were maintained under 5% CO_2/95% humidified air at 37°C in Dulbecco modified Eagle's medium (DMEM), supplemented with 5% horse serum and 10% fetal bovine serum, antibiotics (penicillin and streptomycin), and 2 mM L-glutamine. The culture medium was changed once every three days.

2.4. Cytokine Induction and Determination. Cytokine secretion was induced according to the method described by Inglot et al. [31]. Blood samples were collected in syringes containing sodium heparin. Within 1 h of collection, the blood was diluted 10-fold with RPMI 1640 medium supplemented with penicillin/streptomycin and L-glutamine. One ml portions of the cell suspension were distributed in duplicate into 48-well flat-bottomed tissue culture plates. NP-POL at doses of 1 μg–100 μg was added to 100 μl of RPMI 1640. As a reference, positive inducers were used: 2 μg/ml of leucoagglutinin (PHA) and 2 μg/ml of lipopolysaccharide (LPS). Control wells containing the nontreated blood cell samples were used to measure the spontaneous production of cytokines (negative control). The plates were incubated for 22 h at 37°C in a 5% CO_2 atmosphere. After incubation, the supernatants were collected and used for the determination of cytokines. IL-1β, TNF-α, IL-6, and IL-10 were determined by an enzyme-linked immunosorbent assay using human IL-1β, TNFα, IL-6, and IL-10 ELISA Max™ Deluxe Kit (BioLegend, San Diego, CA) according to the procedure recommended by the manufacturer.

2.5. Determination of Cell Viability. Cell viability was determined using the MTT colorimetric assay [32]. PC12 cells were seeded onto poly-L-lysine-coated 96-well plates (1×10^4/well) and next incubated for 24 h with inducers: NP-POL (1 μg/ml–150 μg/ml) or toxin 6-OHDA (1 μM–200 μM). To measure the neuroprotective effect, NP-POL nonapeptide was applied simultaneously with 6-OHDA (150 μM) or preincubated for 1 h and then exposed to 150 μM 6-OHDA for 24 h. After cell treatment, the supernatant was removed and the cells were incubated with MTT (5 mg/ml) for 4 h at 37°C. The formazan crystals were dissolved by adding 100 μl of DMSO and vigorously shaking to complete resolving. The absorbance was measured by an EnSpire™ 2300 microplate reader (PerkinElmer, Massachusetts, USA) at 570 nm. Cell viability was expressed as a percentage of control.

2.6. Determination of Antioxidant Activity as the Ability to Scavenge DPPH Free Radicals. The antioxidant activity of NP-POL nonapeptide was assessed on the basis of the radical scavenging effect of stable 1,1-diphenyl-2-picrylhydrazyl free radical activity according to Yen and Chen [33], with minor modifications. The tested samples were dissolved in water to a final volume of 1 ml and mixed with 1 ml of ethanol (98%). The reaction was started by adding 0.5 ml of 0.3 mM DPPH in ethanol. The mixtures were left for 30 minutes at room temperature, and the absorbance of the resulting solutions was measured at 517 nm. For calibration, aqueous solutions of known Trolox concentrations ranging from 2 μg to 20 μg (able to scavenge 500 μl of 0.3 mM DPPH radical solution) were used. Radical scavenging activity of the peptide was expressed as μM $Trolox_{eq}$.

2.7. FRAP Method. The FRAP method (ferric-reducing antioxidant power) was used to determine the antioxidative capacity of NP-POL according to Benzie and Strain [34]. 3 ml of FRAP working solution (300 mM acetate buffer

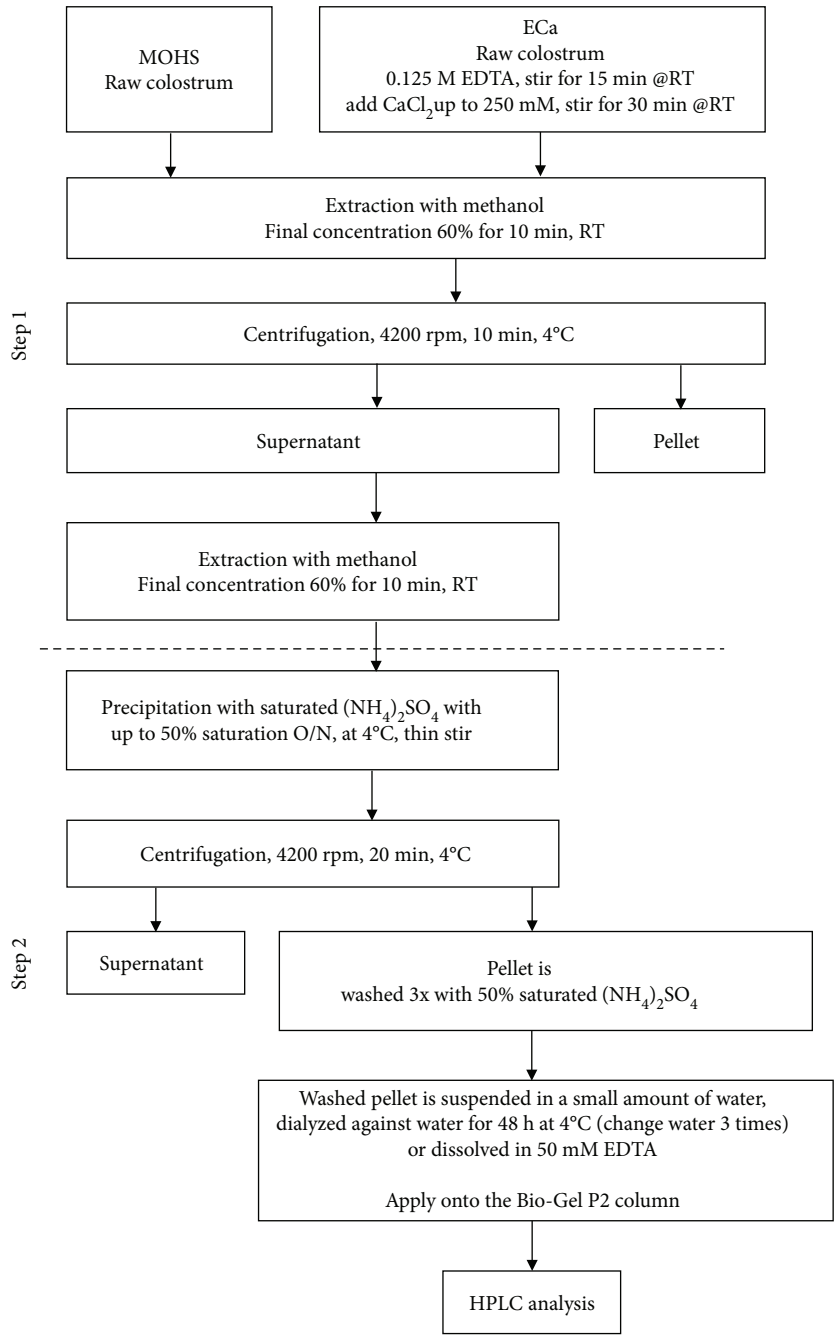

Figure 1: Diagram of the NP-POL peptide isolation process.

pH 3.6; 10 mM 2,4,6-tripyridyl-s-triazine; and 20 mM $FeCl_3 \times 6H_2O$ (10 : 1 : 1 *v*/*v*)) was mixed with 1 ml of sample. After 10 min of reaction, the absorbance was measured at $\lambda = 593$ nm. An aqueous solution of known Fe(II) concentrations was used for calibration (in the range from 100 μg to 1000 μg). Results were expressed as μg Fe^{2+}.

2.8. Determination of Fe(II) Ion Chelation. Chelation of iron ions by the NP-POL peptide was estimated by the method of Xu et al. [35] with some modifications. A 250 μl sample was mixed with 1250 μl of H_2O and 110 μl of 1 mM $FeCl_2$. After 2 min, 1 ml of 500 μM ferrozine aqueous solution was added and the mixture was allowed to react for 10 minutes. The absorbance of the ferrous iron-ferrozine complex was measured spectrophotometrically at 562 nm. A known concentration of $FeCl_2$ (0 μg–20 μg) was used to generate a standard curve, and the ability to chelate iron ions was expressed as μg Fe^{2+}.

2.9. Determination of Intracellular ROS Level. Intracellular ROS was analyzed using a 2′,7′-dichlorofluorescein diacetate (DCFH-DA) fluorescence assay. PC12 cells were plated onto 96-well poly-L-lysine-coated plates in DMEM culture medium 24 h before experiments. The NP-POL

peptide was applied to the PC12 cells at 1 μg/ml, 10 μg/ml, and 100 μg/ml simultaneously with 6-OHDA (150 μM), 1 h before exposure to 6-OHDA, or without exposure to 6-OHDA. 24 hours later, the medium was removed, and the cells were washed with DMEM medium without FBS and then incubated with 25 μM DCFH-DA for 40 min at 37°C. The fluorescence intensity was measured every 30 minutes at 485 nm (excitation) and 527 nm (emission) wavelengths on a microplate reader (PerkinElmer, Massachusetts, USA). Changes in absorbance were expressed as relative fluorescence units (RFU)/min. Data are presented as a percentage of control.

2.10. Determination of Protein Content. Protein concentration in the analyzed samples was determined by a bicinchoninic acid kit according to the manufacturer's suggestions.

2.11. Western Blot Analysis. PC12 cells (1×10^6 cells/ml) were seeded onto poly-L-lysine-coated 6-well culture plates and incubated with NP-POL (100 μg/ml), 6-OHDA (150 μM), or NP-POL and 6-OHDA applied simultaneously, for 5 min, 10 min, 30 min, and 60 min at 37°C. Next, the cells were lysed by RIPA buffer (150 mM NaCl, 50 mM Tris-HCl pH 7.5, 5 mM EDTA, 1% Triton X-100, 0.1% SDS, and 0.5% deoxycholate) supplemented with a protease and phosphatase inhibitor cocktail (Roche), 1 mM NaF, and 2 mM Na_3VO_4 for 30 min in ice. Lysates were centrifuged at 14,000g for 10 min (4°C), and the protein content was measured using a BCA kit. 50 μg of protein samples was separated on 12% sodium dodecyl sulfate- (SDS-) polyacrylamide gel and transferred to a nitrocellulose membrane. The membrane was blocked (Tris-HCl buffer, pH 7.0, 5% Tween 20 (TBS-T), and 5% nonfat dried milk) for 1 h at room temperature and then probed overnight at 4°C with primary antibodies anti-ERK 1/2 and anti-phospho-ERK 1/2 diluted 1 : 1000 in TBST with 5% BSA, and for 1 h at room temperature using secondary antibodies conjugated with alkaline phosphatase (1 : 10,000 in TBST with 5% BSA) according to the standard procedure. Immunocomplexes were visualized using a NBT/BCIP substrate and analyzed in a ChemiDoc MP Imaging System.

2.12. Analysis of Neurite Outgrowth (a) and Protection before 6-OHDA (b)

(a) PC12 cells (1×10^4 cells/well) were plated onto poly-L-lysine-coated chamber slides (Nunc) and cultured in Opti-MEM-reduced serum medium (Gibco) at 37°C and 5% CO_2 for 2 h. NP-POL (1 μg/ml, 10 μg/ml, and 100 μg/ml) was added to the cells as a potential inducer of neuritogenesis. NGF (0.1 μg/ml) was used as a positive control, while untreated PC12 cells were used as a negative control. PC12 cells were maintained at 37°C in 95% humidified atmosphere/5% CO_2 for 3–6 days in the presence of the tested substances. Cells were observed by phase-contrast microscopy, and the number of neurite-positive cells was counted.

(b) PC12 cells (1×10^4/well) were plated onto poly-L-lysine-coated chambered slides and cultured in Opti-MEM-reduced serum medium at 37°C and 5% CO_2 for 2 h. When cells were settled, 0.1 μg/ml of NGF was added into each well. After 96 h, differentiated PC12 cells were preincubated with NP-POL (1 μg/ml–100 μg/ml) for 1 h and then exposed to toxic 6-OHDA (150 μM) for 24 h. NGF-treated cells were used as a positive control, and 6-OHDA-treated cells were used as negative control. Images of the treated cells were captured with a digital camera. The number of neurite-bearing cells was used to evaluate neurite outgrowth and retraction in response to treatment.

2.13. Statistical Analysis. Each experimental procedure was performed in at least three independent cell preparations with two replicates each. One-way ANOVA followed by Dunnett's multiple comparison test was used to compare control and treated groups with $p < 0.05$ considered statistically significant.

3. Results and Discussion

Colostrum and milk are the initial mammalian nourishment. They are the richest reservoir of important nutrients in newborn development. They contain protective and supporting factors, such as immunoglobulins, cytokines, and also lymphokines and peptides which may stimulate the neonate immune system and play a regulatory role [36, 37]. One of them is a proline-rich polypeptide complex (PRP) from ovine colostrum, also known as Colostrinin (CLN). PRP was first discovered over 30 years ago as an IgG_2-PRP complex and was subsequently found in human, bovine, and caprine colostra [22]. The immunomodulatory activity of PRP suggests a therapeutic use in the case of diseases in which changes in innate immunity play a role, including neurodegenerative disorders [23]. The current study presents a method of isolation of a previously unknown NP-POL peptide from PRP and shows its potential biological role in the regulation of cellular mechanisms regulating the survival of nerve cells treated with neurotoxic 6-hydroxydopamine and its potential use as a pharmacological preparation in the treatment of Parkinson's disease.

3.1. NP-POL Peptide Isolation and Identification. PRP was isolated from colostrum using the method described by Janusz et al. [22]. A selective extraction with 60% methanol in a one-step protocol produced a PRP-rich solution, which after ammonium sulfate precipitation gave a final preparation, designated MOHS. Treatment of the colostrum with EDTA to dissolve casein micelles prior to alcohol extraction increased the yield of PRP several times [28]. The employment of EDTA allowed discovery of a previously unknown nonapeptide named NP-POL, which in the presence of a chelate was released from the complex (Figure 1). The nonapeptide precipitation obtained from 100 mg of an ECa sample led to the highest yield. The elution profile obtained after the separation of the ECa sample on Bio-Gel P2 in molecular

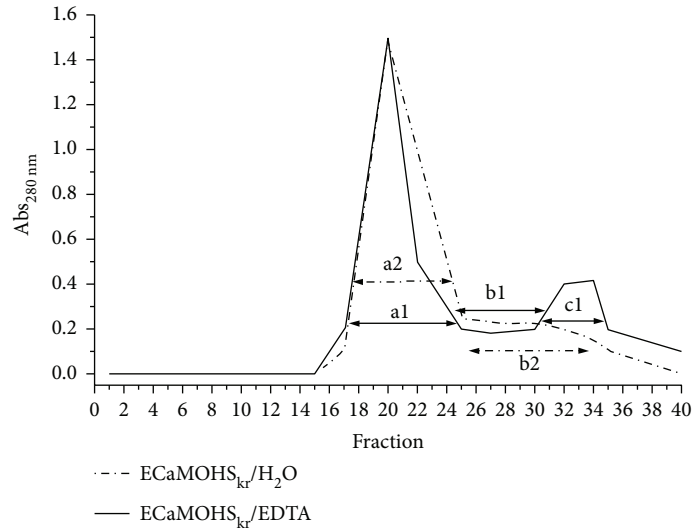

Figure 2: Separation of the NP-POL peptide from Colostrinin by Bio-Gel P2 molecular sieve beads.

sieve chromatography was characterized by the presence of a high peak of "a1" and a much lower peak of "c1" (Figure 2). The fraction "c1" was then fractionated by RP-HPLC. The obtained chromatogram showing the changes in absorbance at $\lambda = 220$ nm over time confirmed the presence in the "c1" peak of peptide fractions differing in hydrophobic/hydrophilic properties, released from C-18 by acetonitrile at a concentration of 15%–60% (see supplementary data available here). Electrophoretic analysis revealed the presence of peptides of MW about 1 kDa in fraction 2. Amino acid sequence analysis of subfraction "c1" revealed the presence of a nonapeptide with the Arg-Pro-Lys-His-Pro-Ile-Lys-His-Gln sequence. Screening of homologous proteins in the UniProt protein sequence database using the Fasta 3 program showed that the newly discovered NP-POL nonapeptide showed 100% homology to the 16- to 24-amino acid fragment of the sheep and beef alpha s1 casein precursor and 88.88% identity with alpha-s casein (see supplementary data).

3.2. Immunoregulatory Activity of NP-POL Nonapeptide. It has been previously shown that PRP modulates cytokine production by human whole blood cell cultures. In addition, PRP inhibits the production of nitric oxide (NO) induced by proinflammatory agents both *in vivo* and *in vitro* [23]. Cytokines are the proteins which stimulate or inhibit the activation, proliferation, and differentiation of various target cells upon antigen activation. They also participate in regulation of inflammation and immunity and are crucial for protection against infection and injury. In turn, NO is an important agent functioning as an effector molecule in biological signaling, connected with regulation of immune responses, cell differentiation, and apoptosis [4].

In the present study, we determined the cytokine- and NO-inducing activity of the previously unknown PRP complex constituent—NP-POL nonapeptide. Human whole blood cultures used as an experimental model ex vivo mimic the natural environment of immunocompetent cells and preserve the various intercellular communications between the different blood cell populations. In our study, we determined the levels of two types of cytokines: IL-1β and TNF-α, secreted by Th1 cells involved in cellular immunity, and IL-6 and IL-10 secreted by Th2 cells, participating in the humoral immune response. Additionally, murine bone marrow-derived macrophages (BMDM) were used as a model to determine the effect of NP-POL on NO production. The results obtained showed that the NP-POL peptide was not a cytokine or NO inducer and also did not inhibit production of nitric oxide (NO) induced by LPS (data not shown).

3.3. Neuroprotective Effect of NP-POL Nonapeptide. Parkinson's disease is one of the most common neurodegenerative movement disorders, caused by a selective loss of dopaminergic neuronal cells in the midbrain region substantia nigra pars compacta [1, 2]. One of the many causes of PD is the accumulation of free radicals and oxidative stress products which lead to selective neuronal loss [4]. Our work was designed to ascertain whether the newly discovered PRP component NP-POL nonapeptide has a potential neuroprotective and antiapoptotic capacity. We mainly focused on its activity against the neurotoxicity of 6-hydroxydopamine on neuronal PC12 cells and its potential antioxidant properties. 6-OHDA, which is a toxic dopamine analog, has been detected in both rat and human brains after long-term L-DOPA administration. It is a redox active neurotoxin which is commonly used to produce a Parkinsonian pattern of neuronal loss [12, 14].

3.3.1. NP-POL Protects PC12 Cells from 6-OHDA-Induced Toxicity. Firstly, the cytotoxic effect of NP-POL on PC12 Tet On cells was determined. Our results indicated that NP-POL at doses ranging from 1 μg/ml to 150 μg/ml neither displayed cytotoxicity nor showed any adverse effect on PC12 cell viability at higher concentrations (Figure 3(a)). Then, we investigated the protective effect of NP-POL against 6-OHDA toxicity. PC12 cell viability was reduced by 6-

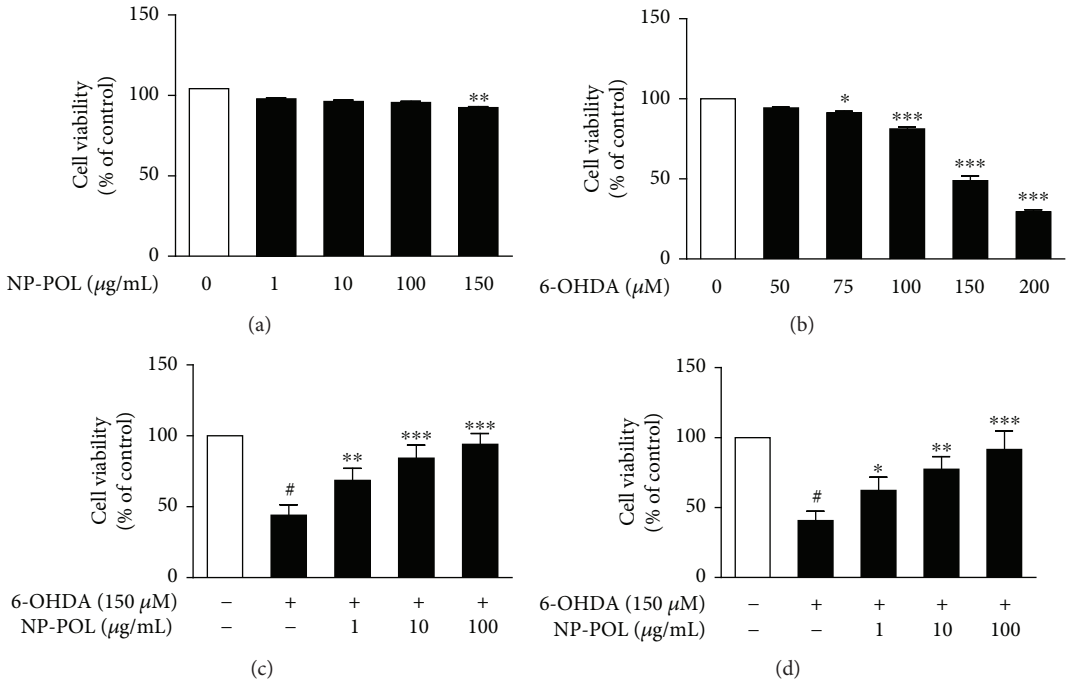

Figure 3: The effects of NP-POL on 6-OHDA-induced cytotoxicity of PC12 cells. PC12 cells (1×10^4/well) were incubated for 24 h with inducers: NP-POL (1 μg/ml–150 μg/ml) (a) or toxin 6-OHDA (1 μM–200 μM) (b). To measure the neuroprotective effect of NP-POL, the nonapeptide was preincubated with cells for 1 h before application of 150 μM 6-OHDA (c) or was applied simultaneously with 6-OHDA (150 μM) (d). Cell viability was measured by the MTT assay. Data are presented as mean ± SD ($n = 3$). Results were considered significant at $^{\#}p < 0.001$ versus untreated cells and at $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ versus 6-OHDA.

OHDA treatment in a dose-dependent manner. However, 150 μM of 6-OHDA decreased PC12 cell viability by about 50%, and this dose was used to measure the neuroprotective activity of NP-POL (Figure 3(b)). It was shown that both 1 h pretreatment (Figure 3(c)) and also simultaneous application (Figure 3(d)) of NP-POL with 6-OHDA significantly increased cell survival in a dose-dependent manner. As was shown previously, PRP possesses neuroprotective activity against toxic amyloid β_{42}, which may suggest a potential antiapoptotic activity [23]. The present work suggests that the NP-POL peptide could effectively protect PC12 cells from 6-OHDA, a dopaminergic neuron-damaging toxin.

3.3.2. NP-POL Protects Differentiated PC12 Cells from 6-OHDA Toxicity. The neuroprotective effect of NP-POL against 6-OHDA toxicity was also determined on an NGF-differentiated PC12 cell model. Firstly, we showed that undifferentiated PC12 cells cultivated with NP-POL for 5 days did not produce neurites (data not shown). Next, PC12 cells were differentiated in the presence of NGF for 5 days and then treated with NP-POL alone, neurotoxin 6-OHDA alone, or with NP-POL applied to the PC12 cells 1 h before the neurotoxin. As shown in Figures 4(b)–4(d), NP-POL did not show any toxic effect on the PC12 cells. When the cells were treated with 6-OHDA, the cell number was reduced, the cells began to swell, the neurites started to retract, the network collapsed, and cell debris appeared (Figure 4(e)). To investigate whether NP-POL had protective activity against 6-OHDA-induced neurite damage, the cells were preincubated for 1 h with NP-POL before 6-OHDA application (Figures 4(f)–4(h)). Compared with the cells exposed to 6-OHDA alone, 1 μg/ml and 10 μg/ml of NP-POL exhibited a weak protective effect (Figures 4(f) and 4(g). However, 100 μg/ml of NP-POL peptide effectively increased cell viability, with significant protection of neurites and reduction of cell debris (Figure 4(h)).

3.3.3. NP-POL Peptide Attenuates 6-OHDA-Induced ROS Generation. Overproduction of ROS and an impaired antioxidative defense system are some of the initial steps in PD pathology [4]. The toxic effect of 6-OHDA, used here to create experimental models of PD, can be linked to the overproduction of ROS in PC12 cells, which may increase the expression of redox-sensitive transcription factors responsible for oxidative and inflammatory reactions in PD and also leads to increased levels of toxic products of proteins and lipid oxidation, and also leads to severe mitochondrial dysfunction and neuronal apoptosis [14, 38].

Therefore, investigation of the effect of NP-POL peptide on ROS generation was the next goal of our study. Firstly, using the DPPH method, FRAP method, and Fe^{2+} scavenging method, we revealed no antioxidant capacity of NP-POL itself (see supplementary data). Next, the effect of NP-POL on 6-OHDA-induced ROS generation was examined. PC12 cells treated with NP-POL showed a significant decrease in DCF fluorescence intensity in a dose-dependent manner (39.8% ± 8%, 36.2% ± 1%, and 31.7% ± 0.9% for 1 μg/ml, 10 μg/ml, and 100 μg/ml of NP-POL, respectively; all data was expressed as % of control) (Figure 5(a)). In comparison, in 6-OHDA-treated cells, a

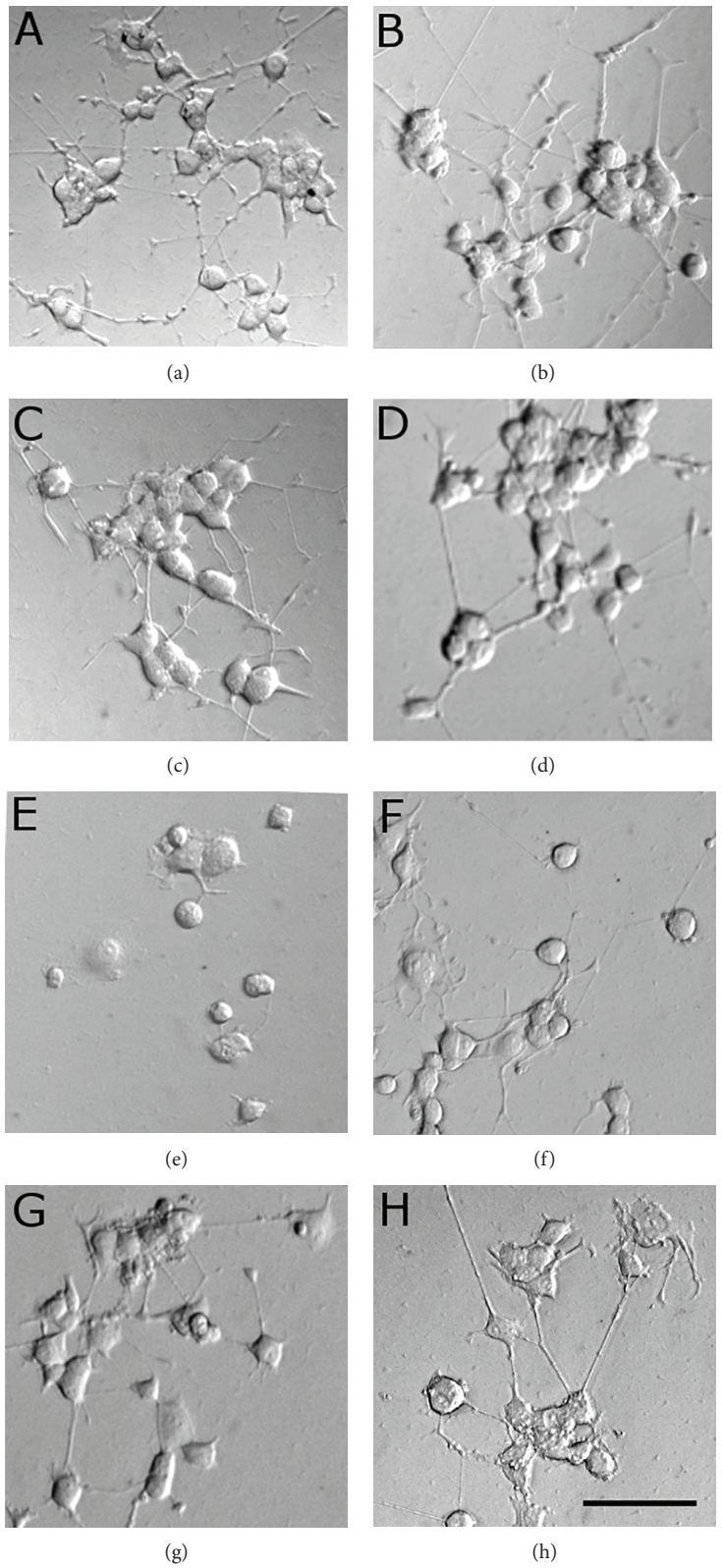

Figure 4: The neuroprotective effect of the NP-POL peptide on NGF-differentiated PC12 cell viability treated with 6-OHDA neurotoxin. (a) Control, (b) NP-POL 1 μg/ml, (c) NP-POL 10 μg/ml, (d) NP-POL 100 μg/ml, (e) 6-OHDA 160 μM, (f) NP-POL 1 μg/ml + 6-OHDA 160 μM, (g) NP-POL 10 μg/ml + 6-OHDA 160 μM, and (h) NP-POL 100 μg/ml + 6-OHDA 160 μM. Scale bar = 50 μM.

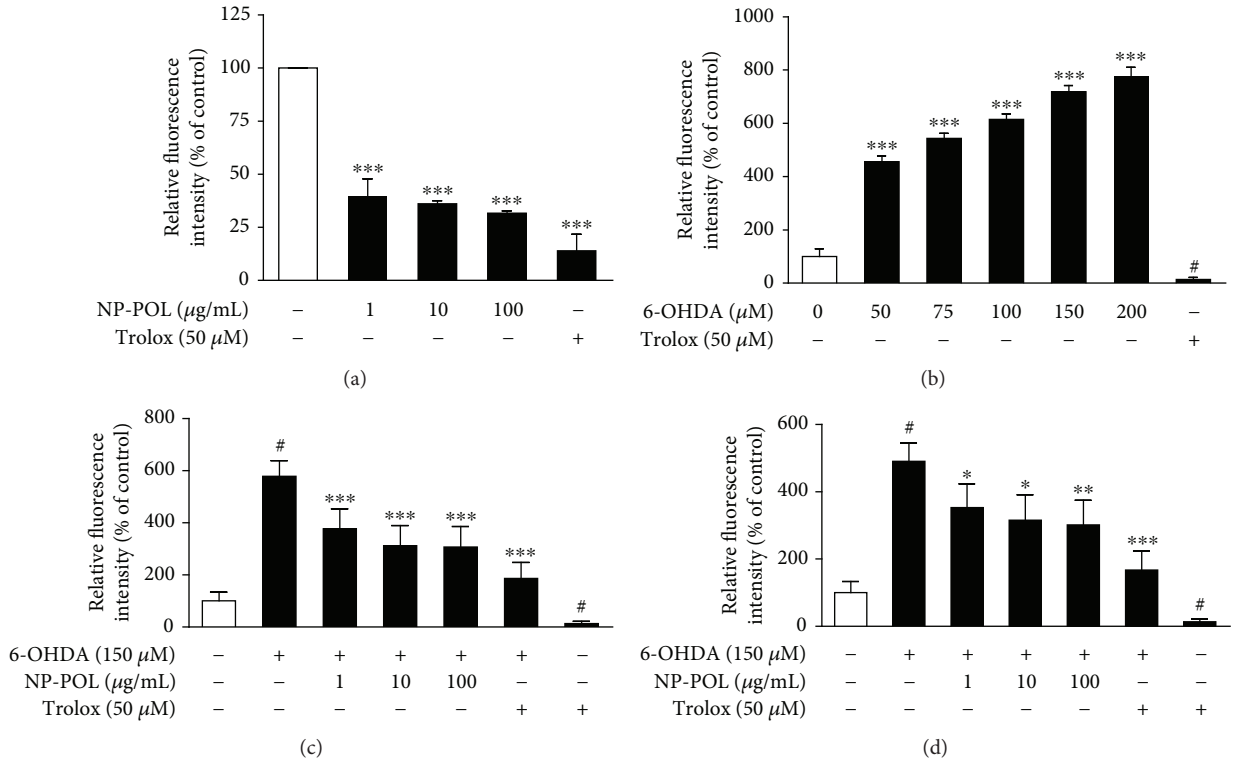

Figure 5: The effect of the NP-POL peptide on intracellular ROS generation induced by 6-OHDA. (a) NP-POL significantly reduced intracellular ROS levels in PC12 cells. (b) 6-OHDA induced an exponential increase in intracellular ROS in exposed cells. Both 1 h preincubation (c) with selected doses of NP-POL before 24 h of 6-OHDA exposure and 24 h of coincubation with NP-POL and 6-OHDA (d) resulted in a significant decrease in intracellular ROS levels. Data are expressed as mean ± SD ($n = 3$). Results were considered significant at $^{\#}p < 0.001$ versus untreated cells and $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ versus 6-OHDA.

significant increase of DCF fluorescence intensity was observed (Figure 5(b)). This effect was also dose-dependent, while a sevenfold increase (719% ± 22.9% versus control 100%) was observed at the dose of 150 μM. However, 1 h preincubation and coincubation of NP-POL with 6-OHDA (150 μM) significantly reduced ROS generation when compared to the 6-OHDA applied alone (1 h preincubation with NP-POL: 6-OHDA alone: 578% ± 60.3%, NP-POL 1 μg/ml and 6-OHDA: 377% ± 76.8%, NP-POL 10 μg/ml and 6-OHDA: 312.5% ± 77.3%, and NP-POL 100 μg/ml and 6-OHDA: 307% ± 78.2% (Figure 5(c)); simultaneous application: 6-OHDA alone: 490.95 ± 54%, NP-POL 1 μg/ml + 6-OHDA: 353% ± 70.9%, NP-POL 10 μg/ml + 6-OHDA 315% ± 75.6%, and NP-POL 100 μg/ml + 6-OHDA 301.6% ± 73.3%) (Figure 5(d)). The same inhibitory effect of NP-POL was observed when H_2O_2 was used as a source of ROS (data not shown).

Previous studies have investigated the inhibitory effects of PRP on oxidative stress and ROS generation. It was confirmed that PRP effectively and specifically reduces the generation of ROS, protein, and lipid oxidation, regulates glutathione metabolism, and improves antioxidant system activity [25, 39]. These findings suggest that PRP maintenance towards intracellular antioxidant homeostasis is due to the biological capacities of its constituent peptides. One of them is the constituent NP-POL peptide.

3.3.4. NP-POL Nonapeptide Regulates Activity of ERK 1/2 MAP Kinases. ERK 1/2 kinase, one of the most well-characterized members of the mitogen-activated protein (MAP) kinase family, regulates a range of processes from metabolism and inflammation to cell death and survival. In the nervous system, ERK 1/2 regulates synaptic plasticity, brain development, and repair, as well as cellular response to stress factors [40, 41]. 6-OHDA-induced oxidative stress mediates the cellular response of survival and apoptosis involving, besides p38 MAPK and JNK kinases, downstream kinases of ERK 1/2 signaling promoting cell survival [42]. A number of reports indicate that transient phosphorylation of ERK 1/2 kinases may protect against 6-OHDA-induced cytotoxicity in neuronal cells via the PKA/Bcl2-dependent pathway [41, 42]. In our present work, we showed that PRP possesses neuroprotective activity in its ability to activate the cGMP/ERK 1/2 signaling pathway in PC12 cells [26]. Therefore, it was important to check whether the neuroprotective effect of NP-POL on 6-OHDA-treated PC12 cells may be related to its influence on ERK 1/2 activation.

It was shown that the NP-POL nonapeptide at 100 μg/ml induced the transient phosphorylation of ERK 1/2 in PC12 cells after 5 min, compared to the control group (Figure 6(a)). PC12 cells treated with 6-OHDA at 150 μM induce sustained ERK 1/2 phosphorylation for 60 min (Figure 6(b)). However, the sustained phosphorylation of

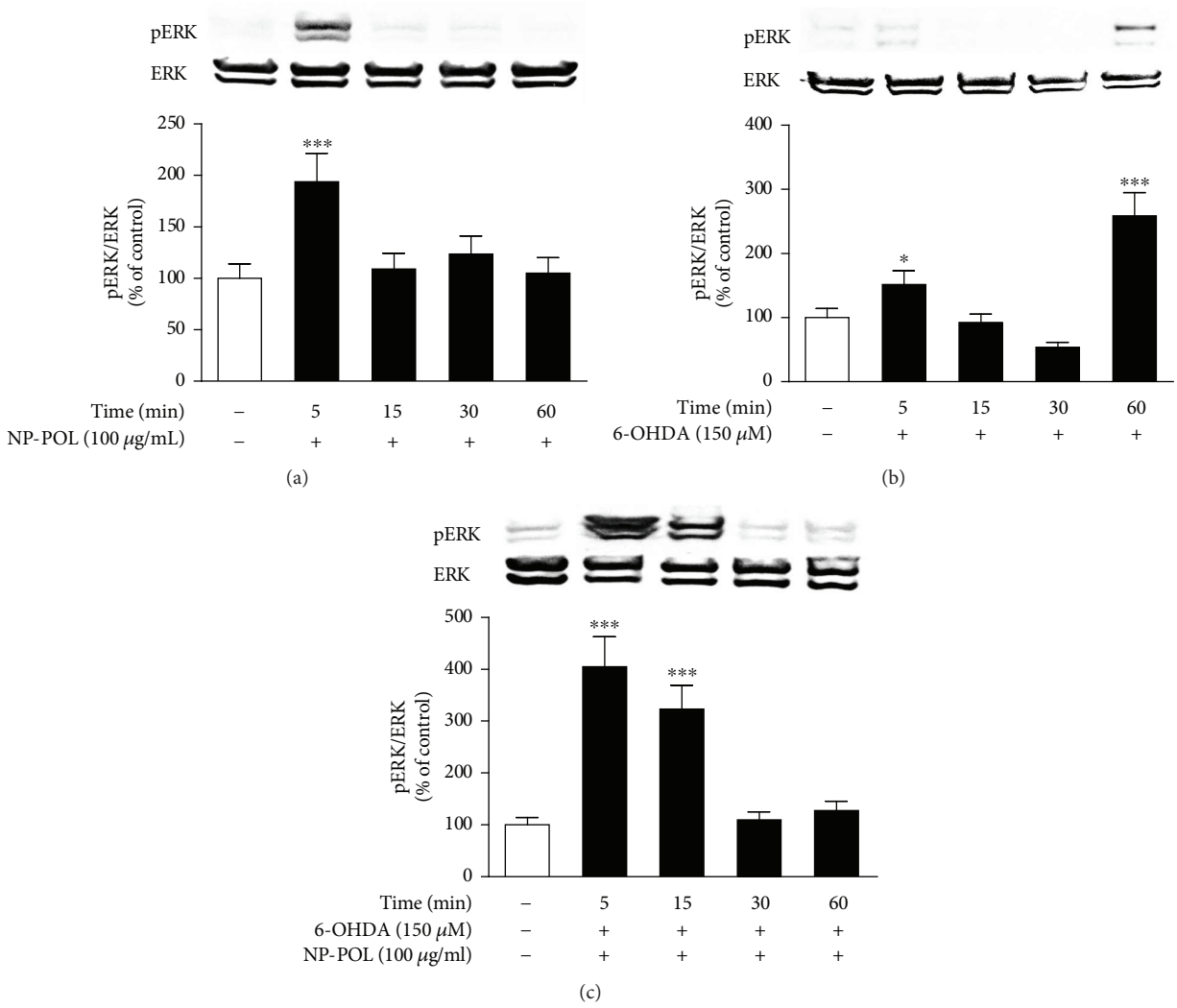

Figure 6: The effect of NP-POL peptide on ERK 1/2 kinase activity. (a) NP-POL transiently enhanced ERK 1/2 activation at the selected dose of 100 μg/ml after 5 min of incubation. (b) Stable 6-OHDA-enhanced ERK 1/2 activation after 60 min of incubation. (c) Coincubation of NP-POL with 6-OHDA resulted in the transient activation of ERK 1/2 after 5 and 15 min. Data are presented as mean ± SD ($n = 3$). Results were considered significant at $^{*}p < 0.05$ and $^{***}p < 0.001$ versus untreated cells.

ERK 1/2 induced by 6-OHDA (150 μM) was reduced by coincubation with 100 μg/ml of NP-POL nonapeptide for 60 min with a simultaneous induction of transient phosphorylation of ERK 1/2 at 5–15 min (Figure 6(c)). Kulich and Chu [43, 44] found that catalase was capable of attenuating 6-OHDA-mediated sustained ERK phosphorylation. Therefore, we can speculate that the NP-POL nonapeptide could inhibit 6-OHDA-mediated sustained ERK 1/2 activation and toxicity by the effect of NP-POL on catalase activation.

4. Conclusions

In the present work, we demonstrated for the first time a method of isolation of the new PRP component, NP-POL nonapeptide with the RPKHPIKHQ sequence. NP-POL was isolated from PRP by Bio-Gel P2 molecular sieve beads using 50 mM EDTA. The NP-POL nonapeptide showed a neuroprotective effect on PC12 cells treated with neurotoxic 6-OHDA (6-hydroxydopamine) exerting a beneficial effect on the regulation of cell survival and the inhibition of ROS overproduction released during 6-OHDA metabolism, probably by its ability to activate the antioxidant system. Additionally, this effect was connected with transient ERK 1/2 kinase activation. Thus, these results suggest that the NP-POL nonapeptide would likely be a promising agent in the treatment of neurodegenerative diseases, such as Parkinson's disease.

Data Availability

The data used to support the findings of this study are available from co-corresponding authors upon request.

Conflicts of Interest

All the authors who took part in this study declare that they have nothing to disclose regarding competing interests of funding from industry with respect to this manuscript.

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Supplementary Materials

Figure S1. Elution profile of peptide fraction "c1" separated by RP-HPLC. Fraction "c1" previously isolated by Bio-Gel P2 was dissolved in 0.1% TFA and loaded on a Nucleosil 100 C-18 column. The proteins were eluted with an acetonitrile linear gradient (0%–60% for 80 min) containing 0.1% TFA at a flow rate of 1 ml/min. Figure S2. SDS-PAGE analysis of peptide fraction "c" obtained by the molecular sieve beads Bio-Gel P2. The samples (10 mg) were loaded on to gel slabs (12%). The gel slabs were cast 24 h ahead of electrophoresis and stained with Coomassie Brilliant Blue R 250 for proteins. NP POL localization: line 2, MWM—molecular size marker. Table S1. Comparison of the amino acid sequence of NP POL nonapeptide with a sequence of homologous proteins. The search for homologous proteins based on UniProt protein sequences was carried out using the FastA3 software package. NP POL nonapeptide showed 100% homology to the 16- to 24-amino acid fragment of the sheep and beef alpha s1 casein precursor and 88.88% identity with the alpha s1 casein. Table S2. Antioxidant capacity of the NP-POL peptide measured in tests conducted *in vitro*. The antioxidant activity of the NP POL nonapeptide (0.1 μg/ml–100 μg/ml) was assessed by DPPH, FRAP, and Fe(II) ion chelation methods (for details see Sections 2.6, 2.7, and 2.8). All data are expressed as mean values ± SD, $n = 3$. *(Supplementary Materials)*

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Review Article

Relevance of Alternative Routes of Kynurenic Acid Production in the Brain

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The catabolism of tryptophan has gained great importance in recent years due to the fact that the metabolites produced during this process, with neuroactive and redox properties, are involved in physiological and pathological events. One of these metabolites is kynurenic acid (KYNA), which is considered as a neuromodulator since it can interact with NMDA, nicotinic, and GPR35 receptors among others, modulating the release of neurotransmitters as glutamate, dopamine, and acetylcholine. Kynureninate production is attributed to kynurenine aminotransferases. However, in some physiological and pathological conditions, its high production cannot be explained just with kynurenine aminotransferases. This review focuses on the alternative mechanism whereby KYNA can be produced, either from D-amino acids or by means of other enzymes as D-amino acid oxidase or by the participation of free radicals. It is important to mention that an increase in KYNA levels in processes as brain development, aging, neurodegenerative diseases, and psychiatric disorders, which share common factors as oxidative stress, inflammation, immune response activation, and participation of gut microbiota that can also be related with the alternative routes of KYNA production, has been observed.

1. Kynurenic Acid (KYNA)

The main tryptophan (Trp) catabolism route is through the kynurenine pathway (KP), where the final product is the nicotinamide adenine nucleotide (NAD+) de novo production. NAD+ plays an essential role in metabolism and cellular energy homeostasis. NAD+/NADH ratio dysfunction is related to mitochondrial disorders, aging, and age-related diseases [1]. In humans, it is estimated that 95% of Trp is catabolized through KP [2]. Along with this pathway, some neuroactive metabolites are produced. One of them is kynurenic acid (KYNA), which is considered a natural antagonist for the glycine-B coagonist site of N-methyl-D-aspartate receptor (NMDAr). However, high micromolar concentrations of KYNA are needed to block NMDAr functions [3–6]. Also, AMPA receptors can be competitively inhibited by KYNA at millimolar concentrations, but in nanomolar to micromolar levels, KYNA induces their facilitation through allosteric modulation [7]. KYNA can also inhibit noncompetitively α7-nicotinic receptors (α7-nAChRs; IC_{50}~7 μM) which can bind to α-bungarotoxin being the most prevalent in the brain [5, 8, 9]. Under

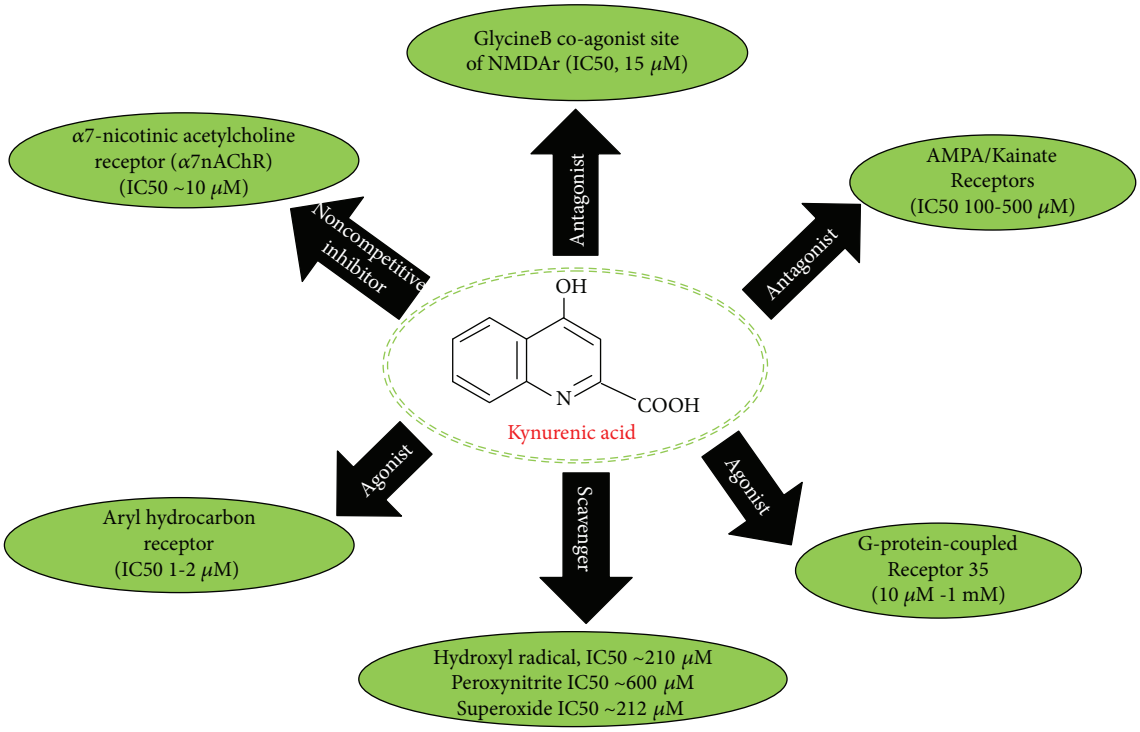

Figure 1: Targets of kynurenic acid (KYNA). AMPA: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; α7nAChR: α7-nicotinic acetylcholine receptor; IC50: half maximal inhibitory concentration; NMDAr: N-methyl-D-aspartate receptor.

physiological conditions, it has been suggested that α7-nAChRs are the primary endogenous target of KYNA [10–12]. Due to KYNA can interact with NMDAr, α7-nAChRs and AMPAr[9,13,14]and since its levels secondarily affect the extracellular concentrations of glutamate, dopamine, acetylcholine and γ-aminobutyric acid (GABA) is considered as neuromodulator[10–19]. Importantly, all these receptors and neurotransmitters are critically involved in neurodevelopment, plasticity, cognition, behavior, and memory process among others [20].

On another hand, it has been shown that G-protein-coupled receptor (GPR35) is activated by KYNA [21]. The stimulation of this receptor is associated with neuronal excitability regulation and transmitter release, since GPR35 activation induces N-type calcium channel inhibition in rat sympathetic neurons [22, 23]. The KYNA effects on glutamate levels and the reduction of excitatory transmission can also be related with the ability of KYNA to activate GPR35 [23, 24]. In this regard, it has been proposed that the KYNA interaction with GPR35 reduces the release of proinflammatory cytokines in cell lines, which can be associated with the analgesic effects of KYNA in inflammatory models [25, 26]. Another target of KYNA is the aryl hydrocarbon receptor (AHR), which is considered a xenobiotic receptor [27], and its activation is associated with the suppression of cellular immune response favoring carcinogenesis and tumor outgrowth [25, 27]. Specifically, stimulation of AHR by KYNA enhances the expression of IL-6, fact by which KYNA was considered as a factor involved in the escape of tumors, via the IL-6-dependent pathway, for immune surveillance [27].

Finally, KYNA can also interact with reactive oxygen species (ROS) in chemical combinatory systems, and it can lead to decrease ROS production and lipid peroxidation induced by prooxidants, in rat brain homogenates. Importantly, this scavenger property of KYNA is independent of its effect on the NMDA and cholinergic receptors [28].

The relevance of KYNA in the brain has been experimentally shown both during development and adulthood. In this context, KYNA levels have been found higher in fetal brain [29–31] and decreased in the postnatal period and in adult age [31]. However, during adulthood, fluctuation of brain KYNA levels provokes a broad spectrum of behavioral and cognitive alterations [18, 32, 33], and when brain KYNA levels decreased, cognitive process improves in mice and rats [18, 34]. These evidences strongly suggest an important role of KYNA during neurodevelopment and adulthood.

As was mentioned before, KYNA is an endogenous metabolite with multiple targets (Figure 1) that can lead to different effects depending on the environment conditions. Until now, the major production of KYNA has been attributed to kynurenine aminotransferases (KATs). Nevertheless, in events such as neurodevelopment, aging, some neurodegenerative diseases, and psychiatric disorders, the production of KYNA cannot be completely explained by the kynurenine aminotransferase activity alone but there are other common factors which could be involved in its production. In this review, we are focused on the alternative mechanisms by which KYNA can be produced since these could be extremely important under certain conditions.

2. Kynurenine Aminotransferase Canonical Way to Produce KYNA

The canonical route of KYNA formation is through the kynurenine pathway by kynurenine aminotransferases. These enzymes catalyze the irreversible transamination of kynurenine to produce KYNA. Until now, 4 isozymes of kynurenine aminotransferases have been described. All the isozymes are pyridoxal-5′-phosphate dependent and require an α-ketoacid molecule as a cosubstrate. KATs have a low affinity for their substrate (approximately 1 mM) so that the rate of KYNA formation is directly controlled by local kynurenine availability [35, 36]. Since the canonical pathway is the most studied, there are many reviews about these isozymes in the literature [37, 38]. Here, we provide a table with the principal biochemical characteristics of kynurenine aminotransferase isozymes (Table 1).

Recently, it has been reported that KATs can also take D-kynurenine (D-Kyn) as a substrate both in rat and in human tissues [39]. The de novo production of KYNA from D-Kyn in the rat prefrontal cortex was 30 times less potent than that from L-Kyn. The production induced by D-Kyn was inhibited just 30% by the KAT inhibitor AOAA, while the production induced by L-Kyn was almost abolished by the same treatment. Similar effects were observed in human homogenates—the production of KYNA from the enantiomer L-Kyn decreased around 98% in presence of the KAT inhibitor in the human brain and liver, while the KYNA production induced by D-Kyn drops at about 70% in presence of AOAA. Considering the low affinity for the substrate shown by the KATs and the evidence that the kynurenic acid produced by D-Kyn is not completely inhibited by the KAT inhibitor, it is feasible to suggest that there are alternative mechanisms by which KYNA can be produced and they could be relevant in physiological conditions as well as in pathological events.

3. D-Amino Acid Oxidase and D-Amino Acids in KYNA Production

During many years, L-amino acids have had more attention than D-enantiomers; however, recently, it has been shown that D-amino acids are present in animals and humans at high concentrations and fulfill specific biological functions, as was demonstrated with a pool of amino acids necessary for protein synthesis; after being enzymatically converted to L-amino acids, they could also act antagonistically to L-amino acids, deactivating their biological site [40, 41]. The presence of D-amino acids in mammals results from microorganisms or racemization of L-amino acids to their D-isomer, in food and other proteins which are pH, time, and temperature dependent [40]. Alterations in the concentrations of D-amino acids might occur in some disorders related to bacterial pathogens and immune activation [42].

Specifically for KYNA production, D-Trp and D-Kyn have been studied for many years. The first evidence showing that D-tryptophan (D-Trp) could be utilized for growth was reported by du Vigneaud and coworkers in 1932 [43]. But, it was Berg in 1953 [44] who demonstrated that D- and L-Trp can be equally effective to support growth in rats. After these findings, more studies on D-Trp were performed. It was found that in rat liver slices, D-Trp and D-Kyn were metabolized slower than L-Trp and L-Kyn, respectively. After incubation with D-Trp, small amounts of L-Kyn, D-Kyn, and KYNA were found [45]. In 1971, it was shown that after feeding or injecting rabbits with D-Trp or D-Kyn, they excreted kynurenic acid as well as indole pyruvic acid [46]. Also, in the normal human subject, it has been observed that, after ingestion of D-Trp, D-Kyn, indole pyruvic acid (IPA), and acetyl-Trp are excreted [47]. Later, it was showed that D-formylkynurenine was the intermediate during the conversion of D-Trp to D-Kyn, and the enzyme that catalyzed this reaction was inhibited by the presence of L-Trp [48]. *In vitro* experiments demonstrated that D-Kyn can be converted to KYNA in kidney preparations (slices and homogenates) and this conversion can be due to the presence of D-amino acid oxidase, since purified D-amino acid oxidase from *Trigonopsis variabilis* rapidly converts D-Kyn to KYNA [46]. These findings confirmed the previous results in which it was proposed that the mechanism by which D-Trp produced KYNA was independent of D-Trp racemization to L-Trp; however, this did not completely exclude the D- to L-Trp conversion possibility. Later, it was known that the conversion of L-Kyn to KYNA was catalyzed by α-ketoglutarate-dependent transaminase, and when L-Trp and L-Kyn were incubated in the presence of α-ketoglutarate-eliminating system, there was no KYNA production. On the contrary, when D-Trp or D-Kyn were incubated in the same conditions, KYNA production was found; however, when D-enantiomers were incubated in anaerobic conditions, KYNA was not detected, suggesting an alternative mechanism for KYNA production from D-enantiomers, which involved an oxidase and discarded the transaminase reaction [49].

Additional experiments showed that when rat liver homogenates were incubated with D-Trp (3 mg) under oxygen conditions, L-Trp, IPA, D-Kyn, KYNA, and anthranilic acid were produced. However, when the homogenates were incubated under nitrogen conditions, no metabolite was formed. To determine whether all inversion processes required oxygen, D-Trp was incubated with liver homogenates under oxygen conditions for 2 h; once IPA has been formed in this period of time, the atmosphere was changed to nitrogen and sodium azide was added to minimize kynurenine production; they could observe that even with the change of atmosphere, L-Trp continued to occur at expenses of IPA, which was formed under the oxidizing conditions. This means that the amination of IPA to L-Trp do not require aerobic conditions suggesting that the transamination occurs, since the reaction was stimulated by addition of glutamic acid and pyridoxal phosphate [50]. But the important point here was that not all D-Trp were inverted to L-Kyn; additionally, considerable amount of D-Kyn was also produced. When slices or homogenates of rat liver were incubated with D-Kyn, KYNA was produced, which was abolished by benzoate addition, indicating that D-amino acid oxidase (DAAO) was involved in the reaction, since benzoate

Table 1: Biochemical characteristic of KATs.

Enzyme	Cellular brain location	Characteristic	Optimum pH	Substrates with higher potency towards	Cosubstrate	Endogenous inhibitors	References
KAT-I/glutamine transaminase K/cysteine conjugate beta-lyase 1	Glia, astrocytes (nuclei and cytosol), and neurons (cytoplasmic)	(i) KAT I and KAT III share similar genomic structures (ii) KAT I immunoreactivity was found in ventral medulla, nucleus ambiguus, nucleus of the solitary tract, and intramedio lateral cell column of the spinal cord (iii) pKa 7.6 (iv) Enzyme partially purified: Km 875 μmol/L, KYN concentrations ranging from 2 μmol/L to 2 mmol/L (v) Relative percentage of brain activity: Mouse: 25.7 Rat: 15.5 Human: 9.8 (vi) Present high activity in the cerebellum	9.5–10	Glutamine Phenylalanine Kynurenine (hKAT-I under physiological pH)	Pyruvate	Glutamine Tryptophan Phenylalanine Indole-3-pyruvic acid Cysteine	[84–93]
KAT-II/α-aminoadipate aminotransferase	Astrocytes	(i) At physiologic KYN concentrations and pH, KAT II catalyzed around 75% of KYNA synthesis in most brain areas (ii) pKa 5.7 (iii) Enzyme partially purified: Km 660 μmol/L, KYN concentrations ranging from 2 μmol/L to 2 mmol/L (iv) Relative percentage of brain activity: Mouse: 12.3 Rat: 58.7 Human: 54.1	7.4	Kynurenine Glutamate Aminoadipate Methionine	α-Ketoglutarate Pyruvate	Aminoadipate Asparagine Glutamate Histidine Cysteine Lysine 3-Hydroxykynurenine phenylalanine	[85, 89, 90, 94, 95]
KAT-III/cysteine conjugate beta-lyase 2		(i) mKAT III shows activity toward a number of amino acids (ii) mKAT III is more active than hKAT I under basic conditions (iii) Northern blot analysis showed a strong transcript in the liver, kidney, and heart and to a less extent in the brain and testis (iv) Has a higher isoelectric point than KAT I	9-10	Glutamine Histidine Methionine Phenylalanine	α-Ketobutyrate Oxaloacetate	Cysteine Glutamine Histidine Methionine Leucine Phenylalanine	[95–97]

Table 1: Continued.

Enzyme	Cellular brain location	Characteristic	Optimum pH	Substrates with higher potency towards	Cosubstrate	Endogenous inhibitors	References
		(v) 3-HK decreases mKAT III-catalyzed kynurenine transamination (vi) pKa 8.7					
KAT-IV/glutamic-oxaloacetic transaminase 2/mitochondrial aspartate aminotransferase	Mitochondria of astrocytes and neurons	(i) mKAT I, III, and IV showed high resistance to heat treatment (ii) pKa 6.9 (iii) Enzyme partially purified: Km 724 μmol/L, KYN concentrations ranging from 2 μmol/L to 2 mmol/L (iv) Relative percentage of brain activity: Mouse: 63.0 Rat: 25.8 Human: 36.1	8.5	Aspartate Glutamate	α-Ketoglutarate	Aspartate Glutamate	[94, 98]

Table 2: Enzymes involved in KYNA production from D- and L-enantiomers under different oxygen conditions.

	D-Trp → D-Kyn → KYNA	L-Trp → L-Kyn → KYNA	D-Trp → IPA	IPA → L-Trp
α-Ketoglutarate-eliminating system	✓	✘		
Aerobic conditions	✓	✓	✓	✓
Anaerobic conditions	✘	✓	✘	✓
Enzyme involved	DAAO	Transaminase	Oxidase	Transaminase

is its inhibitor [50]. An *in vivo* experiment showed that the intraperitoneal (i.p.) administration of D-Trp or D-Kyn increased the levels of KYNA in rat plasma and this production was abolished by DAAO inhibitor, 5-methylpyrazole-3-carboxylic acid (summary in Table 2) [51, 52].

The first evidence that showed that D-Kyn can produce KYNA in rodents and the human brain was showed in 2009 [15, 53], and one year later, Pérez de la Cruz and coworkers found KYNA production from D-Kyn in different human brain regions, showing that in the human cerebellum, the production of KYNA is highest than in other regions. Furthermore, coincubation with benzoic acid inhibited KYNA production [54]. Moreover, microdialysis studies proved an increase in KYNA levels after intraperitoneal administration of D- or L-Trp (100 mg/kg) or direct infusion of D-Kyn in the prefrontal cortex. Interestingly, when the DAAO inhibitor was injected in combination with D-Trp or D-Kyn, the effect in KYNA levels was decreased [55]. Following this line, knowing that DAAO is abundant and has high activity in the cerebellum [56–58], microdialysis studies were designed in this region, showing that the infusion of 100 μM of L-Kyn or D-Kyn produced 17.9 and 10.7 times more KYNA than the baseline, respectively, which was really surprising since a previous study demonstrated that KYNA production from D-Kyn needs 100 μM of this enantiomer, while only 2 μM of L-Kyn was necessary to produce almost the same amount of KYNA in rat cortex. Also, this experimental study showed the importance of DAAO in cerebellum KYNA production since *in vitro* experiments had shown that the production of KYNA from D-Kyn inhibited almost 30% by a KAT inhibitor, while it inhibited almost 70% by DAAO inhibitors [59].

Since these studies showed that KYNA can be produced in the brain from D-amino acids, new studies were focused on elucidating whether the other redox or neuroactive metabolites of the kynurenine pathway were also produced from D-enantiomers. In this context, Notarangelo and coworkers demonstrated that after i.p. D-Trp injection, the levels of L-Trp increased in the plasma, forebrain, and cerebellum, which confirmed that the conversion of D-Trp to L-Trp can take place in the brain and impact it. Then, they showed that D-Kyn increased both in the forebrain and in cerebellum and that at 30 min postinjection of D-Trp (30 mg/kg), KYNA levels were increased just in the cerebellum via DAAO activity, since the coadministration with a DAAO inhibitor decreased KYNA levels. The other branch of the pathway was also studied, and 3-HK and QUIN metabolites were increased in the forebrain after D-Trp injection; 3-HK increased 2-fold in the cerebellum, and any change in QUIN levels was not observed [42]. On the other hand, after i.p. D-Kyn injection, KYNA and 3-HK were found in the plasma, liver, forebrain, and cerebellum [60].

Since D-Trp and D-Kyn can be present in normal conditions by food intake or can be originated from microorganisms that inhabitate the digestive tract [61–64], it appears logical to suggest that D-enantiomers are, in part, responsible of KYNA, 3-HK, and QUIN levels in the brain. Knowing that kynurenine pathway metabolites have been associated with neurological disorders, it is also important to study the role of D-enantiomers since they can be responsible of the kynurenine level alterations in diseases, in which correlation with high DAAO activity or in those that are associated with previous infections is showed [65–67].

4. Indole-3-Pyruvic Acid as a KYNA Precursor

Indole pyruvic acid is a natural compound present in mammals and is the transamination product of tryptophan by the action of aromatic amino acid transaminase [68, 69]. The first studies that proposed that IPA could be a precursor of KYNA were conducted in the 1980s and demonstrated that IPA administration increased the brain content of 5-hydroxytryptamine (5-HT), 5-hydroxyindole-3-acetic acid (5-HIAA), and Trp [70, 71]. It was also demonstrated that Trp and IPA administration produced a dose-dependent increase of KYNA levels in the brain and others organs [71, 72]. Actually, the important point is that the same doses of IPA or Trp (100 mg/Kg) are able to produce almost the same concentrations of KYNA in rat brain (22 ± 2 and 23 ± 3 picomoles/g, resp.). At the same time, studies in parallel were carried out and rats were administrated with probenecid (inhibitor of KYNA's brain transport) and IPA, in order to clarify whether or not the raise in KYNA levels was due to an increased rate of synthesis or to a decreased rate of disposal. However, KYNA levels were significantly higher in animals treated with IPA + probenecid than in controls, suggesting not only that IPA indeed increases the rate of KYNA synthesis in rat brain but also that KYNA disposal occurs through a mechanism sensitive to probenecid. Until that time, it was known that the administration of IPA was able to increase KYNA levels but the mechanism was unknown. One of the hypotheses was that after administering IPA, Trp levels could be increased, which would generate a greater amount of KYNA by the canonical route. However, when ^{3}H-IPA was administered and KYNA and Trp levels were monitored in the brain alkaline extracts, there were 2600 cpm/μmol of KYNA and 380 cpm/μmol of Trp found

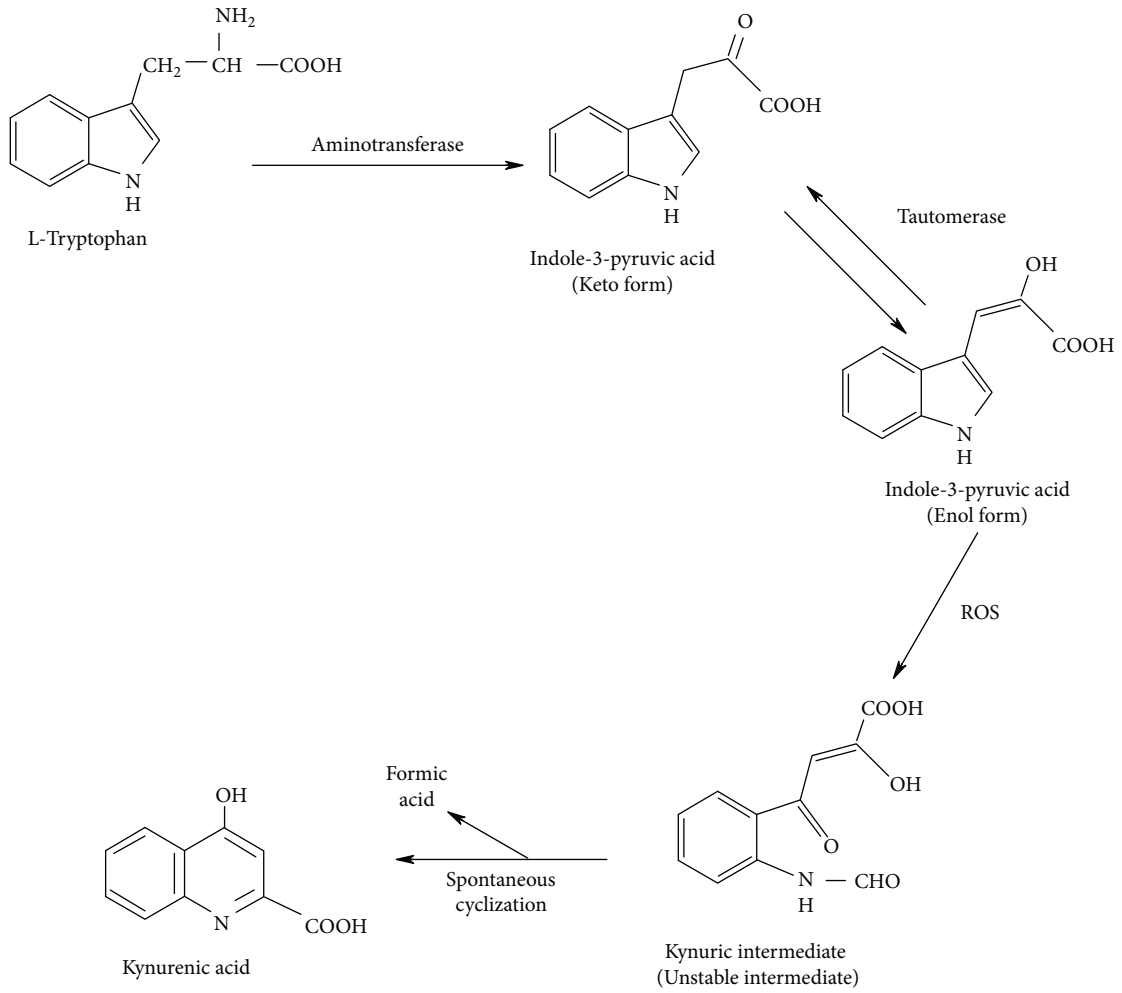

Figure 2: KYNA production from the interaction of indole pyruvic acid (IPA) with ROS (modified from Hardeland[74]).

[72], suggesting that part of IPA could be converted to Trp and then it follows the canonical pathway to produce KYNA, but there was also another mechanism involved in KYNA production by IPA [71].

In this context, Politi and coworkers [73] showed that IPA could be transformed into KYNA in different rat organ homogenates, but in the absence of enzymatic systems and with oxygen in the incubation mixture. They incubated keto and enol forms of IPA in a free enzymatic system observing that the enol form produced more KYNA than the keto form (24 ± 5 ng and 6 ± 2 ng, resp.). Because the chemical transformation of IPA to KYNA needs a radical attack from reactive oxygen species, they also incubated in the same conditions keto and enol forms, but adding a free radical generator system (ascorbate/Fe/hydrogen peroxide). They observed that under these conditions, the enol form produced 251 ± 38 ng of KYNA while the keto form produced 12 ± 5 ng of KYNA. After these results, IPA scavenging properties were demonstrated through the inhibition of chemiluminescence and malondialdehyde formation; in both, the enol form was more efficient than the keto form, which is possibly due to the fact that the enol conformation contains two conjugated double bonds in the carbon frame [73].

In summary, tryptophan can be degraded by tryptophan 2-oxoglutarate aminotransferase, whose primary product is indole-3-pyruvic acid. IPA is either produced in keto or enol tautomer (Figure 2). The enolic form can easily interact with reactive oxygen species and undergoes pyrrole ring cleavage. The kynurenic product formed then spontaneously cyclizes to produce KYNA [74]. This process can be considered in mammals since it has been showed that IPA enol tautomer is rather stable in mammalian tissues and in plasma of mammals and humans treated with IPA, due to the presence of specific tautomerases in circulation, favoring the formation of KYNA in the presence of free radicals [75].

5. Myeloperoxidases Produce KYNA from L-Kyn

The importance of peroxidases in KYNA production was evaluated after knowing that in homogenates of dinoflagellate *Lingulodinium polyedrum*, the KYNA production from L-Kyn was stimulated by oxidants [76]. After incubation of L-Kyn with H_2O_2 in the presence of peroxidases, KYNA production in a linear manner was observed. Taking in mind that hemoperoxidases, including horseradish peroxidase, have a broad substrate specificity for hydrogen donors, a

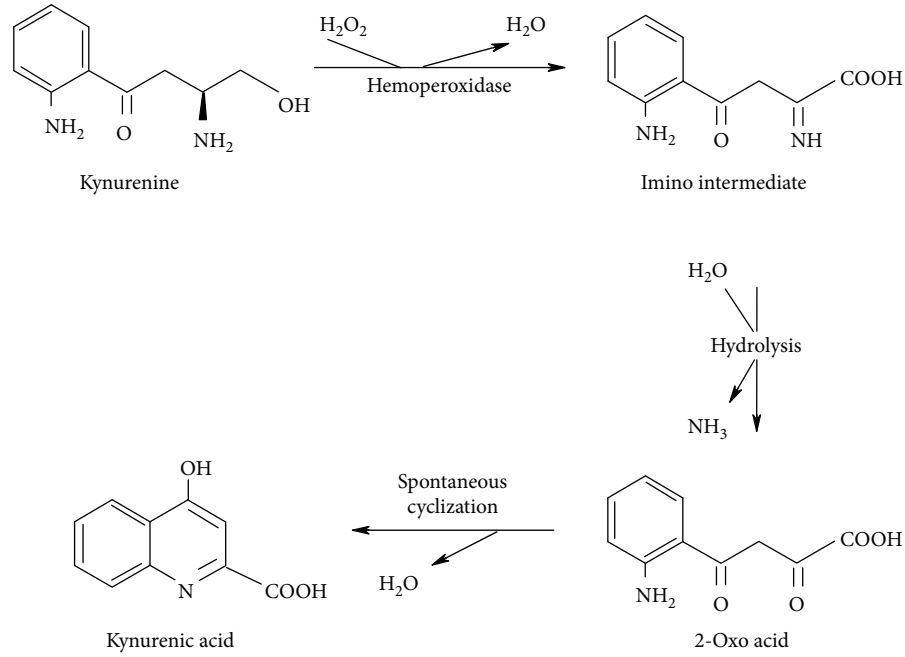

Figure 3: Hemoperoxidase participation on KYNA production from kynurenine (modified from Hardeland[74]).

mechanism by which these enzymes can produce KYNA from L-Kyn was proposed (Figure 3). Kynurenine can donate hydrogen forming an unstable imino acid, which is hydrolyzed to the respective 2-oxo acid and ammonia. Then, the 2-oxo acid formed spontaneously cyclizes and forms KYNA [74, 77]. This process can be considered in mammals, since hemoperoxidases may substantially favor the process in which H_2O_2 stimulates KYNA production.

6. Interaction between D-Kyn and L-Kyn with ROS Induces KYNA Production

As was mentioned before, L-Kyn can be converted to KYNA in the presence of H_2O_2, and this conversion is substantially enhanced by horseradish peroxidase. However, it is important to mention that this production was also observed in the absence of the enzyme. The reaction was monitored at different pHs, and the results showed that in acid pH (5.5), KYNA was not detectable; but when the pH of the medium was 7.4, 8, or 8.6, the KYNA production was increased at around 11- to 17.5-fold [77]. This evidence in the pH effect indicated that the major contribution in KYNA production from L-Kyn is due to H_2O_2 decomposition [78–81].

Later, cells of *Lingulodinium polyedrum* were incubated with kynurenine and KYNA levels were increased in the medium. This effect was highly light dependent. To clarify the relationship between photosynthetically generated oxygens during light and KYNA production from L-Kyn, Zsizsik and Hardeland evaluated the effect of two oxidant generators (carbonyl-cyanide-m-chlorophenylhydrazone (CCCP) and paraquat) and a photosynthesis inhibitor (dichlorophenyldimethylurea (DMCU)) in this paradigm. Incubation of L-Kyn in homogenates of *Lingulodinium polyedrum* exposed to light produced around 50–70 nmol KYNA/mg protein, and this production was stimulated in the presence of CCCP and paraquat (65% and 53%, resp.). However, KYNA production decreased around 42% in the presence of DMCU because this compound blocks the electron transport chain of photosystem II. This data suggested that oxidants (H_2O_2 and superoxide anions) stimulate KYNA production from L-Kyn [76].

Taking previous findings, Blanco Ayala and coworkers showed that the first evidence of the reaction between D-Kyn and L-Kyn with ROS produces KYNA in mammals [59]. By using chemical combinatorial assays, it was demonstrated that both D- and L-Kyn were able to produce KYNA through their interaction with hydroxyl radical and peroxynitrite, the effect with peroxynitrite being more pronounced. Then, cerebellum homogenates were used to evaluate the effect of coincubation of L- or D-Kyn with peroxynitrite. The production of KYNA from L-Kyn and D-Kyn in cerebellum homogenates was 18.1- and 9.8-fold higher, respectively, compared to the basal levels. When the homogenates were incubated with L- or D-Kyn plus peroxynitrite, the production increased by 2.6 and 2.8, respectively, compared with the incubation with the enantiomers alone. Next, through microdialysis experiments, it was demonstrated that the same effect occurs *in vivo*. Here, intracerebellar infusion of L- or D-Kyn produced KYNA level increments of 17.9 and 10.7 times, respectively, compared with baseline at 2 h postinfusion. In addition, basal levels of KYNA were increased in the cerebellum cortex (2.9 nM to 11.4 nM) after 30 min of peroxynitrite infusion, suggesting that the production of kynureninate is influenced by the oxidant environment. When the peroxynitrite was infused previously to both enantiomers, KYNA increased 4.1- and 3.2-fold compared with the animals infused just with L- or D-Kyn [59].

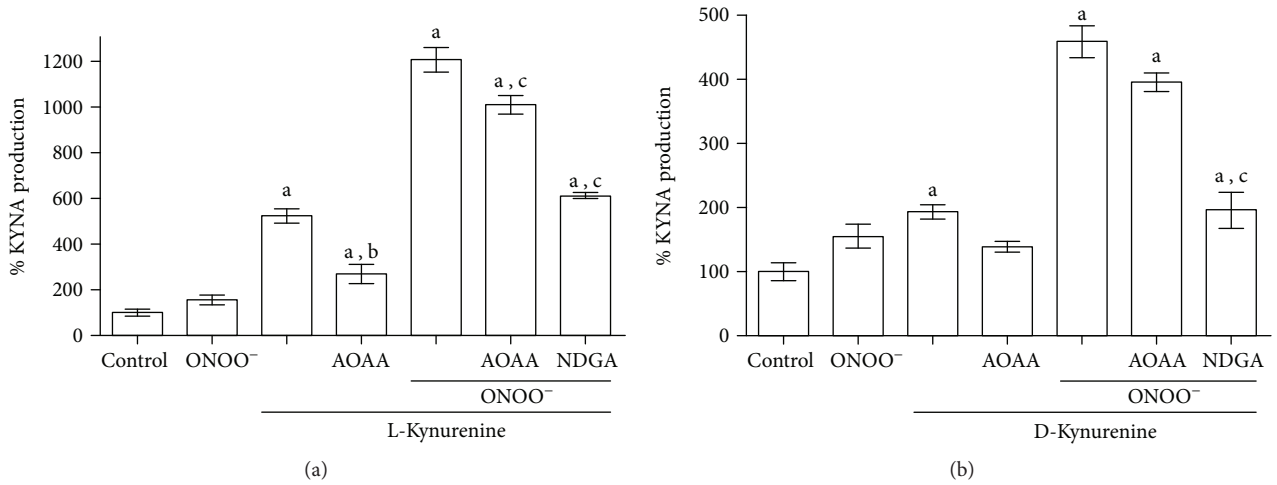

Figure 4: Effect of KAT inhibitor and a ROS scavenger on KYNA production from L-Kyn or D-Kyn in rat brain homogenates. AOAA and NDGA were used at 1 mM and 5 μM, respectively. Homogenates were incubated with (a) L-Kyn or (b) D-Kyn (20 μM and 10 μM of $ONOO^-$ in Krebs buffer) during 1 h at 37°C. Data are expressed as a percentage of endogenous tissue levels of KYNA and represent the mean ± SEM of 5 experiments per group. In both cases $^aP < 0.05$ versus control, $^bP < 0.05$ versus L-Kyn, and $^cP < 0.05$ versus L- or D-Kyn + $ONOO^-$ (one-way ANOVA followed by Tukey's post hoc test). AOAA: aminooxyacetic acid; $ONOO^-$: peroxynitrite; NDGA: nordihydroguaiaretic acid.

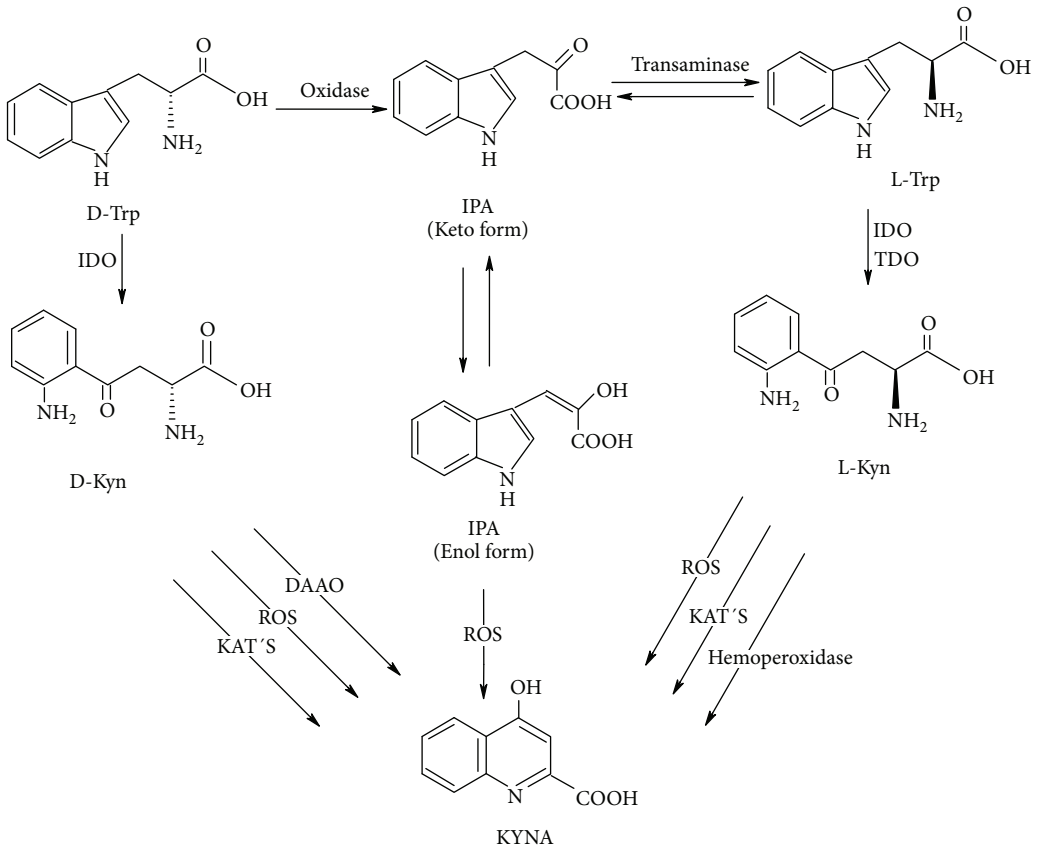

Figure 5: D-Trp can produce KYNA by two different ways: (1) D-Kyn formation by indoleamine dioxygenase (IDO) (since D-Kyn is a poor substrate of tryptophan dioxygenase (TDO). Once D-Kyn is formed, it can be taken as substrate by DAAO and KATs or to interact with ROS to produce KYNA and (2) the inversion of D-Trp to L-Trp, which occurs in two steps: the first one requires oxygen and it is suggested that DAAO can be the enzyme responsible to produce the intermediate IPA and the second step involves the reamination of IPA to yield L-Trp by means of a transaminase. L-Trp can follow the canonical pathway, that is, to produce L-Kyn, which is taken by the kynurenine aminotransferases (KATs) to produce KYNA. However, L-Kyn can also interact with ROS or with peroxidases and promote KYNA formation. The other important way to produce KYNA is through IPA, which in its enolic form can react with ROS producing KYNA.

The importance of the redox environment was also observed in brain homogenates, which were incubated with 20 μM of L- or D-Kyn and peroxynitrite (25 μM) during 1 h at 37°C in Krebs buffer (Figure 4). Under these conditions, L-Kyn and D-Kyn increased KYNA levels 5- and 1.2-fold more, respectively, compared with those of the control. KYNA production from L-Kyn decreased by the use of AOAA, a KAT inhibitor, while KYNA production from D-Kyn in the presence of AOAA was not significantly altered. After coincubation with peroxynitrite, KYNA increases around 11- and 4-fold from L- and D-Kyn, respectively. The combination L-Kyn + $ONOO^-$ + AOAA decreases just 20% KYNA production compared with L-Kyn + $ONOO^-$, suggesting that KAT participation in KYNA production is minimal under these conditions. In the case of D-Kyn + $ONOO^-$ + AOAA, it was not significantly different compared with D-Kyn and $ONOO^-$. However, KYNA production from the enantiomers plus peroxynitrite was decreased around 50% when an antioxidant, NDGA, was used, suggesting that the KYNA production was favored by the oxidant environment.

These data are in accordance with previous evidence showing that L-Kyn and D-Kyn are good ROS scavengers and in this way can produce KYNA [82, 83]. All these findings suggest another pathway to produce KYNA which may have relevance in brain development and aging and in neurological diseases that show redox environment alteration.

7. Concluding Remarks

Although the specific contributions of the alternative routes of KYNA production remain unclear, abundant evidence has shown that the increase of this metabolite is involved in many physiological and pathological processes, in which the redox environment is altered by the presence of free radicals, the decrease of antioxidant defense, and the activation of immune response and inflammatory mediators. All of these factors could be related with KYNA production as was mentioned throughout this review. The challenge for future research is to clarify the precise degree of involvement of these alternative routes (Figure 5), in processes such as neurodevelopment, aging, psychiatric disorders, and aging-related diseases, in which have been described as having high levels of KYNA; but also, it is known that there is high presence of free radicals and inflammatory cytokines. Some of these diseases are also related with previous infections and with DAAO activity alterations; all these factors promote the oxidant environment that could impact directly KYNA production. These new routes are a target of study and represent a new alternative to modulate KYNA levels in the processes in which they are involved.

Disclosure

The authors alone are responsible for the content and writing of the paper.

Conflicts of Interest

The authors report no conflicts of interest.

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Review Article

Antioxidant and Cell-Signaling Functions of Hydrogen Sulfide in the Central Nervous System

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Hydrogen sulfide (H_2S), a toxic gaseous molecule, plays a physiological role in regulating homeostasis and cell signaling. H_2S is produced from cysteine by enzymes, such as cystathionine β-synthase (CBS), cystathionine γ-lyase (CSE), cysteine aminotransferase (CAT), and 3-mercaptopyruvate sulfurtransferase (3MST). These enzymes regulate the overall production of H_2S in the body. H_2S has a cell-signaling function in the CNS and plays important roles in combating oxidative species such as reactive oxygen species (ROS) and reactive nitrogen species (RNS) in the body. H_2S is crucial for maintaining balanced amounts of antioxidants to protect the body from oxidative stress, and appropriate amounts of H_2S are required to protect the CNS in particular. The body regulates CBS, 3MST, and CSE levels in the CNS, and higher or lower levels of these enzymes cause various neurodegenerative diseases. This review discusses how H_2S protects the CNS by acting as an antioxidant that reduces excessive amounts of ROS and RNS. Additionally, H_2S regulates cell signaling to combat neuroinflammation and protect against central neurodegenerative diseases such as Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD), and amyotrophic lateral sclerosis (ALS).

1. Introduction

Hydrogen sulfide (H_2S) is a colorless toxic gas with the characteristic odor of rotten eggs [1]. It is produced by decomposition of organic matters and is found in natural gas, petroleum, and volcanic and sulfur-spring emissions [2] under ambient temperature and pressure. Although H_2S is toxic, it plays a physiological role in the nervous system [3]. H_2S functions in the secretion of corticotrophin-releasing hormone from serotonergic neurons [4, 5] and in the relaxation of smooth muscle [6, 7]. Additionally, H_2S shields neurons and cardiac muscles from oxidative stresses [5, 8–11] and helps to maintain insulin secretion [11–13].

H_2S is produced endogenously from cysteine via enzymes, such as cystathionine β-synthase (CBS), cystathionine γ-lyase (CSE), 3-mercaptopyruvate sulfurtransferase (3MST), and cysteine aminotransferase (CAT). CBS, a pyridoxal-5′-phosphate- (PLP-) dependent enzyme, is expressed mostly in the brain, with marked localization in astrocytes and cerebellar Bergmann glia [3, 14]. Additionally, Northern blot analysis indicates that CBS is expressed in the hippocampus, cerebellum, cerebrum, and brainstem [3]. While CBS is the main source of H_2S in the human brain, CBS and CSE are expressed in the tissues of various organs, such as the kidney and the liver. CSE is the main contributor to H_2S levels in the thoracic aorta, ileum, portal, vein, and uterus. In addition to

generating H_2S from cysteine, CBS catalyzes the condensation reaction of homocysteine, whereas CSE cannot perform this function. 3MST is expressed mainly in the brain, and most of the H_2S produced by 3MST is bound in the form of sulfane sulfur, one of the forms through which endogenous H_2S is stored [15]. Understanding the specific expression patterns of these three enzymes is useful for designing therapeutic drugs.

H_2S acts as a signaling molecule in the cell signal transduction pathways in the nervous system, the circulation system, and in many other organs. In the central nervous system (CNS), H_2S is associated with various physiological processes, including neuroprotection [16] and neurotransmission [17]. H_2S inhalation has a neuroprotective function in a mouse model of Parkinson's disease (PD) [18]. H_2S protects neurons from apoptosis and degeneration [19] by exerting anti-inflammatory effects and upregulating antioxidant enzymes [16]. H_2S protects neurons from oxidative stresses by reducing the level of reactive oxygen species (ROS) and the aggregation of lipid peroxidation products. Furthermore, H_2S inhibits the biological activity of peroxynitrites ($ONOO^-$) formed in the reaction of nitric oxide (NO) with superoxide anion [20]. H_2S functions as an antioxidant by scavenging ROS directly and by reducing glutathione disulfide (GSSG) [21]. Increased levels of ROS are found at inflammation sites. Removal of ROS can occur by supplying homocysteine, and stimulated H_2S synthesis expedites the antioxidant activity [22]. High levels of H_2S cause generation of ROS and reactive nitrogen species (RNS), whereas lower amounts of H_2S react with hydrogen peroxides (H_2O_2), $ONOO^-$, and oxide ion (O^{2-}) [23]. Additionally, H_2S functions as an antioxidant by attaching to particular agents, such as glutathione (GSH), superoxide dismutase (SOD), N-nitroarginine methyl ester (L-NAME), and vitamin C [24].

In this review, we discuss the antioxidant roles of H_2S; the production of H_2S from various enzymes, such as CSE, CBS, and 3MST; the cell signaling role of H_2S in the CNS; the importance of producing appropriate amounts of H_2S from enzymes to maintain proper neuronal function in the CNS; how dysregulation of cell signaling in the production of enzymes responsible for maintaining H_2S levels in the body can lead to central neurodegenerative diseases, such as Alzheimer's disease (AD), PD, Huntington disease (HD), and amyotrophic lateral sclerosis (ALS); and how neuroinflammation and disease conditions can be prevented by reducing oxidative stress conditions via the antioxidant functions of H_2S.

2. H_2S-Producing Enzymes

2.1. H_2S Production by CBS. CBS converts serine and homocysteine to generate cystathionine. Additionally, CBS can produce H_2S from a combination of cysteine and homocysteine. It is unclear whether CBS forms only cystathionine or produces H_2S from cysteine and homocysteine [25, 26]. CBS has an important role in the regulation of homocysteine levels *in vivo*. Mammalian CBS is regulated by posttranslational modifications and contains a redox-sensitive heme cofactor. The ferrous form of CBS, which forms under local oxidizing conditions, is less active than the ferric form [27]. Carbon monoxide (CO) binds CBS in the ferrous state and inhibits the catalytic activity of CBS [28]. As the redox potential of the Fe^{3+}/Fe^{2+} couple in CBS is very low (−350 mV), the availability of CBS entering the ferrous state is controversial. CO reversibly inhibits CBS in the presence of a physiologically relevant reducing system, such as methionine synthase reductase and nicotinamide adenine dinucleotide phosphate (NADPH) [29]; therefore, crosstalk is exhibited between the CO and H_2S systems. S-Adenosylmethionine (SAM), another metabolite that allosterically activates CBS (Figure 1) [30], is a major methyl donor and the precursor of homocysteine. SAM activates CBS by combining with the carboxy-terminal domain of CBS, which increases H_2S production [31].

Activation of astrocyte and microglia cell inflammation reduces expression of CBS, leading to diminished levels of H_2S in the brain. Endogenous H_2S in the brain is generated mainly by CBS, and altering CBS expression can change the H_2S levels. Various endogenous and exogenous compounds, such as epidermal growth factor (EGF), which transforms tumor growth factor-α (TGF-α) and cyclic adenosine monophosphate (cAMP), can increase CBS messenger RNA (mRNA) transcription, which is irregularly maintained in some diseases. These observations suggest several pharmacological targets for combating CNS diseases. CBS expression is threefold higher in patients with Down's syndrome than in normal controls, whereas CBS expression is lower in children with high IQ scores [32]. These findings suggest that overexpression of CBS may have a negative influence on cognitive function. Homocysteinemia is caused by an absence of CBS [32]. In rat hippocampal slices, H_2S generation from CBS is maintained by calcium (Ca^{2+})/calmodulin and increased by L-glutamate. N-Methyl-D-aspartate (NMDA) and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPA) are inhibited by amino-phosphonopentanoate (AP-5) and 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), which demonstrates their involvement in this process. The brains of patients with AD show a ~55% reduction in H_2S levels, whereas L-cysteine levels are unchanged, and CBS activity remains. Chronic H_2S exposure impairs fetal neuronal development and monoamine neurochemistry in rats. Therefore, H_2S may have functional involvement in neurodegenerative diseases. CBS can act as an antioxidant inhibitor of peroxynitrite-mediated processes through activation of NMDA receptors. The involvement of the NMDA receptor, with the resulting alteration of long-term potentiation (LTP) in the hippocampus, was the first biological effect described in patients with Down's syndrome who had enhanced concentrations of H_2S in their cerebral spinal fluid (CSF). This increased concentration may be because CBS is encoded on chromosome 21 and is overexpressed in these patients [33].

CBS is considered the main enzyme in the CNS that produces H_2S in the brain. H_2S is produced by CBS by using cysteine and homocysteine as substrates, and various factors, such as the EGF conversion to TGF-α and cAMP, are related to producing this enzyme. Understanding the regulation of CBS in the brain may lead to the development of potential therapeutic treatments for many CNS diseases.

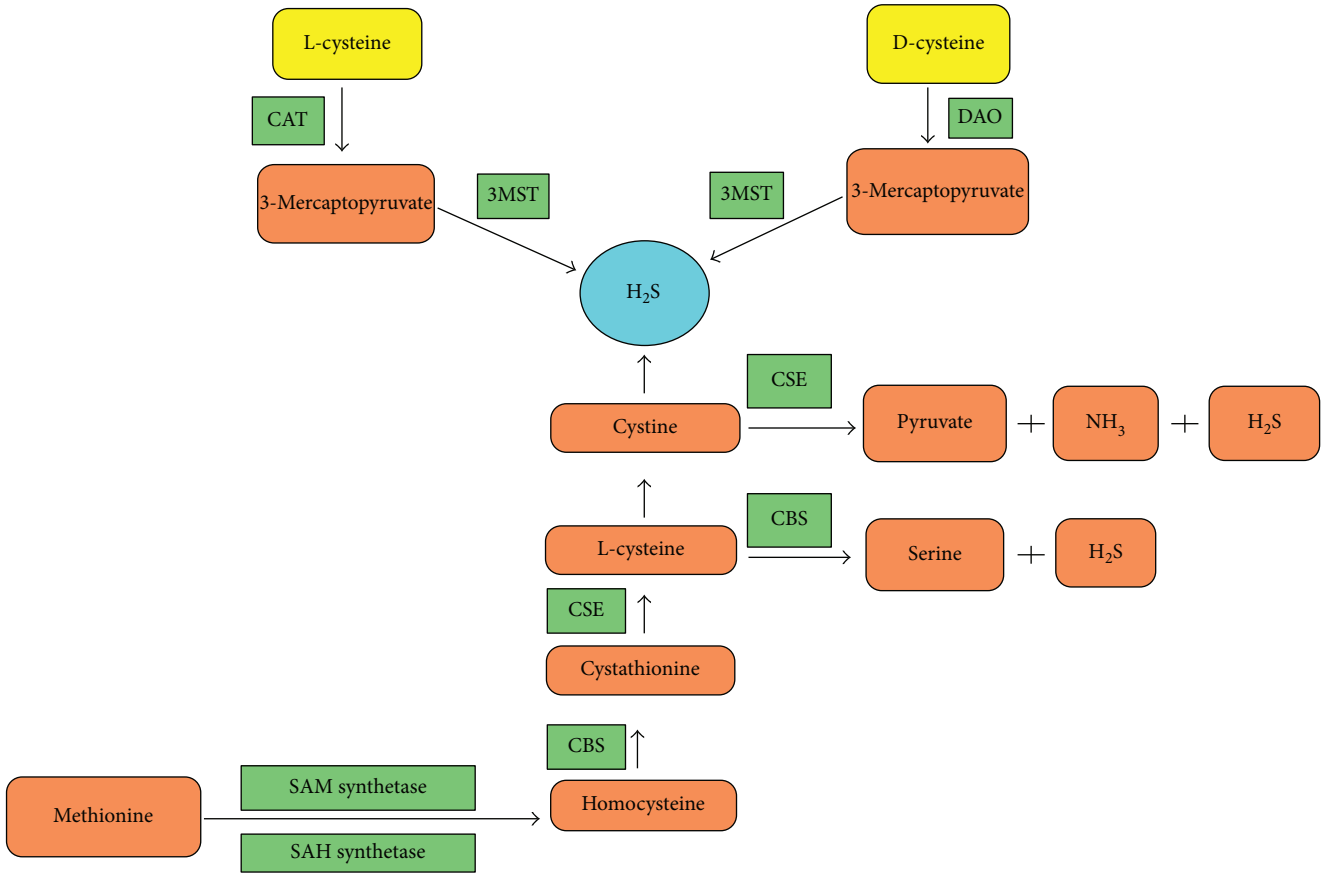

Figure 1: Biosynthesis of hydrogen sulfide (H_2S) from the cystathionine β-synthase (CBS), cystathionine γ-lyase (CSE), cysteine aminotransferase (CAT), and 3-mercaptopyruvate sulfurtransferase (3MST). From the metabolism of methionine, homocysteine is converted to cystathionine and H_2S is produced from the L-cysteine and homocysteine by enzymes CBS and CSE. L-cysteine in converted to serine and H_2S with the enzyme CBS. Cystine is converted to pyruvate, ammonia (NH_3), and H_2S with enzymes CSE. 3MST works in two ways such as 3MST/CAT and 3MST/DAO (D-amino acid oxidase) pathways to produce H_2S from L-cysteine and D-cysteine. SAM: S-Adenosylmethionine synthase; SAH: S-Adenosyl homocysteine hydrolase; DAO: D-Amino acid oxidase.

2.2. H_2S Production by CSE. CSE converts cysteine to H_2S, pyruvate, and ammonia; however, this enzyme can also use homocysteine and cysteine as a substrate for H_2S production (Figure 1) [25, 34]. Rat CSE also uses cysteine (the disulfide form of cystine) as a substrate in H_2S production [35, 36]. In this case, cysteine persulfide is formed in the presence of a reductant to release H_2S. The cysteine concentration is extremely low in the reducing environment of cells, but it contributes to H_2S biogenesis under normal conditions. CSE is thought to be the most prominent enzyme for generating H_2S in mammalian tissues. CSE-deficient mice show a profound depletion of H_2S in peripheral tissues [37]. CSE activity has been detected in brain tissue lysates [38], and murine CSE protein expression has been found in imaging studies of the brain [39, 40]. In contrast, CBS protein is expressed mostly in astrocytes [41]. The production of H_2S from cystine is significantly decreased in brain homogenates from CBS-knockout mice, which demonstrates that CBS is the main source of H_2S in the brain [42].

CSE, a PLP-dependent enzyme, is found mostly in the liver and kidney and in vascular and nonvascular smooth muscles. CSE exists in the small intestine and stomach of rodents [43]. CSE regulation is less well characterized than that of CBS regulation. Upregulation of CSE is caused by S-nitroso-N-acetylpenicillamine (SNAP), a type of NO donor. Another NO donor, sodium nitroprusside (SNP), enhances CSE activity. Additionally, H_2S interacts with NO to facilitate NO function in vasorelaxation [6, 44, 45]. CSE-knockout mice deficient in the H_2S-producing enzyme CSE develop hypertension [37]. Although endogenous production of H_2S is poorly understood, it plays roles in reducing oxidative stress and in posttranslational protein modification [46–48].

CSE produces H_2S in the brain and has various physiological roles in maintaining body functions. Similar to CBS, it serves as an important marker of the progression of many CNS diseases.

2.3. H_2S Production by 3MST and CAT. Recent studies have revealed that 3MST and CAT enzymes produce H_2S from cysteine in the brain [49, 50]. The brains of CBS-knockout mice show the presence of a different H_2S-producing enzyme [51], and the activity of this enzyme requires mitochondrial and cytosolic components. The required mitochondrial components include 3MST and CAT, which acts as a

synaptosome, and the cytosolic components include α-ketoglutarate [32]. However, 3MST and CAT show enzymatic activities at pH 7.4, which is comparatively alkaline, and the intermediate of CAT catalysis, 3-mercaptopyruvate (3MP), is an unstable molecule that affects the generation of 3MST. These observations imply that this pathway can generate H_2S under physiologic conditions (Figure 1) [51]. Aspartate, another substrate for CAT, can associate competitively with CAT to inhibit H_2S generation. A comparison of these enzymes reveals several differences. CBS is found primarily in astrocytes, whereas 3MST is localized mainly in neurons. 3MST generates bound sulfane sulfur more efficiently than does CBS. 3MST transfers sulfur from H_2S to bound sulfane sulfur, whereas CBS has low capacity for this activity. 3MST is also localized in the thoracic aorta. The presence of 3MST, CAT, and α-ketoglutarate in the endothelium suggests that H_2S can be generated in the endothelium [32].

3MST and CAT are other enzymes that produce H_2S from cysteine under physiologic conditions and maintain homeostasis by ensuring the balance of H_2S in the body.

3. Bioactivity of H_2S

There are two possible mechanisms by which H_2S is released; it can be released immediately after the production by the enzymes and it can be stored and released in response to a physiologic signal. Two forms of sulfur stores in cells have been identified [52, 53]. Acidic conditions release H_2S from acid-labile sulfur. Another form of storage, bound sulfane sulfur, releases H_2S under reducing conditions [54]. Acid-labile sulfur is contained in iron–sulfur complexes that play a pivotal role in a wide range of redox reactions in the respiratory chain of mitochondria. H_2S is released from acid-labile sulfur at pH 5.4 [55]. As the pH in mitochondria is between 7 and 8, it is likely that acid-labile sulfur does not release H_2S under normal physiologic conditions.

H_2S can be associated into proteins as bound sulfane sulfur [56]; thus, enzymatically produced H_2S may be stored as bound sulfane sulfur. Cells expressing 3MST and CAT have increased levels of bound sulfane sulfur [55] compared to cells expressing a defective mutant of 3MST that does not produce H_2S [51]. The level of sulfur is intracellularly dependent on the H_2S-generating activity of 3MST; H_2S generated by 3MST is preserved as bound sulfane sulfur in cells. In the presence of major cellular reducing substances GSH and cysteine at their physiologic concentrations, H_2S is released from lysates of cultured neurons and astrocytes at pH 8.4 [55]. Because the reducing activity of thiols is higher under alkaline conditions than at a neutral pH, H_2S release can be detected at pH values higher than 8.4. Although systemic pH changes of up to approximately 0.2 constitute either alkalosis or acidosis, it is possible that the pH can be altered to a greater extent locally. As neurons are excited, sodium ions (Na^+) enter and potassium ions (K^+) exit from cells, which results in high potassium concentrations in the extracellular environment. This depolarizes the membrane of the surrounding astrocytes and activates their Na^+/bicarbonate (HCO_3^-) cotransporters. The entrance of HCO_3^- causes alkalization of the cells. The newly produced H_2S stays in equilibrium with its anionic form bisulfide (HS^-), with an intracellular ratio of H_2S to HS^- of 1 : 4. However, it is unclear whether HS^- anion and free H_2S contribute equally to cell signaling. H_2S has the ability to traverse cell membranes without the need of a facilitator [57], whereas it was previously thought that HS^- anions are not able to cross cell membranes and, hence, could target only intracellular proteins. In contrast, a channel permeable to HS^- anions was recently found in the bacterium *Clostridium difficile*, demonstrating that the signaling role of HS^- anions may be confined to HS^- anion-producing cells [58].

Signaling of H_2S is maintained by rapid clearance of H_2S by various biochemical pathways that metabolize H_2S. A high rate of H_2S generation is maintained by its degradation via oxygen-dependent catabolic processes in mitochondria in murine tissues [59]. Additionally, H_2S can be present in a bound form sulfane, which releases H_2S in the presence of a reducing agent under alkaline conditions; however, there is no evidence for a physiological function of sulfane sulfur in cellular signaling. H_2S also reacts with hemeproteins, such as hemoglobin, neuroglia, and cytochrome c oxidase, which may act as links for this gasotransmitter [59]. Also, H_2S can be methylated in the cytosol by thiol-S-methyl transferase to produce methane thiol, which can be further methylated to become the less toxic compound dimethyl sulfide [60].

After the production from various enzymes, H_2S stays in equilibrium by forming anions in the body, and further generation of H_2S is dependent on the degradation rate needed to maintain the physiological functions of the body.

4. Detection and Measurement of H_2S

Intracellular H_2S levels can be detected and quantified using several methods that have varying levels of sensitivity [61]. H_2S generation from cysteine or homocysteine using very high substrate concentrations leads to inaccurate detection of H_2S levels. The most common method for accurate detection of H_2S levels involves H_2S trapping with zinc or lead, followed by acidification and reaction with N,N-dimethyl-P-phenylenediamine (DMPD) to produce methylene blue, which can be detected by colorimetry. This process is preferred under acidic conditions and results in the release of bound H_2S from stored sources. However, this method does not differentiate between free and bound H_2S. Moreover, this method lacks sensitivity and cannot detect nanomolar amounts of H_2S. In contrast, gas chromatography can detect H_2S levels in the nanomolar range and can distinguish between free sulfide and acid-labile sulfide [62]. Measurement of H_2S in real time [63] is not easily possible; amperometry does allow for monitoring and direct measurement of H_2S production in real time, but the detection electrodes require frequent calibration, which is accompanied by difficulties related to handling small volumes. Lastly, H_2S-specified probes can detect local H_2O_2 generation in live cells, but these probes are sometimes inadequate for the identification of H_2S in the submicromolar range [64].

In conclusion, H_2S can be detected using various methods, such as colorimetry or gas chromatography, but

these methods cannot detect or measure H_2S at the nanomolar or submicromolar range. Although these methods are limited, they can be optimized to detect H_2S within such limitations, whereas amperometry measures H_2S in real time.

5. Signaling Mechanisms

5.1. H_2S as Signaling Molecules in the CNS. Olas et al. experimentally demonstrated that H_2S serves a neuroprotective function, maintaining the intracellular pH in microglial cells and limiting the damage to activated microglia at the site of injury. H_2S inhibits cytochrome c oxidase or causes excessive NMDA receptor stimulation through the secondary transmitter cAMP. NMDA receptors are built from three subunits, NMDAR1, NMDAR2A, and NMDAR2B. Endogenous ligands of the receptor include acid, NMDA, and glutamic acid. After joining the glutamate receptor subunit, phosphorylation occurs inside the NMDAR1 ion channel via protein kinase A (PKA) activity, which is dependent on cAMP [65]. For this reason, the channel opens, and an influx of Ca^{2+} ions is observed. In the next step, the signaling pathway involves changes in the long-term strengthening of synapses, which enhances the efficiency with which nerve impulses travel across synapses. H_2S affects the function of the hypothalamic–pituitary–adrenal glands [66]. H_2S decreases release of potassium hormones stimulated by the hypothalamus by acting as a negative regulator of the hypothalamic–pituitary–adrenal glands. This compound also affects intracellular stores of Ca^{2+}, stimulating their release inside cells, which causes nerve excitation. It has been demonstrated that H_2S reduces the cysteine disulfide bond of the NMDA receptor to increase its activity [67]. Eventually, H_2S-derived polysulfide (H_2S_n) increases the activity by producing bound sulfane sulfur in the cysteine residues of the receptors. H_2S_n also activates the channels in astrocytes to enhance intracellular concentrations of Ca^{2+} that facilitate the release of serine, which in turn increases the activity of NMDA receptors. Wang et al. demonstrated the involvement of H_2S in neuronal cell differentiation [68].

It has been reported that concentrations of H_2S 10 to 130 μM in the CNS not only activate the NMDA receptor-mediated response but also increase the speed with which LTP occurs [3]. At higher concentrations (320 and 640 μM), sodium hydrosulfide (NaHS) inhibits synaptic transmission. In fact, H_2S concentrations from 30 to 400 μM produce the opposite effects on neuronal transmembrane potentials in toxicological studies [69]. Expression of gamma aminobutyric ($GABA_B$) receptor subunits 1 and 2 is upregulated by H_2S, whereas expression of the $GABA_B$ receptor subunits 2 and 1 is inhibited by hydroxylamine, a nonspecific inhibitor of H_2S biogenesis [70]. H_2S affects the levels of epinephrine, norepinephrine, and serotonin in the brain [71]. Additionally, H_2S enhances intracellular Ca^{2+} in neurons, astrocytes, and microglia by upregulating the influx of Ca^{2+} into the cytoplasm from extracellular and intracellular compartments [72]; this affects the interactions among these cells. Indeed, activation of voltage-dependent Ca^{2+} channels or of transient receptor potential channels by H_2S is thought to underpin the intracellular increase in Ca^{2+} [44].

To conclude, H_2S performs a cell-signaling function in the CNS by activating NMDA receptors and increasing intracellular Ca^{2+} by activating voltage-gated sodium channels in neuronal cells. By doing so, it performs antioxidant functions by upregulating generation of GSH and mitigating oxidative stresses in cells.

5.2. Potential Molecular Targets in H_2S Signaling in the CNS. H_2S has recently been understood to act as a signaling molecule in the CNS. Indeed, H_2S is involved in the regulation of the pathways and molecules detailed in the following subsections.

5.2.1. cAMP/PKA Signaling Pathway. Generation of cAMP by adenylyl cyclase (AC) stimulates PKA, which, in turn, phosphorylates various intracellular proteins; hence, it is involved in the maintenance of brain functions. LTP is produced rapidly by high-frequency presynaptic activation that strengthens the postsynaptic response, continuing presynaptic stimulation. Regulation of LTP requires activation of PKA, which may phosphorylate NMDA receptors and enhance Ca^{2+} permeability, facilitating both the early and late phases of LTP (Figure 2) [73]. It has been demonstrated that NaHS, which is a H_2S donor, enhances cAMP generation in primary cultures of the cerebral cortex, cerebellum neurons, and glial cells in a concentration-dependent manner [74]. These studies demonstrated that H_2S may modulate the activity of NMDA receptors through changing intracellular cAMP levels and upregulating the induction of LTP. Activation of the cAMP/PKA pathway also stimulates ryanodine receptors in the brain, leading to calcium-induced calcium release [73].

5.2.2. Tyrosine and Mitogen Kinases. Tyrosine kinase (RTK) receptors are regarded as a part of a large family of cell surface receptors with intrinsic RTK activity [11]. The possibility that H_2S may upregulate the reducing activity and protect neurons against oxidative stress acquired through activation of upstream RTK (Figure 2). It is likely that H_2S stimulates epidermal growth factor receptor (EGFR) type RTK, as experiments with tryphostin A23 inhibited the effect of H_2S or WST-8, a tetrazolium salt, compared with a control analogue trypostin A1 that lacked EGFR inhibitory activity [11]. The activation of EGFR by H_2S is consistent with observations that H_2S promotes NMDA signaling and LTP, which are similar to the effects observed with EGF [73]. Mitogen-activated protein kinases (MAPKs) are a large family of kinases divided into five distinct groups in mammals; they are activated by external stimuli, and their activation stimulates downstream effectors through phosphorylation. MAPKs maintain many cellular activities, including apoptosis, differentiation, metabolism, mobility, cell division, and survival [75]. It has been demonstrated recently that H_2S inhibits LPS-imparted NO production in microglia through inhibition of p38 MAPK. This indicates that H_2S may be useful in the neuroprotection involved in the treatment of cerebral ischemia and neuroinflammatory diseases [76].

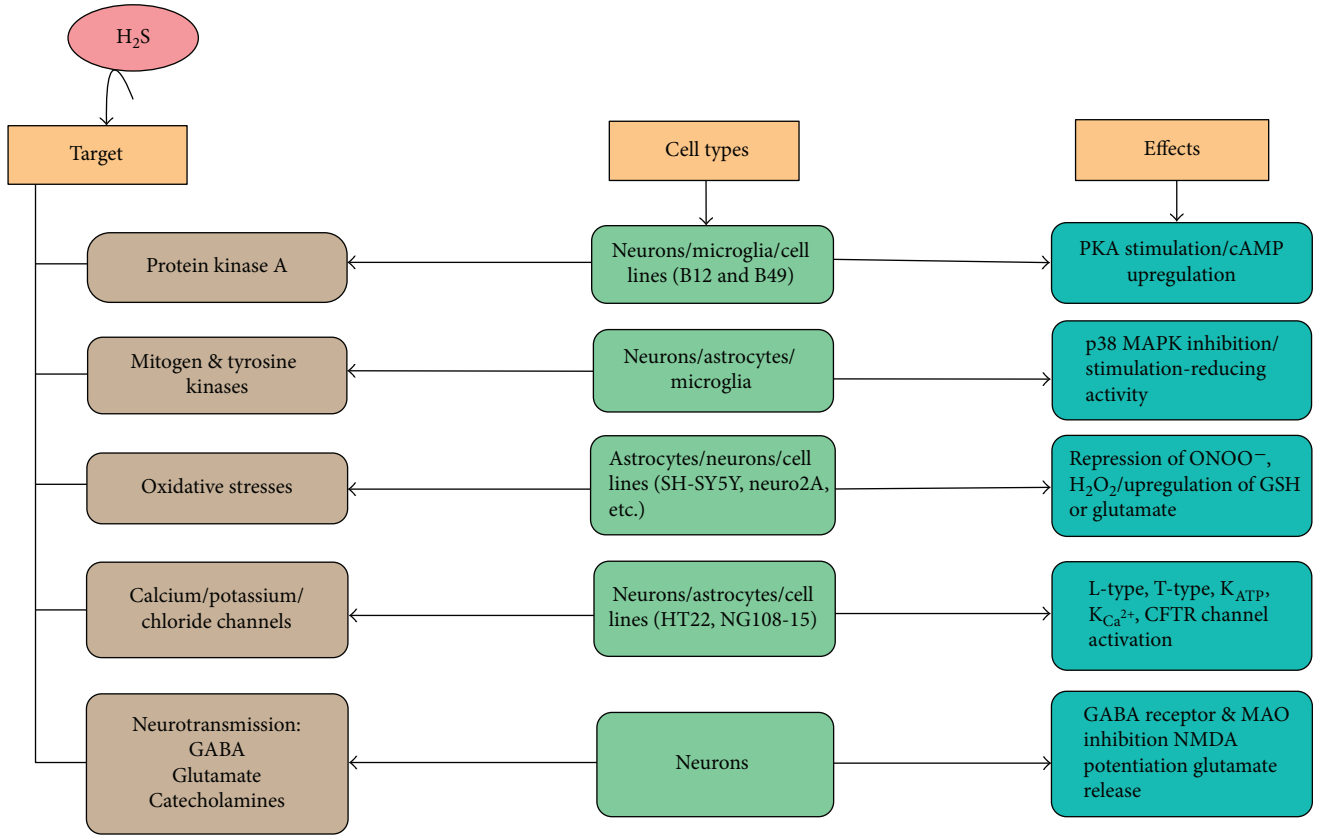

Figure 2: Potential molecular targets in hydrogen sulfide (H_2S) signaling in the central nervous system (CNS). H_2S targets protein kinase A (PKA) and activates PKA stimulation or cyclic adenosine monophosphate (cAMP) upregulation which has effects on neurons, microglia, and the cell lines such as B12 and B49. It also activates mitogen and tyrosine kinases which initiates p38 mitogen-activated protein kinase (MAPK) inhibition as well as stimulation of the reducing activity in neurons, astrocytes, and microglia. Oxidative stress has activity on suppression of peroxynitrites ($ONOO^-$), hydrogen peroxide (H_2O_2), and upregulation of glutathione (GSH) or glutamate. Additionally, H_2S activates on calcium (Ca^{2+}), potassium (K^+), and chloride (Cl^-) channels in neurons, astrocytes, and cell lines such as HT22 and NG108–15. Moreover, H_2S has effects on neurons in neurotransmission such as gamma-aminobutyric acid (GABA) receptor inhibition, N-methyl-D-aspartic acid (NMDA) potentiation glutamate release, and monoamine oxidase (MAO) inhibition. In these ways, H_2S stimulates molecular targets on the CNS to impart their different functions.

5.2.3. GSH and Oxidative Stress. It has been noted that H_2S inhibits peroxynitrite-imparted cytotoxicity, intracellular protein nitration, and protein oxidation in human neuroblastoma SH-SY5Y cells. These studies demonstrate that H_2S has the potential role to act as an inhibitor of peroxynitrite-mediated processes *in vivo* and reflect the potential antioxidant action of H_2S [77]. Hence, H_2S protects against the activity of peroxynitrite-mediated processes *in vivo*, which suggests the potential antioxidant action of H_2S. Hence, H_2S protects against the activity of peroxynitrite in SH-SY5Y neuroblastoma cells, assembly via enhanced GSH production (Figure 2). In the same way, in HT22 neuronal cells and primary cultured immature cortical neurons, H_2S inhibits cell toxicity related to apoptosis, a form of oxidative glutamate toxicity that operates independently from the glutamatergic signaling at ionotropic glutamate receptors [11].

5.2.4. Effects of H_2S on Ca^{2+}, Potassium (K^+), and Chloride (Cl^-) Channels in the CNS. In neurons, physiological concentrations of H_2S generate a biphasic response in dorsal raphe seronergic neurons; this response is characterized by initial, rapid-onset depolarization followed by sustained hyperpolarization. The primary depolarization response is sensitive to inhibition via removal of external Ca^{2+} or blockage using cadmium but not tetradotoxin, which is a sodium channel blocker; this highlights the participation of extracellular Ca^{2+} influx in the initial depolarization response [69]. Plasma membrane voltage-gated channels that may be activated by H_2S include L-type channels and T-type Ca^{2+} channels. L-type Ca^{2+} channels are coded by four different genes in mammals, Cav1.1–Cav1.4. L-type channels are expressed in neurons and endocrine cells and regulate many processes, such as neurohormones and neurotransmitter secretion, gene expression, mRNA stabilization, neuronal survival, synaptic efficiency, and the activity of other ion channels, such as NMDA receptors [78]. Neurons express Cav1.2 and Cav1.3 subtypes. The subtypes differ in their activation thresholds, sensitivity to dehydropyridine antagonists, activation kinetics, and subcellular distributions. H_2S has led to neuronal death and an increase in $[Ca^{2+}]i$ in rat cerebellar granule neurons, but both of these outcomes have been blocked by L-type channel-specific blocker, nifedipine or

nimodipine, demonstrating that H_2S acts on L-type Ca^{2+} channels. In astrocytes, Ca^{2+} waves induced by H_2S were found to be blocked by nifedipine [79].

Using particular K^+ channel blockers, gliclazide and apamin, respectively, researcher has found that physiological concentrations of H_2S activate both adenosine triphosphate- (ATP-) sensitive potassium and calcium (K_{ATP} and $K_{Ca^{2+}}$) channels in the hypothalamus and dorsal raphe serotonergic neurons (Figure 2) [80]. H_2S was also found to stimulate K_{ATP} channels in neuronal cell lines. Blockade of K_{ATP} channels with the blockers glibenclamide and glipizide counteracted the survivability imparted by H_2S during oxytocic insult; this finding was confirmed using the K_{ATP} activator pinacidal [9]. K_{ATP} channels also play roles in seizure control, mediating neurotransmitter release from presynaptic neurons, and mediating neuroprotection during hypoxic challenge; hence, it is tempting to speculate that H_2S plays a neuroprotective role through activation of K^+ channels [81]. Additionally, H_2S has been found to activate cystic fibrosis transmembrane conductance regulator (CFTR) Cl^- channels in HT22 neuronal cell lines, leading to neuroprotection during oxytosis (Figure 2). This was observed via dose-dependent repression of neuroprotection due to H_2S using specific CFTR blockers, 5-nitro-2-(3-phenylpropylamino) benzoic acid (NPPB) and indolylacetic acid (IAA), and was confirmed using the CFTR activator levamisole [9]. Taken together with our recent observation of H_2S stimulating $Cl^-/HCO3^-$ transporters in smooth muscle cells, these studies suggest that H_2S in the CNS is involved in the regulation of inhibitory K^+ channels and therefore plays a pivotal role in mediating excitability [82].

5.2.5. Effect of H_2S on GABA-Mediated, Glutamate-Mediated, and Catecholaminergic Neurotransmission. GABA is the major inhibitory transmitter within the mammalian CNS: 20–30% of all synapses in the CNS employ GABA as their transmitter [83]. GABA-mediated inhibition in the CNS is critical, as loss of GABAergic inhibition leads to seizures and neuronal hyperexcitability. There are three types of receptors for GABA in the CNS: $GABA_A$, $GABA_B$, and $GABA_C$ receptors; these produce slow, prolonged inhibitory signals that modulate the release of neurotransmitters [84]. H_2S has been found to promote amelioration of hippocampal damage caused by recurrent febrile seizures via a reversal of the loss of the GABABR1 and GABAB2 caused by the seizures [85]. This amelioration was traced to the elevated mRNA and protein levels of these GABA receptors, possibly due to acute (H_2S-induced) increases in $[Ca^{2+}]i$, following Ca^{2+}-dependent transcription [85]. This may affect the excitation or inhibition balance that is perturbed during fever by affecting slow, accelerated inhibitory signals and neurotransmitter release. It is possible that H_2S accelerates inhibitory signals on transmitter release and may have potential uses in the treatment of excitatory diseases, such as epilepsy [70].

Although there is no direct evidence of H_2S agonist activity on NMDA receptors, accumulating evidence suggest that H_2S may generate physiological or pathological functions through maintaining NMDA receptors [73]. H_2S stimulates LTP through potentiation of NMDA receptors. This effect is achieved mainly by H_2S-imparted activation of the cAMP/PKA pathway [3]. Excessive activation of NMDA causes calcium overload in cells, leading to cell death [86]. Hence, NMDA receptors play essential roles in certain conditions, such as stroke, neuropathic pain, PD, and so forth. NMDA receptor blockers have been found to inhibit H_2S-imparted cell death in neurons and decrease infarct volume in an *in vivo* rat stroke model [73], demonstrating that H_2S may impart cell death by opening NMDA receptors (Figure 2). In brief, H_2S-imparted NMDA signaling may promote excitation and contribute to whether neurons survive or die [87]. Sublethal or lethal concentrations of H_2S have been reported to inhibit monoamine oxide, leading to an increase in noradrenaline and adrenaline in the hippocampus, striatum, and brainstem but not in the cortex or cerebellum. Because of the myriad effects elicited by catecholamines or adrenoceptors in the CNS, further study is needed to elucidate the importance of the toxicological effects of H_2S [88].

However, H_2S activates different receptors and molecular targets as mentioned above paragraphs either individually or in combination to impart neuroprotective effects in the CNS.

6. Roles of GSH and H_2S as Antioxidants in the CNS

GSH is a nonprotein thiol that is present in millimolar amounts in mammalian cells. It is considered less able to potentiate oxidation than cysteine and is good for regulating intracellular redox potential. The essential function of GSH includes its antioxidant activity [89], particularly its function in regulating protein thiol homeostasis and serving as the reaction partner for the detoxification of xenobiotics [90], as a cofactor in isomerization reactions, and in storage and transport from cysteine [91]. In the brain, GSH is an essential antioxidant that is regarded as highly sensitive to perturbation of the equilibrium between the antioxidant system and ROS. Oxidant species are associated with the pathogenesis and advancement of various neurodegenerative diseases, the regulation of redox status, and the antioxidant capacity of the CNS in the period of oxidative stress, which is essential for neuroprotection [92]. Glutamyl cysteine synthatase (GCS) is regulated physiologically either by competitive nonallosteric inhibition by GSH [93] or by the availability of its precursor amino acids. The availability of cysteine is essential for GSH synthesis. Cysteine is produced via the transsulfuration pathway, whereas dietary methionine is transformed to cysteine. Activation of ATP-dependent methionine promotes the generation of SAM and the gradual demethylation and removal of the adenosyl moiety-generated homocysteine. Homocysteine accumulates with serine to produce CBS. The terminal enzyme of the transsulfuration pathway is CSE, which is a PLP-dependent enzyme. It catalyzes the transition of L-cystathionine into L-cysteine; α-ketobutyrate and ammonia are the rate-limiting enzymes for the synthesis of cysteine from methionine. Hence, cystine levels in cells may also be increased by transport of cysteine via specialized transporter systems [94]. The significance of

the transformation pathway involved in producing cystine for GSH production in the liver is well recognized because any disturbance of this pathway reduces levels of cellular GSH [95].

Moreover, H_2S has the ability to protect neurons from oxidative stress by enhancing levels of GSH. When extracellular concentrations of glutamate are enhanced, a process known as oxidative glutamate toxicity, the import of cysteine in exchange for glutamate by the cysteine/glutamate antiporter is reduced. Because cysteine is converted to cystine in cells for the production of GSH, a reduction in cystine causes a reduction in the production of GSH. H_2S conserves cells under conditions of oxidative stress by two mechanisms, by increasing the generation of GSH, by increasing levels of cysteine/cystine transporters, and by redistributing the localization of GSH to mitochondria. As H_2S is regarded as a reducing substance and as cystine is present in plasma and blood at certain concentrations, H_2S may inhibit the process by which cysteine is reduced to cystine in the extracellular space and may enhance the transmembrane transport of cysteine into cells for GSH generation. Enhanced cysteine transport contributes to increased production of GSH. Enhanced GSH generation by H_2S is important under conditions of oxidative stress caused by glutamate. H_2S enhances both the generation of GSH and its redistribution to mitochondria. Additionally, its generation in mitochondria may occur in the context of reducing oxidative stress [96]. To achieve the protective effect of H_2S, one should test not only for glutamate toxicity but also for other markers of oxidative stress. In cerebral tissues, glutamate is not entirely liable for causing neuronal damage. The results of H_2O_2-imparted oxidative stress should not be ignored. H_2S retrieves GSH levels which is oppressed by H_2O_2, demonstrating that H_2S conserves cells from various oxidative stress stimuli. H_2S can also be restored [96]. In the embryonic brain, GSH levels that have been reduced by ischemia reperfusion and cysteine import are further oppressed by glutamate. In brief, H_2S enhances GSH concentration by intracellularly upregulating the transport of cysteine to a greater extent than it upregulates that of cystine. Additionally, H_2S enhances the redistribution of GSH into mitochondria. Hence, H_2S generated in mitochondria plays a role in the conservation of cells under conditions of oxidative stress [49].

Furthermore, although antioxidant activity is produced through a direct interaction between H_2S and ROS [32], it seems unlikely to be a quantitively efficient mechanism because of the low concentrations of H_2S compared with those of other antioxidants, such as GSH. Intraperitoneal NaHS treatment of pregnant rats protects the fetal brain from damage caused by ischemia reperfusion, which is compensated for by GSH levels [49]. Supplementation with cysteine facilitates the proliferation and differentiation of neuronal stem cells to neurons and astroglia, which is attenuated by knockdown of CBS expression using small interfering RNA (SiRNA) [68]. The neuroprotective effects of H_2S can be imparted by its anti-inflammatory and antiapoptosis activities [15] and its stabilization of membrane potentials [97]. Overall, GSH is a pivotal enzyme that reduces oxidative species in the CNS and maintains the H_2S balance to avoid neurodegenerative conditions in our body.

7. Antioxidant Effects of H_2S in CNS Neurodegenerative Diseases

Several major factors can cause the initiation and progression of neurodegenerative diseases, including oxidative stress, protein misfolding, and protein aggregation. Dysregulation of GSH homeostasis and deactivation of GSH-dependent enzymes are thought to play essential roles in the initiation and advancement of neurodegenerative diseases such as AD, PD, HD, and ALS (Figure 3) [98].

7.1. PD. PD is a neurodegenerative disorder affecting more than four million people all over the world. The brain of PD patient is characterized with loss of dopamine-secreting neurons in an area of the midbrain which is known as the substantia nigra (SN), subsequently causing bradykinesia, postural instability, resting tremor, and rigidity of patients [99]. In PD, H_2S metabolism may be involved. In a mouse model of PD, H_2S levels in the SN and striatum were lower in control mice [100]. In one experiment, H_2S introduced through injection or inhalation [18] prevented PD-like abnormalities, including movement dysfunction and microglial activation, from occurring. ROS is associated with the progression of PD; it is thought that impairment of the protective functions of GSH and related enzymes is involved in PD initiation and progression. As an example, postmortem brain tissue from PD patients' samples contained decreased amounts of GSH compared to controls [101]. In PD, many kinds of proteins are associated with cysteine residues, which are sensitive to oxidation. Hence, redox-sensitive proteins such as α-synuclein, parkin, and DJ-1 are involved in familial PD. α-Synuclein was the first gene found to be involved in familial PD and accumulation of Lewy bodies and subsequent neuronal cell death (Figure 3) [102]. GSSG regulation facilitates this accumulation, and neuronal cell death involved with α-synuclein in *Drosophila* can be rescued by interventions that enhances GSH [103].

Some recent studies have found that H_2S shields neurons against oxidative stress. These studies also found that H_2S has anti-inflammatory effects on brain cells in PD animal models [104]. The current clinical treatment for PD is levodopa (L-DOPA) replacement therapy to improve symptoms; however, this treatment can result in side effects such as dyskinesia and cannot prevent the advancement of PD. Based on previous studies, plasma homocysteine levels in PD are elevated when patients are treated with L-DOPA [105]. Moreover, current studies indicate that treatment with NaHS can significantly reduce the loss of SN neurons and slow the advancement of motor dysfunction in 6-hydroxydopamine hydrobromide-imparted and rotenone-induced PD models [106]. Additionally, inhalation of H_2S hinders the movement disorder resulting from 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-imparted PD. Hence, H_2S is thought to provide new ideas for the pathogenesis and clinical treatment of PD [18]. H_2S plays a role in PD to

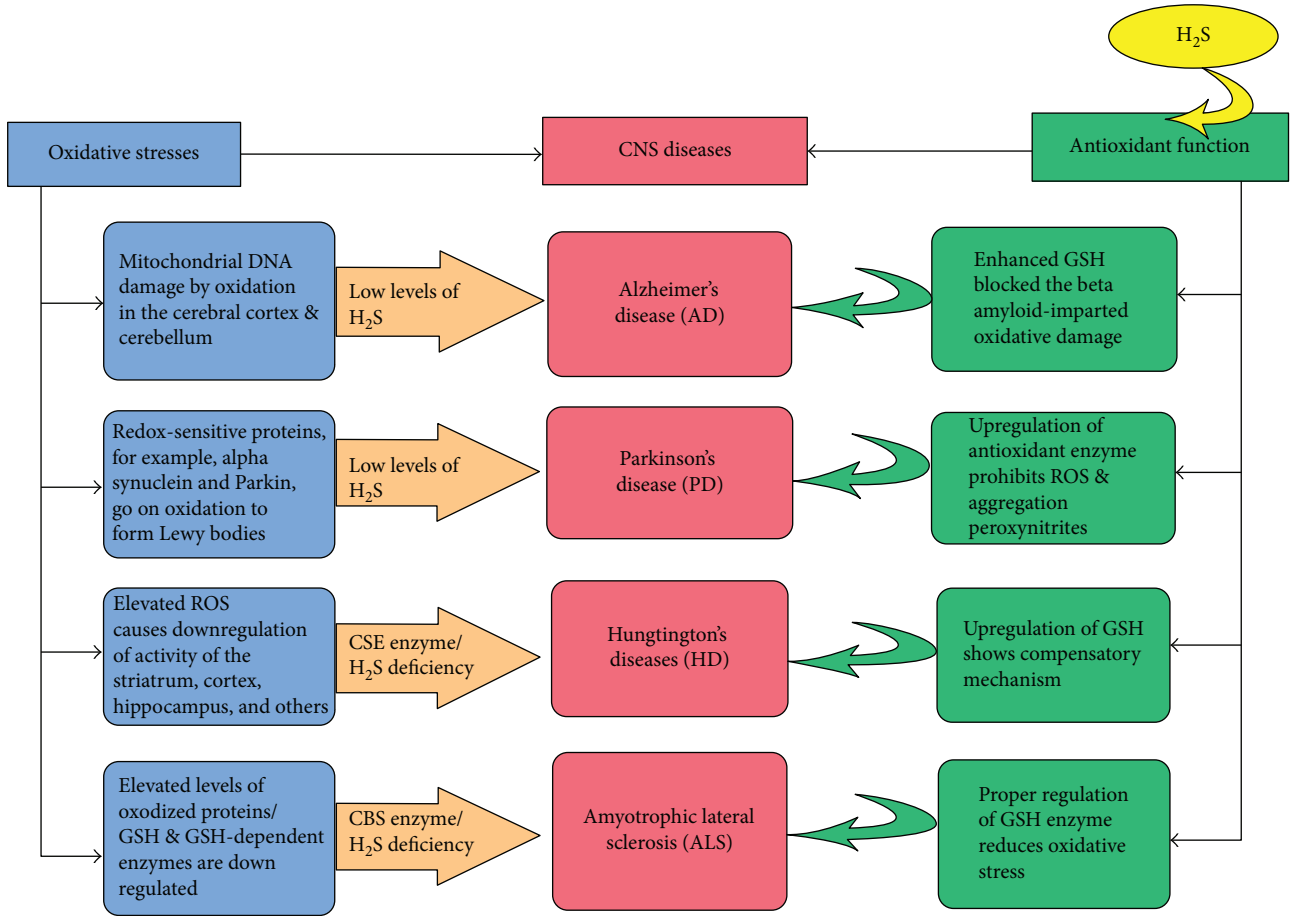

Figure 3: Role of hydrogen sulfide (H_2S) as antioxidant in the neurodegenerative diseases Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD), and amyotrophic lateral sclerosis (ALS). In AD, mitochondrial damage is caused by low levels of H_2S in the cerebral cortex and cerebellum whereas H_2S acts as antioxidant by GSH and amyloid beta-mediated oxidative damage. In PD, redox-sensitive proteins such as α-synuclein, parkin, and so on form Lewy bodies because of low levels of H_2S whereas H_2S imparts antioxidant functions by upregulation of antioxidant enzymes which inhibits reactive oxygen species (ROS) as well as lipid peroxidation products. In HD, elevated levels of ROS causes downregulation of activity in the striatum, hippocampus, and so on because of low supply of H_2S whereas H_2S acts as an antioxidant by reducing the excessive ROS. Lastly, in ALS, excessive amounts of proteins are downregulated because of low levels of H_2S and H_2S acts as antioxidant by proper regulation of antioxidant enzymes in ALS. In this ways, H_2S imparts antioxidant functions by modulating oxidative stress conditions in the neurodegenerative diseases.

combat oxidative stresses, combining with the enzyme GSH that acts as an antioxidant.

7.2. AD. AD is a catastrophic as well as progressive neurodegenerative disorder featured by extracellular accumulation of amyloid beta (Aβ) protein as well as intraneuronal neurofibrillary tangles (NFTs). The deleterious microglial activation in AD has been supported by analysis of postmortem brains of patients with AD where microglial overactivation occurred before neuronal damage demonstrating a crucial role in the advancement of AD [107]. Oxidative stress is also associated with AD progression. Dissection of postmortem AD brains has shown increased oxidative damage to nuclear and mitochondrial DNA in the cerebral cortex and cerebellum compared to age-matched controls (Figure 3) [108]. In addition, Aβ is thought to be a prooxidant itself, and this characteristic is considered partly liable for ROS production [109]. This kind of oxidation functions in neuronal death, leading to advancement of AD [110]; mutations in GSH-dependent enzymes are reported to increase the risk of AD. For example, a polymorphism in the glutathione peroxidase 1 (GPX1) gene has been identified as a possible risk factor in AD advancement [111]. Increasing intracellular levels of GSH is one defensive approach against AD advancement. N-Acetylcysteine (NAC) can act as a precursor for de novo synthesis of GSH. Mice treated with NAC prior to intracerebroventricular injection of Aβ demonstrated an enhanced ability to learn and increased memory function compared to controls [112]; GSH content is enhanced after NAC treatment. In addition, lipid protein and oxidation are reduced [112]. The enhanced GSH may block the prooxidant effects of the Aβ and prevent onset of the AD-like syndrome or may support more efficient repair of Aβ-imparted oxidative damage.

H_2S levels are lower in the brains of AD patients than in age-matched healthy people, although expression levels of

CBS do not differ between the two groups [113]. Although AD is regarded as a result of decreased production of H_2S, there may be an associated decrease in neuronal cytoprotection that enhances the harmful effects of damage and neuroinflammation induced by Aβ and oxidative stress [114–116]. Whether the low levels of H_2S seen in the brain in AD are a cause or a consequence of the disorder is not clear. In an experiment with rats examining whether vascular ischemia was associated with a decrease in viable neuron numbers in the hippocampus, injection with NaHS intraperitoneally markedly protected against neuronal injury and improved learning and memory performance, based on tests using a Morris water maze [117]. Most studies have focused on the pathway by which CBS catalyzes the reaction with substrate homocysteine to produce cystathionine; little attention has been paid to another pathway, in which CBS produces H_2S from L-cysteine as a substrate. SAM enhances CBS function in both metabolic pathways which is much reduced in AD brains. A recent study found that H_2S and SAM were reduced but that homocysteine was upregulated in AD brains [113]. These findings indicate that both H_2S and SAM are reduced; amounts of H_2S may be associated with the cognitive deterioration in this disease.

Furthermore, in AD, neurons are degraded via activated neuroinflammation, oxidative stress, and neuron apoptosis. Homocysteine, a pivotal risk factor for AD, has deleterious effects on cognitive function. A recent study of homocysteine-exposed rats found that H_2S ameliorated homocysteine-imparted cognitive dysfunction; this may play a constructive role via inhibiting reactive aldehyde aggregation, conserving GSH homeostasis, and enhancing aldehyde-dehydrogenase 2 activity and expression in the hippocampus [118]. Additionally, the Aβ cascade theory is considered as a major pathogenesis that may impart AD via oxidative stress and changes in synapses [119]. Hence, H_2S may reverse Aβ-imparted cognitive deficiency by decreasing the generation of Aβ and repressing the downregulation of CBS and 3MST [120]. Moreover, one study found that advancement of AD can be delayed by treatment with H_2S donors or spa waters rich in H_2S content, targeting multiple pathophysiological mechanisms. In that study, decreased TNF-α and B cell lymphoma 2 (Bcl-2) expression resulted in attenuation of morphological alterations in the hippocampus and improved spatial learning and memory ability [121]. In other AD models, the cytotoxic lipid oxidation product 4-hydroxynonenal was scavenged using H_2S therapy, providing a novel hope in the fight against AD via the neuroprotective effects of H_2S [122]. It has been shown that deficiencies in H_2S biosynthesis are involved in AD and that exogenous H_2S may have therapeutic potential by decreasing Aβ protein plaques.

7.3. HD. HD is an autosomal dominant disease associated with a mutation in the gene encoding huntingtin (Htt) following to extended polyglutamine repeats of mutant Htt (mHtt) which elicits oxidative stress, neurotoxicity, motor, and behavioral changes. HD is featured by highly selective as well as serious damage to the corpus striatum that regulates motor function [116]. In HD, as in other neurodegenerative diseases, GSH and GSH-dependent enzymes are dysregulated. Plasma samples of HD patients were found to have lower GSH contents compared to age-matched controls [123]. In addition, GPX activity in erythrocyte samples was lower in HD patients than in age-matched controls [124]. In another study, it was reported that there was no difference in GPX activity in cultured fibroblasts from HD versus non-HD patients [125]. The HD mouse model R6/2 showed an increased GSH content in mitochondria isolated from the cortex and striatum [126]. The authors demonstrated that enhancement of GSH may be a compensatory mechanism for elevated ROS production, although they did not measure ROS or other products of oxidative stress precisely.

Surprisingly, the dominant expression of CBS in the brain in a recent study revealed the importance of CSE in the manifestation of HD, an autosomal-dominant disease associated with a mutation in the gene encoding Htt [127]. Hence, HD is thought to be the result of highly selective and profound damage to the corpus striatum, which maintains motor function. This may reflect selective small G protein Rhes (gene) binding to mHtt, enhancing its neurotoxicity [128]. There is a massive aggregation of CSE, the biosynthetic enzyme for cysteine, in HD-diseased tissues, which may mediate HD pathophysiology. Defects that occur at the transcriptional level seem to reflect the influence of mHtt on specificity protein 1 and transcriptional activation of CSE as a pathogenic mechanism; supplementation with cysteine reverses abnormalities in HD tissue cultures and in intact mouse models of HD, demonstrating therapeutic potential [129]. In this study, CSE deficiency was found in brain tissues but not in the cerebellum of HD patients, in line with the relative susceptibility of these brain regions to HD (Figure 3). Additionally, in Q175 and R6/2HD murine models of HD, CSE expression is downregulated in the striatum, cortex, hippocampus, and brainstem, but not in the cerebellum. CSE-knockout mice display impaired Rota rod performance and an abnormal hindlimb clasping and clenching phenotype that is reminiscent of murine models of HD. These HD-related phenotypic changes are reversed by exogenously supplied cysteine [107]. In mice treated with an H_2S-releasing derivative of naproxen (ATB-346), there was a marked acceleration in the recovery of lost motor function and further enhancement of anti-inflammatory effects [130].

Additionally, H_2S stimulates various cytoprotective pathways [43]. It is not clear whether the pathophysiological influences of CSE aggregation in HD reflect its role in producing cysteine or H_2S. It is thought that treatment with H_2S donors will be useful in the treatment of HD [129]. The capability of CSE and cysteine to reverse oxidative stress and lethality in HD cells demonstrates that cysteine supplementation might be useful in HD treatment. Cysteine deficiency has been found in oxidative stress and aging [131]. In the brains of HD patients, CSE levels are greatly decreased in the striatum, moderately decreased in the cerebral cortex, and unchanged in the cerebellum, reflecting the relative susceptibility of these brain regions damaged by HD. A study based on a CSE model of HD demonstrated therapeutic effects of cysteine and NAC in mice with HD. That study

concluded that NAC supplementation may be useful in treating diseases associated with impaired reverse transsulfuration and oxidative stress [132]. Further studies are needed to find out the exact pathways about the roles of H_2S in HD.

7.4. ALS. ALS is a debilitating neurodegenerative disease that causes muscle atrophy and paralysis leading to death. ALS is the result of selective degeneration of motor neurons. Some studies have shown that astrocytes expressing a mutation in the enzyme SOD can accelerate motor neuron death [133]. While ALS is regarded as a degenerative disease of the upper and lower motor neurons, damage is not confined to motor neurons, with sensory and axonal projections also affected but to a lesser extent [134]. Various SOD mutations have been shown to result in this distinct pathology. This phenomenon has been reported in mice harboring different SOD1 mutations, such as Gly37Arg, Gly85Arg, and Gly93Ala. All three distinct mutations result in neurodegeneration [135]. As an example, Gly93Ala mice, but not Gly37Arg mice, have elevated levels of oxidized proteins related to disease progression in the spinal cord [136]. Besides the oxidative stress involved in reduced scavenging of superoxide ion, other studies reported accumulation of GSH *in vitro* to be associated with motor neuron cell death, which stimulates ALS [137]. GSH and GSH-dependent enzymes appear to be dysregulated in ALS (Figure 3). For example, in one study, erythrocyte GSH content was noticeably lower in ALS patients than in age-matched controls. Levels of H_2S in cerebral tissue in the familial ALS (fALS) mouse model SOD1G93A showed that increased levels of H_2S distorted H_2S metabolism in ALS [98].

H_2S is regarded as an essential biological gaseous transmitter at relatively low concentrations. It acts as a neuromodulator and neuroprotectant and regulates physiological functions to repress oxidative stress. In contrast, some data imply that higher concentrations of H_2S in ALS have toxic effects. L-homocysteine is degraded during H_2S synthesis. Moderate levels of homocysteinemia are seen in patients with spinal cord injury and ALS [138]. Others with neurological diseases, such as AD, dementia and schizophrenia patients, also show increased homocysteine levels [139]. Homocysteine imparts oxidative stresses and deoxyribonucleic acid (DNA) damage, while H_2S has the opposite effect. It can be concluded that decreased amounts of CBS that result in dysregulation of homocysteine metabolism or H_2S synthesis might be a vital factor in the pathogenesis of incidental and late neuronal disorders [140]. From the above discussion, it can be concluded that H_2S is involved in the pathogenesis of ALS, as found in several studies, and it could be an important marker for diagnosis of ALS in patients.

The above discussion of these neurodegenerative diseases clearly shows that H_2S plays a neuroprotective role by combating oxidative stresses in the CNS to protect the body.

8. Role of H_2S in Neuroinflammation

Inflammatory processes have been described in many neurodegenerative diseases, including AD, PD, HD, and ALS. As neuroinflammation is considered as a key factor in neurodegeneration, many therapeutics are aimed at delaying or stopping advancement of inflammation in neurodegenerative diseases [141]. For example, lipopolysaccharide (LPS) causes neuroinflammation, neuronal ultrastructure impairment, and cognitive defects. LPS links to immune cells such as monocytes, dendritic cells, macrophages, and B cells, thereby increasing the secretion of proinflammatory cytokines, NO, and eicosanoids [142]. Treatment with NaHS decreases LPS-induced inflammation in both primary cultured microglia and immortalized murine microglial cells. It is speculated that H_2S inhibits NO synthase and p38 MAPK signaling pathways in a concentration-dependent manner. Suppression of H_2S generation by silencing CSE in LPS-stimulated macrophages results in enhanced generation of H_2S [143]. Levels of proinflammatory cytokines are lower after CSE silencing. Microglia and astrocytes, regarded as the immune cells of the brain and spinal cord, are the main active immune defense of the CNS. They impart inflammatory action by inducing nuclear factor-κB (NF-κB), releasing the inflammatory mediators TNF-α, interleukin (IL-6), and nitrite ions, and downregulating CBS and H_2S [144]. These inflammatory factors are involved in tissue repair but may also stimulate further tissue injury and cause cell death. This effect is slightly reversed in cells pretreated with NaHS, demonstrating the anti-inflammatory effects of H_2S [40]. It is unclear whether the anti-inflammatory mechanism involves a direct effect of H_2S on astrocytes and microglia or an indirect effect via inhibiting the release of proinflammatory factors [145].

Moreover, AMP-stimulated protein kinase (AMPK) is recognized as a central factor in inflammation [146]. One study demonstrated inhibition of neuroinflammation by activation of AMPK by H_2S, supporting earlier findings on the inhibitory effect of activation of AMPK against inflammation. Although AMPK has been described as a therapeutic intervention in various diseases, the discovery of H_2S-imparted AMPK activation via the calmodulin-dependent protein kinase β (CaMkβ) makes H_2S an interesting anti-inflammatory target. It can be concluded that H_2S imparts pivotal anti-inflammatory functions, due to its interaction with inflammation-related LPS, microglia, astrocytes, and AMPK [145].

To sum up, while combating oxidative stresses, H_2S plays a neuroinflammatory role by inhibiting the release of proinflammatory factors in the CNS.

9. Further Investigations

H_2S is regarded as a ubiquitous molecule with essential roles in a wide range of physiological and pathological processes. Various H_2S-mediated therapies have been studied as potential catalysts of this unique mediator in preclinical and early clinical testing. The goal for developing H_2S-based therapeutics is to enhance efficiency and reduce toxicity compared with existing therapies. Ongoing studies range from simple approaches, such as the use of zero valent sulfur, to sophisticated tactics, such as targeted H_2S release to specific organelles. Further advancement of pH, oxygen, and free radical-sensitive donors will be helpful on the way

to achieving selective delivery of H_2S. Agents that stimulate the various H_2S-producing enzymes (CSE, CBS, and 3MST) specifically are attractive therapeutic candidates to study. However, research in the field of H_2S is hindered by a lack of specific inhibitors of the various enzymes involved in the synthesis of this gasotransmitter. Several enzymes, such as CSE, have been identified as substantial therapeutic targets for developing potent and highly selective inhibitors for diagnostic and therapeutic applications. Greater understanding of the mechanism of H_2S release and modulation of synthesis is required to monitor H_2S levels *in vivo* and to improve H_2S-based therapeutics.

10. Conclusions

H_2S, a commonly known toxic gas, plays a homeostatic role in the body by acting as an antioxidant against oxidative species such as ROS and RNS. H_2S is generated from enzymes such as CBS, CSE, CAT, and 3MST. Higher or lower amounts of H_2S are associated with various CNS diseases including AD, PD, HD, and ALS; therefore, H_2S level serves as a marker for detecting these diseases. Considering that H_2S was previously regarded as a poisonous gas, it is surprising that proper amounts of H_2S are required in the body; decreased H_2S levels cause neurodegenerative diseases, and induction of H_2S can ameliorate disease conditions. The proper maintenance of H_2S via biogenesis and catabolism functions in cell-signaling pathways. H_2S contributes as an antioxidant and as an antineuroinflammatory agent. Furthermore, H_2S exerts protective effects in neurological systems by shielding neurons against hypoxic injury, preventing hypochlorous acid-mediated oxidative damage, enhancing GSH generation, and repressing oxidative stress in mitochondria. Further studies are required to develop H_2S-based therapeutics to treat neuroinflammatory diseases.

Abbreviations

AD: Alzheimer's disease
ALS: Amyotrophic lateral sclerosis
AP-5: Amino-phosphonopentanoate
AMPA: α-Amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptor
AMPK: AMP-stimulated protein kinase
ATP: Adenosine triphosphate
Bcl-2: Basal cell lymphoma-2
CBS: Cystathionine β-synthatase
CSE: Cystathionine γ-lyase
CAT: Cystathionine amino transferase
cAMP: Cyclic adenosine monophosphate
CNQX: 6-Cyano-7-nitroquinoxaline-2,3-dione
CNS: Central nervous system
CO: Carbon monoxide
Ca^{2+}: Calcium ion
CSF: Cerebral spinal fluid
CFTR: Cystic fibrosis transmembrane conductance regulator
DMPD: N,N-Dimethyl-P-phenylenediamine
DNA: Deoxyribonucleic acid
EGF: Epidermal growth factor
EGFR: Epidermal growth factor receptor
GSH: Glutathione
GSSG: Glutathione disulfide
GCS: Glutamyl cysteine synthase
GPX1: Glutathione peroxidase
H_2S: Hydrogen sulfide
H_2O_2: Hydrogen peroxide
HS^-: Bisulfide
HCO_3^-: Bicarbonate ion
HD: Huntington's disease
H_2S_n: Polysulfide
L-NAME: L-Nitro arginine methyl ester
3MST: 3-Mercaptopyruvate sulfurtransferase
MAPKs: Mitogen-activated protein kinases
3-MP: 3-Mercaptopyruvate
mRNA: Messenger RNA
NMDA: N-Methyl-D-aspartate
NAD: Nicotinamide adenine dinucleotide
NADPH: Nicotinamide adenine dinucleotide phosphate
NaHS: Sodium hydrosulfide
NAC: Acetylcysteine
PD: Parkinson's disease
PLP: Pyridoxal-5′-phosphate
PKA: Protein kinase constant A
ROS: Reactive oxygen species
RNS: Reactive nitrogen species
SOD: Superoxide dismutase
SAM: S-Adenosylmethionine
SNAP: S-Nitroso-N-acetylpenicellamine
SNP: Sodium nitroprusside
SiRNA: Small interfering RNA
SN: Substantia nigra
TGF-α: Tumor growth factor-α.

Conflicts of Interest

The authors reported no potential conflict of interests.

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Review Article

Omega Class Glutathione S-Transferase: Antioxidant Enzyme in Pathogenesis of Neurodegenerative Diseases

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The omega class glutathione S-transferases (GSTOs) are multifunctional enzymes involved in cellular defense and have distinct structural and functional characteristics, which differ from those of other GSTs. Previous studies provided evidence for the neuroprotective effects of GSTOs. However, the molecular mechanisms underpinning the neuroprotective functions of GSTOs have not been fully elucidated. Recently, our genetic and molecular studies using the *Drosophila* system have suggested that GstO1 has a protective function against H_2O_2-induced neurotoxicity by regulating the MAPK signaling pathway, and GstO2 is required for the activation of mitochondrial ATP synthase in the *Drosophila* neurodegenerative disease model. The comprehensive understanding of various neuroprotection mechanisms of *Drosophila* GstOs from our studies provides valuable insight into the neuroprotective functions of GstOs *in vivo*. In this review, we briefly introduce recent studies and summarize the novel biological functions and mechanisms underpinning neuroprotective effects of GstOs in *Drosophila*.

1. Introduction

Glutathione S-transferases (GSTs) are a superfamily of multifunctional isoenzymes involved in the cellular detoxification of several endogenous and exogenous compounds. GSTs catalyze the nucleophilic attack of glutathione (GSH) on the electrophilic centers of substrates, including insecticides, toxic compounds, metabolites, and organic hydroperoxides. GSTs play a crucial role against carcinogens, therapeutic drugs, and various types of cellular oxidative damage [1]. GSTs also regulate the biosynthesis and intracellular transport of hormones [1]. Based on their sequence similarity and substrate specificities, GSTs are subdivided into at least ten subfamilies: alpha, delta, epsilon, kappa, mu, pi, sigma, theta, zeta, and omega [2].

The omega class GST (GSTO) is the most recently defined GST class and a relatively ancient cytosolic enzyme [3, 4]. GSTOs appear to be widespread in nature and have been identified in bacteria, insects, yeast, mammals, and plants [2, 3, 5–7]. GSTO shares low sequence similarity with other GST classes but exhibits the GST fold. GSTO has interesting characteristics compared with those of other GST types. The active sites of GSTOs have a cysteine residue at the N-terminus that can bind to GSH, whereas other GST classes have tyrosine or serine residues in their active sites [8]. GSTOs have distinct enzymatic properties and thiol transferase and dehydroascorbate (DHA) reductase activities, which are similar to reactions catalyzed by thioredoxin and glutaredoxin [3]. There is increasing evidence that GSTOs are also involved in the detoxification of several exogenous stressors. Silkmoth GSTO was induced in the fat body after exposure to several environmental stressors, including bacteria and ultraviolet-B (UV-B) [9]. GSTO3 from the human pathogenic filarial worm *Onchocerca volvulus* (*Ov*GSTO3) demonstrates stress-resistant effects [10]. Overexpressing GSTO-1 in *Caenorhabditis elegans* exhibits increased resistance during oxidative damage [11]. In addition, GSTOs were shown to scavenge free radicals by regulating DHA reduction and catalyzing the reduction of inorganic arsenic, monomethylarsonate (MMA), and dimethylarsonate (DMA) [12–14]. *In vitro* studies have shown that human GSTO1 participates in modulation of the ryanodine receptor,

which is a Ca^{2+} release channel. In addition, these studies also showed that human GSTO1 is involved in modulation of the signaling pathway during c-Jun N-terminal kinase- (JNK-) mediated apoptosis and in the activation of interleukin-1β, an important mediator of inflammatory response [15–17]. Human GSTO1-1 is a novel regulator of lipopolysaccharide- (LPS-) induced inflammatory responses in macrophages and is required for LPS-mediated signaling in macrophages [18, 19].

Recently, an important role for human GSTO1-1 in glutathionylation of the target proteins has been described [17, 20]. β-Actin has been reported to be deglutathionylation by human GSTO1-1 [20]. GSTO1-1 decreased global protein glutathionylation level in macrophages [18]. These findings indicate a critical role for GSTO1 in redox homeostasis through affecting glutathionylation/deglutathionylation of the target proteins. Furthermore, genetic polymorphisms in the human *GSTO1* and *GSTO2* genes may be associated with the risk of bladder, urothelial, breast, and ovarian cancer [21, 22]. Thus, GSTOs play important roles in decreasing oxidative stress produced by various stressors and cellular processes. Recent studies related to neurodegenerative disorders have implicated polymorphic variants of GSTOs in the age at onset and progression of neurodegenerative diseases such as Alzheimer's disease (AD) and Parkinson's disease (PD) [23–25]. GSTO2 expression levels are decreased in the brains of AD patients. Although GSTO has a protective function against neuronal damage, the molecular mechanisms and physiological functions of GSTOs are still not clear and should be researched further. In this review, we mainly focus on recent studies that have investigated the neuroprotective functions of GSTOs in the *Drosophila* model system.

2. Omega Class Glutathione S-Transferases in *Drosophila*

On the basis of the genome sequences and comparative analyses, the *Drosophila* GST genes can be divided into six subfamilies: delta, epsilon, sigma, omega, zeta, and theta. *Drosophila* harbors 36 GST genes that encode 41 proteins [2, 26]. Four different *GSTO* genes in *Drosophila* are located on chromosome 3L. *GSTO* genes form a cluster spanning approximately 6 kb [2]. This is evidence of internal duplication within the cluster, evolutionally. This duplication gave rise to differentially expressed GSTO isoforms and generated diverse members with differing functionality. These four *GSTO* genes had been named previously as follows: *sepia*, *GstO1*, *GstO2*, and *GstO3* [2]. The sequence identities/similarities are high, at 43–65%/66–82%, based on the amino acid sequence alignment of the different isoforms of GSTO in *Drosophila* [27]. All isoforms of GSTO have N-terminal extensions and cysteine residues in the active site rather than tyrosine or serine residues, which are found in the active sites of other classes of GSTs. Furthermore, all isoforms of GSTOs have high thiol transferase and DHA reductase activities, characteristic of GSTOs, and low activity toward 1-chloro-2,4-dinitrobenzene (CDNB), a general GST substrate [27]. In addition, the tissue distributions of GSTOs were determined by reverse transcription polymerase chain reaction (RT-PCR) and Western blot analysis [27–29]. They have a different tissue distribution in *Drosophila*. Sepia was found only in the eye. GstO1 and GstO2A were highly expressed in the head and abdomen of adult flies. However, GstO2B and GstO3 were expressed at approximately the same level in all tissues. Therefore, these studies suggest that *Drosophila* GSTOs might possess tissue-specific physiological functions.

3. Neuroprotective Functions of GstOs in *Drosophila*

3.1. GstO1 Has a Protective Function against Neuronal Toxicity. Oxidative stress poses a major threat to organisms living in an aerobic environment and plays a critical role in several neurological disease processes [30]. Oxidative stress is widely implicated in neuronal cell death. Hydrogen peroxide (H_2O_2) has been implicated in triggering apoptotic death in several cell types [31, 32]. It may also induce the production of reactive oxygen species (ROS) in neuronal cells. In a recent study, our group determined that GstO1 is highly expressed in the head, and *GstO1* mutant flies are sensitive to ROS, produced under H_2O_2 exposure. Interestingly, H_2O_2-induced lethality and apoptotic cell death of neurons in *GstO1* mutant flies were suppressed by neuron-specific expression of GstO1. These results suggest that GstO1 has a physiological function in neurons, and GstO1 neuronal expression is sufficient to suppress H_2O_2-induced neurotoxicity in *GstO1* mutant flies.

Phosphorylation cascades leading to the activation of MAPK, including ERK, JNK, and p38, are among the major cellular signaling pathways known to influence cell survival under ROS damage [33–35]. Several reports have shown that accumulation of ROS in response to H_2O_2 exposure results in the activation of several stress kinases, involving the ASK1, ERK, and JNK pathways [33–35]. Our studies of H_2O_2-induced neurotoxicity in *GstO1* mutant flies reveal that increased ERK phosphorylation in *GstO1* mutant flies treated with H_2O_2 was rescued by the expression of GstO1 [28]. The mechanism for the suppression of H_2O_2-mediated neurotoxicity appears to be mediated through the suppression of ERK pathway activation. Thus, these findings strongly demonstrate that GstO1 has a critical, protective role against H_2O_2-mediated neurotoxicity by regulating the MAPK pathway.

3.2. GstO2 Regulates Complex V Activity in Neurodegenerative Disease. Previous studies have reported that single-nucleotide polymorphisms in human *GSTO* genes are associated with the age at onset for AD, PD, and stroke [36, 37]. The *GSTO1 D140* allele is associated with a decreased risk of familial PD [23]. In addition, a possible relationship between GSTOs loci and the age at onset of amyotrophic lateral sclerosis (ALS) has been reported [38]. These various studies provide evidence that genetic variation of human *GSTOs* can influence the age at onset of several different neurodegenerative diseases. These studies also suggest that GSTOs may contribute to the pathogenesis of each

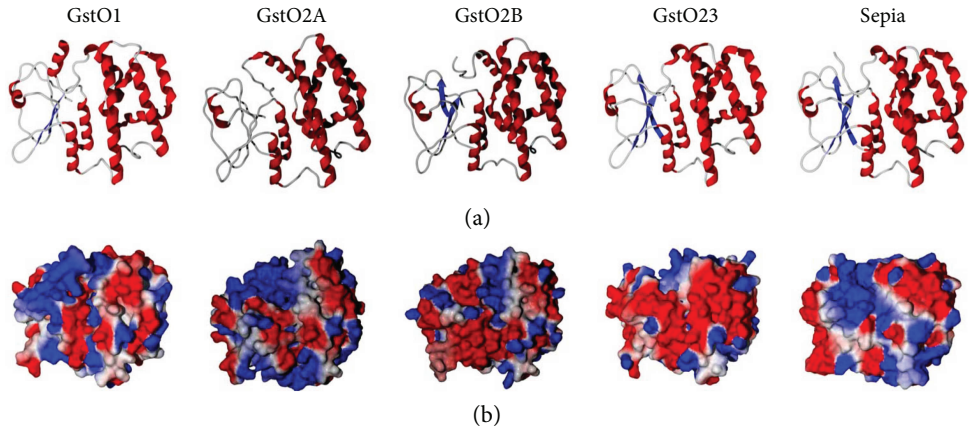

Figure 1: Comparison of the three-dimensional (3D) structures and surface electrostatic potential distributions of *Drosophila* GstOs. The 3D structure predictions of GstOs were generated by I-TASSER server for protein structure prediction, which is based on a threading alignment algorithm (a). Overall 3D ribbon structures of GstOs are shown (b). Negative and positive charges are represented in red and blue, respectively. The figures were generated by the Molegro Molecular Viewer.

neurological disorder and have a protective role in neuronal cells during the development of neurological diseases. However, many studies have failed to demonstrate the molecular function of GSTOs *in vivo*. A speculative mechanism involving the pathogenesis of neurodegenerative disease was recently proposed. Our subsequent study showed that GstO2A is a novel genetic regulator of the *Drosophila parkin* mutant, which is the popular *Drosophila* model of PD [39]. Furthermore, we showed compelling evidence that GstO2A catalyzes the glutathionylation of the ATP synthase β subunit, which is a catalytic component of the mitochondrial ATP synthase complex (complex V). The glutathionylation of the ATP synthase β subunit induced by GstO2A expression in *parkin* mutants is important for the rescue of ATP synthase activity in these mutants [39]. Moreover, human GSTO1 has the capacity to glutathionylate or deglutathionylate target proteins [20]. Although the role GstO2A plays in the glutathionylation of target proteins is not clear, these findings strongly suggest that enhancing the activity of GstO2A in neuronal cells could alleviate neurodegeneration in the *Drosophila* model of PD.

3.3. GstO2 Regulates Ascorbic Acid Recycling. Ascorbic acid (AsA), the reduced form of vitamin C, is an essential cofactor in various enzymatic reactions. AsA is an important antioxidant with multiple cellular functions and plays a role in detoxification against endogenous and exogenous stressors. Interestingly, the brain exhibits one of the highest AsA concentrations in the body. AsA concentrations of 1~2 mM have been detected throughout the brain while intracellular concentrations in neuronal cells are much higher, reaching up to 10 mM [14]. This evidence suggests a critical role of AsA in the brain or neuronal cells [40]. Imbalance of AsA homeostasis has also been demonstrated in neurodegenerative diseases such as AD, PD, and ALS.

In most cells, ascorbic acid is regenerated from the oxidized form of ascorbic acid, DHA [41, 42]. This recycling pathway of DHA to AsA is known to be mediated by specific reductases, such as GSH- or NADPH-dependent DHA reductases [43–45]. AsA is synthesized in the liver of several mammals. However, humans and other primates do not express the specific enzyme for AsA biosynthesis and are unable to synthesize AsA [46]. Therefore, humans require a supply of AsA from food. In this regard, understanding the mechanism for AsA recycling is important for maintaining cellular AsA homeostasis. Previous reports have shown that the DHA reductase activity of human GSTO2 is approximately 70–100-fold higher than that of human GSTO1 using an *in vitro* enzyme assay [12]. The notable feature of human GSTO2 is very high DHA reductase activity, which suggests that human GSTO2 may have a protective role against oxidative stress by recycling AsA [12]. In *Drosophila*, GstO2B has the highest GSH-dependent DHA reductase activity among the GstOs [27]. In addition, we showed that GSH-dependent DHA reductase activity is decreased in *GstO2* mutant flies. Furthermore, the AsA redox state, determined by the AsA/DHA ratio, was also dramatically decreased in *GstO2* mutants [39]. These studies suggest that GstO2B may be critical in the maintenance of AsA concentrations in cells and plays a protective role against oxidative stress by regulating the AsA recycling pathway in *Drosophila*.

4. Structural Difference and Diverse Function of Omega GSTs in *Drosophila*

Binding of the GSH to GSTs is highly conserved in the N-terminal domain. Substrate-binding sites (H-site) in the C-terminal domain of GSTs are variable with different features, hydrophobicities, shapes, and electrostatic potential distributions [47]. Many research groups have shown that residues in the substrate-binding sites of omega class GST homologues are well conserved across species [48–50]. Although most residues in the G-site and H-site of GstOs in *Drosophila* are highly conserved [27], the GstOs have different physiological functions *in vivo* [39, 51]. Therefore, we constructed three-dimensional models of *Drosophila* GstOs using I-TASSER server and analyzed the surface electrostatic

potential distributions (Figure 1). The electrostatic potential distributions of GstO1, GstO2A, and sepia are similar, with only some differences. In contrast, the electrostatic potential distributions of GstO2B and GstO3 differ markedly from those of the other *Drosophila* GstO electrostatic potential distributions. These features are likely to be determinants of interactions between *Drosophila* GstOs and substrates that are still to be discovered. We showed that GstOs have different electrostatic potential distributions and substrate-binding site shapes from each other, by homology modeling analysis. Because these GstO isoforms differ only in the portion of the C-terminal domain that binds the hydrophobic substrate, this region may influence substrate preference. These data may explain the functional differences between each GstO isoforms. However, the differences in the functions and catalytic mechanisms of GstOs have not been fully elucidated. Identification of the differences in the structure and electrostatic potential of the GstO substrate-binding sites helped us understand the catalytic role of GstOs in reaction with different substrates and their ability to perform different functions *in vivo*.

5. Putative Functions of Other GSTOs in *Drosophila*

Although the *in vivo* function of *Drosophila* GstO3 is not yet elucidated, there is some evidence that it may also be involved in antioxidant processes. The *GstO3* transcript of *Drosophila* is expressed at approximately the same level in all tissues. Interestingly, the expression level of *GstO3* transcript increased in response to various stressors such as heat shock, heavy metal stress, and exposure to rotenone [52–54]. However, little is known with regard to the exact mechanism responsible for increased GstO3 expression. Thus, GstO3 may have a wide range of antioxidant activities. Further studies are required to understand the physiological function and molecular mechanism by which GstO3 protects cells from various oxidative stressors.

6. Conclusions and Perspective

GSTs are ubiquitously expressed enzymes belonging to the GSH-mediated antioxidant. Sequence alignment analyses revealed that GSTs, including GSTOs, exist in a wide range of organisms. The broad distribution of several GSTs among all living organisms may reflect its important and diverse physiological functions. Particularly, the role exerted by GSTOs in neuronal cells appears to be relevant. Although various studies suggest that oxidative stress plays an important role in the pathogenesis of several human diseases, including neurodegenerative diseases, the exact mechanism of GSTOs in neuroprotective effects against several oxidative stressors that provide a pharmacological basis for the relationship between GstOs and the development of neurodegenerative diseases has not been elucidated. As discussed in this review, our research on *Drosophila* GstOs has shown that GstOs are involved in protective effects against various neurotoxic conditions. For instance, GstO1 has a protective function against H_2O_2-induced neurotoxicity by regulating the MAPK signaling pathway. In addition, GstO2 is required for the activation of mitochondrial ATP synthase in the *Drosophila* model of PD. This finding suggests that enhancing the activity of GstO2 in neuronal cells could alleviate neurodegeneration in the *Drosophila* model of PD. Furthermore, GstO2 has a DHA reductase activity and is required for the recycling of AsA. Thus, GstO2 may play a critical role in the maintenance of AsA concentrations in neuronal cells and plays a protective role against oxidative stress by regulating the AsA recycling pathway in neuronal cells. A comprehensive understanding of various neuroprotection mechanisms of *Drosophila* GstOs and a further investigation of their pharmacological and antitoxicological properties will enhance our understanding of their role in neuronal cells and the pathogenesis of neurodegenerative diseases.

Previously, our studies provided several evidences for novel diverse roles of GstOs, based on genetic and molecular studies using the *Drosophila* model system. The C-terminal domain, including the H-site of *Drosophila* GstOs, is less similar than the N-terminal domain, which contains a cysteine residue in the G-site of GstOs. Thus, the structural differences in the C-terminal domain of GstOs may be responsible for the differences in the functions of various *Drosophila* GstOs and may influence substrate preference. Further studies are required to identify the *in vivo* substrates of GSTOs, which are related to neuroprotection, in order to better understand the functional diversity of GSTOs. Because various biological processes are conserved in *Drosophila* and mammals, we expect that the elucidation of diverse *in vivo* functions of *Drosophila* GstOs will have broad biological implications in understanding neuroprotection mechanisms.

Conflicts of Interest

The authors declare no conflicts of interest.

Acknowledgments

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Review Article

Ischemic Retinopathies: Oxidative Stress and Inflammation

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Ischemic retinopathies (IRs), such as retinopathy of prematurity (ROP), diabetic retinopathy (DR), and (in many cases) age-related macular degeneration (AMD), are ocular disorders characterized by an initial phase of microvascular changes that results in ischemia, followed by a second phase of abnormal neovascularization that may culminate into retinal detachment and blindness. IRs are complex retinal conditions in which several factors play a key role during the development of the different pathological stages of the disease. Increasing evidence reveals that oxidative stress and inflammatory processes are important contributors to the pathogenesis of IRs. Despite the beneficial effects of the photocoagulation and anti-VEGF therapy during neovascularization phase, the need to identify novel targets to prevent initial phases of these ocular pathologies is still needed. In this review, we provide an update on the involvement of oxidative stress and inflammation in the progression of IRs and address some therapeutic interventions by using antioxidants and anti-inflammatory agents.

1. Introduction

Ischemic retinopathies (IRs), such as retinopathy of prematurity (ROP), diabetic retinopathy (DR), and age-related macular degeneration (AMD), are the main causes of severe visual impairment and sight loss in children, adults (with diabetes), and elderly population, respectively [1, 2]. IRs are biphasic diseases characterized by loss of the preexisting vessel bed and sustained hypoxia that leads to a secondary vasoproliferative phase characterized by anarchic vessel proliferation into the vitreous humor, which can result in retinal detachment and blindness [3]. Importantly, the ensuing ischemic events secondary to initial vessel loss can also have devastating effects on neuronal homeostasis and function [4].

Several factors contribute to the pathogenesis of IRs; however, oxidative stress [5, 6] and inflammatory processes [7, 8] stand as major ones. Oxidative stress is defined as an imbalance favoring generation over the removal of reactive oxygen species (ROS), such as free radicals, nitric oxide ($^{\bullet}NO$), superoxide anion ($O_2^{-\bullet}$), and hydrogen peroxide (H_2O_2). Free radicals are short-lived reactive molecules that disrupt molecular nature of lipids, amino acids, and nucleic acids. ROS are normal by-products of aerobic metabolism; inefficient removal by antioxidants leads to redox injury and cellular damage [9]. Factors that participate in the pathogenesis of IRs, such as hyperoxia in ROP, hyperglycemia in DR, and lipid accumulation in AMD, are important amplifiers of oxidative stress that cause dysregulation of cell metabolism and participate in limiting antioxidant defenses during the development of the IRs [9–13].

Inflammation and oxidant stress are tightly intertwined. Inflammation is a cellular response to factors (including those due to oxidant stress) that challenge the homeostasis of the tissues, but this process also acts as a defense mechanism to maintain the equilibrium of the functions. Cytokines and chemokines are signaling proteins that travel throughout the body to exert specific functions in inflammation. However, sustained inflammation can be detrimental to tissue integrity. Increasing evidence shows that a local and/or systemic augmentation of ROS or inflammatory molecules is

implicated in the pathogenesis of IRs. Current therapies only target late phases of these ocular pathologies, specifically the vasoproliferative phase. Yet, there is an urgency to tackle the initial ischemic phases. We hereby review prominent concepts that involve oxidative stress and inflammation in the genesis and progression of IRs.

2. Retinopathy of Prematurity (ROP)

ROP is the major cause of visual impairment and blindness in neonates worldwide. A demographic census in 2010 reported ~184,700 preterm babies worldwide with ROP; 20,000 of them were blind or severely visually impaired [14]. This problem is reaching epidemic proportions in middle-income and developing countries; the survival of extremely premature infants is increasing without a significant change in morbidity [15].

2.1. Pathogenesis of ROP. ROP is a disease that affects the immature retinal vascular system and thus occurs in premature infants with an incompletely vascularized retina. Therefore, the incidence and severity of retinopathy are directly proportional to the degree of prematurity. It is widely accepted that the development of ROP progresses through two phases. The first phase begins when retinal vascular growth ceases after premature birth. During this time, the retinal cytoprotective factors, such as insulin-like growth factor-1 (IGF-1), diminish [16] and the vessels become particularly vulnerable to injury caused by any number of stressors, including the amount of oxygen supply. Premature infants are exposed to higher oxygen tension after birth compared to those in utero. This leads to a downregulation of the major hypoxia-driven vascular endothelial growth factor (VEGF), as well as an increase in vasoobliteration of immature retinal capillaries through the actions of oxidant stress and intertwined inflammation [17]. The loss of blood vessels, associated with an increase in maturation-dependent metabolic demand, causes the retina to become gradually hypoxic. In order to ensure an adequate perfusion to the hypoxic retina, an overproduction of hormones and growth factors stimulates an excessive vessel formation at the junction between the vascular and avascular retina. This sets the beginning of the second phase of ROP. Interestingly, these new vessels fail to reperfuse the avascular retina, as instead of growing into areas of need, they grow chaotically into the vitreous, where traction and detachment of the retina as well as bleeding can occur, ultimately resulting in blindness. This critical stage of ROP (defined in humans as stage 4-5) occurs most frequently around 34–36 weeks after conception [18].

2.2. Nitrooxidative Stress, Lipid Oxidation and Nitration, and ROP. The pathogenesis of ROP is related to many causative factors, including low gestational age, low birth weight, genetic components, and relative hyperoxia. Hyperoxia is one of the major environmental predisposing factor to ROP, as it is the molecular basis for generation of reactive oxygen species (Figure 1). Nitric oxide formation also requires oxygen, and based on the redox state of the retina, it can have either beneficial or detrimental effects to the retina. Using the experimental model of oxygen-induced retinopathy (OIR) which shares many features of ROP, it was shown that endothelial nitric oxide synthase expression and activity increase when the redox state is shifted towards an oxidative environment [19]. Under these conditions, nitric oxide reacts with reactive oxygen species resulting in generation of nitrites, nitrates, and most damaging peroxynitrite that cause retinal microvascular degeneration [20] by a process called nitrooxidative stress. Genetic ablation [21] and pharmacologic inhibition of endothelial nitric oxide synthase [22] have been shown to attenuate hyperoxia-induced retinal microvascular degeneration, demonstrating the importance of nitrooxidative stress in ROP.

Lipid peroxidation of cell membranes secondary to inadequately high oxygen tension is pivotal to the pathogenesis of ROP (Figure 1). The retina is highly susceptible to lipid peroxidation, being composed of lipids with elevated levels of polyunsaturated fatty acids (PUFAs), such as docosahexaenoic acid (DHA), *cis*-arachidonic acid, and choline phosphoglyceride. Prostanoids are synthesised from arachidonic acid by the sequential action of phospholipase A_2 and cyclooxygenase, which are triggered by oxidant stress and peroxidation. The accumulation of peroxides eventually favours thromboxane A_2 production, which is a potent cytotoxic agent in microvessels [23]. Inhibitors of cyclooxygenase and thromboxane A_2 synthase selectively curtail oxygen-induced retinal vasoobliteration in mice [23]. Nitrative stress results in *cis*- to *trans*-isomerisation of arachidonic acid, and this was shown to contribute to retinal vascular degeneration in a mouse model of ROP [24]. Circulating levels of plasma *trans*-arachidonic acid are increased in oxygen-induced microvascular degeneration and are known to be secondary to nitrative stress. More specifically, *trans*-arachidonic acid formation has been shown to be abrogated in mice treated with nitric oxide synthase inhibitors and in mice deficient in endothelial nitric oxide synthase [22]. The endothelial cytotoxicity induced by *trans*-arachidonic acid results from the formation of the antiangiogenic and proapoptotic thrombospondin-1 [22] via activation of long-chain fatty acid receptor GPR40 [25].

Platelet-activating factor and lysophosphatidic acid are other lipids generated during peroxidation that act as proinflammatory mediators and contribute to microvascular injury in the retina. Platelet-activating factor is abundantly generated during oxidant stress, and its cytotoxic effects are mediated to a large extent by thromboxane A_2 [26]. Along the same lines, lysophosphatidic acid is released from lysophosphatidylcholine by the action of lysophospholipase D and can play a role in retinal inflammation leading to microvascular cytotoxicity in OIR [27].

2.2.1. Antioxidant Agents in ROP. Susceptibility of the immature retina to hyperoxia-triggered oxidative stress lies in the incomplete development of its antioxidant system [28, 29]. In order to circumvent this hurdle, supplementation with antioxidants has been attempted. Vitamin E is a naturally occurring free radical scavenger that decreases lipid peroxidation and helps to maintain membrane integrity in retinal

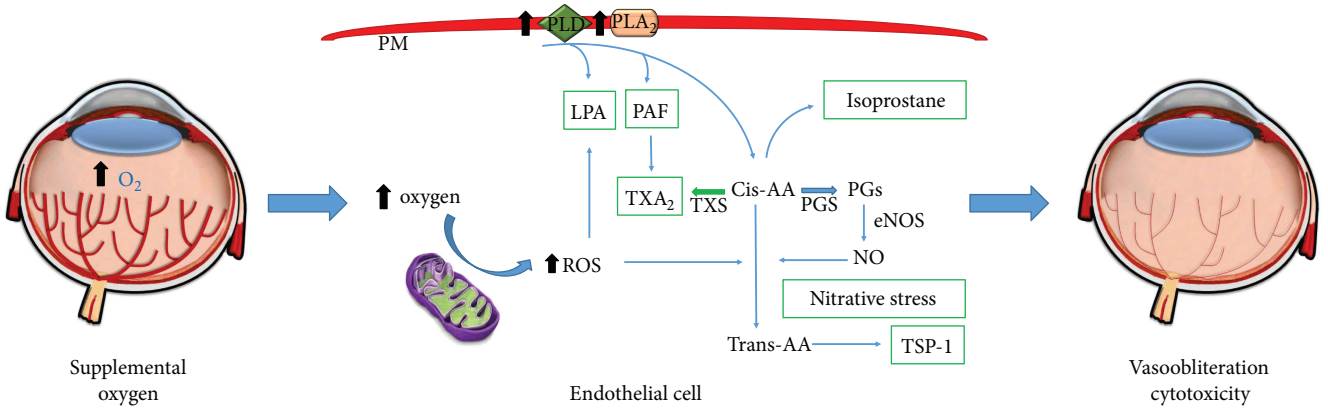

Figure 1: The effects of oxidant stress on premature retinal vasculature. The premature retina is relatively deficient in antioxidants. Consequently, oxidant stress is more likely to induce peroxidation and nitration that is cytotoxic to retinal microvasculature. Downstream mediators of peroxidation, notably the phospholipids PAF and LPA, the nonenzymatically derived prostanoids, isoprostanes, and nitration products such as *trans*-arachidonic acids (TAAs), are all cytotoxic to retinovascular endothelium, causing vasoobliteration. PLD: phospholipase D; PLA_2: phospholipase A2; PM: plasma membrane; PGS: PG synthase; TXS: thromboxane synthase; TSP-1: thrombospondin-1.

cells. Vitamin E normally occurs in its highest concentration in the retina; however, premature infants are born with approximately 10% of adult levels [30]. A few clinical trials have showed a benefit of vitamin E supplementation on the incidence and severity of ROP [31, 32], but these effects have mostly been marginal [33]. In addition, adverse effects from vitamin E supplementation have been reported, resulting in an increased risk of life-threatening infections and bleeding in the brain when injected intravenously [34]. Hence, prophylactic supplementation with vitamin E remains controversial and is currently not regularly utilized.

Vitamin C is another important aqueous phase antioxidant in cells and plasma [35]. Vitamin C has a number of important metabolic functions and is actively transported across the placenta [36]. Vitamin C concentrations in cord plasma are higher than the mother's and, in term infants, plasma concentrations fall considerably over the first 24 hours of life [37]. Preterm infants generally have higher cord vitamin C concentrations than term infants, and concentrations then decline over a few days [38]. Most preterm infants receive vitamin C as part of a multivitamin supplement, but there are few data on which to base optimum concentrations [39]. In fact, the relation between vitamin C concentration and morbidity in very preterm infants remains controversial. Silvers et al. [40] reported that high plasma vitamin C concentrations were associated with a low antioxidant status and poor outcome in premature infants, as well as, a greater risk of developing bronchopulmonary dysplasia [41]. In contrast, Moison et al. [42] reported lower plasma vitamin C concentrations in preterm infants who developed bronchopulmonary dysplasia compared with those who did not. In a pilot observational study in very low birth weight infants, an increased risk of ROP with higher plasma vitamin C concentrations at day 7 and an increased risk of bronchopulmonary dysplasia with lower concentrations at 28 days were found [43]. Darlow et al. [44] thus hypothesized that maintaining a lower plasma vitamin C concentration in the first week of life and a higher concentration in weeks 3-4 would be accompanied by improved clinical outcome and least morbidity (chronic lung disease and ROP) in very low birth weight infants.

2.3. Inflammation and ROP. The role of inflammation in ROP has been poorly investigated. Recent evidences suggested that prenatal, perinatal, and postnatal inflammation might contribute to a gradual increase in the risk for ROP [7]. Clinical studies found that inflammatory stimuli such as bacteria in the placenta [45] and late bacteremia [46] were risk factors for developing ROP. Moreover, systemic inflammation in animal models in neonates has been shown to perturb retinal vessel development and to induce pathological features of ROP [47, 48]. Furthermore, recent studies by using genetically modified mice with a deficiency in tetrahydrobiopterin (BH4), an essential cofactor implicated in multiple metabolic process, showed that BH4 plays an essential role in maintaining the inflammatory and neurovascular retinal homeostasis [49] and is involved in the development of retinopathy [50].

Cytokines and chemokines are small proteins secreted by immune cells that play a central role in distinct inflammatory processes including the progression of ROP [51]. Cytokines such as IL-1β, TNFα, and IL-6 act as primary initiators of inflammation following infection or tissue damage [52]. Interestingly, IL-1β and TNFα produced by retinal microglia cells following exposure to hypoxia have been associated with deleterious effects in the retina [53]. In OIR model, IL-1β has been associated with retinal microvascular degeneration by inducing semaphorin3A in neurons [54], while in the choroid IL-1β causes direct cytotoxicity to choroidal blood vessels, which results in a hypoxic subretina and consequently loss of retinal pigment epithelium (RPE) and photoreceptor integrity [55].

IL-10 is generally considered an anti-inflammatory cytokine [56], capable of protecting the developing retina

against ongoing inflammation. Although a study showed that IL-10 can be implicated in promoting pathological angiogenesis in an OIR mice model [57], in another study, IL-10 was able to inhibit the expression of proinflammatory cytokines on microglial cells [58]. Furthermore, in pregnant rats exposed to systemic inflammation, IL-10 treatment reduced the occurrence of brain damage in their newborn pups [59]. Infants with an IL-10 high-producer allele showed a trend (albeit not significant) towards a lower prevalence of severe ROP [60].

On the other hand, chemokines which induce chemotaxis and regulate movements of immune cells such as microglia to sites of inflammation are of special interest for pathophysiology of ROP. For instance, the chemokine interleukin-8 (IL-8) is implicated in both inflammation and pathological neovascularization in the eye [61]. In humans, higher serum concentration of IL-8 right after birth was associated with later ROP [62]; concordantly in rats, increased levels of an IL-8 homologue were detected during the peak of pathological neovascularization in a model of ROP [63].

An important player in innate immunity called RANTES is suggestive of participating in the development of ROP. Although the role of RANTES in ROP is not known, low concentrations of RANTES have been detected in the vitreous humor of patients with vasoproliferative ROP [64]; low serum levels have also been detected in infants who later developed severe ROP [65, 66]. Further investigations are needed to establish a more convincing role for RANTES in ROP.

MCP-1 is an attracting factor in a variety of immune cells and is expressed in a wide range of tissues including activated microglia in the neuroretina [67]. Preterm infants who later developed ROP tended to have higher cord serum concentrations of MCP-1 than healthy preterm infants [68]. Elevated levels of MCP-1 in the vitreous humor of patients with retinopathy have been documented [69, 70]. In animal models, MCP-1 was associated with retinal neovascularization, possibly by attracting macrophages/microglia during the ischemic phase of retinopathy [71, 72].

2.3.1. Anti-Inflammatory Agents in ROP. Few pharmacological interventions using anti-inflammatory drugs have been tried in ROP. Ketorolac is a nonsteroid anti-inflammatory drug (NSAID) derived from indomethacin that inhibits the synthesis of prostaglandins by competitively blocking the activity of COX 1 and COX 2 [73]. Upon topical application, ketorolac diminishes prostaglandin E_2 concentration in aqueous humor [74]. A recent preliminary report suggests that ketorolac in the form of an ophthalmic solution can reduce the risk of developing severe ROP in very preterm newborns, without producing significant adverse side effects [75]. Interestingly, the authors showed that the incidence of severe ROP was significantly lower in very preterm newborns treated with ketorolac, when compared to the controls not receiving such treatment. These results suggest that administration of ketorolac as an ophthalmic solution might be an effective preventive strategy in patient at risk of developing severe ROP.

Dexamethasone, a steroidal anti-inflammatory agent, has also shown to reduce the incidence of ROP [76]. However, its use is associated with significant side effects [77, 78], and its efficacy in preventing ROP is controversial. A prospective randomized, controlled multicenter clinical trial to investigate the benefits of COX inhibitors for prevention of ROP is proposed (NCT02344225).

An anti-IL-1 treatment using a proprietary drug candidate labelled 101.10 (amino acid sequence: rytvela) has shown promising results in animal models. Rivera et al. demonstrated pronounced beneficial effects of 101.10 in a rodent model of ROP [54]. Interestingly, 101.10 markedly reduced retinal inflammation and preserved retinovascular architecture in the animals exposed to the OIR model. However, human clinical trials are still needed to investigate the benefits of 101.10 in preventing ROP progression.

2.3.2. Promising Therapeutic Treatments in ROP. The Caffeine for Apnea of Prematurity (CAP) trial found that caffeine was beneficial in reducing the incidence of severe ROP [79]. Mechanisms implicated in caffeine actions in reducing ROP are still unknown, yet effects on sonic hedgehog [80], matrix metalloproteinases (MMPs) [81], and oxidative stress [82] could be involved. Up to now, a prospective randomized, controlled multicenter clinical trial to investigate the benefits of caffeine on ROP is ongoing (NCT02344225).

Beneficial properties of omega-3 supplementation in ROP have been suggested. Connor et al. [83] showed that dietary omega-3 fatty acids protect against pathologic neovascularization by increasing the formation of cytoprotective and anti-inflammatory metabolites. A meta-analysis showed that long-chain polyunsaturated fatty acid supplementation of infant formulas improves infants' visual acuity up to 12 months of age [84]. The impact of omega-3-PUFA supplementation specifically on human ROP has started to be addressed; omega-3-containing fish oil emulsion supplementation to premature infants has been shown to be associated with reduced risk for ROP [85]. At present, a trial examining the effects of omega-3 PUFA supplementation to very low birth weight infants on prevention of ROP is ongoing at the University of California (NCT02486042).

3. Diabetic Retinopathy (DR)

DR is a leading cause of vision loss in working adult population and one of the most common complications of diabetes mellitus [1, 86]. It is estimated that the prevalence of diabetes mellitus in adults (aged 20–79 years) will continue to rise in the following years [87]. Therefore, as a result of urbanization and aging population, the number of DR patients is projected to increase from 37.3 million to 56.3 million by 2030 [88].

3.1. Pathogenesis of DR. DR is a progressive disease that develops in stages, from mild nonproliferative DR to moderate severe nonproliferative DR and finally to the ultimate stage of proliferative DR, which is characterized by the growth of abnormal leaky retinal blood vessels and consequently to the detachment of the retina [89]. Throughout the different stages, patients with DR may develop diabetic

macular edema (DME) which is due to the breakdown of the blood-retinal barrier (BRB) leading to a vascular leakage of fluid and plasma components at the retina [90]. The inner BRB consists of a single layer of tightly connected endothelial cells which is supported by pericytes. A preserved BRB plays a key role in supporting and maintaining the integrity of the retina and prevents the retinal vessels from leaking [91, 92]. Different factors have been shown to contribute to the BRB breakdown (Figure 2). During diabetes, high glucose levels cause an impairment of the tight junctions which become loosened, and endothelial cells and pericytes undergo apoptosis and thus allow an outward flow of plasma components, including lipid and proteins, into the vitreous. BRB leakage results in the swelling of the macula associated with improper perfusion and development of areas of retinal ischemia [92, 93]. In addition, circulating leukocytes, which become less deformable, adhere with the activated endothelial and participate in capillary occlusion and ischemia [94].

Several studies have shown that vascular endothelial growth factor (VEGF) has a primordial role in the BRB breakdown. VEGF levels are increased in patients with proliferative DR and contribute to retinal vascular permeability [95]. Moreover, VEGF upregulation occurs even before the onset of hypoxia. Indeed, it has been shown that VEGF levels are increased at the early stages of DR, and this could be a consequence of an inflammatory environment characterized by the release of proinflammatory cytokines (i.e., IL-1β and IL-6) and the formation of advanced glycosylation end products (AGEs) [96, 97]. In response to hypoxia and inflammation, VEGF in association with angiopoietin 2 (Ang2) plays a key role in neovascularization and affects the integrity of preexisting vasculature [98]. VEGF antagonists attenuate vascular leakage in DR [99].

Pericyte recruitment to the microvessel wall is primordial for the formation of BRB, and this recruitment is controlled by the platelet-derived growth factor (PDGF) B and PDGF receptor β (PDGFRβ) [100]. Human and animal studies have shown that the loss of pericytes in diabetes is triggered by the activation of nuclear factor kappa B (NF-κB) with an increase in Bax expression causing pericyte apoptosis [101]. Another study has shown that high levels of glucose activated protein kinase Cδ (PKCδ) and p38α mitogen-activated protein kinases (MAPK) which increase the expression of Src homology 2 domain-containing phosphatase (SHP-1), a protein tyrosine phosphatase, resulting in the dephosphorylation of PDGFRβ to induce pericyte apoptosis and acellular capillaries in an NF-κB-independent pathway [102]. Pericyte loss is one of the main characteristics of DR with the formation of microaneurysms and acellular capillaries [103].

A new hypothesis suggests that photoreceptors in the outer retina might also play an important role in the development of diabetic retinopathy [104]. Du and collaborators have proposed that photoreceptors contribute to diabetes-induced degeneration of retinal capillaries [105]. Accordingly, diabetes causes oxidative stress in photoreceptors in part through alteration in ion flux. These abnormalities might affect intermediate cells such as Müller cells and leukocytes which result in characteristic pathologic alteration to the retinal vasculature including increased permeability and nonperfusion [104]. In support to this hypothesis, it was demonstrated that DR was less severe in a group of patients with retinitis pigmentosa [106] and in mice lacking photoreceptors [107].

3.2. Oxidative Stress and DR. The retina is rich in polyunsaturated fatty acids and characterized by a high-energy demand and an exposure to light; together, these conditions favor oxidative stress. Oxidative stress is involved in the pathogenesis of DR, and high levels of ROS have been found in patients with DR [108].

Mitochondria are the major source of ROS or reactive nitrogen species (e.g., superoxide and peroxynitrite). In presence of high glucose, oxidation of carbohydrates leads to an impairment of the electron transport chain and results in the accumulation of electrons at coenzyme Q leading to the generation of superoxide anion from oxygen, which in turn generates other ROS [109]. ROS play a role in the production of cytokines to promote inflammation and facilitate the recruitment of neutrophils to the site of inflammation [110].

ROS production induces major mitochondrial DNA damages which result in defects in transcription of electron transport chain subunits and further exacerbate ROS production [111]. In addition, ROS induce mitochondrial lipid membrane deterioration which leads to the release of cytochrome C and Bax translocation to the mitochondria. These manifestations drive apoptosis in pericytes and endothelial cells in diabetes [112]. Other sources of ROS generation are NAD(P)H oxidase (NOX), cytochrome p450, and nitric oxide synthase [113]. Superoxide anion can also be generated by the uncoupled nitric oxide synthase, and by reacting with NO generates peroxynitrite, which contributes to IR [114].

One of the metabolic manifestations of hyperglycemia that enhance oxidative stress is the polyol pathway, which corresponds to the conversion of glucose to sorbitol by aldose reductase. The aldose pathway has been suggested to contribute in the pericyte loss [115]. The accumulation of AGEs also contributes to retinal damages in DR. AGEs bind with their receptors (RAGEs) in endothelial cells, pericytes, and RPE to induce NADPH-mediated oxidative stress, which in turn induces NF-κB activation and cytokine formation [116].

The hexosamine pathway is another pathway that mediates the high glucose-driven oxidative stress and ensued complications observed in DR. Fructose 6-phosphate is deviated from the glycolytic pathway to be converted to glucosamine 6-phosphate and then to uridine diphosphate *N*-acetylglucosamine (UDPGlcNAc). UDPGlcNAc attaches to Ser/Thr residues leading to posttranslational modifications of proteins. The hexosamine pathway leads to the activation of plasminogen activator inhibitor-1 (PAI-1) which participates in the pathogenesis of diabetic complications [117, 118].

3.2.1. Antioxidants in DR. Given the role for oxidative stress in the genesis of DR, potential effective interventions can be achieved. Under normal conditions, endogenous antioxidant systems such as superoxide dismutase (SOD), catalase, thioredoxin reductase, glutathione reductase, glutathione peroxidase, GSH, thioredoxin, and tocopherol (vitamin E) ensure the clearance and detoxification

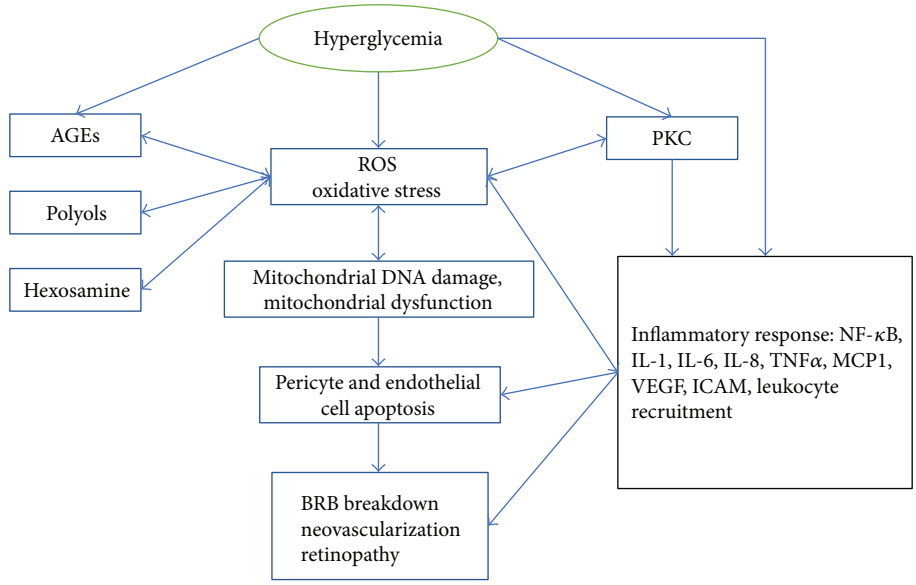

Figure 2: The role of oxidative stress and inflammation in diabetic retinopathy. Hyperglycemia activates PKC, AGEs, polyol, and hexosamine pathways which contribute to oxidative stress and mitochondrial dysfunction leading to pericyte and endothelial cell apoptosis. Upregulation of inflammatory mediators results in cell death and BRB breakdown.

of ROS and free radicals and prevent their accumulation. In diabetic rats, these antioxidant molecules have been found to be diminished in the retina [110]. Conversely, overexpression of mitochondrial SOD can be protective against oxidative stress in the retina under diabetes conditions [119].

A number of antioxidant compounds have been shown to be protective in diabetic retinopathy animal models; however, some clinical studies have failed to demonstrate the efficacy of these antioxidants in DR [120–122]. Vitamins C and E supplementation may protect against progression of DR [123, 124]. Vitamins C and E enhance the enzymatic activities of glutathione reductase, glutathione peroxidase, and SOD and decrease pericyte dropout in diabetic rats [125]. Lipoic acid, an antioxidant which can scavenge ROS, was found to have beneficial effects in the development of DR by inhibiting capillary cell apoptosis and inhibiting oxidative damage in the retina of diabetic rats [126]. Nicanartine is a lipid-lowering compound which has antioxidant properties and was found to be effective in inhibiting pericyte loss in diabetic rats although it fails to prevent the increase in acellular capillary formation [127].

Polyphenols contained in green tea have potent antioxidant properties. Green tea supplementation increases retinal GSH levels and the enzymatic activities of catalase and SOD and decreased acellular capillaries in diabetic rats [125, 128]. Benfotiamine, a thiamine derivate (vitamin B1), has been shown to inhibit three ROS production pathways (hexosamines, PKC, and AGEs pathways) implicated in DR pathogenesis, as well as NF-κB activation, and to prevent the increase in acellular capillaries in the retina of diabetic rats [129]. Some lipid-lowering drugs such as fenofibrate have been shown to be beneficial in preventing the progression of DR, given that dyslipidemia and high circulating fatty acids are associated with increased oxidative stress in the retina. Two placebo-controlled randomized trials, the Fenofibrate Intervention and Event Lowering in Diabetes and Early Treatment Diabetic Retinopathy Study (FIELD) and Action to Control Cardiovascular Risk in Diabetes (ACCORD) eye studies have shown that fenofibrate retards the progression of DR in adult patients with type 2 diabetes [130, 131]. The mechanism by which fenofibrate exerts its protective effect remains to be determined and may involve oxidative stress, apoptosis, inflammation, and BRB preservation [132], possibly by inducing anti-inflammatory effects via sirtuin 1-dependent signaling pathway inhibition of NF-κB in human retinal endothelial cells [133].

3.3. Inflammation and DR. Inflammation has been also suggested to contribute to the development and progression of DR. Activation of Toll-like receptors by pathogen-associated molecular patterns (PAMPs) leads to the release and the nuclear translocation of NF-κB which triggers the transcription of several cytokines and chemokines such as TNFα, IL-1, IL-6, and MCP-1. In diabetes, there is an increase in the activity of TLR2 and TLR4 which participate in microvascular complications [134]. High concentrations of glucose induce the expression of TLR2 and 4 via PKCα and PKCδ, and a knockdown of both TLR2 and TLR4 reduces high glucose-induced NF-κB activation [135]. Some studies have reported that increased plasma levels of free fatty acids (FFAs) can activate TLR2 and TLR4 [136, 137]. Monocytes have been shown to respond to the presence of FFAs by increasing TLR2 and TLR4 expression resulting in increased NF-κB activation [138].

Activated microglial cells are considered to be a major source of proinflammatory cytokines in damaged tissues. In diabetic rats, an increase in the number of activated microglia

in the retina was associated with increased production of inflammatory cytokines, ROS and MMPs, and a concomitant loss of neuronal cells in ganglion cell layer and inner nuclear layer [139]. Likewise, several studies have shown that DR is accompanied with an augmentation of inflammatory mediators including ICAM1, VEGF, IL-6, IL-8, and MCP-1 and angiogenic factors such as angiotensin II, angiopoietin-1, angiopoietin-2, and erythropoietin (reviewed in [140]). For instance, high levels of TNFα have been detected in the vitreous humor and serum from DR patients [141–143]. It has been shown that TNFα directly contributes to BRB breakdown in DR by activating PKC-ζ/NF-κB pathway which reduces the expression of tight junction proteins claudin-5 and ZO-1 and increases endothelial cell permeability [144]. Blocking TNFα actions by using the specific TNFα inhibitor etanercept [145] disrupts NF-κB activation and inhibits BRB dysfunction.

Other than microglia, Müller cells can also contribute to the inflammatory response in DR, as these cells produce a variety of inflammatory factors [146–148]. Besides, several lines of evidence have shown that the adherence of leukocytes contributes directly to the death of endothelial cells via Fas-FasL-mediated mechanism [149]. The adhesion of leukocytes to the diabetic endothelium is mediated by the intracellular adhesion molecule-1 (ICAM-1) and CD18. Neutralization of ICAM-1 and CD18 with specific antibodies attenuates leukocyte adhesion and prevents retinal endothelial cell injury [150].

3.3.1. Anti-Inflammatory Agents in DR. Despite glycemic control, blood pressure control, and lipid-lowering therapy in diabetic patients, the prevalence of DR is increasing and therapeutic approaches are limited. The development of new anti-inflammatory strategies to prevent and treat DR is being proposed. Etanercept is an FDA-approved recombinant fusion protein for the treatment of psoriasis because of its anti-TNFα properties [151]. Acting as a competitive inhibitor of TNFα, etanercept reduces leukocyte adhesion, suppresses BRB breakdown, and decreases their activation [145, 152]. Although other TNFα inhibitors such as pegsunercept have displayed efficacy in animal models, by reducing pericyte dropout and capillary degeneration [153, 154], etanercept failed to demonstrate efficacy in patients with diabetic macular edema refractory to anti-VEGF therapy [155].

Resveratrol (3,5,4$'$-trihydroxystilbene), a naturally occurring polyphenol found in grapes and red wine, is known for its antioxidant and anti-inflammatory properties. Oral resveratrol administration (5 mg/kg) has been shown to improve glucose tolerance, decrease NF-κB activation, and lower TNFα levels in preclinical diabetic models [156]. Resveratrol also exerts its neuroprotective effects on retinal ganglion cells following intravitreal injection, improving their survival by activating sirtuin 1 [157]. Its effects have not been tested in humans with diabetes.

IL-1β [141, 158, 159] and the enzyme caspase-1 generating IL-1β are considered important targets to prevent DR [160]. Accordingly, inhibition of caspase-1 using minocycline or by blocking IL-1R1 receptor prevented diabetes-induced increase in IL-1β and degeneration of retinal capillaries [158]; interestingly, similar effects were seen with exogenous antioxidants [159, 161].

4. Age-Related Macular Degeneration (AMD)

Age-related macular degeneration (AMD) is the most common cause of vision loss in the elderly population and accounts for 8.7% of all blindness worldwide [162]. Its prevalence is increasing as a consequence of an exponential aging in the population. According to a recent systematic review and meta-analysis study [162], the projected number of people with AMD in 2020 is estimated at 196 million and approximately 288 million by 2040.

4.1. Pathogenesis of AMD. Two types of AMD are clinically recognized: dry AMD which is characterized by the formation of extracellular deposits called drusen, followed by RPE and photoreceptor death, and geographic atrophy (GA) and wet AMD which is characterized by choroidal neovascularization [163, 164]. Both forms of AMD result in loss of central vision. To date, laser photocoagulation and anti-VEGF therapy are the most common treatments for wet AMD [165]. However, mechanisms and, therefore, treatments for dry AMD remain largely elusive.

AMD is a multifactorial disorder wherein a complex interplay of genetic and environmental factors contributes to its pathogenesis (Figure 3). Multiple genes involved in lipid metabolism, complement pathway, and extracellular matrix remodeling have been found to be associated with AMD progression [6]. Furthermore, RPE senescence [166], oxidative stress [9], and immune dysfunction [167] are also involved. In the following sections, we will focus our discussion on the role of oxidative stress and inflammation in AMD.

4.2. Oxidative Stress and AMD. The outer retina, composed by RPE and photoreceptors, is constantly exposed to an oxidative environment, on one hand arising from the high oxygen delivery from the choroid and, on the other hand, due to constant photic bombardment. Photoreceptors are the main source of ROS in the outer retina due to light exposure and their high metabolic rate-associated oxygen consumption [168]. Outer segments of the photoreceptors which are rich in polyunsaturated fatty acid (PUFA) are sensitive to autooxidation and prone to oxidative stress. To maintain the balance in the proportion of oxidant species, photoreceptors have efficient antioxidant defense mechanisms such as SOD1 and SOD2 which transform superoxide (O_2^-) to hydrogen peroxide (H_2O_2) and superoxide (O_2^-), and glutathione peroxidase (GPx), and/or glutathione reductase (GR) and catalase which convert O_2^- into H_2O and O_2 [9]. In addition to its enzymatic defenses, photoreceptors count on its interlink with the multifunctional RPE cells. RPE cells ensure the correct functioning of the outer retina, for instance, by maintaining the structure of the external blood-retinal barrier, secreting growth factors, absorbing excess of light, participating in the photoreceptor outer segment phagocytosis, and cycling of retinoids. RPE daily phagocytes the oxidized

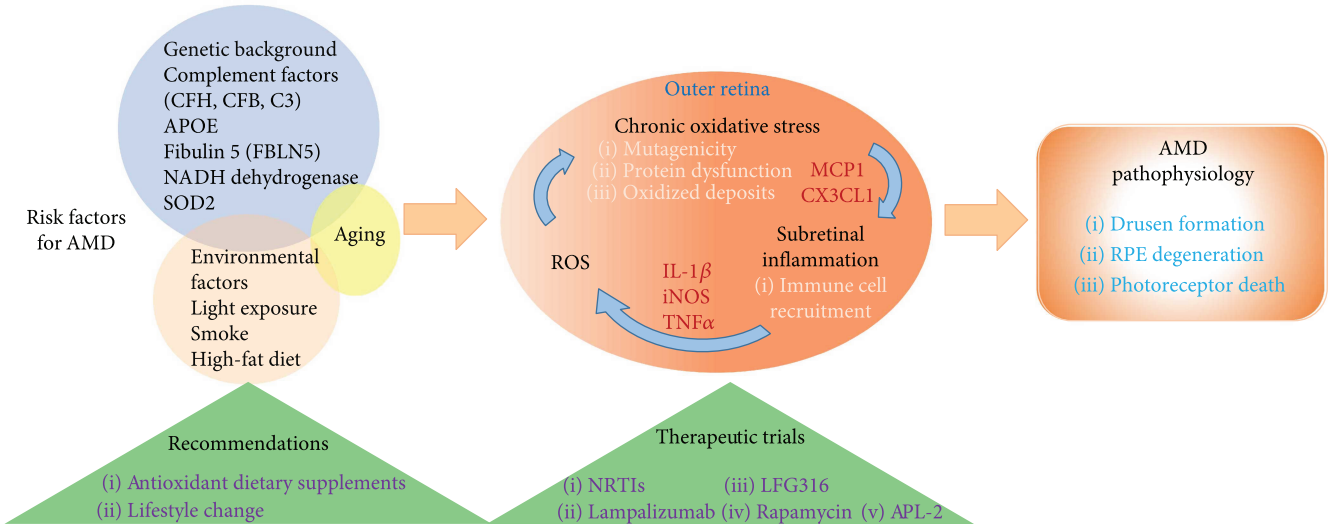

Figure 3: Scheme summarizing the risk factors of AMD, the link between oxidative stress and inflammatory factors involved in the pathogenesis of dry AMD and the current antioxidant/anti-inflammatory therapeutic trials and recommendations. CFH: complement factor H; CFB: complement factor B; C3: complement 3; APOE: apolipoprotein E; NADH: nicotinamide adenine dinucleotide H; SOD2: superoxide dismutase 2; ROS: reactive oxygen species; MCP1: monocyte chemotactic protein 1; CX3CL1: C-X3-C motif chemokine ligand 1; IL-1β: interleukine 1 beta; iNOS: inducible nitric oxide synthase; TNFα: tumor necrosis factor alpha; NRTI: nucleoside reverse transcriptase inhibitor; RPE: retinal pigment epithelium.

membranes of outer segments, contributing in this way, to decrease the oxidative stress in photoreceptors. However, the degradation products of these phagocytized outer segments generate lipofuscin in RPE cells. Due to its molecular composition, lipofuscin is photoreactive and thus increases the susceptibility of RPE to light damage with aging. In addition, phagocytosis process may also generate high level of H_2O_2 through NADPH oxidase activity and peroxisomal-oxidation, which exposes the RPE to the risk of oxidative stress [169, 170]. Interestingly, photoreceptors and RPE cells can modulate oxidative damage induced by oxidized cellular components with an autophagy process involving activation of p62/Nrf2 pathway [171, 172].

Many factors such as aging, environmental stress, smoke cigarette, and genetic factors are involved in the development of AMD (Figure 3). These elements may also contribute to increase the production of prooxidants and reduce antioxidant defenses. For instance, cigarette smoke contains high level of ROS [173], which has demonstrated to reduce the levels of endogenous antioxidants, such as glutathione, cysteine [174], and SOD [175].

Mutations of genes encoding proteins involved in the mitochondrial respiratory chain (NADH complex, cytochrome complex) have also been shown to lead to pathological oxidative stress increasing the risk for AMD [176]. A similar paradigm also applies to complement factor H defense protein (CFH) [177], and for polymorphisms of NADH dehydrogenase and SOD2 [178].

4.2.1. Antioxidant Agents in AMD. A number of antioxidants have been suggested to confer protection of RPE and photoreceptors. This is the case for carotenoids (lutein, zeaxanthin, and mesozeazanthin) which act as scavengers of ROS [179, 180], alpha-lipoic acid which enhances GPx activity [181], curcumin which augments the expression of the cytoprotective and antioxidant enzyme heme oxygenase-1 in RPE cells, and caffeic acid phenethyl ester (CAPE) which confers photoreceptor protection against H_2O_2-mediated cell death by increasing the expression of heme oxygenase-1 and supressing NF-κB activation [182, 183]. However, despite the promising effects of these antioxidant molecules in animal models, two large multicenter investigations, the Age-Related Eye Disease Study (AREDS and AREDS2), failed to show convincing efficacy of these types of supplementation for AMD [184, 185].

4.3. Inflammation and AMD. RPE cells exposed to oxidative stress can elicit inflammation [186, 187]. The accumulation of lipids, particularly in the form of drusen, is associated with a chronic inflammation. RPE cell function is crucial for retinal homeostasis, and loss of its integrity and/or function increases the risk of progression of AMD. When oxidative stress exceeds the antioxidant defense capacities, RPE cells release inflammatory chemokines such as MCP-1 and fractalkine (CX3CL1) playing a key role in microglia recruitment in subretinal space [188]. Physiologically, microglial cells are not present in the outer retina; however, their migration increases upon damage to the photoreceptors and/or RPE cells and also when insoluble debris from phagocytosis are accumulated in RPE cells and subretinal space during aging. These oxidized deposits containing advanced glycation end products (AGEs) and lipoxidation end products (ALEs) are recognized and cleared by macrophages [189, 190]. When oxidative stress is sustained, monocytes are recruited from blood circulation. These activated immune cells present in the subretinal space release IL-1β, iNOS, and TNFα, which in turn induce ROS production in RPE through NADPH oxidase activation and increase oxidative damages [191, 192].

Even more, proinflammatory cytokines secreted by infiltrating lymphocytes or macrophages exert senescence and dysfunction to RPE [193, 194]. RPE senescent cells in turn may secrete a range of pleiotropic factors that recruit inflammatory cells and exacerbate inflammation and damage to the outer retina [195]. Accordingly, accumulation of microglia in the outer retina has been associated with RPE and photoreceptor damage in AMD [196, 197]. All these evidences highlight the interwoven relationship between oxidative stress and inflammation in the development of AMD.

4.3.1. Anti-Inflammatory Agents in AMD. Robust evidence suggests that modulation of inflammation could attenuate AMD progression. Rapamycin (sirolimus), an mTOR inhibitor used for its immunosuppressive effect in organ transplantation, has been proposed for AMD therapy. Sirolimus has been shown to preserve RPE and photoreceptors from cell death in a mouse model of retinal degeneration [198]. However, in a phase II clinical trial, the subconjunctival or intravitreal injections of sirolimus every 3 months for 24 months did not reduce geographic atrophy progression in the patients with AMD [199, 200].

Targeting the complement system provides another strategy to tackle inflammation and reduce AMD progression [201]. Encouraging results were observed with the humanized anti-factor D monoclonal antibody complement inhibitor, lampalizumab (Roche Inc.). These results from phase II clinical trial showed a 24% reduction in geographic atrophy after monthly intravitreal injections for 18 months [202]. Along the same lines, the C3 inhibitor APL-2 (Apellis Pharmaceuticals) and the anti-C5 monoclonal antibody LFG316 (Alcon) are currently in phase II clinical trial [203].

In the perspective of targeting inflammation to treat AMD, NLRP3-inflammasome, a key component of the innate immunity, has shown to play an important role in the development of AMD [204]. A number of studies have shown a strong association in the activation of the NLRP3-inflammasome and the development of geographic atrophy in patients with AMD [205, 206]. Interestingly, nucleoside reverse transcriptase inhibitors (NRTIs), administered for human immunodeficiency virus (HIV) patients, have proven to block P2X7-dependent NLRP3 inflammasome activation [207]; currently, clinical trials are under preparation using NRTIs for AMD patients.

5. Conclusion

Oxidative stress and inflammation play an important role in the development of IRs. A better understanding of the mechanisms implicated in early stages should identify new targets that allow the development of new therapeutic approaches. Along these lines, a more profound elucidation of the complex interplay of oxidative stress and inflammatory mediators is required. Although, several epidemiological and animal studies have revealed beneficial effects of antioxidants, results from clinical trials have been at best tepid, possibly because of the complexity in targeting oxidants and more importantly the absence of strategies to deal with biologically active stable product peroxidation such as isoprostanes, neuroprostanes, and isofurans [208]. Specific anti-inflammatory approaches may turn out to be more promising.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this article.

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