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MOLECULAR TOXICOLOGY

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Nitric oxide modulates high-energy phosphates in brain regions of rats intoxicated with diisopropylphosphorofluoridate or carbofuran: prevention by *N-tert*-butyl-α-phenylnitrone or vitamin E

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Abstract Acute effects of seizure-inducing doses of the organophosphate compound diisopropylphosphorofluoridate (DFP, 1.25 mg/kg s.c.) or the carbamate insecticide carbofuran (CF, 1.25 mg/kg s.c.) on nitric oxide (NO) were studied in the brain of rats. Brain regions (pyriform cortex, amygdala, and hippocampus) were assayed for citrulline as the determinant of NO and for high-energy phosphates (ATP and phosphocreatine) as well as their major metabolites (ADP, AMP, and creatine). Rats, anesthetized with sodium pentobarbital (50 mg/kg i.p.), were killed using a head-focused microwave (power, 10 kW; duration, 1.7 s). Analyses of brain regions of controls revealed significantly higher levels of citrulline in the amygdala (289.8 ± 7.0 nmol/g), followed by the hippocampus (253.8 ± 5.5 nmol/g), and cortex (121.7 ± 4.3 nmol/g). Levels of energy metabolites were significantly higher in cortex than in amygdala or hippocampus. Within 5 min of CF injection, the citrulline levels were markedly elevated in all three brain regions examined, while with DFP treatment, only the cortex levels were elevated at this time. With either acetylcholinesterase (AChE) inhibitor, the maximum increase in citrulline levels was noted 30 min post-injection (> 6- to 7-fold in the cortex, and > 3- to 4-fold in the amygdala or hippocampus). Within 1 h following DFP or CF injection, marked declines in ATP (36–60%) and phosphocreatine (28–53%) were seen. Total adenine nucleotides and total creatine compounds were reduced (36–58% and 28–48%, respectively). The inverse relationship between the increase in NO and the decease in high-energy phosphates, could partly be due to NO-induced impaired mitochondrial respiration leading to depletion of energy metabolites. Pretreatment of rats with an antioxidant, the spin trapping agent *N*-*tert*-butyl-α-phenylnitrone (PBN, 200 mg/kg i.p.), prevented DFP- or CF-induced seizures, while the antioxidant vitamin E (100 mg/kg i.p. per day for 3 days) had no anticonvulsant effect. Both antioxidants, however, significantly prevented the increase of citrulline and the depletion of high-energy phosphates. It is concluded that seizures induced by DFP and CF produce oxidative stress due to a marked increase in NO, causing mitochondrial dysfunction, and thereby depleting neuronal energy metabolites. PBN pretreatment provides protection against AChE inhibitor-induced oxidative stress mainly by preventing seizures. Additional antioxidant actions of PBN may contribute to its protective effects. Vitamin E has direct antioxidant effects by preventing excessive NO production.

Keywords Anticholinesterase agent · Diisopropylphosphorofluoridate · Carbamate · Citrulline · Nitric oxide · Nitric oxide synthase · Energy-rich phosphates · ATP · Neuronal oxidative stress · *N*-*tert*-Butyl-α-phenylnitrone · Vitamin E

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Introduction

It is well established that organophosphate and carbamate compounds exert brain hyperactivity, such as convulsions and seizures, because of accumulation of acetylcholine (ACh) as a consequence of acetylcholinesterase (AChE) inhibition. However, the mechanisms involved in pathogenesis of neuronal damage appear to be linked to free radical-mediated injury. Lipid peroxidation, mitochondrial dyshomeostasis or dysfunction or damage, reduction of neuronal energy level, and reduced

cytochrome *c* oxidase (COx) activity support the contention that AChE inhibitors, such as diisopropylphosphorofluoridate (DFP) and carbofuran (CF) cause neuronal injury by excessive formation of reactive oxygen species (ROS) (Yang and Dettbarn 1998; Gupta et al. 1998, 2000a, 2000b; Milatovic et al. 2000a, 2000b, 2001a, 2001b). Our earlier findings clearly demonstrate that both DFP and CF are neurotoxic (Gupta et al. 1985, 2000a, 2001; Misulis et al. 1987; Gupta and Kadel 1989; Yang et al. 2000).

The brain is highly susceptible to oxidative stress because of its:

1. great consumption of O_2, energy, and glucose
2. large amount of peroxidizable fatty acids
3. relatively low antioxidant capacity, and
4. ease of peroxidation of brain membranes

(Zaleska and Floyd 1985; Floyd and Carney 1992; Floyd 1999). In recent studies, we have demonstrated that acute toxicity of DFP and CF caused marked depletion of high-energy phosphates in rat brain, due to greater utilization and reduced synthesis of such phosphates, and probably because of excessive generation of reactive oxygen/nitrogen species (Gupta et al. 2001).

Nitric oxide (NO), a free radical that has been widely regarded as a messenger molecule or neurotransmitter in the central nervous system (Bredt and Snyder 1992, 1994; Dawson et al. 1992), has also been considered as a toxic molecule implicated in seizures (Lallement et al. 1996; Kim et al. 1999) and neurotoxicity (Nowicki et al. 1991; Dawson et al. 1993; Dawson 1995; Dawson and Dawson 1996; Szabo 1996; Burney et al. 1997; Gupta et al. 2000c). Excessive production of NO is primarily triggered by increased Ca^{2+} influx associated with *N*-methyl-D-aspartate (NMDA) receptor activation (Garthwaite 1991; Gunasekar et al. 1995; Zhang and Snyder 1995). NO causes depletion of cellular energy stores by prolonging poly-(ADP-ribose) polymerase (PARP) activation (Zhang et al. 1994), and by inhibiting enzymes involved in mitochondrial respiration (Brorson et al. 1999) and glycolysis (Molina y Vedia et al. 1992; Erecinska et al. 1995). Recent studies demonstrated that NO, like cyanide and azide, directly and specifically inhibits mitochondrial respiration by competing with molecular O_2 for binding to cytochrome *c* oxidase (COx) (Giulivi 1998). Even nanomolar concentrations of NO can directly inhibit COx activity, and cells producing large quantities of NO can inhibit their own respiration as well as the respiration of neighboring cells (Brown and Cooper 1994). The COx complex is a primary generator of energy in a tissue such as brain, which depends on oxidative phosphorylation. Neuronal nitric oxide synthase (nNOS) binds directly to phosphofructokinase, a key regulatory enzyme in glycolysis, and thus represents a possible mechanism for NO-mediated inhibition of glycolysis (Firestein and Bredt 1999). The net result is a reduced neuronal energy status.

Generally, the seizures induced with directly or indirectly acting (AChE inhibitors) cholinomimetics can be prevented by pretreatment with cholinergic antagonists such as atropine and/or 2-pyridine aldoxime methochloride (2-PAM). However, once the seizures are initiated, noncholinergic systems are progressively recruited (Solberg and Belkin 1997; Raveh et al. 1999), and the seizures become refractory to cholinergic antagonists (McDonough and Shih 1993). Moreover, against carbamate toxicity, oximes such as 2-PAM are contraindicated (Gupta and Kadel 1989). In recent years, various protective strategies have been developed, including one based on the prevention of ROS generation and on ROS scavenging by antioxidant pretreatment (Zivin et al. 1999a; Dettbarn et al. 2000; Milatovic et al. 2000a, 2000b; Gupta et al. 2001).

The present investigation was undertaken with two specific objectives: firstly, to determine a possible correlation between alterations in NO levels and changes in high-energy phosphates (ATP and phosphocreatine) in brain regions of rats acutely intoxicated with DFP or CF, and secondly, to determine whether antioxidant [*N-tert*-butyl-α-phenylnitrone (PBN) or vitamin E] pretreatment prevents the induced changes in NO and energy metabolites.

Materials and methods

Animals

Male Sprague-Dawley rats weighing 180–200 g, purchased from Harlan (Chicago, Ill.), were used in this investigation. They were housed five per cage (large size) in a room with controlled conditions: temperature 21 ± 1°C, humidity 50 ± 10%, and light/dark cycle 12 h/12 h. Every third day, rats were placed in clean cages. They had free access to pelleted food (Rodent Laboratory Chow, Purina Mills, Inc., St. Louis, Mo.) and tap water. The animals were acclimatized to these conditions for 7–10 days before being used. During the treatment, rats were placed in individual cages (small size). The animal facility is approved by the Institutional Animal Care and Use Committee (IACUC). All experiments were conducted in accordance with guidelines from the National Institutes of Health, with adequate measures taken to minimize any discomfort to the rats.

Chemicals

Carbofuran (2,3-dihydro-2,2-dimethyl-7-benzofuranyl *N*-methylcarbamate), in crystalline form with a technical purity 99%, was supplied by FMC Corp. (Agricultural Chemical Group, Princeton, N.J.). *N-tert*-Butyl-α-phenylnitrone was purchased from Aldrich Chemical Co. (Milwaukee, Wis.) and vitamin E (DL-α-tocopherol) was purchased from ICN Biomedical, Inc. (Aurora, Ohio). Diisopropylphosphorofluoridate, lithium chloride, L-citrulline, adenosine 5'-triphosphate (ATP), adenosine 5'-diphosphate (ADP), adenosine 5'-monophosphate (AMP), phosphocreatine (PCr), and creatine (Cr) were purchased from Sigma Chemical Co. (St. Louis, Mo.). All other chemicals used were of highest purity and purchased from Fisher Scientific (Fair Lawn, N.J.).

Experimental design and animal treatment

About 150 rats, with 4–6 rats per group, were used in this project. The investigation was conducted in two series of experiments. In the first series of experiments, the animals were used for citrulline

(nitric oxide) studies, and in the second series of experiments, for study of high-energy phosphates and their major metabolites. Animals in the control group received normal saline (1 ml/kg s.c.). All rats receiving DFP or CF were pretreated with lithium chloride (LiCl, 127 mg/kg i.p.) 12 h prior to the DFP or CF administration. LiCl is known to facilitate the coupling of cholinergic-induced excitation and phosphoinositol 1-phosphate transmembrane signaling, thus reducing the concentration of cholinergic agents needed to induce seizures (Morrisett et al. 1987; Savolainen et al. 1988).

The rationale for selecting the doses of DFP and CF was based on our previous reports (Gupta and Kadel 1989; Gupta et al., 1985, 2001). In brief, the administration of DFP or CF produced signs of the onset of cholinergic hyperactivity within 5–7 min. Signs of maximal severity, including convulsions and seizures, were attained within 15–30 min, and lasted for more than 2 h. The rationale for selecting the doses of PBN and vitamin E was also based on previous reports (Marubayashi et al. 1986; Punz et al. 1998; Zivin et al. 1999a; Gupta et al. 2000a, 2001; Milatovic et al. 2000a). The doses chosen were sufficient for in vivo trapping of ROS.

Determination of citrulline as a marker of NO synthesis

Rats receiving an acute dose of DFP (1.25 mg/kg s.c.) or CF (1.25 mg/kg s.c.) were killed 5 min, 15 min, 30 min, 60 min, or 24 h after treatment to establish the time-course of changes in citrulline levels in the brain regions. Rats receiving pretreatment with an antioxidant, such as the spin trapping agent PBN (200 mg/kg i.p., single dose) or vitamin E (100 mg/kg i.p. per day for 3 days), were treated with DFP (1.25 mg/kg s.c.) or CF (1.25 mg/kg s.c.) 30 min after the last antioxidant pretreatment, and were killed 30 min later (30 min was long enough to induce maximum changes in citrulline levels). All rats were anesthetized with sodium pentobarbital (50 mg/kg i.p.), prior to being killed by irradiation using a head-focused microwave. Microwave conditions were as follows: instrument, Cober Metabolic Vivostat, model S6F (Cober Electronics, Inc., South Norwalk, Conn.); microwave frequency, 2450 MHz; power, 10.0 kW; and duration, 1.7 s. The IACUC strongly recommended the use of anesthesia prior to microwave irradiation to minimize any discomfort or stress. In preliminary studies, it was noted that sodium pentobarbital (50 mg/kg s.c.) did not alter the levels of citrulline in brain regions (data not shown).

The brains were removed from the skulls and the pyriform cortex, amygdala, and hippocampus were dissected, and stored (for not more than 3 days) at –80°C until assayed for citrulline. The areas of the brain selected represent sites of onset and propagation of seizures induced by AChE inhibitors in LiCl-pretreated rats (Schliebs et al. 1989; Wasterlain and Shirasaka 1994; Planas et al. 1995; Zivin et al. 1999a, 1999b). Each brain region, weighing 40–60 mg, was homogenized in 1.5 ml perchloric acid (0.4 M) for 1 min using a Brinkmann homogenizer (setting 5) with a PT-10 probe, followed by sonication for 10 s with a Biosonic Cell Disruptor equipped with a microprobe (setting 40). The homogenates were allowed to extract for 30 min at ice-cold temperature, followed by centrifugation (10,000 rpm for 20 min at 4°C) using a Sorvall centrifuge (RC 26 Plus). The supernatants were aspirated into beakers and neutralized to pH 7 with 1 M KOH before being centrifuged again at 10,000 rpm for 20 min at 4°C to remove fine precipitates of perchlorate ($KClO_4$). The supernatants were assayed for citrulline concentrations according to the modified method of Bagetta et al. (1995), based on reversed-phase high performance liquid chromatography (HPLC) coupled with a scanning fluorescence detector (excitation at 334 nm and emission at 440 nm). The concentrations of citrulline in brain regions were determined as nmol/g wet weight.

Determination of high-energy phosphates

Rats receiving an acute dose of DFP (1.25 mg/kg s.c.) or CF (1.25 mg/kg s.c.) were killed 60 min after injection. Rats receiving pretreatment with PBN (100 mg/kg i.p., single dose) or vitamin E (200 mg/kg i.p. per day for 3 days) were injected with DFP (1.25 mg/kg s.c.) or CF (1.25 mg/kg s.c.) 30 min after last antioxidant pretreatment, and killed 60 min later. All rats were anesthetized with sodium pentobarbital (50 mg/kg i.p.), prior to being killed by irradiation using a head-focused microwave. The microwave conditions were the same as described above for the citrulline assay. Sodium pentobarbital (50 mg/kg i.p.) does not alter the levels of high-energy phosphates or their metabolites in the brain or muscles of rats intoxicated with AChE inhibitors (Gupta et al. 1991, 1994, 2000a, 2000b).

The brains were removed from the skulls and the pyriform cortex, amygdala, and hippocampus were dissected, and stored (for not more than 3 days) at –80°C until assayed for high-energy phosphates and their metabolites. Each brain region, weighing about 40–60 mg, was extracted in 2.0 ml perchloric acid (1 M), and after adjusting the pH to neutral, the supernatant was analyzed for adenine nucleotides (ATP, ADP, AMP) and creatine compounds (PCr and Cr) according to the isocratic reversed-phase HPLC method (Gupta et al. 2000a, 2000b, 2001), using Waters HPLC System coupled with a dual λ-absorbance detector (model 2487). A computer system with a Waters Millennium^{32} Software program (Workstation v. 3.2) was used to control the HPLC system and integrate the data. The absorbance at 214 nm (for PCr and Cr) and 254 nm (for ATP, ADP, and AMP) were monitored simultaneously, and the concentrations were determined in the low nanomolar range and expressed as μmol/g wet weight. From these values, total adenine nucleotides (TAN = ATP + ADP + AMP) and total creatine compounds (TCC = PCr + Cr) were calculated.

Statistical analysis

All data are presented as mean ± SEM of 4–6 rats in each group. Statistical significance of differences was determined by analysis of variance (ANOVA) followed by the Tukey-Kramer test. Differences of $P < 0.05$ were considered statistically significant.

Results

Rats receiving DFP or CF showed typical signs of anticholinesterase toxicity, including tremors, convulsions, wet dog shakes, mild to moderate seizures with rearing and falling over, and progressing to severe seizures within 7–15 min. PBN or vitamin E treatment alone produced no effects, but when given as pretreatment, PBN prevented the development of seizures and other signs of toxicity, whereas vitamin E had no such effect.

Data for citrulline levels in brain regions of control rats and those intoxicated with an acute dose of DFP (1.25 mg/kg s.c.) or CF (1.25 mg/kg s.c.) are shown in Fig. 1 and Fig. 2, respectively. Control levels of citrulline were markedly higher in the amygdala (289.8 ± 7.0 nmol/g), followed by the hippocampus (253.8 ± 5.5 nmol/g), and lowest in the cortex (121.7 ± 4.3 nmol/g). Preliminary data revealed that LiCl (127 mg/kg i.p.) alone had no significant effect on citrulline levels in any of the three brain regions examined (data not shown).

Within 5 min of CF injection, the citrulline levels were elevated more than 4-fold in the cortex, and more than 2-fold in the amygdala and hippocampus. At this time point, after DFP treatment, only the cortex levels of citrulline were elevated (224%). Within 15 min, with either DFP or CF, the levels of citrulline were significantly higher in all three brain regions, and were maximally elevated at 30 min post-injection (> 6- to 7-fold in

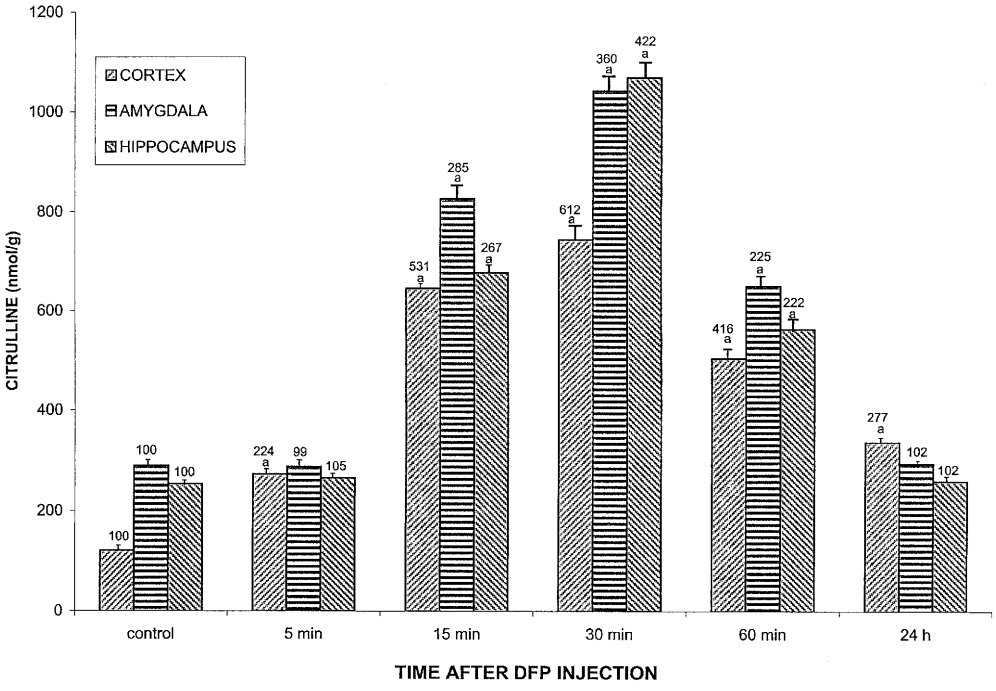

Fig. 1 Citrulline levels in brain regions of rats intoxicated with an acute dose of diisopropylphosphorofluoridate (DFP, 1.25 mg/kg s.c.). Values of citrulline (nmol/g) are presented as means ± SEM ($n = 4$–6). *Numbers above each bar* are percentages of controls (100%); *a* significant difference between values from control rats and DFP-treated rats ($P < 0.05$)

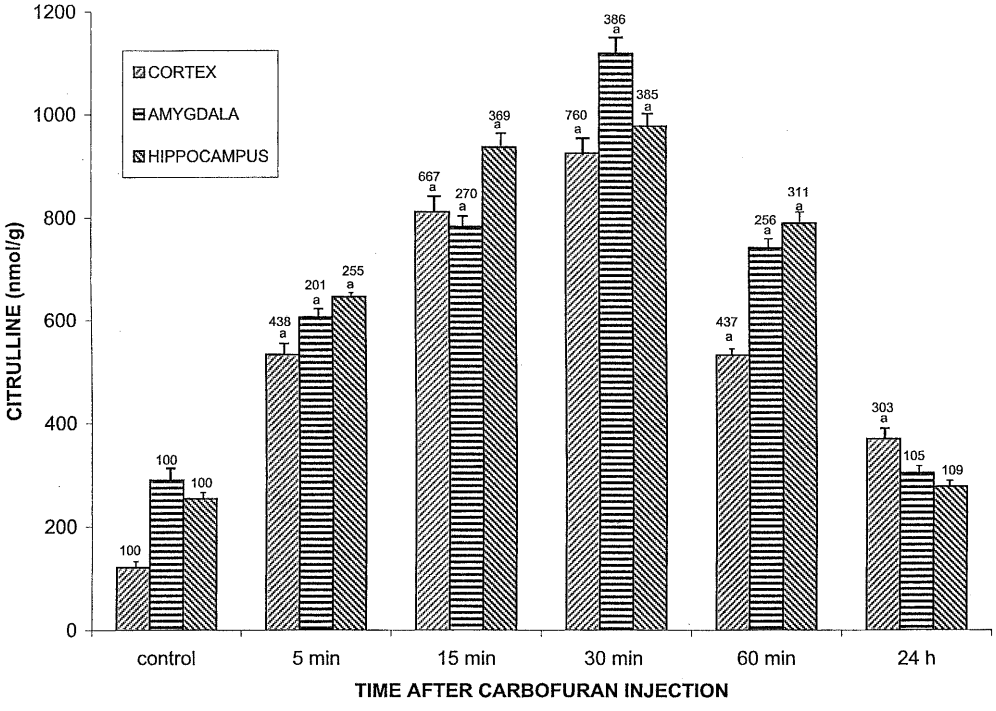

Fig. 2 Citrulline levels in brain regions of rats intoxicated with an acute dose of carbofuran (CF, 1.25 mg/kg s.c.). Values of citrulline (nmol/g) are presented as means ± SEM ($n = 4$–6). *Numbers above each bar* are percentages of control values (100%); *a* significant difference between values from control rats and CF-treated rats ($P < 0.05$)

the cortex, and > 3- to 4-fold in the amygdala or hippocampus), and remained elevated up to 60 min. When measured 24 h later, the citrulline levels were at control level in the amygdala and hippocampus, whereas the cortex values remained increased.

When given alone, PBN or vitamin E did not alter the levels of citrulline in any of the three brain regions examined, but when given as pretreatment to DFP or CF they provided significant protection against DFP- or CF-induced increase in citrulline (Figs. 3 and 4, respectively). With each of the antioxidants, protection was greater against DFP than against CF in the cortex, and was equally effective in amygdala and hippocampus.

Data on high-energy phosphates and their metabolites in the brain regions of control rats and those intoxicated with DFP (1.25 mg/kg s.c.) or CF (1.25 mg/kg s.c.) are shown in Fig. 5 (cortex), Fig. 6 (amygdala), and Fig. 7 (hippocampus). Control values of energy

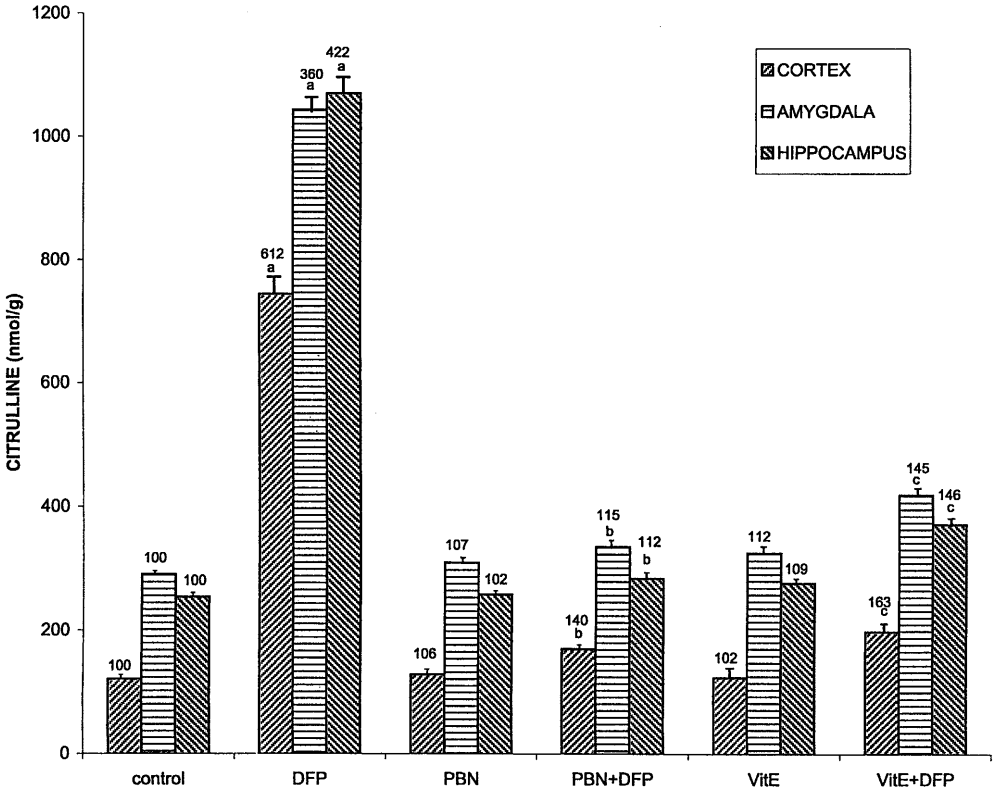

Fig. 3 Protection from DFP-induced changes in citrulline levels in rat brain regions by antioxidant treatment with *N-tert*-butyl-α-phenylnitrone (PBN) or vitamin E. For the details of dosages and treatment of DFP, PBN, and vitamin E, see Materials and methods section. Values of citrulline (nmol/g) are presented as means ± SEM ($n = 4$–6). *Numbers above each bar* are percentages of control values (100%); *a* significant difference between values from control rats and DFP-treated rats ($P < 0.05$), *b* significant difference between values from DFP-treated rats and PBN + DFP treated rats ($P < 0.05$), *c* significant difference between values from DFP-treated rats and vitamin E + DFP treated rats ($P < 0.05$)

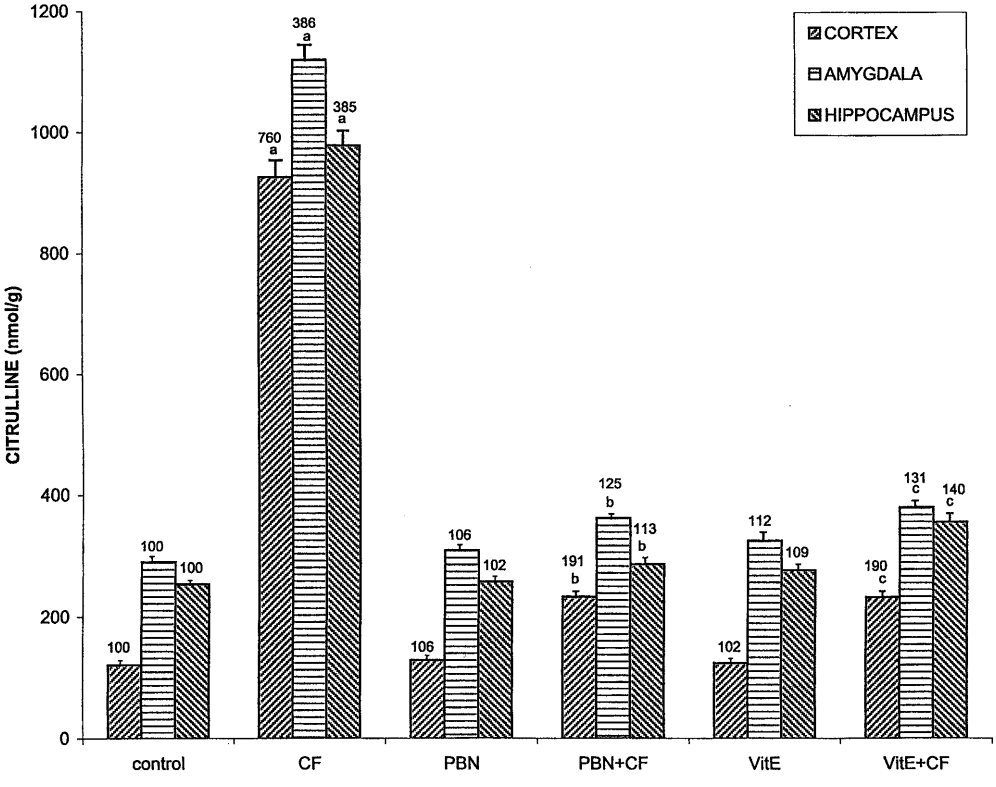

Fig. 4 Protection from CF-induced changes in citrulline levels in rat brain regions by antioxidant treatments (PBN or vitamin E). For the details of dosages and treatment of CF, PBN, and vitamin E, see Materials and methods section. Values of citrulline (nmol/g) are presented as means ± SEM ($n = 4$–6). *Numbers above each bar* are percentages of control values (100%); *a* significant difference between values from control rats and CF-treated rats ($P < 0.05$). *b* significant difference between values from CF-treated rats and PBN + CF-treated rats ($P < 0.05$), *c* significant difference between values from CF-treated rats and vitamin E + CF-treated rats ($P < 0.05$)

metabolites were significantly greater in the cortex than in the amygdala or hippocampus. One hour after DFP or CF injection, the levels of ATP, TAN, PCr, and TCC were significantly reduced in all three brain regions (Figs. 5, 6, 7). With either DFP or CF, the reductions in ATP and TAN levels were greater in the cortex than in the amygdala or hippocampus. With DFP, the levels of PCr were reduced to the same degree in all three brain

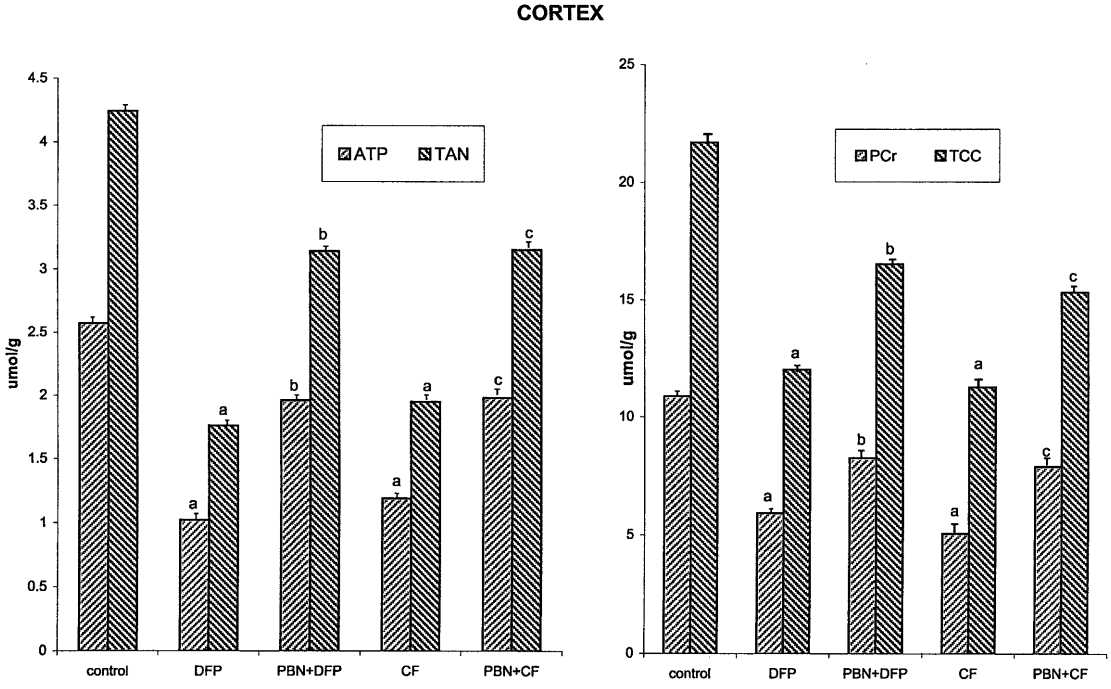

Fig. 5 Protection from DFP- or CF-induced changes in ATP, total adenine nucleotides (TAN), phosphocreatine (PCr), and total creatine compounds (TCC) in rat brain cortex by PBN treatment. For the details of dosages and treatment of PBN, DFP, and CF, see Materials and methods section. Values of energy metabolites (μmol/g) are presented as means ± SEM ($n = 4$–5). *a* Significant difference between values from control rats and DFP- or CF-treated rats ($P < 0.05$), *b* significant difference between values from DFP-treated rats and PBN- + DFP-treated rats ($P < 0.05$), *c* significant difference between values from CF treated rats and PBN + CF-treated rats ($P < 0.05$)

regions (52–55%), whereas with CF treatment the PCr levels were reduced significantly more in the cortex (47%) than in the amygdala or hippocampus (71–72%). PBN treatment alone had no effect on the levels of high-energy phosphates or their major metabolites (data not shown), but when given as pretreatment, it provided significant protection against DFP- or CF-induced depletion of energy metabolites. Protection was greater in the amygdala and hippocampus than in the cortex.

Discussion

The present investigation was carried out with two major objectives: firstly to measure the changes in citrulline (determinant of NO) levels and to explore a possible relationship with AChE inhibitor-induced depletion of high-energy phosphates (ATP and PCr) in selected rat brain regions, and, secondly, to determine whether antioxidant pretreatment prevents the induced changes. Brain regions of the control rats revealed a wide variation in NO levels, as indicated by the amygdala containing more than twice the NO level of the cortex, while the hippocampus value was in the middle of those of the other two regions. This is in contrast to the values for high-energy phosphates and their major metabolites, which are significantly higher in the cortex (23–32%) than in the amygdala or hippocampus (Gupta et al. 2000a, 2000b, 2001). Within the brain, regional variations in the levels of energy metabolites were found to be due to differences in the activity of creatine kinase (CK), which reversibly catalyzes the Lohmann reaction (creatine + ATP ↔ phosphocreatine + ADP). In the forward reaction, CK catalyzes the synthesis of phosphocreatine and ADP, while in reverse reaction, it catalyzes the synthesis of ATP and creatine (Watts 1971; Saks et al. 1978). CK activity was reported to be approximately 25% higher in the cortex than in the hippocampus (Gupta et al. 2000b). Therefore, it is plausible that the higher NO level in the amygdala could be due to a higher activity of nNOS; however, the exact explanation is yet to be elucidated.

In recent studies, NO has been considered as a toxic molecule because it has been implicated in seizures (Lallement et al. 1996; Kim et al. 1999) and neurotoxicity (Nowicki et al. 1991; Dawson et al. 1993; Dawson 1995; Dawson and Dawson 1996; Burney et al. 1997). NO exerts neurotoxic effects primarily by depletion of cellular energy stores through multiple mechanisms:

1. by prolonging PARP activation (Zhang et al. 1994)
2. by inhibiting mitochondrial enzymes such as COx (Brown and Cooper 1994; Cleeter et al. 1994; Brown 1995; Torres et al. 1995; Giulivi 1998; Brorson et al. 1999; Brunori et al. 1999) and CK (Gross et al. 1996; Kaasik et al. 1999), and
3. by inhibiting glycolytic enzymes, such as phosphofructokinase (Molina y Vedia et al. 1992; Erecinska et al. 1995; Firestein and Bredt 1999).

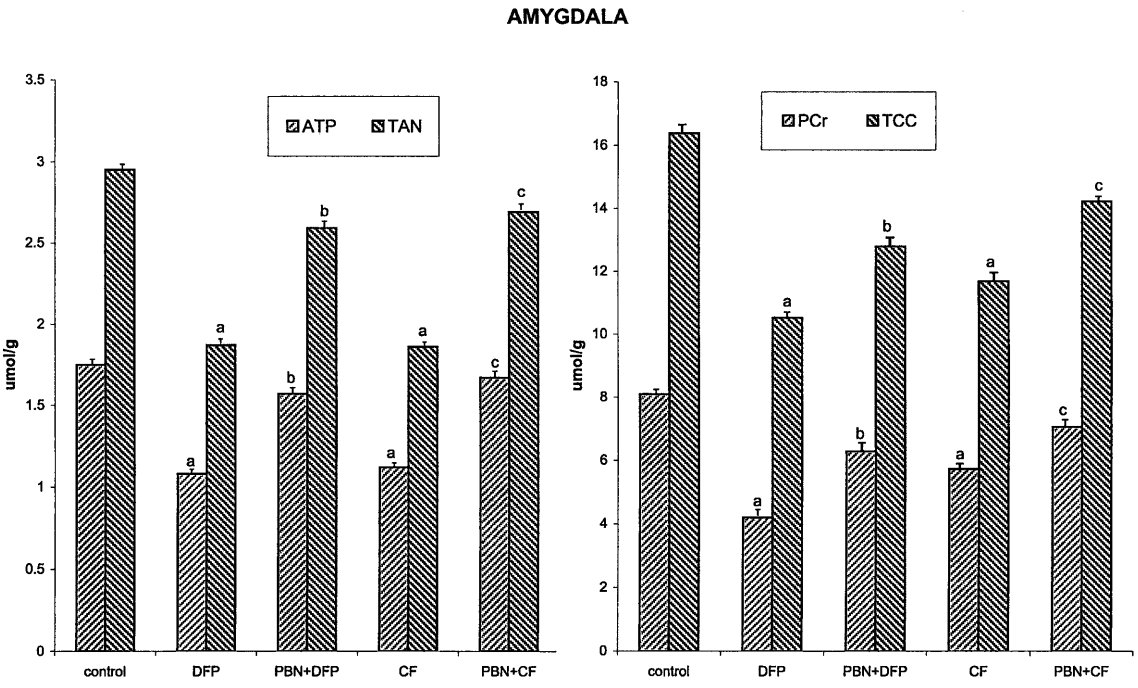

Fig. 6 Protection from DFP- or CF-induced changes in ATP, TAN, PCr, and TCC in rat brain amygdala by PBN treatment. For the details, see Fig. 5 and Materials and methods section. Values of energy metabolites (μmol/g) are presented as means ± SEM ($n = 4$–5). *a* Significant difference between values from control rats and DFP- or CF-treated rats ($P < 0.05$). *b* significant difference between values from DFP treated rats and PBN + DFP-treated rats ($P < 0.05$), *c* significant difference between values from CF-treated rats and PBN- + CF-treated rats ($P < 0.05$)

These reports clearly provided evidence that NO impairs mitochondrial/cellular respiration and other functions by inhibiting the activities of several key enzymes, particularly COx, and thereby causing ATP depletion. These data support our findings of a correlation between NO and energy metabolites. Inhibition of COx may be the primary mechanism for ATP depletion, since COx is the terminal and rate-limiting enzyme of the mitochondrial respiratory chain, which generates ATP by oxidative phosphorylation. In fact, almost all intracellular ATP is known to be generated in the mitochondria (Pedersen 1999; Saraste 1999). Therefore, a reduced capacity of COx can lead to an incomplete reduction of O_2, increased production of ROS and reduced ATP synthesis, producing more oxidative damage to mitochondrial membranes (Bose et al. 1992; Bondy and Lee 1993; Yang and Dettbarn 1998; Milatovic et al. 2001a, 2001b). This is further supported by the findings that AChE-inhibiting organophosphorus insecticides cause inhibition of oxidative phosphorylation in the rat brain (Fukushima et al. 1997).

With each of the AChE inhibitors examined, NO levels were significantly elevated, while energy metabolites were reduced in all three brain regions. The increase of NO was significantly greater in the cortex, and correlated well with a greater depletion of high-energy phosphates in this brain region than in the amygdala or hippocampus. Other factors will contribute to the energy changes, including mitochondrial and neuronal damage, and a higher rate of ATP utilization needed to generate NAD in the ADP-ribosylation of nuclear proteins (Gupta et al. 1998, 1999, 2000a, 2000b), resulting in a decline of TAN and TCC (Figs. 5, 6, 7). Thus, the combination of impaired synthesis of ATP with its greater utilization during brain hyperactivity appears to result in a significant depletion of ATP.

From our recent studies, an important question that emerged was whether brain hyperactivity, such as seizure activity and the decrease in ATP generate increases in ROS, or whether hyperactivity produces ROS leading to a decrease in ATP. The present findings revealed that within 5–15 min after CF or DFP injection (the time required for onset and development of clinical signs), NO levels increased > 5- to 6-fold in the cortex and > 2- to 3-fold in the amygdala and hippocampus (Figs. 1 and 2). With each AChE inhibitor, the maximum increase in NO occurred at 30 min post-injection in all three brain regions. The data also revealed that maximum decline in high-energy phosphates occurred 1 h after DFP or CF injection (Figs. 5, 6, 7, time-course data not shown). Therefore, the findings suggest that in the case of AChE inhibitors, the increase in ROS (measured as increased NO synthesis) preceded the decrease of ATP in the brain.

In contrast to vitamin E, pretreatment with the spin trapping agent PBN prevented DFP- or CF-induced convulsions and seizures. This could primarily be due to a direct, reversible interaction of PBN with AChE, which is sufficient to protect a critical fraction of AChE against phosphorylation by DFP or carbamylation by CF (Zivin et al. 1999a; Milatovic et al. 2000a). The data presented in Figs. 3 and 4 show that AChE inhibitor-

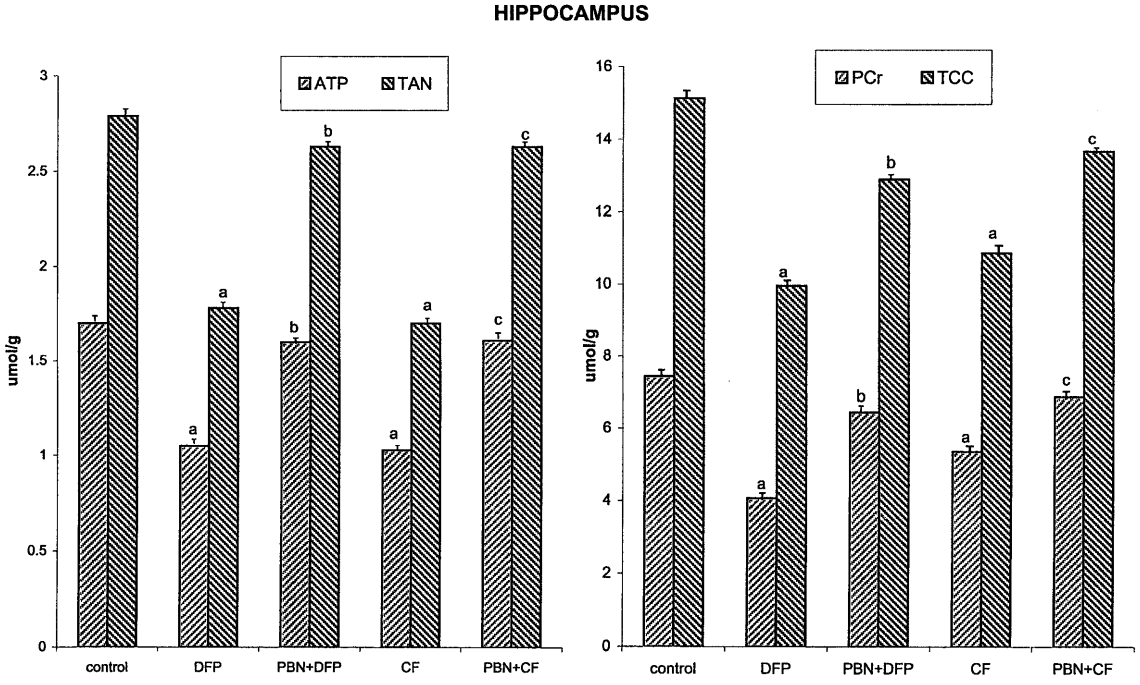

Fig. 7 Protection from DFP- or CF-induced changes in ATP, TAN, PCr, and TCC in rat brain hippocampus by PBN treatment. For the details, see Fig. 5 and Materials and methods section. Values of energy metabolites (μmol/g) are presented as means ±SEM ($n=4$–5). *a* Significant difference between values from control rats and DFP or CF treated rats ($P<0.05$), *b* significant difference between values from DFP-treated rats and PBN + DFP-treated rats ($P<0.05$), *c* significant difference between values from CF-treated rats and PBN + CF-treated rats ($P<0.05$)

induced increases in NO were significantly prevented by PBN as well as by vitamin E. There is evidence that suggests that PBN inhibits the induction of inducible nitric oxide synthase (iNOS) by reducing the expression of iNOS protein (decrease in mRNA expression), and thus prevents the overproduction of NO (Miyajima and Kotake, 1995, 1997). The findings presented in this paper (Figs. 5, 6, 7) and elsewhere (Gupta et al. 2001) demonstrate that AChE inhibitor-induced depletion of high-energy phosphates are, in part, also prevented by antioxidants (PBN or vitamin E), supporting the suggestion that increased generation of ROS may contribute to depletion of energy phosphates.

Both PBN and vitamin E are known to accumulate in the mitochondria (Bjorneboe et al. 1991; Folbergrovà et al. 1995), which is also the major site for the production of ATP (Pedersen 1999; Saraste 1999) as well as for generation of ROS (Turrens 1997; McLennan and Esposti 2000; Staniek and Nohl 2000). In addition to its anticonvulsant effect, PBN has some other pharmacological actions, such as:

1. reversible Ca^{2+} channel blockade (Anderson et al. 1993)
2. reversible inhibition of AChE and protection of this enzyme against critical prolonged inhibition by organophosphates and carbamates (Zivin et al. 1999a; Milatovic et al. 2000a, 2000b)
3. protection of COx activity (Milatovic et al. 2001a, 2001b), and
4. induction of hypothermia (Pazos et al. 1999).

PBN has also been shown to decrease ischemia-reperfusion injury in the brain (Folbergrovà et al. 1995; Floyd 1999). From studies of an ischemic model, Folbergrovà et al. (1995) reported that PBN accumulates in the mitochondria where it could prevent ROS damage to the respiratory chain and thus maintain the level of high-energy phosphates. Our recent studies have shown that vitamin E was less effective in protecting energy levels in brain (Gupta et al. 2001).

Previous studies have demonstrated that mitochondria contain the highest concentration of vitamin E (Bjorneboe et al. 1991) and that vitamin E accelerates ATP resynthesis in tissues subjected to ischemia-reperfusion (Marubayashi et al. 1986; Punz et al. 1998). In addition, vitamin E prevented changes in lipase activity and lipid peroxidation in the brain and spinal cord of rats otherwise caused by the organophosphorus insecticide metasystox (Tayyaba and Hasan 1985). Vitamin E acts mainly as a chain-breaking antioxidant and radical scavenger, protecting cell membranes against oxidative damage (Marubayashi et al. 1986; Bast et al. 1991; van Acker et al. 1993) by regulating ROS production (Chow et al. 1999), maintaining oxidative phosphorylation in mitochondria, and accelerating restitution of high-energy phosphates (Kotegawa et al. 1993; Punz et al. 1998). Although the protective effects of PBN and vitamin E against DFP- or CF-induced elevation of NO and depletion of energy-rich phosphates in rat brain are of the same degree, the two antioxidants may act by different mechanisms (Barclay and Vinqvist 2000; Gupta et al. 2001). The main difference being that PBN prevents seizure activity and thus inhibits the cascade of

ROS generation and loss of energy metabolites (Zivin et al. 1999a). That PBN also acts as a typical antioxidant has been shown in experiments with kainic acid, which is a potent convulsant acting by stimulation of glutamatergic receptors. PBN pretreatment without anticonvulsant action prevents the rise in citrulline and the loss of energy metabolites otherwise seen in only kainic acid-treated rats (Milatovic et al. 2001b).

It can be concluded from the present findings of the early and rapid increase in NO that increased ROS generation may be the first step in the mechanisms involved in DFP- or CF-induced loss of energy metabolites, causing mitochondrial dysfunction and neuronal injury, which can be prevented by pretreatment with antioxidants with or without anticonvulsant actions. Following a similar protocol, our future studies will examine whether other ROS, such as hydroxyl radicals, have similar effects on neuronal energy metabolites, and whether the changes induced can be prevented by antioxidants.

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