FACTA UNIVERSITATIS
Series: **Medicine and Biology** Vol.9, No 2, 2002, pp. 162 - 165 UC 616.851

EFFECTS OF NITRIC OXIDE SYNTHASE INHIBITORS, 7-NI AND L-NAME, ON CONTENT OF REDUCED GLUTATHIONE IN EXPERIMENTAL MODEL HUNTINGTON'S DISEASE

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Summary. *The etiology of neuronal death in neurodegenerative diseases, including Huntington's disease (HD), is still unknown. There could be a complex interplay between altered energy metabolism, excitotoxicity and oxidative stress. Our aim was to examine the effects of intrastriatal injection of a selective inhibitor of neuronal nitric oxide synthase, 7 nitro indazole (7-NI), and non-specific potent nitric oxide synthase inhibitor, Nω-nitro-l-arginine methyl ester (l-NAME), to investigate the possible involvement of nitric oxide in quinolinic acid (QA)-induced striatal toxicity in the rat. Unilateral administration of QA in rat striatum in a single dose of 150 nM was used as model of HD. The second and third group were treated with 7-NI and QA and l-NAME and QA, respectively. The control group was treated with 0.9% saline solution likewise. The content of reduced glutathione, a key antioxidant, was increased in the hippocampus and basal forebrain of 7-NI- and QA-treated animals, compared to the QA-treated animals. These results support the hypothesis that oxygen free radicals contribute to the excitotoxic neuronal injury, and also that neuronal nitric oxide synthase inhibitor 7-NI, but not l-NAME could be potential neuroprotective agents in HD.*

Key words: *Huntington's disease, quinolinic acid, Nω-nitro-l-arginine methyl estar, 7-nitro indazole, reduced glutathione*

Introduction

Huntington's disease (HD) is an inherited (autosomal dominant) disorder resulting in progressive brain degeneration, which usually becomes manifest in mid-life. It leads to progressive dementia and severe involunary sudden jerky (choreiform) movements, which make speech and feeding progressively more difficult (1). The nature of the primary biochemical defect remains undetermined. The disease causes a widespread loss of cortical cells, which is presumably responsible for producing dementia, and also loss of cells from the corpus striatum, which causes the motor disturbance (2).

The N-methyl-D-aspartate (NMDA) class of glutamate receptors is belived to play a prominent role in the pathogenesis of CNS excitotoxicity (3). There are many possible ways in which Ca^{2+} overload may kill cells, including activation of intracellular proteases and lipases, impaired mitochondrial function as the mitochondria take up Ca^{2+} from the cytosol, generation of free radicals, etc (4). Two additional mechanisms triggered by the rise in Ca^{2+} may also contribute to excitotoxicity. First, nitric oxide (NO) synthesis appears to be important, since inhibitors of nitric oxide synthase (NOS) are capable of protecting against glutamate-induced neurotoxicity; the mechanism may be related to free radical production. Secondly, production of arachidonic acid may inhibit the glutamate uptake carrier, thus causing the action of glutamate to be enhanced (5).

The enzymatic metabolism of tryptophan in the central nervous system via the kynurenine pathway yields several intermediates that have the ability to influence excitatory aminoacid (EAA) neurotransmission (6). One such product, quinolinic acid (QA), a pyridine dicarboxylic acid, behaves as an agonist at NMDA receptor sites. Neuronal application of QA produces excitatory responses and causes excitotoxic damage to neurons bearing NMDA receptors and receiving glutamatergic innervation (7). QA is derived from the catabolism of kynurenine, the first metabolic product of tryptophan degradation via the kynurenine pathway. The phenomenon of excitotoxicity, involving neuronal damage due to overstimulation of NMDA and non-NMDA ionotropic receptors has been implicated in acute neuronal disorders such as HD. Excitotoxic striatal lesions induced by QA were used to testify neuroprotective actions of NOS inhibitors in the basal forebrain and hippocampus (8).

NO and its toxic metabolite peroxynitrite ($ONOO^-$) can inhibit components of the mitochondrial respiratory chain leading, if damage is severe enough, to a cellular energy deficiency state. Within the brain, the susceptibility of different brain cell types to NO and $ONOO^-$

exposure may be dependant on factors such as the intracellular reduced glutathione (GSH) concentration and the ability to increase glycolytic flux in the face of mitochondrial damage (9). Inhibiting NOS, an essential enzyme for production of NO, as a means of reducing NMDA-induced neurotoxicity has produced conflicting effects. Some researchers have found neuroprotection with NOS inhibitors, while others report no neuroprotection or exarcebation of NMDA-induced toxicity (10, 11). These discrepancies may stem from at least two methodological problems. First, some compounds may affect endotelial NOS (eNOS), as well as neuronal (nNOS). Second, variability in the neurotoxic effects of acute QA injection, commonly used to induce NMDA receptor mediated lesions, may reduce reliability in assessing potentially neuroprotective compounds (12).

Our aim was to examine the neuroprotective potential of two separate NOS inhibitors. 7-nitroindasole (7-NI) is known to be relatively specific for nNOS, whereas Nomega-nitro-L-arginine (l-NAME) affects both nNOS and endotelial (eNOS) (13, 14).

Methods

Investigations were made on adult Wistar rats of both sexes, with body weight of about 250 g. Animals were divided into four groups and were put in macrolen cages (Erath, FRG). Animals had free access to food and water. Anarage microclimate conditions were as follows: room temperature 23±2°C, air humidity 55±10%, the air was conditioned by 10-50 exchanges per hour, and light regime was in cycle of 12 hours from 7-19 hours.

QA was administered unilaterally into striatum in the single dose of 250.7μg (150nM) using stereotaxic instrument for small animals with coordinates for striatum (8.4;2.4;5.0 mm). The second and third group were treated with 7 nitro indazole (7-NI) and QA (7-NI in dose of 1×10^{-4}g) and Nω-nitro-l-arginine methyl estar (l-NAME) and QA (l-NAME in dose of 1×10^{-4}g). 7-NI was immediately applied before the neurotoxin, in contrast to l-NAME that was applied thirty minutes before QA. Control groups of animals were treated with 0.9% saline solution in the same manner. For all treated animals injected intracerebral volumen was 10×10^{-6} ml.

Before the treatment animals were anesthetized by pentobarbital sodium i.p. in a dose of 0.0405g/kg b.w.

Animals were sacrificed by decapitation 7 days after the treatment.

Brain tissue of basal forebrain and hippocampus was prepared in 10% sulfosalicylic acid for GSH-determination (15).

Content of reduced glutathione (GSH) was determined by using 5,5-dithiobis-2-nitrobenzoic acid (DTNB, 36.9mg in 10ml of methanol) which reacts with aliphatic thiol compounds in tris-HCl buffer (0.4M, pH-8.9) making yellow coloured p-nitrophenol anion. Intensity of colour was used for spectrophotometrical measurement of GSH concentration at 412 nm (16).

The means and SD were calculated for each parameter of interest. Differences between groups were examined using Student′s independent *t*-test. Statistical significance was accepted at $p<0.05$.

Results

There was no significant difference in content of reduced glutathione between ipsi- and contralateral sides of basal forebrain and hippocampus of control animals (Fig 1,2).

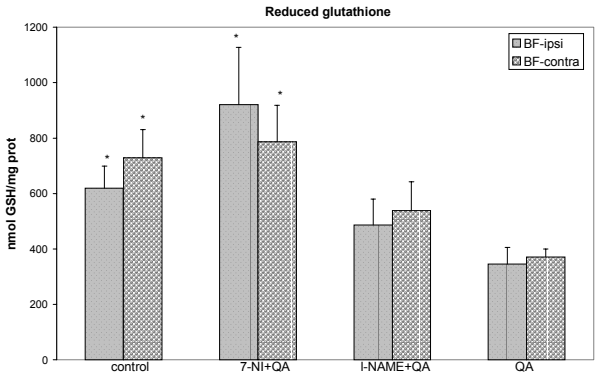

Fig. 1. Content of reduced glutathione in the ipsi- and contralateral basal forebrain of QA- and NOS inhibitors-treated Wistar rats. (BFi, BFc = basal forebrain ipsi-, contralateral). Values are given as nmol GSH/mg prot. Mean ± S.D. ∗ - Significance to corresponding values of QA-treated group. (Student′s t-test, $p<0.05$).

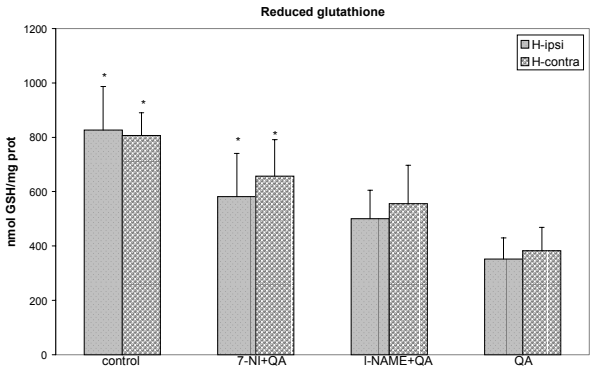

Fig. 2. Content of reduced glutathione in the ipsi- and contralateral hippocampus of QA- and NOS inhibitors-treated Wistar rats. (Hi, Hc = hippocampus ipsi-, contralateral). Values are given as nmol GSH/mg prot. Mean ± S.D. ∗ - Significance to corresponding values of QA-treated group. (Student′s t-test, $p<0.05$).

The content of reduced glutathione was evidently decreased in the ipsi- and contralateral basal forebrain and hippocampus QA-treated animals compared with control (basal forebrain ipsilateral $p<0.0016$ and contralateral $p<0.0276$; hippocampus ipsilateral $p<0.0313$ and contralateral $p<0.0001$). The difference of content of reduced glutathione in basal forebrain and hippocampus 7-NI and QA-treated animals compared to the QA-treated

animals was highly significant (basal forebrain ipsilateral $p<0.0052$ and contralateral $p<0.0068$; hippocampus ipsilateral $p<0.0279$ and contralateral $p<0.0024$), but in forebrain cortex and hippocampus L-NAME and QA-treated animals there was no difference (Fig 1, 2).

In both groups of animals treated with nitric oxide synthase inhibitors there was no significant change in content of reduced glutathione compared with control animals (Fig 1, 2).

Discussion

Bioenergetic defects and oxidative stress could be critical links in excitotoxic mechanisms of neuronal death. Imbalance between production and removal of free radicals would be abrasive for a neuron. Increase in the intracellular levels of reactive oxygen species (ROS), frequently referred to as oxidative stress, represents a potentially toxic insult which if not counteracted will lead to membrane dysfunction, DNA damage and inactivation of proteins (17). Glutathione-associated metabolism is a major mechanism for cellular protection against agents generated in oxidative stress. The glutathione tripeptide is central to a complex multifaced detoxification system, where there is substantial inter-dependence between separate component members. Glutathione provides the cell with multiple defences not only against ROS, but also against their toxic products. Reduced form of these nonprotein thiols represents the main endogenous antioxidative compound responsible for control of redox state in the cell as well as in the extracellular space (18).

QA kills hippocampal neurons via an oxidative pathway associated with reduced glutathione depletion. NMDA receptor activation increases NO synthesis probably by increasing intracellular calcium which binds to calmodulin, allowing it to activate NOS. Striatal application of QA is associated to neuronal damage through NMDA receptor activation, increased NOS activity and decrease in the reduced glutathione content (19). Decreased reduced glutathione content in the basal forebrain and hippocampus, the structures distant from ipsilateral treated striatum, could indicate the spatial propagation of oxidative stress, not only in the selectively vulnerable striatal GABA-ergic neurons, but in the structures included in the motor and cognitive loops of basal ganglia.

In the present study we report that the selective nNOS inhibitor 7-NI increased the content of reduced glutathione both in the ipsi- and contralateral basal forebrain and hippocampus. Further, the less selective nNOS inhibitor l-NAME has not altered the content od reduced glutathione when the two compounds were actually appled to the rat striatum. Excessive NOS dependent NO release during high levels of NMDA receptor stimulation results in production of toxic hydroxyl radicals and nitrogen dioxide. In the present report 7-NI was effective in elevation of reduced glutathione content, while l-NAME was ineffective. One possible explanation for these results is the relative specificity of these two compounds for nNOS. While the neuroprotective properties of 7-NI probably represent antagonism of nNOS, the trend toward increased lesion with l-NAME may have resulted from nonspecific antagonism of eNOS. The relative effectiveness of NOS antagonists as neuroprotectants may depend in part on their relative specificity for the neuronal form of NOS (20).

The age-related onset and progressive course of HD may be due to a cycling process between impaired energy metabolism and oxidative stress (21). The deleterious networks in the brain of HD are formed on the basis of the intimate interactions among the key pathogenic factors, including oxidative damage, aberrant calcium homeostasis and metabolic compromise (22). In our study the *in vivo* cytoprotective effects of NOS inhibitors against striatal excitotoxic lesions suggest that 7-NI, but not l-NAME could be used as potential neuroprotective agents in HD, which has been suggested to involve excitotoxicity.

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UTICAJ INHIBITORA SINTAZE AZOTNOG OKSIDA, 7-NI I L-NAME NA SADRŽAJ REDUKOVANOG GLUTATJONA U EKSPERIMENTALNOM MODELU HANTINGTONOVE BOLESTI

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Kratak sadržaj: *Etiologija selektivnog umiranja neurona u neurodegenerativnim bolestima je nepoznata, iako postoje dokazi o defektu energetskog metabolizma, ekscitotoksičnosti i oksidativnom oštećenju. Verovatno je da ključnu ulogu ima kompleksna interakcija između ovih mehanizama. Cilj našega rada bio je da se ispitaju efekti intrastrijatne primene selektivnog inhibitora neuronske azot oksid sintaze-7 nitro indazola (7-NI), kao i nespecifičnog inhibitora azot oksid sintaze-Nω-nitro-l-arginin metil estra (l-NAME), zbog moguće uključenosti azot oksida u toksičnost strijatuma izazvanu hinolinskom kiselinom (HK), kod pacova. Unilateralna aplikacija HK u strijatum pacova u pojedinačnoj dozi od 150 nM korišćena je kao model HB. Druga i treća grupa tretirane su 7-NI i HK, odnosno l-NAME i HK. Kontrolna grupa dobijala je 0.9% fiziološki rastvor na isti način. Sadržaj redukovanog glutationa, ključnog antioksidanta, povećan je u hipokampusu i bazalnom prednjem mozgu životinja tretiranih 7-NI i HK, u poređenju sa životinjama tretiranim samo HK. Ovi podaci pokazuju da kiseonični slobodni radikali učestvuju u ekscitotoksičnom oštećenju neurona, kao i da inhibitor neuronske azot oksid sintaze 7-NI, za razliku od l-NAME, može biti potencijalni neuroprotektivni agens u HB.*

Ključne reči: *Hantingtonova bolest, hinolinska kiselina, Nω-nitro-l-arginin metil estar, 7-nitro indazol, redukovani glutation*