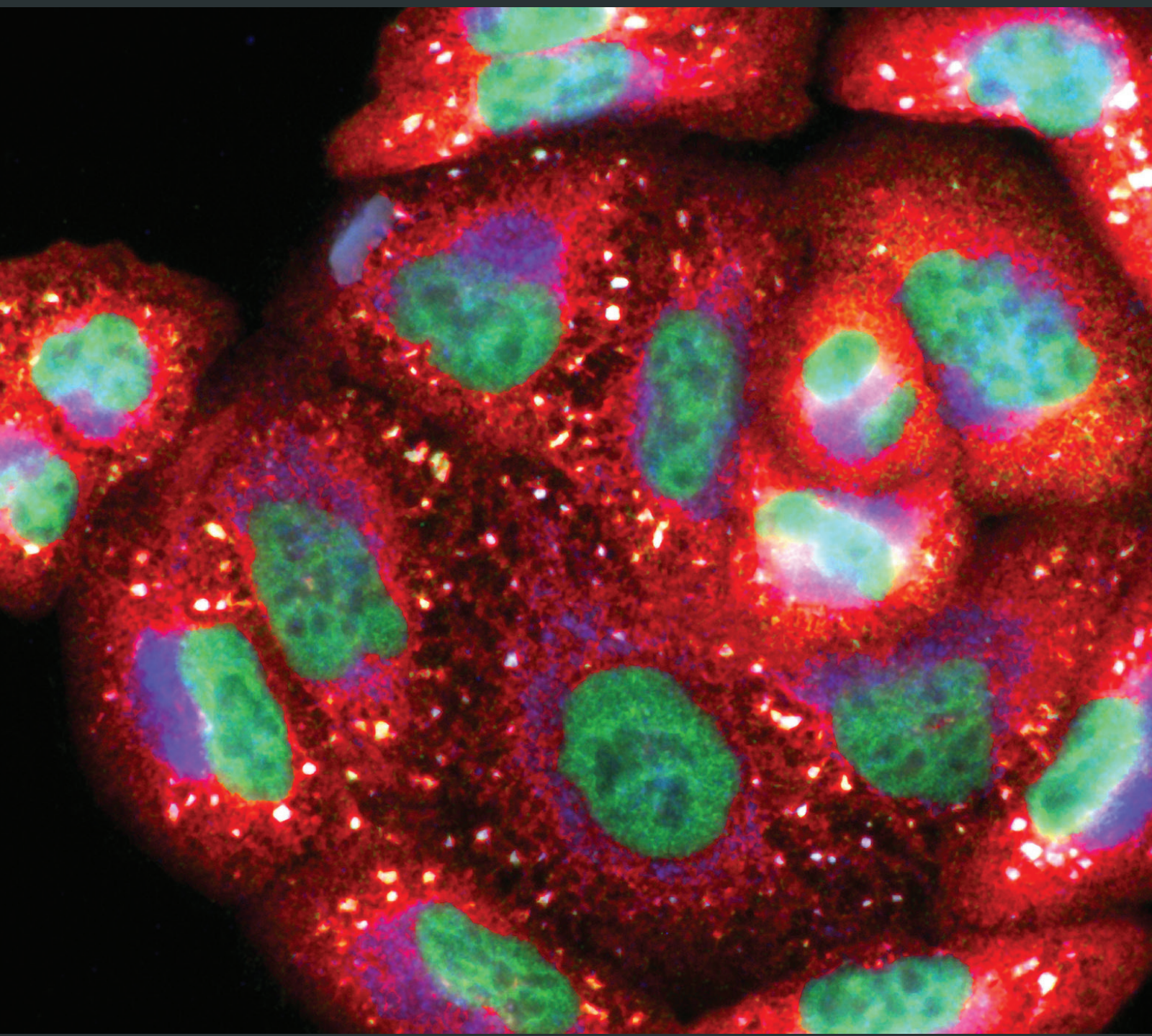
Oxidative Medicine and Cellular Longevity

Nitric Oxide: A Regulator of Cellular Function in Health and Disease

Guest Editors: Joern R. Steinert, Lezanne Ooi, Luis Sobrevia, and Scott Ryan



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Editorial Board

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Hindawi Publishing Corporation
Oxidative Medicine and Cellular Longevity
Volume 2016, Article ID 9782346, 2 pages
http://dx.doi.org/10.1155/2016/9782346

Editorial

Nitric Oxide: A Regulator of Cellular Function in Health and Disease

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Received 4 October 2015; Accepted 4 October 2015

Nitric oxide (NO) is a gaseous messenger molecule synthesized from L-arginine and molecular oxygen by three different NO synthases, that is, neuronal (nNOS), endothelial (eNOS), and inducible (iNOS) form [1]. Since its discovery in the early 1980s by the three Nobel Laureates Furchgott, Ignarro & Murad [2], NO has been widely recognised as an important signalling molecule in many physiological processes. The initial identification of NO as an endothelium-derived relaxing factor (EDRF) [3] generated great interest in its function in vascular biology. Over the following years, however, the focus on NO research rapidly expanded from the vascular system to its role in immunity and inflammation, the nervous system, pregnancy, aging, and cell death.

Many studies suggest that excessive or abnormal production of NO plays a crucial role in neuronal cell death associated with neurodegenerative disorders such as Alzheimer's and Parkinson's diseases, as well as various conditions of vascular dysfunction. At physiological levels, NO is essential for neuronal function, differentiation and survival through activation of signalling pathways that include the cyclic guanosine monophosphate (cGMP)/soluble guanylyl cyclase pathway [4] and S-nitrosylation, in which NO reversibly binds to thiol groups of proteins [5]. The vast network of NO-associated signalling paradigms further includes acetylation/deacetylation and methylation/demethylation modifications and peroxynitrite formation (leading to nitrotyrosination of protein residues), as well as modulation of gene expression via epigenetic changes [5–8]. How controlled S-nitrosylation/nitrotyrosination of proteins and activation of the NO/cGMP signalling pathway promote cellular survival and induce epigenetic changes while uncontrolled signalling promotes cell death and dysfunction remains to be elucidated. The aim of this Special Issue is to gather information encapsulating the above signalling pathways.

The articles published in this Special Issue largely cover (1) NO signalling in neuronal function and disease as well as (2) vascular targets in endothelial function and dysfunction, both of which involve the broad range of actions of this signalling molecule. In order to understand the contribution of NO to neuronal dysfunction, one has to consider that NO is a crucial molecule in cellular physiology. Numerous studies show the involvement of NO in neuronal development, plasticity, excitability, and transmission [8–12]. However, the common mechanisms across several neurodegenerative disorders relate to the neurotoxicity of NO and its downstream reactive nitrogen species (RNS). Enhanced nitrotyrosine

immunoreactivity is evident in brains from patients with Alzheimer's and Parkinson's disease. Nitrated proteins are associated with β-amyloid deposition and nitrotyrosination of Tau protein and synaptophysin has been reported in brain samples from patients with Alzheimer's disease. The potential downstream signalling pathways of these posttranslational modifications are discussed in the review by S. A. Bradley and J. R. Steinert in this Issue. The cellular roles of NO in neurodegenerative disease, such as Alzheimer's, are reviewed by R. Balez and L. Ooi with a focus on neurotoxicity versus neuroprotection while the role of aberrant NO signalling in neurodevelopmental disorders such as Fragile X syndrome is investigated in the study by E. Lima-Cabello et al.

Other topics of this issue focus on aspects of vascular NO signalling with particular interest in fetoplacental dysfunction caused by abnormal eNOS regulation (as discussed by A. Leiva et al.). Endothelial function and eNOS regulation are essential for healthy cardiovascular responses but are also critical for adaptations during pregnancy. Several diseases associated with vascular dysfunction such as atherosclerosis, diabetes mellitus, hypertension, or preeclampsia involve altered NO signalling [13–15].

The paper by A. Leiva et al. reviews the mechanisms leading to abnormal NO signalling, which include reduced bioavailability of L-arginine (NOS substrate) and tetrahydrobiopterin (BH_4), abnormal calcium-calmodulin signalling, and activation and inhibition of eNOS activity through phosphorylation of Ser^{1177} or Thr^{495}, respectively [16]. NO has also been reported to play a crucial role in the transition of fetoplacental endothelial cells from a mitogenic to a metabolic phenotype in the macrocirculation, compared with a change from a metabolic to a mitogenic phenotype in the microcirculation in response to insulin in gestational diabetes mellitus [17]. Thus, the actions of NO are not only important during vascular adaptations to pregnancy and related dysfunctions but also essential during vascular signalling induced by physical training resulting in elevated nitrite and nitrate levels as reported in the study by A. M. Jacomini et al.

Together, this Special Issue highlights the tremendous diversity of NO signalling pathways with regard to its function in health and disease. All contributing publications have emphasised that NO represents an important signalling molecule and future research required in this field will extend the understanding of the broad actions of NO.

Luis Sobrevia
Lezanne Ooi
Scott Ryan
Joern R. Steinert

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Hindawi Publishing Corporation
Oxidative Medicine and Cellular Longevity
Volume 2016, Article ID 8548910, 12 pages
http://dx.doi.org/10.1155/2016/8548910

Research Article

An Abnormal Nitric Oxide Metabolism Contributes to Brain Oxidative Stress in the Mouse Model for the Fragile X Syndrome, a Possible Role in Intellectual Disability

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Received 11 June 2015; Accepted 15 September 2015

Academic Editor: Joern R. Steinert

Background. Fragile X syndrome is the most common genetic cause of mental disability. Although many research has been performed, the mechanism underlying the pathogenesis is unclear and needs further investigation. Oxidative stress played major roles in the syndrome. The aim was to investigate the nitric oxide metabolism, protein nitration level, the expression of NOS isoforms, and furthermore the activation of the nuclear factor NF-κB-p65 subunit in different brain areas on the fragile X mouse model. *Methods.* This study involved adult male Fmr1-knockout and wild-type mice as controls. We detected nitric oxide metabolism and the activation of the nuclear factor NF-κBp65 subunit, comparing the mRNA expression and protein content of the three NOS isoforms in different brain areas. *Results.* Fmr1-KO mice showed an abnormal nitric oxide metabolism and increased levels of protein tyrosine nitrosylation. Besides that, nuclear factor NF-κB-p65 and inducible nitric oxide synthase appeared significantly increased in the Fmr1-knockout mice. mRNA and protein levels of the neuronal nitric oxide synthase appeared significantly decreased in the knockout mice. However, the epithelial nitric oxide synthase isoform displayed no significant changes. *Conclusions.* These data suggest the potential involvement of an abnormal nitric oxide metabolism in the pathogenesis of the fragile X syndrome.

1. Introduction

Fragile X syndrome (FXS) is a rare genetic disorder, mostly characterized by moderate to severe mental retardation, autistic and hyperactive behaviour, macroorchidism, large ears, a prominent jaw, and high-pitched jocular speech [1]. Neuropathological features of the fragile X syndrome are long, thin, and sinuous dendritic spines, increased intracranial volume, enlarged ventricles, increased volumes of selective subcortical grey matter regions, decreased size of

the posterior cerebellar vermis, tonic-clonic seizures, and an altered brain glucose metabolism [2, 3]. It is caused by the lack of expression of the fragile X mental retardation protein (FMRP), an mRNA-binding protein encoded by the fragile X mental retardation 1 (FMR1) gene, which is believed to play a role in the regulation of local protein synthesis and possibly mRNA trafficking in the brain [4].

Nitric oxide (NO) is an important signalling molecule that is widely used in the nervous system. NO is synthesized by three different NO synthase (NOS) isoenzymes, all of which are present in the central nervous system (CNS). It is suggested that nitric oxide plays an important role regulating cellular adaptations, and controlling a range of processes in the body, including intracellular signalling, immune function, tissue turnover, expression of antioxidant enzymes, and cellular inflammation. Its involvement in learning, memory, behavioural processes, and cognition is clearly described [5].

With recognition of its roles in synaptic plasticity (long-term potentiation; long-term depression) and elucidation of calcium-dependent NMDAR-mediated activation of neuronal nitric oxide synthase (nNOS), numerous molecular and pharmacological tools have been used to explore the physiology and pathological consequences for nitrergic signalling. In addition, the inability to constrain NO diffusion suggests that spillover from endothelium (eNOS) and/or immune compartments (iNOS) into the nervous system provides potential pathological sources of NO, where control failure in these other systems could have broader neurological implications [6, 7]. However, high levels of NO production also lead, by reaction with reactive oxygen species (ROS), to the formation of peroxynitrite, a highly reactive species contributing to brain oxidative damage and protein nitration. Abnormal NO signalling could therefore contribute to a variety of neurodegenerative pathologies such as intellectual disabilities, stroke/excitotoxicity, Alzheimer's disease, multiple sclerosis, and Parkinson's disease. iNOS is primarily induced by ROS and cytokines through activation of the nuclear factor κB (NF-κB), a ubiquitous transcription factor that plays a key role in regulating immune and inflammatory responses. Under normal conditions, NF-κB is present in the cytoplasm as an inactive heterotrimer [8].

Moreover, oxidative stress leads to increases in ROS, which activates an IκB kinase complex (IKKs), triggering its degradation and allowing free NF-κB to translocate to the nucleus and activate gene expression [9, 10]. NF-kappa-B is a ubiquitous pleiotropic transcription factor that regulates many biological processes such as inflammation, immunity, differentiation, cell growth, tumorigenesis, and apoptosis. NF-kappa-B is a homo- or heterodimeric complex and the heterodimeric p65-p50 complex is the most abundant in the cell. The dimers bind at kappa-B sites in the DNA of their target genes and the individual dimers can bind with different affinity and specificity [11]. Concerning the brain, NF-κB has been implicated in normal processes of synaptic plasticity and memory but also in molecular mechanisms involved in neurological diseases [12].

In this study we analysed whether FXS could be caused by an alteration of NO metabolism, by studying the activation of the NF-κB-p65 subunit, the most abundant activated form in the nucleus, and also 3-nitrotyrosine and NO level, together with NOS isoform proteins detection in different brain areas from Fmr1-KO compared with wild-types of different ages. This work presents NO metabolism abnormalities in the Fmr1-KO mouse brain; these findings seem to suggest that NO may be involved in the pathogenesis of FXS and may represent a new therapeutic pathway for research.

2. Material and Methods

2.1. Experimental Animal Model. Animal studies were approved by the Animal Care and Use Committee of the Carlos Haya Hospital in accordance with the National Institute of Health guidelines for animal care. The Fmr1-knockout FVB-129 mouse line, donated by B. Oostra (Erasmus University Rotterdam), was used for the experiments. The animals were housed as a mouse colony in the Experimental Animal House of the University of Malaga. All experimental protocols met the guidelines of the University of Malaga Animal Welfare Committee and the European Communities Council Directive (86/609/EEC) regarding the handling of experimental animals. The mice were housed under controlled conditions of temperature and humidity, with a 12 h light/dark cycle with free access to standard food and water. Wild-type (WT) and Fmr1-knockout (Fmr1-KO) mice at 3 and 6 months of age were used in these experiments. Mouse littermates were obtained from crossings of heterozygous (wild-type/Fmr1-knockout) females and hemizygous (Fmr1-knockout) males and randomly located in different experimental groups, with 8–12 animals per group. The animals were sacrificed by cervical dislocation. Mouse brains were dissected immediately onto a cold plate, frozen, and stored at −80°C for biochemical analysis.

2.2. Genotype Analysis. DNA from the tail tissue was isolated by the Salting Out protocol to analyse genotype. The knockout mutation was distinguished from the normal gene by PCR analysis following the procedure used in previous publications [13]. Diluted DNA (2 μL) and 5 pmol of appropriate primers were used with the Ready Mix Kit (Sigma, S. Louise, MO). The PCR reactions were carried out on a thermocycler (Perkin-Elmer, Fremont, CA) and the products were visualized using ethidium bromide in a 1.5% agarose gel electrophoresis.

2.3. Brain Dissection. Animals were sacrificed by cervical dislocation. The brains were removed immediately, frozen promptly in liquid nitrogen, and the different regions, hippocampus, prefrontal cortex, and cerebellum, were dissected following Paxino's atlas coordinates and stored at −80°C.

2.4. LPS-Activated Organotypic Culture Brain Slice. For *ex vivo* experiments, the brain was cut into 100 μm thick slices and transferred onto plates containing sterile cold KR-HEPES buffer for assays. Organotypic slices culture of the central nervous system was performed following the standardized protocol. Incubations of the organotypic slice culture in the presence of 30 mg/mL or 60 mg/mL *Escherichia coli* lipopolysaccharide (LPS) exotoxin were carried out to

induce inducible nitric oxide synthase (iNOS) expression. Organotypic culture slices were incubated for 1 hour in the presence of 300 mM aminoguanidine (AG) to inhibit the iNOS, and then 30 mg/mL or 60 mg/mL LPS was added. The NO production was measured in the culture medium in untreated or treated organotypic slices culture for 10 min with LPS in the absence or presence of 300 μM AG and the results obtained were expressed as μM of nitrite/nitrate production.

2.5. Determination of Nitrate Concentration. Nitrate concentration was measured in the different brain areas by using a commercial assay kit (Abcam, Cambridge, UK).

2.6. Relative Quantification of nNOS, iNOS, and eNOS mRNAs. Real-time quantitative PCR technology was used to assay nNOS, iNOS, and eNOS mRNAs expression on 10 samples from each experimental group. Total RNA was isolated from the different brain areas by using the RNeasy Lipid Tissue RNA isolation kit (Quiagen, Netherlands). First strand cDNA was synthesized using High-Capacity cDNA Archive Kit (Applied Biosystems, Weiterstadt, Germany). For gene expression assays, cDNA was amplified using the 7500 Fast Real-Time PCR System (Applied Biosystems). Primers and probes were used from the commercially available TaqMan Gene Expression Assays (Mm 00607939_s1, Mm00435217_m1, Mm00440502_m1, and Mm00435175_m1, Applied Biosystems). (Relative changes in gene expression levels were determined using the $2^{-\Delta\Delta Ct}$ method). The cycle number at which the transcripts were detectable (CT) was normalized to the cycle number of GAPDH detection, referred to as ΔCT. PCR efficiency was determined by TaqMan analysis on a standard curve for targets and endogenous control amplifications, which were highly similar.

2.7. Western Blot Analysis. Western blot analyses were performed on brain extracts. Mouse brains were dissected into main structures comprising the cortex, hippocampus, and cerebellum. Samples were homogenized using a motorized pellet pestle in 1 mL of homogenization buffer containing 20 mM HEPES and 100 mM KCl pH 7.0 with protease inhibitor cocktails (SigmaFast, cat# 8830, Sigma, Saint Louis, MO). Cytosolic fractions were isolated by centrifugation. Protein concentration in each sample was measured by the Bradford method. Aliquots of tissue samples corresponding to 40 μg (cytosol) and 20 μg (nucleus) were heated to 100°C for 5 min with an equivalent volume of sample buffer (containing 2% SDS and 5% mercaptoethanol, bromophenol blue, and 20% glycerol) and loaded onto 10% and 12% polyacrylamide gels. Proteins were electrotransferred to a PVDF membrane, blocked for 1 h at 37°C in a blocking solution containing 3% BSA, 0.05% Tween-20, and PBS (pH 7.4), and incubated overnight at 4°C with primary antibodies in the blocking solution. An antibody against 3-nitrotyrosine was purchased from BIOMOL International (Plymouth Meeting, PA); antibodies against nNOS (155 kDa), iNOS (130 kDa), and eNOS (133 kDa) were purchased from Abcam (Cambridge, UK) and an antibody against p65 (65 kDa) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Membranes were rinsed three times with 0.05% Tween-20 in PBS for 10 min each, followed by incubation for 1 h at room temperature in a 1 : 5000 dilution of goat anti-rabbit IgG-HRP, purchased from Santa Cruz Biotechnology (Santa Cruz, CA), with 3% BSA in PBS. The blot was washed three times for 10 min each and then processed for analysis using an enhanced chemiluminescence detection kit (Biorad, cat# 170-5070) and visualized with a digital luminescent image analyser (Biorad).

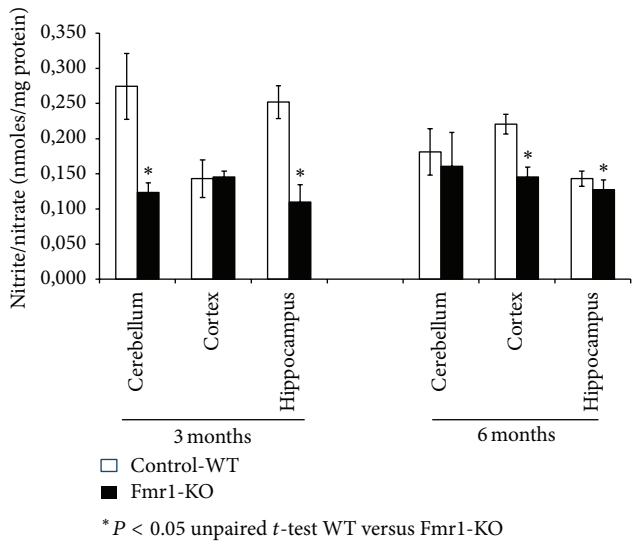


Figure 1: Nitrite/nitrate concentration in the cytosolic fraction of different brain areas, such as hippocampus, cortex, and cerebellum, and from fragile X mental retardation 1-knockout (Fmr1-KO) and wild-type (WT) mice of different ages. Data are described as the mean values ± standard deviation of eight experiments ($^*P < 0.05$ versus WT).

2.8. Statistical Analysis. Data are expressed as means ± SEM of 5-6 mice for group. Two-tailed Student's t-test was used to compare the two groups. One-way ANOVA followed by Dunnett's test was used to compare among three or more study groups. $P < 0.05$ is regarded as statistically significant.

3. Results

3.1. Basal Levels of Nitrite/Nitrate Were Reduced in the Cytosolic Fraction of Different Brain Areas. Figure 1 shows the NO concentration in different brain areas, such as hippocampus, prefrontal cortex, and cerebellum. A total of 6 mice samples were grouped by age at different stages (3 and 6 months). The results showed that the production of NO in each of the studied areas and at the different ages from Fmr1-KO mice displayed a lower production of NO when compared to WT-controls. NO production was significantly lower in Fmr1-KO mice compared to WT in cerebellum and hippocampus of the 3-month-old mice, and no changes occurred in cerebellum from 3-month-old mice. Moreover, NO production was significantly reduced in cortex and hippocampus of 6-month-old Fmr1-KO mice compared to WT.

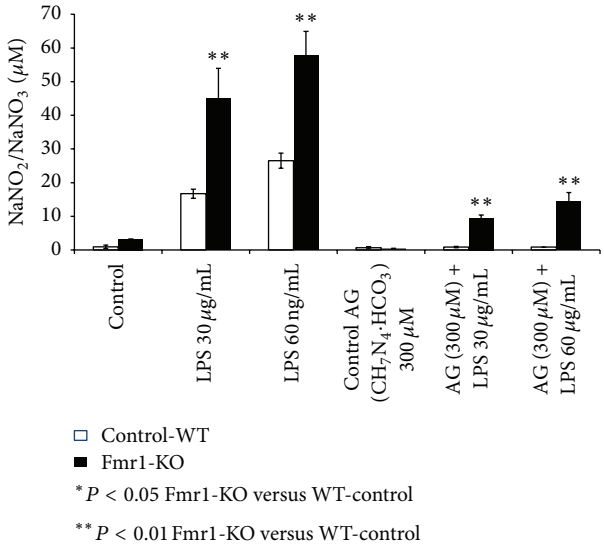


Figure 2: NO production is enhanced after lipopolysaccharide incubation and inhibited by aminoguanidine (AG) treatment of organotypic cultured brain slices from the Fmr1-KO mice. NO analysis was performed in culture brain slices by a nitrate/nitrite colorimetric test kit in the culture medium. Data represent mean ± SEM. $^{*}P < 0.05$ FXS versus control. $^{**}P < 0.01$ FXS versus control.

3.2. Nitric Oxide Metabolism Was Increased in LPS-Activated Organotypic Culture Brain Slices Obtained from the Fmr1-KO Mouse. Inducible nitric oxide metabolism was studied in the central nervous system using organotypic culture brain slices, under different incubation conditions to analyse if the NOS2 enzyme was involved in NO production in the Fmr1-KO mouse brain.

The incubation of culture slices in the presence of LPS increased the production of NO in a concentration dependent manner in the Fmr1-KO mouse, as can be observed in Figure 2.

The inhibition of the NO production by a NOS2 inhibitor, such as AG, confirms the extremely significant reduction of NO production in culture slices incubated for one hour in AG before the incubation in the presence of LPS. However, the complete inhibition was not seen within our experimental conditions.

3.3. 3-Nitrotyrosine Is Enhanced in Brain Proteins. NO is an important signalling molecule that is widely used for signalling during normal physiological processes of the nervous system. Superoxide and NO are known to bind to form peroxynitrite. Peroxynitrite and/or peroxynitrite-derived intermediates can free nitrate or protein-bound tyrosine residue to form 3-nitrotyrosine. The detection of protein-bound 3-nitrotyrosine is often used as a marker of inflammation and NO overproduction. Figure 3 shows the pattern of nitrated proteins in different brain areas, such as hippocampus, prefrontal cortex, and cerebellum. Detected immunoreactive bands indicate a statistically significant increase of endogenous tyrosine nitration in each studied area from the Fmr1-KO mice when compared to WT-controls at the two different adult stages.

3.4. NF-κB-p65 Subunit Is Upregulated in the Brain. Most of the NF-κB target genes encode proteins participating in stress and inflammatory responses, such as iNOS which is known to have NF-κB binding sites on its promoter. Thus NF-κB seems to be a key molecular mechanism for iNOS mRNA induction, and its activation seems to be a beneficial mediator to cellular stress. To determine the possible activation of the NF-κB-p65 subunit, Western blot analyses were performed in cortex, hippocampus, and cerebellum tissues. Figure 4 shows that, in the three studied regions, NF-κB-p65 protein content was significantly higher in Fmr1-KO mice when compared to WT mice.

3.5. nNOS and iNOS Protein Levels Are Divergent in the Brain. In order to further investigate the role of NO in the fragile X mouse pathology, we analysed whether NOS isoforms are activated in the brain from fragile X syndrome mice. Here we studied the activation of three different proteins: nNOS, iNOS, and eNOS by using the immunoblotting technique and also real-time PCR to measure mRNA expression levels. We first analysed NOS isoforms activation in different brain areas, such as hippocampus, prefrontal cortex, and cerebellum, and in different ages from Fmr1-KO animals and WT. Fmr1-KO mice displayed a significant decrease in nNOS protein content in all the different areas of 3- and 6-month-old Fmr1-KO mice when compared to WT-controls. However, this decrease was more evident in the cortex at both ages than in the hippocampus and in the cerebellum (Figure 5(a)). The iNOS isoform was highly expressed in the three studied areas from Fmr1-KO mice at both ages when compared to same aged WT-controls (Figure 5(b)). Regarding eNOS protein content from 3- and 6-month-olds, no significant differences were found between the three studied areas of Fmr1-KO mice compared to WT-control group (Figure 5(c)).

3.6. Abnormal Expression Pattern of NOS Isoforms in the Brain. To further characterize the involvement of these proteins in the fragile X phenotype, we analysed the mRNA expression level of the three proteins in the same studied areas and in whole brain. Figure 6 shows real-time PCR experiments of the three proteins normalized by GADPH mRNA levels. Figures 6(a) and 6(b) demonstrate a significant decrease in the levels of mRNA nNOS expression in the whole brain and in the three selected areas, when comparing Fmr1-KO samples at both age groups compared with WT-control samples. The decrease was particularly pronounced in cerebellum of 3-month-old Fmr1-KO compared to WT (Figure 6(a)). The mRNA level of the iNOS was significantly increased in whole brain and in the three selected areas, when comparing 3- and 6-month-old Fmr1-KO with WT-controls (Figures 6(c) and 6(d)). Surprisingly, this effect was more evident in the hippocampus of 6-month-old Fmr1-KO compared to WT than in the rest of the studied areas of this same group (Figure 6(d)). Regarding gene expression of the eNOS isoform, no changes were observed in whole brain and in the three different selected areas of 3- and 6-month-old mice of the Fmr1-KO and WT group (Figures 6(e) and 6(f)).

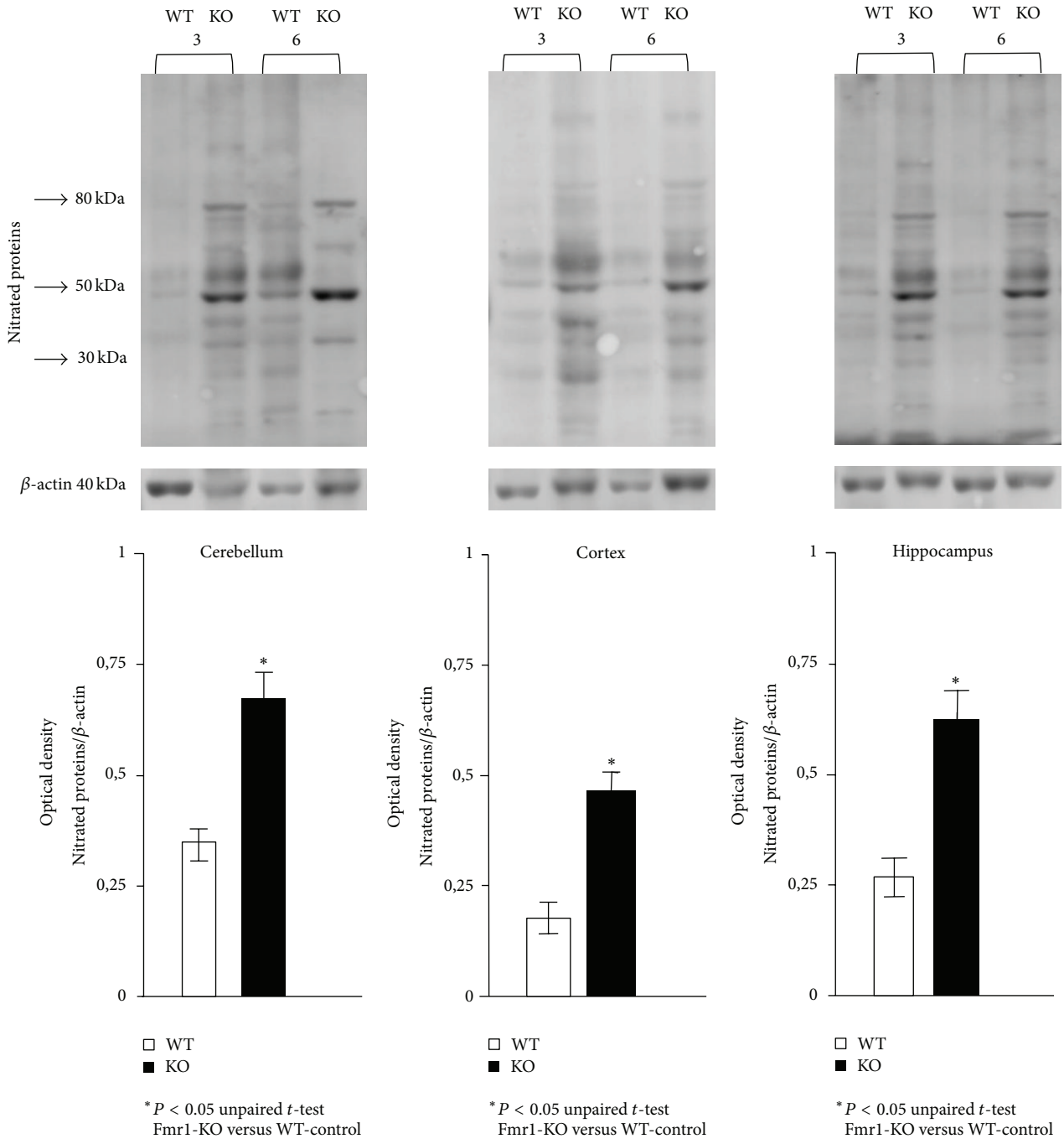


Figure 3: Western blot analysis of nitrated proteins in the cytosolic fraction of different brain areas, such as hippocampus, cortex, and cerebellum from fragile X mental retardation 1-knockout and wild-type (WT) mice. Molecular weight markers (kDa) are indicated on the left. Photographs are representative of six independent experiments. Data are described as the mean values ± standard deviation of six experiments ($^{*}P < 0.05$ versus WT).

4. Discussion

ROS are emerging as important players in the aetiology of neurologic and neurodevelopmental disorders including FXS. Out of several ROS-generating systems, the enzymes nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and NOS are believed to play major roles; and Figure 7 illustrates a diagram including key components of the molecular signalling mediated by this pathway. Mounting evidence suggests that activation of NADPH oxidase and the expression of NOS are directly linked to the generation of highly reactive free radicals, which affect various cellular components. Our previous studies carried out on the Fmr1-KO indicated that brain oxidative stress (increased generation of ROS, activation of the enzyme NADPH oxidase) is involved in several pathological manifestations of

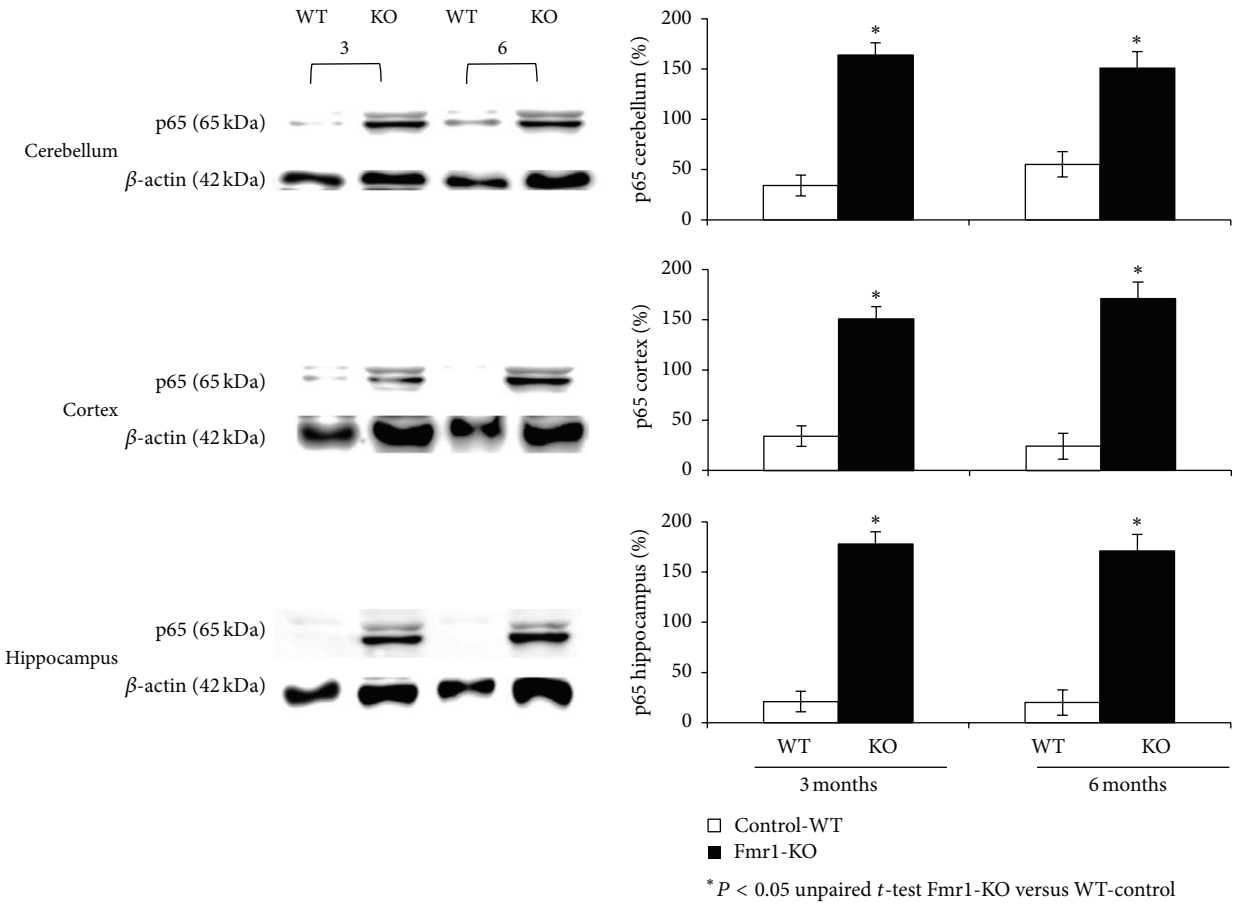
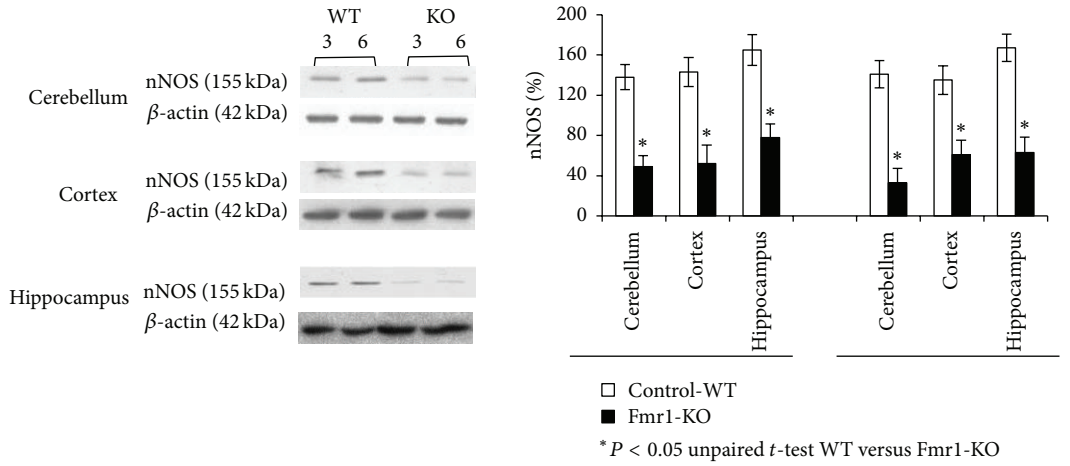


Figure 4: Western blot analysis of p65 protein levels in the nuclear fraction of different brain areas, such as hippocampus, cortex, and cerebellum from fragile X mental retardation 1-knockout (Fmr1-ko) and wild-type (WT) mice of different ages. Densitometry analysis of specific bands expressed as percentage relative to WT samples (100%). β-actin levels were used as loading control. Molecular weight markers (kDa) are indicated on the left. Photographs are representative of six independent experiments. Data are described as the mean values ± standard deviation of six experiments ($^{*}P < 0.05$ versus WT).

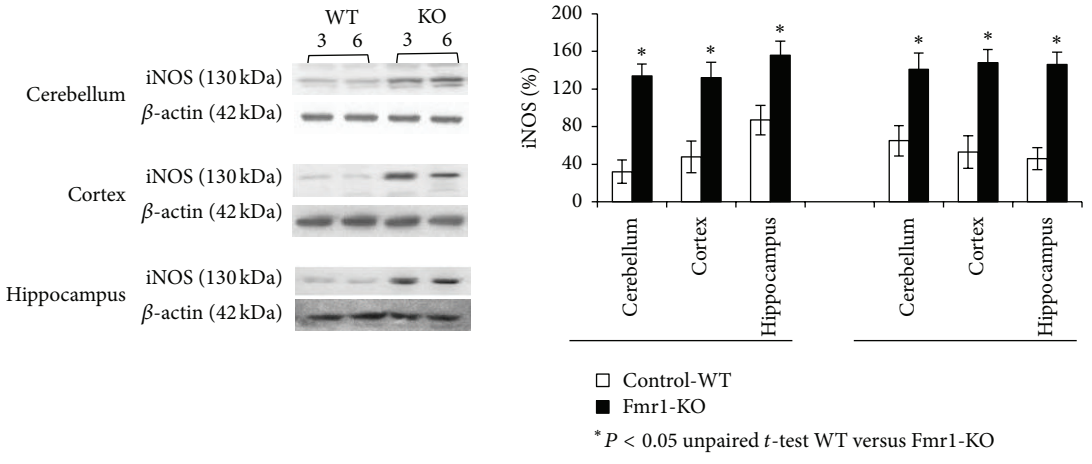
the syndrome [14–16]. In the present study, we demonstrate that adult Fmr1-KO mice develop altered NO homeostasis. In addition NO is a physiological messenger molecule; NO has been shown to participate in many physiological processes as signalling molecule and in long term it is involved in several pathophysiological mechanisms in the brain. Various forms of neuronal injury are frequently associated with increased or de novo overexpression of NOS [17]. On the basis of animal experiments, NO has been implicated in molecular mechanisms of epileptic seizures, ranging from mediation of an excitotoxic cascade to modulation of the central nervous system blood flow during epileptic episodes, and finally to participation in subsequent neuronal injury [18]. During pathologically increased overproduction of NO, much of the newly synthesized NO will be converted into peroxynitrite, which is an extremely potent free radical [19]. This substance subsequently interferes with mitochondrial energy metabolism and may even cause death of neurons by necrosis or apoptosis [20]. In this scenario, we have described an overproduction of NO after lipopolysaccharide incubation and the increased NO level was inhibited by aminoguanidine treatment of organotypic cultured brain slices from Fmr1-KO mice. Further studies must be carried out to understand if the inhibition of nNOS, by an excessive NADPH oxidase free radical overproduction, could constitute a cytoprotective mechanism against the negative consequences of the continual enhancement of oxidative molecules, which provides a novel clue to extend the physiological and pathophysiological roles of oxidative stress in the fragile X syndrome.

In our study, the level of 3-nitrotyrosine, another marker related to nitroxidative stress, was significantly elevated in cortex cerebellum and hippocampus homogenates of Fmr1-KO mice when compared to WT-controls. Most brain pathologies are accompanied by inflammation, during which the production of NO (mainly from iNOS) and/or superoxide (O_2^-) plus H_2O_2 (mainly from NOX) is increased [21].

iNOS is not normally expressed in the brain, but inflammatory mediators such as lipopolysaccharide and cytokines cause its expression in microglia and astrocytes, and possibly in neurons [22]. Indeed, we found a statistically significant increase in inducible iNOS isoform levels in Fmr1-KO mice compared to WT in young and adult mice in the three different studied areas.



(a)



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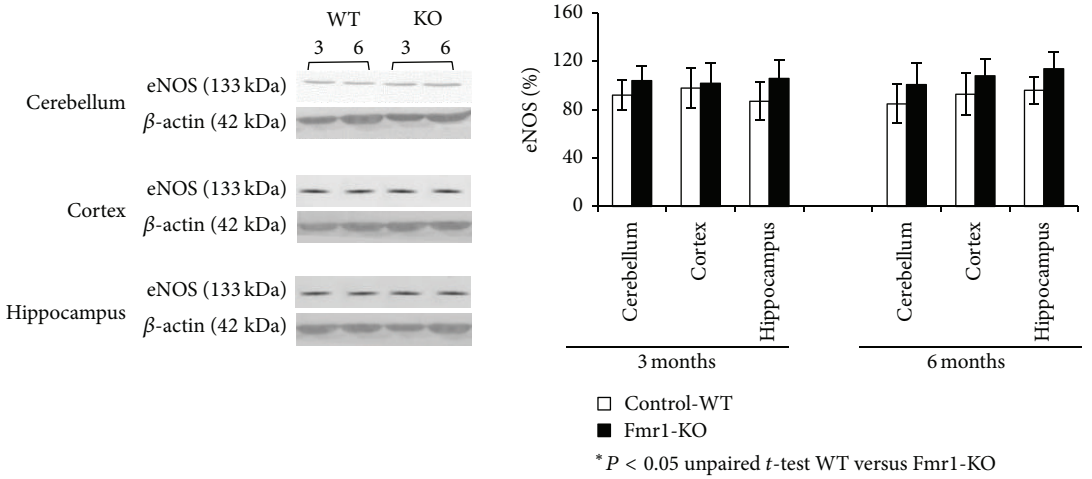

(c)

Figure 5: ((a), (b), and (c)) nNOS, iNOS, and eNOS protein levels in the cytosolic fraction of different brain areas, such as hippocampus, cortex, and cerebellum from fragile X mental retardation 1-knockout (Fmr1-KO) and wild-type (WT) mice of different ages, were analysed by Western blot. Densitometry analysis of specific bands expressed as percentage relative to WT samples (100%). β-actin levels were used as loading control. Molecular weight markers (kDa) are indicated on the left. Photographs are representative of six independent experiments. Data are described as the mean values ± standard deviation of six experiments ($^*P < 0.05$ versus WT).

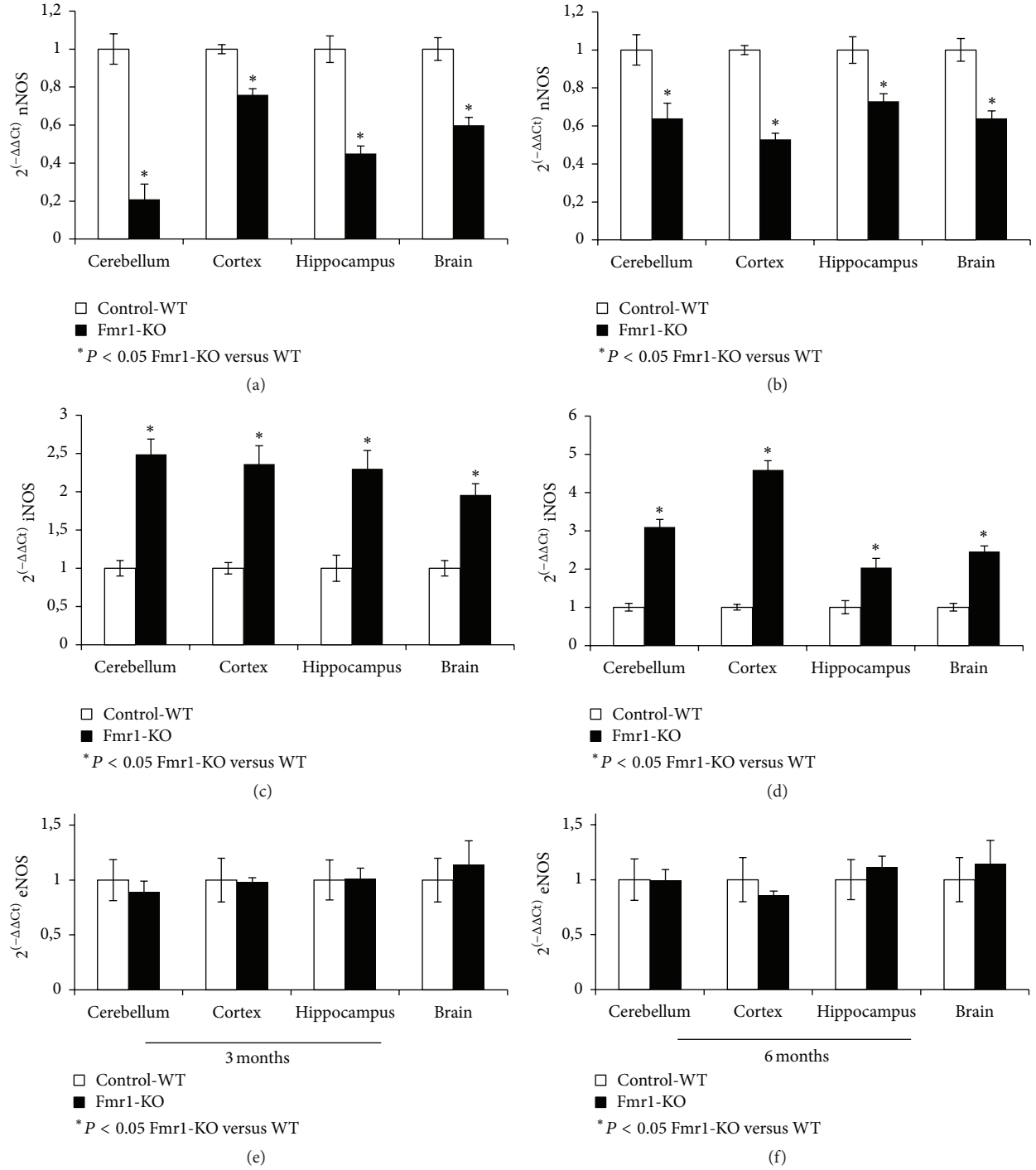

Figure 6: mRNA levels determined by real-time qRT-PCR of the three NOS isoforms in (a), (b), (c), (d), (e), and (f). Bar graph shows nNOS, iNOS, and eNOS in the different brain areas, such as hippocampus, cortex, and cerebellum from fragile X mental retardation 1-knockout (Fmr1-KO) and wild-type (WT) mice of different ages (* $P < 0.05$ versus WT).

Our results indicate an overall increased level of NF-κB-p65 in the brain from Fmr1-KO mice. Our main goal attempts to suggest a cooperative role of NO and NF-κB by regulating or contributing to the oxidative stress described in the brain of the Fmr1-KO mouse. Nuclear transcription factor kappa B is a stress inducible transcription factor, and it was demonstrated previously by others [23] that mice lacking a subunit of NF-κB-p65 show a selective learning deficit in the spatial version of the radial arm maze. These observations suggest that long-term changes of this factor affect adult neuronal

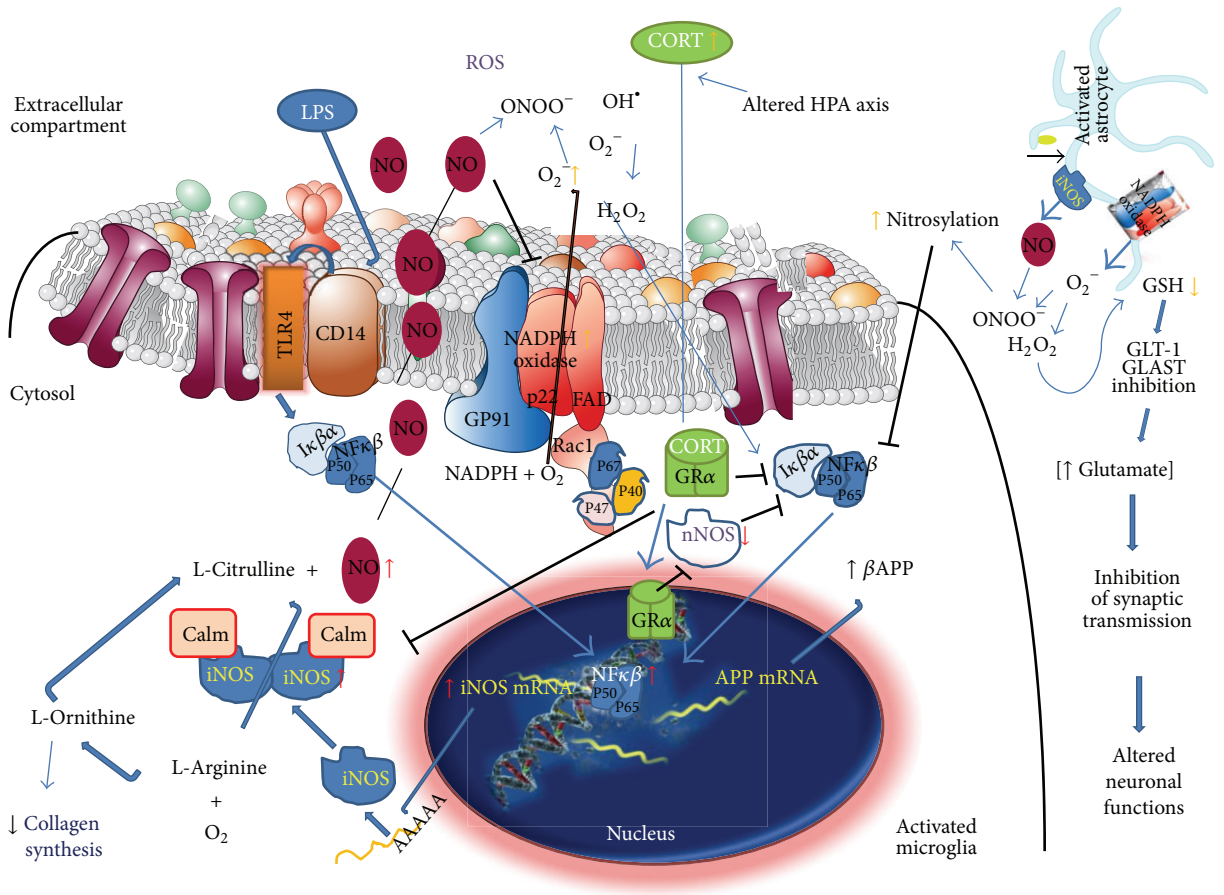

Figure 7: Glucocorticoids (Cortisol/Corticosterone) act as a nNOS inhibitor, and both molecules induce the releases of the NF-κB transcription factor that translocate to the nucleus to activate the synthesis of iNOS. Also other extracellular signals, such as lipopolysaccharide, act to induce NO production. NO acts as neurotransmitter and neuromodulator that regulates the NMDA receptors in the nervous systems. nNOS production of NO in CNS is associated with pain perception, control of sleep, appetite, thermoregulation, neural development, and synaptic plasticity. Activated microglia secrete inflammatory mediators to coactivate astrocytes and to induce cellular damage, which in turn will lead to abnormal synaptic transmission and neuronal dysfunction. NO antagonises NADPH oxidase (NOX2) assembly that in turn leads to a reduction in ROS production. Thus, as NO levels decline, oxidative mechanisms increase. Oxidative and nitrosative stress can also decrease intracellular GSH (reduced form) levels, resulting in a reduced antioxidant capability of the cells and an abnormal neuronal function. NO enhances glutamate release to influence memory processes by long-term potentiation (LTP). NO has been suggested as the retrograde molecule that activates the glutamate release. A compensatory mechanism, involving nNOS, is present in order to maintain LTP. The effect of NO on glutamate release depends on the NO level. Thus, when NO concentrations are low there is a decrease in glutamate release, but when NO increases, the inhibitory effect on glutamate release is reversed. S-Nitrosylation is a posttranslational regulatory mechanism that reduces the activity of target proteins, such as ERK, GAPDH, caspases, transglutaminases, and GTPase proteins (p21, RAC1, or cdc42). It has also been demonstrated that NO can induce S-nitrosylation and downregulate the NMDA receptor. S-Nitrosylation inhibits NF-κB, which could be an autoregulatory mechanism of NO production, since iNOS is induced by this transcription factor. NMDA receptor activation leads to the activation of nNOS, which could play a protective role by S-nitrosylation of the NMDA receptor; this inhibitory mechanism is regulated in order to prevent toxic effects. However iNOS activation and NO overproduction have also been suggested as an activator of NMDA-dependent neurotoxicity. Also, NO synthesis is involved in collagen physiology leading to connective tissue abnormalities.

function after synaptic stimulation, and it can be regulated by NF-κB nuclear translocation leading to gene activation, such as iNOS. It has been suggested that NF-κB activity following hippocampal learning may contribute to consolidation-associated synaptic reorganisation [24]. Moreover, the possible role of NO and NF-κB in the locomotor activity of rats has been described [25], and Fmr1-KO mice showed significant hyperactivity during development is frequently found in fragile X patients [13]. Kaltschmidt and cols. [26] have speculated that changes during cerebellar development may be controlled by glutamate-induced gene expression involving NF-κB. Similarly, the important role of NO in cell development was described in several animal models and humans [27].

The pathological effects of abnormal expression of NF-κB may result in many diseases. Those molecular mechanisms have not yet been thoroughly studied in the fragile X syndrome. We provide data to support the role of NF-κB in the Fmr1-KO mouse model. To the best of our knowledge,

no study has yet been reported on NF-κB in children or in the Fmr1-KO mouse model. In our study, we have found that there was a significant increase in the amount of the NF-κB-p65 subunit in nuclear extracts, from cortex, cerebellum, and hippocampus in both ages from Fmr1-KO mice when compared to WT. Elevated amounts of p65 in the Fmr1-KO mouse model can strengthen the conceptual frameworks of the role of free radicals (ROS and RONS) in the etiopathology of this condition. Naik and cols. [28] noted a significant increase in NF-κB DNA binding activity in peripheral blood samples of children with autism. NF-κB regulates a vast number of genes including those encoding cytokines, death and survival proteins, adhesion molecules, cyclooxygenase-2, manganese superoxide dismutase, and, of course, inducible nitric oxide synthase [29]. Both increased expressions of iNOS and NF-κB have been shown to participate in neurodegenerative diseases such as Parkinson's disease, Huntington's disease, or Alzheimer's. Inhibition of NF-κB decreased inducible nitric oxide synthase and cyclooxygenase-2 expression and restored working memory in the Alzheimer's disease mouse model [30].

nNOS is predominantly active in central and peripheral neurons, where production of NO is very important for cell communication [31]. nNOS produces low concentrations of NO over long periods and is activated by calcium ions (Ca^{2+}) through transient binding to the calcium-binding protein calmodulin. The results presented here demonstrate that protein and mRNA levels of nNOS of 3- and 6-month-old Fmr1-KO mice were relatively low in the three studied areas compared to WT mice. However, we suggest that the attenuated nNOS expression probably results in downregulation of NO production in Fmr1-KO mice. However, in our study, the production of NO was significantly lower in Fmr1-KO than in WT, in both ages, and in the three studied areas.

This deficiency probably results in an inadequate production of NO during nervous system development. nNOS regulation differs from iNOS; it is tightly regulated by calcium-activated calmodulin, specific phosphorylation, or interaction with plasma membrane ionotropic receptors [32]. This tight regulation makes NO generation an ideal signalling molecule in neurons that can regulate physiological processes such as differentiation and plasticity in the nervous system [33, 34]. L-Arginine is the only endogenous substrate of NOS and thus governs the production of NO during nervous system development. The suppression of nNOS gene expression has been attributed to large amounts of cytokines released during inflammation [35]. It has also been noted that the amount of NO produced by iNOS can depend greatly on the amount of the substrate, l-arginine (l-Arg) [36]. In addition, it has been suggested that NO derived from nNOS activity keeps iNOS suppressed under normal conditions, whereas downregulation of nNOS is a necessary condition to facilitate the expression of iNOS and the release of large amounts of NO [35]. To our knowledge, this is the first study demonstrating dysregulation of iNOS expression in the Fmr1-KO mouse model.

Recently published research has demonstrated that nNOS-knockout mice exhibit higher locomotor activity than their wild-type counterparts in a novel environment, as measured in the open field (OF) test, and also significantly shorter step-through latencies after training in a passive avoidance paradigm. Furthermore, abnormal spontaneous motor activity rhythms were found in the KO during the dark phase of the day, indicating dysregulation of rhythmic activities. These data indicate that nNOS-KO mimics certain ADHD-like behaviours [37]. It has been extensively demonstrated that Fmr1-knockout mice display hyperactivity [13], attentional and learning deficits consistent with a possible implication of a reduction in nNOS expression, and reduced nitric oxide basal production as has been shown in this work.

eNOS produces NO mainly in endothelial tissue of blood vessels, where NO causes vasodilation and endothelial relaxation of muscles and soft tissue [38]. However, in our study no differences were found in the eNOS isoform between different ages or between the Fmr1-KO and WT-control group.

The levels of nitroxidative stress were significantly higher in persons with ID such as Down syndrome and West syndrome [20, 39]. Our results are in agreement with previously published research demonstrating that nitroxidative stress is involved in intellectual disabilities. Xu et al. suggested that nNOS was reduced on postnatal day 21 in the hippocampus of Fmr1-knockout and impaired NO production may retard spine maturation in FXS [40]. Kwan et al. found that neocortical nNOS protein levels are severely reduced in developing human FXS cases [41]. Thus, alterations in regulation of nNOS in brain circuits may contribute to cognitive dysfunction in FXS.

Oxidative stress is involved in the fragile X syndrome as it was previously proposed [13, 42, 43]; it was also demonstrated that antioxidants and free radicals scavengers will be useful as experimental therapeutic approach to treat fragile X pathology [14, 44]. This work proposed that abnormal nitric oxide metabolism is involved in oxidative stress, and this mechanism may benefit antioxidant treatment. Also, selective nitric oxide synthase inhibitors may be investigated to determine the therapeutic possibilities in fragile X syndrome.

5. Conclusion

NOS abnormal expression observed in the Fmr1-KO mouse brain leads to changes in the NO metabolism, protein nitration, and NF-κB activation that may be involved in the pathogenesis of intellectual disability in the FXS and may represent a new therapeutic target for this rare disorder. In conclusion, the regulation of both iNOS and nNOS may play a fundamental role in the pathophysiological pathways contributing to FXS. Thus, the cross-talk of iNOS, nNOS, and NF-κB is suggested in neurological processes. Several neurological and inflammatory disorders have been linked to NF-κB, therefore restoring the nitric oxide/nitrosative status, and also to REDOX equilibrium on the FXS which addresses a promising approach for further investigation on treatment trials for the disease as has been also suggested in previous recent publications [45, 46].

Abbreviations

Calm: Calmodulin
CORT: Cortisol/Corticosterone
NF-κB: Nuclear factor κB
iNOS: Inducible nitric oxide synthase
ROS: Reactive oxygen species
NO: Nitric oxide
GLAST: Glutamate/aspartate transporter
GLT-1: Glutamate transporter subtype 1
GSH: Glutathione
ROS: Reactive oxygen species
LPS: Lipopolysaccharide.

Disclosure

CIBERobn is an initiative of the ISCIII (Instituto de Salud Carlos III), from the Spanish Ministry of Health, Spain. Yolanda de Diego-Otero is recipient of a "Nicolas Monarde" contract from the "Servicio Andaluz de Salud. Consejería de Salud. Junta de Andalucía."

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

The authors thank D. W. E. Ramsden for the English language revision of the text. Grant sponsor of this paper is Spanish Ministry of Science and Innovation through Grant no. SAF2008-00486, Andalusian Regional Ministry of Health, Grant no. PI2009-0507, and Andalusian Regional Ministry of Economy and Knowledge, Grants no. CTS546 and no. P10-CTS-05704. The paper is partially funded by FEDER "Fondos Europeos de Desarrollo Regional."

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Hindawi Publishing Corporation
Oxidative Medicine and Cellular Longevity
Volume 2016, Article ID 4867124, 15 pages
http://dx.doi.org/10.1155/2016/4867124

Review Article

Nitroxyl (HNO): A Reduced Form of Nitric Oxide with Distinct Chemical, Pharmacological, and Therapeutic Properties

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Received 5 May 2015; Revised 14 August 2015; Accepted 1 September 2015

Academic Editor: Lezanne Ooi

Nitroxyl (HNO), the one-electron reduced form of nitric oxide (NO), shows a distinct chemical and biological profile from that of NO. HNO is currently being viewed as a vasodilator and positive inotropic agent that can be used as a potential treatment for heart failure. The ability of HNO to react with thiols and thiol containing proteins is largely used to explain the possible biological actions of HNO. Herein, we summarize different aspects related to HNO including HNO donors, chemistry, biology, and methods used for its detection.

1. Nitric Oxide (NO)

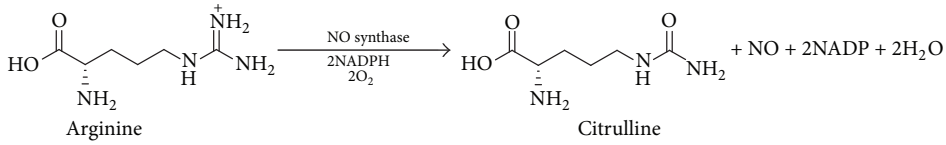
Biological activities associated with nitrogen oxide species are the subject of intense and current research interest. Much of this interest stems from the discovery of endogenous NO generation [1], a significant event that represents a fundamentally new paradigm in mammalian cell signaling. Prior to this finding, the idea that a small, freely diffusible, reactive molecule (known more for its toxicity) could be biosynthesized in a highly regulated fashion and elicit specific biological functions was unheard of and, for some at the time, heretical [2]. Most attention has been focused on NO since it is generated directly in mammalian cells by a family of enzymes referred to as the NO synthases (NOS) which converts the terminal guanidino nitrogen of L-arginine into NO (Scheme 1) [3, 4].

NO was described more than 30 years ago as a ligand of the NO-sensitive soluble guanylate cyclase (NO-sensitive sGC), the enzyme that catalyzes the conversion of guanosine 5′-triphosphate to guanosine 3′,5′-cyclic monophosphate (cGMP) [5]. NO activation of sGC involves coordination to a regulatory ferrous heme on the protein resulting in an increase in catalysis and a subsequent increase in intracellular cGMP [2]. Increased levels of cGMP within vascular smooth muscle result in vasodilatation [5]; thus continual release of NO regulates vascular tone and assists in the control of blood pressure [6]. In addition, NO increases the level of cGMP in platelets, and this is thought to be the mechanism by which it inhibits platelet function [7]. In the vasculature, NO also prevents neutrophil/platelet adhesion to endothelial cells, inhibits smooth muscle cell proliferation and migration, regulates programmed cell death (apoptosis), and maintains endothelial cell barrier function [8].

Along with NO, significant interest also exists for its oxidized congeners nitrogen dioxide (NO_2), peroxynitrite ($ONOO^-$), and nitrite (NO_2^-), among others. This attention may be a function of the facility by which NO is oxidized in aerobic systems and the toxicity of these resulting strong oxidants [9]. On the other hand, reduced nitrogen oxides (relative to NO) such as hydroxylamine (NH_2OH), nitroxyl (HNO), and ammonia (NH_3) have received much less attention [10] although there are several reports that suggest they can be generated endogenously [11]. These compounds were studied in the past with regard to their biological activity/toxicity [12]. Among the reduced forms, nitroxyl (HNO) is the least understood but is rapidly emerging as a novel entity with distinct pharmacology and therapeutic advantages over the common nitrogen oxide species [13].

2. Nitroxyl (HNO)

In 1896, the Italian scientist Angeli published the synthesis of the inorganic salt $Na_2N_2O_3$ [14] and several years later



Scheme 1: Enzymatic production of NO from L-arginine.

proposed its aqueous degradation produced nitrite and NOH [15]. The decomposition mechanism of Angeli's salt (AS) was revised more than a century later; and the products are now well established to be nitrite and the more stable HNO isomer [16]. Nitroxyl (variously called nitrosyl hydride, hydrogen oxonitrate (IUPAC), nitroso hydrogen, or monomeric hyponitrous acid) is a compound related to NO by 1-electron reduction and protonation [17]. Nitroxyl has a very unique biological profile which is distinct from that of its redox cousin NO [18]. Nitroxyl is growing as a potential therapeutic agent for congestive heart failure (CHF) as will be discussed [19, 20]. Nitroxyl appears to be a simple triatomic species, but it possesses novel and atypical chemistry. Prior to 2002, the biologically relevant form of HNO was thought to be the deprotonated anion, NO^-, since the generally accepted pK_a for HNO was 4.7 [21]. However, recent studies revised the pK_a significantly upward, to approximately 11.4, making HNO the exclusive species present at biological pH [22–24]. In addition, the different spin states of the two species HNO/NO^- complicate the acid-base relationship between them [17]. NO^-, which is isoelectric to molecular oxygen O_2, has a triplet ground state and a singlet lowest excited state, while the HNO ground state is a singlet. Thus, the HNO acid-base equilibrium species possess different electronic spin states (i.e., protonation-deprotonation is spin forbidden) and it is expected that the rates of protonation and deprotonation will be extremely slow compared to normal acid-base reactions [24]. This spin forbidden transformation supports the idea that the observed biological activity is attributed to the protonated form HNO [17, 24].

Another complex feature of HNO that complicates the study of the production, detection, and its chemistry is the high reactivity. Chemically, HNO is a highly reactive electrophile that spontaneously dimerizes to give hyponitrous acid, which dehydrates to give nitrous oxide (N_2O) and water (see (1)) [17, 25, 26]. Detection of N_2O as an end product may serve as a marker for the involvement or at least the presence of HNO in biological systems [27]. This reaction has been studied both theoretically and experimentally and several rate constant values have been offered with the accepted value, determined by flash photolysis techniques at room temperature, being $k = 8 \times 10^6\ M^{-1}\ s^{-1}$ [24]:

$$HNO + HNO \longrightarrow HON{=}NOH \longrightarrow N_2O + H_2O \quad (1)$$

Thus, unlike most commonly used nitrogen oxides, HNO cannot be stored or concentrated and is typically studied using donor species that release HNO as a decomposition product.

3. Nitroxyl Donors

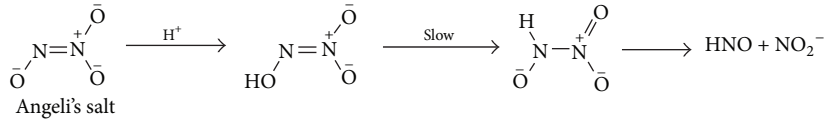
3.1. Angeli's Salt. As mentioned, Angeli's salt (AS) or sodium trioxodinitrate ($Na_2N_2O_3$) is the most common HNO donor currently used. Angeli's salt releases HNO with a half-life of approximately 2-3 min at physiological pH and temperature. Nitroxyl release from this compound is observed between pH values 4 and 8 [28], through a first-order process, and the generally accepted mechanism involves protonation of the dianion followed by tautomerization and heterolytic cleavage of the N–N bond to produce HNO and nitrite (Scheme 2) [16]. ^{15}N-labelling confirms that HNO originates solely from the nitroso group while the nitrite's origin is from the nitro group [29].

Interestingly, when the pH drops below 4, the rate of AS decomposition significantly accelerates, and NO becomes the only nitrogen-containing end product. At higher pH (pH > 8), the decomposition rate decreases. Such a decay profile is extremely useful for practical purposes, as stock solutions of AS are relatively stable at high pH, while the release profile is pH insensitive near physiological conditions. However, the fact that 1 equivalent of nitrite is coproduced during AS decomposition may disturb the experimental interpretation considering the fact that nitrite has its own physiological profile [16].

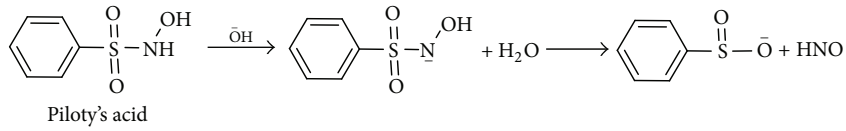
3.2. Piloty's Acid. *N*-Hydroxysulfenamides (Piloty's acid and its derivatives) are another class of HNO donors that have been examined in detail. Piloty's acid shares some similarities with AS including pH-dependent first-order decomposition. Piloty's acid is stable at low pH and its decomposition rate increases at higher pH (Scheme 3). Significant HNO release occurs at pH values higher than biological conditions, while at physiological pH many of Piloty's acid analogs are oxidized and subsequently release NO, not HNO. This pH restriction has limited the use of these compounds as HNO donors in biological studies [30].

3.3. Cyanamide. Cyanamide, an antialcoholic drug used in Canada, Europe, and Japan, was found to be bioactivated via oxidation by the enzyme catalase leading to an *N*-hydroxycyanamide intermediate. This intermediate species spontaneously decomposes to release cyanide and HNO (Scheme 4), which targets a thiol containing enzyme in the metabolic pathway of ethanol (aldehyde dehydrogenase, ALDH) [31, 32].

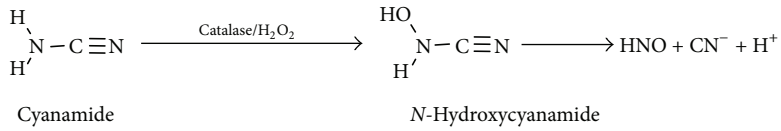
The release of cyanide during this reaction has restricted the use of cyanamide and its derivatives in biological settings. However, this drug demonstrates the feasibility of clinical use



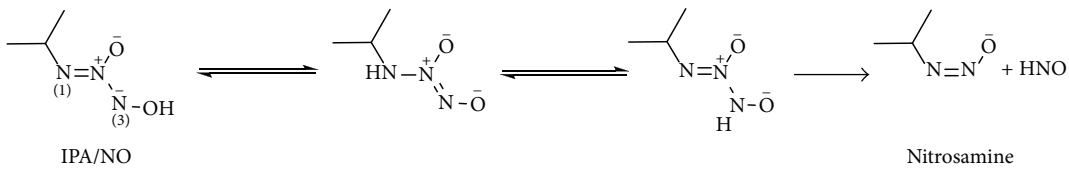
Scheme 2: Nitroxyl release from AS.



Scheme 3: Nitroxyl release from Piloty's acid.



Scheme 4: Nitroxyl release from cyanamide.



Scheme 5: Nitroxyl release from IPA/NO.

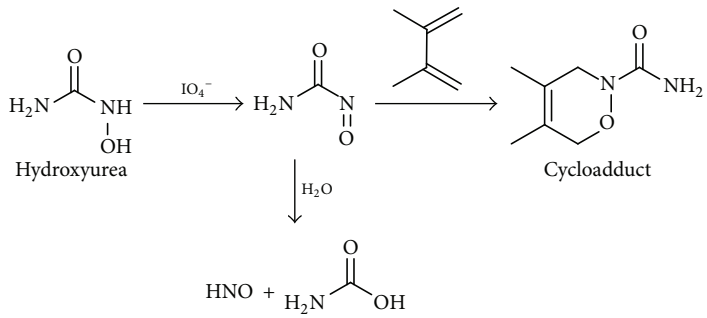
of HNO donors and the ability to selectively target protein thiols as will be seen later [9].

3.4. Diazeniumdiolates as HNO Donors. Diazeniumdiolates (NONOates) which are complexes of secondary amines and NO share the same chemical class as AS but they fragment back to NO under the same conditions in which AS decomposes to HNO [33–35]. In contrast to these NO donors, decomposition in neutral solution of the amine NONOate produced with primary amines (isopropylamine, IPA/NO) results in HNO release, while NO forms only at lower pH values, similar to AS [36]. Dutton et al. [37] showed that the mechanism of HNO formation is similar to that of AS, starting from protonation of N(1), followed by slow tautomerization forming a higher-energy isomer protonated at N(3). This tautomer will be a very minor component of the equilibrium but once formed it will spontaneously and rapidly dissociate to produce HNO and the deprotonated nitrosamine because of the very small barrier to N–N bond cleavage (Scheme 5). The resulting nitrosamine is expected to be unstable and rapidly decompose to the primary alcohol and N_2 [38].

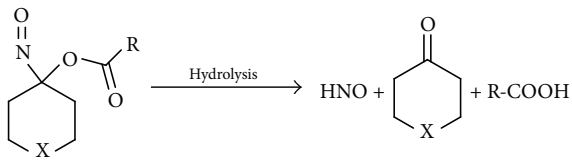
3.5. Acyl Nitroso Compounds. Acyl nitroso compounds were observed as an oxidation product of hydroxyurea (a drug that is used for sickle cell disease), and they rapidly hydrolyze to yield N_2O as evidence for HNO release. Cycloadducts with conjugated 1,3-dienes and N–O heterodienophiles stabilize the acyl nitroso compounds and then they can undergo retro-Diels Alder reactions to yield the parent acyl nitroso compound that rapidly hydrolyses to yield HNO (Scheme 6) [39].

3.6. Acyloxy Nitroso Compounds. A new class of HNO donors, acyloxy nitroso derivatives of cyclohexane, has been developed by King's group [40, 41]. They are easily synthesized from oxidation of cyclohexanone oxime or dihydro-2H-pyran-4(3H)-one oxime by either lead tetraacetate (LTA) [40] or (diacyloxyiodo)benzene [42]. They release HNO upon cleavage of the ester bond (Scheme 7). Modifying the electronic and/or steric properties of the acyl group position changes the rate of ester cleavage and thus HNO release. One of the major benefits of these HNO donors, besides controlling the HNO release rate, is that nitrite is not a product as is the case with AS. Another advantage of these compounds that makes them very useful in biochemical studies is the blue color that is a result of the $n \rightarrow \pi^*$ electronic transition of the N–O bond, allowing biochemical reaction kinetics to be easily monitored [40].

Changing the substitution in such group of compounds greatly determines their properties. 1-Nitrosocyclohexyl trifluoroacetate decomposes immediately releasing HNO upon addition of water or buffer solution in a manner that resembles Angeli's salt ability to release HNO, while 1-nitrosocyclohexyl pivalate is considerably stable with $t_{1/2}$ of 2260 min in a 1 : 1 mixture of MeOH : Tris buffer

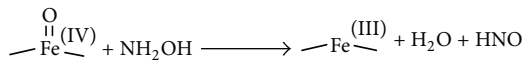


Scheme 6: Nitroxyl release from acyl nitroso compounds.



Acyloxy nitroso compound
R = CH_3, $C(CH_3)_3$, CF_3
X = CH_2, O

Scheme 7: Nitroxyl release from acyloxy nitroso compounds.



Scheme 8: Oxidation of hydroxylamine to HNO by high-valent iron-oxo complexes.

(50 mM, pH 7.6) and hence it is not considered as a common HNO donor [43].

4. Endogenous Production of HNO

Since the discovery of endogenous NO generation, carbon monoxide (CO) [44] and hydrogen sulfide (H_2S) [45] have also joined the ranks of gaseous/small molecule signaling species. The potency by which HNO elicits many of its pharmacological actions and the apparent selectivity of HNO towards several of its established biological targets suggests that the actions of pharmacologically administered HNO are the result of preexisting signaling pathways which responds to HNO. Some reports even assumed that HNO can serve as the endothelium-derived relaxing and hyperpolarizing factor that might contribute to NO claimed vasorelaxing actions [46]. However, no confirmed evidence exists for HNO endogenous generation and this might be due to the lack of a specific and sensitive trap for HNO that can be applied for biological systems. Numerous *in vitro* chemical studies provide possible chemical pathways for that to occur.

Several reports showed that HNO can be produced by NOS under certain conditions [47–50] especially in the absence of its vital prosthetic group, tetrahydrobiopterin [47, 51], and via the metabolism of the NOS product *N*-hydroxy-L-arginine (NOHA) under oxidative stress [52]. Nitroxyl can be also formed under nitrosative stress (e.g., reaction of NO with hydroxylamine yields HNO) [53] and by thiolysis of *S*-nitrosothiols (RSNO, a well characterized species formed in biological systems as a result of NO generation) [2] following thiol nucleophilic attack on the SNO sulfur (see (2)) [11, 26]. Hence,

$$\mathrm{RSNO + RSH \longrightarrow RSSR + HNO} \quad (2)$$

The direct reduction reactions of NO by mitochondrial cytochrome *c* [54], xanthine oxidase [55], Cu- and Mn-containing superoxide dismutase (CuMnSOD) [56], and ubiquinol [57] are among other reactions reported to generate HNO.

Oxidation of NH_2OH can serve as an alternative source for HNO production. The generation of high-valent iron-oxo complexes from the reaction of ferric hemes (usually peroxidases or catalases) with hydrogen peroxide can oxidize NH_2OH to HNO (Scheme 8) [27, 58].

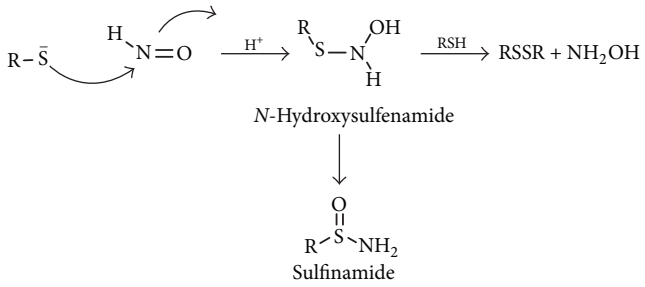
5. Biological Chemistry of HNO

Nitroxyl is a highly reactive electrophile, and the literature on the chemistry of HNO includes multiple examples of reactivity with nucleophiles, oxidants, and metalloproteins [59]. Bartberger et al. [22] used quantum mechanical calculations to predict that HNO reacts somewhat selectively with nucleophiles. Theoretical examination predicts that HNO will not significantly hydrate or react with alcohols; however, it appears to be very reactive towards thiols, with the reaction with amines intermediate between water/alcohol and thiols. These reactions are different from reactions of NO with those nucleophiles. Table 1 summarizes the main possible reactions of NO and HNO with biological reactants.

5.1. Reaction with Thiols. To date, thiols appear to be a major site of HNO biochemical reactivity. Doyle et al. [59] produced one of the earliest reports of thiol reactivity with HNO showing a 98% yield of disulfide and NH_2OH as the final products of the reaction of HNO with thiophenol in 40% aqueous acetonitrile (see (3)). This yield suggested that

Table 1: Comparison of NO and HNO reactivity with biological reactants [28].

Biological reactant	NO	HNO
NO	No reaction	Forms $N_2O_2^-/HN_2O_2$ unknown chemistry
HNO	Forms $N_2O_2^-/HN_2O_2$ unknown chemistry	Dimerization and decomposition to N_2O
O_2	Autoxidation leading to nitrosative species, that is, NO_2 and N_2O_3	Forms a potent $2e^-$ oxidant that is not $ONOO^-$
RSH/RS−	No direct reaction	Forms sulfinamide or disulfide + hydroxylamine
Fe(II) heme	Very stable Fe(II)-NO	Forms coordination complex
Fe(III) heme	Forms unstable electrophilic nitrosyl, first step in reductive nitrosylation	Very stable Fe(II)-NO except sGC
Cu^{2+}	No reaction	Reduces Cu^{2+} to Cu^+ yielding NO
Lipid radical	Yields lipid-NO	Yields lipid-H + NO



Scheme 9: Reaction of HNO with thiols.

the rate of this reaction significantly exceeds the rate of HNO dimerization (see (1)) [17]:

$$2C_6H_5SH + HN_2O_3^- \longrightarrow C_6H_5SSC_6H_5 + NH_2OH + NO_2^- \quad (3)$$

Further thiol reactivity was reported with other biologically relevant thiols such as reduced glutathione (GSH) [26], *N*-acetyl-L-cysteine (NAC) [60], and dithiothreitol (DTT) [61]. The mechanism for this reaction was suggested to include an attack of the nucleophilic sulfur on the electrophilic nitrogen atom of HNO leading to the formation of an *N*-hydroxysulfenamide intermediate. The *N*-hydroxysulfenamide intermediate can further react with excess thiol (or a vicinal protein thiol) to give the corresponding disulfide and hydroxylamine (Scheme 9) [59] or rearrange to sulfinamide in a competing reaction (Scheme 9) [26].

Significantly, disulfide formation is considered to be biologically reversible because disulfides are easily regenerated while the generation of the sulfinamide apparently represents an irreversible thiol modification [62]. The rate of some of these reactions was also investigated and for GSH the rate constant was found to be $k = 2 \times 10^6\ M^{-1}\ s^{-1}$ [25]. The HNO-thiol reaction was also reported to be condition dependent (under high thiols concentration the disulfide is the main product while under low reduced thiol concentration the *N*-hydroxysulfenamide intermediate rearranged to the sulfinamide) [17, 26, 60].

This reactivity with thiols indicates that protein sulfhydryl groups may be a major target for HNO. Shen and English characterized a similar type of chemistry using electron spray ionization mass spectrometry (ESI-MS) upon the reaction of AS with thiol proteins, such as bovine serum albumin (BSA), glyceraldehydes-3-phosphate dehydrogenase (GAPDH), and human brain calbindin (HCalB). They used MS characters of whole proteins and their digests to demonstrate the ability of HNO to induce disulfide bond formation when these reactions were done in the presence of excess cysteine, while, with no excess thiols, the sulfhydryl group present in these proteins was converted mainly to sulfonamide [63]. Other reports suggest the ability of these sulfinamides to be hydrolyzed to sulfinic acids (SO_2H) [64, 65]. The rate of the reaction between HNO and thiol proteins appears to be faster than that with small thiols, as the cysteine in BSA reacts with a rate constant of $k = 6 \times 10^6\ M^{-1}\ s^{-1}$. It can be estimated that GAPDH, with a low pK_a thiol, reacts at a rate $>10^9\ M^{-1}\ s^{-1}$. Thus, thiol pK_a and hydrophobicity of the thiol environment influence the reactivity [66].

Hoffman and coworkers used a tandem mass spectrometric analysis including MS/MS with collision induced dissociation (CID) and electron capture dissociation to identify the sulfinamide modification introduced by AS to thiol proteins. These studies revealed a characteristic neutral loss of $HS(O)NH_2$ fragment (65 Da) that is liberated from the modified cysteine upon CID monitored by mass spectrometry. Upon storage, partial conversion of the sulfinamide to sulfinic acid was observed, leading to neutral losses of 65 and 66 Da (HS(O)OH) [64].

These experimental data are supported by theoretical calculations for the free energies involved in the reaction of HNO with 5 different thiols. The reaction was found to be dependent on the loss of the S–H proton, a requirement for S–N bond formation. These findings were consistent with proteins whose environment favors the existence of thiolate anions being selectively inhibited by HNO. This work also showed that protonation of the HNO oxygen atom may also be required before or during the rate limiting step. The authors strongly suggest that the competition between the two possible pathways would be kinetically controlled. Additionally, the calculated values of ΔG indicate that the preferred reaction pathway depends upon the hydrophobicity

of the environment, the availability of a local base, and the identity of the thiol substituent. In a hydrophobic environment, the calculated activation barriers strongly indicate that formation of a disulfide is favored, consistent with the ability of GSH to prevent the irreversible inhibition of proteins *in vitro*. The formation of the sulfinamide would be expected to become more favorable if a base was present in the local environment and with stronger electron-withdrawing substituents on the thiol. In solution, a greater competition between the two pathways is predicted, but formation of the disulfide is expected to be favored in most conditions [67].

5.2. Reaction with Metals/Metalloproteins. Another aspect of HNO chemistry important for its biological activity is its ability to react and/or form coordination complexes with metalloproteins, particularly iron heme proteins [68]. Nitroxyl reacts with oxidized metals to form reductive nitrosylation products in a single step reaction; this reaction was originally observed upon exposure of metmyoglobin (metMb) or methemoglobin (metHb) to AS (see (4)) [59, 69]:

$$\text{Fe (III)} + \text{HNO} \longrightarrow \text{Fe (II) NO} + \text{H}^{+} \quad (4)$$

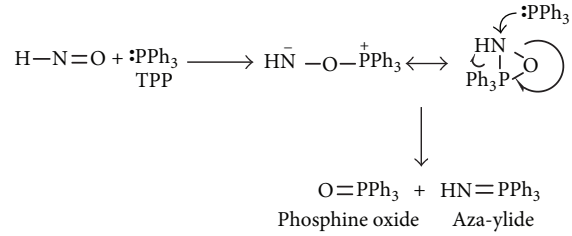
Other ferric proteins including cytochromes, peroxidases [69], and catalases [70] also undergo reductive nitrosylation by HNO, indicating lack of specificity toward nitrogen (histidine), sulfur (cysteine or methionine), and oxygen (tyrosine) as the protein part that binds to iron next to the HNO binding site. The protein environment around this site may have significant impact on the kinetics of reductive nitrosylation [17].

The suggested mechanism for this reaction is either an inner sphere mechanism, whereby direct coordination of HNO to the metal center occurs followed by electron transfer and deprotonation, or via an outer sphere mechanism involving initial electron transfer to the metal center followed by coordination of NO to the reduced metal center. The existence of an outer sphere process is evident in the reaction of HNO with ferricytochrome c, which only produces the ferrous species and NO [59]. The rate constant of these reactions was estimated for the reaction of synthetic ferric porphyrin with AS to be $k = 3 \times 10^5\ \text{M}^{-1}\ \text{s}^{-1}$ (comparable to that with metMb $k = 8 \times 10^5\ \text{M}^{-1}\ \text{s}^{-1}$) [71].

Nitroxyl also reacts with oxyhemes (e.g., oxymyoglobin, MbO_2, and Fe(II)O_2) converting two MbO_2 to two metMb (Fe(III)) with a rate constant of 10^7. A two-step reaction was proposed with the intermediacy of NO and formation of peroxide (see (5)-(6)); the formed peroxide can react to generate a ferryl-*p*-cation radical. As this reaction has not been confirmed, there is a possibility that HNO is reduced to H_2NO rather than being oxidized to NO with rapid dissociation of the resulting Fe(III)O_2 complex (see (7)-(8)) [17, 66]:

$$\text{Fe (II)}\ \text{O}_2 + \text{HNO} \longrightarrow \text{Fe (III)} + \text{NO} + \text{HO}_2^{-} \quad (5)$$

$$\text{Fe (II)}\ \text{O}_2 + \text{NO} \longrightarrow \text{Fe (III)} + \text{NO}_3^{-} \quad (6)$$



Scheme 10: Reaction of HNO with triphenylphosphine.

$$\text{Fe (II)}\ \text{O}_2 + \text{HNO} \longrightarrow \text{Fe (III)} + \text{H}_2\text{NO} + \text{O}_2 \quad (7)$$

$$\text{Fe (II)}\ \text{O}_2 + \text{H}_2\text{NO} \longrightarrow \text{Fe (III)} + \text{NH}_2\text{OH} + \text{O}_2 \quad (8)$$

Other metalloproteins also have been reported to react with HNO. CuZnSOD reacts with HNO to yield NO [69], while reaction with MnSOD results in a Mn–NO complex [72].

5.3. Reaction with Phosphines. Previous reports showed reactions of organic phosphines with various nitroso compounds. C-Nitroso and S-nitroso compounds react with triphenylphosphine (TPP) and similar triarylphosphines to form phosphine oxides and aza-ylides [73–75]. Reisz et al. reported a similar type of phosphine reactivity toward HNO (viewed as a simplified nitroso compound) (Scheme 10) [76].

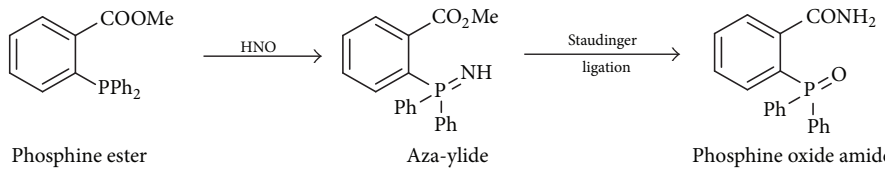
The proposed mechanism for this reaction was P addition to N or O of the nitroso group forming a three-membered ring intermediate, which upon reaction with additional equivalent of phosphine would give the corresponding aza-ylide and phosphine oxide.

This work also showed that, in the presence of an electrophilic ester, properly positioned on the phosphine, the aza-ylide undergoes Staudinger ligation to yield an amide with the nitrogen atom derived from HNO (Scheme 11) [76].

5.4. Other HNO Reactions. Nitroxyl reacts with O_2 to form an RNS (reactive nitrogen species) which does not appear to be peroxynitrite (ONOO^{-}) but has some similar chemistry [77, 78]. This species is a strong $2e^{-}$ oxidant and hydroxylating agent and is cytotoxic at low millimolar concentration and causes DNA strand breaks [53, 77]. The rate of this autoxidation of HNO is relatively slow ($k = 1000\ \text{M}^{-1}\text{s}^{-1}$) compared to the rate of HNO reactivity with other biological reactants like GSH, and the lower concentration of O_2 relative to the other biomolecules severely limits the formation of this species *in vitro* and consequently limits the oxidative damage due to the exposure to HNO *in vivo* [17].

Other RNS could be formed indirectly from HNO, after its oxidation to NO with superoxide dismutase (SOD) [66]. N_2O_2^{-} also can be formed from the reaction of HNO and NO at a rate constant of $5 \times 10^6\ \text{M}^{-1}\text{s}^{-1}$ [66]. Nitroxyl can react with other nucleophilic nitrogen oxides such as hydroxylamine (see (9)) and nitrite (reverse of Scheme 2):

$$\text{NH}_2\text{OH} + \text{HNO} \longrightarrow \text{N}_2 + 2\text{H}_2\text{O} \quad (9)$$



Scheme 11: Staudinger ligation of HNO with triarylphosphines.

Nitroxyl reduction to NH_2OH can easily occur under physiological conditions. This was confirmed by calculation of the reduction potential for the HNO, $2H^+/NH_2OH$ couple at pH 7 to be 0.3 V [24]. It was proposed that NH_2OH might be the product of HNO oxidation of NADPH by two electrons to $NADP^+$ though this has not been confirmed [79].

6. Detection of HNO

Several methods implicate the presence or fleeting existence of HNO. As mentioned, the detection of N_2O has been used as evidence for HNO generation since HNO spontaneously dimerizes to hyponitrous acid ($H_2N_2O_2$) that then decomposes to N_2O and water (see (1)). N_2O is usually detected by gas chromatography-mass spectrometry (GC-MS). However, this cannot be applied to biological samples as an absolute proof of HNO intermediacy since N_2O can be generated in ways not involving free HNO [80]. Moreover, N_2O formation is second order in HNO and requires fairly high concentrations for this reaction to be significant.

As discussed earlier, HNO reacts readily with thiols. In fact, Pino and Feelisch [81] used thiol reactivity as a means of trapping HNO and distinguishing its actions from those of NO. Furthermore, the identification of the HNO based thiol modification could serve as a useful method for HNO detection. Donzelli et al. [27] used HPLC techniques to identify both the sulfinamide [$GS(O)NH_2$] and the oxidized glutathione (GSSG) as evidence of GSH exposure to AS.

Transition metals can also be used to trap HNO, with the highest applicability of metMb or synthetic analogs and trapping to produce EPR active species [82, 83]. Other methods involved the oxidation of HNO to NO using ferricyanide and then detecting the formed NO with chemiluminescence or electrochemical analysis techniques [84].

Shoeman and Nagasawa [85] reported that nitrosobenzene could trap HNO to give cupferron (Scheme 12), a species that complexes copper forming a colored complex. However, this has not yet been exploited for the detection of HNO in a biological system, and the efficiency of this trap has not yet been determined.

The use of water soluble phosphines represents a powerful way to trap HNO in biological samples by tracking the aza-ylide (Scheme 10) that is formed upon the reaction of HNO with phosphines using simple ^{31}P NMR techniques [76].

HPLC and colorimetric methods were developed by Reisz et al. to quantify HNO release from donor compounds using phosphine derivatives. Ligation of HNO using carbamate containing phosphine derivatives yielded HNO derived urea (Scheme 13) and the corresponding phenol. Starting with a nitro derivatized phosphine trap yielded *p*-nitrophenol that can be monitored calorimetrically [86].

HNO reaction with GSH was also used for HNO detection. HNO was trapped by GSH followed by labeling of GSH with the fluorogenic agent, naphthalene-2,3-dicarboxaldehyde (NDA), and subsequent quantitation by fluorescence difference [87].

A prefluorescent probe, 4-((9-acridinecarbonyl)amino)-2,2,6,6-tetramethylpiperidin-1-oxyl (TEMPO-9-AC), has been used to detect HNO in aqueous solution and to differentiate it from nitric oxide (NO). TEMPO-9-AC reacts with HNO via net hydrogen abstraction to produce the highly fluorescent TEMPO-9-AC-H and NO. The utility of TEMPO-9-AC as a probe for HNO has been shown using the common HNO donors Angeli's salt and Piloty's acid (PA) along with a recently reported HNO donor, 2-bromo-PA [88].

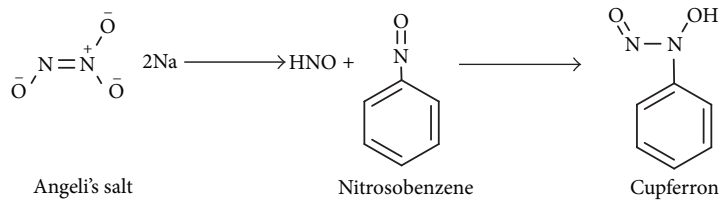
Recently, a near-infrared fluorescent turn-on sensor for the detection of nitroxyl (HNO), using a copper-based probe, CuDHX1, was recently developed for selective quantitation of HNO in biological samples. The probe contains a dihydroxanthene (DHX) fluorophore and a cyclam derivative as a Cu(II) binding site. Upon reaction with HNO, CuDHX1 displays fivefold fluorescence turn-on in cuvettes and is selective for HNO over thiols and reactive nitrogen and oxygen species [89].

Clearly, the development of sensitive, specific, and biologically compatible traps/detectors for HNO is essential in determining whether HNO is physiologically relevant. Moreover, such methodology will be valuable for future investigations distinguishing the specific actions of HNO from those of other nitrogen oxides.

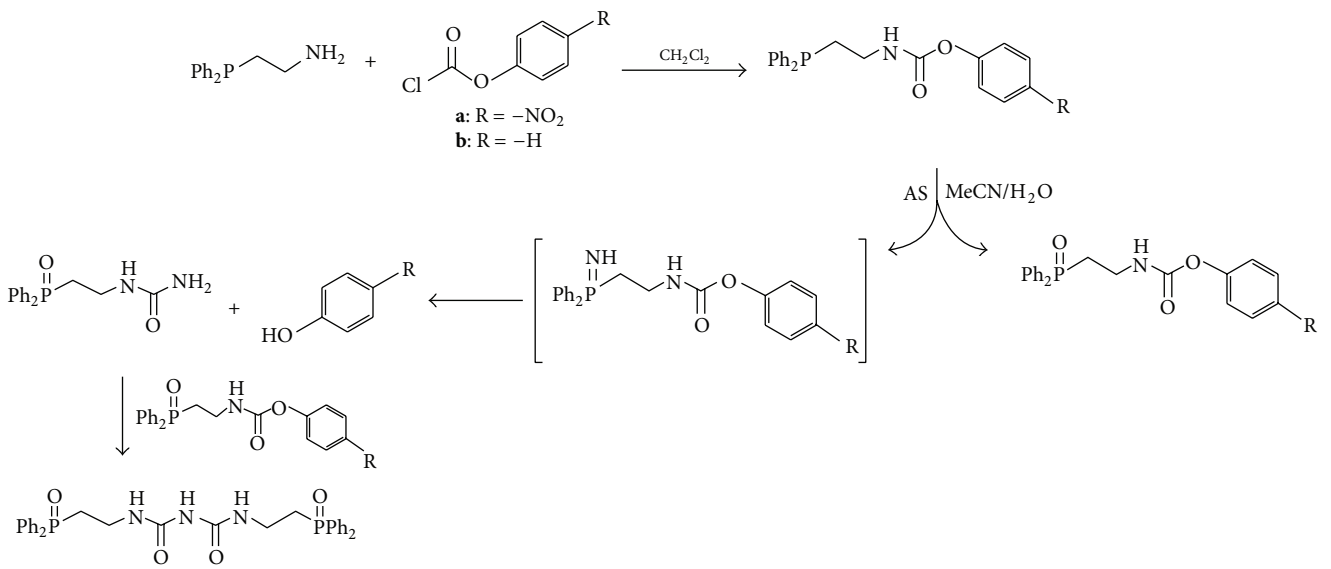
7. Biological Activity of HNO

Endogenous HNO generation remains uncertain in mammalian cells. Therefore, the question of whether HNO is a natural signaling/effect or species remains open. However, numerous studies indicate that exogenous HNO administration results in interesting, novel, and potentially important pharmacology and toxicology [62].

7.1. HNO and Antialcoholism. The first report of HNO serving as a drug was from the Nagasawa research group who were examining the action of the antialcoholic drug cyanamide [31, 32, 90]. The utility of this drug lies in its ability to inhibit ALDH, an enzyme involved in the metabolism of ethanol to acetate. Cyanamide has no inherent activity and must be oxidatively bioactivated to elicit enzyme inhibition.



Scheme 12: Reaction of AS with nitrosobenzene.



Scheme 13: Ligation of AS with arylphosphines producing phenols that can be monitored calorimetrically.

Oxidation of cyanamide by catalase/H_2O_2 generates an *N*-hydroxy intermediate that decomposes to HNO and cyanide as shown in Scheme 4. Inhibition of ALDH was proposed to occur via reaction of HNO with the active site cysteine thiolate of the enzyme. This finding was among the first indications that HNO was thiophilic and could alter the actions of thiol proteins.

7.2. HNO and Vascular Function. Similar to NO, HNO acts as a potent vasorelaxant [91]. Angeli's salt elicits vasorelaxation in isolated large conduit [92, 93], small resistance arteries [94], and intact coronary [95] and pulmonary [96] vascular beds, in both rabbits [97] and dogs [98, 99]. Acyloxy nitroso compounds also caused dilatation in thoracic aorta isolated from Sprague-Dawley rats in a dose-dependent manner [43]. Paolocci et al. showed that HNO shows selectivity being a venodilator in contrast with NO donors that equally dilate both arteries and veins. In addition, they showed HNO's ability to decrease mean arterial blood pressure [99]. Recently, HNO is introduced as a vasodilator for humans that is not susceptible to tolerance [100].

Because NO elicits vasodilation via activation of sGC [101], it is possible that HNO was being converted to NO in these experiments. Such conversion seemed especially likely because HNO itself has been reported to be incapable of activating sGC [102], although these studies were performed under high thiol conditions that probably scavenged HNO. Like NO, vasorelaxant responses to HNO are accompanied by an increase in cGMP [103] and are impaired by the sGC inhibitor, 1*H*-[1,2,4]oxadiazolo[4,3-a]quinoxaline-1-one (ODQ) [92, 95, 103], indicating that HNO targets sGC. Whether HNO itself directly activates sGC or first requires oxidation to NO, or if the preference of HNO for Fe(III) versus Fe(II) targets its actions to the oxidized, NO-insensitive sGC isoform, which predominates in disease, remains to be determined [104]. Zeller et al. [105] support the idea of HNO being oxidized first to NO by the enzyme SOD before activating sGC, but further investigations are required.

Nitroxyl, but not NO, increases the plasma level of the vasodilatory neuropeptide calcitonin gene related peptide (CGRP) [98, 99]. Calcitonin gene related peptide stimulates endothelial NO release, increases vascular smooth muscle cAMP, and activates K^+ channels to elicit vasodilatation [25, 106]. However, the use of antagonist, CGRP, does not prevent *in vivo* vasodilatation of HNO, suggesting that CGRP may not contribute to HNO's actions [99]. Although CGRP seems to contribute to AS mediated coronary vasodilation *ex vivo* [95], it remains unclear how HNO stimulates *in vivo* CGRP release and whether CGRP plays a similar role in other vascular beds. Recent report suggested that HNO endogenously produced via the reaction of NO with H_2S can form a disulfide bond in the sensory chemoreceptor channel TRPA1 resulting in sustained calcium influx that causes release of CGRP [107].

HNO also targets K^+ channels to modulate vascular function. Multiple studies show that both K_v and K_{ATP} channels

were affected in HNO-mediated vascular and nonvascular smooth muscle relaxation [46, 94]. By contrast, NO only activates K_{Ca} channels in this preparation [108]. Nitroxyl activation of K^+ channels may be direct or cGMP-dependent and HNO-thiol interactions could enable direct modulation of K_v and K_{ATP} channels independently of cGMP [94].

The vasodilatory capacity of HNO donors and their ability to target signaling pathways distinct from NO might offer therapeutic advantages over traditional nitrovasodilators. Several reports demonstrated that, unlike organic nitrates such as glyceryl trinitrate (GTN), AS does not develop tolerance after either acute or chronic use [100, 103]. Given that AS inhibits the thiol protein ALDH-2 [9], which has an important role in GTN biotransformation to NO [109], cross-tolerance between AS and GTN is not likely to happen which may be of interest [103]. Nitroxyl donors could have novel utility as vasodilators, alone or in conjunction with conventional nitrovasodilators, in the treatment of vascular disorders such as angina and heart failure [13].

7.3. HNO and Myocardial Function. Originally, the pharmacological utility of HNO in the cardiovascular system was considered limited to vasodilation. More recently, HNO donors have been shown to be capable of profoundly affecting myocardial contractility [18].

Nitroxyl has been shown to have positive inotropic (force of muscle contraction) and lusitropic (relaxation of cardiac muscle) properties; both properties contribute to increased cardiac output in both normal and failing canine hearts. In this study, Paolocci et al. utilized a pressure-volume relationship approach to examine primary effects of HNO on the heart from changes in vascular loading conditions and demonstrated for the first time that AS was capable of increasing the inotropic status of the heart while fastening ventricular relaxation and unloading of the heart [99].

Nitroxyl-mediated inotropy was not mimicked by an NO donor and is prevented by the HNO scavenger NAC indicating a direct HNO effect. In dogs with tachypacing-induced congestive heart failure (CHF), HNO improved both contractility and relaxation to an extent similar to that found for control dogs [98]. Nitroxyl inotropy was not dependent on beta-adrenergic pathways and was additive to beta-adrenergic agonists in enforcing myocardial contractility. Positive inotropy, unloading cardiac action, and peripheral vasodilation are all desirable outcomes in CHF subjects. Current CHF cases are usually treated via beta-agonists or phosphodiesterase inhibitors, giving inotropic support to the heart, and through nitrosovasodilators, to unload the heart. That HNO donors provide both effects together is a unique advantage.

Tocchetti et al. [110] and others [19] have explained the increased myocardial contractility by showing that HNO elicits the prompt release of Ca^{2+} from the sarcoplasmic reticulum (SR) in both cardiac and skeletal muscle via activation of ryanodine receptors (RyR2) and skeletal Ca^{2+} release channels (RyR1). These effects appear to occur through modifications of certain thiol residues in these receptors. In both cases, the effects were reversible upon the addition of a sulfhydryl reducing agent, DTT.

Froehlich et al. [111] suggested that HNO donated by AS can also activate Ca^{2+} uptake by the cardiac SR Ca^{2+} pump (SERCA2a) by targeting the thiols in phospholamban (PLN, a regulatory protein that controls SERCA2a). They concluded that HNO produces a disulfide bond that alters the conformation of PLN, relieving inhibition of the Ca^{2+} pump. This increase in uptake keeps the net diastolic Ca^{2+} low.

In addition to these changes in Ca^{2+} cycling, HNO also acts as a cardiac Ca^{2+} sensitizer, enhancing myofilament responsiveness to Ca^{2+} and augmenting maximal force of myocardial contractility. This effect is likely to be due to modulation of myofilament proteins that have many reactive thiolate groups and are potential targets of HNO. Altering intracellular redox conditions with DTT prevented HNO-induced augmentation in muscle force development, consistent with the idea of HNO affinity for strategically located thiols [112].

These combined mechanisms suggesting the orchestrated action of HNO with cardiac myofilaments and SR to enhance inotropy and lusitropy at potentially lower energetic cost make HNO an attractive candidate for CHF cases.

7.4. HNO and Ischemia Reperfusion Injury. Nitroxyl also elicits effects in ischemia reperfusion (IR) injury. Ischemia reperfusion injury occurs when tissue is deprived of adequate blood flow for a period of time causing hypoxia, which is the ischemic event, and other effects. Reperfusion of oxygenated blood causes the injury and results in necrosis of the tissue. Ischemia reperfusion injury can be alleviated by preconditioning the tissue, which involves brief occlusions of the vasculature prior to the actual cessation of blood flow. Administration of AS to ischemic myocardium was found to be detrimental, as opposed to the beneficial effects exerted by equieffective doses of NO donors [97]. No clear-cut mechanisms for the HNO exacerbation of injury were identified, though a possible explanation may be the ability of HNO to induce neutrophil accumulation [9]. Pagliaro and coworkers later showed that HNO is capable of inducing early preconditioning-like effects in isolated rat hearts to an extent even greater than that induced by NO donor [113]. Again this protective signaling cascade is not clear, with Shiva and coworkers suggesting that mitochondrial thiols are possible HNO targets [114]. Additionally, CGRP may mediate a part of HNO ability to induce protection, as CGRP has been reported to be protective in this context [115].

Furthermore, the distinct role of HNO in cardiovascular system made it a very promising strategy in treatment of cardiomyopathy associated with various diseases such as diabetes. The HNO donor 1-nitrosocyclohexylacetate (1-NCA) was proven to limit cardiomyocyte hypertrophy and LV diastolic dysfunction in a mouse model of diabetes *in vivo* [116].

7.5. HNO and Platelet Aggregation. Treatment of platelets with micromolar concentrations of AS leads to inhibition of platelet aggregation in both time- and dose-dependent fashion [117]. Hoffman et al. [64] identified the ability of HNO

to induce modifications in 21 proteins within human platelets, with 10 of those proteins showing dose-dependent modification. The function of these proteins ranges from metabolism and cytoskeletal rearrangement to signal transduction.

7.6. HNO and Anticancer Activity. In early 2008, Brooks reported the anticancer activity of HNO by demonstrating the ability of HNO to suppress the proliferation of both estrogen receptor- (ER-) positive and ER-negative human breast cancer cell lines, in a dose-dependent manner. This inhibition was accompanied by a significant decrease in the blood vessel density in HNO-treated tumors. Both *in vitro* and *in vivo* models of breast cancer were used to evaluate AS. One explanation for this activity is the ability of HNO to inhibit the thiol protein glyceraldehydes-3-phosphate dehydrogenase (GAPDH) blocking the glycolytic cycle inside the malignant cell and leading to a cascade of signaling mechanisms resulting in the inhibition of angiogenesis [118]. Nitroxyl inhibition of GAPDH was previously reported leading to inhibition of glycolysis in yeast cells [119, 120].

In the next year, Froehlich also reported the ability of HNO to suppress the growth of both hormone dependent and independent cancers such as prostate, breast, pancreatic, and lung cancer that overexpress a protein called MAT-8 protein (mammary tumor 8 kDa protein) [121, 122]. MAT-8 is member of the family of FXYD regulatory proteins; it inhibits the plasma membrane Na^+/K^+ pump causing collapse of the transmembrane Na^+ gradient [123], affecting cellular Ca^{2+} levels that protect tumor cells from apoptosis [124]. Froehlich demonstrated that HNO inhibited the MAT-8 protein via disulfide bond formation between transmembrane cysteine residues, relieving the Na^+/K^+ pump inhibition and triggering endoplasmic reticulum stress, rendering the cell susceptible to apoptosis and destruction [125].

7.7. HNO and Antioxidant Activity. Chemistry predicts that HNO can serve as an antioxidant, with its relatively weak H–N bond strength of ~50 Kcal/mol, making it a good hydrogen atom donor, with the ability to quench reactive radical species [28]. HNO capably inhibits lipid peroxidation in both yeast and *in vitro* model systems [126]. Nitroxyl inhibits free radical lipid oxidation by interacting with the initiating agent, lipid radical or lipid peroxy radical, thus terminating the radical chain chemistry (see (10)–(13)). Oxidation of HNO gives NO that can also quench radical intermediates [9]:

$$\mathrm{L{-}H + In^{\bullet} \longrightarrow L^{\bullet} + In{-}H} \quad (\mathrm{L = lipid,\ In = free\ radical\ initiator}) \tag{10}$$

$$\mathrm{In^{\bullet} + HNO \longrightarrow In{-}H + {}^{\bullet}NO} \tag{11}$$

$$\mathrm{L^{\bullet} + HNO \longrightarrow L{-}H + {}^{\bullet}NO} \tag{12}$$

$$\mathrm{LOO^{\bullet} + HNO \longrightarrow LOO{-}H + {}^{\bullet}NO} \tag{13}$$

7.8. HNO and Central Nervous System (CNS). Nitroxyl also interacts with the thiol residues on the *N*-methyl-D-aspartate (NMDA) receptor that plays a vital role in neuronal communication and synaptic function in the CNS [127]. Since overstimulation of the NMDA receptor has been implicated in the excitotoxicity associated with glutamate, the physiological outcome of this HNO action affords protection against glutamate-based excitotoxicity. In contrast, Colton and coworkers [128] reported that HNO was also capable of blocking glycine-dependent desensitization of the NMDA receptor resulting in net sensitization of the receptor. The suggestion was made that the divergent outcomes of these studies are at least in part a function of O_2 depletion upon long term use of AS, which consumes O_2 during decomposition. Thus, careful attention needs to be paid to experimental conditions, particularly with regard to O_2 and metal content. However, both studies indicate that HNO can modify the activity of the NMDA receptor, in either a positive or a negative fashion, depending on the oxygen tension of the system [9].

7.9. HNO and Other Thiols. In addition to the thiol proteins mentioned in the previous sections, HNO is a potent inhibitor of some cysteine proteases like papain [129] and cathepsin P [130, 131]. It also inhibits the metal responsive yeast transcription factor Ace1 [132] and depletes intracellular GSH levels [53].

7.10. HNO Toxicity. Cyanamide is the only clinically approved drug that functions exclusively through release of HNO. The therapeutic use of cyanamide induces little inherent toxicity at prescribed doses indicating that administration of HNO donors is not inherently toxic.

However, some *in vitro* experiments showed some toxicity associated with the use of AS in very high concentrations compared to those used for biological studies. AS was found to significantly reduce cellular viability, due to severe depletion of cellular GSH levels. Nitroxyl can also induce double-stranded DNA breaks. The cytotoxicity of Angeli's salt was substantially diminished under hypoxic conditions, indicating a role for O_2 in the toxicity of HNO. These observations were observed at 1–3 mM AS, which significantly exceeds the concentrations of AS utilized in pharmacological applications. The effects observed with cultured cells may not be applicable to *in vivo* systems with intact redox buffering capacity [53].

High concentrations of HNO have been proposed to be responsible for the neurotoxicity observed following administration of AS to rats via intranigral infusion. The effects were both acute and progressive (occurring after the dissipation of HNO). The acute phase was attributed to direct interactions of HNO with cellular components while the delayed phase may be a result of excitotoxicity [133]. Intrathecal administration of Angeli's salt to the lumbar spinal cord of rats resulted in motor neuron injury without effect on sensory neurons [134].

Nitroxyl shows some interactions with mitochondria as a redox active species, though these interactions were not

reported to be toxic, and no pharmacological utility was reported also [9]. Inhibition of GAPDH by HNO inhibits cellular glycolysis [119].

In summary, HNO seems to hold very promising therapeutic potential for cardiovascular diseases (especially CHF) due to its activity profile on the heart and vasculature. Enhancement of intracellular Ca^{2+} cycling and myofilament Ca^{2+} sensitization appear to be unique, in addition to vasodilation and reduction in cardiac load. HNO also provides a promising strategy in fighting cancer due to its ability to inhibit GAPDH. Moreover, the bioactivity of HNO appears distinct compared to NO, making biological targeting by HNO localized and specific facilitating the use of HNO as a novel therapeutic agent.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Hindawi Publishing Corporation
Oxidative Medicine and Cellular Longevity
Volume 2016, Article ID 8262383, 9 pages
http://dx.doi.org/10.1155/2016/8262383

Research Article

Training Status as a Marker of the Relationship between Nitric Oxide, Oxidative Stress, and Blood Pressure in Older Adult Women

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Received 12 June 2015; Revised 14 August 2015; Accepted 24 August 2015

Academic Editor: Luis Sobrevia

The purpose of this study was to evaluate the influence of functional fitness and oxidative capacity on the nitric oxide concentration associated with hemodynamic control in older adult women. The sample consisted of 134 women (65.73 ± 6.14 years old). All subjects underwent a physical examination to assess body mass index, waist-hip ratio, body fat measurement by dual energy X-ray absorptiometry, and blood pressure (BP). Training status (TS) was evaluated by indirect determination of maximal oxygen uptake by a treadmill test using Balke protocol modified for older adults. Functional fitness was also evaluated through a "Functional Fitness Battery Test" to determine the general fitness functional index (GFFI). All participants were separated according to the functional fitness (TS1, very weak and weak; TS2, regular; TS3, good and very good). Plasma blood samples were used to evaluate prooxidant and antioxidant activity and nitrite and nitrate concentrations. The general results of this study showed that good levels of TS were related to lower levels of lipoperoxidation and protein damage, higher levels of antioxidant, and higher concentration of nitrite and nitrate. This combination may be responsible for the lower levels of BP in subjects with better TS.

1. Introduction

Considered as a chronic-degenerative disease, hypertension (HT) has a high incidence among elderly people and has several factors involved in its etiology, such as environmental, genetic, psychological, and humoral factors [1, 2]. Among them, low concentration of nitric oxide (NO) and high exposure to oxidative stress (OS) have been receiving special attention due to the impairment in the vasodilatation mechanism which can compromise the normal values of blood pressure (BP).

Nitric oxide is produced by endothelial cells and is being considered as a potent vasodilator due to the effects in the vascular smooth muscle relaxation [3]. So, low concentration of NO is associated with increased values of BP, especially in older people [4, 5].

Oxidative stress is also considered in the HT etiology because it affects many functions in the organism. Actually, reactive oxygen species (ROS), such as superoxide anions ($O_2^{\cdot-}$), hydroxyl radical ($^{\cdot}OH$), and hydrogen peroxide (H_2O_2), are physiologically produced in low concentrations and work as a molecular signaling to maintain vascular

integrity and as a regulator of endothelial function [6]. However, in high concentration, $O_2^{-\cdot}$ can induce a deleterious effect in the organism [7]. For example, the interaction between $O_2^{-\cdot}$ and NO has an important role in vascular homeostasis [8] because of the formation of peroxynitrite molecules (ONOO−) [9, 10], which are largely responsible for the process of endothelial dysfunction and decrease of NO bioavailability [6, 11].

In short, both mechanisms can compromise the vasodilatation, resulting in elevated values of BP. However, it has been shown that regular physical exercise has an important function against these deleterious effects. The main mechanism for that is related to shear stress by increased blood flow during the physical activity [12, 13]. This in turn contributes to improving endothelial function due to the stimulus for the production of NO and the endothelial superoxide dismutase (ecSOD), an antioxidant that has a high affinity with $O_2^{-\cdot}$, counteracting the reaction between NO and $O_2^{-\cdot}$, consequently increasing the NO bioavailability [6, 10, 14–16].

Although many studies have been demonstrating that physical exercise promotes an improvement in NO concentration by increased endothelial nitric oxide synthase (eNOS) activity and oxidative stress [17, 18] through the increased stimulus of superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) [16, 19], the effectiveness of physical exercise is still controversial. For example, Trapé et al. demonstrated that good level of training status improved the levels of nitrite concentration but with no change in TBARS [20]. Gomes et al. [21] found decreased levels in malondialdehyde (MDA) and an increased level of whole blood nitrite concentration but no difference in plasma nitrite after 3 months of physical exercise in patients with metabolic syndrome. And finally, Bouzid et al. compared young and elderly people and showed that some oxidative stress markers (SOD, GPx, and glutathione reductase) improved after acute exercise only in the young groups and just the MDA increased in the elderly group [22]. These results demonstrated low antioxidant efficiency with aging.

All these differences can be mainly due to the differences among intensity, type, duration, and frequency in which physical exercise can be practiced, especially those related to elderly people [23–25]. The study of Santana et al. confirms this statement. The authors evaluated the plasma nitrite in hypertensive older women after the peak of incremental test and after 90% of anaerobic threshold acute exercise and only in the incremental test the values of nitrite increased. These results showed that there is a difference in nitrite concentration according to the intensity of exercise [26].

Independent of the physical exercise practiced, exercise should be good enough to raise fitness level of the practitioner, thus bringing the benefits previously described. However, there are still few studies seeking the establishment of the relationship through the current TS of the practitioner and the benefits of the practice. With this background the hypothesis of this study was that elderly people with good level of TS, regardless of the type of exercise performed, will present better relationship between BP, NO concentration, and OS. Therefore, the purpose of this study was to investigate if the relationship between BP, NO, and OS can be modulated by the TS in older adult women.

2. Methods

2.1. Sample Selection. All procedures were previously approved by Institutional Review Board of University of São Paulo State (CEP/FC-UNESP n° 323.427) and all subjects provided written consent before the beginning of experiments. Extension programs linked to universities and associations of retirees related to the elderly community were visited and all individuals were invited to participate in this study with the same chance to be included once they met the inclusion criteria.

2.2. Inclusion and Exclusion Criteria. Subjects should be nonsmoking, nonalcoholic, and nondiabetic, should be within the age of 50–80 years, should not have cardiovascular peripheral, cerebrovascular, neurologic, or psychiatric diseases (angina, vascular disease, etc.), should not have maximal systolic blood pressure (SBP) > 160 mmHg and maximal diastolic blood pressure (DBP) > 100 mmHg or other health conditions which could compromise the achievement of motor tests, and should have normal values of lipid and glycemic profile. The subject medical history was reviewed on their first visit.

2.3. Blood Collection. Plasma samples were prepared from whole blood samples obtained from fasting individuals by venous puncture with heparinized vials for further analysis of oxidant and antioxidant profile and nitrite and nitrate concentration. All participants were informed to avoid foods rich in nitrate (beet, cabbage, spinach, and lettuce) in the last day before the blood collection.

2.3.1. Protein Assay. Proteins were quantified by the method of Lowry et al., which uses a bovine albumin solution in the concentration of 1 mg/mL as standard, using 10 μL of sample. Such quantification was used to correct the calculation of the following analysis: SOD, TBARS, and oxidatively modified proteins [27].

2.3.2. Thiobarbituric Acid Reactive Substances (TBARS). Plasma lipid peroxide levels were determined by measuring TBARS, a common method for measuring the concentration of malondialdehyde, the main breakdown product of oxidized lipids. For the TBARS assay, using 250 μL of sample, trichloroacetic acid (10%, w/v) was added to the homogenate to precipitate proteins and to acidify the samples. This mixture was then centrifuged (4000 rpm, 10 min), the protein-free sample was extracted, and thiobarbituric acid (0.67%, w/v) was added to the reaction medium. The tubes were placed in a water bath (100°C) for 30 min. The absorbencies were measured at 535 nm using a spectrophotometer [28].

2.3.3. Oxidatively Modified Proteins. The protein damage was determined by protein carbonyls measurements, using 200 μL of sample. Plasma samples were incubated with

2,4-dinitrophenylhydrazine (DNPH 10 mM) in a 2.5 M HCl solution for 1 h at room temperature in the dark. Samples were vortexed every 15 min. Subsequently, a 20% trichloroacetic acid (w/v) solution was added and the solution was incubated on ice for 10 min and centrifuged for 5 min at 1000 g to collect protein precipitates. An additional wash was performed with 10% trichloroacetic acid (w/v). The pellet was washed three times with ethanol/ethyl acetate (1 : 1) (v/v). The final precipitates were dissolved in 6 M guanidine hydrochloride solution and incubated for 10 min at 37°C, and the absorbance was measured at 360 nm [29].

2.3.4. Nitrite (NO_2^-) Concentrations. The nitrite content of the samples was analyzed using an ozone-based reductive chemiluminescence assay. For this, 50 μL of plasma samples was injected into a solution of acidified triiodide, purging with nitrogen in line with a gas-phase chemiluminescence NO analyzer (Sievers Model 280 NO Analyzer, Sievers, Boulder, CO, USA). Approximately 8 mL of triiodide solution (2 g of potassium iodide and 1.3 g of iodine dissolved in 40 mL of water with 140 mL of acetic acid) was placed in the purge vessel into which plasma samples were injected. The data were analyzed using the software Origin Lab 6.1 [30].

2.3.5. Nitrate (NO_3^-) Concentrations. To determine the nitrate concentration, 40 μL of plasma was incubated with the same volume of nitrate reductase buffer (0.1 M potassium phosphate, pH 7.5, containing 1 mM β-nicotinamide adenine dinucleotide phosphate and 2 Uf nitrate reductase/mL) in individual wells of a 96-well plate. Samples were allowed to incubate over night at 37°C in the dark. Eighty microliters of freshly prepared Griess reagent (1% sulfanilamide and 0.1% naphthyl ethylenediamine dihydrochloride in 5% phosphoric acid) was added to each well and the plate was incubated for an additional 15 min at room temperature. A standard nitrate curve was obtained by incubating sodium nitrate (0.2–200 mM) with the same reductase buffer [30].

2.3.6. Measurement of SOD Activity. The technique used to measure SOD activity is based on inhibition of the superoxide radical reaction by pyrogallol, a compound that oxidizes itself with the pH variation, leading to formation of a colored product, detected spectrophotometrically at 420 nm for 2 minutes. The percentage of inhibition of initial reaction rates depends on the pH and the quantity of SOD present in the reaction mixtures. Thus, the quantity of enzyme required to inhibit the reaction by 50% is defined as one unit of SOD. 968 μL of Tris-phosphate buffer was added to 50 mM/L (pH 8.2), 8 μL of pyrogallol to 24 mM/L, and 4 μL CAT 30 mM/L in 20 μL of samples. Three different concentrations of SOD (0.25 U, 0.5 U, and 1 U) were used to make the standard curve, which provided an equation of the line for purposes of the calculation. Thus, the SOD activity was determined by measuring the rate of formation of the oxidized pyrogallol [31].

2.3.7. Measurement of GPx Activity. The GPx activity was measured by reaction with glutathione reductase, measuring the consumption of NADPH in the reduction reaction coupled to the GPx reaction, using an enzymatic-colorimetric method by commercial kit (Cayman Chemical Company, Ann Arbor, MI, USA).

2.4. Blood Pressure (BP). Blood pressure was measured after 5 min of rest on three separate days according to the VI Brazilian Hypertension Guidelines [32], using an adequate aneroid sphygmomanometer to the circumference of the arm and a stethoscope placed over the brachial artery.

2.5. Physical Examination. All subjects underwent a physical examination to assess training status (TS), body composition, and anthropometric measurements. Participants were also questioned about the current habits of physical exercise. The baseline testing was performed at least 24 h after the last exercise session.

2.5.1. Training Status (TS). Indirect determination of maximal oxygen uptake ($\dot{V}O_2$max) was performed by a treadmill test using Balke protocol modified for older adults [33], which allowed an estimation of participant's $\dot{V}O_2$max. The "Battery Test" proposed by the "American Alliance for Health, Physical Education, Recreation and Dance" (AAHPERD) was also used to assess the functional fitness of the participants, evaluating the following physical capabilities: coordination, flexibility, muscular strength and endurance, dynamic agility, and cardiovascular endurance as previously described [34–36]. The sum of the percentiles of each test was used to calculate the GFFI. All participants were divided according to the TS (TS1, very weak and weak GFFI: 0 to 199 points; TS2, regular GFFI: 200 to 299 points; TS3, good and very good GFFI: 300 to 500 points). Due to low frequency of participants in the groups "very weak" and "very good," the GFFI classification led to adjustments as the division of groups. Respecting the minimum of 10% of participants in each group to perform the statistical analysis [37], it was decided to unite the groups "very weak" and "weak" and unite the groups "good" and "very good."

2.5.2. Body Composition. Body composition was measured by dual energy X-ray absorptiometry scan (DEXA, Discovery Wi/HOLOGIC Inc., Bedford, USA). The equipment was calibrated before the data collection, according to the manual instructions. After completion of the scans, the body composition was analyzed using the software settings, thus providing the results of total body fat mass, android and gynoid fat mass, and bone mineral density.

2.5.3. Anthropometric Indicators. After anthropometric measurement of waist and hip circumference, body weight, and height, the waist-hip ratio was calculated dividing the waist circumference (cm) by the hip circumference (cm), while body mass index (BMI) was calculated dividing body weight (kg) by the square of the height (m).

2.6. Statistical Analysis. Descriptive statistics was calculated and Pearson's correlation coefficient was performed to detect

Table 1: Subjects' characteristics.

Variables	TS1 (n = 54)	TS2 (n = 40)	TS3 (n = 40)	Ranges of all participants	
				Minimum	Maximum
Age (years)	66.85 ± 5.89	66.47 ± 5.31	63.47 ± 6.76[a]	50.00	79.00
Anthropometry and body composition variables					
Body mass index (kg/m^2)	29.46 ± 5.11	27.22 ± 6.75	27.23 ± 3.83	19.10	35.00
Waist-hip ratio	0.89 ± 0.09	0.88 ± 0.06	0.89 ± 0.08	0.34	1.33
Total body fat mass (%)	42.34 ± 5.07	40.53 ± 5.84	39.71 ± 4.59	28.20	51.95
Android fat mass (%)	43.59 ± 6,44	39.66 ± 7.68[a]	40.68 ± 6.43	20,72	51.77
Gynoid fat mass (%)	42.92 ± 4.65	41.42 ± 5.81	41,30 ± 5.74	25.9	51.07
Bone mineral density (g/cm^2)	0.86 ± 0.10	0.84 ± 0.07	0.79 ± 0.09[a]	0.57	1.10
Functional fitness variables					
Coordination (s)	16.35 ± 4.71	12.26 ± 2.29[a]	10.59 ± 1.74[a]	8.30	37.16
Flexibility (cm)	51.19 ± 10.91	58.24 ± 10.59[a]	61.76 ± 9.23[a]	21.00	80.5
Muscular strength (rep)	18.96 ± 4.05	23.77 ± 3.77[a]	27.35 ± 3.53[ab]	10.00	37.0
Agility (s)	31.54 ± 4.68	24.32 ± 2.69[a]	20.52 ± 2.32[ab]	16.17	45.77
Endurance (s)	584.42 ± 76.39	533.73 ± 52.96[a]	473.05 ± 35.02[ab]	355.00	802.00
GFFI (score)	130.47 ± 42.73	249.40 ± 28.25[a]	361.50 ± 40.21[ab]	35	441
$\dot{V}O_2$max (mL/kg/min)	24.65 ± 6.85	27.54 ± 7.78	30.94 ± 7.46[a]	12.4	49.20
Number of exercises performed	1.24 ± 0.82	1.50 ± 0.81	1.70 ± 1.06[a]	0	5

GFFI, general functional fitness index; TS1, very weak and weak GFFI; TS2, regular GFFI; TS3, good and very good GFFI. Values are mean (SE).
[a] $p < 0.05$ versus TS1.
[b] $p < 0.05$ versus TS2.

correlation among variables. One-way ANOVA with Tukey's post hoc test was performed to assess statistically significant differences between groups ($p < 0.05$). TS was considered as independent variable. The data were analyzed by SPSS 17.0 statistical package. Power analysis was calculated using the prevalence of 30% of hypertension in the Brazilian population and 80% of power and significance level of 5% [32].

3. Results

From all clusters visited, 134 older adult women (65.75 ± 6.14 years old) met our initial inclusion criteria. Figure 1 presents the correlation between $\dot{V}O_2$max and GFFI. It can be observed that good levels in the GFFI were associated with high values of $\dot{V}O_2$max. Even though both variables have a moderate correlation, the authors opted to use the GFFI as independent variable, because the GFFI comprises a multicomponent assessment which is in agreement with American College of Sports Medicine Guidelines for the elderly [24].

Subjects' characteristics are shown in Table 1. TS3 was 3 years younger than other groups. For anthropometrics variables, android fat mass was lower in TS2 and bone mineral density was lower in TS3 compared to the other respective groups. Despite these differences, the sample of this study was considered homogeneous for body composition parameters.

About the TS, as expected, TS3 presented higher values of GFFI and $\dot{V}O_2$max compared to the other groups (Table 1). These results are in accordance with the number of physical exercises that the participant performed.

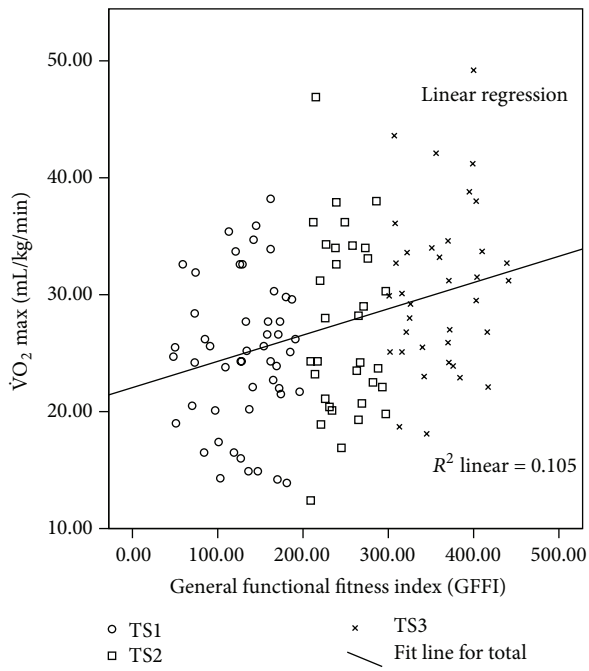

Figure 1: Pearson correlation coefficient between general functional fitness index (GFFI) and $\dot{V}O_2$max. *Correlation is significant at the 0.01 level.

Figure 2 demonstrates the oxidative profile between groups. The lipoperoxidation (TBARS) was lower in TS3 and TS2 compared to TS1 (a), and lower damage to protein was

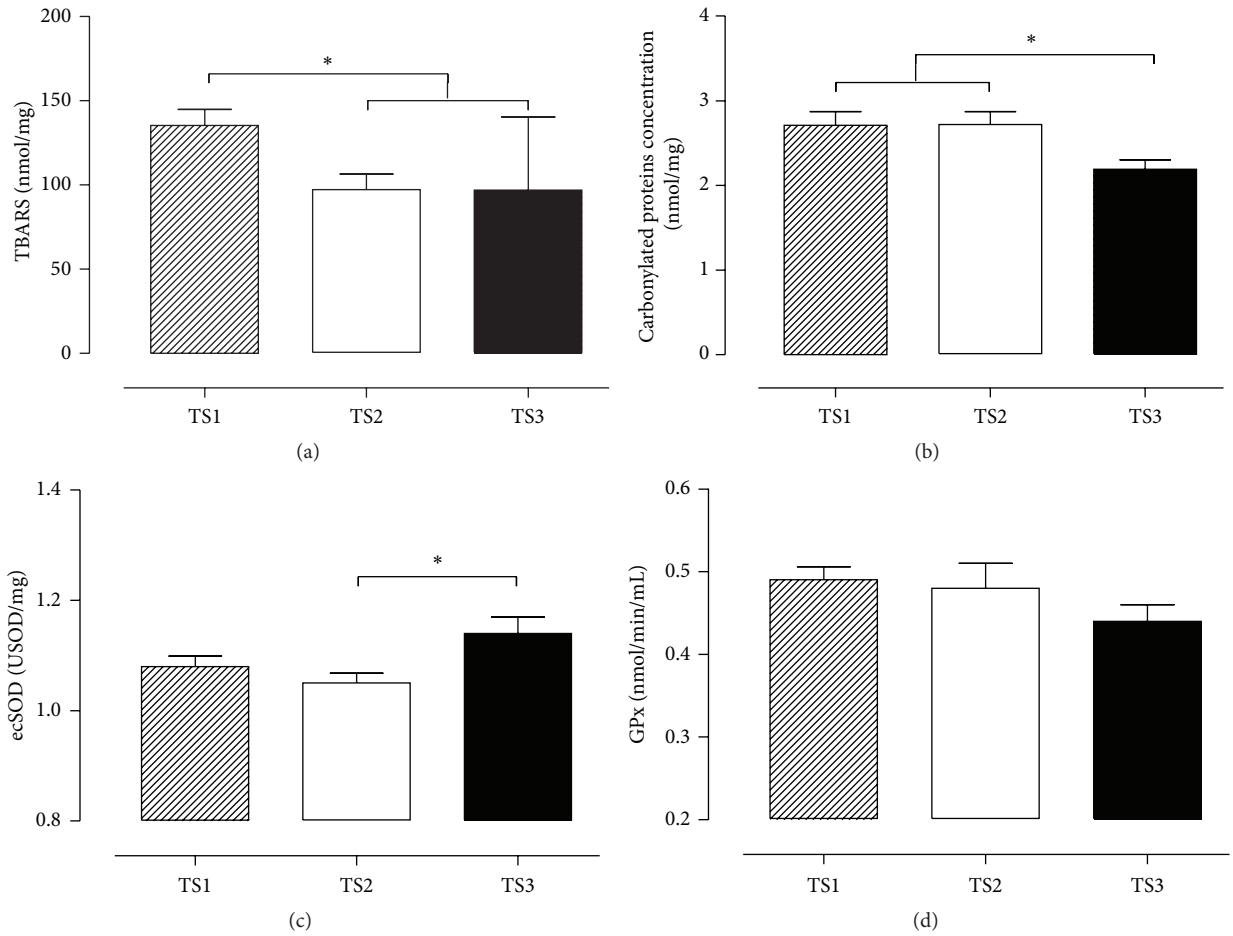

Figure 2: Lipoperoxidation (TBARS) values expressed in nmol/mg protein (a); protein oxidation (carbonyls) values expressed in nmol/mg protein (b); ecSOD activity values expressed in USOD/mg protein (c); GPx activity values expressed in nmol/min/mL (d); TS1, very weak and weak GFFI; TS2, regular GFFI; TS3, good and very good GFFI. $^{*}p < 0.05$.

observed in TS3 (b). For antioxidant capacity, higher SOD activity was observed in TS3 compared to TS2 (c). However, no difference was found in the GPx activity between groups (d).

The results presented in Figure 3 demonstrate that individuals with better TS (TS3) have higher plasma nitrite concentrations compared to TS1 and TS2 groups (a), while TS2 and TS3 show higher values of nitrate concentrations compared to TS1 (b).

For hemodynamic variables, it can be observed that TS3 presented lower values of SBP and DBP compared with TS1 (Figure 4). There was no difference between groups about the use of the number of antihypertensive drugs (TS1 = 0.72 ± 1.07; TS2 = 0.77 ± 0.83; TS3 = 0.75 ± 0.92).

4. Discussion

With the purpose to evaluate if the relationship between BP, NO, and OS can be modulated by TS in older adult women, the general results of this study showed that good levels of TS were related to lower levels of ROS damage (TBARS and protein carbonyls, responsible for measuring membrane and protein damage), higher levels of antioxidant (ecSOD), and higher concentration of nitrite and nitrate. This combination was related to the lower levels of BP in older adult women with good level of training status.

GFFI was chosen to represent the training status of the participants although the correlation between GFFI and $\dot{V}O_2$max was moderate ($r = 0.324$; $p < 0.01$); however previous study of our laboratory demonstrated a good correlation between GFFI and $\dot{V}O_2$max [20]. In addition, the GFFI represents a multicomponent assessment which is in agreement with ACSM guidelines [24].

Regarding OS profile, our results showed that subjects with good level of TS demonstrated lower prooxidant profile and higher antioxidant profile. These results are in accordance with Traustadottir et al., who compared physically fit older adults, men and women, with sedentary age-matched controls, demonstrating that greater physical fitness is associated with lower oxidative stress and greater antioxidant capacity in older adults [33].

Physical exercise is the main stimulus to supply the energy demand required to perform certain activity. Krebs cycle and electron transport chain are stimulated for ATP

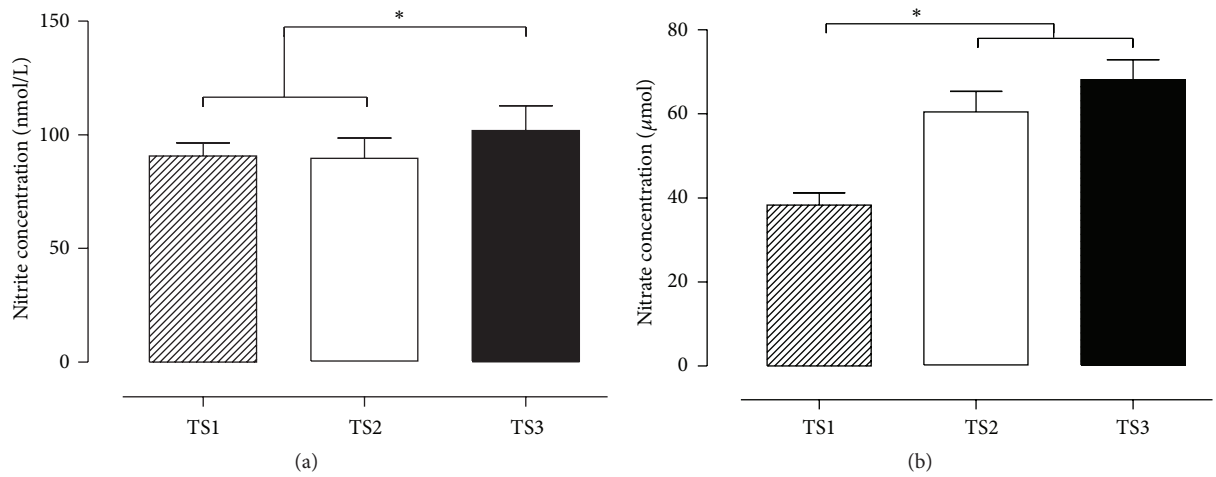

Figure 3: Nitrite values expressed in nmol/L (a); nitrate values expressed in μmol (b); TS1, very weak and weak GFFI; TS2, regular GFFI; TS3, good and very good GFFI. $^{*}p < 0.05$.

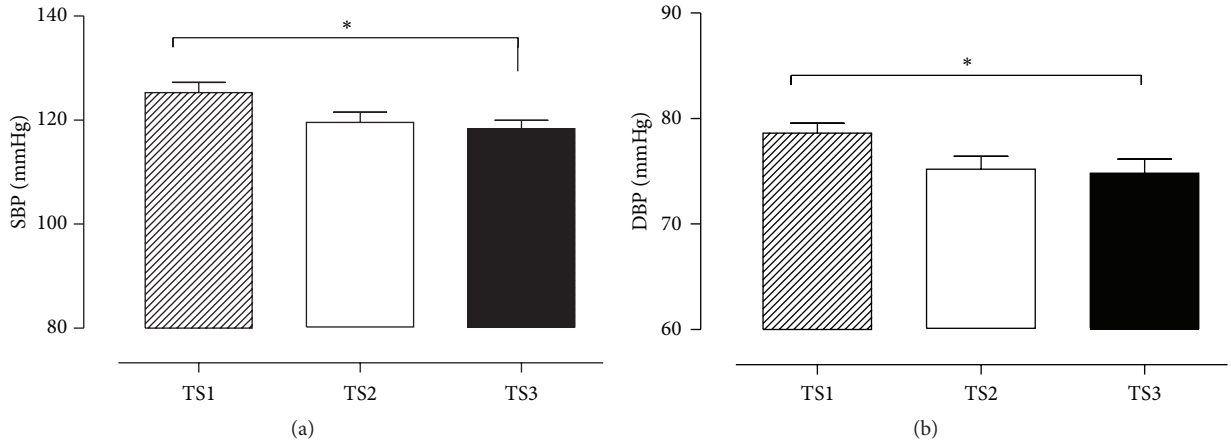

Figure 4: Systolic blood pressure (SBP, (a)) and diastolic blood pressure (DBP, (b)) values expressed in mmHg; TS1, very weak and weak GFFI; TS2, regular GFFI; TS3, good and very good GFFI. $^{*}p < 0.05$.

resynthesis, resulting in a marked increase of ROS, especially $O_2^{-\cdot}$. However, physical exercise also activates some genes responsible for the generation of antioxidant components, such as SOD, CAT, and GPx, as described previously [17, 18].

Together, these results take an important function for blood pressure control due to the increased vasodilatation mechanism. Although there are several conditions that can contribute to endothelial dysfunction, OS levels and aging are involved in hypertension, both leading to an increased production of ROS. Judge et al. compared young and old male Fischer-344 rats and observed a significant increase in OS levels in older animals, which showed a higher concentration of H_2O_2, protein carbonyls, and TBARS and also a significant age-related increases in MnSOD, GPx, and CAT activities [34]. However, in the current study, TS3 was associated with lower OS effect which was associated with higher NO concentration. Considering that the reactions between NO and superoxide anion are most likely a major cause of impaired endothelium dependent vasorelaxation in hypertension [35, 36], the reduction in OS may be responsible for increase in NO bioavailability.

The SOD is one of the major defense systems for removal of $O_2^{-\cdot}$, acting as a catalyst for the dismutation of $O_2^{-\cdot}$ ($O_2^{-\cdot} + O_2^{-\cdot} + 2H^+ \rightarrow O_2 + H_2O_2$), while GPx and CAT play an important role in the elimination of hydrogen peroxide (H_2O_2), promoting its catalysis by water, avoiding the accumulation of $O_2^{-\cdot}$ and H_2O_2, so there is no production of hydroxyl radical, against which there is an enzymatic defense system [37–39]. A lower activity of SOD and GPx enzymes has been observed, besides a high production of H_2O_2 in hypertensive subjects newly diagnosed and untreated, being inversely correlated to BP [40].

The study of Bouzid et al. divided older adults according to their TS level based on answer to a physical activity questionnaire and observed that, during postexercise period, antioxidant activity (ecSOD, GPx, and α-tocopherol) increased in both groups (low and high fitness level), but it was not observed in the sedentary group [41].

Although no difference was found in the GPx activity between groups, the current study showed that better levels of TS (TS3) were associated with higher SOD activity and NO concentration, thus suggesting higher NO bioavailability. According to this finding, a previous study of Trapé et al. also showed that nitrite concentration was higher in better TS group, although there was no difference for TBARS [20]. Additionally, the study of Vasconcelos et al. showed that hypertensive patients showed higher oxidative damage and lower antioxidant markers, suggesting that they were under oxidative stress [42]. Thus, this relationship seems to be responsible for the better BP control.

Besides the fact that all participants have taken the same number of antihypertensive drugs and all values of BP had been considered normal, TS3 presented low values of BP in this study.

Thereby, physical exercise has been related to increased eNOS activity and antioxidant system due to stimulus of peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α). By modulating the ratio of ATP/ADP, which is essential to maximize the energy associated with muscular contraction [43], physical exercise has an important function in the regulation of enzyme adenosine monophosphate kinase (AMPK) activity [44–47]. The activation of AMPK increases the expression of PGC-1α in skeletal muscle [18, 48, 49], which induces the expression of lipid catabolism genes (β-oxidation), electron transport chain [17, 18, 50], and activation of some genes responsible for generation of antioxidant components such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) [17, 18]. Thus, increased expression of PGC-1α by physical exercise is associated with increased antioxidant enzyme activity, thereby opposing the OS.

5. Conclusions

In general, the results of the present study suggest that the relationship between BP, NO, and OS can be modulated by the TS because better results in the TS are associated with lower ROS molecular damage and higher NO bioavailability, both contributing to an improved blood pressure control in older adult women.

It is important to note that the novelty aspect of this study is the way of assessment of fitness. The physical activity can be practiced by different forms promoting different adaptations in human body. Adding that to the ACSM recommendations for multicomponent exercises for elderly people, the general training status seems more adequate to establish the relationship with health. However this statement still has not been established by the literature, as was demonstrated by the current study.

6. Limitations of the Study

The authors understand that ambulatory BP measurements are more accurate than office BP measurements. However, we did not have this technique available at the time of data collection. No specific control of diet was performed; however, participants were requested to have a light meal before the test. The hormonal therapy was not controlled in this study. Although it can be considered one of the factors that affect the endothelial function by altering degradation of NO, the information in the anamneses questionnaire was not enough to be included in this study. The use of antihypertensive drugs was not interrupted during the study.

Conflict of Interests

The authors declare that they have no competing interests.

Acknowledgments

This study was supported by FAPESP "São Paulo Research Foundation" (Grant no. 2013/13146-3) and CAPES research grants.

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Hindawi Publishing Corporation
Oxidative Medicine and Cellular Longevity
Volume 2016, Article ID 3806157, 8 pages
http://dx.doi.org/10.1155/2016/3806157

Review Article

Getting to NO Alzheimer's Disease: Neuroprotection versus Neurotoxicity Mediated by Nitric Oxide

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Received 3 July 2015; Accepted 16 August 2015

Academic Editor: Javier Egea

Alzheimer's disease (AD) is a neurodegenerative disorder involving the loss of neurons in the brain which leads to progressive memory loss and behavioral changes. To date, there are only limited medications for AD and no known cure. Nitric oxide (NO) has long been considered part of the neurotoxic insult caused by neuroinflammation in the Alzheimer's brain. However, focusing on early developments, prior to the appearance of cognitive symptoms, is changing that perception. This has highlighted a compensatory, neuroprotective role for NO that protects synapses by increasing neuronal excitability. A potential mechanism for augmentation of excitability by NO is via modulation of voltage-gated potassium channel activity (Kv7 and Kv2). Identification of the ionic mechanisms and signaling pathways that mediate this protection is an important next step for the field. Harnessing the protective role of NO and related signaling pathways could provide a therapeutic avenue that prevents synapse loss early in disease.

1. Alzheimer's Disease

Dementia is a form of neurodegenerative disorder, generally characterized by a disease specific loss of synapses and neurons which leads to memory impairment, cognitive decline, and eventually death [1]. Alzheimer's disease (AD) is the most common form of dementia, estimated to affect 36 million people worldwide, with this number predicted to triple by 2050 [2]. As the leading cause of disability and with the need for care in older people, the global economic cost associated with AD was estimated to be $604 billion in 2010 [3]. Currently, there is no known cure for AD, with available drugs only effective in mild to moderate cases and limited to treating the symptoms rather than the underlying cause of the disease [4]. As the world's population ages, AD will soon reach epidemic proportions; thus, there is an ever-increasing need for viable treatment options or a cure.

For the majority of AD cases, known as sporadic or late-onset AD, the precise etiology is currently unknown; however, a combination of advanced age and the inheritance of the $\varepsilon 4$ allele of the apolipoprotein E gene can act as significant risk factors [5]. In the rare and inherited form of AD, known as familial or early-onset AD, several genetic mutations have been identified. The most common familial AD mutations occur in either the presenilin-1 or presenilin-2 genes (*PSEN1*, *PSEN2*), with duplications and mutations in the amyloid precursor protein (encoded by *APP*) also linked to the disease [6, 7]. The average age of onset for sporadic AD patients is between 65 and 80 years, while familial patients experience a drastically reduced age of onset, sometimes as early as the mid-20s.

The major neuropathological hallmarks of AD are the accumulation and aggregation of two proteins: β-amyloid (Aβ), in the form of extracellular plaques, and hyperphosphorylated tau, as intracellular neurofibrillary tangles [1, 8]. A pathogenic shift in the processing of the APP by two enzyme complexes, β-secretase and γ-secretase (of which the presenilins are catalytic subunits), results in the production of Aβ peptides [7]. These can form aggregates that disrupt cell signalling, trigger inflammatory immune responses, and cause oxidative stress [9]. When tau, a microtubule-associated protein, becomes hyperphosphorylated, it loses the ability to stabilise neuronal microtubules and abnormally accumulates in axons, dendrites, and cell bodies [10]. This disrupts vital transportation systems within the neuron and can trigger the activation of signaling pathways that lead to

neuronal death [11]. A major problem in the field is that the models used to study AD provide only limited representations of this complex disease. The differences between rodent AD models and the human condition, coupled with a lack of clear understanding of disease progression, have contributed to the limitations of drugs in the clinic for AD.

2. Multifactorial Disease and the Failure of Drugs in the Clinic

AD is a complex and multifactorial disorder, which has made studying disease pathogenesis problematic. Studying snapshots of AD, through the window of postmortem tissue, has led to a complicated and at times uninterpretable mass of data. The key to understanding the disease must lie in engaging in longitudinal studies. Central to this has been the development of agents that can accurately image disease progression, through the analysis of biomarkers. Emerging data from long-term studies suggest that disease pathogenesis commences decades before cognitive decline [12, 13]. Oxidative and nitrosative stress, the result of increased levels of reactive oxygen and nitrogen species, respectively, have been reported in AD brains before the accumulation of Aβ and phosphorylated tau [14, 15]. The production of reactive oxygen and nitrogen species is both exacerbated by and can induce the formation of Aβ and phosphorylated tau [9]. In addition, disruptions to neuronal calcium signalling, mitochondrial dysfunction, and inflammation caused by the activation of microglia have all been reported to contribute to AD pathogenesis [16, 17]. Collectively, these pathogenic mechanisms result in synaptic loss and neuronal death, especially for cholinergic neurons found in the brain regions responsible for memory and language [18]. Ultimately, the disease spreads throughout the brain contributing to cognitive decline and eventually leading to death.

The complex pathogenesis of AD, coupled with the inaccessible nature of human brain tissue, has hindered the identification and development of prospective pharmaceuticals. During the period of 1998 to 2011, it is estimated that over 100 potential compounds targeting the treatment of AD have failed in the clinic, leaving only a handful of approved therapeutics addressing the cognitive symptoms but not the disease itself [19]. The primary pharmaceuticals currently available to AD patients are cholinesterase inhibitors (Donepezil, Rivastigmine, and Galantamine) and NMDA receptor antagonists (Memantine). These drugs have been shown to reduce memory loss and slow disease progression temporarily in some patients by 6–12 months [20]. With the development of imaging agents that can measure amyloid deposition, along with an improved knowledge of genetic risk factors, the possibilities for discerning the early events in disease pathogenesis are becoming a reality. Now it is essential that there is investment in longitudinal studies to investigate genetic contributions to disease processes in patients. In considering the development of effective drugs for AD, we need to identify early events that could afford protection to neurons and synapses. Recent findings suggest that one signaling molecule that warrants further investigation is nitric oxide.

3. Nitric Oxide in Alzheimer's Disease: Mechanisms and Effects

As a gasotransmitter that is freely diffusible across membranes, nitric oxide (NO) makes for a powerful signaling molecule with far-reaching cellular consequences that can be both protective and maladaptive. The multiple physiological effects of NO as a vasodilator, inflammatory mediator, and neuromodulator allow for a coordinated effect on brain function. NO is synthesized by three distinct genes, *NOS1*, *NOS2*, and *NOS3*, that encode the neuronal, inducible, and endothelial NO synthases (nNOS, iNOS, and eNOS), respectively. Both nNOS and eNOS are constitutively expressed, with their activation dependent on Ca^{2+}/calmodulin, whilst iNOS expression is induced in inflammatory cells and is not dependent on Ca^{2+}/calmodulin. Each of these cell types (neurons, endothelial cells, and inflammatory cells) is altered in Alzheimer's brain. There are deficits in the cerebrovasculature, characterized by the breakdown of the blood-brain barrier, as well as increased inflammatory signaling and alterations in neuronal signaling, all key components of AD [21]. Each of the three NOS isoforms has been postulated to play a role in either AD progression or prevention, leading to a seemingly conflicting message about the role of NO in AD and whether NO is neuroprotective or neurotoxic.

The signaling pathways of NO converge on three main cellular effects, all of which have been identified to play a role in AD: signaling via soluble guanylate cyclase and the cyclic guanosine monophosphate (cGMP) pathway [22]; direct S-nitrosylation of protein cysteine residues (addition of a nitrosyl ion NO^- to generate a nitrosothiol, RS-N=O) (reviewed in [23]); and protein tyrosine nitration (addition of nitrogen dioxide NO_2 to generate 3-nitrotyrosine) [24]. Diversion of NO signaling towards one of these pathways over another depends on the local cellular microenvironment, including levels of transition metal complexes and redox status [25]. In addition, at high concentrations, NO reacts with superoxide anion that is formed as a by-product of respiration, to generate peroxynitrite ($ONOO^-$), a highly reactive oxidant and cytotoxic species [26]. Thus, the production of peroxynitrite links high levels of NO release with oxidative stress. The numerous effects of NO in the multicellular environment of the brain have complicated the analysis of NO in the etiology of AD. Previous studies in postmortem tissue and animal models have yielded a complex proposition of NOS expression changes and NO signaling in AD (Table 1).

Synthesizing this data to assess a meaningful role for NO and NOS activity in AD is an impossible task due to the study of different brain regions and disease states, in addition to using different techniques and markers to quantitate NO. In the context of a multicellular environment, such as the brain, the source of NO (i.e., from which NOS enzyme, the signaling molecule, is derived) is important when considering normal physiological roles versus pathological effects. For example, iNOS releases higher levels of NO (up to the micromolar range), compared to nanomolar levels by eNOS or nNOS [27]. Because of its short half-life, there is a limited ability for NO to diffuse in three-dimensional space [28]. Thus, the source of NO directly affects its local concentration. Even in

Table 1: Alterations in the expression and activity of nitric oxide synthase (NOS) enzymes in Alzheimer's disease (AD) tissue and animal models.

Author	Methods	Tissue type/control	Results
Hyman et al., 1992 [65]	Immunocytochemistry staining of NOS in neurons using rabbit polyclonal antibody and peroxidase linked secondary antibody.	Hippocampus and temporal neocortex from AD and control postmortem brains. AD mean age: 80.85 ± 2.0 years. Control mean age: 60.4 ± 5.9 years. Five control subjects had brain abnormalities upon postmortem examination.	No significant difference between the expressions of NOS in AD neurons in comparison to controls.
Dorheim et al., 1994 [66]	L-Citrulline (coproduct of NO) was used as a marker of NOS activity in microvessels.	Brain microvessels from AD and control patients.	Significant increase in NOS activity in AD brain microvessels.
Benzing and Mufson, 1995 [67]	Nicotinamide adenine dinucleotide phosphate-diaphorase (NADPH-d) used as a marker for NOS in neurons.	AD and control postmortem brains (age, sex, brain weight, and postmortem interval matched).	Significantly higher levels of NADPH expression in AD neurons in comparison to controls.
Norris et al., 1996 [68]	mRNA expression levels of nNOS and NADPH-d staining used as markers for nNOS expression.	Frontal cortex, visual cortex, and hippocampus of AD and control postmortem brains.	A decrease (not significant) in cellular abundance of nNOS in AD brains in comparison to controls. A significant decrease in the number of cells expressing nNOS in distinct brain regions.
Gargiulo et al., 2000 [69]	Immunohistochemistry staining with monoclonal antibodies for NOS and protein kinase C (PKC) expression.	Regions of the temporalis gyrus from AD and control postmortem brains.	A significant decrease in NOS levels from AD brains, no change in PKC expression levels.
Lüth et al., 2001 [70]	Immunohistochemistry and western blotting of iNOS and eNOS expression levels in three tissue types.	Sporadic AD postmortem brains, human APP transgenic mice, and electrolytic cortical lesions in rat tissue. Age and postmortem interval matched for human controls. Aged and nontransgenic mice matched controls.	Increased expression of iNOS and eNOS in both human AD and transgenic mice reactive astrocytes in comparison to controls.
Venturini et al., 2002 [71]	Optical, fluorescence, and NMR spectroscopy was used to determine Aβ_{25-35} interaction with NADPH-d and downstream effects on NOS activity.	Neuronal and glioma-like rat cell lines and appropriate controls.	Aβ_{23-35} interacts with NADPH-d, decreasing the availability of the substrate for cNOS and strongly reducing cNOS activity.
Stepanichev et al., 2008 [72]	NADPH-d histo- and immunocytochemistry used as a marker for nNOS and iNOS expression.	Cerebral and hippocampus Aβ_{25-35} administered rat tissue and non-Aβ rat tissue as controls.	Aβ_{25-35} did not influence nNOS or iNOS mRNA or protein expression. Aβ_{25-35} increased nNOS activity but not iNOS.

the case of a single neuron, events at the cell body do not necessarily translate to signaling at the synapse. New studies need to be carried out which can address the precise temporal and spatial NO signaling at the synapse and at extrasynaptic sites. This precise localization of NO most likely underlies the differential neuroprotective versus neurotoxic effects.

4. Neuroprotective versus Neurotoxic Effects of Nitric Oxide

An area of controversy in regard to the involvement of NO in AD pathogenesis is the extent to which the molecule is neuroprotective or neurotoxic [29–31]. Several studies have demonstrated that NO holds neuroprotective properties through its induction of the cGMP pathway [32–34]. This triggers vasodilation and consecutive increases in the cerebral blood supply to neurons, reducing the potential for oxidative stress, in addition to minimizing excess Ca^{2+} influx through inhibition of NMDA receptors at glutamatergic synapses [32–34].

Within the brain approximately 15% of the oxygen consumed is reduced in a one-electron transfer to superoxide, the main downstream component of oxidative stress. The ability of NO to easily cross local membranes thus allows it

to react with free superoxide from cellular respiration [35]. The resultant peroxynitrite from the NO/superoxide pathway has been shown to induce lipid peroxidation, which can result in Ca^{2+} dysfunction, as well as functional alterations to proteins through S-nitrosylation of cysteine residues and nitration of tyrosine residues, both molecular markers of AD [36–38]. Further evidence suggests that the upregulation of constitutive NOS leads to the uncoupling of the enzyme, with the resultant formation of peroxynitrite overriding the neuroprotective cGMP pathway [34, 39]. Discrepancies could be due in part to the challenges of measuring NO and peroxynitrite concentrations in situ (half-life < 3 s and half-life < 1 s, resp.), which prevents a clear distinction of the formation of neurotoxic peroxynitrite at the expense of protective NO [26]. Developments in more precise nanotechnology based measurements for NO and peroxynitrite have helped to demonstrate that the hypothesized cytotoxic effects of NO in AD are only observed once NO has been converted to peroxynitrite [26, 40, 41]. Further developments in the accuracy of methods to measure NO and peroxynitrite are required to fully appreciate the roles of these signaling molecules in AD.

A primary activator of nitrosative stress in AD is the release of excess Ca^{2+} into the cytosol from the overstimulation of NMDA receptors, a concept known as excitotoxicity [29, 42]. Under physiological conditions, repetitive stimulation of NMDA receptors is considered to strengthen long-term potentiation (LTP), enhancing synaptic plasticity in neurons and the encoding of memory and learning [43]. However, prolonged, high intensity activation of extrasynaptic NMDA receptors triggers cell death pathways [44]. It has been demonstrated that nNOS is colocalized with NMDA receptors in the postsynaptic density and that after Ca^{2+} influx into postsynaptic neurons NO acts as a retrograde messenger providing a positive feedback mechanism to maintain glutamate release through the NMDA receptors, strengthening LTP [35, 45]. However, NO has also been found to inhibit NMDA receptors through cGMP induction [33]. A significant reduction in NMDA receptors in the hippocampus and cortex of postmortem AD brains has also been observed [46, 47]. The reduction in NMDA receptors is postulated to underlie the cognitive decline of AD, with the upregulation of NO a compensatory yet potentially neurotoxic mechanism to increase glutamate release in attempts to maintain LTP [48, 49]. Close associations of nNOS and NMDA receptors are central to this compensatory role of NO. Understanding the signaling pathways of synaptic versus extrasynaptic receptors is the next challenge for the field.

5. The Contribution of Nitrosative Stress to Aβ and Tau Pathology

A rare consensus in the literature regarding NOS and NO in AD is that iNOS expression is increased in microglia and astrocytes during Aβ elicited inflammatory and immune responses [30, 50, 51]. This increased expression of iNOS in microglia and astrocytes generates elevated levels of NO and peroxynitrite through the NO/superoxide pathway, in addition to ROS and other neurotoxic molecules that can lead to neuronal death [50, 51]. Removal of iNOS in transgenic AD mice or the use of iNOS inhibitors to block NO production has been shown to protect against Aβ induced neurotoxicity, indicating that nitrosative stress may be one of the key factors mediating Aβ pathogenesis in AD [50, 52]. The involvement of NO in facilitating the neuropathogenesis of Aβ highlights the potentially significant role of NO in disease progression.

Increased NO synthesis due to overactivation of neuronal NMDA receptors and microglial activation, in combination with the properties that allow retrograde messenger activity, has implicated both intracellular and extracellular sources of pathogenic NO to neurons [31]. In aging rat hippocampal neurons nitrotyrosination of presenilin-1 caused it to increase Aβ1-42 production in a similar manner observed by *PSEN1* mutations associated with familial AD [37]. This finding points to a specific mechanism for how nitrosative stress could potentially induce one of the hallmarks of AD [37]. Furthermore, although the presence of S-nitrosylated proteins and tau has been demonstrated in AD, prolonged NO exposure can induce the formation of cytoplasmic tau oligomers in SH-SY5Y cells, providing evidence of a potential mechanism underlying tau neuropathogenesis in AD [53]. Experiments in cell lines using overexpression of AD markers have provided clues to mechanism but this research has a limited capacity to model AD effectively. Recapitulation of findings in clinically relevant samples is now essential. Induced pluripotent stem cells derived from sporadic Alzheimer's patients provide that opportunity.

6. Potassium Channels and Nitric Oxide Augmentation of Synaptic Plasticity and Neuronal Excitability

One of the major problems with the study of postmortem tissue is that the very cells required for study (i.e., vulnerable neurons) have been lost in the disease, which can lead to difficulties in assessing the changes identified between cases and controls. An important level of study in AD research is identifying early changes in neurons that may lead to degeneration or survival. The manipulation of these pathways may then provide potential targets for interventions.

Recent studies using AD mouse models have been used to identify early changes in neurons that could be targeted. The 3x Tg-AD mouse model bears mutations in three genes involved in familial AD: *APP*; *PSEN1*; and *MAPT*, encoding amyloid precursor protein; presenilin-1 (part of the γ-secretase complex); and tau. Consequently, these mice exhibit progressive neuropathology, including plaques and tangles, in addition to hippocampal synaptic dysfunction. An early characteristic of Alzheimer's brain is the loss of synapses and this occurs prior to memory loss. Synapse density therefore provides a better correlate with cognitive deficits than the classic hallmarks of plaques or tangles [54, 55]. A study of presymptomatic AD mice (i.e., before the development of cognitive behavioral changes) indicated that NO functions to maintain both LTP and long-term depression, while increasing the probability of neurotransmitter release [56].

In this way, NO signaling pathways are altered as a means to promote synaptic plasticity. Together these data suggest that NO increases the excitability of presynaptic neurons to promote neurotransmitter release in a pathologically dampened system. It remains unclear how NO mediates increased excitability of presynaptic neurons. However, the most likely mechanism is modulation of presynaptic ion channel activity. M-channels (Kv7 channels), as critical regulators of neuronal excitability, provide a possible candidate for this role. M-channels are voltage-gated outward potassium channels that remain open at the resting membrane potential of neurons. As such, increases in M-current reduce neuronal excitability, while M-current inhibition increases action potential firing. In sensory neurons NO is a potent neuromodulator with the ability to increase excitability by inhibiting M-current [57, 58]. NO-mediated changes in excitability have been identified in the mouse hippocampus, modulating outward potassium currents [31]. These effects included potentiation of Kv2 currents and suppression of Kv3 currents, which together promoted sustained action potential firing [31]. Indeed M-channel modulators are being touted as therapeutic possibilities for AD, amongst other neuronal excitability disorders [59]. Modifiers of potassium channel activity and neuronal excitability could therefore yield effective drug targets.

7. Nitric Oxide Suppression of Inflammatory Signaling

Other recent studies have also challenged the view that NO and proinflammatory factors drive disease progression in AD. Manipulating the effect of inducible NO in mice to levels equivalent to those in humans has led to some interesting results. These data suggest that local immune suppression, rather than immune activation, leads to degeneration of specific brain regions [60]. Elevated cerebrovascular NO levels increase NFκB/p65 signaling in epithelial cells, preventing leukocyte trafficking [61]. The AD mouse model termed 5xFAD overexpresses mutant human *APP* with the Swedish (K670N, M671L), Florida (I716V), and London (V717I) mutations along with human *PSEN1* bearing two mutations (M146L and L286V). Consequently, in 5xFAD mice, scavenging NO boosts the trafficking capacity of epithelial cells and enhances recruitment of monocytes/macrophages into the brain from the periphery [61]. Consistent with this, systemic administration of the NO scavenger, rutin, reduces amyloid plaques in various mouse models of AD [62, 63], potentially via increased macrophage recruitment to the CNS and more rapid clearing of Aβ. In assessing the reasons why numerous AD drugs have failed in the clinic, first we have to address the extensive time difference in disease progression in humans compared to the mouse models in which the drugs were tested. Development of clinically representative models and the timing of interventions need to be further considered. As a field we need to establish universality in sampling selection and longitudinal studies that represent a timespan appropriate to disease processes (e.g., 20 years). Central to this is a commitment to long-term funding of such projects.

8. Summary

It is difficult to identify the specific contribution of NO and the extent to which NO synthases influence the development of AD. Human postmortem brain tissue has provided a snapshot of the final stages of the disease but has failed to represent early changes in the AD brain. Meanwhile, animal models have provided a more dynamic insight into disease pathogenesis but do not represent the complexities of the sporadic human disease. Future studies need to utilize human patient-derived neuronal cell models to elucidate the contribution of NO to neuroprotection and neurotoxicity in AD [64]. The development of inhibitors of the specific NOS isoforms for use in AD models will help to elucidate the neuroprotective and neurotoxic sources of NO. An emphasis on identifying the ionic mechanisms that cause alterations in NO-mediated excitability in AD may lead to the development of new drug targets.

New data suggest that alterations in NO signaling function as a compensatory mechanism to coordinate neuroprotective responses at the failing synapse and that the loss of local immune responses and amyloid clearance are likely more relevant to disease pathogenesis than increases in proinflammatory neurotoxic signaling. Identifying suitable NO or voltage-gated potassium channel modulatory drugs could therefore provide preventive action against synaptic loss and AD pathology.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Hindawi Publishing Corporation
Oxidative Medicine and Cellular Longevity
Volume 2016, Article ID 5346327, 10 pages
http://dx.doi.org/10.1155/2016/5346327

Review Article

Role for Tetrahydrobiopterin in the Fetoplacental Endothelial Dysfunction in Maternal Supraphysiological Hypercholesterolemia

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Received 3 July 2015; Accepted 23 August 2015

Academic Editor: Joern Steinert

Maternal physiological hypercholesterolemia occurs during pregnancy, ensuring normal fetal development. In some cases, the maternal plasma cholesterol level increases to above this physiological range, leading to maternal supraphysiological hypercholesterolemia (MSPH). This condition results in endothelial dysfunction and atherosclerosis in the fetal and placental vasculature. The fetal and placental endothelial dysfunction is related to alterations in the L-arginine/nitric oxide (NO) pathway and the arginase/urea pathway and results in reduced NO production. The level of tetrahydrobiopterin (BH_4), a cofactor for endothelial NO synthase (eNOS), is reduced in nonpregnant women who have hypercholesterolemia, which favors the generation of the superoxide anion rather than NO (from eNOS), causing endothelial dysfunction. However, it is unknown whether MSPH is associated with changes in the level or metabolism of BH_4; as a result, eNOS function is not well understood. This review summarizes the available information on the potential link between MSPH and BH_4 in causing human fetoplacental vascular endothelial dysfunction, which may be crucial for understanding the deleterious effects of MSPH on fetal growth and development.

1. Introduction

Hypercholesterolemia is considered one of the most important risk factors for the development of cardiovascular disease (Adult Treatment Panel ATP III) [1, 2]. Pregnancy is a physiological process that can involve the development of maternal pathologies, such as preeclampsia, gestational diabetes mellitus (GDM), and metabolic disorders, including maternal pregestational obesity, supraphysiological gestational weight gain (spGWG) [3, 4], and hypercholesterolemia [5, 6]. These alterations may compromise the health of the mother and/or the fetus [5–7]. In normal pregnancies, the mother exhibits a physiological (i.e., normal) increase (30–50%) in the plasma total cholesterol (TCh) level in a process that is referred to as maternal physiological hypercholesterolemia (MPH) [5, 6, 8]. However, in some cases, for mostly unknown

reasons, the TCh level is elevated far beyond the physiological range, which is referred to as maternal supraphysiological hypercholesterolemia (MSPH) [5, 6, 9]. Although studies have shown the potential adverse effects of MSPH on the early development of atherosclerosis [10, 11] and endothelial dysfunction in the fetoplacental vasculature [5, 6], the global prevalence of MSPH remains unknown [12].

Endothelial cells synthetize nitric oxide (NO), a potent vasodilator that is generated by NO synthases (NOS; i.e., the L-arginine/NO pathway), following the oxidation of L-arginine in a process that depends on the bioactivity of several cofactors, including tetrahydrobiopterin (BH_4) [13–15]. In pregnant women with MSPH, the umbilical vein dilation and endothelial NOS (eNOS) activity are reduced, and arginase (ARG) activity is increased compared with MPH [5, 6]. Remarkably, ARG inhibition results in a partial restoration of human umbilical vein dilation, suggesting that other factors play a role in this phenomenon. In nonpregnant women, hypercholesterolemia reduces the NO bioavailability by mechanisms that include a reduction in BH_4 levels [16]. Reduced activity and/or level of BH_4 favor(s) the generation of a superoxide anion ($O_2^{\bullet-}$) instead of NO, resulting in endothelial dysfunction [14, 17, 18]. Altogether, this indicates that elevated plasma TCh levels may result in reduced NO synthesis via several mechanisms, leading to endothelial dysfunction. The potential effect of MSPH on BH_4 availability and regulation of the biosynthesis of this cofactor, as well as its consequences in the modulation of fetal endothelial function, are unknown [5, 6]. This review summarizes the findings regarding a potential link between MSPH and BH_4 and human fetoplacental vascular endothelial dysfunction.

2. Hypercholesterolemia

In nonpregnant women, hypercholesterolemia is mainly related to genetic mutations involving genes that are related to cholesterol traffic, such as lipoprotein receptors and cholesterol transporters, metabolic disorders, and an imbalanced diet [2, 19]. According to the Third Report of the National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III), this condition is the main risk factor for the development of cardiovascular disease (CVD) [1, 2]. Therefore, the proper management of plasma cholesterol levels can reduce the risk of developing CVD [20]. For this reason, the clinical *cut-off* point for normal blood TCh in nonpregnant women is rigorously controlled and suggested to be <200 mg/dL [1].

A higher risk of hypercholesterolemia-associated CVD also results from a reduced blood level of high-density lipoprotein cholesterol (HDL, i.e., <40 mg/dL) and/or an increased blood level of low-density lipoprotein cholesterol (LDL, i.e., >100 mg/dL); the latter depends on the global cardiovascular risk, as recently recommended in the Guideline on the Treatment of Blood Cholesterol to Reduce Atherosclerotic Cardiovascular Risk in Adults from the American Heart Association [21]. Although CVD is normally diagnosed in adults, some studies indicate that endothelial dysfunction (i.e., an initial phenomenon in the development of atherosclerosis) and early atherosclerotic lesions (i.e., fatty streaks) begin during intrauterine life, in fetal vessels, as a consequence of increased levels of maternal cholesterol [5, 6, 10]. This indicates the relevance of monitoring the potential adverse effects of maternal cholesterolemia during pregnancy on the fetal vasculature [22]. This is even more important because the current global prevalence for a high plasma concentration of TCh (>200 mg/dL) in nonpregnant women is ~40% [23]. It is conceivable that a significant number of pregnant women will have increased plasma levels of cholesterol, exposing them to the inherent consequences of this condition as well as the associated fetal vascular alterations [12].

Hypercholesterolemia during Pregnancy. Pregnancy is a physiological condition that is characterized by progressive weeks of gestation-dependent increases (increasing 40–50%) in the maternal blood level of cholesterol and triglycerides [24, 25]. MPH is considered an adaptive response of the mother to satisfy the high cholesterol demand of the growing fetus for membrane and hormone synthesis [26]. Unfortunately, there are no established clinical reference values for the total and lipoprotein cholesterol levels during pregnancy in the global population, including the pregnant Chilean population [5, 6, 8]. A summary of the literature on the TCh, lipoprotein cholesterol, and triglyceride concentrations per trimester of pregnancy for different populations is shown in Table 1. Based on the literature, the estimated mean values for TCh are 179, 226, and 257 mg/dL for the 1st (1–13 weeks of pregnancy), 2nd (14–28 weeks of pregnancy), and 3rd (28–40 weeks of pregnancy) trimesters of pregnancy, respectively. For HDL and LDL, the mean values were 62 and 101, 73 and 131, and 68 and 149 mg/dL for the 1st, 2nd, and 3rd trimesters of pregnancy, respectively. MSPH has been defined by considering an at-term *cut-off* point for TCh of 280–300 mg/dL [5, 11, 27], and it is associated with vascular alterations at birth [5] and during childhood [28]. Additionally, increased oxidative stress in the maternal and fetal blood and placenta [27] as well as reduced expression of placental LDL receptors [29] was found in pregnancies that had maternal TCh levels that were higher than this *cut-off* point.

Although lipid trafficking through the placenta is restrictive and children born to mothers with MSPH have normal blood cholesterol levels [5, 31], a positive correlation between the maternal and fetal blood cholesterol in the 2nd and 3rd trimesters of pregnancy has been established [11, 32]. Moreover, increased early atherosclerotic markers, such as fatty streaks and lipid peroxidation, were found in the aortas of human fetuses [11] as well as in 7- to 14-year-old children [12] who were born to mothers with MSPH. Furthermore, endothelial dysfunction in the human umbilical vein from pregnancies with TCh values that were higher than this *cut-off* point has been proposed to be associated with reduced endothelium dependent vascular relaxation, due to lower eNOS and higher ARG activity [5]. These results provide evidence for the potential effect of MSPH in the placenta, leading to adverse consequences for the fetal vasculature. Interestingly, there is limited information on the prevalence of MSPH in the global population, which is mainly because

Table 1: Reported maternal plasma lipid concentration in pregnancy.

Studied population (number of pregnant women)	Trimester of pregnancy	TCh	HDL	LDL	Tg	Observations	Reference
USA (142)	1	180	70	110	112	Maternal overweight and obesity association with lipid concentration during pregnancy	[37]
	2	230	80	137	162		
	3	260	76	162	212		
Brazil (288)	1	186	54	108	97	Maternal lipid concentration during pregnancy as a risk factor for GDM	[38]
	2	228	62	143	150		
	3	243	62	135	177		
Argentina (101)	1	160	58	78	90	Measurement of maternal plasma lipids during pregnancy	[39]
	2	201	62	107	140		
	3	244	61	144	202		
Chile (265)	1	178	60	102	108	Maternal lipid concentration association with impaired endothelium dependent dilation of the human umbilical vein	[6]
	2	232	73	124	179		
	3	269	75	147	244		
Chile (74)	1	—	—	—	—	Cut-off point for TCh in maternal plasma from where fetoplacental vascular dysfunction is seen	[5]
	2	—	—	—	—		
	3	238	72	120	232		
UK (178)	1	215	67	124	125	Measurement of maternal plasma lipids and apolipoproteins during pregnancy	[40]
	2	252	81	126	180		
	3	281	69	159	252		
UK (17)	1	164	—	—	77	Measurement of maternal plasma lipids and markers of oxidative stress in normal and GDM pregnancies	[41]
	2	212	—	—	133		
	3	261	—	—	233		
Ireland (222)	1	197	65	104	—	Reference values for maternal lipids during pregnancy	[42]
	2	224	75	128	—		
	3	278	68	147	—		
Italy (22)	1	178	68	97	93	Measurement of maternal plasma lipids during pregnancy	[43]
	2	247	73	153	155		
	3	282	68	168	230		
Sweden (18)	1	182	69	104	99	Measurement of maternal plasma lipids during pregnancy	[44]
	2	238	79	140	165		
	3	248	69	153	215		
Spain (45)	1	166	—	—	71	Measurement of maternal plasma LDL oxidation in normal, GDM, and obese pregnancies	[45]
	2	193	—	—	106		
	3	228	—	—	150		
Spain (25)	1	170	68	89	60	Maternal lipases activity and hormones concentrations during pregnancy	[46]
	2	234	82	136	117		
	3	254	71	153	184		
Serbia (50)	1	190	75	97	85	Maternal lipid concentration association with newborn size	[47]
	2	245	89	126	151		
	3	267	79	144	219		
Turkey (801)	1	166	53	94	93	Maternal lipid concentrations with fetal growth and development in GDM and preeclampsia	[48]
	2	—	—	—	—		
	3	271	63	155	274		
Israel (3938)	1	175	58	88	100	Association of maternal lipid concentration with preeclampsia and GDM	[49]
	2	225	63	121	175		
	3	238	63	137	237		
Nigeria (60)	1	172	41	112	93	Atherosclerotic risk in pregnant women	[50]
	2	203	51	126	128		
	3	232	63	136	171		

Women were subjected to lipids determination at 1st trimester (0–14 weeks of gestation), 2nd trimester (14–28 weeks of gestation), or 3rd trimester (28–40 weeks of gestation) of pregnancy. TCh: total cholesterol; LDL: low-density lipoprotein; HDL: high-density lipoprotein; Tg: triglycerides. —: not reported; GDM: gestational diabetes mellitus. Values are mean in mg/dL.

the maternal blood cholesterol level is not routinely evaluated during pregnancy. Moreover, in a group of pregnant Chilean women, the prevalence of this maternal condition was ~30% [5, 6]. As a result, a significantly higher number of pregnant women will potentially present with an adverse intrauterine condition that could result in the development of vascular alterations in the growing fetus, such as endothelial dysfunction and early atherosclerosis.

3. Fetoplacental Endothelial Dysfunction in MSPH

The placenta is a physical and metabolic barrier between the fetal and maternal circulations, and it is a crucial organ that supports proper fetal development [33]. Because the placenta and umbilical cord lack autonomic innervation [34], a balance between circulating vasodilators and vasoconstrictors is crucial to maintaining normal fetoplacental function [33, 35]. Endothelial dysfunction is defined as an imbalance between vasodilator and vasoconstrictor molecules that are produced by or acting on endothelial cells [36] and that are critical for normal fetal development.

3.1. L-Arginine/NO Pathway. NO is a gas derived from the metabolism of L-arginine via the enzyme NOS, in a metabolic reaction where there is equimolar generation of L-citrulline and NO (as in the L-arginine/NO pathway) [7]. NOS are a group of enzymes with at least three isoforms that are encoded by different genes in mammals [57], that is, neuronal NOS (nNOS or type 1), inducible NOS (iNOS or type 2), and endothelial NOS (eNOS or type 3). eNOS is the main form that is expressed in endothelial cells [3], and reduced activity of this enzyme may result from lower expression, reduced activation, or increased inactivation [58, 59]. eNOS activity is modulated by different agents, including the level of its cofactor BH_4 and posttranslational phosphorylation/dephosphorylation. For example, phosphorylation of serine 1177 (Ser^{1177}) via PI3 kinase/Akt is associated with higher activity [60]; however, phosphorylation of threonine 495 (Thr^{495}) via protein kinase C (PKC) maintains a low activity of this enzyme [58, 61]. Remarkably, hypercholesterolemia is associated with reduced eNOS expression, an effect that is reversed by restoring the cholesterol levels with the use of statins, for example, [62–64]. Additionally, it has been shown that cholesterol regulates the phosphorylation of eNOS. In mice and pigs, there is a negative correlation between the TCh level and the activation-phosphorylation of Ser^{1177} [65, 66]. On the other hand, HDL also induces Ser^{1177} phosphorylation of eNOS [67]. It was recently shown that, in fetoplacental macrovascular endothelial cells from pregnant women with MSPH, eNOS activity, but not its protein abundance, is reduced [5]. However, $Ser^{1177}$and Thr^{495}phosphorylation was reduced compared to cells from pregnant women with MPH. As a result, an altered maternal cholesterol level may modify the eNOS activity in pregnancy.

3.2. ARGs/Urea Pathway. ARGs (ARG-I and ARG-II) are a family of enzymes that compete with NOS for the substrate L-arginine, leading to the synthesis of L-ornithine and urea [68]. Interestingly, hypercholesterolemia is associated with increased ARG activity in animal models [69, 70] and humans [71, 72]. The activity of ARGs is also increased in HUVECs from pregnancies with MSPH compared with MPH pregnancies [5]. Because the pharmacological blockade of ARG with *S*-(2-boronoetil)-L-cysteine (BEC) partially reverses the reduced eNOS activity observed in HUVECs in MSPH pregnancies, ARGs are likely involved in modulating eNOS activity in this cell type [4].

4. BH_4 Metabolism in MSPH

4.1. BH_4. BH_4 is a cofactor required for NOS activity because this molecule stabilizes the enzyme as an active dimer, allowing for optimal oxidation of L-arginine into NO [13, 15]. A reduction in the BH_4 level leads to reduced eNOS activation, which is likely due to the uncoupling that results in the generation of superoxide anion ($O_2^{\bullet-}$) rather than NO, promoting vascular oxidative stress and endothelial dysfunction [14]. In the endothelium, BH_4 is synthesized by at least two metabolic pathways: *de novo* biosynthesis from guanosine triphosphate (GTP) and the salvage pathway from sepiapterin to BH_2 and BH_4 [14] (Figure 1(a)). *De novo* biosynthesis involves the sequential action of GTP cyclohydrolase 1 (GTPCH1), 6-piruvoil tetrahydropterin synthase (PTPS), and sepiapterin reductase (SR). The GTPCH1 step is the limiting step of the pathway, and it is highly regulated at the transcriptional, translational, and posttranslational levels [73]. For the salvage pathway, the reduction of BH_2 to BH_4 is the limiting step and requires the enzyme dihydrofolate reductase (DHFR) [73]. The BH_4 level could be reduced by decreased synthesis and by the oxidation of BH_4 to BH_2 via oxygen-derived reactive species and peroxynitrite ($ONOO^-$), resulting in eNOS uncoupling (Figure 1(b)) [13, 18, 74]. The latter is a phenomenon that occurs in a variety of clinical conditions that are associated with vascular disease, including diabetes mellitus, hypertension, atherosclerosis [75–77], and hyperglycemia [18].

4.2. BH_4 Metabolism in Hypercholesterolemia. Patients with hypercholesterolemia have low NO availability [78, 79] as well as a lower BH_4 level (Table 2). Interestingly, oral or local supplementation with BH_4 restores the impaired NO-dependent vasodilation in subjects with hypercholesterolemia [13, 52, 55, 80]. The association between human hypercholesterolemia and a reduced level of GTPCH1 has not yet been addressed [14, 18]. However, reduced eNOS activity is reversed by supplementation with the BH_4 substrate sepiapterin or by GTPCH1 overexpression in mice [81–83]. As a result, this enzyme likely plays a role in hypercholesterolemia. Incubation with human LDL reduces NOS and GTPCH1 expression in rat vascular smooth muscle cells [84, 85]. Additionally, a reduced level of GTPCH1 due to hyperglycemia in human aortic endothelial cells decreases the BH_4 level and NO synthesis, which is reversed by GTPCH1 overexpression [86]. Interestingly, and in corroboration with these findings, there are results showing similar changes in HUVECs that

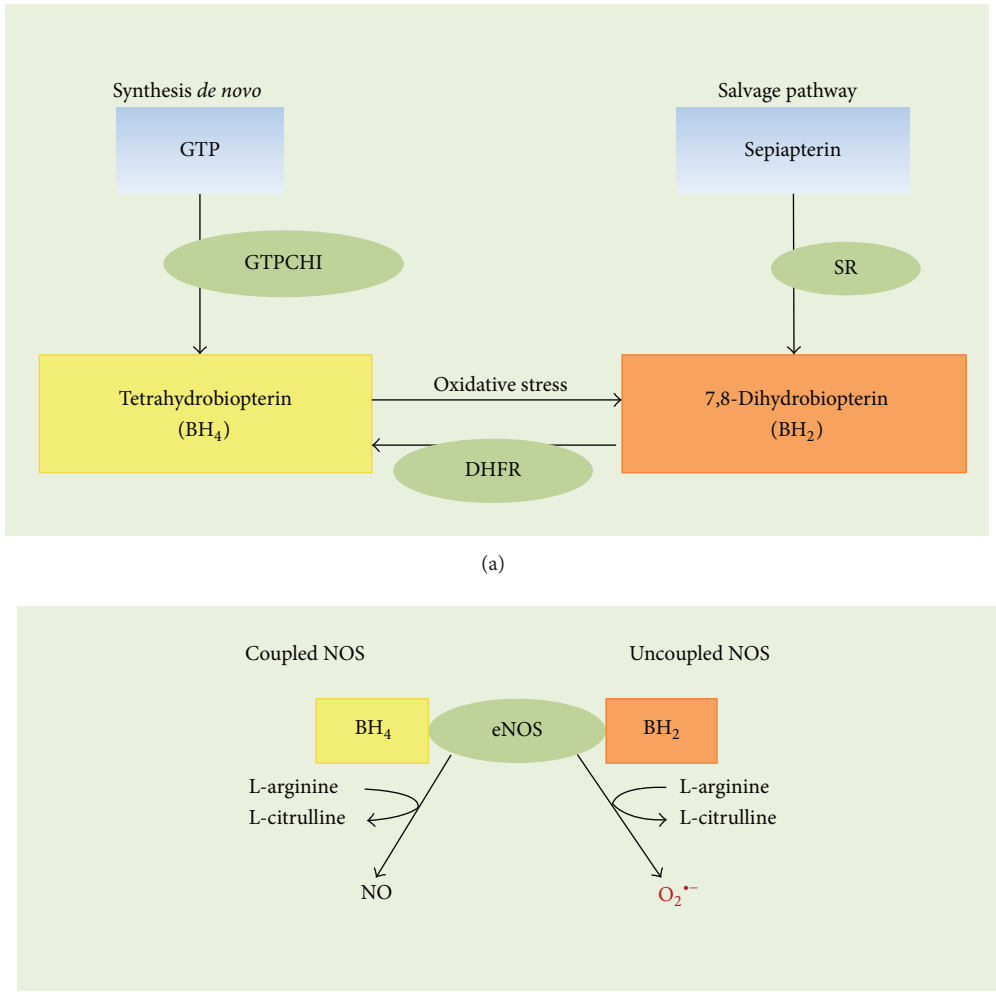

FIGURE 1: Tetrahydrobiopterin metabolism and endothelial nitric oxide synthase uncoupling. (a) The first step in the *de novo* synthesis of tetrahydrobiopterin (BH_4) is the rate limiting reaction involving the enzyme GTP cyclohydrolase 1 (GTPCH1), whose substrate is GTP. An alternative *salvage pathway* for BH_4 synthesis is the reduction of 7,8-dihydrobiopterin (BH_2) to BH_4 by the enzyme dihydrofolate reductase (DHFR). BH_2 is generated from sepiapterin by the sepiapterin reductase enzyme (SR). *Oxidative stress* may be an environmental condition that promotes the oxidation of BH_4 to BH_2, decreasing the bioavailability of BH_4. (b) Under physiological conditions, nitric oxide synthases (NOS, *coupled NOS*) generate nitric oxide, following the metabolism of L-arginine into L-citrulline in the presence of BH_4. However, uncoupling NOS (*uncoupled eNOS*) with these enzymes may result in the generation of a superoxide anion ($O_2^{\bullet -}$). This phenomenon results from a deficiency in BH_4 and an increased BH_2 bioavailability (from data in [6, 17, 30]).

were subjected to the pharmacological induction of GTPCH1 expression [87].

The potential effect of MSPH on BH_4 availability in the regulation of the synthesis of this cofactor and its effect on the modulation of fetal endothelial function are unknown [5, 6]. Because NO synthesis is reduced in the fetal endothelium from pregnancies with MSPH via mechanisms involving increased ARG but reduced eNOS activity, it is hypothesized that this maternal condition could also result in reduced BH_4 bioavailability for NO synthesis. As a result, these potential mechanisms could explain the endothelial dysfunction and reduced vascular relaxation observed in MSPH. Preliminary results show that the BH_4 level is reduced in HUVECs from MSPH [88] (Leiva A., Sobrevia L., *unpublished*). However, it is unknown whether BH_4 metabolism is altered in human fetoplacental endothelial cells in pregnancies with MSPH or whether restoration of the BH_4 level improves the endothelial dysfunction in MSPH [4–6, 33].

5. Concluding Remarks

The prevalence of MSPH in the global population has not been evaluated, although it is estimated as approximately 30% [5, 6]. MSPH is a factor that favors the development of vascular changes in the growing fetus and, eventually, in children [12]. These vascular disorders include endothelial

Table 2: Effect of hypercholesterolemia on tetrahydrobiopterin availability and endothelial function.

Study model	Tissue or cell type	Experimental condition	BH_4 level	Parameter	Effect	Reference
Hypercholesterolemia	Human brachial artery	Basal	Reduced	Endothelium dependent vasodilation	Reduced	[13]
		BH_4 infusion	Increased	Endothelium dependent vasodilation	Increased	
Hypercholesterolemia	Human coronary artery	Basal	Reduced	Coronary artery diameter and flow	Reduced	[51]
		BH_4 infusion	Increased	Coronary artery diameter and flow	Increased	
Hypercholesterolemia	Human brachial artery	Basal	Reduced	Endothelium dependent vasodilation	Reduced	[52]
		BH_4 supplementation	Increased	Endothelium dependent vasodilation	Increased	
Hypercholesterolemia	Human coronary microcirculation	Basal	nr	Myocardial blood flow	Reduced	[53]
		BH_4 infusion	nr	Myocardial blood flow	Increased	
Hypercholesterolemia	Human skin	Basal	nr	Endothelium dependent vasodilation	Reduced	[54]
		R-BH_4 infusion	nr	Endothelium dependent vasodilation	Increased	
		S-BH_4 infusion	nr	Endothelium dependent vasodilation	Reduced	
Hypercholesterolemia	Human skin	Basal	nr	Endothelium dependent vasodilation	Reduced	[55]
		BH_4 infusion	nr	Endothelium dependent vasodilation	Increased	
Cell culture	Human mesenteric microvascular endothelial cells	Incubation with oxLDL	Reduced	NO generation	Reduced	[56]
			Reduced	Superoxide generation	Increased	
		Incubation with oxLDL + sepiapterin	Increased	NO generation	Increased	
			Increased	Superoxide generation	Reduced	
Cell culture	Human aortic endothelial cells	Incubation with LDL	Reduced	NO generation	Reduced	[52]
		Incubation with LDL + BH_4	nr	NO generation	Increased	

Basal corresponds to no treatment. BH_4: tetrahydrobiopterin; R-BH_4: R-tetrahydrobiopterin (NO synthase cofactor and antioxidant); S-BH_4: stereoisomer of BH_4 (antioxidant); oxLDL: oxidized low-density lipoprotein; LDL: low-density lipoprotein; NO: nitric oxide; nr: not reported.

dysfunction in the fetus and placenta, disrupting the equilibration between the ARG/urea and L-arginine/NO signaling pathways. However, it is unknown whether these alterations correlate with the degree of MSPH in pregnancy or the alterations in BH_4 metabolism and eNOS function (Figure 2). Drugs that control the TCh plasma level in adult, nonpregnant subjects are not used during pregnancy. This condition limits our present knowledge regarding the correlation between the mother's and fetus's TCh level and the vascular function of the fetus during pregnancy. However, based on the available evidence from subjects with hypercholesterolemia, we propose that restoration of the BH_4 level will improve the fetoplacental endothelial function in humans. Therefore, it is essential to focus future studies on exploring the dynamics of the BH_4 metabolism in MSPH pregnancies and the possible contribution that restoring this cofactor could have on this maternal condition and vascular function.

Conflict of Interests

There is no conflict of interests.

Authors' Contribution

Andrea Leiva and Luis Sobrevia designed research study; Andrea Leiva, Bárbara Fuenzalida, and Carlos Salomón collected clinical data; Andrea Leiva, Bárbara Fuenzalida, Francisco Westermeier, Fernando Toledo, Jaime Gutiérrez, Carlos Sanhueza, and Fabián Pardo collected and analyzed literature

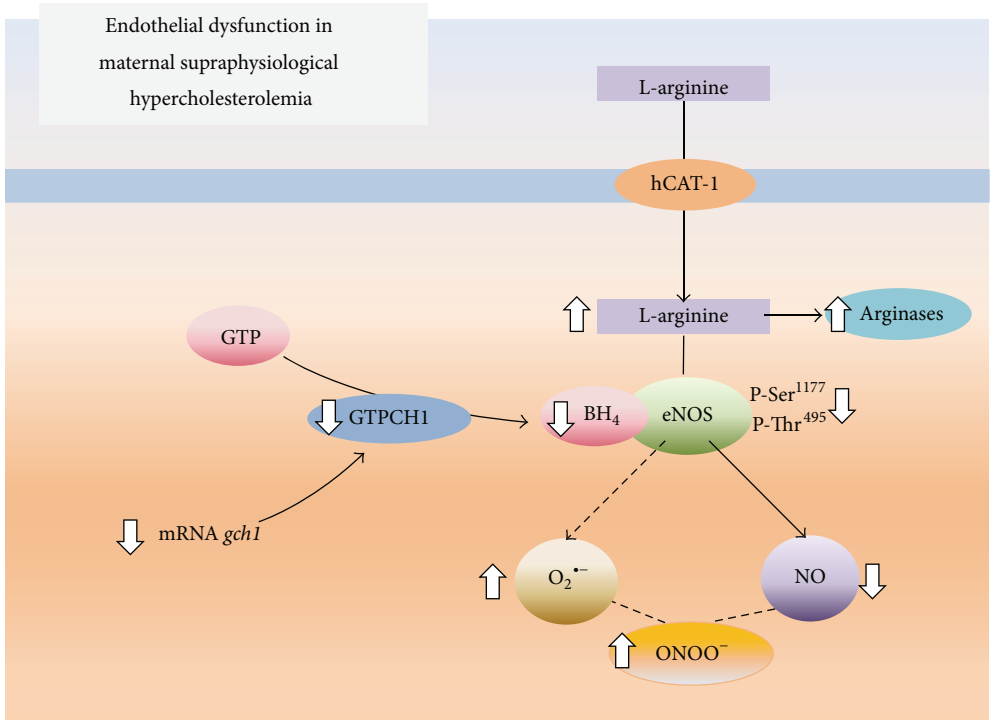

Figure 2: Effect of maternal supraphysiological hypercholesterolemia on the endothelial L-arginine/NO signaling pathway. In umbilical vein endothelial cells from pregnancies complicated by maternal physiological hypercholesterolemia, the amino acid L-arginine is taken up by the human cationic amino acid transporter 1 (hCAT-1) and metabolized by endothelial nitric oxide synthase (eNOS) and, to a lesser extent, arginases. This phenomenon occurs in the presence of tetrahydrobiopterin (BH_4), resulting in NO generation. BH_4 is generated by the enzyme GTP cyclohydrolase 1 (GTPCH1), which is coded by the *gch1* gene and whose substrate is GTP. In cells from pregnancies where the pregnant women had maternal supraphysiological hypercholesterolemia, hCAT-1-mediated L-arginine transport is increased (⇑), increasing the availability of this amino acid for eNOS and arginases. In this pathological condition, L-arginine is mainly used by arginases, limiting the formation of NO via eNOS. In addition, eNOS has reduced (⇓) activity because of the lower phosphorylation of Ser^{1177} and the bioavailability of BH_4. The reduction in the BH_4 concentration results from a reduced expression of *gch1*, leading to eNOS uncoupling and the generation of a superoxide anion ($O_2^{\bullet-}$). The $O_2^{\bullet-}$ reacts with NO to form peroxynitrite ($ONOO^-$; from data in [5–7, 14]).

information; Andrea Leiva and Luis Sobrevia designed the figures; Andrea Leiva, Bárbara Fuenzalida, Fernando Toledo, and Fabián Pardo constructed the tables; Andrea Leiva, Bárbara Fuenzalida, and Luis Sobrevia wrote the paper.

Acknowledgments

The authors thank Mrs. Amparo Pacheco and Mrs. Ninoska Muñoz from CMPL, Pontificia Universidad Católica de Chile (PUC), for excellent technical and secretarial assistance, respectively. This work was supported by Fondo Nacional de Desarrollo Científico y Tecnológico (FONDECYT 1150344, 1150377, 3130583, 3140516, and 3140532), Chile.

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Hindawi Publishing Corporation
Oxidative Medicine and Cellular Longevity
Volume 2016, Article ID 5681036, 9 pages
http://dx.doi.org/10.1155/2016/5681036

Review Article

Nitric Oxide-Mediated Posttranslational Modifications: Impacts at the Synapse

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Received 15 May 2015; Accepted 28 June 2015

Academic Editor: Vittorio Calabrese

Nitric oxide (NO) is an important gasotransmitter molecule that is involved in numerous physiological processes throughout the nervous system. In addition to its involvement in physiological plasticity processes (long-term potentiation, LTP; long-term depression, LTD) which can include NMDAR-mediated calcium-dependent activation of neuronal nitric oxide synthase (nNOS), new insights into physiological and pathological consequences of nitrergic signalling have recently emerged. In addition to the canonical cGMP-mediated signalling, NO is also implicated in numerous pathways involving posttranslational modifications. In this review we discuss the multiple effects of S-nitrosylation and 3-nitrotyrosination on proteins with potential modulation of function but limit the analyses to signalling involved in synaptic transmission and vesicular release. Here, crucial proteins which mediate synaptic transmission can undergo posttranslational modifications with either pre- or postsynaptic origin. During normal brain function, both pathways serve as important cellular signalling cascades that modulate a diverse array of physiological processes, including synaptic plasticity, transcriptional activity, and neuronal survival. In contrast, evidence suggests that aging and disease can induce nitrosative stress *via* excessive NO production. Consequently, uncontrolled S-nitrosylation/3-nitrotyrosination can occur and represent pathological features that contribute to the onset and progression of various neurodegenerative diseases, including Parkinson's, Alzheimer's, and Huntington's.

1. Introduction

Since its characterisation in the early 1980s by Furchgott, Ignarro and others [1–3], nitric oxide (NO) has been widely recognised as an important signalling molecule in many physiological processes. The initial identification of NO as endothelium-derived relaxing factor (EDRF) [4] generated a great interest in its function in vascular biology. Over subsequent years, the focus on NO research rapidly expanded from the vascular system to its role in immunity and inflammation, cell death, cell survival, and aging, to name but a few. Of particular interest is its role within the nervous system and its function in neuronal signalling. NO was first identified to be present in the central nervous system by the discovery of one of its synthesising enzymes, neuronal nitric oxide synthase (nNOS), within the mammalian brain [5]. Aside from its production through nNOS, NO can also be synthesised through activation of either one of the two other nitric oxide synthases termed endothelial nitric oxide synthase (eNOS) and inducible nitric oxide synthase (iNOS) [6]. After synthesis, NO can bind to its predominant physiological receptor soluble guanylyl cyclase (sGC) to catalyse the conversion of guanosine-5′-triphosphate (GTP) to cyclic guanosine monophosphate (cGMP). From here cGMP can regulate the activity of many downstream targets such as the modulation of protein kinases and ion channels, demonstrating that relatively low amounts of generated NO can be amplified substantially through this signalling pathway. Following the initial characterisation of NO, its diverse function was soon recognized throughout the nervous system [7]. NO generation *via* nNOS in response to NMDAR activation was one of the earliest pathways characterised in the brain [7, 8] and it became evident that NO could serve as an important signalling molecule within neurons. Involvement

of NO ranges from synaptic plasticity and activity modulation [9], such as LTP/LTD, to pathological actions seen in many neurodegenerative conditions [10].

It is now generally acknowledged that, in addition to the canonical sGC/cGMP pathway mentioned above, NO has additional roles in modulating protein function *via* induction of posttranslational modifications. NO can lead to thiol nitrosylation of cysteine residues termed S-nitrosylation (–SNO, covalent and reversible attachment of an NO molecule to a thiol group [–SH]) and tyrosine nitration termed 3-nitrotyrosination (NO_2-Tyr *via* peroxynitrite formation [$ONOO^-$], Figure 1). These modifications impact on protein-protein interactions, protein structure, and function and are largely generated through the excessive production of NO which occurs through overactivation of nNOS or induction of iNOS *via* neuroinflammatory stimuli or additional toxins. Although S-nitrosylation is an important modulator of protein function under physiological conditions, it is largely detrimental under pathophysiological conditions due to the high levels of reactive oxygen species and reactive nitrogen species present. Similarly, tyrosine nitration is predominantly damaging due to its occurrence in environments where toxic peroxynitrite is generated. An important and differing characteristic of the two processes is that S-nitrosylation is a reversible mechanism, the equilibrium of which can be shifted by the activities of reductases, namely, thioredoxin or S-nitrosoglutathione reductase [11, 12], whereas 3-nitrotyrosination is an irreversible modification. Furthermore, the equilibrium between nitrosylation and denitrosylation can be differentially affected during disease and aging which may then further perpetuate these processes making it an important signalling pathway in physiology and pathology.

The above modifications have been implicated in many cellular processes, such as modulation of transcription factors, membrane receptors, and general effects on neuronal development, health, and survival or differentiation [10, 11, 13–16]. The mechanisms by which nitrergic activity can regulate gene expression and thereby determine the fate of a neuron can be widespread [17]; however, this review focuses specifically on direct nitrergic effects related to synaptic function and transmitter release.

As the likelihood of S-nitrosylation increases in a hydrophobic environment [11, 12], proteins attached to the membrane or localized within cellular membrane microdomains are highly susceptible to these modifications. This therefore applies not only, for instance, to postsynaptic density proteins and neurotransmitter receptors, but also to membrane-associated molecules of the presynaptic release machinery. Although NO targets include ion channels, which are involved in setting neuronal excitability and calcium homeostasis [18–23] and are thereby indirectly involved in synaptic function, we do not cover this area as this topic has been reviewed elsewhere [24, 25].

2. Impacts of NO Signalling at the Synapse

Glutamate is the major excitatory neurotransmitter in the mammalian central nervous system and its downstream

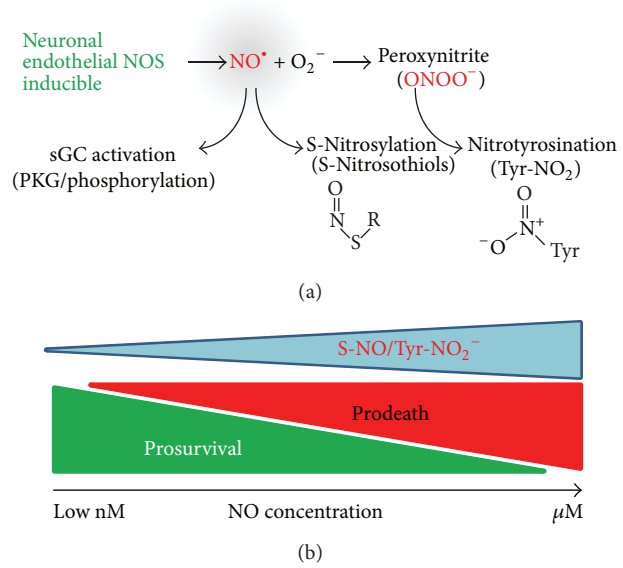


Figure 1: Nitric oxide profile and posttranslational modifications. This figure indicates pathways of NO generation and posttranslational modifications. (a) Generation of NO by the three different NO synthases leads to activation of the sGC and thiol nitrosylation forming S-nitrosothiols. Further reaction of NO with oxygen radicals leads to the formation of peroxynitrite and subsequent irreversible modification of tyrosine residues. (b) Concentration dependency between NO levels and the amount of posttranslational modifications with associated dominance of prosurvival or prodeath signalling.

functions are mediated by metabotropic and ionotropic glutamate receptors. Several groups have reported nitrergic modulation of glutamate signalling. It has been shown that NO was able to stimulate glutamate release in hippocampal slices [26] and increase glutamate release in the rat and mouse hippocampus or rat dorsomedial medulla oblongata [27–29]. However, the molecular mechanisms responsible for these nitrergic modifications are not fully understood and in addition to the canonical pathways posttranslational modifications at the synaptic level may explain some of the above findings [30].

The release of GABA, an inhibitory neurotransmitter, has been shown to be negatively affected by NO in the internal granule cell layer of the cerebellum [31] and in auditory cortical neurons [32]. However, NO can induce an increase in GABA release, as systematically found in other parts of the brain [33]. Furthermore, it has been shown that NO can regulate GABA release differentially in a concentration-dependent manner. There is evidence that basal or low NO concentrations decrease GABA release, whereas high NO concentrations could augment GABA release [34, 35]. In the other brain regions, data are inconsistent with NO mediating an increased GABA release from synaptic vesicles into the cytosol rendering vesicles to be GABA depleted [36], while, in other studies, NO, *via* peroxynitrite formation, induced a potentiation of evoked GABA release [37], and nNOS inhibition resulted in enhanced GABAergic inputs into CA1 pyramidal neurons [38]. These unexplained discrepancies in

nitrergic effects are most likely due to its multiple modes of action depending on the cellular environment and illustrate the broad range of NO signalling pathways.

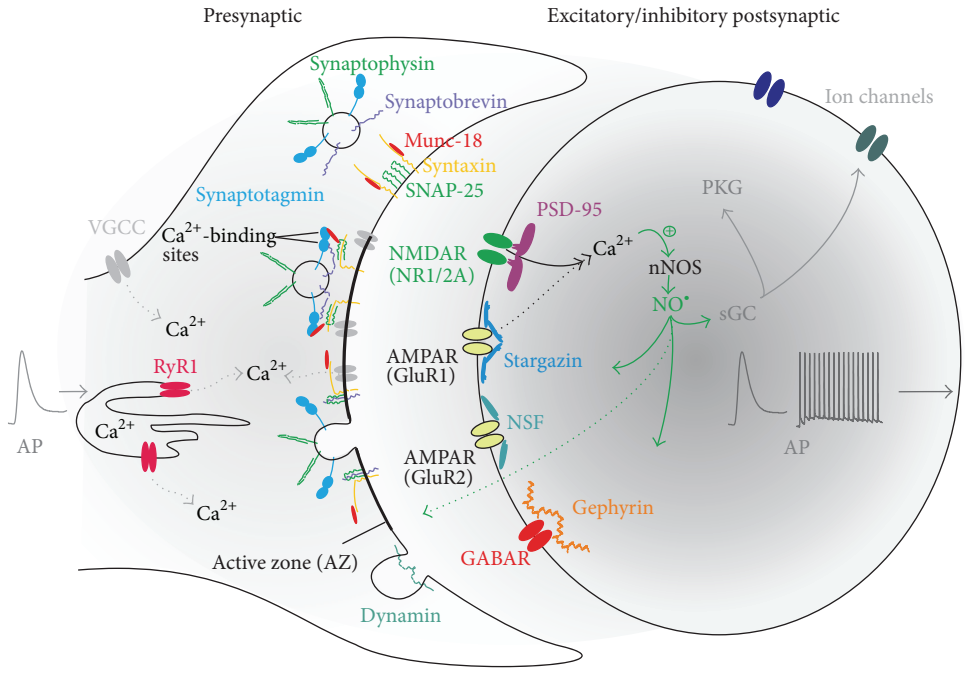
Dopamine (DA) is a neurotransmitter present at high concentrations in specific brain regions of the central nervous system, that is, in the mesencephalon (substantia nigra) [39], and NO-mediated enhanced DA release was initially reported *in vitro* and *in vivo* in the striatum [40, 41]. However, in the intact bovine retina, NO significantly decreased basal or potassium-induced DA release, while NOS inhibition stimulated basal release of DA [42]. As a decrease in DA release is a key contributor to Parkinson's disease, the link between oxidative stress/NO/$ONOO^-$ and deficient DA release could relate nitrergic posttranslational modifications to the pathology observed in this disease.

Many of the above data suggest that NO can bidirectionally, possibly in a concentration-dependent manner, regulate transmitter release and postsynaptic responsiveness. Although some of these data indicate cGMP involvement, this is not always the case and posttranslational modifications, as investigated in more recent years, could well explain the observed discrepancies. We discuss below some specific synaptic nitrergic effects related to overall transmitter release and neuronal communication.

2.1. Presynapse: NO at the Synaptic Release Machinery. The presynaptic site represents a crucial part in synaptic transmission. Here, release of vesicles, either filled with excitatory or inhibitory neurotransmitters, by exocytosis and reuptake *via* endocytosis, is essential for neuronal communication [43–45]. There are numerous presynaptic signalling molecules whose regulation determines the amount and availability of released transmitter [43–46]. In general, Ca^{2+} triggers release in a highly cooperative manner [47] within a few hundred microseconds of an incoming action potential [48]. There are several key steps involved in this process which enable molecular mechanisms to deliver fast vesicle fusion at a synapse (see below); however, due to the complexity and specificity of these mechanisms, any modulation of involved molecules contributing to release, that is, by NO, will affect synaptic vesicular fusion. To name just a few involved in these cascades, the Ca^{2+} sensor synaptotagmin, following Ca^{2+} binding, triggers vesicular release by stimulating its interaction with a core fusion machinery composed of SNARE (soluble *N*-ethylmaleimide sensitive factor attachment protein receptor) and synaptic membrane proteins that mediate fusion during exocytosis (Figure 2). Complexin adaptor proteins assist synaptotagmin by activating and clamping this core fusion mechanism so that synaptic vesicles containing synaptotagmin are positioned at the active zone ready for release. Through the specific binding of these specialised proteins (SNARE and Sec1/Munc-18 proteins) a connection is formed between synaptic vesicles and the synaptic membrane resulting in them being brought to close proximity to one another. This enables both rapid fusion and release of synaptic vesicles [49]. There are two types of SNARE proteins that constitute the core complex and these are known as vesicular v-SNAREs and target t-SNAREs. v-SNAREs, such as synaptobrevin/VAMP, are located largely on the synaptic vesicles, whereas t-SNAREs, such as syntaxin or SNAP-25, are located mainly on the plasma membrane. During fusion both t-SNAREs and v-SNAREs form α-helical trans-SNARE complex that facilitates the tight association of the two membranes. The three SNARE proteins SNAP-25, syntaxin-1, and synaptobrevin are essential components of the synaptic vesicle fusion machinery [44]. Any modulation of any of these proteins could potentially have dramatic impacts on release. Studies have shown that a lack of synaptobrevin-2 activity in mouse hippocampal knockout neurons resulted in a drastic reduction of evoked release with virtually no ready releasable pool available in these neurons [50]. Similarly, modifications of SNAP-25 either by removing the 9 C-terminal residues (SNAP-25Δ9) or by substituting C-terminus amino acids with more positively charged residues resulted in reduced release frequencies, slower release kinetics, and prolonged fusion pore duration [51] confirming the tight regulatory role of these proteins in vesicle fusion. Additionally mutant mice expressing a constitutively open syntaxin-1, which enhances SNARE complex formation, showed an increased speed of evoked release, enhanced Ca^{2+} affinity of release, and accelerated fusion pore expansion [52]. Fast neurotransmission depends not only on coordinated release but also on replenishment of vesicle pools which depends on endocytotic pathways. One molecule involved in endocytosis is the large GTPase dynamin which induces fission of the vesicle from the plasma membrane as shown by using the temperature-sensitive allele of *dynamin* that blocks all synaptic endocytosis at restrictive temperatures [53, 54]. Below we discuss studies reporting nitrergic modulation of several of the above synaptic molecules; however, the literature only partially offers insights into how posttranslational modifications (PTM) can induce functional changes and are largely observational.

2.1.1. S-Nitrosylation. In an earlier study, S-nitrosylation has been investigated in purified synaptic vesicle fractions and it was found that NO donor application led to widespread thiol modifications of proteins [55]; however, this study did not identify any specific residues but concluded that syntaxin-1, SNAP-25, and synaptobrevin of the SNARE complex of synaptic molecules were affected. One of them, syntaxin, is participating in exocytosis and is believed to be stabilized by the classical binding of Munc-18. This critical binding mode of Munc-18 to the closed conformation of syntaxin has been shown to be disrupted following Cys 145 S-nitrosylation [56], thereby reversing the inhibition and facilitating interaction with the membrane fusion machinery. This provides scope on the possibility of S-nitrosylation forming a regulatory mechanism to modulate this inhibitory interaction and subsequently allowing the binding of syntaxin with other SNARE proteins to induce membrane fusion [56]. Interestingly the sequence surrounding the Cys 145 (syntaxin-1) is highly conserved in all neuronal syntaxins implying a general modulatory function of this modification as shown in adrenal chromaffin cells [56] but also in β-cells where S-nitrosylation of Cys 141 (syntaxin-4) augments insulin exocytosis [57].

Another important regulatory protein which is predominantly involved in the scission of newly formed vesicles



(a)

S-Nitrosylation		3-Nitrotyrosination	
SNAP-25		SNAP-25	
Dynamin	Cys 86, Cys 607	Munc-18	
Syntaxin-1	Cys 145	Synaptophysin	Tyr 250
Syntaxin-4	Cys 141	Dynamin	Tyr 354
Synaptobrevin		Synaptotagmin	Tyr 151, Tyr 216, Tyr 229, Tyr 311, Tyr 364, Tyr 380
Stargazin			
PSD-95			
Gephyrin			
GABAR			
NMDAR1	Cys 744, Cys 798		
NMDAR2A	Cys 399		
RyR1			
NSF			

(b)

Figure 2: Simplified pathways of synaptic transmission and effects of nitrergic posttranslational modifications. (a) Left, presynapse; right, postsynapse: showing signalling steps involved in transmitter release and neurotransmitter receptor function. Ca^{2+}-dependent activation of postsynaptic nNOS leads to nitrergic modifications of molecules (colour-coded) which are involved in synaptic transmission in pre- and postsynaptic compartments. NO diffuses within the postsynaptic cell but also into the presynapse. Presynaptic assembly of the prefusion SNARE complex (such as the vesicular v-SNARE complex protein synaptobrevin assemblies with the plasma membrane SNARE proteins syntaxin and SNAP-25) and membrane proteins in close proximity to the active zone (AZ) results in a prefusion state of the vesicle. In a further step, Munc-18 associates with syntaxin-1 when syntaxin-1 is in a closed conformation; as syntaxin-1 opens during SNARE complex assembly it enables subsequent vesicle fusion and transmitter release following Ca^{2+} influx. Ca^{2+} release from intracellular stores *via* ryanodine receptors (RyR1) as well as influx through voltage-gated Ca^{2+} channels (VGCC, in grey) in response to incoming action potentials (AP) leads to the accumulation of intracellular Ca^{2+}levels and promoting of vesicle fusion. After fusion pore opening, the resulting SNARE complexes are disassembled and vesicles are recycled (dynamin-mediated), refilled with neurotransmitter, and reused for release. Postsynaptic receptors, excitatory NMDAR, and inhibitory GABAR are S-nitrosylated. Furthermore, the scaffolding proteins PSD-95, Stargazin, NSF, and Gephyrin are also nitrosylated with various functional outcomes. Ultimately, these modifications in addition to the canonical sGC/cGMP pathway (in grey) will alter the synaptic response and change AP firing characteristics following nitrergic signalling. (b) All colour-coded proteins have been shown to be subject to nitrergic posttranslational modifications with their specific residues indicated.

from the membrane is dynamin. This mechanism is crucially involved in presynaptic endocytosis but can also mediate internalisation of postsynaptic receptors from the membrane, thus regulating vesicular trafficking. S-Nitrosylation of dynamin at Cys 86 and Cys 607 has been shown to inhibit its activity in different cellular environments [58, 59], therefore providing evidence that these posttranslational modifications of dynamin could be modulating membrane trafficking.

A more general way to affect synaptic release is *via* upstream modulation of intracellular Ca^{2+} levels. Here, influx of Ca^{2+} through depolarisation-induced activation of voltage-gated Ca^{2+} channels and Ca^{2+} store release, *via* IP_3 and ryanodine receptors, provides an important signalling mechanism. Enhanced Ca^{2+} store release *via* ryanodine receptor activation is associated with LTP and it has been shown that S-nitrosylation of the ryanodine receptor 1 enhances Ca^{2+} store release [60]. The authors did not further investigate the roles of presynaptic versus postsynaptic ryanodine receptor modulation; however, they concluded from their data that this posttranslational modification contributes to physiological and pathological signalling at the synapse.

2.1.2. 3-Nitrotyrosination. Although nitrotyrosination has not been widely investigated at a synaptic level, some studies indicate that SNAP-25 undergoes 3-nitrotyrosination. SNAP-25 is a t-SNARE that is involved in the specificity of membrane fusion and directly executes fusion by forming a tight trans-SNARE complex with the v-SNAREs that brings the synaptic vesicle and membranes together. A study by Michela Di Stasi et al. showed that peroxynitrite induced tyrosine nitration of SNAP-25 and Munc-18 [61], two of the major proteins involved in protein-protein interactions in the docking/fusion steps of vesicle release. Another regulator of vesicle release is synaptophysin which is one of the most abundant integral proteins of vesicular membranes and is involved in several steps of synaptic function including synapse formation, biogenesis, and exocytosis/endocytosis of synaptic vesicles [62–64]. Although functional data are lacking, it has been shown that synaptophysin is nitrated (Tyr 250) and the formation of the synaptophysin/dynamin complex is impaired following peroxynitrite exposure [65, 66]. Dynamin I on the other hand is also nitrated on Tyr 354 which possibly interferes with tyrosine phosphorylation and results in further diminished complex formation [65] showing that activity of this molecule is affected by both S-nitrosylation and tyrosine nitration in a negative manner.

A further member of the SNARE protein family is the Ca^{2+} sensor synaptotagmin. Synaptotagmins are transmembrane proteins that have two cytoplasmic C2 Ca^{2+}-binding domains, C2A and C2B. There are 16 different isoforms of synaptotagmin and depending on the isoform they are localized to either synaptic/secretory vesicles or the plasma membrane. A study which characterised nitrergic effects on synaptotagmins expressed synaptotagmin 1 in Hi5 insect cells and detected tyrosine nitration in 6 of 11 surface accessible tyrosine residues, three in the C2A domain (Tyr 151, Tyr 216, and Tyr 229) and three in the C2B domain (Tyr 311, Tyr 364, and Tyr 380) [67]. Although the above studies implicate that posttranslational modifications by NO have functional consequences, evidence supporting a functional role is rare. However, it is conceivable to suggest that the conformational changes induced by NO have strong impacts on protein-protein interactions of this complex release machinery. Therefore, depending on the concentration of NO and the reversibility of these posttranslational modifications, the consequences for neuronal signalling will be important and relevant in physiology and pathology.

2.2. Postsynapse: NO as a Modulator of Neurotransmitter Receptors. In addition to the reported presynaptic mechanisms, synaptic function also relies on the signal transmission through postsynaptic receptors. The postsynapse is a dynamic system, which can undergo potentiation and depression in response to changes in activity and demand resulting in changes in receptor expression or function [68, 69]. These synaptic plasticity phenomena can be mediated by changes in postsynaptic strength, that is, receptor densities or postsynaptic excitability in response to ion channels adaptations, and in general by changing the balance between excitation and inhibition [70]. These mechanisms are crucially involved in physiology and their disturbance contributes to pathology. Here, nitrergic posttranslational modifications can play a role in modulating these homeostatic plasticity signalling pathways by directly affecting receptor function or indirectly by modulating receptor translocation to the membrane.

2.2.1. S-Nitrosylation. The most prominent postsynaptic receptor which has been shown to undergo S-nitrosylation by both exogenous and endogenous NO is the NMDAR. Specifically, the critical cysteine residue Cys 399 on the NR2A subunit is modulated leading to receptor inhibition [71] and hypoxia-induced stress conditions cause two residues on the NR1 subunit to be S-nitrosylated (Cys 744, Cys 798) further promoting channel inhibition [72]. NR1 and NR2A receptor subunits are also endogenously S-nitrosylated by prion protein signalling which requires the presence of copper. This basal and inhibitory S-nitrosylation of the NMDAR suggests a beneficial action of prion proteins, the lack of which, in prion disease, might contribute to the pathology [73]. Postsynaptic receptors, such as the NMDAR, are located within high postsynaptic density regions containing the scaffolding protein postsynaptic density-95 (PSD-95). Here, synaptic strength can be modulated, not only through direct receptor interaction but also through its localisation *via* PSDs. As such, PSD-95 has been shown to be physiologically S-nitrosylated and this modification further leads to a decreased PSD-95 clustering at synaptic sites [74].

Similarly, gephyrin is a postsynaptic scaffolding protein at the inhibitory synapse and its S-nitrosylation reduces the size of gephyrin clusters. This culminates in reduced cell surface expression of synaptic $GABA_A$ receptors and thus modulates hippocampal inhibitory synaptic transmission [75]. Synaptic plasticity strongly depends on the ability of postsynaptic receptors to be incorporated in or removed from the postsynaptic membrane. Here, stargazin, which regulates AMPA receptor surface expression, is S-nitrosylated

in NMDAR-dependent manner promoting binding to the AMPA receptor subunit GluR1 and thus enhances receptor surface expression [76]. Another AMPA receptor binding protein is N-ethylmaleimide sensitive factor (NSF), which, following S-nitrosylation, promotes AMPA receptor subunit GluR2 surface translocation [77]. In general, nitrergic signalling leads to a reduction in NMDAR activity, either directly by receptor or indirectly *via* PSD-95 S-nitrosylation and thus this could be interpreted as a negative feedback to reduce NO-mediated cytotoxicity. On the other hand, excitatory glutamate receptor signalling is enhanced and inhibitory $GABA_A$ signalling reduced following NO activation with both modulations resulting in sufficient excitatory neurotransmission without necessarily leading to elevated NMDAR-mediated Ca^{2+} entry.

2.2.2. 3-Nitrotyrosination. Although it has been shown that general nitrotyrosination of cellular proteins is increased following NO donor application or in response to certain stress conditions [78, 79], specific neuronal postsynaptic receptors as targets have not yet been identified. However, one study investigated the ultrastructural localisation of nitrotyrosine signals using immunogold labelling in untreated rat brain. The main signals were seen in the outer mitochondrial membrane, in dendritic spines, and also at synaptic vesicles in axon terminals [80], corroborating above findings related to the presynaptic release machinery. No labelling was detected specifically at postsynaptic sites; however, astrocytes showed a strong positive nitrotyrosination signal. As astrocytes are important components of the tripartite synapse and are reportedly involved in neurodegenerative conditions [81], this nitrergic modulation could be modulating the postsynaptic signalling cascade. This data suggests that physiologically, under unstimulated conditions, there is some basal nitrotyrosination present, which, due to its irreversible nature, will inevitably accumulate during aging, disease, and oxidative stress conditions. It becomes apparent that nitrergic activity can induce a variety of functional changes at the synapse and depending on the target protein and specific PTM it can modulate neuronal activities resulting in opposing effects on synaptic transmission.

3. Conclusions

Many neurodegenerative disorders have been associated with abnormal nitrergic signalling. In particular, enhanced levels of NO and related nitrosylation and nitrotyrosination events are evident in many cases. Several pathways in neurodegeneration by which NO has detrimental impacts on neuronal function have been reported including nitrosylation of dynamin-related protein 1 (DRP1), apolipoprotein E (ApoE), parkin, peroxiredoxin 2 (Prx2), X-linked inhibitor of apoptosis (XIAP), protein-disulphide isomerase (PDI), or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (reviewed in [10, 13]). Although nitrotyrosination seems to be much less explored there is strong evidence that this irreversible modification, which is mediated by the formation of peroxynitrite, has important contributions to pathology, particularly related to mitochondrial dysfunction [82]. Nitrotyrosination of cytochrome *c* provides an interesting example that this modification can result in a gain of protein function [83], whereas, on the other hand, nitrotyrosination can inhibit enzymes with essential tyrosine residues located in the active centre, as shown for mitochondrial MnSOD [84], a modification which is reported in several pathologies [85]. Direct evidence, for example, for the involvement of NOS-generated NO and subsequent nitrotyrosination in cell death, comes from studies using nNOS knock-out mice, in which NMDA-mediated cytotoxicity and neuronal cell death were diminished following the insult [86].

Together, there is accumulating evidence that highlights the importance of protein S-nitrosylation and nitrotyrosination in perturbing cell functions, including mitochondrial activities, protein folding, ubiquitination, synaptic transmission, and other signal transduction pathways. Alteration of one or several of these mechanisms can contribute to neuronal dysfunction and the development of neurodegenerative disorders. In this review we discussed specifically posttranslational modifications which occur directly at the synapse, including the presynaptic release machinery and postsynaptic transmitter receptor signalling. In addition to various high throughput proteomic approaches, which are essential to detect oxidative and other forms of protein modifications of general signalling molecules [87–90], it is also necessary to evaluate the physiological functional changes that occur as a result of these posttranslational modifications. The use of different model systems including mouse and *Drosophila* for degeneration as well as the knowledge of defined levels of nitric oxide as released by various donors [91] will allow further exploration of nitrergic pathways and their contributions to diseases and physiology.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgment

This work was supported by the Medical Research Council.

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Hindawi Publishing Corporation
Oxidative Medicine and Cellular Longevity
Volume 2015, Article ID 157876, 8 pages
http://dx.doi.org/10.1155/2015/157876

Research Article

Selective Nitric Oxide Synthase Inhibitor 7-Nitroindazole Protects against Cocaine-Induced Oxidative Stress in Rat Brain

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Received 30 December 2014; Revised 24 March 2015; Accepted 26 March 2015

Academic Editor: Joern Steinert

One of the mechanisms involved in the development of addiction, as well as in brain toxicity, is the oxidative stress. The aim of the current study was to investigate the effects of 7-nitroindazole (7-NI), a selective inhibitor of neuronal nitric oxide synthase (nNOS), on cocaine withdrawal and neurotoxicity in male Wistar rats. The animals were divided into four groups: control; group treated with cocaine (15 mg/kg^{-1}, i.p., 7 days); group treated with 7-NI (25 mg/kg^{-1}, i.p., 7 days); and a combination group (7-NI + cocaine). Cocaine repeated treatment resulted in development of physical dependence, judged by withdrawal symptoms (decreased locomotion, increased salivation and breathing rate), accompanied by an increased nNOS activity and oxidative stress. The latter was discerned by an increased formation of malondialdehyde (MDA), depletion of reduced glutathione (GSH) levels, and impairment of the enzymatic antioxidant defense system measured in whole brain. In synaptosomes, isolated from cocaine-treated rats, mitochondrial activity and GSH levels were also decreased. 7-NI administered along with cocaine not only attenuated the withdrawal, due to its nNOS inhibition, but also reversed both the GSH levels and antioxidant enzyme activities near control levels.

1. Introduction

Cocaine is a potent psychostimulant, recognized as one of the most significant examples of drug abuse due to intense feeling of euphoria and increased concentration and energy. The psychostimulant effects of cocaine appear to be mediated by its ability to enhance the dopaminergic activity in the mesocorticolimbic circuit through binding to the dopamine, serotonin, and noradrenalin transport proteins and directly prevent their reuptake into presynaptic neurons [1]. The repeat intake of cocaine is related to tolerance and development of dependence and serious injuries of the central nervous system, heart, and liver. One of the metabolic pathways of cocaine, N-oxidation mediated by cytochrome P 450 enzymes and flavin adenine (FAD) containing monooxygenases, leads to production of reactive oxygen species (ROS), namely, nitroxide, nitrosonium, and iminium ions, which have been recognized to be involved in cocaine-induced organ toxicity [2]. Increased reactive oxygen species production in the central nervous system has been identified to play a pivotal role in the neuropathology induced by drugs of abuse, including cocaine [3]. Oxidative stress may occur during or after drug exposure and/or during the withdrawal from the drug [4]. Not only has the role of nitric oxide and related N-methyl-D-aspartate (NO/NMDA) cascade been discussed in the process of development of tolerance and withdrawal from different drugs of abuse [5] but also it is considered an important source of oxidative stress, induced by psychostimulants [6]. Although nitric oxide (NO) plays an important physiological role as a neurotransmitter in the central nervous system (CNS), excessive neuronal nitric oxide synthase- (nNOS-) dependent NO release during high levels of NMDA receptor stimulation results in production of hydroxyl ($HO^{\bullet}$) and peroxynitrite ($ONOO^{-}$) radicals that are responsible for oxidative injury [7].

7-Nitroindazole (7-NI) is a heterocyclic compound, which inhibits nNOS by competing with both L-arginine and tetrahydrobiopterin [8] and has been used extensively as

a selective inhibitor of nNOS [9, 10]. There are a number of studies showing the beneficial effect of inhibiting nNOS activity as a means of reducing NO/NMDA-induced neurotoxicity and attenuating tolerance and withdrawal to psychoactive agents [9, 11, 12].

In the context of this information, the objective of the following study was to investigate the effect of 7-NI on withdrawal symptoms and neurotoxicity induced by multiple administration of cocaine to male Wistar rats.

2. Materials and Methods

2.1. Drugs and Chemicals. All the reagents used were of analytical grade. Cocaine, 7-nitroindazole, and other chemicals, sucrose, Tris, DL-dithiothreitol, phenylmethylsulfonyl fluoride, potassium phosphate, calcium chloratum ($CaCl_2$), magnesium chloratum ($MgCl_2$), L-arginine, L-valine, bovine hemoglobin, beta-nicotinamide adenine dinucleotide 2′-phosphate reduced tetrasodium salt (NADPH), ethylenediaminetetraacetic acid (EDTA), bovine serum albumin (fraction V), reduced glutathione (GSH), oxidized glutathione (GSSG), glutathione reductase (GR), cumene hydroperoxide, Percoll, and MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide), were purchased from Sigma Chemical Co. (Taufkirchen, Germany). 2,2′-Dinitro-5,5′-dithiodibenzoic acid (DTNB) and D-glucose were obtained from Merck (Darmstadt, Germany). Dimethyl sulfoxide (DMSO) was purchased from Valerus (Sofia, Bulgaria).

2.2. Animals. Male Wistar rats (6–8 weeks of age, body weight 200–250 g) were used. The rats were housed in Plexiglass cages (3 per cage) in a 12/12 light/dark cycle, under standard laboratory conditions (ambient temperature 20°C ± 2°C and humidity 72% ± 4%) with free access to water and standard pelleted rat food 53–3, produced according to ISO 9001:2008. Animals were purchased from the National Breeding Center, Sofia, Bulgaria. A minimum of 7-day acclimatization was allowed before the commencement of the study and their health was monitored regularly by a veterinary physician. All performed procedures were approved by the Institutional Animal Care Committee and the principles stated in the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (ETS 123) [13] were strictly followed throughout the experiment.

2.3. Experimental Design. Animals were divided into four groups ($n = 12$) as follows:

group 1: control animals, treated with saline for 7 days, which were involved in the experiment from the very beginning and housed under the same standard laboratory conditions as the treated animals;

group 2: animals, receiving 15 mg/kg^{-1} i.p. of cocaine for 7 days [14];

group 3: animals, receiving 25 mg/kg^{-1} i.p. 7-NI for 7 days [10];

group 4: animals, treated with 7-NI (25 mg/kg^{-1} i.p.) and 30 min later with cocaine (15 mg/kg^{-1} i.p.) for 7 days.

Twenty-four hours after the last administration of the compounds the animals were observed for behavioral changes related to the withdrawal syndrome. Then the animals were sacrificed through decapitation and brains were extracted. Brains of six animals from each group were taken for isolation of synaptosomes and brains from the other six animals of each group were used for measurement of nNOS and antioxidant enzymes.

2.4. Behavioral Observation Test. Quantitative assessment of behavioral changes in the animals was performed 24 hours after the last administration of cocaine, alone and in combination with 7-NI. The observations and changes were recorded on the basis of the standardized observation grid, derived from that of Irwin test [15], adjusted to the conditions and objectives of our study. Briefly, 24 hours after the last administration, six rats of each group were placed in separate cages and observed for 30 min. The rats were observed simultaneously with the control group, given vehicle for the following symptoms: decreased locomotor activity, excitation, changes in coordination, salivation, and respiration. The symptoms were evaluated by their presence or absence and were rated on a 3-point scale (slight, moderate, and marked). Being mainly the quantitative procedure, no formal statistical analysis was conducted.

2.5. Assessment of Biochemical Parameters in Whole Brain. The first part of our experiment was carried out in whole brain. Therefore no particular brain structures were identified and isolated. Briefly, the procedure we followed was as follows: after decapitation of six ($n = 6$) rats from each group, the brains were taken out, measured, and divided into four parts, one for measurement of nNOS activity, one for assessment of MDA quantity, one for GSH levels assessment, and one for measurement of antioxidant enzymes. The brain samples were consequently homogenized with the respective buffers.

2.5.1. Preparation of Brain Tissue Extracts and Assessment of nNOS Activity. The brains were minced and homogenized in 10 volumes of buffer, containing 320 mmol L^{-1} sucrose, 50 mmol L^{-1} Tris, 1 mmol L^{-1} DL-dithiothreitol, and 100 μg/L phenylmethylsulfonyl fluoride (pH = 7.2) according to the method used by Knowels and Moncada [16]. The homogenates were then centrifuged at 17 000 ×g for 60 min. The protein content was measured by the method of Lowry [17] with bovine serum albumin as a standard.

nNOS activity was measured spectrophotometrically using the oxidation of oxyhemoglobin to methemoglobin by NO. The incubation medium contained 40 mM potassium phosphate buffer, pH = 7.2, 200 μmol L^{-1} $CaCl_2$, 1 mmol L^{-1} $MgCl_2$, 100 μmol L^{-1} L-arginine, 50 mmol L^{-1} L-valine, 2.6 μmol L^{-1} oxyhemoglobin, 100 μmol L^{-1} NADPH, and brain extract. The change in the difference in absorbance at 401 nm and 421 nm was monitored with a double split

beam spectrophotometer (Spectro UV-VIS Split), at 37°C. The activity of the enzyme was expressed in nmol/min/mg, using the millimolar extinction coefficient of methemoglobin 77.2 M^{-1} cm^{-1}.

2.5.2. Preparation of Brain Homogenate for Assessment of Malondialdehyde (MDA). The brains were homogenized with 0.1 M phosphate buffer and EDTA, pH = 7.4 (1 : 10). Lipid peroxidation was determined by measuring the rate of production of thiobarbituric acid reactive substances (TBARS) (expressed as malondialdehyde (MDA) equivalents) as described by Deby and Goutier [18] with slight modifications. Briefly one volume of the brain homogenate was mixed with 1 mL 25% trichloroacetic acid (TCA) and 1 mL 0.67% thiobarbituric acid (TBA). Samples were then mixed thoroughly, heated for 20 min in a boiling water bath, cooled, and centrifuged at 4000 rpm for 20 min. The absorbance of supernatant was measured at 535 nm against a blank that contained all the reagents except the tissue homogenate. MDA concentration was calculated using a molar extinction coefficient of 1.56×10^5 M^{-1} cm^{-1} and expressed in nmol/g wet tissue.

2.5.3. Preparation of Brain Homogenate for GSH Assessment. GSH was assessed by measuring nonprotein sulfhydryls after precipitation of proteins with trichloroacetic acid (TCA), using the method described by Bump et al. [19]. Briefly, brains were homogenized in 5% TCA (1 : 10) and centrifuged for 20 min at 4 000 ×g. The reaction mixture contained 0.05 mL supernatant, 3 mL 0.05 M phosphate buffer (pH = 8), and 0.02 mL DTNB reagent. The absorbance was determined at 412 nm and the results were expressed as nmol/g wet tissue.

2.5.4. Preparation of Brain Homogenates for Antioxidant Enzyme Activity Measurement. Measured amounts of brain were rinsed in ice-cold physiological saline and minced with scissors. 10% homogenates were prepared in 0.05 M phosphate buffer (pH = 7.4) and centrifuged at 7,000 ×g and the supernatant was used for antioxidant enzymes assay. Analyses were performed in triplicate and the average values were taken. Protein content was measured by the Lowry method [17].

(1) Catalase Activity (CAT). Catalase activity (CAT) was assessed following the method of Aebi et al. [20]. Briefly, 10 μL of homogenate was added to 1990 μL of H_2O_2 solution (containing 6.8 μL of 30% H_2O_2 + 1983.2 μL 0.05 M phosphate buffer, pH = 7.4). CAT activity was determined by monitoring the H_2O_2 decomposition which was measured spectrophotometrically by the decrease in absorbance at 240 nm. Enzyme activity was calculated using a molar extinction coefficient of 0.043/mM^{-1}/cm^{-1} and expressed as μM/minute/mg protein.

(2) Superoxide Dismutase Activity (SOD). Superoxide dismutase activity (SOD) was measured according to the method of Misra and Fridovich [21], following spectrophotometric autoxidation of epinephrine at pH = 10.4, 30°C, using the molar extinction coefficient of 4.02/mM^{-1}/cm^{-1}. The incubation mixture contained 50 mM glycine buffer, pH = 10.4. The reaction is started by the addition of epinephrine. SOD activity is expressed as nmol of epinephrine prevented from autoxidation after addition of the sample.

(3) Glutathione Peroxidase Activity (GPx). GPx was measured by NADPH oxidation, using a coupled reaction system consisting of glutathione, glutathione reductase, and cumene hydroperoxide [22]. Briefly, 100 μL of enzyme sample was incubated for 5 minutes with 1.5 mL 0.05 M phosphate buffer (pH = 7.4), 100 μL 1 mM EDTA, 50 μL 1 mM GSH, 100 μL 0.2 mM NADPH, and 1 unit of glutathione reductase. The reaction was initiated by adding 50 μL cumene hydroperoxide (1 mg/mL) and the rate of disappearance of NADPH with time was determined by monitoring absorbance at 340 nm. Results were expressed as nmol/min/mg protein using the molar extinction coefficient of 6.22 mM^{-1} cm^{-1}.

(4) Glutathione Reductase Activity (GR). GR was measured according to the method of Pinto et al. [23] by following NADPH oxidation spectrophotometrically at 340 nm and using an extinction coefficient of 6.22 mM^{-1} cm^{-1}. The incubation mixture contained 0.05 M phosphate buffer, pH = 7.4, 2.5 mM GSSG, and 125 μM NADPH at 30°C. Results were expressed as nmol/min/mg protein.

(5) Glutathione-S-transferase (GST). Glutathione-S-transferase (GST) activity was measured using 1-chloro-2,4-dinitrobenzene (CDNB) as the substrate [24]. The incubation mixture containing 1.6 mL 0.05 M phosphate buffer, 100 μL 1 mM GSH, 100 μL 1 mM EDTA, and 100 μL homogenate was incubated for 15 minutes at 37°C. After the incubation, 100 μL 1 mM CDNB was added and the increase in absorbance with time was recorded at 340 nm. Enzyme activity is measured using an extinction coefficient of 9.6 $\times$ 10^3/M^{-1}/cm^{-1} and is expressed as nmol of CDNB-GSH conjugate formed/minute/mg protein.

2.6. Isolation of Synaptosomes. Rats (n = 6 from each group) were decapitated, and the brains were taken out for synaptosomes isolation as described by Taupin et al. [25]. Briefly, the brains were homogenized in 10 volumes of cold Buffer 1, containing 5 mM HEPES and 0.32 M sucrose (pH = 7.4). The brain homogenate was centrifuged twice at 1000 ×g for 5 min at 4°C. The supernatant was collected and centrifuged three times at 10 000 ×g for 20 min at 4°C. The pellet was resuspended in ice-cold Buffer 1. The synaptosomes were isolated by using Percoll reagent to prepare the gradient and then were resuspended and incubated in Buffer 2, containing 290 mM NaCl, 0.95 mM $MgCl_2 \cdot 6H_2O$, 10 mM KCl, 2.4 mM $CaCl_2 \cdot H_2O$, 2.1 mM NaH_2PO_4, 44 mM HEPES, and 13 mM D-glucose. Incubations were performed in a 5% CO_2 + 95% O_2 atmosphere. The content of synaptosomal protein was determined according to the method of Lowry et al. [17] using serum albumin as a standard. Synaptosomes viability was measured by mitochondrial activity (MTT reduction), described by Mungarro-Menchaca et al. [26]. The formed

Table 1: Quantitative assessment of some behavioral changes observed 24 hours after the last administration of cocaine and 7-NI.

Behavioral changes	Control	Cocaine	7-NI	7-NI + cocaine
Decreased locomotor activity	−	++	−	−
Excitation	−	++	−	−
Changes in coordination	−	−	−	−
Salivation	−	+++	−	+
Respiration-enhanced breathing	−	+++	−	+

$N = 6$ (for each treatment group).
+++ means severe; ++ means moderate; + means slight; − means no effect.
Observations were performed 24 hours after the last administration of the compounds. The symptoms were observed for 30 min. The observations and changes were recorded on the basis of the standardized observation grid, derived from that of Irwin test [15], adjusted to the conditions and objectives of our study.

formazan crystals were dissolved in DMSO. The extinction was measured spectrophotometrically at $\lambda = 580$ nm. GSH levels in synaptosomes were assessed, using Ellman reagent (DTNB) [27], which forms color complexes with –SH group at pH = 8 with maximum absorbance at 412 nm.

3. Statistical Analysis

Statistical program "MEDCALC" was used for analysis of the data. The data are expressed as mean ± SEM of six rats in each group. The significance of the data was assessed using the nonparametric Mann-Whitney U test. Values of $P \leq 0.05$ were considered statistically significant.

4. Results

4.1. Behavioral Observation Test. The effect of 7-NI on cocaine withdrawal symptoms is shown in Table 1. In the animals experiencing cocaine deprivation, the withdrawal was manifested by moderate decrease in locomotor activity, excessive salivation, discerned by dampness visible around mouth, and enhanced breathing. Deprivation of 7-NI did not induce any behavioral changes. In the animal group treated with 7-NI in combination with cocaine the withdrawal symptoms were attenuated. No changes in food and water consumption were observed.

Being an inhibitor of nNOS 7-NI administered alone led to a significant decrease in the enzyme activity by 40% ($P < 0.05$). Cocaine multiple administration resulted in statistically significant ($P < 0.005$) increase in nNOS activity by 59%. 7-Nitroindazole coadministration with cocaine restored the enzyme activity nearly to control levels. In addition, compared to cocaine only group, 7-NI decreased nNOS activity by 43% ($P < 0.05$). Results are shown in Table 2.

4.2. MDA Quantity and GSH Levels. A significant increase by 31% ($P < 0.05$) in MDA quantity and a marked decrease in GSH levels by 44% ($P < 0.05$) were observed in the brains of rats after 7-day administration of cocaine. Compared to the nontreated controls, 7-NI coadministration decreased MDA production and restored GSH levels. Compared to cocaine only group, 7-NI decreased MDA production by 21% ($P < 0.05$) and increased GSH levels by 64% ($P < 0.05$). Results are shown in Table 2.

4.3. Assessment of Antioxidant Enzyme Activity. The results are presented in Figure 1. Compared to the control group, cocaine toxicity is presented by increased activities of SOD (36%, $P < 0.05$) and GPx (78%, $P < 0.05$) and by decreased activities of other brain antioxidant enzymes, as follows: CAT (40%, $P < 0.05$), GR (39%, $P < 0.05$), and GST (45%, $P < 0.05$). Pretreatment with 7-NI prevented cocaine-induced toxicity by restoring the activities of antioxidant enzymes. When comparing the data obtained from 7-NI + cocaine group versus cocaine only group, a significant decrease in SOD activity by 17% ($P < 0.05$) and in GPx activity by 24% ($P < 0.05$) and a significant increase in CAT activity by 58% ($P < 0.05$) and in GST activity by 59% ($P < 0.05$) were observed.

4.4. Mitochondrial Activity (MTT Reduction) and GSH Levels. The results of cocaine and 7-NI administration alone and in combination on mitochondrial activity, discerned by MTT reduction and GSH levels, showed that cocaine multiple treatment decreased MTT reduction by 53% ($P < 0.05$) and reduced GSH levels by 55% ($P < 0.05$). 7-NI alone administration did not change these parameters; however in combination with cocaine it showed protective effect discerned by preserved mitochondrial activity and GSH levels by 57% ($P < 0.05$) and by 60% ($P < 0.05$), respectively, compared to the cocaine only group. The results are depicted in Figure 2.

5. Discussion

Psychostimulants' abuse is a serious problem in the modern societies not only due to the development of drug addiction, which according to the criteria of the Diagnostic and Statistical Manual of Mental Disorders (DSM IV) [28] is considered a brain disease, but also due to the induced neurotoxicity. Cocaine is a well-known and widespread psychostimulant, notorious with its potential for tolerance and dependence development, as well as with its toxicity to the brain. One of the neurobiological mechanisms underlying these processes is thought to be activation of NMDA/NO cascade that results in an increase of nNOS activity and excessive production of NO that plays an important role both as a neurotransmitter and as a neurotoxicant [7]. In our study we investigated the possible neuroprotective effect of 7-NI, a selective inhibitor of the neuronal NOS after multiple cocaine administration alone and in combination with the inhibitor.

The results of our study indicate that repeated administration of cocaine for 7 consecutive days to male rats

Table 2: Changes in nNOS activity, MDA quantity, and GSH levels in brain homogenate after multiple administration of cocaine, alone and in combination with 7-NI.

Group	nNOS activity (nmol/min/mg)	MDA nmol/g/wet tissue	GSH nmol/g/wet tissue
Control	0.604 ± 0.04	3.55 ± 0.20	1.68 ± 0.15
Cocaine	0.935 ± 0.14*	4.65 ± 0.32*	0.94 ± 0.10*
7-NI	0.360 ± 0.07*	3.50 ± 0.10	1.59 ± 0.10
7-NI + cocaine	0.535 ± 0.10+	3.68 ± 0.15+	1.55 ± 0.08+

Data are expressed as mean ± SEM of six rats. *Significant difference from control values (Mann-Whitney U test, $P < 0.05$); +significant difference from cocaine-treated group (Mann-Whitney U test, $P < 0.05$).

resulted in development of physical dependence, judged by withdrawal symptoms (decreased spontaneous locomotion, increased salivation and breathing rate), accompanied by an increased nNOS activity and development of oxidative stress. Cocaine exposure resulted in an increased formation of the lipid peroxidation product MDA and in the impairment of the nonenzymatic (GSH) and enzymatic (SOD, CAT, GR, GPx, and GST) potential in whole brain. In synaptosomes, isolated from cocaine-treated rats, the mitochondrial activity measured by MTT reduction and GSH levels were also decreased. Our results are in good correlation with the results of Bashkatova et al. [29], who observed high levels of lipid peroxidation in the hippocampus of rats exposed *in utero* to cocaine and with the observations of Poon et al. [30] that reported an oxidation of proteins in cocaine-exposed human neuronal progenitor cells. Moreover, the detected increased formation of thiobarbituric acid reactive substances (TBARS) (expressed as MDA) by cocaine corroborates the data obtained from the clinical study, carried out by Sordi et al. [31] in crack cocaine users during early withdrawal. The authors reported higher TBARS levels in severe crack users.

Cocaine exposure has been reported to increase hydrogen peroxide (H_2O_2) and lipid peroxide production in the prefrontal cortex and in the striatum of rats [32], brain structures with numerous dopaminergic nerve terminals. The present results showed that multiple cocaine administration led to impairment of the enzyme antioxidant defense, discerned by increased SOD and GPx activities and by a decrease in CAT, GR, and GST activities. In the available literature there are several proposed mechanisms underlying the changes of the oxidative status by cocaine. One of these mechanisms is related to the massive increase of dopamine release due to the cocaine binding to the transporter sites of monoamines that result in an inhibition of their uptake in the presynaptic neuron [1]. Enhanced neurotransmitter levels, primarily dopamine, in the synaptic cleft have been related to ROS formation [33]. Dopamine is further metabolized (by autooxidation) to generate hydrogen peroxide (H_2O_2) and superoxide anion (O_2^-) that can explain the observed increase in SOD and GPx activities. Our results support the data reported by Dietrich et al. [32] that determined an increase in SOD and GPx activities in brain cortex and striatum. At the same time cocaine administration decreased CAT, GR, and GST activities. Catalase is the main enzyme responsible for further detoxification of H_2O_2 to H_2O and O_2 [34]. The excessive amount of H_2O_2, produced on one hand by autooxidation of dopamine and on the other by cocaine itself, may explain the reduced CAT activity by the generated hydrogen peroxide. The increased GPx activity may be regarded as a compensatory mechanism in order to get rid of the excess peroxides due to lower CAT activity. The increased GPx activity is probably further responsible for the detected GSH depletion and decreased GR and GST activities.

The heterocyclic compound 7-NI that inhibits nNOS by competing with L-arginine and tetrahydrobiopterin [8] has been used extensively as a selective inhibitor of nNOS. Several studies have indicated that 7-NI affects different physical processes and behaviors, related to drug abuse, such as tolerance, withdrawal, neurotoxicity, psychomotor stimulation, and reward [35]. Our results showed that 7-NI administered along with cocaine attenuated the withdrawal, which is probably due to the detected decrease in nNOS activity, enhanced by cocaine (see Table 2). These data support the behavioral studies carried out by Haracz et al. [36] and Itzhak [9] in which the authors proved that administration of 7-NI reduced the hyperactivity and attenuated the induction of behavioral sensitization to cocaine. Along with its beneficial effects in drug addiction, antioxidant properties of 7-NI were also reported [37]. Chu et al. [38] found that 7-NI increased the SOD levels, decreased by hypoxic brain injury. In our study 7-NI administered along with cocaine decreased the formation of MDA and reversed both the GSH levels and antioxidant enzyme activities near control levels. Neuroprotective effects of 7-NI could be explained by its inhibition on nNOS activation and further formation of $ONOO^-$, as well as by its MAO-B inhibition [39]. Furthermore, antioxidant properties of 7-NI, which are not nNOS dependent were suggested by Di Monte et al. [40] when MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) treated mice showed a reduced brain MPP^+ level after being injected with 7-NI.

6. Conclusions

Under the conditions of this study and on the basis of the obtained results we can conclude that in the experimental model of cocaine multiple administration in rats the selective nNOS inhibitor 7-nitroindazole not only attenuated the behavioral changes induced by cocaine deprivation but also exerted an antioxidant and neuroprotective activity. The possible mechanisms underlying the neuroprotective effects

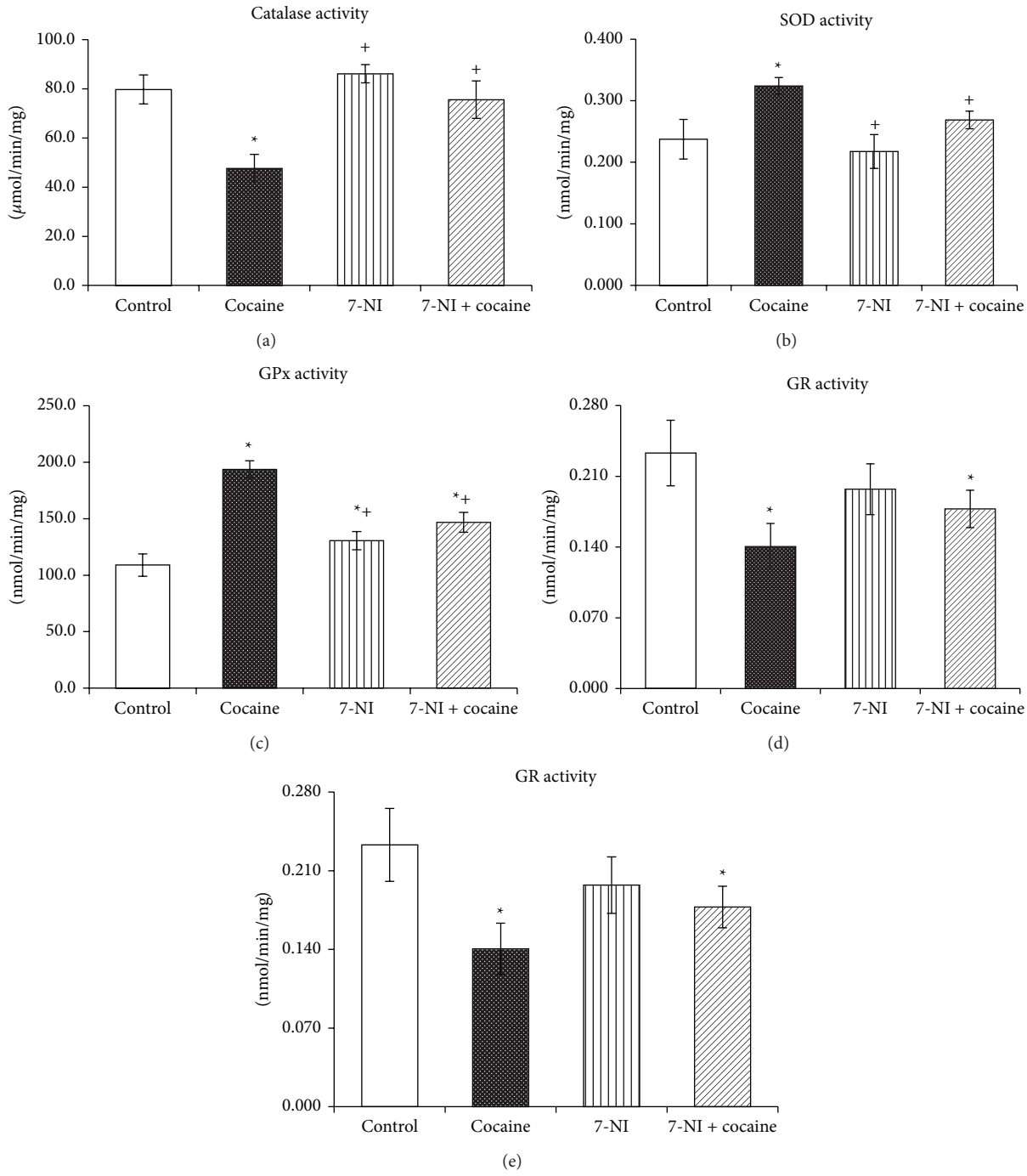

Figure 1: CAT, SOD, GR, GPx, and GST activity measured in rat brain from cocaine group (15 mg/kg^{-1}, i.p., 7 days), 7-NI group (25 mg/kg^{-1}, i.p., 7 days), and cocaine + 7-NI group. Parameters: CAT (catalase), SOD (superoxide dismutase), GR (glutathione reductase), GPx (glutathione peroxidase), GST (glutathione-S-transferase). Data are expressed as mean ± SEM of six animals (Mann-Whitney U test). *Significant difference from control values (Mann-Whitney U test, $P < 0.05$); $^{+}$significant difference from cocaine-treated group (Mann-Whitney U test, $P < 0.05$).

of 7-NI could be due to the combination of its inhibitory effect of nNOS and its direct free radical scavenging properties. The beneficial effect of 7-NI in restoration of the antioxidant cell defense in the brain, impaired by multiple cocaine administration, and along with it the attenuation of the physical dependence, induced by cocaine, once again confirm the role of the oxidative stress in the development of addiction to psychoactive compounds.

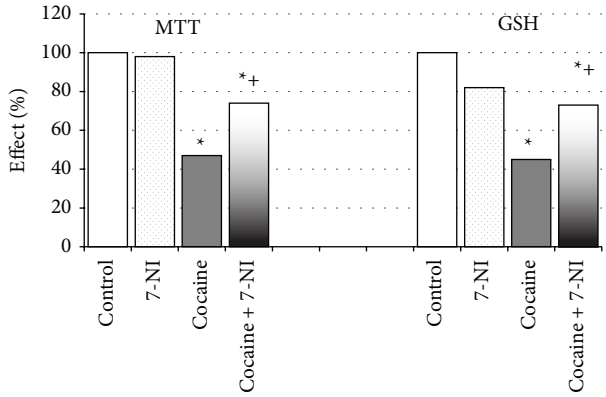

Figure 2: Effect of 7-nitroindazole (7-NI) in combination with cocaine on synaptosomal mitochondria activity (MTT reduction) and GSH level. Data are expressed as mean ± SEM of six animals (Mann-Whitney U test). *Significant difference from control values (Mann-Whitney U test, $P < 0.05$); $^{+}$significant difference from cocaine-treated group (Mann-Whitney U test, $P < 0.05$).

Conflict of Interests

The authors declare that there is no conflict of interests.

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