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Angeli's Salt Induces Neurotoxicity in Dopaminergic Neurons *In Vivo* and *In Vitro*

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In this study, we investigated the hypothesis that the pro-oxidative properties of Angeli's salt (AS), a nitroxyl anion (HNO/NO^-) releasing compound, cause neurotoxicity in dopaminergic neurons. The pro-oxidative properties were demonstrated *in vitro* by measuring hydroxylation products of salicylate and peroxidation of lipids under various redox conditions. AS (0–1000 μM) released high amounts of hydroxylating species in a concentration dependent manner. AS also increased lipid peroxidation in brain homogenates at concentrations below 100 μM, while inhibiting it at 1000 μM concentration. The AS induced pro-oxidative effects were completely suppressed by copper (II), which converts nitroxyl anion to nitric oxide, as well as by a potent nitroxyl anion scavenger glutathione. Neurotoxicity towards dopaminergic neurons was tested in rat nigrostriatal dopaminergic system *in vivo* and by using primary mesencephalic dopaminergic neuronal cultures *in vitro*. Intranigral infusion of AS (0–400 nmol) caused neurotoxicity reflected as a dose dependent decrease of striatal dopamine seven days after treatment. The effect of the 100 nmol dose was more pronounced when measured 50 days after the infusion. Neurotoxicity was also confirmed as a decrease of tyrosine hydroxylase positive neurons in the substantia nigra. Neither sulphononoate, a close structural analog of AS, nor sodiumnitrite caused changes in striatal dopamine, thus reflecting lack of neurotoxicity. In primary dopaminergic neuronal cultures AS reduced [^{3}H] dopamine uptake with concentrations over 200 μM confirming neurotoxicity. In line with the quite low efficacy to increase lipid peroxidation *in vitro*, infusion of AS into substantia nigra did not cause increased formation of fluorescent products of lipid peroxidation. These results support the hypothesis that AS derived species oxidize critical thiol groups, rather than membrane lipids, potentially leading to protein oxidation/dysfunction and demonstrated neurotoxicity. These findings may have pathophysiological relevance in case of excess formation of nitroxyl anion.

Keywords: Angeli's salt; Dopamine; Free radicals; Lipid peroxidation; Neurotoxicity; Nitroxyl anion

Abbreviations: AS, Angeli's salt; CYS, L-cysteine; DOPAC, 3,4-dihydroxyphenylacetic acid; GSH, Glutathione; HPLC-EC, High pressure liquid chromatography with electrochemical detection; NO, Nitric oxide; NO^-, Nitroxyl anion; NOS, Nitric oxide synthase; RFU, Relative fluorescent intensity unit

INTRODUCTION

Nitric oxide synthase (NOS) generates nitric oxide (NO), which has been shown to have important biological functions as a cell modulator.[1] In addition several authors have proposed that NOS also produces a reduced form of nitric oxide, nitroxyl anion (NO^-).[2–4] Other proposed pathways for nitroxyl anion production *in vivo* are via reaction of S-nitrosothiols with excess thiols[5] or by reversible reduction of nitric oxide by Cu(I)/Zn containing superoxide dismutase (SOD).[6] Nitroxyl anion has been proposed to exist in two different spin states with different reaction chemistries.[7] It has also been demonstrated that HNO is only a weak acid and therefore up to 50 % of the NO^- may exist in the protonated form under

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physiological conditions.[8] Therefore, nitroxyl anion or its protonated form (HNO) can be defined as a NOS or nitric oxide derived reactive compound, which may have actions different from those of nitric oxide.

Many studies have focused on either neurotoxic or neuroprotective properties of NOS and nitric oxide in various animal models. However, the biological effects of nitroxyl anion have been reported only in few studies.[7,9–11] Nitroxyl anion has been shown to possess pro-oxidant properties *in vitro*.[11–13] Direct interaction of nitroxyl or nitroxyl anion with biological compounds could lead to oxidative modification of proteins specially on their sulphydryl groups.[5,8,13] Moreover, after reaction of oxygen with nitroxyl anion, a distinct profile of oxidative reactions takes place compared to other reactive nitrogen species.[13] These species could cause oxidative and nitrosative stress leading to neurotoxicity.

Glutathione (GSH) is an important endogenous antioxidant and decrease of GSH levels may be a critical step leading to oxidative stress induced neuronal damage.[14] Also nitroxyl anion is scavenged by thiols in biological systems.[11,15] Because of the extremely high reactivity of nitroxyl anion/nitroxyl with thiols[5,8,13] excess formation of nitroxyl anion could lead to lowered levels of GSH and subsequently increased vulnerability to other oxidants. Another endogenous factor affecting the balance between nitroxyl anion and nitric oxide is Cu/Zn SOD, which can convert nitric oxide to nitroxyl anion and vice versa.[6] Therefore, GSH and Cu (II) can be used as a tool to monitor the effect of redox environment on nitroxyl anion induced oxidative stress.

In the present study, we investigated the hypothesis that nitroxyl anion/nitroxyl causes oxidative stress and neurotoxicity towards dopaminergic neurons. Neurotoxicity of Angeli's salt (AS), a known generator of nitroxyl anion, was studied on rat dopaminergic cells in intact animals as well as in primary cultured mesencephalic neurons. The impact of redox environment on prooxidative effects was studied by using *in vitro* test systems.

MATERIALS AND METHODS

In Vivo Experiments

Wistar rats (male 250–350 g, Han/Kuo, Institute of Biomedicine, University of Helsinki) were anesthetized with chloral hydrate (350 mg/kg, i.p.) and prepared for stereotaxic infusion of 1 μl of vehicle (10 mM NaOH) or drug solution (AS 25, 100, 200, 400 nmol, sulphononoate 400 nmol or $NaNO_2$ 400 nmol). Stereotaxic coordinates (A: 3.2 mm, L: 2.1 mm, H: 2.0 mm and mouth bar at −3.5 mm) from interaural line for right substantia nigra compacta area were used as per Paxinos and Watson.[16] After slow infusion of the drug solution the injection needle was kept in place for 5 min. The body temperature of the animals was maintained at approximately 37°C during and after the operation by using a thermoblanket and heating lamp, respectively. After recovery from anesthesia the rats were kept in plastic cages with free access to water and food. Rats were sacrificed by decapitation 2, 7, 8 or 50 days after the drug delivery and striatum and substantia nigra were promptly dissected and frozen in liquid nitrogen. Tissue samples were stored at −80°C. Striatal dopamine and 3,4-dihydroxyphenylacetic acid (DOPAC) were measured using high pressure liquid chromatography with electrochemical detection (HPLC-EC) method as previously described.[17]

For immunohistochemical demonstration of neurofilament protein and tyrosine hydroxylase positive neurons the animals were decapitated and the caudal part of the brain including the injection area was frozen down in dry ice and cut in 10 μm cryostat sections in coronal sections. The sections were fixed in 0.4% *p*-benzoquinone (Fluka, Switzerland) in PBS for 15 min and processed for immunhistochemistry as described in detail previously.[18] A purified rabbit polyclonal antibody against tyrosine hydroxylase (Chemicon International, Temecula, CA) was used at 1:500 dilution for 24 h at +4C followed by the FITC-conjugated anti-rabbit secondary antibody at a 1:40 dilution (Cappel Laboratories, Cochranville, PA) for 1 h at room temperature. The 68 kDa neurofilament protein was detected using mouse monoclonal antibodies against the 68 kDa neurofilament protein (Sigma, St. Louis, MO) at 1:1000 dilution for 24 h at +4C. The immunostained sections were viewed and photographed using an Olympus BH2 microscope equipped with appropriate filters.

In Vitro Cell Culture Experiments

Mixed mesencephalic neuronal/glial cultures were prepared as previously described.[19] Confluent mesencephalic glial monolayers were established from 7 day old rat pups after 14–21 days of culture in 24-well Costar tissue culture plates at 37° in humidified 95% air/5% CO_2. These cultures were essentially devoid of neurons. Fetal mesenchephalic cells from gestational day 16 rats were seeded (100,000 cells/well) onto either the glial monolayers for mixed cell cultures or onto polylysine-coated wells for neuron-enriched cultures, and treated 4–6 days after seeding. The cells were exposed to AS (0–5 mM) for a period of 24 h and capacity for [^{3}H]dopamine uptake was assessed after incubating the cells with 0.2 μCi [^{3}H]dopamine in media

containing 0.1% ascorbate for 4 h. Cells were washed twice with saline and then allowed to equilibrate for 4 h with 0.1 mM dopamine. [^{3}H]Dopamine released into the media was measured by scintillation counting.

Measurement of Hydroxyl Radical Generation *In Vitro*

In order to measure hydroxyl radical generation of AS (0–1000 μM) or sulphononoate (0–400 μM) either of these drugs was incubated (5–60 min) in phosphate buffered saline solution containing sodium salicylate (1 mM) as a hydroxyl radical trapping agent in an atmospheric oxygen tension. Hydroxylation products of salicylate, such as 2,3- and 2,5-dihydroxybenzoic acid were assayed by using HPLC-EC system as previously described.[20]

Assay of Lipid Peroxidation in Brain Homogenates *In Vitro* and in the Substantia Nigra *In Vivo*

Brain homogenates were made from rat brain cortical tissue (50 mg/ml, in phosphate buffered saline solution, pH 7) using an ultrasonic cell disrupter. Homogenates were incubated at 37°C in a shaking water bath for 2 h. Fluorescent products of lipid peroxidation, which are cross-linked aldehyde species with primary amines,[21] were determined according to a microassay procedure by using a Perkin Elmer LS 50B spectrofluorometer (activation/emission wavelengths were 356/426 nm) as previously described.[12,22] As the effect of both 100 μM and 1 mM concentrations of AS on the detection of previously formed products of lipid peroxidation was tested, no interference with the detection method used was observed (data not shown).

The AS induced brain lipid peroxidation was assayed *in vivo* by using a Perkin Elmer LS 50B spectrofluorometer (activation/emission wavelengths were 356/426 nm) as previously described.[23]

Chemicals

Angeli's salt (sodium trioxodinitrate) and sulphononoate (diazenesulphonic acid, hydroxy-, 1-oxide, disodium salt), purchased from Cayman Chemicals (Ann Arbor, MI, USA), were diluted in 10 mM NaOH. The concentrations of stock solutions of Angeli's salt and sulphononoate were verified by absorbances according to the supplier ($A_{237}\varepsilon = 6100\,M^{-1}cm^{-1}$ for AS and $A_{259}\varepsilon = 8500\,M^{-1}cm^{-1}$ for sulphononoate). The other chemicals were obtained from Sigma. For cell culture Dulbecco's modified eagle's medium nutrient mixture F-12 HAM supplied by Sigma was used.

Statistical Analysis

Data are presented as mean ± SEM values of indicated numbers of observations. Results were analyzed by one way analysis of variance and p values were calculated using Neuman-Keuls test (p values less than 0.05 were considered statistically significant).

RESULTS

In Vivo Neurotoxic Effects of Angeli's Salt after Intranigral Infusion

Intranigral infusion of AS (0–400 nmol) caused a dose dependent decrease in dopamine levels in the rat striatum when measured 7 days after the drug delivery (Fig. 1A). The high dose of AS (400 nmol) caused a 79% loss of dopamine in the ipsilateral striatum as compared to the contralateral striatum. Smaller dose of AS (100 nmol) caused a 24% decrease in striatal dopamine levels. The time course of injury was further studied by sacrificing rats at 2, 8 and 50 days after intranigral infusion of smaller doses of AS (25 and 100 nmol). There was a 15% decrease of dopamine levels in striatum when measured 8 days after the infusion of a 100 nmol dose (Fig. 1B). At 50 days after the infusion striatal dopamine levels were further decreased to 56%, demonstrating that AS causes a progressive time dependent injury. The smallest dose of AS (25 nmol) did not cause injury at any time point studied (data not shown). AS (0–100 nmol) did not decrease significantly DOPAC levels (data not shown). However, the 400 nmol dose caused a 30% fall in striatal DOPAC levels, as compared to the control side (data not shown).

As the degradation of AS is known to produce NO_2^- we tested the effect of equimolar doses of $NaNO_2$ and AS on striatal dopamine. To further specify our study of the neurotoxicity of Angeli's salt derived nitroxyl anion we tested also an equimolar dose of sulphononoate, a close structural analog of Angeli's salt. A 400 nmol dose was chosen for all treatments. At 7 days after the drug delivery AS (400 nmol) showed a 85% decrease in striatal dopamine (Fig. 1C). Neither the $NaNO_2$ group nor the sulphononoate group showed decrease of dopamine indicating that nitrite or sulphononoate derived nitrous oxide are not toxic to dopaminergic neurons in our model.

The AS (200 nmol) induced injury was further verified by immunocytochemistry using neuronal markers, such as antibodies against neurofilament proteins (data not shown). We found that numbers of neurofilament positive neurons were clearly reduced on the ipsilateral ventral mesencephalon as compared to the contralateral side. Immunocytochemical demonstration further indicated that the number of

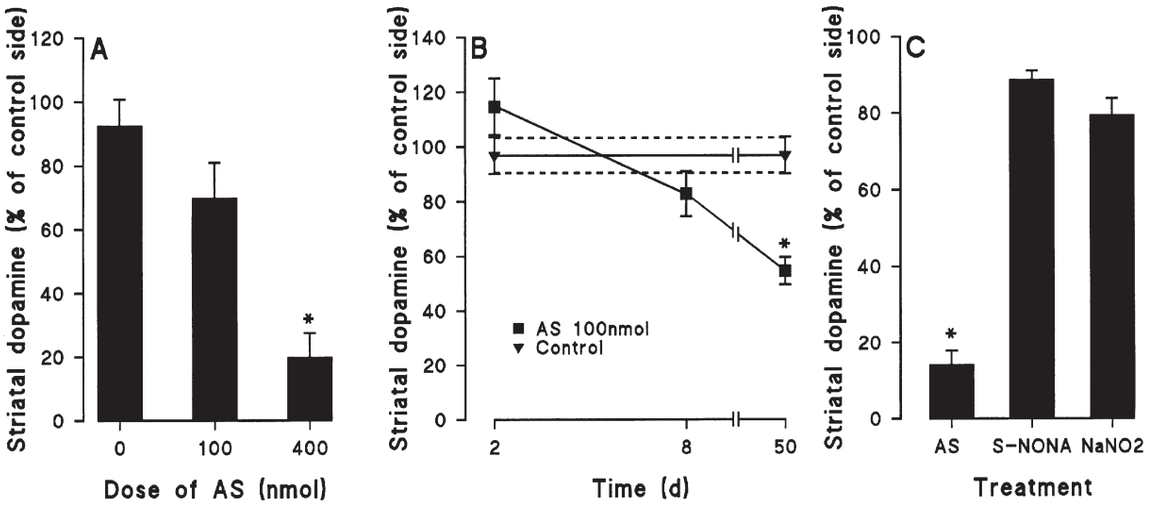

FIGURE 1 The neurotoxic effect of intranigral infusion of Angeli's salt (AS) on striatal dopamine levels *in vivo*. Freshly prepared AS (0–400 nmol in 10 mM NaOH), sulphononoate (S-NONA; 400 nmol in 10 mM NaOH) or sodiumnitrite ($NaNO_2$; 400 nmol) was infused in a volume of 1 μl into the midbrain substantia nigra of anesthetized rats. The animals were sacrificed at indicated time points and the caudate nucleus of both hemispheres were dissected and processed for the measurement of dopamine by using HPLC-EC. (A) The animals were sacrificed 7 days after the infusion of 0–400 nmol dose of AS. Bars show mean + SEM ($n = 4–6$) values of dopamine levels (% of control side) in the nerve terminal area. (B) The animals were sacrificed either 2, 8 or 50 days after the infusion of 100 nmol dose of AS. Points show mean ± SEM ($n = 8$) values of dopamine levels (% of control side). (C) The animals were sacrificed 7 days after the infusion of the drug solution. Bars show mean + SEM ($n = 8$) values of dopamine levels (% of control side). *$p < 0.05$ compared to vehicle (10 mM NaOH) injected rats.

tyrosine hydroxylase immunoreactive neurons was significantly reduced as compared to the contralateral control side (compare Fig. 2A and B). Injection of 10 mM NaOH vehicle to the substantia nigra showed no destruction of the tissue nor difference in the tyrosine hydroxylase positive cells on the injection side compared to the control side (Fig. 2C and D).

When brain lipid peroxidation was measured *in vivo* from tissue samples from the substantia nigra, no effect was found in contrast to our *in vitro* brain lipid peroxidation results. Both 100 and 400 nmol resulted in 1.0 relative fluorescent intensity unit (RFU) production of fluorescent lipid peroxidation products whereas a 10 nmol dose of ferrous citrate caused a production of 11.0 RFUs under testing conditions. On average 0.8 RFUs was measured on the contralateral control side in all experiments.

In Vitro Neurotoxic Effects of Angeli's Salt in Mesencephalic Dopaminergic Cell Cultures

In enriched neuronal cultures treatment with AS (0–1000 μM) caused a concentration dependent decrease of dopamine uptake, which was used as an index of neurotoxicity (Fig. 3A). In mixed glial/neuronal cultures AS (0–5 mM) was also toxic to dopaminergic cells (Fig. 3B). These results suggest that glial cells do not prevent AS induced neurotoxicity *in vitro*. Under these conditions glutathione even in high concentrations (0–20 mM) did not protect neurons against AS induced neurotoxicity (data not shown).

Potential Neurotoxic Mechanism of Angeli's Salt Hydroxyl Radical Generation

Incubation of AS (0–1000 μM) with salicylate (1 mM) caused a time and concentration dependent formation of hydroxyl adducts of salicylate, such as 2,3- and 2,5-dihydroxybenzoic acid (Fig. 4A). The generation rate of 2,3- and 2,5-dihydroxybenzoic acid reached its maximum within 15 min and declined to near baseline at 60 min (data not shown). This relatively short-lasting formation reflects a rapid release of nitroxyl anion and its derived oxidative species from AS. AS mimics the biological effects of nitric oxide, such as vasodilatation,[10] since nitroxyl anion released from AS is bio-transformed to nitric oxide by oxidants such as Cu (II).[15] In the presence of Cu (II) (10 μM) the generation of hydroxyl adducts of salicylate by AS (100 μM) was nearly totally suppressed (Fig. 3B). Also cysteine has been reported to abolish the AS induced vasodilation,[24] possibly through the competing reaction of thiol group with nitroxyl anion.[5,25] In the present study glutathione (100 μM) and L-cysteine (100 μM) suppressed the AS induced hydroxylating species generation (Fig. 4B).

As sulphononoate did not show neurotoxicity *in vivo*, we further compared the production of hydroxyl radical adducts of salicylate in the presence

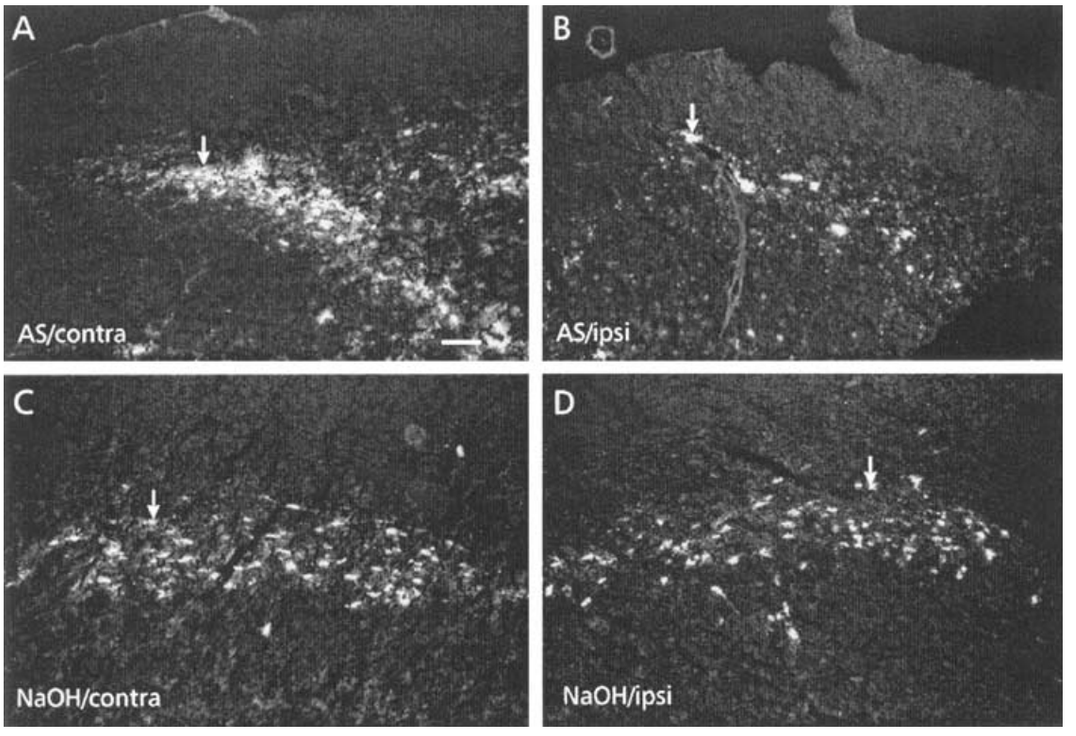

FIGURE 2 Immunocytochemical demonstration of tyrosine hydroxylase immunoreactivity in the substantia nigra injected with 200 nmol dose of Angeli's salt (A, B) or with 10 mM NaOH (1 μl) vehicle (C, D) 18 days after the injections. Scale bar = 50 μm. (A) The control side (AS/contra) of the animal which received AS shows that most of the dopaminergic neurons of the pars compacta of the substantia nigra are intact and show extensive expression of tyrosine hydroxylase (arrow). (B) The injection side (AS/ipsi) of the animal which received AS demonstrates the destructive effect of AS. This section reveals a similar area as in A, but here the numbers of tyrosine hydroxylase positive neurons (arrow) are markedly reduced. Thus, AS not only destroys cells directly at the site of the injection but also affects the dopaminergic neurons close to the injection site. These effects occur even if the animals showed only a 35% loss of striatal dopamine compared to the control side measured by HPLC-EC. Note that part of the substantia nigra was totally destroyed as shown in the lower right corner of this section. (C) The control side (NaOH/contra) of the vehicle (10 mM NaOH) injected rat shows large numbers of tyrosine hydroxylase positive neurons in the pars compacta of the substantia nigra (arrow). (D) A similar view of the vehicle injected side (NaOH/ipsi) confirms that the vehicle itself does not cause an injury. Thus, large numbers of tyrosine hydroxylase positive neurons (arrow) can be seen.

of either AS or sulphononoate. In this experiment AS (0–400 μM) showed a concentration dependent generation of hydroxylation adducts of salicylate up to 11.22 ± 0.59 nmol/ml ($p < 0.01$, $n = 3$) during 60 min incubation. However, even the highest concentration of sulphononoate (400 μM) generated only minute quantities of hydroxyl radical adducts of salicylate (0.034 ± 0.019 nmol/ml, n = 3).

Brain Lipid Peroxidation

We further studied the effects of AS on tissue disruption induced lipid peroxidation. This method is considered a sensitive method to study both pro-oxidant and antioxidant properties of compounds in brain homogenates.[12,26,27] AS caused a biphasic concentration dependent response on the peroxidation of brain lipids (Fig. 5A). Tissue disruption induced brain lipid peroxidation was constantly increased on concentrations below 100 μM, while suppressed at a 1000 μM concentration. This biphasic effect could be due to the conversion of nitroxyl anion to nitric oxide in brain homogenates, the nitric oxide production being significant with high concentrations of AS under conditions present in this experiment. Therefore the effect of AS was studied in the presence of Cu (II), which is known to enhance the conversion of nitroxyl anion to nitric oxide.[15] Cu(II) (100 μM) alone increased lipid peroxidation from 0.708 ± 0.105 RFU without copper to 1.295 ± 0.138 RFU in the presence of copper. In the presence of 100 μM Cu(II), the pro-oxidant effects of AS completely disappeared. Also in the presence of 100 μM Cu(II) the antioxidant properties of AS were uncovered even with low concentrations of AS and were further pronounced with higher concentrations (Fig. 5A) reflecting the previously shown antioxidant properties of nitric oxide.[22]

Effect of Thiols on AS Mediated Brain Lipid Peroxidation

Based on the reactivity of nitroxyl anion with thiol group of cysteine and GSH,[5,13,25] the effects of AS

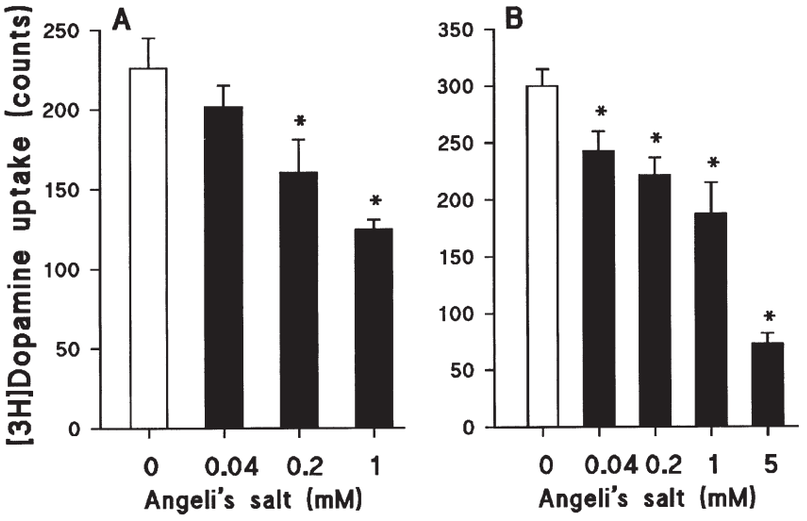

FIGURE 3 Effect of Angeli's salt (AS) in mixed primary mesencephalic neuronal cultures. E16 neurons were plated on polylysine-coated wells or on confluent P7 glia. AS was applied to cell culture and 24 h later cells were suspended and assayed for [^{3}H]dopamine uptake. The dopamine uptake was used as an index of cell viability. (A) The effect of AS (0–1 mM) on dopamine uptake in primary neuronal cultures of mesencephalic dopamine neurons ($n = 4$). (B) The effect of AS (0–5 mM) on dopamine uptake in mixed glial/neuronal cultures ($n = 6$–7). The mean + SEM [^{3}H]dopamine uptake is shown. *$p < 0.05$ for decrease in [^{3}H]dopamine uptake compared to control.

were further evaluated in the presence of thiols. Without the addition of AS, 1 mM GSH slightly inhibited the tissue disruption-induced lipid peroxidation and totally abolished the pro-oxidative effect of AS (10 μM) in agreement with the recent report that AS mediated two-electron oxidation is nearly stoichiometrically inhibited by GSH.[13] In the presence of 100 μM AS, GSH (1 mM) was a more potent antioxidant than in the absence of it. L-Cysteine (1 mM) alone acted as a pro-oxidant but it abolished the pro-oxidant effect of Angeli's salt (10 μM) in brain homogenate (Fig. 5B).

DISCUSSION

To the best of our knowledge these results provided the first *in vivo* evidence that AS or nitroxyl/nitroxyl

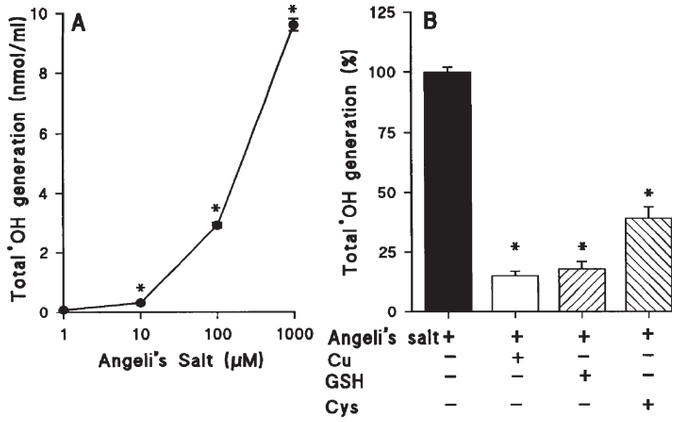

FIGURE 4 (A) Concentration dependent generation of hydroxyl radicals by Angeli's salt (AS). AS (1–1000 μM) induced hydroxyl radical formation was measured after 60 min incubation. Nitroxyl anion (NO^-) generating Angeli's salt was incubated in phosphate buffered saline solution with salicylate (1 mM), a hydroxyl radical trapping agent. The results show mean ± SEM ($n = 3$) values of the total amount of hydroxyl radical adducts of salicylate, such as 2,3- and 2,5-dihydroxybenzoic acid, which were accumulated during incubation time and measured by HPLC-EC procedure. *$p < 0.05$ compared to control. (B) The effect of copper (Cu^{2+}), glutathione (GSH) and L-cysteine (Cys) on AS induced hydroxyl radical generation. AS (100 μM) was incubated with or without Cu^{2+} (10 μM), GSH (100 μM) or Cys (100 μM) in phosphate buffered saline containing salicylate (1 mM) for 60 min. Mean + SEM ($n = 3$–4) values of total amount of 2,3- and 2,5-dihydroxybenzoic acids, the hydroxyl adducts of salicylate, measured by using HPLC-EC procedure are shown. *$p < 0.05$ compared to AS.

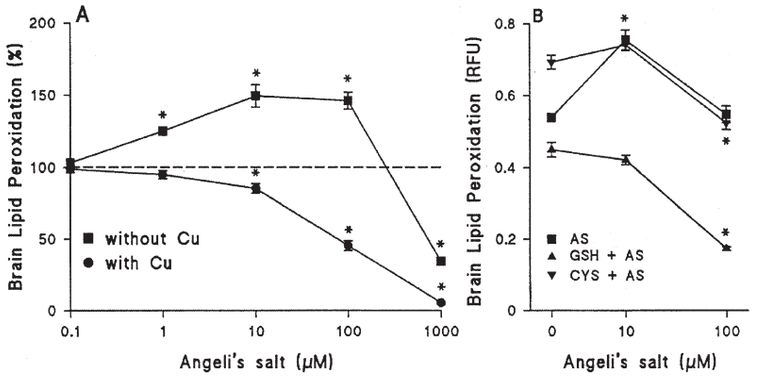

FIGURE 5 (A) The effect of Angeli's salt (AS) on the lipid peroxidation in brain homogenates. AS (0.1–1000 μM) was added to brain homogenates (50 mg/ml) and incubated with or without copper (Cu^{2+} 100 μM) for 2 h at 37°C. The accumulation of fluorescent products of lipid peroxidation, assayed by spectrofluorometer was used as the marker of oxidation of brain lipids. The control group fluorescent products of lipid peroxidation increased from 0.153 ± 0.012 relative fluorescent intensity units (RFU) to 0.708 ± 0.105 RFU without copper and to 1.295 ± 0.138 RFU with copper (100 μM) during 2 h incubation. The results show mean ± SEM percentage of lipid peroxidation compared to the corresponding control group (100%) ($n = 3$). (B) The effect of glutathione (GSH) and L-cysteine (Cys) on the pro-oxidant effects of AS in brain homogenates. AS (10 or 100 μM) was added to brain homogenates (50 mg/ml) and incubated without or with GSH (1000 μM) or Cys (1000 μM) for 2 h at 37°C. The accumulation of fluorescent products of lipid peroxidation, assayed by spectrofluorometer was used to mark oxidation of brain lipids. The mean ± SEM of relative fluorescent intensity units (RFU) is shown ($n = 3$). All experiments were carried out in PBS containing 1 mM NaOH. *$p < 0.05$ compared to corresponding control.

anion could cause neurotoxicity to dopaminergic neurons. The AS induced injury in substantia nigra was reflected as a delayed and progressive decrease of striatal dopamine. Dopaminergic cell death was further confirmed by a decrease of tyrosine hydroxylase positive neurons in the substantia nigra. The neurotoxicity was also observed in primary mesencephalic neuronal cultures. Results from cell free systems *in vitro* support the hypothesis that AS or nitroxyl/nitroxyl anion generated oxidative species may react with thiols leading to protein modification and neurotoxicity.

To study the neurotoxicity of AS we employed a stereotaxic infusion of the compound into the rat substantia nigra. This method has been previously used to demonstrate the neurotoxicity of other compounds such as MPP^+ and ferrous citrate.[28,29] The neurotoxic effect was noted with a 400 nmol dose as early as 7 days after the infusion of the drug whereas the toxicity of a 100 nmol dose was further augmented between 8 and 50 days after the drug delivery. The long-lasting decrease of dopamine levels suggests that AS causes neuronal death rather than temporary dysfunction of neurons. Histological evaluation confirmed that AS caused marked cell death close to the injection site where the concentration of AS is highest. This rapid AS induced cytotoxicity in cell body region leads to destruction of nerve terminals and subsequent decrease of dopamine levels in striatum during few days. Concentration gradient of AS will decrease very fast due to its very short halflife when moving away from the injection site. However, even 1 mm away from the injection site there was decreased amounts of tyrosine hydroxylase positive neurons. Therefore, we propose that the lower concentrations of AS may cause in these areas partial dysfunction or initiate a reaction cascade which leads to delayed neuronal death as was shown with 100 nmol dose in the present study.

The toxicity is affiliated with reactive nitroxyl anion or its consecutive reaction product, since the same dose of nitrite ion, one of the final breakdown products of AS, did not cause injury. Sulphononate, a compound with structure that resembles that of AS, has been previously used as a control substance for NO donating NONOate type drugs. This compound releases nitrous oxide which is also one of the final degradation products of AS derived nitroxyl. The relatively high local concentration of nitroxyl anion present immediately after the stereotaxic delivery of AS into the substantia nigra could potentially result in local nitrous oxide production and physical damage to the cells. However, sulphononoate does not produce damage even on a concentration which releases theoretically double concentration of nitrous oxide as compared to the dose of AS used in our experiment. Also in *in vitro* experiments sulphononoate does not generate hydroxylating species. Therefore, we conclude that the AS mediated toxicity is not caused by nitrous oxide and that sulphononoate might release its final breakdown product nitrous oxide via a reaction pathway not involving nitroxyl anion in a radical generating form. These results support the hypothesis that AS induced neurotoxicity is related to the generation of oxidative species.

Recent studies on the chemistry of AS derived oxidants have concluded that nitroxyl anion derived oxidative reactions are augmented by the presence of

oxygen.[13] Also AS has been shown to potentially consume equimolar quantity of oxygen under *in vitro* conditions.[13] Based on these reports AS could cause temporary depletion of oxygen and hypoxia induced cell death in the proximity of the injection site. However previous reports on fibroblast cultures under hypoxic vs. aerobic conditions actually confirm that hypoxic cells are significantly less vulnerable to AS mediated stress.[11] The outcome of AS mediated oxidative reactions is indeed dependent on the local concentration of oxygen and the effect of oxygen tension at the injection site can therefore not be neglected. However, local depletion of oxygen at the injection site can be expected to result in immediate diffusion of oxygen into the injection site from the surrounding normoxic tissue. Therefore, we hypothesize that the neurotoxicity observed in our experiments is not due to hypoxemia but rather the result of pro-oxidant stress mediated by the presence of oxygen and AS derived reactive species.

Another possible mechanism of neurotoxicity could be nitroxyl anion induced oxidative stress mediated damage to cellular lipid components. However, AS did not increase the formation of fluorecent products of lipid peroxidation in the substantia nigra, suggesting that AS mediated neurotoxicity is not caused by oxidation of brain lipid membranes. AS derived oxidants have a high reactivity with thiols.[5,8,13] Therefore, we propose that AS induced thiol depletion or protein oxidation may be a more important mechanism than lipid peroxidation in mediating AS or nitroxyl anion induced neurotoxicity.

Our *in vitro* dopaminergic neuron studies clearly confirm the neurotoxic effect of AS (Fig. 3A and B). Our results are in good agreement with previous reports on the cytotoxicity of Angeli's salt towards fibroblasts.[11] The presence of glial cells did not protect against neurotoxicity. Intracellular GSH has been shown to protect against cytotoxicity of AS,[11] however, in our experiment GSH even with high 20 mM concentrations did not protect against AS induced neurotoxicity. Because GSH does not diffuse easily through cell membranes[30] this result may reflect that AS or AS derived reactive product penetrates to cells where it causes oxidative stress. This is also supported by the finding that AS decreases intracellular GSH levels in lung fibroblast,[11] thus potentially exposing cells to oxidative stress.

The present results confirm that AS generates radicals which efficiently hydroxylate aromatic compounds such as salicylate and benzoic acid as recently shown.[13] AS induced hydroxyl radical generation was blocked by copper(II) which has been shown to convert nitroxyl anion to nitric oxide.[15] Therefore, nitroxyl anion rather than nitric oxide leads to radical generation and oxidative stress. Thiols, especially glutathione, efficiently abolished AS induced radical generation in cell free system *in vitro*, supporting previous findings that endogenous thiols protect against AS induced oxidative stress and cytotoxicity.[11]

The pro-oxidant properties of AS were further assayed using brain tissue homogenates. AS had a small but significant effect on lipid peroxidation even with the 1 μM concentration in the present study. AS has a biphasic effect on the peroxidation of brain lipids. The increased formation of fluorescent products of lipid peroxidation with low concentrations of AS is in line with the shown generation of hydroxyl radicals. However, higher doses inhibit, lipid peroxidation. Nitroxyl anion may be converted to nitric oxide in biological systems by for example copper ions[15] or copper containing SOD enzyme.[6] Therefore, the antioxidant effect of AS may result from oxidation of nitroxyl anion to nitric oxide which has been shown to block tissue disruption-induced brain lipid peroxidation by micromolar concentrations.[22] GSH abolished the pro-oxidant effect of AS further emphasizing the role of thiols as an important defence mechanism against nitroxyl anion mediated toxicity.[11,13]

This is the first time it has been shown that AS or nitroxyl anion is toxic in the rat brain to the best of our knowledge. This toxicity was noted on only 25 times higher concentration compared to equipotent dose of dopaminergic neurotoxin MPP^+.[28] Taking into consideration the extremely short halflife of AS under *in vivo* conditions we believe that these results provide a new and important piece of information on the biochemical properties of AS derived nitroxyl. Thiol levels as well as the redox environment affected the pro-oxidative effects of AS, suggesting the importance of GSH when nitroxyl anion formation is increased. We hypothesize that AS may decrease GSH levels and oxidize critical thiols in proteins, which might lead to demonstrated neurotoxicity.

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