Research report

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

Michael A. La Fontaine [a,c], James W. Geddes [b,d], Andrea Banks [a], D. Allan Butterfield [a,c,d,*]

[a] Department of Chemistry, University of Kentucky, Lexington, KY 40506, USA
[b] Department of Anatomy and Neurobiology, University of Kentucky, Lexington, KY 40506, USA
[c] Center of Membrane Sciences, University of Kentucky, Lexington, KY 40506, USA
[d] Sanders-Brown Center in Aging, University of Kentucky, Lexington, KY 40506, USA

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 W/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. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: 3-Nitropropionic acid; Huntington's disease; Oxidative stress; Synaptosomal membrane; Spin labeling

1. Introduction

Huntington's disease (HD) is an autosomal dominant neurodegenerative disorder characterized by progressive loss of striatal neurons [2,15]. 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]. One 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 Kreb's cycle [3,9,16,17,20,27,37,44,45].

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-nitrotryrosine, 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

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 sucrose, 4-maleimido-tempo (MAL-6) and Anti-Rabbit IgG antibody were obtained from Sigma. The protease inhibitors aprotinin, leupeptin, and pepstatin A, were obtained from Calbiochem. The OxyBlot™ oxidized protein detection kit was obtained from Oncor. All remaining chemicals were obtained from Sigma in the highest possible purity.

2.2. Animals

All animal protocols have been approved by the University of Kentucky Animal Care and Use Committee. Male rats were purchased from Harlan, and housed in the Sanders-Brown Center for Aging Animal Care Facility. The Sprague–Dawley rats were exposed to 12-h light/12-h dark conditions and were fed Purina Rodent Laboratory Chow with no restrictions to feed or water. At 4 months of age, rats treated with 3-NP were injected i.p. daily for 4 or 3 days with 3-NP dissolved in physiologic saline at a dose of 20 mg/kg, pH 7.4. Control animals received corresponding injections of physiologic saline. Animals were anesthetized with sodium pentobarbital and decapitated 24 h after the final 3-NP injection.

2.3. Whole brain studies

Animals were decapitated 24 h after the final injection. The whole brain was removed and suspended in approximately 20 ml of isolation buffer (0.32 M sucrose, 10 mM Hepes, 4 mg/ml leupeptin, 4 mg/ml pepstatin, 5 mg/ml aprotinin, 20 mg/ml type II-S soybean trypsin inhibitor, 0.2 mM phenylmethylsulfonyl fluoride, 2 mM EGTA, 2 mM EDTA, pH 7.4). The individual brains were homogenized as previously described [25,30].

2.4. Regional differences in 3-NP-induced oxidative stress

To determine if oxidative stress is limited to the striatum, the whole brain was removed and the neocortex and striatum were isolated and suspended in approximately 20 ml of isolation buffer. The individual striata and cortex were homogenized as described above.

To determine if oxidation precedes striatal lesions, first observed following four consecutive days of 3-NP administration (Ref. [4], our unpublished observations), the whole brain was removed and the striatum was isolated and suspended in approximately 20 ml of isolation buffer. The individual striata were homogenized as described above.

2.5. Synaptosome preparation

Synaptosomes were purified as previously described [11,28]. The homogenate was re-spun at $20,000 \times g$ at 4°C for 10 min. The resulting pellet was suspended in approximately 1-ml isolation buffer and layered on a discontinuous sucrose gradient (10 ml of 1.18 M sucrose, pH 8.5/10 ml 1.0 M sucrose, pH 7.4/10 ml 0.85 M sucrose, pH 7.4, each containing 2 mM EDTA, 2 mM EGTA, and 10 mM Hepes). The samples were then spun at $82,500 \times g$ for 2 h at 4°C in a Beckman swinging-bucket rotor. Synaptosomes were collected from the 1.18/1.0 M sucrose interface and resuspended in 20 ml of lysing buffer (10 mM Hepes, 2 mM EDTA, 2 mM EGTA, pH 7.4). The samples were then spun at $32,000 \times g$ at 4°C for 10 min. The pellet was resuspended in lysing buffer and spun twice more. After the third spin, the protein concentration was determined by the Lowry method [36].

2.6. Spin labeling

Each sample was separated into 4-mg aliquots. Spin labeling of synaptosomal membrane proteins was done as previously described [28,54]. Lysed synaptosomal membranes were labeled with 20-μg MAL-6/mg protein. After 18-h incubation at 4°C, samples were washed six times with lysing buffer in order to remove excess spin label. Each 4-mg pellet was resuspended in 1-ml lysing buffer. EPR spectra were acquired on a Bruker model 300 EPR spectrometer operating at an incident microwave power of 16 mW, a modulation amplitude of 0.4 G, a time constant of 1.28 ms, and a conversion time of 10 ms.

The W/S ratio (Fig. 1) of EPR spectra from MAL-6 labeled membrane proteins has been extensively studied in both brain synaptosomal membranes and erythrocyte membranes [13,24–26,28,29,32,54]. Increased steric hindrance of the protein-bound spin label will cause a decrease in the W/S ratio. This can be caused by a variety of changes in the environment of the spin label, including altered protein conformation, a decrease in segmental motion in spin labeled proteins and/or changes in the interactions between proteins. Several oxidative conditions employed in our laboratory, including Fenton chemistry to produce hydroxyl radicals [28], hyperoxia [30,32] ischemia–reperfusion [24–26], accelerated aging [13], β-amyloid-derived free radicals [12,29,31] and menadione [52], have shown that an increase in protein oxidation is associated with a decrease in the W/S ratio.

2.7. Protein carbonyl measurements

To determine the level of protein oxidation, an oxidized protein detection kit based on immunochemical detection
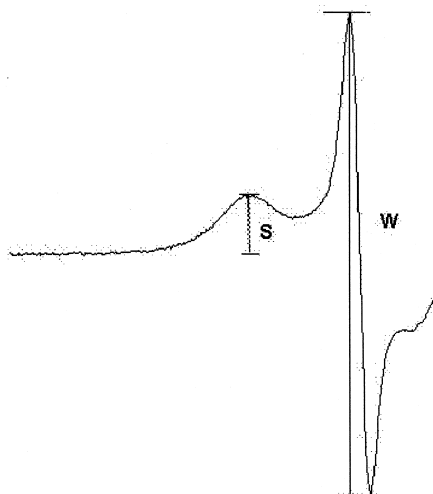
Fig. 1. A typical EPR spectrum of MAL-6-labeled synaptosomal membrane proteins depicting the W and S components of the low field resonance line, from which the W/S ratio is calculated.

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 ($0.75 \times 60 \times 70$ mm, 12% acrylamide) according to the method of Laemmli [34]. Following elec-
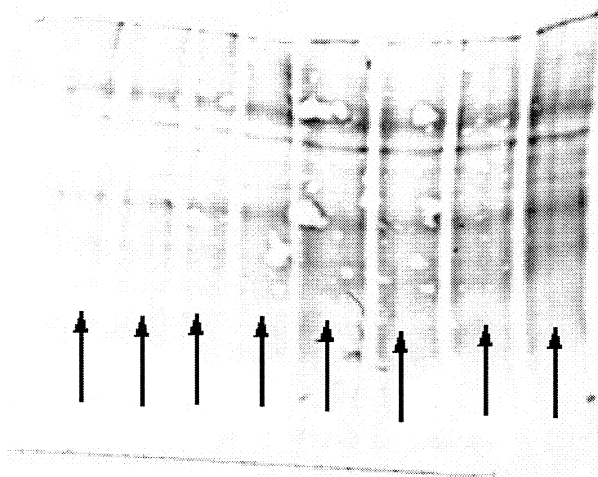
trophoresis, proteins were transferred to nitrocellulose paper (0.45-μm pore size) according to the procedure adapted from Glenney [22]. Tris–glycine and 20% methanol at a pH of 8.5 was used as the transfer buffer. Following transfer, nitrocellulose paper was blocked in 3% BSA (in PBS with sodium azide, 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, MCID/M4, provided by Imaging Research, Ontario, Canada.
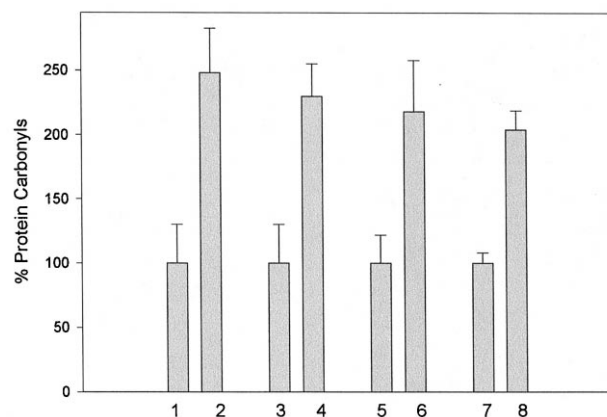

Fig. 3. Protein carbonyls, an index of protein oxidation, in brain following 3-NP administration as described in Section 2. Whole brain synaptosomal membrane proteins were obtained from rats treated with 3-NP for 4 days and from control rats after 4 days. Synaptosomal membrane proteins were also obtained 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 ($p < 0.05$) was shown for whole brain of treated animals after 4 days. For both control and treated animals, $N = 6$. A significant increase in protein carbonyls was observed ($p < 0.001$, $p < 0.01$ for striatum and cortex, respectively) in striatum and cortex from treated animals when compared to controls. Control $N = 6$, treated sample $N = 6$. Protein carbonyl levels of animals treated for 3 days were increased significantly when compared to control animals ($p < 0.01$). $N = 5$ for both control and treated sample. (Columns on graph are as follows: column 1 = whole brain control; column 2 = whole brain 3-NP-treated; column 3 = striatum control; column 4 = striatum 3-NP-treated; column 5 = cortex control; column 6 = cortex 3-NP-treated; column 7 = 3-day striatum control; column 8 = 3-day striatum 3-NP-treated).

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.

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-
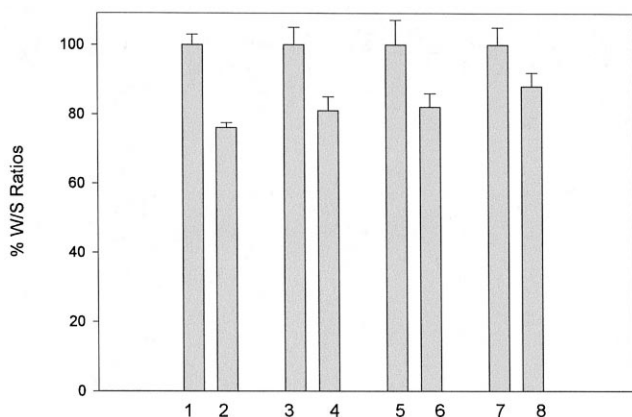

Fig. 4. MAL-6-labeled synaptosomal membrane proteins were obtained from the whole brain of rats treated with 3-NP and the W/S ratios were measured. Significant decreased W/S ratios were observed ($p < 0.001$) when compared to control samples. Control $N = 8$, treated sample $N = 7$. MAL-6-labeled synaptosomal membrane proteins were isolated from rat brain cortex and striatum from animals treated with 3-NP and the W/S ratios were measured. Significant decrease in W/S ratios were observed ($p < 0.001$) when compared to control samples. Control $N = 6$, treated sample $N = 6$. MAL-6-labeled synaptosomal membrane proteins were isolated from rat brain striatum from animals treated with 3-NP for 3 days and the W/S ratios were measured. Significant decrease in W/S ratios were observed ($p < 0.01$) when compared to control samples. Control $N = 5$, treated sample $N = 5$. (Columns on graph are as follows: column 1 = whole brain control; column 2 = whole brain 3-NP-treated; column 3 = striatum control; column 4 = striatum 3-NP-treated; column 5 = cortex control; column 6 = cortex 3-NP-treated; column 7 = 3-day striatum control; column 8 = 3-day striatum 3-NP-treated).

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 synaptsomal 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 stratum, 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 [38], and acetyl-L-carnitine [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-sulfophenyl)-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-

creased and protein carbonyls are increased in cortical synaptasomes suggest this brain region is also oxidized. Furthermore, the presence of protein oxidation assessed by EPR and protein carbonyl measurement in striatum after 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 [4]. 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 [33,57]. 3-NP is an irreversible inhibitor of complex II of the electron transport chain and of the Kreb's cycle. The major mechanism of 3-NP toxicity is thought to be via inhibition of the citric acid cycle [4].

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 [35]. Other evidence has also linked free radical involvement with excitotoxicity [18]. By impairing ion pumps and reducing ATP levels, 3-NP results in neuronal depolarization and removal of the Mg^{2+} block of the NMDA receptor ion channel. This results in ambient levels of glutamate becoming excitotoxic [42,56]. Calcium influx through the NMDA receptor results in impaired mitochondrial function and increased oxidative stress [35,46]. The increase in intracellular Ca^{2+} 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 (C3b/C4B4) [40]. In addition, 3-NP leads to increased levels of tumor necrosis factor-α [21], a pro-inflammatory cytokine, and increased expression of inducible nitric oxide synthase [41].

Several groups have studied the effect of 3-NP administration on behavior, physical dexterity, and neuropathology. Borlongan et al. [8] 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. [23] showed a correlation between severity of 3-NP-induced striatal lesions and motor deficits, including bradykinesia, gait length, and gait velocity. Tsai et al. [53] demonstrated a decrease in glutamine synthetase (GS) activity upon administration of 3-NP in a dose-dependent manner. In 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 motor skills associated with increased brain protein oxidation [19], and, based on analogous studies with amyloid β-peptide-associated oxidative stress [1,13,29], the decrease of GS activity and age-dependent susceptibility also suggest an underlying oxidative process for 3-NP toxicity. Studies of agents to attenuate the effects of 3-NP are currently underway in our laboratory.

Acknowledgements

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