



## Research report

## 3-Nitropropionic acid induced in vivo protein oxidation in striatal and cortical synaptosomes: insights into Huntington's disease

Michael A. La Fontaine<sup>a,c</sup>, James W. Geddes<sup>b,d</sup>, Andrea Banks<sup>a</sup>, D. Allan Butterfield<sup>a,c,d</sup><sup>a</sup> Department of Chemistry, University of Kentucky, Lexington, KY 40506, USA<sup>b</sup> Department of Anatomy and Neurobiology, University of Kentucky, Lexington, KY 40506, USA<sup>c</sup> Center of Membrane Sciences, University of Kentucky, Lexington, KY 40506, USA<sup>d</sup> Sanders-Brown Center in Aging, University of Kentucky, Lexington, KY 40506, USA

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## Abstract

3-Nitropropionic acid (3-NP) administered systemically daily for 4 days to rats inhibits mitochondrial oxidative phosphorylation and induces selective lesions in the striatum in a manner reminiscent of Huntington's disease (HD). To investigate the potential oxidative nature of these lesions, rats were injected with 3-NP (20 mg/kg i.p.) daily for 4 days and subsequently isolated brain synaptosomal membranes were examined for evidence of oxidative stress. Brain synaptosomal membrane proteins from rats injected with 3-NP exhibited a decreased in  $M/S$  ratio, the relevant electron paramagnetic resonance (EPR) parameter used to determine levels of protein oxidation (76% of control), and Western blot analysis for protein carbonyls revealed direct evidence of increased synaptosomal membrane protein oxidation (248% of control). Similar results were obtained in synaptosomes isolated from striatum and from cerebral cortex, demonstrating that the oxidative changes are not restricted to the lesion site. Moreover, increased oxidative stress was evident prior to the appearance of morphological lesions. These data are consistent with the hypothesis that 3-NP-induced striatal lesions, and perhaps those in HD, are associated with oxidative processes.

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## 1. Introduction

Huntington's disease (HD) is an autosomal dominant neurodegenerative disorder characterized by progressive loss of striatal neurons [1,2]. Although the mechanisms of selective striatal damage in HD are not known, the activation of excitatory amino acid receptors have been implicated [3]. In addition, various toxins have been found to cause striatal lesions reminiscent of the neurochemical and anatomical changes associated with this disorder [4].

Such toxin is 3-nitropropionic acid (3-NP), a naturally occurring plant mycotoxin that is an irreversible inhibitor of succinate dehydrogenase, a subunit of complex II of the electron transport chain and a component of the Krebs cycle [5,9,16,17,20,27,37,44,45]. Previous evidence for the involvement of oxidative stress in 3-NP neurotoxicity includes protection against 3-NP toxicity by antioxidants and increased conversion of salicylate to 2,3-dihydroxybenzoic acid (DHBA) and 2,5-DHBA as well as increased 3-nitrotyrosine, a marker for peroxy nitrite-mediated damage [48]. However, striatum was the only brain region studied and markers of oxidation were assessed only after the appearance of lethargy in the animals, a time point that coincides with the appearance of morphologic lesions. It is not known if 3-NP results in oxidative stress in brain regions other than striatum. It is also not known if the oxidative stress precedes or follows striatal lesions induced by 3-NP. This was examined in the present study in rat brain synaptosomal preparations using

Abbreviations: 3-Nitropropionic acid (3-NP); Electron paramagnetic resonance (EPR); Reactive oxygen species (ROS); Huntington's disease (HD); 2,2,6,6-Tetramethyl-4-malimidopiperidin-1-oxyl (MAL-6); 2,4-Dinitrophenylhydrazine (DNPH).

<sup>1</sup> Corresponding author. Department of Chemistry and Center of Membrane Sciences, University of Kentucky, Lexington, KY 40506-0055, USA. Fax: +1-606-257-5876; e-mail: dabcs@pop.uky.edu

electron paramagnetic resonance (EPR) and Western blot analysis for protein carbonyls to assess protein oxidation following 3-NP administration.

## 2. Materials and methods

### 2.1. Chemicals

3-NP was obtained from Aldrich Chemical. Ultra-pure

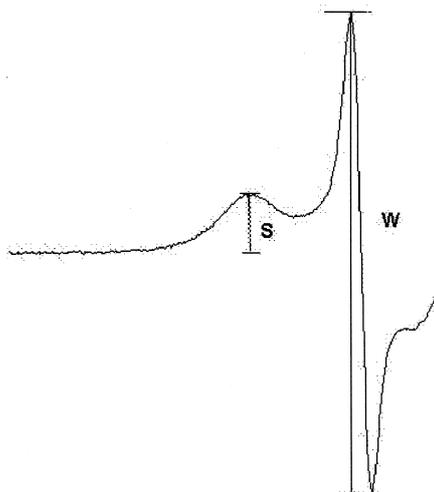


Fig. 1. A typical EPR spectrum of MAL-6-labeled synaptosomal membrane proteins depicting the W and S components of the low field resonance line, from which the W/S ratio is calculated.

of protein carbonyl groups derivatized with 2,4-dinitrophenylhydrazine DNPH was used. Synaptosomal membrane proteins were isolated as above and treated with 20-mM DNPH in 10% trifluoroacetic acid and Derivatization-Control solution and incubated for 20 min. Derivatization was neutralized with OxyBlot neutralization solution 2 M Tris 30% glycerol and 19% 2-mercaptoethanol. Proteins were separated by SDS-PAGE. Experiments were arranged such that both control samples and samples from rats treated with 3-NP were loaded on the same gel Fig. 2.

Polyacrylamide gel electrophoresis was performed in mini-slabs 0.75×60×70 mm, 12% acrylamide according to the method of Laemmli [34]. Following elec-

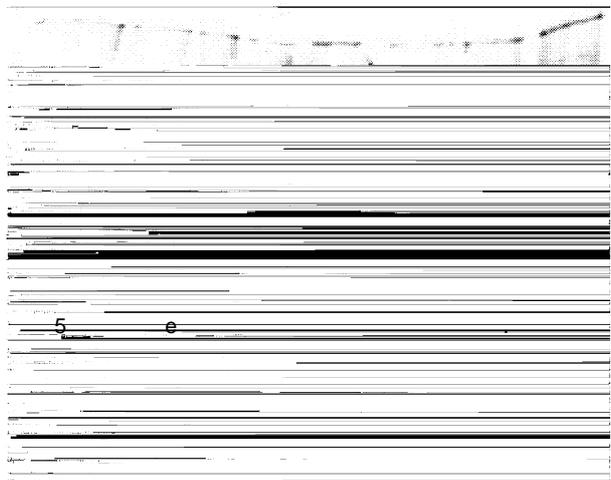


Fig. 2. Developed OxyBlot gel transferred onto nitrocellulose paper. From left to right, lanes 1±4 are synaptosomes from control animals, lanes 5±8 are synaptosomes from animals treated with 3-NP for 4 days.

trophoresis, proteins were transferred to nitrocellulose paper 0.45µm pore size according to the procedure adapted from Glenney [22]. Tris±glycine and 20% methanol at a pH of 8.5 was used as the transfer buffer. Following transfer, nitrocellulose paper was blocked in 3% BSA in PBS with sodium azide, 0.01% and Tween 20, 0.2% for 1 h at room temperature. Membranes were washed three times with washing buffer sodium chloride, 1%, PBS, 2%, sodium azide, 0.01%, and Tween 20, 0.1%. To the membranes, rabbit anti-DNP antibody 1:150 dilution in 90% washing buffer, 10% blocking buffer was added and incubated at room temperature for 1 h under mild shaking. Following incubation, membranes were washed three times with washing buffer. Anti-Rabbit IgG 1:15,000 dilution in blocking buffer was added to the membrane and incubated at room temperature for 1 h under mild shaking. Following incubation, membranes were washed three times then developed using BCIP-NBT solution one SigmaFast tablet per 10-ml deionized water.

Western blots were analyzed using computer-assisted imaging software, MCI-D4, provided by Imaging Research, Ontario, Canada.

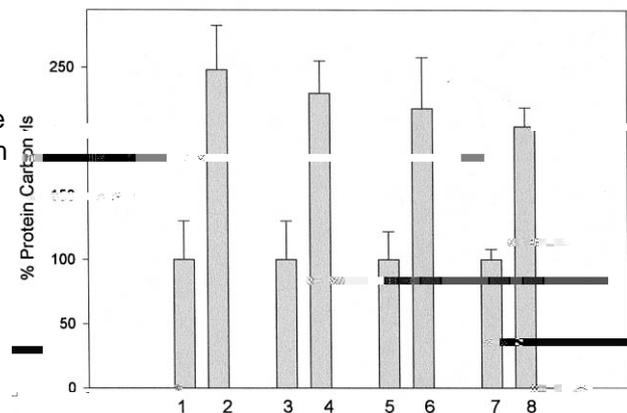


Fig. 3. Protein carbonyls, an index of protein oxidation, in brain following 3-NP administration as described in Section 2. Whole brain synaptosomal membrane proteins were obtained from rats treated with 3-NP for 4 days and from control rats after 4 days. Synaptosomal membrane proteins were also obtained from striatum and cortex from rats treated with 3-NP for 4 days, striatum from rats treated with 3-NP for 3 days, striatum and cortex from control rats after 4 days, and striatum from control rats after 3 days. Protein carbonyl levels were determined using the Oxyblot technique see Section 2. An increase in protein carbonyls 0.05 was shown for whole brain of treated animals after 4 days. For both control and treated animals Ns 6. A significant increase in protein carbonyls was observed p < 0.001, p < 0.01 for striatum and cortex Ns 5 for bo

## 2.8. Statistical analysis.

One-way analysis of variance was used for comparison of the means. Student's *t*-test was used where applicable. Values are expressed as the mean  $\pm$  S.E.M.

## 3. Results

Carney and Carney [14], Oliver et al. [43], Starke-Reed and Oliver [51], Smith et al. [49] and Stadtman [50] have shown that cytosolic protein carbonyl levels are a measure of and are increased in protein oxidation. Protein carbonyl levels were measured by Oxyblot analysis to determine if an increase of carbonyls were present on synaptosomal membranes isolated from 3-NP-treated animals. Consistent with the oxidative stress hypothesis of 3-NP toxicity, a significant increase in carbonyls was present in whole brain synaptosomal membrane proteins isolated from rats treated for 4 days with 3-NP (248% of control,  $p < 0.05$ , Fig. 3). Significant increases of protein carbonyls were also present in synaptosomal membranes isolated from the striatum and cortex of rats injected with 3-NP (230% of control,  $p < 0.001$  for striatum, 218% of control,  $p < 0.01$  for cortex, Fig. 3). Consistent with the hypothesis that oxidation occurs prior to neuronal loss, striatal synapto-

somes isolated from rats injected only 3 days with 3-NP also exhibited increased protein carbonyl levels (204% of control,  $p < 0.01$ , Fig. 3).

Consistent with the protein carbonyl data, the W/S ratio of whole brain synaptosomal membrane proteins from rats injected with 3-NP is significantly decreased (76% of control,  $p < 0.001$ , Fig. 4). In addition, the W/S ratios of MAL-6-labeled synaptosomal membrane proteins isolated from either the striatum or cortex of rats injected for 4 days with 3-NP is significantly decreased when compared to those of control animals (81% of control in striatum,  $p < 0.001$ ; 82% of control in cortex,  $p < 0.001$ , Fig. 4). Striatal synaptosomal membrane proteins labeled with MAL-6 from rats injected with 3-NP for only 3 days exhibit a W/S ratio that is also decreased (88% of control,  $p < 0.01$ , Fig. 4), consistent with the notion that oxidative stress precedes neuronal loss and consistent with the protein carbonyl results cited above.

## 4. Discussion

Chronic 3-NP administration in rats results in progressive metabolic impairment and selective damage to the striatum. This has been utilized as a model of neuronal damage in HD [8]. However, it is not known if the 3-NP-induced oxidative stress precedes or accompanies neuronal damage, and if the oxidative stress is restricted to the striatum, the site of morphologic lesions.

There is extensive indirect evidence of a role of oxidative stress in 3-NP toxicity, including depletion of glutathione pools [7], increased free-fatty acid release [6], neuroprotection by the spin trap 5,5-dimethyl-1-pyrroline-*n*-oxide (DMPO) [48], creatine [88] and acetylcholine [55], caloric restriction [10] and over-expression of copper/zinc superoxide [5]. In contrast, the free radical spin traps  $\alpha$ -phenyl-*N*-tert-butyl-nitrone (PBN), *N*-tert-butyl- $\alpha$ -2-sulphophenyl-nitrone (S-PBN) exacerbated 3-NP toxicity [44] due to interference with 3-NP metabolism [39]. Despite the indirect evidence that antioxidant mechanisms protect against 3-NP toxicity, there is little direct evidence of 3-NP-induced oxidative stress. Schulz et al. [47] did report increased conversion of salicylate to 2,3-dihydroxybenzoic acid (DHBA) and 2,5-DHBA following 3-NP administration as well as an increase in 3-nitrotyrosine, a marker for peroxynitrite-mediated damage. However, striatum was the only brain region studied and only after the appearance of lethargy in the animals, a time point at which morphological damage could be observed.

In the current study, protein oxidation determined by direct measures of protein carbonyls and by EPR of striatal synaptosomes obtained from 3-NP treated rats after 4 days corresponds to observed striatal lesions. That the W/S ratio of spin-labeled cortical synaptosomal proteins is de-

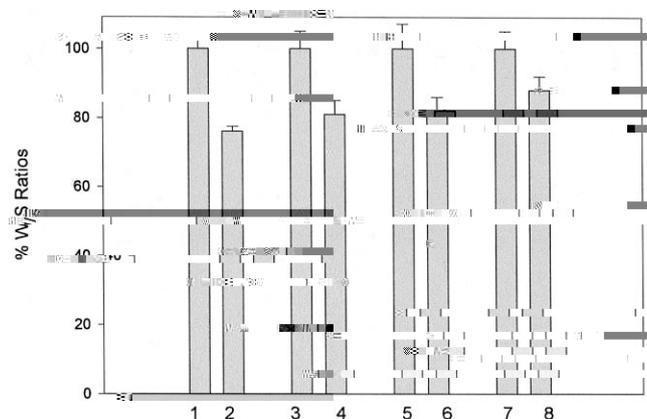


Fig. 4. MAL-6-labeled synaptosomal membrane proteins were obtained from the whole brain of rats treated with 3-NP and the W/S ratios were measured. Significant decreased W/S ratios were observed ( $p < 0.001$ ) when compared to control samples. Control samples: 1, whole brain control; 2, whole brain 3-NP-treated; 3, striatum control; 4, striatum 3-NP-treated; 5, cortex control; 6, cortex 3-NP-treated; 7, 3-day striatum control; 8, 3-day striatum 3-NP-treated. Significant decrease in W/S ratios were observed ( $p < 0.001$ ) when compared to control samples. Control samples: 1, whole brain control; 2, whole brain 3-NP-treated; 3, striatum control; 4, striatum 3-NP-treated; 5, cortex control; 6, cortex 3-NP-treated; 7, 3-day striatum control; 8, 3-day striatum 3-NP-treated.

creased and protein carbonyls are increased in cortical addition, they showed an age-dependent increase in susceptibility towards 3-NP toxicity. These deficiencies in behavior and motor control are reminiscent of the loss of EPR and protein carbonyl measurement in striatum after motor skills associated with increased brain protein oxidation. 3-days of 3-NP treatment suggests that oxidative stress precedes the motor abnormalities and striatal lesions, first observed after 4 days of 3-NP administration. These results provide direct evidence that oxidative stress occurs following 3-NP administration, that the oxidative damage is more widespread than the morphologic lesion, and that oxidation occurs prior to the appearance of morphologic lesions.

3-NP could potentially induce oxidative stress via at least three mechanisms: increased oxygen flux through the electron transport chain; indirect excitotoxic mechanisms; and inflammatory responses to neuronal degeneration.

The flux of oxygen through the electron transport chain results in free radical production. 3-NP is an irreversible inhibitor of complex II of the electron transport chain and of the Krebs's cycle. The major mechanism of 3-NP toxicity is thought to be via inhibition of the citric acid cycle.

3-NP administration also results in indirect excitotoxicity. Previously, EPR was used to show that activation of NMDA receptors can lead to generation of superoxide radicals. Other evidence has also linked free radical involvement with excitotoxicity. By impairing ion pumps and reducing ATP levels, 3-NP results in neuronal depolarization and removal of the Mg block of the NMDA receptor ion channel. This results in ambient levels of glutamate becoming excitotoxic. Calcium influx through the NMDA receptor results in impaired mitochondrial function and increased oxidative stress. The increase in intracellular Ca concentration also results in activation neuronal nitric oxide synthase, which produces NO.

Oxidative stress could also occur in association with an inflammatory response induced by 3-NP toxicity. There is evidence that 3-NP induces an inflammatory response, for example, 3-NP-induced striatal lesions have neutrophil infiltration and are associated with immunoreactivity to serum immune complement factors. In addition, 3-NP leads to increased levels of tumor necrosis factor- $\alpha$ , a pro-inflammatory cytokine, and increased expression of inducible nitric oxide synthase.

Several groups have studied the effect of 3-NP administration on behavior, physical dexterity, and neuropathology. Borlongan et al. showed that 3-NP can lead to hyperactivity as well as hypoactivity, making 3-NP unique among excitotoxin models in mimicking the two-stage progression of HD behavioral alteration. Guyot et al. showed a correlation between severity of 3-NP-induced striatal lesions and motor deficits, including bradykinesia, gait length, and gait velocity. Tsai et al. demonstrated a decrease in glutamine synthetase activity upon administration of 3-NP in a dose-dependent manner. In

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