Role of Nitric Oxide in Neurodegeneration and Vulnerability of Neuronal Cells to Nitric Oxide Metabolites and Reactive Oxygen Species

Debomoy K. Lahiri, Chandramallika Ghosh, Jack T. Rogers, Stephen Bondy, and Nigel H. Greig

Abstract Nitric oxide (NO) is produced during the oxidative deamination catalyzed by nitric oxide synthase (NOS) that converts L-arginine to L-citrulline. NO can also be released chemically from a group of compounds called NO donors, such as sodium nitroprusside (SNP). NO directly or through its metabolites is believed to be involved in several disorders, including Alzheimer's disease (AD). This chapter summarizes the role of NO and oxidative stress in neurodegeneration and describes the experimental evidence of increased vulnerability of neuronal cells to NO metabolites and other reactive oxygen species (ROS). As NO is a highly labile, unstable free gas, levels of the stable end products, such as nitrite and nitrate (NOx), were measured. When different cell types were treated with SNP, a significant level of NOx was detected in a time- and dose-dependent manner, which was more than the spontaneous release by SNP. Astrocytic, glial, and epithelial cell lines released significantly higher levels of NOx compared with neuronal cell lines after SNP treatment. Neuronal cells were more sensitive to SNP-induced cytotoxicity, as determined by lactate dehydrogenase assay. SNP-mediated toxicity is known to be due, in large part, to the accumulation of cyanide ions, and the ability of cells to protect themselves against this toxicity depends upon their levels of NO metabolites. Cell lines that generate more NOx, such as astrocytic and epithelial, are better protected against the SNP-induced toxicity than are less NOx-protecting neuronal cell lines. Our results suggest that various cell types metabolize SNP differently and that neuronal cell lines are more vulnerable than other cell types to SNP treatment. As neuronal cell lines lack an NO-generated protective mechanism, these cells are potentially primary targets for neurodegeneration by toxic agents including the free radicals and peroxynitrites.

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1 Introduction

Alzheimer's disease (AD) currently afflicts more than 5 million people within the United States of America, and this number will likely triple in the forthcoming 40 years given the increasing numbers of aged citizens. At present, the few agents that are FDA-approved for the treatment of AD have demonstrated only modest effects in modifying clinical symptoms for relatively short periods, and none has shown a clear effect on disease progression [1, 2]. The process of discovering and developing new drugs is lengthy and expensive, and more mechanistic-based studies are currently needed to expand available therapeutic options [1]. Neurodegeneration in AD is believed to be mediated by multiple events, including neurotransmitter imbalance, free-radical formation, and abnormal protein depositions, mostly induced by amyloid beta peptide (Aβ) and hyperphosphorylated tau protein [3–5]. Moreover, recent evidence suggests that oxidative stress and nitric oxide–mediated events play important roles in the pathogenesis of neurodegenerative disorders, such as AD and Parkinson's disease (PD) [6–8]. Nitric oxide (NO) is a small, diffusible, lipophilic, and transient free radical gas that mediates significant and diverse signaling functions in nearly every organ system within the body [9]. This chapter is presented in two parts: The first reviews the role of NO and free radicals in neurodegeneration, and the second summarizes experimental evidence for the increased vulnerability of neuronal cells to metabolites of NO and reactive oxygen species.

1.1 Neuronal Death and Survival Under Oxidative Stress in AD and PD

Current research indicates that several known gene mutations causing familial AD (Aβ precursor protein [APP], presenilin-1, or presenilin-2 gene) and familial PD (Parkin, PINK-1, or DJ-1) are associated with increased oxidative stress [6]. Further, several known genetic (e.g., apolipoprotein E-epsilon 4 variant [APOEε4]) and environmental (e.g., metals or pesticides exposure) risk factors of sporadic AD and/or PD are associated with increased oxidative stress. At the clinical level, patients at the early stages of AD and PD together with cellular and animal models of these diseases provide consistent evidence that oxidative insult is a major early event in the pathologic cascade leading to AD and PD. Research on the molecular mechanisms of longevity studies suggests that prolongevity gene products, such as forkhead transcription factors and sirtuins, may participate in the insulin-like signaling pathway and oxidative stress resistance against aging. Therefore, an enhancement of prolongevity signaling (e.g., caloric restriction) has been suggested to be a

promising approach as antioxidative strategy against age-associated neurodegenerative diseases [6].

1.2 Superoxide and NO in Senescence and Aging

It has been known for some time that although they themselves are mostly harmless species, superoxide and NO are precursors of the reactive species hydroxyl radical and peroxynitrite and thereby are initiators of aging and various pathologies. In addition, dietary modulation of age-related changes in cerebral pro-oxidant status has been previously suggested to be both feasible and potentially valuable [10]. Furthermore, the role of the physiologic free radicals, superoxide and NO, in senescence and aging development and the mechanisms of processes mediated by these radicals has recently been reviewed [11].

1.3 Role of Reactive Oxygen Species and Reactive Nitrogen Species in Oxidative and Nitrosative Stress and in Aging

The free radical theory of aging attributes cellular pathology to the cumulative accumulation of reactive oxygen species (ROS), although it remains controversial. Molecular interactions between ROS and reactive nitrogen species (RNS), such as NO, suggest that in biological systems one effect of increased ROS is the disruption of protein S-nitrosylation, a ubiquitous posttranslational modification system [12]. In this manner, ROS may not only damage cells but also disrupt widespread signaling pathways. Hence, the interrelationship between oxidative and nitrosative stress in the context of aging and the cardiovascular system are currently being investigated [12].

1.4 Role of NO in Aging, AD, Obesity, and Heart Disease

NO may play a role in the progression of AD and PD [13]. Lipopolysaccharides (LPSs), as well as other bacterial and viral products, cause inducible nitric oxide synthase (iNOS) synthesis that, in turn, produces copious amounts of NO. LPSs similarly activate cytokine and iNOS production in the cardiovascular system and thereby lead to coronary heart disease. Fat is a major supply of NO stimulated by leptin. As fat stores enlarge, the release of leptin and NO rises in parallel in a circadian rhythm with a peak at night. Hence, NO could be to blame for increased coronary heart disease in the presence of obesity. Aging of the anterior pituitary and pineal, with resultant lowered secretion of pituitary hormones and the pineal hormone melatonin, respectively, may be caused by NO. Thus, antioxidants, such as melatonin, vitamin C, and vitamin E, may likely play important roles in reducing or eliminating the oxidant damage produced by NO [13].

1.5 Role of NO and Cellular Stress Response in Brain Aging and Neurodegenerative Disorders

NO and other ROS and RNS exert important roles in the brain, such as neuromodulation, neurotransmission, and synaptic plasticity, but are also involved in pathologic processes such as neurodegeneration and neuroinflammation [14]. Acute and chronic inflammation induces an increased nitrogen monoxide formation and nitrosative stress. NO and its toxic metabolite, peroxynitrite, inhibit components of the mitochondrial respiratory chain leading to cellular energy deficiency, dysfunction, and, ultimately, to cell death. Within the brain, the vulnerability of different brain cell types to NO and peroxynitrite exposure are influenced by factors such as the intracellular reduced glutathione and cellular stress resistance signal pathways. Hence, neurons, contrary to astrocytes, appear exceptionally susceptible to the effect of nitrosative stress. Recent studies support this position for neurologic disorders such as AD, PD, amyotrophic lateral sclerosis (ALS), multiple sclerosis, and Huntington's disease. To endure different types of injuries, brain cells have developed integrated adaptive responses, termed *longevity assurance processes*, encompassing a number of genes dubbed *vitagenes* and components of the heat shock protein (HSP) system, including HSP70 and HSP32, to identify and control assorted forms of stress. Calabrese and colleagues [14] suggest that maintenance or recovery of the activity of vitagenes may delay the aging process and lower the occurrence of age-related diseases thereby providing prolongation of a healthy life span.

1.6 Interplay Between Superoxide and NO in Aging and Diseases via Many Physiologic Functions

Free radicals, superoxide and NO, are critical signaling molecules that mediate a wide variety of physiologic functions, including phagocytosis and vasorelaxation [15]. Nonetheless, regulation errors may result in free radical–mediated damaging processes in cells and tissues. Afanas'ev [15] has suggested that an interplay between superoxide and NO may impact and be responsible for the development of aging and diseases. In particular, the superoxide-mediated proton leak may lead to the inhibition of oxidative phosphorylation, and the competition between NO and superoxide ($O_2^{-\bullet}$) in their reactions with cytochrome oxidase may be a cause of mitochondrial aging [15].

2 NO and Its Physiologic Role

NO is a labile and highly reactive species that is enzymatically generated during the oxidation of L-arginine to L-citrulline by nitric oxide synthase (NOS) [9, 16, 17] (Fig. 1). NO can also be generated by a group of compounds that are collectively known as NO donors, such as sodium nitroprusside (SNP). SNP, a prototypical nitrovasodilator, is considered to produce its vasorelaxant action by releasing NO.

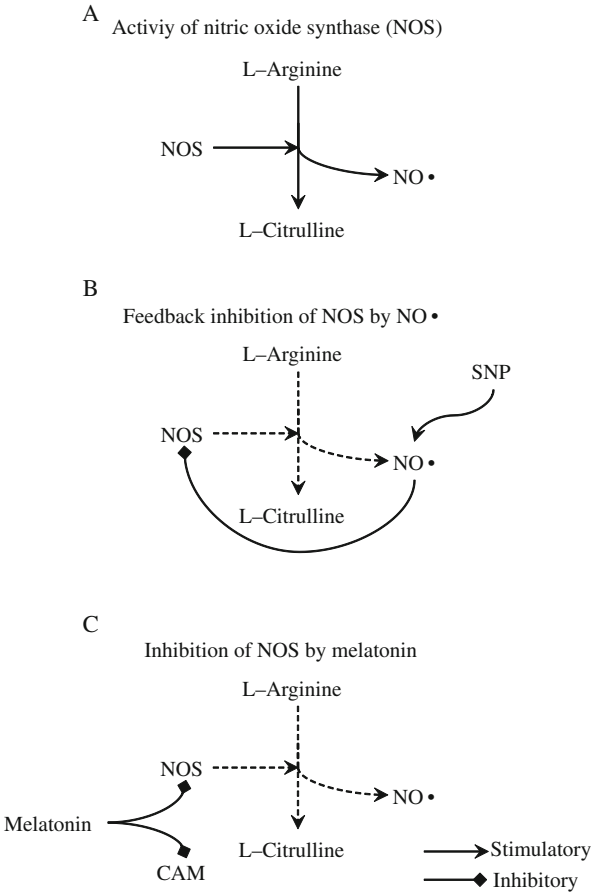

Fig. 1 Regulation of NOS activity: Schematic diagram showing the activity of NOS and its regulation by NO and melatonin. (**a**) Activity of NOS, (**b**) feedback inhibition of NOS by NO, and (**c**) inhibition of NOS by melatonin

The compound is hence used to examine pathways induced by NO in cell lines of different origins and to study the susceptibility of cells to SNP-mediated toxicity. NO plays a fundamental role as a neuronal messenger involved in neurotransmitter release, long-term potentiation, and gene transcription. Under specific conditions, however, NO and its metabolites become neurotoxic [18, 19]. In addition, a recent study suggests that NO mediates toxicity in paraquat-exposed SH-SY5Y cells and that 7-nitroindazole exerts a protective role [20].

2.1 Aβ, NO, and Synaptic Plasticity

The peptide Aβ plays a major role in AD. Consequently, much research is under way to understand the mechanisms by which Aβ is involved in AD pathogenesis. Aβ has

been shown to markedly impair hippocampal long-term potentiation (LTP), a commonly studied cellular model of synaptic plasticity that is considered to underlie learning and memory. Notably, the NO pathway is involved in synaptic dysfunction after Aβ elevation in AD [21] brain. Hence, researchers are currently investigating the exact role of the NO/cGMP/cAMP-regulatory element binding (CREB) pathway in Aβ-induced changes of basal neurotransmission and synaptic plasticity in the hippocampus, a structure within the temporal lobe of the brain fundamental for memory storage [21].

2.2 Aβ Fragment Impairs Memory and Increases NO in the Temporal Cortex of Rats

Aβ(25–35) has been demonstrated to impair memory and increase NO in the temporal cortex of rats [22]. In particular, injection of the fraction Aβ(25–35) caused an increase of neuronal nitric oxide synthase (nNOS) and iNOS immunoreactivity in the temporal cortex and hippocampus. In addition, there was a significant increase of reactive astrocytosis, which was accompanied by neuronal damage in the temporal cortex and hippocampus of rats injected with Aβ(25–35).

3 The Vulnerability of Different Cell Types to ROS and RNS Insults

The vulnerability of different cell types to ROS and RNS is incompletely understood. This issue has been addressed by studying the susceptibility of different cell types to SNP-mediated damage and by quantitatively analyzing the level of secreted derivatives of APP, specific forms of which have previously been shown to be neuroprotective [23–25]. SNP has been used as an exogenous source of NO to define the role of free radicals, superoxides, and ROS in general metabolism and viability of astrocytic and neuroblastoma cell cultures. Consequent to the instability of NO, its stable end products such as nitrite and nitrate (NOx) are generally measured, rather than the parent compound. An additional reason for selecting SNP is that, in addition to NO, SNP also chemically generates KCN, which is toxic to cells. Unlike cyanide, NO is labile and converts to its stable end products, such as nitrite and nitrate, through a series of chemical steps involving the participation of superoxides. As each cell type generates various levels of superoxides, the production of NOx therefore varies among different cell lines. The vulnerability of a specific cell type depends, in an inverse manner, upon the accumulation of nitrite and nitrate. For example, astrocytic and epithelial cell lines generate a considerable amount of NOx, and these cell lines are more resistant against the SNP-mediated damage than neuronal cell lines. These results suggest that cell lines of different origins responded differentially to NO-induced cellular insults, that APP processing was altered as a

consequence of SNP treatment, and that neuronal cell lines lack an effective protective mechanism against free radicals–mediated insult compared with astrocytic cell lines.

4 Use of SNP to Study the Susceptibility of Different Cell Types Toward Free Radicals

In the absence of SNP, nitrite concentrations proved to be below the detection limit of all the cell lines tested at every time point. However, SNP was found to release NOx when incubated with the cell culture medium alone. When the SNP-containing medium was added to the cells, there was an increased release of NOx in the conditioned medium in a time- and dose-dependent manner. This observed increase was significantly higher than the spontaneous NOx released from SNP in the medium. This finding indicates that cellular processes participate in the generation of NOx under these conditions.

5 Different Cell Types Generate NOx Differently When Treated with SNP

Measurement of NOx levels in conditioned medium from different cell lines demonstrated that SNP has different NOx generating capacity depending on the cell type. In all cell types, NOx continued to accumulate in a time-dependent fashion. Two groups of cell types were observed: one with a high level of NOx release, epitomized by astrocytic, glial, and epithelial cells, and one with a low NOx release, as found in neuronal cells. As an example, the SNP-induced NOx release from C6 and U-138 cells was significantly higher at all measurement points (6, 12, 24, 32, and 48 h) than that determined in PC12, IMR-32, and N1E-115 cells under the same conditions. Compared with the conditioned medium, the level of NOx could not be detected in intracellular extracts of different cell types after SNP exposure.

5.1 Increased Levels of NOx Release in SNP-Treated Astrocytic and Epithelial Cell Lines

The SNP-mediated release of NOx at 100 μM SNP is described below. In the conditioned medium of astrocytic U-138 cells, the NOx release was 10 μM at 12 h and 25 μM at 24 h. In C6 cells, NOx release was 24 μM at 12 h and 38 μM at 24 h. In the conditioned medium of HeLa cells, NOx release was 34 and 45 μM at 12 and 24 h, respectively (Table 1). The SNP-mediated release of NOx at 300 μM SNP is the following: In U-138 cells, the NOx release was 13 μM at 12 h and 36 μM at 24 h. In C6 cells, NOx release was 32 μM at 12 h and 60 μM at 24 h. In the conditioned medium of HeLa cells, NOx release after 300 μM treatment was 46 and

Table 1 Effect of 100 μM SNP on the release of nitrite and nitrate (NOx) in different cell lines[a]

	SNP (100 μM) effect on release			
Cell lines	NOx (M) 12 h	LDH (%) 12 h	NOx (M) 24 h	LDH (%) 24 h
C6	24.12 ± 0.20	3.60 ± 1.00	37.51 ± 0.21	4.50 ± 1.25
HeLa	34.06 ± 0.31	5.80 ± 1.60	45.16 ± 1.64	15.50 ± 1.60
N1E-115	16.80 ± 1.20	52.60 ± 4.72	24.80 ± 0.96	49.66 ± 1.96
U-138	10.00 ± 0.95	4.00 ± 0.66	24.69 ± 1.16	4.60 ± 0.66
IMR-32	4.10 ± 0.75	0.00 ± 0.00	15.25 ± 0.73	0.00 ± 0.00

[a]Cell lines of different origins were cultured and treated with 100 μM SNP for various time periods, and levels of NOx and LDH were measured as described previously [23]. Cytotoxicity was expressed as the percentage of LDH activity from the plain conditioned medium (0%) to total LDH activity in the medium when cells were completely lysed (100%).

Table 2 Effect of 300 μM SNP on the release of nitrite and nitrate (NOx) in different cell lines[a]

	SNP (300 μM) effect on release			
Cell line	NOx (M) 12 h	LDH (%) 12 h	NOx (M) 24 h	LDH (%) 24 h
C6	31.95 ± 0.32	5.70 ± 0.45	59.09 ± 0.08	4.10 ± 1.80
HeLa	45.65 ± 0.51	2.90 ± 2.60	76.22 ± 1.10	26.70 ± 2.10
N1E-115	18.60 ± 1.10	46.88 ± 16.30	23.10 ± 0.50	56.48 ± 9.24
U-138	13.40 ± 0.25	4.30 ± 1.60	35.90 ± 3.70	12.60 ± 0.83
IMR-32	4.79 ± 0.89	0.00 ± 0.00	20.75 ± 1.33	3.86 ± 0.64

[a]Cell lines of different origins were cultured and treated with 300 μM SNP, and levels of NOx and LDH were measured as described previously [23]. Cytotoxicity was expressed as the percentage of LDH activity from the plain conditioned medium (0%) to total LDH activity in the medium when the cells were completely lysed (100%).

76 μM at 12 and 24 h, respectively (Table 2). Hence, there is an increased NOx release over time and by dose of the treatment in these cell lines, however the release did not follow a simple linear formula.

5.2 Reduced Levels of NOx Release in SNP-Treated Neuronal Cell Lines

In N1E-115 cells at 100 μM SNP treatment, NOx release was 17 and 25 μM at 12 and 24 h, respectively. However, in these cells at 300 μM, NOx release after SNP treatment was only 19 and 24 μM at 12 and 24 h, respectively (Tables 1 and 2). Similar to the neuroblastoma cells, lower levels of NOx were released in the conditioned medium of PC12 cells than that in astrocytic and glial cell lines at all time points and SNP doses studied (data not shown).

6 SNP-Induced Cell Death

SNP-induced damage in different cell types was assessed by assaying for elevations in lactate dehydrogenase (LDH) levels. In this regard, the studied cell lines varied to different degrees in their sensitivity to SNP. For example, glial C6 cells were found to be about 90% viable with 70 μM NOx release, whereas neuroblastoma N1E-115 cells were about 50% viable with only 15 μM NOx release. Thus, the neuronal cell line N1E-115 was most sensitive to cell death in the face of a low level of NOx production. On the contrary, C6 and U-138 cells (and to some extent HeLa) were resistant to NOx-mediated damage up to 48 h after 300 μM SNP treatment (Tables 1 and 2).

7 NOS in Different Cell Types

In neurons, NO is mainly generated by a calcium-dependent activation of constitutive neuronal NOS [9]. Specifically, neurons express a constitutive type of NOS, and its activity is regulated by stimulation of the *N*-methyl-D-aspartate (NMDA) receptor, muscarinic receptor [26], or an increase in Ca^{2+} levels inside the cells [27]. Additionally, neurons can express iNOS [28]. In glial cells, NO is synthesized in a calcium-independent manner via induction of NOS [29]. Astrocytes express Ca^{2+}-independent iNOS [30]. There are several reports describing the cytokine induction of NOS activity in astrocytoma cells [31]. In addition, the endothelial form of NOS is also present within the CNS and is associated with the brain vasculature [32]. Recently, NO-mediated modulation of synaptic activity by astrocytic P2Y receptors has been shown [33].

7.1 Lack of NOS Activity in U-138, C6, and HeLa Cells

NOS activity was quantified in U-138 cells because they were used as a prototype for the astrocytic cell line. Moreover, in these cells a significant release of NOx occurred over time after SNP treatment with very little cell toxicity. NOS activity was assayed by the conversion of L-[^{3}H]arginine to L-[^{3}H]citrulline. No intrinsic NOS activity was detected in U-138 cells. None of the doses of SNP attenuated or stimulated the enzyme activity when C6 or HeLa cells were incubated with SNP for 48 h.

7.2 Presence of NOS Activity in N1E-115 Cells

NOS activity in the neuroblastoma cell line N1E-115 was assessed as it expresses endogenous NOS. When N1E-115 cells were treated with 100 μM and 300 μM SNP in serum-free dulbecco's modified eagle's medium (DMEM) medium (in vivo), no change in NOS activity was detected. The basal level of NOS activity was very low

after 48 h of serum deprivation. However, when the cells were incubated with SNP in DMEM medium containing 10% fetal bovine serum (FBS), the basal level of NOS activity was significantly elevated (data not shown).

7.3 Inhibition of NOS Activity by SNP

An addition of SNP into a cytosolic preparation of N1E-115 cells elicited a concentration-dependent inhibition of the formation of L-[^{3}H]citrulline. For example, compared with untreated N1E-115 cell extracts, there was a decrease of 38% NOS at 300 μM SNP and 73% NOS at 3 mM SNP. However, when the cytosolic preparations from C6, HeLa, or U-138 cells were incubated with SNP for 1 h, there was no NOS activity observed [23].

8 Effects of Different Agents on NOx Production and LDH Release in Astrocytic Cells

To evaluate the pathway involved in NOx production, the effects of a cytokine, a free radical scavenger, and an antioxidant, an NOS inhibitor were studied [23]. Astrocytes are the major cell type within the CNS, and involvement of astrocytes in AD brain pathology has been well documented, therefore U-138 cell lines were used to study the effects of different agents.

8.1 High Dose of IL-1β Decreased Nitrite Production in C6 Cells When Stimulated with SNP

Cytokines like IL-1β were shown to stimulate NOS expression in various cell types [34, 35]. Incubation of U-138 cells with a low dose of IL-1β in the assay did not stimulate nitrite production. This is in agreement with a study by Feinstein et al. [31], which showed no induction of NOS in another glioma cell line, C6, after treatment with IL-1β. However, in our system, a higher dose of IL-1β (30 ng/ml) showed a decrease in nitrite production when stimulated with SNP compared with the control [23].

8.2 Inhibition of NOx Release with Carboxyl-PTIO Treatment

The compound carboxyl-PTIO (2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide) is a water-soluble, stable NO radical scavenger that reacts with free NO radical in a stoichiometric manner. Carboxyl-PTIO was hence used to test the specificity of NO production from SNP. A significant inhibition of

NOx production was observed when U-138 cells were treated with 100 μg/ml carboxyl-PTIO.

8.3 Inhibition of NOx Release with SOD-1 Treatment

NO reacts with superoxide radicals to generate peroxynitrite [9]. As superoxide dismutase (SOD) is a scavenger of superoxide ions and prevents peroxynitrite formation, SOD-1 treatment should decrease NOx release in the medium. In this regard, the addition of SOD-1 to U-138 cells produced a decrease in NOx accumulation.

8.4 L-NAME Treatment Did Not Change the Release of NOx Release

A well-studied NOS inhibitor, L-NAME (L-nitro-arginine methyl ester), was used to study the involvement of NOS in SNP-mediated NOx release in U-138 cells. Incubation of the cells with 300 μM L-NAME did not change the amount of NOx production by SNP. This observation suggests that the action of SNP does not involve NOS. The measurement of LDH demonstrated that, compared with controls, none of the agent described above showed a significant level of toxicity in cell culture.

9 Pathway of NOx Production in Various Cell Types

Different cell types generate varying amounts of NOx from SNP-treated cells, signifying a cell type–specific release of NOx. It remains to be elucidated how cells influence this process, but the following pathway can be suggested from the work of several laboratories (Fig. 2). SNP generates NO after liberating cyanide ions. Peroxynitrite formation results from the reaction of a superoxide ion and NO, which decompose to NO_2 and hydroxyl radicals and subsequently results in the formation of NO_2/ NO_3. As NO was not directly measured in experimental studies but rather is followed through generation of its oxidative metabolites (NOx) that are derived from peroxynitrite, it follows then that more superoxides should lead to an increased peroxynitrite formation. As cellular processes result in the generation of ROS and superoxide ions, these results suggest that the ability of astrocytic and neuroblastoma cells to produce a different level of NOx is due to their different capacity in superoxide generation. Most of the biological effects of SNP are mediated through NO, which activates guanylate cyclase and increases cGMP levels in many cell systems. The mechanism of NO release from SNP is not clear, but several studies [36, 37] indicated that spontaneous release of NO from SNP does not account for the total amount of NO generated from SNP. The dominant site for metabolic activation of SNP to NO in vascular smooth muscle resides in the membrane fractions [38]. As

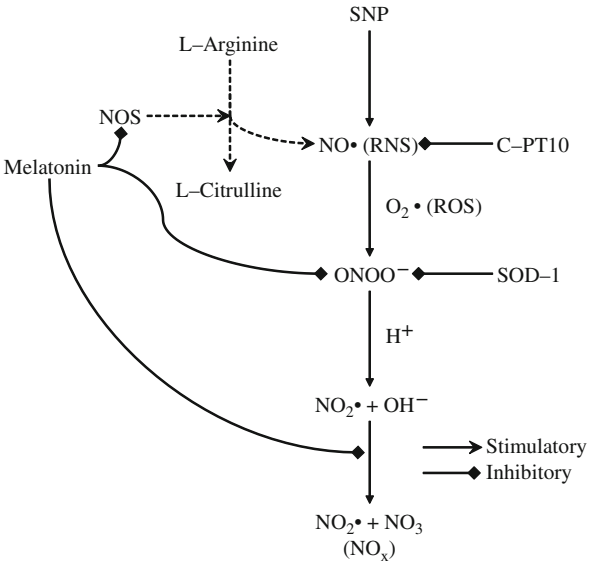

Fig. 2 Generation NOx by SNP in cultured cells. A schematic diagram illustrating the interaction of SNP, NO/its metabolites, and other regulators. SNP chemically releases NO after liberating cyanide ions. Peroxynitrites are formed as a result of the reaction between the superoxide ion and nitric oxide. Peroxynitrites then decompose to NO_2 and hydroxyl radicals, which subsequently result in the formation of NO_2/NO_3 (NOx). As the cellular process results in the formation of ROS including superoxides, astrocytic and neuroblastoma cells produce various levels of NOx due to their different capacity of superoxide generation. The protective effect of NOx against SNP-mediated toxicity differs in cell types, which generate varying degree of NOx from the SNP-treated cells. The generation of NO is regulated by melatonin, SOD-1, and other agents as indicated within the figure and described in the text

our study showed differential release of NOx from different cell types, we hypothesize that the metabolic interaction of SNP with different cell lines may account for this difference in NOx release [23].

10 Mechanism of NOx Production from SNP

The specificity of NOx production from SNP was tested by three different agents: (i) an NO scavenger, (ii) an NOS inhibitor, and (iii) a superoxide scavenger (Fig. 2). When cells were treated with carboxy-PTIO, a characterized and stable NO radical scavenger, a significant inhibition of NO production was observed. Treatment of U-138 and N1E-115 cells with L-NAME, a well-studied NOS inhibitor, did not change the amount of nitrite production by SNP. This observation suggests that the action of SNP does not involve NOS. SOD, which enhances the biological half-life of NO by removing peroxide ions, did not increase nitrite accumulation in our study. The mechanism of the interaction of SOD and NO is not fully understood.

However, it is known that SOD increases the generation of NO from L-arginine in the presence NOS. The mechanism by which SOD increases NOS-mediated NO signal cannot be fully explained by dismutation of $O_2^{-\bullet}$, and the effect of SOD on free NO was different from the effect of SOD on NO that is generated by NOS.

11 Levels of NOx and Cellular Viability

The level of NOx is important for cellular viability, and this determines how each cell line differs in its ability to handle SNP-mediated toxicity. For example, C6 cells were found to be the most and N1E-115 cells the least viable after a similar level of SNP-mediated toxicity. Although the toxic effect of SNP has been suggested to be due to the formation of peroxynitrite generated via the interaction between NO and superoxide, this could also potentially involve the accumulation of either cyanide, NO, or any of these combinations. Nonetheless, these results clearly demonstrate that the neuronal cell lines studied were more vulnerable to NO-mediated cytotoxicity than were astrocytic or glial cell lines, and this finding is of considerable potential in vivo relevance.

12 Protective Cellular Mechanism to Reduce the SNP-Induced Toxicity

Activated astrocytes and macrophages in vivo produce a high amount of NO. It is believed that some protective mechanism may be operative in these cells to avoid the SNP-induced cyanide toxicity. Differential susceptibility of neurons and astrocytes to NO might be due to their different content of reduced glutathione (GSH). The GSH content of neurons has been reported to be drastically decreased after peroxynitrite exposure, whereas the GSH content of the astrocytes was not affected [39]. It has also been reported that neurons containing NOS are more resistant to NO toxicity [18] by an unknown mechanism. Endogenous NO is synthesized from L-arginine by NOS in the various cells described. The astrocyte and microglial cells play an important role in brain pathology and are in close contact with neurons. Elevated NO production from astrocytic cells could potentially damage the surrounding neurons that are more vulnerable to NO-mediated toxicity and may occur during disease processes. In this regard, the role of Aβ in the induction of NOS activity in astrocytic cells has been investigated: Aβ was able to induce the expression of iNOS and NO production in C6 cell lines in the presence of various cytokines [40]. These activated astrocytes may cause neuronal damage via the indirect NO mechanism and thereby impact or drive pathologic processes.

13 Relationship Between the Enzymatic and Nonenzymatic Pathways of NO Release

The release of NOx from SNP in cell culture appears to proceed via a nonenzymatic pathway from the following observations. First, the SNP-derived NOx release was not inhibited by cotreatment with L-NAME, an NOS inhibitor. Second, most of the cell lines described herein (except N1E-115) lack a basal level of NOS activity. Third, other inducers of NOS (such as IL-1 and LPS) have not been found to exert any significant effect on SNP-mediated release of NOx. Although nonenzymatic, the release of NOx involves the intermediate step of NO because an NO scavenger, such as carboxy-PTIO, inhibits it. Additionally, the formation of peroxynitrite with superoxide is critical in final NOx release and is supported by the inhibitory effect of superoxide dismutase (Fig. 2). We have also investigated the relationship between the enzymatic and nonenzymatic pathways of NO release. Our results demonstrate that nonenzymatic release of NOx could inhibit the activity of NOS, resulting in a decreased accumulation of NOx. This could be due to the inhibition of NOS by ROS generated by SNP. This assertion is in agreement with previous reports showing a feedback inhibition of NOS activity with excess NOx. In addition, it is supported by our previous results on differentiated PC12 cells [23].

14 Cellular Participation for the Generation of NOx

According to our working model, a direct interaction of the released NO with superoxide anions and other ROS leads to the formation of nitrite. As ROS are a natural by-product of normal metabolism, cell type–dependent generation of ROS could directly influence the nitrite measurement in these cells. As the half-life of NO is approximately 30 s and that of SNP can be several hours, the effect of SNP over 48 h may be exclusive of NO production but secondary to other metabolites such as KCN. We have not measured the ratio of nitrite to nitrate production, but rather have focused on the release of total NOx. Depending upon the aqueous phase examined, nitrite production usually is significantly increased over nitrate production during the release of NO. These results suggest that NOx release does not solely depend on the growth rate of different cell lines and that SNP-mediated damage caused by the combination of NO and ROS results in severe damage to the neuronal cell lines. As there is a buildup of ROS during normal aging and particularly in AD, the selective loss of neuronal cells should be expected from the apparent lack of any efficient neuroprotective mechanism against superoxides, free radicals, and other ions.

15 Role of Melatonin in the Inhibition of NOx Release

Melatonin hormone, a well-characterized antioxidant, could play an important protective role in aging and AD based on cellular and animal studies [41, 42]. Inhibition

of NO production may be a further means whereby melatonin reduces oxidative damage under conditions, such as in ischemia/reperfusion and sepsis, where NO seems to be important in terms of the resulting damage [43]. Previously, we have shown that melatonin can promote neuronal differentiation [41, 44]. We have additionally explored whether the treatment of cultured cells with melatonin can reduce the release of free radicals and other ROS. Indeed, in neuroblastoma cells, the release of NOx as mediated by SNP was significantly inhibited by treatment with melatonin [23]. These results suggest that SNP-mediated NOx release was mediated by superoxide ions and/or free radicals that, in turn, can be inhibited by melatonin (Figs. 1 and 2). We have also characterized the effects of different neuroprotective compounds, including melatonin [35, 44], to reduce and/or prevent the formation of this cascade pathway.

16 Relationship Between NO Metabolites, Antioxidants, and AD

These results suggest that the release of cell type–specific ionic metabolites could be the target for generation of toxic products, like peroxynitrite, which lead to cell injury, damage, and, eventually, to death. It is conceivable that in neurodegenerative disorders epitomized by AD, the likely increased release of superoxides and other ROS can potentially initiate a feedback loop involving the formation of toxic products that are capable of producing further insult and damage to cells. Thus, the ROS-scavenging function of compounds exemplified by melatonin, along with their neuroprotective and neurodifferentiating roles, can potentially be used for the prevention of neurodegenerative disorders such as AD. The mechanism(s) underpinning such actions warrant further study as such pathways are likely shared among neurodegenerative diseases and provide potential for a common therapeutic/preventative strategy. In conclusion, studies described herein demonstrate that the exposure of neuronal cells to SNP makes them more vulnerable to injury. Furthermore, astrocytic and neuronal cell cultures can be used as valuable model systems to study the involvement of nitric oxide metabolites, peroxynitrites, superoxides, and other reactive oxygen species in health, aging, and neurodegeneration.

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