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Biphasic modulation by nitric oxide of caspase activation due to malonate injection in rat striatum

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Abstract

The present study examined caspase activation and its modulation by nitric oxide (NO) in a model of oxidative stress induced by injection of malonate (3 μmol), a mitochondrial toxin, into rat striatum. Caspase-3-like enzymatic activity was maximal 6 h after malonate while NO production evaluated by its metabolites nitrites and nitrates was increased at 3 h. The neuronal NO-synthase inhibitor 7-nitroindazole reduced malonate induced-NO production by 50% at 25 mg/kg and enhanced by 32% caspase activation. This result suggests that a moderate production of NO potentiates caspase activation, an effect counterbalanced by NO itself at higher concentrations. Accordingly, complete inhibition of NO production by 7-nitroindazole at 50 mg/kg did not modify malonate-induced caspase activity. Thus NO production by the neuronal isoform of NO-synthase is not the major event leading to caspase activation due to malonate. However, NO seems to have pro- and anti-caspase effects that neutralize each other.

Keywords: Caspase; Nitric oxide (NO); 7-Nitroindazole; Malonate; Oxidative stress

1. Introduction

Caspases are cysteine aspartases implicated in apoptotic cell death by cleaving, thus inactivating, molecules involved in DNA repair and cell integrity. Recently, caspase activation has been reported to play a critical role in neurodegenerative diseases and acute neurological injuries including cerebral ischemia (Sastry and Rao, 2000; Mattson et al., 2001). Activation of caspases has been demonstrated in experimental models of transient focal cerebral ischemia (Benchoua et al., 2001; Davoli et al., 2002), and inhibition of caspase-3-like proteases (Fink et al., 1998; Li et al., 2000) or deletion of the caspase-3 gene has led to neuroprotection (Schulz et al., 1999; Le et al., 2002). Induction of caspase-3 has also been reported in post mortem brains of stroke patients (Love et al., 2000).

Members of the caspase family involved in apoptosis can be divided into two groups: the apoptotic initiators (caspase 8 and caspase 9) that in turn activate the apoptotic effectors (caspase-3, -6, and-7) (Köhler et al., 2002). Two pathways can lead to the initiator caspase activation. The first one involved mitochondria and the Bcl-2 family of proteins and leads via cytochrome *c* release in the cytoplasm to caspase-9 activation (Troy and Salvesen, 2002). The second pathway involves death receptors (e.g. Fas) whose stimulation activates caspase-8 (Sartorius et al., 2001; Kaufmann and Hengartner, 2001). Interaction between the two pathways exists as caspase-8 cleaves the Bcl-2 family member Bid, allowing its migration into the mitochondria where it induces cytochrome *c* release (for a review: Zimmermann et al., 2001; Espoti, 2002).

Multiple mechanisms have been reported to regulate caspase activation and activity in vitro. Among them, oxidative stress and nitric oxide (NO) are well known actors in cerebral ischemia. In vivo studies linking them to caspase activation are, however, still limited. A few in vivo studies have demonstrated that oxygen free radicals produced after transient focal cerebral ischemia contribute to cytochrome *c* release (Fujimura et al., 2000) and caspase-8 activation (Morita-Fujimura et al., 2001) and may thus participate in both pathways involved in effector caspase activation. With regard to NO, in vitro studies suggested that it may interact at various steps in the caspase activation pathways leading either to activation or inhibition, according to its concentration, cell type, and the redox potential of the cell (Kim et al.,

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2001, 2002; Brune et al., 1998). In vivo, caspase activation induced by permanent focal cerebral ischemia was delayed in neuronal NO-synthase null mice compared with wild-type animals (Elibol et al., 2001).

In this context, the present study examined caspase activation and its modulation by NO in an in vivo model of cerebral oxidative stress. Oxidative stress was induced by intrastriatal injection of malonate, a reversible inhibitor of succinate dehydrogenase/mitochondrial complex II and three experimental series were carried out. First, we determined the changes in caspase-3 activity from 1 h after malonate injection, up to 24 h after. Second, we determined the time course and source of nitric oxide production. Finally, we examined the effect of inhibiting NO production on caspase activity and on the striatal lesion induced by malonate.

2. Materials and methods

2.1. Animals

All animal experiments were performed in compliance with French regulations on the protection of animals used for experimental and other scientific purposes (D2001-486), as well as with the EEC regulations (O.J. of ECL358 12/18/1986). Male Sprague–Dawley rats (300–330 g) were housed under standard laboratory conditions with a 12-h light/dark cycle and provided with food and water ad libitum.

2.2. Surgical procedure

Rats were anesthetized with chloral hydrate (400 mg/kg, 10 ml/kg, i.p.) and placed in a stereotaxic frame. Malonic acid (0.3 or 3 μmol), dissolved in distilled water and adjusted to pH 7.4 with HCl, was injected into the left striatum via a cannula inserted at the following coordinates: 0 mm anterior to the bregma, 3.5 mm lateral to the bregma and 7 mm below to the surface of the skull. Infusions (1 μl) were carried out over 5 min with the injection cannula left in place for a further 5 min to minimize the risk of retrograde leakage of the injected solution. Sham animals received intrastriatal injection of distilled water. Body temperature (rectal) was maintained at 36–37 °C throughout the surgical procedure using heating pad and lamp. After the surgical procedure and until recovery from anesthesia, rats were placed in a brooder maintained at 29 °C. For subsequent biochemical and histological studies, animals were killed with an overdose of sodium pentobarbitone. Whole brains or left striata were dissected out and used for the following experiments.

2.3. Caspase-3-like activity assay

Left striata were homogenized, using a potter, on ice in a buffer (A) containing 10 mM N-Tris (hydroxymethyl) methyl-2-aminoethane sulfonic acid (TES/NaOH pH 7.4), 1 mM ethylenediamine-tetra-acetic acid (EDTA), 250 mM sucrose, 2 mM dithiothreitol, 0.1% 3-[(3-cholamidopropyl) dimethylammonio]-1 propane sulfonate (CHAPS), 0.2 μM phenylmethylsulfonyl fluoride and 0.1% ethanol. Tissue homogenates were immediately centrifuged for 10 min at 12,000 × *g* at 4 °C. The protein content of the supernatant was determined by the method of Bradford (1976) and the supernatant was diluted with the buffer (A) to obtain 100 μg of protein in 40 μl. Caspase-3-like activity of the supernatant was detected in duplicates by measuring the proteolytic cleavage of the fluorogenic substrate *N*-acetyl-Asp-Glu-Val-Asp-7-amino-4 trifluoromethyl coumarin (DEVD-AFC, 12.5 μM). AFC liberation was monitored over time with a FLx 800 TBI fluorimeter (Biotek, Fisher Bioblock, Illkirch, France) using 400-nm excitation and 505-nm emission wavelength. Fluorescence units were converted to pmol of AFC using a calibration curve generated with free AFC. Samples pretreated with 5 μM caspase-3 inhibitor Ac-Asp-Glu-Val-Asp-CHO (DEVD-CHO) were also included. Results are given as DEVD-CHO inhibitable AFC liberation in pmol AFC/mg protein/min.

2.4. Nitrite/nitrate determination

Nitrite and nitrate (NO_x) in the left striatum samples were measured by a colorimetric assay based on the Griess reaction after reducing nitrate to nitrite (Green et al., 1982) as previously described (Grandati et al., 1997). NO_x content was expressed as nmol/g tissue.

2.5. Quantification of lesion volume

Brains were removed and frozen in methyl-2 butane at −40 °C. Coronal brain sections, 50 μm thick, were cut in a cryostat (−15 °C) at 500-μm intervals, beginning at the level 11.2 mm anterior to the interaural line which corresponds to the beginning of the striatum. After cresyl violet staining, the lesion appeared as unstained tissue easily contrasted with areas of viable tissue stained in violet. Lesioned areas were measured using an image analyser (IMSTAR, Paris, France) and corrected for the edema according to Golanov and Reis (1995). Lesion volumes expressed in mm^3 were calculated by summing the cross-sectional area of the lesion in each section and multiplying this value by the distance between sections.

2.6. Experimental groups

2.6.1. Time course of caspase-3-like activity after malonate injection

This study was divided into two experiments. In the first one, changes in caspase-3-like activity were examined at early times, 1, 2, 4 and 6 h after malonate at 0.3 μmol ($n = 4–5$ per time) or 3 μmol ($n = 5$ per time) or water

injection (sham rats, $n=5$ per time). Naive rats ($n=5$) were used for basal caspase-3-like activity.

In the second experiment, caspase-3-like activity was measured at delayed time points, 6, 15 and 24 h after the injection of malonate at 0.3 μmol ($n=6–9$) or 3 μmol ($n=6–8$). Sham animals were killed at the same times after intrastriatal water injection ($n=6–9$ per time). Naive rats ($n=12$) were used for basal caspase-3-like activity.

2.6.2. Production of NO_x and its source after malonate injection

First nitrite/nitrate contents were determined 3 and 6 h after malonate (3 μmol, $n=12$ per group) or water injection ($n=6$ per group) and in naive rats ($n=11$). In a second experiment, NO_x content was measured 3 h after intrastriatal injection in the four following groups: a sham group receiving intrastriatal injection of water and intraperitoneal (i.p.) injection of peanut oil (the vehicle of 7-nitroindazole, $n=9$), and three groups receiving an intrastriatal malonate injection (3 μmol) and i.p. injection of peanut oil ($n=12$), 7-nitroindazole at 25 mg/kg ($n=12$) or 7-nitroindazole at 50 mg/kg ($n=12$). Treatment with 7-nitroindazole or its vehicle was given 30 min before the intrastriatal injection (10 ml/kg).

2.6.3. Effect of 7-nitroindazole on caspase-3-like activity and lesion volume

In both experiments, treatments with 7-nitroindazole (25 or 50 mg/kg) or its vehicle were given (10 ml/kg, i.p.) 30 min before and 3 h after intrastriatal injections. For caspase activity assay, rats were divided into four groups: a sham group receiving intrastriatal injection of water and injections (i.p.) of peanut oil ($n=9$), and three groups receiving an intrastriatal malonate injection (3 μmol) and injections (i.p.) of peanut oil ($n=16$), 7-nitroindazole at 25 mg/kg ($n=9$) or 7-nitroindazole at 50 mg/kg ($n=8$). Caspase activity was measured as above 6 h after intrastriatal injections. The lesion volume was measured 24 h after malonate in separate animals given either 7-nitroindazole at 25 ($n=11$) or 50 mg/kg ($n=9$), or its vehicle ($n=17$).

2.7. Reagents

N-acetyl-Asp-Glu-Val-Asp-7-amino-4-trifluoromethyl coumarin (DEVD-AFC) was from Biomol (TEBU, Le Perray en Yvelines, France) and Ac-Asp-Glu-Val-Asp-CHO (DEVD-CHO) was from Alexis Biochemicals (Coger, Paris, France). Pentobarbitone was from Sanofi (Libourne, France). All other reagents were purchased from Sigma (Saint Quentin Fallavier, France).

2.8. Statistics

Data are expressed as means ± S.E.M. For caspase activity and NO_x content, the impact of surgery was evaluated by comparing the values of naive and sham animals using one-way analysis of variance (ANOVA) followed by Dunnett's test. Influence of malonate on both factors (comparison between animals given malonate and sham animals) was then analysed by a two-factorial ANOVA followed by protected least significant difference (PLSD) Fisher's test on sham animals and animals given malonate. The effect of 7-nitroindazole on NO_x content, caspase activity and lesion volume was analysed by one-way ANOVA followed by PLSD Fisher's test. P values <0.05 were considered statistically significant.

3. Results

3.1. Time course of caspase-3-like activity after malonate injection

Changes in caspase-3-like activity at early and delayed time points after malonate injection are given in Fig. 1A and B, respectively. Caspase-3-like activity of sham animals did not differ from that of naive animals in both experiments. Malonate at 0.3 μmol did not modify the caspase-3-like

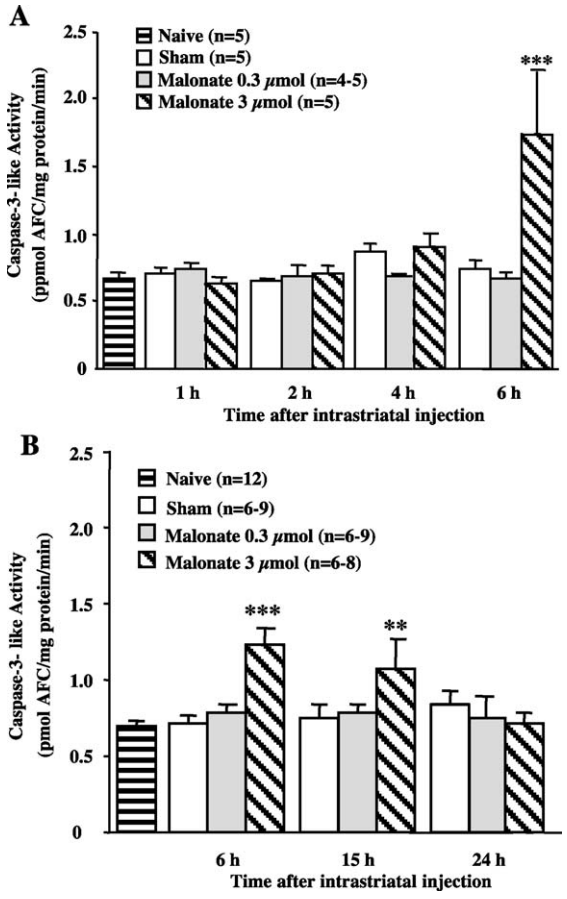

Fig. 1. Changes in striatal caspase-3-like activity at early (A) and delayed (B) time points after malonate injection. Caspase-3-like activity was measured in naive rats and in rats receiving intrastriatal injections of malonate or its vehicle (sham). Data are means ± S.E.M. $^{**}P<0.01$, $^{***}P<0.001$ vs. corresponding sham rats.

activity as compared with sham animals, at early and delayed time points. By contrast, 3 μmol of malonate induced a 137% increase in caspase-3-like activity 6 h after its injection (1.73 ± 0.47 pmol AFC/mg protein/min vs. 0.73 ± 0.06 pmol AFC/mg protein/min in corresponding sham animals, $n = 5$, $P < 0.001$; Fig. 1A). In the second experiment, the increase in caspase-3-like activity observed 6 h after 3 μmol of malonate reached 71% and was maintained at 15 h (44%, $P < 0.001$). Caspase-3-like activity in rats given malonate had returned to sham levels at 24 h (Fig. 1B).

3.2. Production and sources of NO_x after malonate injection

Changes in striatal NO_x content after malonate injection (3 μmol) are given in Fig. 2. NO_x content in sham rats at both times studied did not differ from that of naive rats. The NO_x content of animals given malonate was increased by 29% at 3 h as compared with sham animals (127 ± 7 nmol/g tissue, $n = 12$, vs. 98 ± 9 nmol/g tissue, $n = 6$, $P < 0.05$). No difference was observed at 6 h.

The effect of 7-nitroindazole on the NO_x production after malonate (3 μmol) is represented in Fig. 3. A 60% increase in NO_x production was observed 3 h after malonate (163 ± 10 nmol/g tissue, $n = 12$, vs. 102 ± 14 nmol/g tissue in sham animals, $n = 11$, $P < 0.001$). This increase is reduced to 29% in animals treated with 7-nitroindazole at 25 mg/kg (132 ± 11 nmol/g tissue, $P < 0.05$) and was abolished by 7-nitroindazole at 50 mg/kg (100 ± 12 nmol/g tissue).

3.3. Effects of 7-nitroindazole on caspase-3-like activity and lesion size

The effect of 7-nitroindazole on caspase-3-like activity 6 h after malonate is given in Fig. 4. Malonate induced a

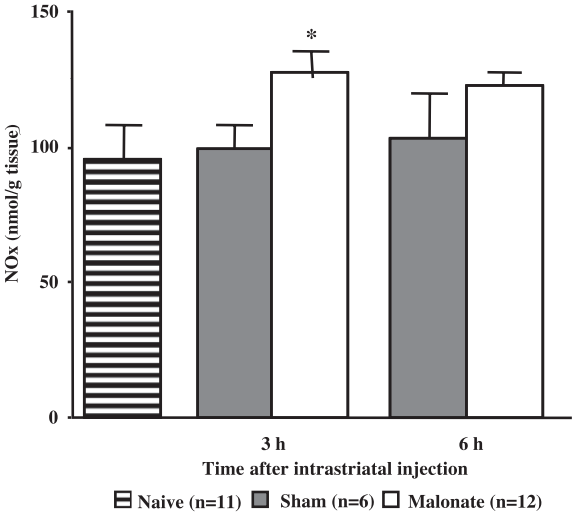

Fig. 2. Changes in striatal NO_x content 3 and 6 h after intrastriatal injection of malonate (3 μmol). Data are means ± S.E.M. *$P < 0.05$.

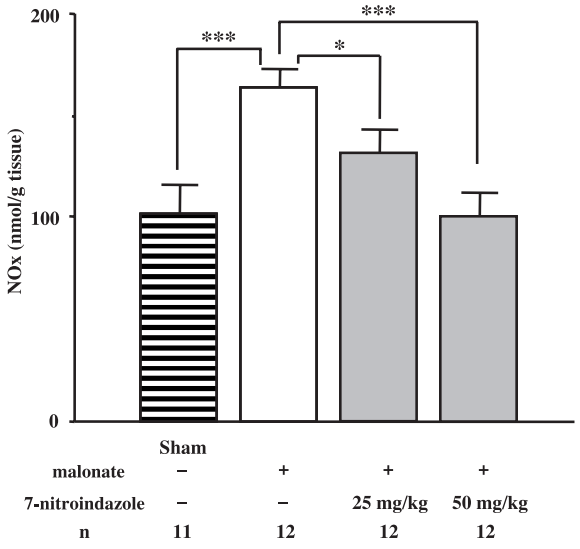

Fig. 3. Effect of 7-nitroindazole on NO production 3 h after malonate injection. Rats were given intrastriatally 3 μmol of malonate or water. Treatments with 7-nitroindazole (25 or 50 mg/kg) or its vehicle (peanut oil) were given (10 ml/kg, i.p.) 30 min before intrastriatal injections. Data are means ± S.E.M. *$P < 0.05$, *** $P < 0.001$.

175% increase in caspase-3-like activity ($P < 0.001$ vs. sham animals). At 25 mg/kg, 7-nitroindazole increased by 32% the induction of caspase-3-like activity compared with animals given malonate alone (2.00 ± 0.27 pmol AFC/mg protein/min, $n = 9$, vs. 1.51–0.15 pmol AFC/mg protein/min, $n = 16$, $P < 0.05$). By contrast, the dose of 50 mg/kg of 7-nitroindazole had no effect.

The lesion volume measured 24 h after intrastriatal injection of malonate is given in Fig. 5. Treatment with 7-

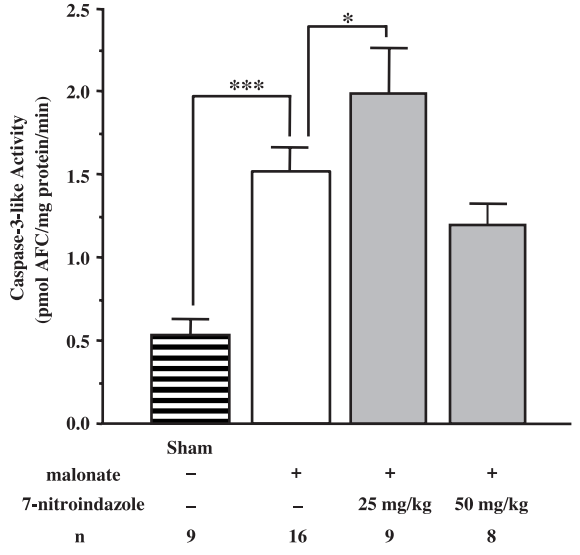

Fig. 4. Effect of 7-nitroindazole on caspase-3-like activity 6 h after malonate injection. Rats were given intrastriatally malonate (3 μmol) or its vehicle (sham). Treatments with 7-nitroindazole (25 or 50 mg/kg) or its vehicle (peanut oil) were given i.p. 30 min before intrastriatal injections. Data are means ± S.E.M. *$P < 0.05$, ***$P < 0.001$.

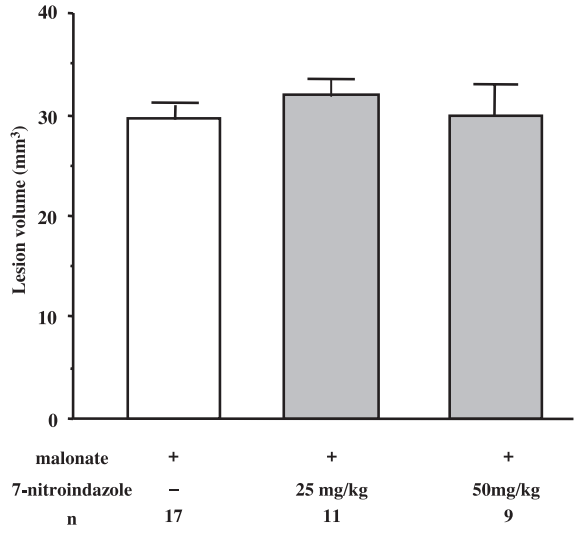

Fig. 5. Effect of 7-nitroindazole on the striatal lesion volume after injection of malonate. The lesion volume was measured 24 h after the injection of malonate (3 μmol) in animals given 7-nitroindazole (25 or 50 mg/kg) or its vehicle (peanut oil). Data are means ± S.E.M.

nitroindazole at both doses (25 and 50 mg/kg) did not modify the size of the lesion.

4. Discussion

Our results show that the mitochondrial toxin malonate injected intrastriatally at 3 μmol induced a transient increase in caspase-3-like protease activity 6 and 15 h after its injection. This increase is preceded by an increase in nitrites/nitrates 3 h after malonate injection due to neuronal NO-synthase (nNOS). Finally, treatment with 7-nitroindazole at 25 mg/kg that partially inhibited NO production enhanced caspase-3 activity while at 50 mg/kg, a dose that completely abolished malonate induced-NO production, it had no effect. None of the 7-nitroindazole dosages modified the striatal lesion size measured 24 h after malonate.

The present study first examined the changes in the caspase activity after malonate injection. Fluorescent coumarin derivatives of tetrapeptides are widely used to determine caspase activity in tissue. The sequence DEVD is the most specific sequence for caspase-3 cleaving activity but it can also be cleaved by caspase-6, -7, -8, and -10 (Talanian et al., 1997). Therefore DEVDase activity is referred to caspase-3-like activity. In our study, the lowest dosage of malonate, 0.3 μmol, was devoid of effect. By contrast, 3 μmol of malonate increased the caspase-3-like protease activity 6 and 15 h after its administration. It is noteworthy that the same dose of malonate increased caspase-3-like protease activity identified by DEVD biotin affinity labeling in mice, although the increase was slightly delayed between 12 and 24 h (Schulz et al., 1998).

Caspase activity is regulated by multiple mechanisms. Among them, nitric oxide appears to have a complex role. On the one hand, NO is able to inhibit caspase activity. This effect may be due to direct inhibition through *S*-nitrosylation of cysteine at the active site of caspase-3-like proteases (Li et al., 1997) or to indirect inhibition via (1) cGMP-dependent mechanism (Kim et al., 1997, 1999) or (2) decrease in ATP content by inhibition of mitochondria (Nicotera et al., 2000). On the other hand, NO was shown to promote caspase activation (Brockhaus and Brune, 1999) notably in neurons (Leist et al., 1997; Maiese and Vincent, 1999; Tamatani et al., 1998). This effect appears to be mediated via p53 accumulation and/or down-regulation of Bcl-2 and up-regulation of Bax proteins, with subsequent cytochrome *c* release and caspase 9 activation that in turn activates caspase-3, -6 and -7. In this context we studied whether NO modulates the early activity of caspase-3-like proteases seen after malonate injection.

According to the time course of caspase-3-like protease activation after 3 μmol of malonate, NO production was examined 3 and 6 h after the intrastriatal injection of the toxin. An increase in the striatal concentration of NO metabolites and nitrites/nitrates was observed 3 h after the injection of malonate thus demonstrating that NO production precedes caspase-3-like protease activation.

Three isoforms of NO-synthase (NOS) have been identified: the constitutive neuronal and endothelial isoforms and the inducible isoform originally isolated from macrophages (Alderton et al., 2001). The early NO production after malonate more likely results from constitutive NOS activation. It is now well established that malonate induces secondary excitotoxic lesions in the striatum (Beal et al., 1993; Greene et al., 1993) as (1) extracellular concentration of glutamate is increased 1 h after intrastriatal injection of 2 μmol of malonate (Messam et al., 1995) and (2) the activation of *N*-methyl-D-aspartate (NMDA) receptors contributes to the damage elicited by malonate (Henshaw et al., 1994; Greene and Greenamyre, 1995; Schulz et al., 1995a). Activation of NMDA receptors leads to an increase in intracellular calcium concentrations that causes the activation of calcium-dependent enzymes including nNOS (Dawson et al., 1991). Thus it was conceivable that nitric oxide produced in response to malonate was synthetised by nNOS.

To confirm this hypothesis, we next examined the effect of 7-nitroindazole, a selective inhibitor of nNOS (Babbedge et al., 1993; Moore et al., 1993b; Yoshida et al., 1994) on NO_x concentration after malonate injection. The dosing regimen was based (1) on the initial kinetics study by MacKenzie et al (1994) showing that 7-nitroindazole given i.p. at 30 mg/kg exerts maximal inhibition on striatal neuronal NOS 30 min after its injection and (2) on a previous study showing a neuroprotective effect of both 25 and 50 mg/kg of 7-nitroindazole, administered i.p. 30 min before 3 μmol of malonate on the striatal lesion (Schulz et al., 1995b). Our treatment with 7-nitroindazole 30 min before malonate injection dose-dependently inhibited the increase in NO_x seen 3 h after malonate, with a complete inhibition at 50 mg/kg. As even 80 mg/kg of 7-nitroindazole

was shown to selectively inhibit neuronal NOS without altering endothelial NOS activity (Moore et al., 1993a; Connop et al., 1994), it appears that in our conditions, NO produced in response to malonate is due to the neuronal isoform of NOS. As mentioned above, activation of nNOS likely results from Ca^{2+} entry through NMDA receptors during secondary excitotoxicity due to malonate.

We next examined the role of nNOS derived NO in the increase in caspase-3-like protease activity induced by malonate at 6 h. When NO production was inhibited by 50% (with 7-nitroindazole at 25 mg/kg), the increase in caspase-3-like protease activity was potentiated. This result suggested that the remaining moderate NO production due to malonate is able to enhance caspase-3-like protease activity. By contrast, when NO production was totally abolished (with 7-nitroindazole at 50 mg/kg), the increase in caspase-3-like protease activity due to malonate was not modified, showing that a high production of NO counterbalances the facilitating effect of the moderate production. Thus, although NO is not the major mediator of malonate-induced-caspase-3-like protease activity, it may have both activating and inhibitory effects that counterbalance each other. Additionally, the production of peroxynitrite, the reaction product of NO with superoxide anion, was reported after malonate injection (Schulz et al., 1996). In in vitro studies, peroxynitrite was shown to induce caspase activation notably by causing the release of cytochrome *c* (Radi et al., 2002). Thus peroxynitrite may mediate NO-induced caspase activation after malonate. The formation of peroxynitrite moreover requires an optimal ratio of superoxide anion to NO. This may contribute to explain why 7-nitroindazole dose-dependently altered caspase-3-like protease activity after malonate.

The striatal lesion size produced 24 h after 3 μmol of malonate is in the same range as that observed by other authors (Schulz et al., 1998; Demougeot et al., 2001). In our study, 7-nitroindazole failed to reduce this striatal lesion suggesting that NO produced by nNOS in response to malonate is not implicated in striatal damage. By contrast, Schulz et al (1995a) showed in the same model a neuroprotective effect of 7-nitroindazole with both dosages (25 and 50 mg/kg). This discrepancy might be due to the fact that the times of administration of 7-nitroindazole differ, as Schulz et al. (1995a) began the treatment 4 h before malonate. Thus although the two injections are repeated at the same interval, we started the treatment later (30 min before malonate). As already mentioned above, injection of 7-nitroindazole leads to maximal nNOS inhibition 30 min later (Mackenzie et al., 1994) and 7-nitroindazole at 50 mg/kg does abolish NO production in the first hours following malonate in our study. Thus lack of neuroprotection with 7-nitroindazole is unlikely due to a lesser inhibitory effect. Both studies also differ by the time when the lesion is measured: 24 h after malonate in our study vs. 7 days in the study of Schulz et al. (1995a). Thus early inhibition of nNOS may have beneficial effect on delayed but not on early striatal damage. Clearly, further experiments are needed to confirm this hypothesis.

In conclusion, we showed that a transient increase in caspase-3-like protease activity occurs in the model of oxidative stress induced by malonate. Our studies also demonstrate in vivo that NO produced by the neuronal isoform of NOS is a modulator of caspase activity, with a dual role depending on its amount. Further experiments will be required to understand fully the mechanism(s) whereby NO exerts its effects.

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