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The role of mitochondrial dysfunction and neuronal nitric oxide in animal models of neurodegenerative diseases

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Abstract

Excitotoxicity, mitochondrial dysfunction and free radical induced oxidative damage have been implicated in the pathogenesis of several different neurodegenerative diseases, such as amyotrophic lateral sclerosis, Parkinson's disease (PD), Alzheimer's disease (AD), and Huntington's disease. Much of the interest in the association of neurodegeneration with mitochondrial dysfunction and oxidative damage emerged from animal studies using mitochondrial toxins. Within mitochondria 1-methyl-4-phenylpyridinium (MPP^+), the active metabolite of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), acts to inhibit NADH-coenzyme Q reductase (complex I) of the electron transport chain. MPTP produces Parkinsonism in humans, primates, and mice. Similarly, lesions produced by the reversible inhibitor of succinate dehydrogenase (complex II), malonate, and the irreversible inhibitor, 3-nitropropionic acid (3-NP), closely resemble the histologic, neurochemical and clinical features of HD in both rats and non-human primates. The interruption of oxidative phosphorylation results in decreased levels of ATP. A consequence is partial neuronal depolarization and secondary activation of voltage-dependent NMDA receptors, which may result in excitotoxic neuronal cell death (secondary excitotoxicity). The increase in intracellular Ca^{2+} concentration leads to an actiation of Ca^{2+} dependent enzymes, including the constitutive neuronal nitric oxide synthase (cnNOS) which produces NO·. NO· may react with the superoxide anion to form peroxynitrite. We show that systemic administration of 7-nitroindazole (7-NI), a relatively specific inhibitor of cnNOS *in vivo*. attenuates lesions produced by striatal malonate injections or systemic treatment with 3-NP or MPTP. Furthermore 7-NI attenuated increases in lactate production and hydroxyl radical and 3-nitrotyrosine generation *in vivo*, which may be a consequence of peroxynitrite formation. Our results suggest that neuronal nitric oxide synthase inhibitors may be useful in the treatment of neurologic diseases in which excitotoxic mechanisms play a role. (Mol Cell Biochem **174:** 193–197, 1997)

Key words: nitric oxide, MPTP, 3-nitropropionic acid, malonate, 3-nitrotyrosine, free radicals

Introduction

The pathogenesis of nerve cell death in neurodegenerative diseases is unknown. Neurodegenerative illnesses are characterized by gradually evolving, slow, relentless neuronal death, not accompanied by an intense tissue reaction or inflammatory response. There is selective loss of certain defined groups of neurons which may be related either anatomically or physiologically. The diseases are exemplified by Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD), amyotrophic lateral sclerosis (ALS) and cerebellar degenerations. Each of these illnesses has a characteristic pattern of pathology, but there is occasional overlap, e.g. seen with the Parkinsonism-Dementia-ALS complex of Guam in which pathologic features of PD, AD and ALS can occur in various combinations. In addition striatonigral and cerebellar degeneration are part of multiple system atrophy. This raises the possibility that some common underlying mechanisms may play a role in these disorders.

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Mitochondrial dysfunction and secondary excitotoxicity in neurodegenerative diseases

Excitotoxicity, mitochondrial dysfunction and free radical induced oxidative damage have been implicated in the pathogenesis of several different neurodegenerative diseases, such as ALS, PD, AD and HD [1]. But, how can a neurotoxic action of glutamate result in an insidious slowly evolving process of neuronal loss occuring over many years? One possibility is that a progressive impairment of energy metabolism may secondarily result in slow excitotoxic neuronal death [2]. Several studies have reported decreased glucose metabolism and abnormalities in mitochondrial electron transport enzymes in neurodegenerative diseases.

Much of the interest in the association of neurodegeneration with mitochondrial dysfunction and oxidative damage emerged from animal studies using mitochondrial toxins. Within mitochondria l-methyl-4-phenylpyridinium (MPP^+), the active metabolite of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) acts to inhibit NADH-coenzyme Q reductase (complex I) of the electron transport chain. MPTP produces Parkinsonism in humans, primates, and mice. Similarly, lesions produced by the reversible inhibitor of succinate dehydrogenase (complex II). malonate. and the irreversible inhibitor, 3-nitropropionic acid (3-NP), closely resemble the histologic, neurochemical and clinical features of HD in both rats and non-human primates. The interruption of oxidative phosphorylation results in decreased levels of ATP in the ventral mesencephalon (MPTP) or the striatum (3-NP and malonate). A consequence is partial neuronal depolarization and secondary activation of voltage-dependent NMDA receptors, which may result in excitotoxic neuronal cell death (secondary excitotoxicity). Consistent with this hypothesis the administration of NMDA antagonists attenuated the neurotoxic effects of MPTP in mice [3] and primates [4, 5] and the effects of malonate in rats [6].

NMDA-receptor mediated cascade of cell death

In neurons, the cytoplasmatic Ca^{2+} concentration is maintained several magnitudes lower than that outside the cell by ATPases that actively transport Ca^{2+} out of the cell, or into intracellular storage organelles such as the endoplasmic reticulum. Slow excitotoxicity may occur as a consequence of a defect in energy metabolism. This results in membrane depolarization due to ATP depletion, followed by relief of the voltage dependent Mg^{2+} block of the NMDA receptor, leading to an ion influx of Na^+ and Ca^{2+}. The increase in intracellular Ca^{2+} concentration leads to an activation of Ca^{2+} dependent enzymes. including the neuronal nitric oxide synthase (nNOS) which produces NO·. Initially, studies of Dawson and colleagues implicated nitric oxide (NO·) in excitotoxic cell death following the activation of NMDA receptors [7]. However, the role of NO· in neuronal injury *in vitro* and *in vivo* remained controversial (for review [8]). The absence of consensus may have been due to the prior lack of NO· synthase inhibitors with specificity for various isoforms of the enzyme. Three major isoforms have now been identified which are the constitutive neuronal (cnNOS) and endothelial (ceNOS) forms, and the inducible form (iNOS) mainly produced by macrophages.

Blockade of cnNOS protects against striatal lesions produced by malonate and NMDA

7-Nitroindazole (7-NI) is a recently described relatively specific inhibitor of cnNOS *in vivo* [9–11]. We examined whether pretreatment of rats with 7-NI can attenuate striatal lesions produced by stereotaxic injections of the succinate dehydrogenase inhibitor malonate [12]. Lesion volumes were determined using the triphenyltetrazolium chloride monohydrate (TTC) method. Pretreatment with 7-NI significantly protected against striatal lesions produced by malonate. The administration of the natural substrate L-arginine (300 mg/kg), which competes with 7-NI for binding to the prosthetic heme group of cnNOS, completely blocked the neuroprotective effects of 7-NI, whereas D-arginine had no effect (Table 1).

We also examined whether pretreatment with 7-NI can attenuate striatal excitotoxic lesions produced by the direct acting excitatory amino acid receptor agonists N-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methylisoxazole-4-proprionic acid (AMPA) and kainic acid (KA). 7-NI significantly attenuated excitotoxic lesions produced by NMDA, but not those by KA and AMPA (Table 2). The protective effects of pretreatment with 7-NI were more pronounced on striatal lesions produced by malonate than on lesions produced by NMDA.

NO· is popularly regarded as being highly reactive but chemists point out, that this is not in fact the case [13]. There is little evidence in the chemical literature that it reacts with more than a small range of compounds. It is not destructive in the same way as the hydroxyl radical, and the suggestion that its cytotoxicity is simply the consequence of its radical nature cannot be correct. However, several potential cytotoxic

Table 1. Effects of pretreatment with 7-NI (50 mg/kg i.p.) or vehicle on striatal lesions produced by 3 µmol of malonate (Mean ± SEM)

Treatment	Lesion volume [mm^3]
Vehicle	38.5 ± 4.8
7-NI	20.2 ± 4.2*
7-NI & 300 mg/kg L-arginine	36.2 ± 4.4
7-NI & 300 mg/kg L-arginine	36.2 ± 4.9*

*$p < 0.01$ as compared to vehicle treated animals.

Table 2. Effects of pretreatment with 7-NI (2 × 50 mg/kg) or vehicle on striatal lesions produced by 200 nmol ol NMDA, 5 nmol of KA or 30 nmol of AMPA

Excitotoxin	Treatment	Lesion volume [mm^3]	3-nitrotyrosine/tyrosine [* 1000]	2,5 DHBA/salicylate [* 1000]	2,3 DHBA/salicylate [* 1000]
NMDA	Vehicle	30.7 ± 4.1	2.9 ± 0.4	4.1 ± 0.6	3.1 ± 0.3
	7-NI	19.9 ± 2.5*	2.0 ± 0.3**	2.3 ± 0.5*	2.9 ± 0.9
KA	Vehicle	34.8 ± 4.1	3.3 ± 0.6	3.6 ± 0.6	4.1 ± 0.9
	7-NI	35.9 ± 5.0	3.2 ± 0.5	4.0 ± 0.8	2.8 ± 0.3
AMPA	Vehicle	30.5 ± 5.7	n.d.	n.d.	n.d.
	7-NI	37.8 ± 3.3	n.d.	n.d.	n.d.

*p < 0.05; **p < 0.01; n.d. – not done.

pathways for NO· have been proposed. The majority of evidence indicates that the toxic effects of NO· occur through a non-enzymatic reaction of NO· with the superoxide anion to form peroxynitrite ($ONOO^-$) [14, 15]. This reaction occurs at an extremely fast rate of 6.7×10^9 M^{-1} s^{-1}, outcompetes superoxide dismutase for the substrate superoxide, and does not require transition metals. Peroxynitrite is a highly reactive molecule, a potent oxidizing agent known to initiate lipid peroxidation in biological membranes, hydroxylation and nitration of aromatic amino acid residues, and sulfhydryl oxidation of proteins [16, 17].

One way to demonstrate peroxynitrite formation *in vivo* is to detect the presence of stable by-products of its reaction with various biological compounds. 3-Nitrotyrosine, the product of addition of a nitro-group (NO_2) to the ortho position of the hydroxyl group to tyrosine, is such a stable compound. So far peroxynitrite is the only species known to be capable of tyrosine nitration in sufficient amounts [18].

To assess whether administration of 7-NI is associated with attenuation of peroxynitrite production we measured the generation of 3-nitrotyrosine by HPLC [12]. One hour after intrastriatal administration of 200 nmol NMDA there was a significant increase in the formation of 3-nitrotyrosine. This increase was attenuated by pretreating with 7-NI, consistent with a block of NMDA receptor mediated NO· and peroxynitrite production (Table 2).

Peroxynitrite may decompose homolytically to yield nitrogen dioxide (NO_2), and species with hydroxyl-like reactivity, without the need of metal catalysis [19]. We used the salicylate method to detect hydroxyl (·OH) radicals [20]. The reaction of salicylate with ·OH radicals results in the formation of 2,3 and 2,5 dihydroxybenzoic acid (DHBA). One hour after intrastriatal administration of 200 nmol NMDA there was a significant increase in both of 2,3 DHBA/salicylate and 2,5 DHBA/salicylate ratios. The increase in 2,5 DHBA was significantly attenuated by pretreatment with 7-NI, confirming that ·OH radicals are produced downstream from NO· production after NMDA receptor activation (Table 2).

An alternative mechanism of NO· or peroxynitrite toxicity is the inhibition of mitochondrial function [21–24] or the depletion of ATP by activation of poly(ADP-ribose) synthase [25]. As compared to vehicle treated animals pretreatment with 7-NI significantly attenuated decreases of striatal ATP concentrations measured 3 h after injections of 3 µmol of malonate (Table 3). Similarly, 7-NI pretreatment attenuated the increase of localized lactate concentrations after striatal malonate lesions as measured *in vivo* using ^1H magnetic resonance spectroscopy [12]. These results contrast with those of a free radical spin trap which exerted neuroprotective effects but had no effect on malonate induced depletions of ATP [26].

Blockade of cnNOS protects against striatal lesions produced by 3-nitropropionic acid (3-NP)

3-Nitropropionic acid (3-NP) is a naturally occurring plant and mycotoxin which is an irreversible inhibitor of complex II. It produces disease in livestock and illness occured in humans safter ingestion of mildewed sugar can in China. The illness results in delayed onset dystonia and putaminal necrosis [27]. Systemic administration of 3-NP to rats and non-human primates produces selective striatal lesions, which share characteristic histologic features with Huntington's disease [28].

After systemic 3-NP treatment in rats there is an increased production of the peroxynitrite by-product 3-nitrotyrosine, of 8-hydroxy-2-deoxyguanosine (a marker of oxidative DNA damage), and of dihydroxybenzoic acids in the striatum [12]. Treatment with the specific nNOS inhibitor 7-NI showed marked protection against striatal lesions, production of 3-nitrotyrosine, 8-hydroxy-2-deoxyguanosine, and dihydroxybenzoic acids. Furthermore, mice overexpressing SOD were protected against 3-NP toxicity and the increase of 3-nitrotyrosine [29].

Blockade of cnNOS protects against MPTP-neurotoxicity

1 -Methyl-4-phenyl-1 ,2,3,6-tetrahydropyridine (MPTP) is used to produce a model of Parkinson's disease in primates and mice. The neurotoxic effects of MPTP are thought to be initiated by MPP^+, which is a metabolite formed by the monoamine oxidase B mediated oxidation of MPTP. In mitochondria, MPP^+ disrupts oxidative phosphorylation by

Table 3. Effects of pretreatment with 50 mg/kg of 7-NI on ATP depletions at 3 h and lactate increases detected by ^{1}H magnetic resonance spectroscopy at 1.5 h induced by striatal injection of 3 µmol of malonate as compared with unlesioned striata

Treatment	ATP [nmol/mg protein]		Lactate [mM]	
	unlesioned	lesioned (malonate)	unlesioned	lesioned (malonate)
Vehicle	18.0 ± 1.6	11.6 ± 0.9**	0.39 ± 0.12	2.22 ± 0.38***
7-NI	17.2 ± 0.8	14.8 ± 1.0*,#	0.41 ± 0.08	1.16 ± 0.15**,##

*p < 0.05, **p < 0.01, ***p < 0.001 as compared with the unlesioned striata (paired Student's *t*-test). # p < 0.05, ## p < 0.01 as compared with ehicle treated, lesioned animals (unpaired Student's *t*-test).

inhibiting complex I of the mitochondrial electron transport chain. In our studies, 7-NI dose-dependently protected against MPTP induced dopamine depletions at 7 days. At a dose of 50 mg/kg of 7-NI there was almost complete protection [30]. This effect was not due to an inhibition of monoamine oxidase B activity or the dopamine transporter. MPTP treatment of mice leads to a significant increase of 3-nitrotyrosine in the striatum, which is abolished by 7-NI treatment [30].

Similarly, Smith *et al*. [31] found that treatment with the non-specific NOS inhibitor L-nitro-arginine blocked MPTP toxicity without interfering with inhibition of complex I. Furthermore, inhibition of NOS reduced MPP^+ induced hydroxyl radical formation in striatum, which may also be a consequence of peroxynitrite formation [31]. These results suggest that NO· plays an important role in MPTP toxicity and that this toxicity may be mediated through peroxynitrite.

Conclusion

These results provide evidence that NO· plays a role in NMDA, malonate, 3-NP and MPTP neurotoxicity *in vivo*. They show for the first time, that NO· and peroxynitrite production occurs *in vivo* in established animal models of neurodegenerative diseases. In lesions produced by mitochondrial toxins (MPTP, 3-NP, malonate) leading to secondary excitotoxic cell death blockade of cnNOS is more efficacious than in lesions produced by the direct acting excitotoxin NMDA. In conjunction with our result that blockade of nNOS attenuates ATP depletion and lactate production following malonate injections this implicates that Ca^{2+} induced activation of nNOS and subsequent generation of NO· and peroxynitrite add to the disruption of oxidative phosphorylation through mitochondrial toxins. As such these results suggest that production of NO· may contribute to the impairment of energy metabolism reported in PD, HD and AD.

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