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A study on the role of nitric oxide and iron in 3-morpholino-sydnonimine-induced increases in dopamine release in the striatum of freely moving rats

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1 We showed previously that interaction between NO and iron (II), both released following the decomposition of sodium nitroprusside (SNP), accounted for the late SNP-induced dopamine (DA) increase in dialysates from the striatum of freely moving rats; in addition, we showed that co-infusion of iron (II) with the NO-donor S-nitroso-N-acetylpenicillamine mimicked SNP effects on striatal DA release.

2 In the present study, intrastriatal co-infusion of iron (II) (given as $FeSO_4$, 1 mM for 40 min) with the NO-donor and potential peroxynitrite generator 3-morpholinosydnonimine (SIN-1) (0.2, 0.5, 1.0 or 5.0 mM for 180 min), potentiated the SIN-1-induced increase in DA concentration in dialysates from the striatum of freely moving rats. Neither alone nor associated with iron (II) did SIN-1 induce changes in dialysate ascorbic acid or uric acid concentrations.

3 Neither co-infusion of a superoxide dismutase mimetic nor uric acid affected SIN-1-induced increases in dialysate DA concentration.

4 Infusion of the iron chelator deferoxamine (0.2 mM for 180 min) decreased dialysate DA and attenuated SIN-1-induced increases in dialysate DA concentrations.

5 These results suggest that iron plays a key role in SIN-1-induced release of striatal DA and do not support any role for either peroxynitrite or superoxide anion in SIN-1-induced release of striatal DA.

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Keywords: NO-donor; dopamine; *in vivo* release; iron; peroxynitrite; microdialysis; rat striatum

Abbreviations: ANOVA, analysis of variance; DA, dopamine; DOPAC, dihydroxyphenylacetic acid; HVA, homovanillic acid; MnTBP, manganese(III) tetrakis (4-benzoic acid) porphyrin; NAC, N-acetylcysteine NO, nitric oxide; SIN-1, 3-morpholinosydnonimine; SNAP, S-nitroso-N-acetylpenicillamine; SNP, sodium nitroprusside; SOD, superoxide dismutase

Introduction

Nitric oxide (NO) is a multifunctional and ubiquitous biological messenger molecule. NO signalling plays an important role in the functioning of the central nervous system (Gartwaite & Boulton, 1995). Several *in vivo* studies have demonstrated that NO modulates extracellular levels of dopamine in the striatum and in the hippocampus. In these studies, either NO-generating drugs [sodium nitroprusside (SNP), 3-morpholinosydnonimine (SIN-1), S-nitroso-N-acetylpenicillamine (SNAP)] (Guevara-Guzman *et al*., 1994; West & Galloway, 1996; 1997; 1998; Segieth *et al*., 2000), NO-synthase inhibitors (Silva *et al*., 1995; Segovia & Mora, 1998; Desvignes *et al*., 1999; Segieth *et al*., 2000; Wegener *et al*., 2000), or NO-synthase substrates (Strasser *et al*., 1994; West & Galloway, 1997; Wegener *et al*., 2000), were employed. The results of these studies have often been conflicting. West & Galloway (1996; 1997) showed that SNAP increased DA efflux from the striatum of chloral hydrate-anaesthetized rats. In contrast, Guevara-Guzman *et al*. (1994) showed that SNAP decreased extracellular DA concentration in urethane-anaesthetized rats, as did NO gas, given directly by dissolution in degassed perfusion fluid. Segieth *et al*. (2000) showed that SNAP promoted DA release from the rat hippocampus *in vivo* at low concentrations, whilst high concentrations induced long-lasting DA decreases. More recently, Trabace & Kendrick (2000) have shown that short-lasting intrastriatal infusion of SNAP induced increases in dialysate DA at low concentrations, and decreases in dialysate DA at high concentrations; these DA changes were attributed to SNAP-induced peroxynitrite formation; at high levels, peroxynitrite may reduce extracellular DA concentration through oxidation, while at low levels it may increase DA levels in a cyclic GMP-dependent manner. We confirmed (Serra *et al*., 2001) that, at high concentrations, SNAP intrastriatal infusion induces non-enzymatic DA oxidation, which is inhibited by co-infusion of N-acetylcysteine (NAC) or uric acid, while, at low concentrations, it increases dialysate DA.

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In a previous study (Serra *et al*., 2000), we showed that intrastriatal infusion of either SIN-1 or SNP produced a NO-mediated increase in DA concentration in dialysates from the striatum of freely moving rats. This effect was attenuated by exogenous ascorbic acid. In addition, SNP greatly increased dialysate DA concentration at the end of the infusion period. This effect appeared to be the consequence of an interaction between NO and iron (II), both released from the ferricyanide moiety of SNP, since it was inhibited by the iron chelator deferoxamine. In addition, we showed (Serra *et al*., 2001) that iron (II) greatly increased DA dialysate concentration when co-infused with either high or low concentrations of SNAP.

Iron is a transitional metal involved in many catalytic and regulatory neuronal processes (Connor, 1997), which readily reacts with NO (Le Brun *et al*., 1997). Iron (II) promotes oxidative stress through reactive oxygen species (ROS) formation (Jellinger, 1999). Several *in vivo* experimental studies have linked iron (II)-induced oxidative stress to iron (II)-induced degeneration and death of nigro-striatal neurons (Sengstock *et al*., 1994; 1997; Mohanakumar *et al*., 1996). When infused intrastriatally, iron (II) induces a delayed and infusion time-related DA increase in dialysates from the striatum of freely moving rats (Han *et al*., 1999). This effect has been related (Han *et al*., 1999) to iron (II)-induced hydroxyl radical formation (Xie *et al*., 1995). Interestingly, both nitric oxide (NO)-donors, with the exception of SNP, and NO gas in Ringer's solution, protected nigral neurones from iron (II)-induced oxidative stress (Rauhala *et al*., 1996; Mohanakumar *et al*., 1996) and degeneration (Lin, 1999).

SIN-1 is not a simple NO donor, since it also generates the superoxide anion, being thus a potential peroxynitrite generator (Menconi *et al*., 1998). Further studies on the role of NO, iron and peroxynitrite in SIN-1-induced DA release from dopaminergic terminals in the striatum of freely moving rats were therefore deemed of interest.

Methods

Animals

Male Wistar rats (Morini, R. Emilia, Italy), weighing between 280–330 g were used in all experiments. The rats were maintained under standard animal care conditions (12 : 12 h light/dark cycle, lights coming on at 7 a.m.; room temperature 21°C), with food and water *ad libitum*. Prior to the start of any experiment, the health of the rat was assessed according to published guidelines (Morton & Griffiths, 1985). All procedures were specifically licensed under the European Community directive 86/609 included in Decreto No. 116/1992 of the Italian Ministry of Public Health.

Drugs

SIN-1, deferoxamine, N-acetylcysteine (NAC), uric acid, and ferrous sulphate [$FeSO_4$, iron (II)] were purchased from Sigma-Aldrich (Milano, Italy); manganese (III) tetrakis (4-benzoic acid) porphyrin (MnTBP) from Calbiochem (Darmstadt, Germany).

Drug administration

Iron (II), MnTBP, NAC and uric acid concentrations were chosen according to Serra *et al*. (2000; 2001); deferoxamine according to Desole *et al*. (1998).

Microdialysis probe construction

The striatal probe combined two independent microdialysis probes of concentric design with two separate inlets and a shared outlet, as previously described (Miele *et al*., 2000). The probes were constructed using two sections of plastic-coated silica tubing (diameter 0.15 mm; Scientific Glass Engineering, Milton Keynes, U.K.) each placed in the centre of a semi-permeable polyacrylonitrile dialysis fibres (molecular cut-off weight of 12 KD, Filtral 16 Hospal Industrie, France). Each probe had a final diameter of 0.22 mm. The tips of the dialysis fibre were sealed and joined using quick-drying epoxy glue. The two sections of silica tubing served as inlets; the outlet was made also with a section of plastic-coated silica tubing, positioned in the centre of polythene tubing. The semi-permeable membrane was coated with epoxy leaving an active length of 4 mm. The diameter of the final probe was approximately 0.50 mm. The striatal probe combining two microdialysis probes of concentric design with two separate inlets and a shared outlet, allowed separate co-infusion of drugs.

Surgery

Stereotaxic surgery was performed under chloral hydrate (400 mg kg^{-1} i.p.) anaesthesia. The microdialysis probes were implanted in the right striatum using the following co-ordinates from the atlas of Paxinos & Watson (1986): A/P +0.5 mm from bregma, +2.5 mm M/L, and −6.0 mm D/V from dura. Body temperature during anaesthesia was maintained at 37°C by means of an isothermal-heating pad (Harvard Apparatus, Kent, U.K.). Following surgery the animals were placed in large plastic bowls (50 × 55 cm), and maintained in a temperature- and light-controlled environment, with free access to food and water. Experiments were carried out 24 h after probe implantation with the animal in its home bowl. This arrangement allowed the rats free movement.

Microdialysis procedure

The composition of the Ringer solution used was as follows, in (mM) NaCl 147, KCl 4, $CaCl_2$ 1.2, $MgCl_2$ 1 (pH 6.0). A microinfusion pump (CMA/100, Microdialysis, Sweden) pumped Ringer solution at a flow rate of 1.0 μl min^{-1} using two separate syringes connected to the inlets by a length of polythene tubing; every 20 min, 40 μl dialysate samples were collected manually in 250 μl micro-centrifuge tubes (Alpha Laboratories, U.K.) attached to the outlet. Subsequently, a 20 μl aliquot of collected dialysate was injected into the analytical system. Drugs were added to the Ringer solution and infused *via* the striatal probe implanted in the striatum.

Chromatographic analysis

DA, dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), ascorbic acid and uric acid were quantified by high performance liquid chromatography with electrochemical detection (HPLC-EC) as previously described (Miele *et al.*, 2000; Serra *et al.*, 2000), using an Alltech 426 HPLC pump equipped with a Rheodyne injector, column 15 cm × 4.6 mm i.d. Alltech Adsorbsphere C18 5U, electrochemical detector Antec CU-04-AZ and Varian Star Chromatographic Workstation. The mobile phase was citric acid 0.5 M, Na acetate 1.0 M, EDTA 12.5 mM, MeOH 10% and sodium octylsulphate 650 mg l^{-1} (pH 3.0); the flow rate was 1.3 ml min^{-1}. The first sample was collected after 60 min of stabilization (time 0), then dialysates were collected, at 20 min intervals, for 40 min prior to the start of experiments.

Histology

Following the experiments, rats were killed with an overdose of chloral hydrate (800 mg kg^{-1} i.p.). The location of each microdialysis probe was confirmed by post-mortem histology. Brains were fixed in formal saline and 50 μm coronal sections were made with a cryostat. The slices were stained with cresyl violet and examined under a microscope.

Statistical analysis

The concentrations in the dialysate were expressed in nM (DA) or μM (DOPAC, HVA, ascorbic acid, uric acid) and given as mean ± s.e.mean. Drug effects on neurochemicals were statistically evaluated in terms of changes in absolute dialysate concentrations. Statistical significance was assessed using analysis of variance (ANOVA) for difference between groups and over time. Difference within or between groups were determined by paired or unpaired *t*-tests with Bonferroni multiple comparison adjustment.

Results

Effects of intrastriatal infusion of increasing concentrations of SIN-1 on dialysate concentration of DA, DOPAC + HVA, ascorbic acid and uric acid

Intrastriatal infusion of SIN-1 0.2 mM ($n=3$) or 0.5 mM ($n=3$) for 180 min did not induce changes in dialysate concentrations of DA (Figure 1A), DOPAC + HVA (baseline levels 2.11 ± 0.42 and 1.45 ± 0.19 μM, respectively), ascorbic acid (baseline levels 7.63 ± 1.12 and 8.32 ± 1.15 μM, respectively) and uric acid (baseline levels 1.70 ± 0.28 and 2.33 ± 0.44 μM, respectively) (data not shown).

Intrastriatal infusion of SIN-1 1 mM ($n=4$) for 180 min induced increases in dialysate DA, with a peak (about six times baseline levels) 60 min after the start of infusion. (Figure 1B). Dialysate DA returned to baseline within 60 min after SIN-1 discontinuation. DOPAC + HVA (baseline levels 2.00 ± 0.34 μM), ascorbic acid (baseline levels 7.58 ± 2.37 μM) and uric acid (baseline levels 1.82 ± 0.17 μM) dialysate concentrations baseline levels were unaffected (data not shown).

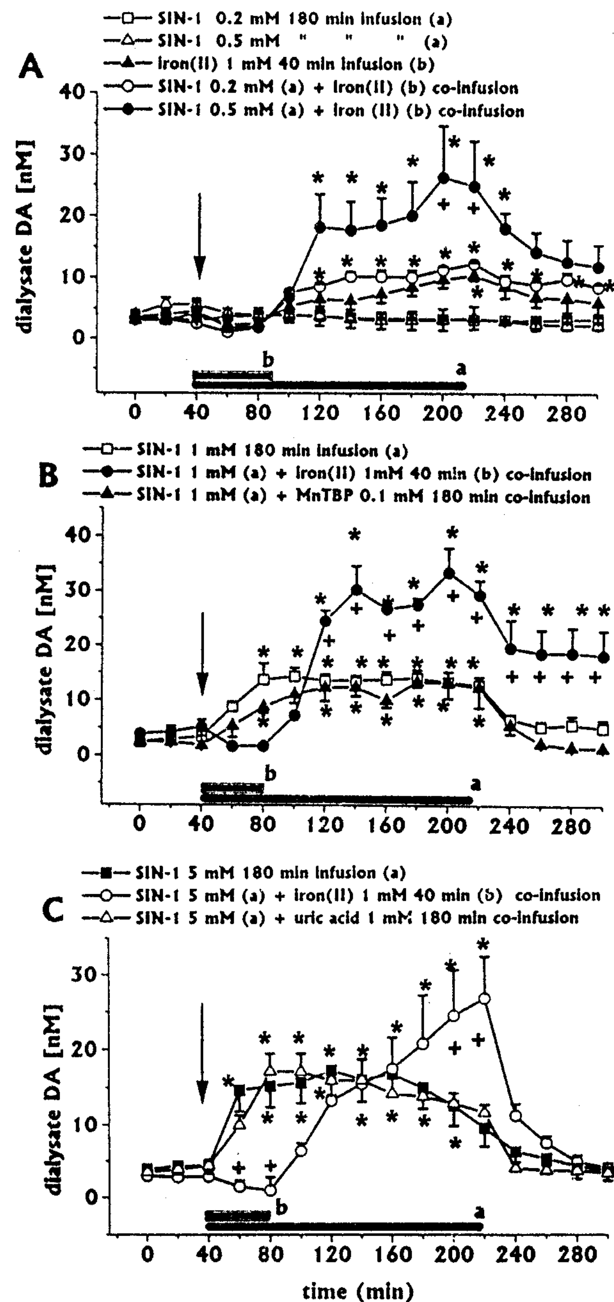

Figure 1 Effects of intrastriatal infusion of increasing concentrations of SIN-1 (0.2 mM ($n=3$, A), 0.5 mM ($n=3$, A), 1.0 mM ($n=4$, B) and 5.0 mM ($n=4$, C) on DA dialysate concentrations, and effects of co-infusion of either iron (II) with SIN-1 (0.2 mM ($n=3$, A), 0.5 mM ($n=3$, A), 1.0 mM ($n=4$, B) and 5.0 mM ($n=4$, C)), MnTBP with SIN-1 (1 mM ($n=3$, B)), or uric acid with SIN-1 (5.0 mM ($n=4$, C)) on SIN-1-induced changes in DA dialysate concentrations. Dialysates were collected, at 20 min intervals, for 180 min during drug infusion (horizontal black bars) and for 80 min after discontinuation of drug infusion. Values are given as mean ± s.e.mean. $*P<0.05$ compared with baseline values. $+P<0.05$ iron (II) + SIN-1 0.5 mM group (A), 1.0 mM group (B) and 5.0 mM group (C) compared with respective SIN-1 groups.

Intrastriatal infusion of SIN-1 5 mM ($n=4$) for 180 min induced increases in dialysate DA, with a peak (about five times baseline levels) 80 min after the start of infusion. (Figure 1C). Dialysate DA returned to baseline within 20 min

after SIN-1 discontinuation. Dialysate DOPAC + HVA concentrations were significantly decreased (Figure 2), while ascorbic acid (baseline levels 8.97 ± 2.42 μM) and uric acid (baseline levels 1.89 ± 0.22 μM) were unaffected (data not shown).

Functional integrity of dopaminergic terminals exposed to SIN-1 5.0 mM

The SIN-1 5.0 mM-induced increase in dialysate DA in the range of that obtained with SIN-1 1.0 mM infusion, and the SIN-1 5.0 mM-induced decrease in dialysate DOPAC + HVA, raised the question as to whether exposure to SIN-1 5.0 mM might compromise the functional integrity of dopaminergic terminals. To address this important point, additional experiments were done. All the questions concerning repeated dialysis procedures within the same animal have been thoroughly addressed in a previous paper (Enrico *et al*., 1997).

SIN-1 5.0 mM was infused intrastriatally for 180 min ($n = 3$). Baseline levels of dialysate neurochemicals were the following: DA, 3.83 ± 0.51 nM; DOPAC + HVA, 1.47 ± 0.27 μM; ascorbic acid, 11.28 ± 0.86 μM; uric acid 1.74 ± 0.23 μM. Four days after the intrastriatal infusion of SIN-1 5.0 mM, dopaminergic terminals were challenged with a d-amphetamine 2.0 mM 15 min infusion, according to Miele *et al*. (2000). Baseline dialysate levels of DA (2.68 ± 0.58 mM) and DOPAC + HVA (0.927 ± 0.17 μM) did not statistically differ from baseline levels detected before SIN-1 infusion. On the contrary, dialysate ascorbic acid (7.65 ± 0.90 μM) and uric acid (5.95 ± 1.56 μM) resulted statistically ($P < 0.05$) lower and, respectively, higher than baseline levels detected before SIN-1 infusion. The increase in dialysate uric acid may reflect a periprobe tissue reaction (Enrico *et al*., 1997).

Following d-amphetamine 2.0 mM 15 min infusion, dialysate DA concentrations increased up to 800–1600% of baseline value. These increases are in the range of those obtained in the previous study (Miele *et al*., 2000) with d-amphetamine systemic (2.0 mg kg^{-1}) administration or intrastriatal (2.0 mM) 15 min infusion.

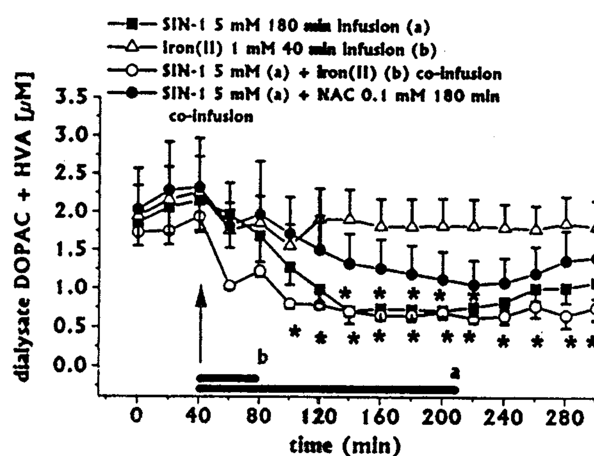

Figure 2 Effect of intrastriatal infusion of SIN-1 ($n = 4$) or iron (II) ($n = 3$) on DOPAC + HVA dialysate concentrations, and effects of iron (II) ($n = 4$) or NAC + iron (II) ($n = 3$) co-infusion with SIN-1, on SIN-1-induced changes in DOPAC + HVA concentrations. Dialysates were collected, at 20 min intervals, for 180 min during drug infusion (horizontal black bars) and for 80 min after discontinuation of drug infusion. Values are given as mean ± s.e.mean. *$P < 0.05$ compared with baseline values.

Effect of iron (II) co-infusion of SIN-1-induced changes in dialysate neurochemicals concentrations

Intrastriatal infusion of iron (II) (1 mM for 40 min, $n = 3$) induced a late moderate increase in dialysate DA concentration, with a peak (about three times baseline levels) 140 min after iron (II) discontinuation (Figure 1A), whilst all other neurochemicals (DOPAC + HVA, ascorbic acid, uric acid) dialysate concentrations were unaffected (data not shown).

Iron (II) (1 mM for 40 min) co-infusion with SIN-1 (0.2 mM for 180 min, $n = 3$) induced an early (40 min after iron (II) discontinuation) and long-lasting increase (even after SIN-1 discontinuation) in dialysate DA, with a peak (about four times baseline levels) at the end of SIN-1 infusion (Figure 1A). During the 40 min of iron (II) and SIN-1 co-infusion, dialysate DA concentrations fell below control values (Figure 1A). All other neurochemicals (DOPAC + HVA, ascorbic acid, uric acid) dialysate concentrations were unaffected (data not shown).

Iron (II) (1 mM for 40 min) co-infusion with SIN-1 (0.5 mM for 180 min, $n = 4$) induced an early (40 min after iron (II) discontinuation) and long-lasting increase (even after SIN-1 discontinuation) in dialysate DA, with a peak (about seven times baseline levels) 20 min before SIN-1 discontinuation (Figure 1A). During the 40 min of iron (II) and SIN-1 co-infusion, dialysate DA concentrations fell below control values (Figure 1A). All other neurochemicals (DOPAC + HVA, ascorbic acid, uric acid) dialysate concentrations were unaffected (data not shown).

Iron (II) (1 mM for 40 min) co-infusion with SIN-1 (1.0 mM for 180 min, $n = 4$) induced an early (40 min after iron (II) discontinuation) and long-lasting increase (even after SIN-1 discontinuation) in dialysate DA, with a peak (about nine times baseline levels) 20 min before SIN-1 discontinuation (Figure 1B). During the 40 min of iron (II) and SIN-1 co-infusion, dialysate DA concentrations fell below control values (Figure 1B). All other neurochemicals (DOPAC + HVA, ascorbic acid, uric acid) dialysate concentrations were unaffected (data not shown).

Iron (II) (1 mM for 40 min) co-infusion with SIN-1 (5.0 mM for 180 min, $n = 3$) induced a late (80 min after iron (II) discontinuation) increase in dialysate DA, with a peak (about nine times baseline levels) at the end of SIN-1 infusion (Figure 1, panel 5). During the 40 min of iron (II) and SIN-1 co-infusion, dialysate DA concentrations fell below control values (Figure 1B). Dialysate DA returned to baseline within 40 min after SIN-1 discontinuation. Dialysate DOPAC + HVA concentrations decreased significantly (Figure 2), while ascorbic acid and uric acid were unaffected (data not shown).

Effect of NAc-cysteine co-infusion on SIN-1 + iron (II)-induced changes in DA and DOPAC + HVA dialysate concentrations

We showed previously (Serra *et al*., 2001) that intrastriatal infusion of NAC protected DA from SNAP-induced non-enzymatic oxidation. In the present study, the increase in dialysate DA given by co-infusion of iron (II) with SIN-1

1.0 mM was greater than that given by co-infusion of iron (II) with SIN-1 5.0 mM (Figure 1B,C). These data suggest that, at high concentrations, SIN-1 might also induce DA non-enzymatic oxidation. Therefore, the study of NAC co-infusion on SIN-1-induced changes in DA and DOPAC+HVA dialysate concentrations was deemed of interest.

Intrastriatal co-infusion of NAC 0.1 mM+SIN-1 5.0 mM (for 180 min) with iron (II) (1 mM for 40 min, $n=3$) induced a significant early and long-lasting increase in dialysate DA, compared with iron (II)+SIN-1 5.0 mM group; in addition, return of dialysate DA to baseline was significantly delayed (Figure 3). Also, NAC co-infusion inhibited iron (II)+ SIN-1-induced decreases in dialysate DOPAC+HVA (Figure 2). Ascorbic acid and uric acid dialysate concentrations were unaffected (data not shown).

Effect of superoxide dismutase (SOD) mimetic MnTBP co-infusion on SIN-induced increases in dialysate DA concentration

Peroxynitrite is formed by reaction of NO with superoxide anions, thus SIN-1 is a potential peroxynitrite generator, since it releases both NO and superoxide anions (Menconi *et al*., 1998). A role for peroxynitrite in SNAP-induced increases in striatal DA has been suggested by Trabace & Kendrick (2000). Ascorbic acid is a natural inhibitor of peroxynitrite generation (Jackson *et al*., 1998; Kirsch & de Groot, 2000), while uric acid is a natural scavenger of peroxynitrite (Hooper *et al*., 1998); however, in this study, SIN-1 infusion affected neither ascorbic acid nor uric acid dialysate levels. SOD is too large a molecule to cross the dialysis membrane used; therefore, we used the cell-permeant SOD mimetic MnTBP (Patel & Day, 1999), in order to assess the role of peroxynitre in SIN-1-induced increases in dialysate DA.

Co-infusion of MnTBP (0.1 mM for 180 min, $n=3$) did not affect SIN-1 (1 mM)-induced increases in dialysate DA (Figure 1B).

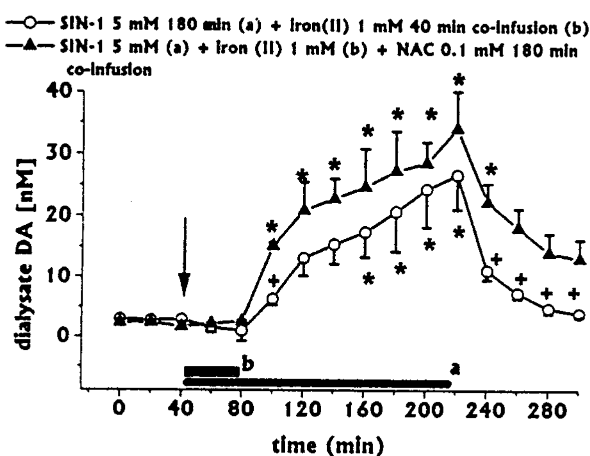

Figure 3 Effect of intrastriatal co-infusion of SIN-1 with iron (II) on dialysate DA (same group as in Figure 1C) and effects of NAC co-infusion ($n=3$) on iron (II)+SIN-1 induced changes in dialysate DA. Dialysates were collected, at 20 min intervals, for 180 min during drug infusion (horizontal black bars) and for 80 min after discontinuation of drug infusion. Values are given as mean± s.e.mean. *$P<0.05$ compared with baseline values; $+P<0.05$ compared with iron (II)+SIN-1.

Effect of uric acid co-infusion on SIN-induced changes in DA and ascorbic acid dialysate concentrations

Uric acid is a strong natural scavenger of peroxynitrite (Hooper *et al*., 1998). Although in the present study SIN-1 infusion failed to modify dialysate uric acid concentrations, the study of uric acid co-infusion on SIN-1-induced changes in DA and dialysate concentrations was deemed of interest.

Co-infusion of uric acid 1 mM with SIN-5 mM for 180 min ($n=4$) did not affect SIN-1-induced increases in dialysate DA (Figure 1C). In addition, uric acid co-infusion did not affect SIN-1-induced decreases in dialysate DOPAC+HVA concentrations (data not shown).

Effect of intrastriatal infusion of deferoxamine on SIN-1 induced increases in dialysate DA

The finding that exogenous iron (II) potentiated SIN-1-induced increases in dialysate DA prompted us to study the effect of the endogenous iron chelator deferoxamine, to evaluate any potential role of endogenous iron in the SIN-1 induced increase in striatal DA release.

Infusion of deferoxamine (0.2 mM) for 180 min ($n=3$) decreased dialysate DA (Figure 4A), whilst it did not affect dialysate DOPAC+HVA (Figure 4B), ascorbic acid or uric acid concentrations (data not shown).

Co-infusion of deferoxamine 0.2 mM for 180 min ($n=4$) attenuated the SIN-1-induced increase in dialysate DA concentration (Figure 4A). Over time ANOVA revealed that the increase was still statistically significant ($P<0.001$), but no significant point resulted with the Bonferroni test.

Deferoxamine co-infusion did not affect SIN-1-induced decreases in dialysate DOPAC+HVA (Figure 4B).

Discussion

The results of the present study have shown that intrastriatal infusion of the NO-donor and potential peroxynitrite generator SIN-1 induces concentration-unrelated increases in dialysate DA. In fact, at lower concentrations (0.2–0.5 mM), SIN-1 does not induce changes in dialysate DA, while the increase given by the higher concentration (5.0 mM) tested does not differ from the increase given by the concentration of 1.0 mM. In addition, the higher SIN-1 concentration (5.0 mM) decreased dialysate DOPAC+HVA levels. At all concentrations tested, SIN-1 infusion did not affect dialysate ascorbic acid and uric acid concentrations.

The SIN-1 5.0 mM-induced increase in dialysate DA in the range of that obtained with SIN-1 1.0 mM infusion, and the SIN-1 5.0 mM-induced decrease in dialysate DOPAC+HVA, raise the question as to whether exposure to SIN-1 might compromise the functional integrity of dopaminergic terminals. West & Galloway (1998) claim that it is unlikely that exposure of striatal dopaminergic terminals to SIN-1 1.0 mM might compromise the functional integrity of nerve terminals *via* a neurotoxic disruption of plasma membrane. In the present study, instrastriatal d-amphetamine infusion 4 days after exposure to SIN-1 5.0 mM increased dialysate DA levels up to 800–1600% of baseline value. These increases are in the range of those obtained in a previous study with systemic or intrastriatal d-amphetamine administrations (Miele *et al*.,

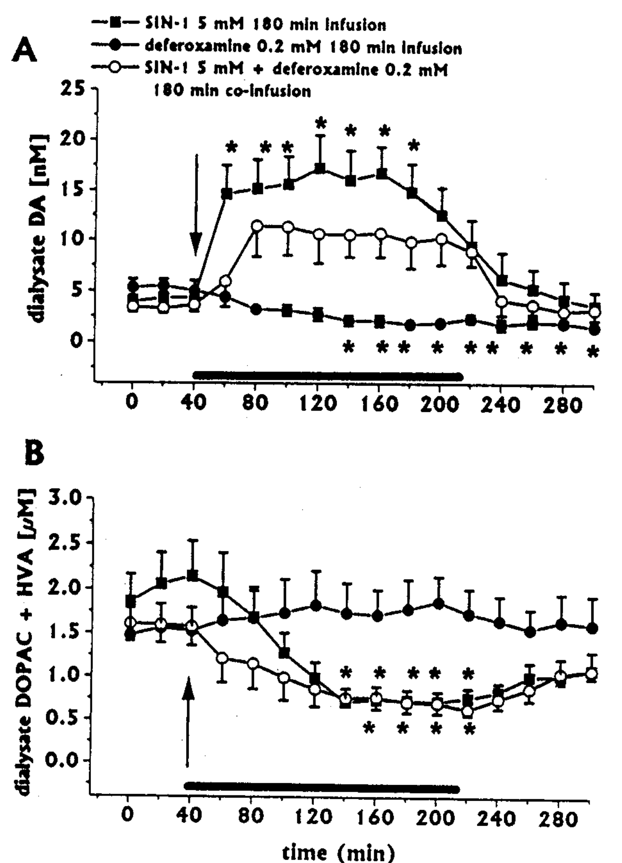

Figure 4 Effect of intrastriatal infusion of deferoxamine ($n=4$) on dialysate DA (A) and DOPAC + HVA concentrations (B), and effects of deferoxamine co-infusion on SIN-1-induced changes in dialysate DA (same group as in Figure 1C) and DOPAC + HVA (same group as in Figure 2). Dialysates were collected, at 20 min intervals, for 180 min during drug infusion (horizontal black bar) and for 80 min after discontinuation of drug infusion. Values are given as mean ± s.e.mean. *$P<0.05$ compared with baseline values.

2000), and should be considered as a reliable test of functional integrity of dopaminergic terminals previously exposed to SIN-1.

SIN-1, besides NO, generates superoxide anions, being thus a potential peroxynitrite generator (Menconi *et al.*, 1998). According to Trabace & Kendrick (2000), peroxynitrite plays a key role in NO-donors (SNAP or (Z)-1-[2-(2-aminoethyl)-N-(2-ammonioethyl)amino]diazen-1-ium-1,2 diolate (NOC-18))-induced changes in striatal DA release. At high extracellular levels, peroxynitrite reduces extracellular DA and DOPAC through oxidation, while at low concentrations it increases striatal DA release in a cyclic GMP-dependent manner. In the present study, however, we were not able to detect SIN-1-induced changes in dialysate DA or DOPAC attributable to peroxynitrite formation. In fact: (i) co-infusion of the SOD mimetic MnTBP failed to inhibit SIN-1-induced increases in dialysate DA; (ii) uric acid is a natural strong scavenger of peroxynitrite (Hooper *et al.*, 1998). However, co-infusion of uric acid with SIN-1 did not affect SIN-1-induced changes in dialysate DA. In addition, at all concentrations tested, SIN-1 failed to induce changes in endogenous uric acid dialysate concentrations. This finding might represent indirect evidence for lack of peroxynitrite formation; (iii) at all concentrations tested, SIN-1 failed to induce changes in dialysate ascorbic acid concentrations. Again, this finding might be indirect evidence for lack of peroxynitrite formation, since ascorbic acid is highly effective to counteract the oxidizing properties of peroxynitrite (Jackson *et al.*, 1998; Kirsch & de Groot, 2000). On the whole, all these findings cast doubt on SIN-1-induced *in vivo* intrastriatal generation of peroxynitrite.

The present study was designed mainly to ascertain the role of iron in the NO donors-induced increase in striatal DA release. In a previous study (Serra *et al.*, 2000), we showed that interaction between NO and iron (II), both released following decomposition of SNP, accounted for the late SNP-induced dopamine (DA) increase in dialysates from the striatum of freely moving rats. This conclusion was based on the observation that the iron chelator deferoxamine greatly inhibited the late SNP-induced increase in dialysate DA; in addition, we showed (Serra *et al.*, 2001) that the late SNP-induced increase in dialysate DA could be mimicked by co-infusing iron (II) with either high or low concentrations of SNAP. In the present study, the amount of NO released by SIN-1 0.2 or 0.5 mM failed to trigger dopamine release; however, a concentration-related increase in dialysate DA occurred when iron (II) was co-infused with SIN-1 0.2 or 0.5 mM. Also, co-infusion with iron (II) potentiated SIN-1 1.0 and 5.0 mM effects on dialysate DA. However, the increase obtained with SIN-1 5.0 mM was lower than that with SIN-1 1.0 mM. In addition, iron (II) co-infusion with SIN-1 5 mM resulted in an early and long-lasting decrease in dialysate DOPAC + HVA. These effects seem to be the consequence of extracellular non-enzymatic oxidation of both DA and DOPAC, which are catechol-containing molecules (Miller *et al.*, 1996); in fact, NAC co-infusion resulted in a greater and long-lasting increase in dialysate DA and restored DOPAC + HVA dialysate levels.

The finding that infusion of deferoxamine decreased dialysate DA concentrations suggests that endogenous iron might have a role in striatal DA release. Deferoxamine is also a Ca^{2+} chelator, therefore it might inhibit DA release by chelating Ca^{2+}; however, the very low *in vivo* affinity for calcium allows the ruling out of this hypothesis (Anghileri *et al.*, 1992). The ability of deferoxamine to decrease DA release might be related to inhibition of tyrosine hydroxylase activity, since iron is essential for enzyme activity. Very recently, however, Liu *et al.* (2001) demonstrated that iron-binding affinity of iron-chelator drugs is not correlated with tyrosine hydroxylase inhibitory activity; this finding allows the ruling out of the above hypothesis. In contrast, the hypothesis that endogenous iron might have a role in striatal DA release is supported by the finding that deferoxamine co-infusion attenuated SIN-1-induced increases in dialysate DA, probably by chelating endogenous free iron.

Understanding the chemistry of NO is important in order to clarify the activity of NO in *in vivo* striatal DA release. NO is a simple hydrophobic gaseous molecule that is highly diffusible and reactive. The following forms are important for its biological action: *NO radical, which can be oxidized to nitrosonium cation (NO^+), or reduced to nitronyl anion (NO^-). NO readily reacts with either iron (II), to form Fe (III)-NO^- complexes, or with iron (III), to form Fe (II)-NO^+ complexes (Le Brun *et al.*, 1997). NO may also interact with ascorbic acid (Millar, 1995); in addition, ascorbic acid may

either protect NO from destruction by superoxide anions (Dudgeon *et al*., 1998; Jackson *et al*., 1998), or scavenge it (Whiteman & Halliwell, 1996). Ascorbic acid may trigger decomposition of the NO-donor SNP both in biological tissue *in vitro* (Bates *et al*., 1991; Reiser *et al*., 1999) and in the striatal extracellular space *in vivo* (Serra *et al*., 2000). In addition, ascorbic acid potentiates decomposition SNAP *in vitro* in striatal slices (Reiser *et al*., 1999) and in the striatal extracellular space *in vivo* (Serra *et al*., 2001), but not that of SIN-1 *in vitro* (Reiser *et al*., 1999). In the present study, SIN-1-induced increases in dialysate DA occurred without changes in dialysate ascorbic acid concentrations. This finding suggests that endogenous ascorbic acid does not interfere with decomposition *in vivo*; in addition, it prompts us to speculate that either scavenging properties of ascorbic acid were not involved in SIN-1-induced changes of dialysate DA, or, if they were, oxidative stress did not exceed the homeostatic regulation of extracellular ascorbic acid (Miele & Fillenz, 1996; Rice, 2000).

The results of the present study allow merely an insight into the mechanism by which the NO-donors increase striatal DA release from dopaminergic terminals. West & Grace (2000) have shown that intrastriatal infusion of either a NO-generator, or a NO-synthase substrate, increased the mean firing rate of nigral DA neurons in anaesthetized rats, suggesting that striatal NO-synthase-containing interneurones play an intermediary role in modulating the striatal efferent regulation of nigral DA neurones. The results of this study do not allow the recognition of a nigral component in the NO-donor-induced increase in striatal DA release; however, they strongly suggest that iron plays a key role in exogenous NO-induced release of striatal DA.

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