Original Article

Oxidative stress induced in rat brain by a combination of 3-nitropropionic acid and global ischemia

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Abstract: In our investigation, we describe the complex model of brain oxidative stress consisted of combination of experimental brain ischemia and energy metabolism violation induced by irreversible inhibitor of mitochondrial succinate dehydrogenase, 3-nitropropionate (3-NPA). 3-NPA causes selective degeneration of striatum neurons, which is extremely sensitive to energy deficit. This complex model allows revealing not only biochemical but also neurological symptoms in experimental animals that permits proper estimation of protective effect of different drugs on animal status. Combination of global ischemia induced by 3-vessel occlusion of major arteries supplying rat brain and subsequent 5-day reperfusion with intraperitoneal injection of 3-nitropropionic acid induces vigorous oxidative stress in brain tissues accompanied by evident neurological symptoms in Wistar rats. Such a combination of damaging factors may be considered as a new complex experimental model of brain oxidative stress permitting the evaluation of neuroprotective effect of potential therapeutic agents. Using this model, protective effect of neuropeptide carnosine was demonstrated which is in agreement with previous data.

Keywords: Brain global ischemia, 3-nitropropionic acid, oxidative stress, carnosine

Introduction

It has long been recognized that 3-NPA causes selective degeneration of striatum neurons, which is extremely sensitive to energy deficit [1]. The protective effect of neuropeptide carnosine has also been demonstrated in previous studies [2, 3]. For the development the new therapeutic strategies for treatment of the cerebrovascular diseases more than 100 different experimental models of brain ischemia on animals are used [4]. However these models are not completely match to clinical situation, because in human cerebrovascular diseases many pathological factors (like atherosclerosis, hypertension, etc.) are involved in brain damage development. For example, in spite of considerable biochemical disorders and high mortality of animals subjected to global ischemia, only poor neurological symptoms were revealed [5, 6]. In such cases, the neurochemical and morphological defects displayed in their brain cannot be verified by behavioral features. This circumstance embarrasses the evaluation of novel therapeutic drugs being potentially able to normalize brain metabolism after ischemic episode using both neurochemical and neurological parameters [6, 7]. Probably, it is one of the reasons why drugs that were effective on experimental models didn’t show protective effect on clinical trials [8, 9]. Oxidative stress makes an important contribution in ischemic injury. Our goal was to make a model of brain oxidative stress, that allows revealing neurological symptoms and estimating protective action of new pharmacological drugs.

Recently we have noted that experimental brain ischemia aggravated by energy deficit induced by simultaneous administration of 3-nitropropionic acid, 3-NPA, reveals both neuro-
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chemical deviations in the brain and a neurological deficit [10]. 3-NPA as an uncompetitive inhibitor of mitochondrial succinate dehydrogenase [11, 12] causes rapid suppression of energy metabolism in the brain, thus resulting in the release of excitotoxic neuromediators, development of oxidative stress, and death of the brain neurons [13]. During systematic administration of the neurotoxin, degeneration of neurons of the striatal area was noted being accompanied with specific locomotor and cognitive disorders close to those described in the Huntington disease [14, 15].

In this article, we characterized rat brain global ischemia aggravated with systemic administration of 3-NPA using both behavioral and biochemical parameters. In order to evaluate the meaning of such a model of brain ischemia we have evaluated the therapeutic effect of neuropeptide carnosine known as the antioxidant brain protector [2, 16].

Materials and methods

Experiments were made using 68 male Wistar rats of 23-26 weeks old (315-350 g weight) maintained under standard conditions. Animals were treated and handled according to the guidelines of the European Union Council. The animals were housed in a well-ventilated animal room under a light/dark cycle of 12:12 h (light on from 06:00 to 18:00 h). The animals had free access to food and water.

Three-vessel global ischemia of rat brain [5, 7] was performed as a 2-step procedure: at the first day, the left arteria vertebralis was electrocauterized, and at the next day, 15 min occlusion of both carotid arteries was carried out with a subsequent 5 days reperfusion. On the last day of the experiment, the animals were decapitated under light anesthesia.

At the beginning of the experiment, the animals were randomly distributed into the following groups: 1 – intact animals (n=8); 2 – sham-operated animals (n=8); 3 – sham-operated animals treated with 3-NPA (30 mg/kg per day intraperitoneally starting from 3rd day of experiment) (n=10); 4 – the animals subjected to global brain ischemia (n=11); 5 – the animals subjected to global brain ischemia in combination with 3-NPA injection (30 mg/kg per day starting from 3rd day of experiment) (n=15); 6 – the animals subjected to global brain ischemia in combination with 3-NPA (30 mg/kg per day starting from 3rd day of experiment) and carnosine (100 mg/kg) (n=14).

The following protocol for carnosine treatment was used: the above mentioned dose was injected intraperitoneally in the very beginning of the re-perfusion, and after 4, 8, and 24 hrs after operation, then once a day until the end of experiment. All substances were dissolved in physiological solution, and the same amount (0.5 ml) of physiological solution was injected to the animals of the control groups.

Neurological symptoms were evaluated by using a scale of features [7] (Table 1) revealing in rats after 3-NPA injection [11, 12]. It was made immediately after the animals were woken up from anesthesia, then after 4, 8, and 24 hrs and every other day during the entire reperfusion period.

On the 7th day of the experiment, the animals were decapitated and the brain prepared using iced physiological solution. The homogenate for Fe²⁺-induced chemiluminescence was obtained from 0.3 g of both hemispheres using phosphate buffer (60 mM KH₂PO₄ and 105 mM KCl, pH 7.45). The mitochondrial fraction was isolated using the differential centrifugation procedure [17] and the samples obtained were

Table 1. Scale of neurological symptoms

<table>
<thead>
<tr>
<th>Huntington’s disease symptoms</th>
<th>Points</th>
</tr>
</thead>
<tbody>
<tr>
<td>Decrease of moving activity, pose defects</td>
<td>1</td>
</tr>
<tr>
<td>Weakness, languor</td>
<td>2</td>
</tr>
<tr>
<td>Rigidity of back extremities and the tail, rotation</td>
<td>3</td>
</tr>
<tr>
<td>Paresis of back and later extremities</td>
<td>4</td>
</tr>
<tr>
<td>Total suppression of locomotion</td>
<td>5</td>
</tr>
</tbody>
</table>
stored (no more than 2 weeks before analyses) at -80°C. Grey matter of both cerebral hemispheres (about 0.8 g) was homogenized in 0.32 M sucrose containing 5 mM HEPES pH 7.4 at a ratio of 9 ml of homogenization medium/1 g of tissue in a Potter homogenizer with a Teflon pestle. The homogenate was centrifuged at 3600 rpm for 10 min to obtain the supernatant. 3 ml of supernatant was pipetted over 4 ml 1.2 M sucrose (each sample per 2 tubes) and centrifuged at 25000 rpm for 20 min in Beckman SW41T centrifuge. The two residues were suspended in 0.5 ml of 0.32 M sucrose and frozen at -80°C.

In the mitochondrial fraction superoxide dismutase, SOD, monoamine oxidase B, MAO B, and succinate dehydrogenase, SDG were measured. SOD was determined by the suppression of nitro blue tetrazolium reduction in the presence of superoxide anion generated by xanthine oxidase; enzyme units corresponded to the amount of the enzyme providing 50% inhibition of nitro blue tetrazolium reduction [18]. MAO B was monitored using benzyl amine as a substrate [6]. SDG was determined as a rate of reduction of 2,6-dichlorophenolindophenol in the presence of phenazine methosulfate during enzymatic oxidation of sodium succinate [19].

In the brain homogenate, the Fe²⁺-induced chemiluminescence was monitored giving the ability to determine the level of lipoperoxides preformed, the rate of Fe²⁺-induced oxidation and the oxidative resistance of the samples (by measuring the lag-period between the addition of Fe²⁺-ions and the beginning of the chemiluminescence reaction) [20].

Protein was measured by Lowry [21]. Statistic analysis of the data was made using one-way analysis of variance (ANOVA) followed by the Tukey-Kramer and Duncan test. Results were expressed as a mean ± S.D.

Results

In the experiments presented, control (sham-operated) animals (Group 2) as well as the animals subjected to global brain ischemia (Group 4) possessed no neurological symptoms. Both appearance and behavior of these animals were not distinct from those of intact rats (Figure 1). Mortality was noted only in Groups 3, 5, and 6 (Table 2). In the Group 3 rats, mortality appeared on the 4th day after the 3-NPA injection. In the Group 5 rats (combination of global ischemia with 3-NPA injection), the mortality rate was only a little bit higher (33% compared with 30%, statistically insignificant) than in Group 3, suggesting that the main reason for the mortality was the 3-NPA injection. Animal mortality was observed at the 4th-5th days of 3-NPA injection. However, the Group 5 animals were characterized as having the highest level of neurological symptoms (Figure 1). Typical neurological symptoms (pose defects, rigidity of back extremities and the tail, paresis of back and later on upper extremities, rotation, total suppression of locomotion and loss of body weight) appeared on
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Table 2. Mortality in the animal groups studied

<table>
<thead>
<tr>
<th>Groups of animals</th>
<th>Mortality</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>amount</td>
</tr>
<tr>
<td>1. Intact (n=8)</td>
<td>0</td>
</tr>
<tr>
<td>2. Sham-operated (n=8)</td>
<td>0</td>
</tr>
<tr>
<td>3. Sham-operated+3-NPA (n=10)</td>
<td>3</td>
</tr>
<tr>
<td>4. Ischemia (n=11)</td>
<td>0</td>
</tr>
<tr>
<td>5. Ischemia+3-NPA (n=15)</td>
<td>5</td>
</tr>
<tr>
<td>6. Ischemia+3-NPA+carnosine (n=14)</td>
<td>1</td>
</tr>
</tbody>
</table>

Figure 2. SDG activity (nmole succinate/mg protein per min) in brain mitochondria of the animals studied. 1 – intact animals, 2 – sham-operated animals, 3 – sham-operated+3-NPA, 4 – ischemia, 5 – ischemia+3-NPA, 6 – ischemia+3-NPA+carnosine; ** corresponds to statistically significant difference (p<0.05) from group 1.

the 3rd day after the 3-NPA injection and became stronger with time (Figure 1).

Carnosine treatment was very efficient – it protected the ischemic animals from both mortality (see Table 2) and marked neurological symptoms (Figure 1).

The analysis of enzyme activity showed the following changes. Succinate dehydrogenase in brain mitochondria was unchanged in Group 2 animals (compared to intact control) but sufficiently increased under experimental ischemia (Group 4, Figure 2). When 3-NPA was injected, significant 4 fold suppression of mitochondrial succinate dehydrogenase was found confirming that an energy deficit was an evident cause of the neurological deficit which appeared. Such an effect of 3-NPA does not depend on experimental conditions (experimental ischemia or carnosine treatment).

In Figure 3 the values of mitochondrial SOD in several groups of animals studied are presented. It is seen that in the sham operation (Group 2), 3-NPA treatment (Group 3) or the experimental ischemia (Group 4) revealed little effect on SOD, whereas a combination of experimental ischemia with 3-NPA (Group 5) sufficiently increased SOD activity. Carnosine normalized the SOD level (Group 6).

Global ischemia increased the mitochondrial MAO B (by 15% compared with sham-operated rats) and 3-NPA did not affect this enzyme. In
Group 6 animals, MAO B did not differ from that in Groups 1 and 2 (Figure 4).

Table 3 contains the results of chemiluminescence analysis of Fe\(^{2+}\)-induced oxidation of lipids in brain samples from several experimental groups. It is seen that the Group 3 animals (sham-operated and 3-NPA treated) did not differ from Groups 1 and 2. At the same time, Groups 4 and 5 rats were characterized by an elevated level of hydroperoxides, a shortened lag-period of oxidation and a dramatic increase in the rate of oxidation compared to the animals in Groups 1 and 2. For Group 5 rats, these parameters were much more pronounced which was consistent with the stronger suffering of these animals under the combined damage induced by both ischemia and 3-NPA. This data indicates more pronounced symptoms in these rats (see Figure 1).

Group 6 animals being treated with both 3-NPA and carnosine did not significantly differ statistically from the Groups 1 and 2 animals (intact
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and sham-operated) while for these animals the slightly increased level of lipid hydroperoxides was found. This data proves that carnosine compensates to the antioxidant stability of the brain exhausted by the combined action of experimental ischemia and 3-NPA. This conclusion is in total agreement with the positive effect of carnosine on neurological symptoms (Figure 1) and the viability (Table 2) of the animals.

Discussion

Development of free radical damage of brain tissues during ischemia is accompanied by neuronal degeneration [5, 22, 23]. Rats usually demonstrate definite resistance to ischemic damage. Thus, although in most cases of experimental brain ischemia there are indications of sufficient metabolic changes, they are not accompanied by neurological symptoms or mortality [7].

The combination of 3-NPA and the experimental brain ischemia resulted in stronger neurological symptoms (Figure 1), which correlated with metabolic disorders in the brain. Under these conditions, the level of lipid hydroperoxides was increased in the brain (by 63% compared to Group 2), the lag-period of Fe²⁺-induced lipid peroxidation was shortened (by 26% compared to Group 2) and the rate of oxidation was correspondently increased (see Table 3).

All these observations pointed to the decrease in the efficiency of the antioxidant defense in the brain under the combined attack of ischemic processes and 3-NPA. The effect on the brain of experimental ischemia itself developed in a similar direction but was much less pronounced.

The neurological symptoms, which appeared under the combined action of the experimental brain ischemia and 3-NPA are indicative of the degeneration of the dopaminergic neurons in the striatum, along with an energy deficit which is characteristic of 3-NPA action. This model, therefore, may be considered as a useful tool in estimating the efficiency of drugs potentially suitable for treating the Huntington disease and relative pathologies.

It is important to note that experimental ischemia (Group 4) is accompanied by the increase in the level of SDG and MAO B in the brain mitochondria (Figure 2) while the combination of ischemia and 3-NPA treatment in addition increases SOD. These modulations of metabolism however do not prevent the accumulation of lipid peroxides (Table 3). Elevated levels of oxidized lipid products in the brain are also in correlation with the high MAO B activity in Group 4 compared to Group 3. It is known that MAO B activation occurs during the reperfusion period [24], which can accelerate lipid peroxidation by the products of MAO B reaction – semialdehyde and superoxide anion (or hydrogen peroxide) [13]. The increased expression of MAO B is induced by the accumulation of extracellular dopamine during the disorder of its re-uptake and the death of dopaminergic neurons [24]. These facts may be interpreted to be an insufficient

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**Table 3. Parameters of Fe²⁺-induced oxidation of brain homogenate of the animals studied**

<table>
<thead>
<tr>
<th>№</th>
<th>Groups of animals</th>
<th>Parameters of Fe²⁺-induced lipid peroxidation in brain homogenates</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Hydroperoxides pre-formed, arbitrary units</td>
</tr>
<tr>
<td>1</td>
<td>Intact (n=8)</td>
<td>161±28</td>
</tr>
<tr>
<td>2</td>
<td>Sham-operated (n=8)</td>
<td>167±30</td>
</tr>
<tr>
<td>3</td>
<td>Sham-operated + 3-NPA (n=7)</td>
<td>201±32</td>
</tr>
<tr>
<td>4</td>
<td>Ischemia (n=8)</td>
<td>248±12(*)</td>
</tr>
<tr>
<td>5</td>
<td>Ischemia + 3-NPA (n=7)</td>
<td>272±29(*)</td>
</tr>
<tr>
<td>6</td>
<td>Ischemia + 3-NPA + Carnosine (n=10)</td>
<td>200±20</td>
</tr>
</tbody>
</table>

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adaptive response of the ischemic brain to attempt to neutralize the oxidative stress induced by the experimental ischemia.

In the literature there were several articles published demonstrating an increase in activity of both cytosolic and mitochondrial SOD in response to 3-NPA injection [25-27]. However, in terms of high mortality of the animals treated with 3-NPA, such adaptive reaction proves to be unsuccessful. At the same time, support of the antioxidant system with the injection of natural neuropeptide carnosine seems to be very effective. Carnosine significantly decreases the mortality of the rats (Table 2), decreases the tissue level of lipid peroxides and restores resistance of the brain towards Fe2+-induced lipid peroxidation (Table 3), although it is not able to prevent SDG inhibition by 3-NPA (Figure 2). Only carnosine may restore the antioxidant state of the brain very efficiently so that SOD does not receive the signal to be stimulated so that it really does not take place (Figure 3).

Carnosine was found to possess a number of useful properties suggesting that it is the natural brain protector against oxidative stress [3]. In our experiments, carnosine also demonstrated high protection efficiency, which permits the recommendation of this compound as an effective neuroprotector. On the whole, the complex research of oxidative stress on the brain using the model described gives an opportunity to estimate possible therapeutic effects on the brain using a number of novel perspective antioxidants.

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