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Angeli's Salt and Spinal Motor Neuron Injury

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Nitroxyl anion or its conjugate acid (NO^-/HNO) and nitric oxide (NO) may both have pro-oxidative and cytotoxic properties. Superoxide dismutase (SOD) enzyme has been shown to convert reversibly HNO to NO. Mutations found in the SOD enzyme in some familial amyotrophic lateral sclerosis (ALS) patients affect redox properties of the SOD enzyme in a manner, which may affect the equilibrium between NO and HNO. Therefore, we studied the effects of HNO releasing compound, Angeli's salt (AS), on both motor and sensory functions after intrathecal administration in the lumbar spinal cord of a male rat. These functions were measured by rotarod, spontaneous activity, paw- and tail-flick tests. In addition, we compared the effect of AS to NO releasing papanonoate, old AS solution and sulphononoate in the motor performance test. The effect of intrathecal delivery of AS on the markers of the spinal cord injury and oxidative/nitrosative stress were further studied.

Results: Freshly prepared AS (5 or 10 μmol), but not papanonoate, caused a marked decrease in the rotarod performance 3–7 days after the intrathecal administration. The peak motor deficiency was noted 3 days after AS (5 μmol) delivery. Old, degraded, AS solution and nitrous oxide releasing sulphononoate did not decrease motor performance in the rotarod test. AS did not affect the sensory stimulus evoked responses as measured by the paw-flick and tail-flick tests. Immunohistological examination revealed that AS caused injury related changes in the expression of glial fibrillary acidic protein (GFAP), fibroblast growth factor (FGF-2) and laminins in the spinal cord. Moreover, AS increased nitrotyrosine immunoreactivity in the spinal motor neurons.

Therefore, we conclude that AS, but not NO releasing papanonoate, causes motor neuron injury but does not affect the function of sensory nerves in behavioural tests.

Keywords: Amyotrophic lateral sclerosis; Angeli's salt; HNO; Nitric oxide; Nitroxyl; Oxidative stress

INTRODUCTION

The role of nitric oxide (NO) as a neural transmitter has been well established.[1] In addition to the known transmitter function, NO has been suggested to have both cytoprotective and cytotoxic properties, which have been linked to antioxidant and pro-oxidative properties, respectively.[2–4] Excess production of NO has been proposed to lead in the production of peroxynitrite type oxidative species in the presence of superoxide.[5] Although NO has been linked with production of potentially neurotoxic species, several studies have concluded that NO can have neuroprotective properties under certain conditions.[6–8] The exact role of NO in neurodegeneration or neurotoxicity remains to be clarified.

Recently another reactive nitrogen species, nitroxyl anion or its conjugate acid (NO^-/HNO), has been proposed to participate in the reactive nitrogen species mediated oxidative reactions.[9–11] There are also some studies suggesting that HNO derived from Angeli's salt (AS) is cytotoxic[10–12] as well as neurotoxic.[13] HNO is a one electron reduction product of NO. Several authors have reported of possible reactions leading to formation of HNO under conditions available also *in vivo*. Recent literature suggests that the most likely source of HNO *in vivo* is via direct formation of HNO by NO synthase (NOS)[14–16] or reaction of S-nitrosothiols with excess thiols.[17] Also Cu/Zn containing superoxide dismutase (SOD) mediated reversible conversion of NO into NO^-/HNO has been suggested.[18] Based on the recent data, the reduction of free NO to NO^- is not likely to take place under physiological conditions.[19]

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However, binding of NO at the active site of the SOD enzyme has been suggested by Liochev and Fridovich to change the properties of this reaction leading into production of bound nitroxyl followed by reaction with oxygen to yield peroxynitrite.[20]

Oxidative species mediated neurodegeneration has been proposed to participate in the pathogenesis of several neurodegenerative diseases such as Parkinson's disease, Alzheimer's disease and amyotrophic lateral sclerosis (ALS).[21] ALS is a neurodegenerative disease leading into selective motor neuron death affecting both upper and lower motor neurons. As with most neurodegenerative diseases its exact pathophysiology remains unknown. However, the familial form of the disease has been linked with a gain of function mutation of the Cu/Zn SOD enzyme,[22,23] possibly resulting in an increased reduction tendency of the enzyme's Cu-ion.[24] Because SOD enzyme has been shown to reversibly convert nitroxyl to NO under experimental *in vitro* conditions,[18] the Cu/Zn SOD harbouring reduced Cu-ion could potentially decrease the conversion of HNO to NO and increase formation of HNO shifting the equilibrium between HNO and NO as also suggested recently by Liochev and Fridovich.[25]

The neurotoxicity of either HNO or NO has not been previously studied at the spinal level *in vivo* in rat. Therefore, we tested whether intrathecal delivery of a bolus dose of AS, a HNO releasing compound or papanonoate, a NO releasing compound into the rat lumbar spinal cord could lead into symptoms of motor neuron toxicity as measured by decreased performance on a rotarod test and reduction of spontaneous movement. The effects of some other possible HNO derived products were also tested. Effects of AS on sensitivity to painful stimulus were tested with paw-flick and tail-flick tests. We further studied the effects of intrathecal delivery of AS on molecular markers of spinal cord injury as well as on markers of oxidative/nitrosative stress such as protein carbonyl and nitrotyrosine formation.

MATERIALS AND METHODS

Intrathecal Catheterization and Intrathecal Drug Delivery

Male Hannover Wistar rats (Harlan, Neatherlands) were housed in 12 h/12 h light–dark cycle and received food and water *ad libitum*. Rats weighting approximately 250 g were anesthetized by subcutaneous injection of fentanyl (0.315 mg/kg), fluanisone (10 mg/kg) and midazolam (5 mg/kg) combination for chronic intrathecal catheterization. A thin (outer dimension approximately 0.25 mm) polyethylene cannula (PE-10; Meadox surgimed A/S, Stenløse, Denmark) was inserted through an incision made into the cisterna magna.[26] Cannula was inserted 5–8 cm into the spinal subarachnoid space while care was taken not to harm the spinal cord during the insertion procedure. The length of the cannula was adjusted to reach the cranial end of the lumbar enlargement. Cannula was fixed by non-resorbable suture into superficial neck muscles through a previously made knot in the cannula. One week after the surgery the animals were injected intrathecally 5 μl of hyperbaric lidocaine (Lidocain Pond, Medipolar, Oulu, Finland) and 10 μl of saline was used to flush the cannula (approximately volume 7.5 μl). Those rats used for the histological or biochemical analysis were not given lidocaine and the location of the cannula was verified at autopsy. Only rats, which showed temporary symmetrical paralysis of the hind limbs after the lidocain delivery were admitted for further testing. Animals were given a 7 days rest period after the lidocaine test before any drug treatment. All animals were adapted for testing situation for 10 min on at least two consecutive days to minimize the effect of stress on measurements.

All animal experiments were approved by the appropriate local institutional and governmental authorities.

Rotarod Performance

Motor performance was tested on a fixed speed rotarod machine (Palmer electric recording drum; diameter 80 mm, speed 10 RPM). All rats used in this study were trained to walk for 120 s on the drum prior to testing. The rotarod test was performed five times and the longest performance time was recorded (cut-off 120 s).

Spontaneous Activity

Spontaneous movement was recorded in a $70 \times 70 \times 35\,cm^3$ dark, noise insulated box equipped with light beam sensors (Kungsbacka Mät- & Regelteknik AB, Kungsbacka, Sweden).[27] Total activity consisting of horizontal activity over a period of 20 min was recorded.

Paw-flick

Changes in nociception were assessed by paw-flick test (Ugo Basile, Comerio, Italy; heat intensity set at 40 arbitrary units (scale 0–90), cut-off time 11 s).[28] The measurement was conducted on both hind paws three times and a mean of latency times was recorded.

Tail-flick

Tail-flick test (Ugo Basile, Comerio, Italy, cut-off time 8 s) was used to test the response to noxious stimulus

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in the tail while the animal was restrained in a transparent plexiglass tube. A mean of three measurements was recorded.

Histological Analysis

For biochemical and histological analysis of tissue samples the tested drugs were injected 2 days after the cannulation and tissue samples were taken 7 days after the drug delivery to minimize the time after cannulation as the intrathecal cannulation process itself produces marked tissue reaction.[29] The animals were killed by decapitation and lumbar spinal cord was promptly dissected and frozen on dry ice. For immunohistochemical analysis the tissue sample was cut in 10 μm cryostat sections few millimetres caudal from the tip of the intrathecal catheter and processed for immunohistochemical analysis as previously described.[30] Briefly, the sections were fixed in 0.4% benzoquinone (Fluka, Switzerland) and dehydrated prior to incubation with normal goat serum (1:30) overnight at + 4°C. Primary antibodies were rabbit polyclonal anti-laminin-1 antiserum (1:2000 in 1:30 NGS), anti-γ1-laminin (1:2000), anti-basic fibroblast growth factor-2 (FGF-2; 1:2000; Sigma, St Louis, MO), anti-nitrotyrosine (2.5 μg/ml; Upstate Biotechnology, Lake Placid, NY, USA) or mouse monoclonal anti-glial fibrillary acidic protein (GFAP, Sigma, 1:5000 in 1:30 NGS). The specificities of all antibodies have been confirmed,[30,31] and they were applied at + 4°C overnight followed by incubation with polyclonal anti-rabbit IgG-FITC or polyclonal anti-mouse IgG-TRIZ (Chappel Laboratories, Cochranville, PA) conjugate for 1 h at room temperature. The sections were viewed and photographed under Olympus microscope equipped with appropriate filter combinations.

Biochemical Analysis

Measurement of Protein Carbonyl Groups

The presence of carbonyl groups in protein extracts from the spinal cord was assayed by sacrificing animals which had received a test substance (HEPES buffer, AS (1 or 5 μmol)), sulphononoate (3 μmol) or papanonoate (5 μmol) intrathecally either 3 days or 1 week before sacrifice as described above. The animals were sacrificed by decapitation and an approximately 80 mg sample of the spinal cord was promptly dissected and frozen on liquid nitrogen. The tissue samples were stored at − 80°C until analysis.

The tissue sample was homogenized by sonication into 10-fold volume of homogenization buffer (5% SDS, 1% Triton-X, 320 mM sucrose, 0.01% BHT in 10 mM Tris-HCl pH 7.2) supplemented with a protease inhibitor cocktail (Complete Protease Inhibitor, Roche, Mannheim, Germany; used according to manufacturers instructions). Debris was pelleted by centrifugation (1500 *g*, 15 min) and the supernatant was stored at − 80°C.

Due to limited quantity of the available sample we used an ELISA method based on that developed by Buss *et al.*[32,33] with some modifications. Briefly, the biotin conjugated anti-DNP antibody (Molecular Probes Inc, Eugene, OR) was used at 1:5000 dilution and the streptavidin–biotin linked horseradish peroxidase (Amersham International, Buckinghamshire, UK) was used at a 1:3000 dilution. The BSA solutions used for blocking and standards were prepared as previously described[33] and the protein concentration of the standards was determined spectrophotometrically from A280 and referenced to a BSA standard curve.

A 30 μl aliquote of the tissue homogenate was treated with 1% (final concentration) streptomycin sulphate (Hoechst AG, Frankfurt, GmbH; a generous gift of professor T. Haltia) for 10 min in the room temperature followed by centrifugation at 11,000*g* for 10 min to pellet the insoluble debris and the nucleic acids in the sample. Thereafter, the proteins in the supernatant were precipitated with 10% TCA for 10 min in the room temperature and the precipitate was pelleted by centrifugation at 11,000*g* for 10 min followed by washes with 10% TCA and ethanol/ethylacetate (1:1), respectively. The final pellet was dissolved in 6 M guanidine HCL (in 20 mM potassium phosphate buffer, pH 2.3) and the protein quantity was determined by the BCA method (Sigma) using interference corrected BSA solution as the standard and adjusted to 2 mg/ml. Following derivatization with three volumes of DNPH solution as previously described,[32] a 10 μl aliquote (containing 5 μg of protein) of the derivatized sample solution was diluted in 1 ml of phosphate buffered saline (150 mM, pH 7.4) and coated onto ELISA plates (Nunc Immunoplate Maxisorp, Nunc, Denmark) with the rest of the assay performed as previously described.[32] This variation of the previously documented ELISA assay gave excellent results with a linear standard plot ($R^2 = 0.997$) from 0 to 8 nmol of carbonyls/mg of protein. The intra-assay coefficient of variation was 10.7% for eight samples containing on average 0.98 nmol of carbonyls/mg of protein.

Chemicals

AS (sodium trioxodinitrate; CAS 13826-64-7), sulphononoate (diazenesulphonic acid, hydroxy-, l-oxide, disodium salt; CAS 61142-90-3) and

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papanonoate (1-propanamine, 3-(2-hydroxy-2-nitroso-1-propylhydrazino)-propylamine; CAS 146672-58-4) were purchased from Cayman Chemicals, Ann Arbour, MI, USA. For anesthesia a combination of fentanyl-fluanisone and midazolam was used (Hypnorm (1.0 ml/kg), Janssen Pharmaceutica, Beerse Belgium; Dormicum (5.0 mg/kg), Roche, Basle, Switzerland). All other chemicals were obtained from Sigma and were of highest quality available.

AS is a chemical commonly used to study the effects of HNO. Its structure closely resembles those of sulphononoate and papanonoate but on degradation AS releases one molecule of HNO and one nitrite ion, whereas papanonoate degrades into two molecules of NO and a polyamine backbone and sulphononoate dissociates into nitrous oxide and sulfate ion.[34] As HNO derived from AS can dimerize into nitrous oxide and the exact mechanism of nitrous oxide release from sulphononoate is not known it could be possible that an intermediate released by sulphononoate would be HNO. Should this be the case one mole of sulphononoate would be expected to release two moles of HNO. We have thus compared the effects of equimolar doses of hypothetical degradation intermediates HNO and NO, respectively. For AS a 1 mol/l HEPES buffer (pH 6.1) was used as a diluent because of the extreme irritation caused by intrathecal delivery of AS in 10 mM NaOH, possibly caused by the alkaline nature of the solution. The pH of the AS solution in 1 M HEPES was measured to be 9.1 and as AS is not reported to be stable under such conditions we dissolved a fresh portion of AS into ice-cold 1 M HEPES immediately prior to infusion into the intrathecal space. The half-life of AS in this solution was measured by UV-spectroscopy (according to supplier A_{237} 6100/M/cm, the presence of HEPES did not change the basal spectrum upon 1:5000 dilution into 20 mM NaOH) to be 24 min at 0°C and 9.5 min at 37°C. Both sulphononoate and papanonoate were diluted in 10 mM NaOH. MK-801 was diluted in neutral saline (100 nmol/10 μl) and infused slowly intrathecally 10 min prior to AS treatment.

Statistical Analysis

For paw-flick and tail-flick measurements data are presented as percentage of baseline to eliminate the variation of baseline values. Data are presented as mean ± SEM for indicated number of observations and p values were calculated using Neuman–Keuls test. Values of p less than 0.05 were considered statistically significant.

RESULTS

Behavioural Effects of AS

Intrathecal delivery of AS (10 μmol) caused an extensive fall in the rotarod performance when measured 7 days after the delivery (Fig. 1). There was no significant effect on rotarod performance when measured at 2 and 24 h after the drug administration with any of the tested doses. With the highest 10 μmol dose there was a progressive deterioration of motor functions up to 1 week leading to total paralysis of hind limbs in two and with nearly total paralysis in the other two of the four rat group. Due to the ethical reasons those animals having extensive paralysis were sacrificed. The rest of the animals were followed 1 week more and motor performance was recovered during that time (data not shown).

Intrathecal delivery of AS causes a decrease of spontaneous locomotor activity reaching the statistically significant effect with the highest dose of 10 μmol when measured 24 h and 7 days after drug delivery (Table I).

The effect of AS on motor and sensory functions was further compared. Because the marked motor paralysis with the highest 10 μmol dose might affect evaluation of sensory stimulus evoked responses we selected a 5 μmol dose for this study. In line with the first dose-response study the 5 μmol dose did not affect acutely the rotarod performance. However, 3 days after the administration the rotarod performance was significantly reduced (Fig. 2A). The motor performance recovered during the next few days. The effect of AS on sensory functions was measured by using paw-flick and tail-flick latencies. AS did not affect the paw-flick

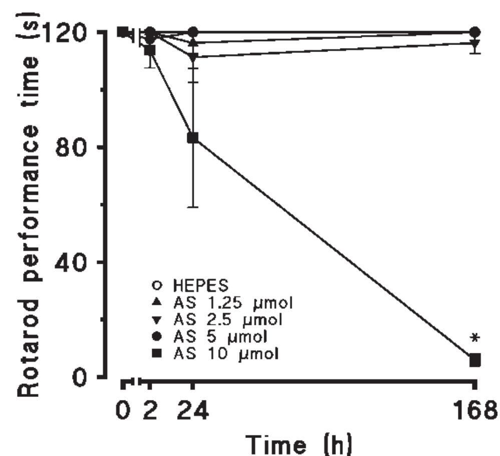

FIGURE 1 The effect of various doses of Angeli's salt (AS) on rat rotarod performance at different time points. AS (0–10 μmol) was delivered into the lumbar intrathecal space and rotarod performance was measured at shown time points after the treatment. AS was dissolved immediately prior to delivery in 1 M HEPES buffer (pH 6.1), which was also used as a control solution. Mean ± SEM values of 4 to 5 rats are shown. *$p < 0.05$ compared to 1 M HEPES-treated control group.

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TABLE I The effect of different doses of Angeli's salt (AS) on the spontaneous locomotor activity when measured 24 h and 7 days after intrathecal treatment (mean ± SEM)

AS dose (μmol)	24 h	7 days
0	1223 ± 87	1126 ± 213
1.25	889 ± 116	1194 ± 179
2.5	820 ± 112	1346 ± 109
5	713 ± 39	881 ± 86
10	307 ± 81*	441 ± 132*

One and seven days after intrathecal delivery of AS (1.25–10 μmol in 1 M HEPES buffer; pH 6.1) or vehicle rats were placed in dark boxes and spontaneous locomotor activity was recorded. Mean ± SEM values of total locomotor activity counts during 20 min period are shown. $n = 8$, *$p < 0.05$ compared to control.

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(Fig. 2B) or tail-flick (data not shown) latencies at any measured time point up to 10 days.

Behavioural Effects of other "NONOates" and Breakdown Products of AS

To determine the role of AS derived oxidants on the fall of rotarod performance, the effect of AS (10 μmol) was compared with sulphononoate (5 μmol) and papanonoate (5 μmol), in equimolar doses in respect to their potential degradation products nitrous oxide and NO, respectively. In addition the effect of 96 h old AS solution (10 μmol), which contains the stable breakdown products of AS, namely sodium nitrite, was also tested. AS caused a fall in the rotarod performance both at 24 h (data not shown) and 7 days after the administration (Fig. 3). Sulphononoate, papanonoate and the old AS solution did not affect the rotarod performance at any studied time point. Only fresh AS solution had effect on spontaneous activity when measured 24 h after the drug delivery (Table II).

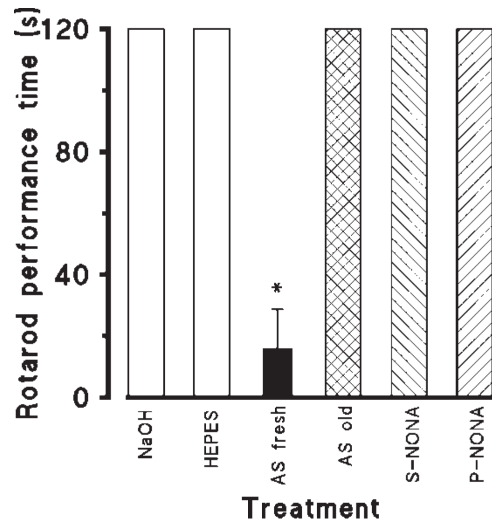

FIGURE 3 Comparison of the effects of fresh Angeli's salt (AS), breakdown products of AS, sulphononoate (S-NONA) and papanonoate (P-NONA) on rat motor performance. The effects of fresh AS salt solution (10 μmol in 1 M HEPES buffer; pH 6.1), nitroxyl exhausted old AS salt solution (10 μmol in 1 M HEPES; 96 h old), sulphononoate (5 μmol in 10 mM NaOH) and papanonoate (5 μmol in 10 mM NaOH) on the rotarod performance were measured at 7 days after the intrathecal drug delivery into the rat lumbar space. The rotarod performance was measured 5 times from each rat and best result was recorded. Cut-off time was 120 s. Mean ± SEM values of 4 rats are shown. *$p < 0.05$ for the fresh AS group compared to all other groups.

Effects of Repeated AS Treatment

The effect of 5 days repeated administration of a 2 μmol dose of AS was further tested on motor and sensory functions. On the 3rd day, 24 h after the second dose of AS, a significant fall in rotarod performance was seen (Fig. 4A). This decline of rotarod performance was maintained at a similar level during the course of AS administration. This effect was reversible and 7 days after the last dose of AS there was no difference between

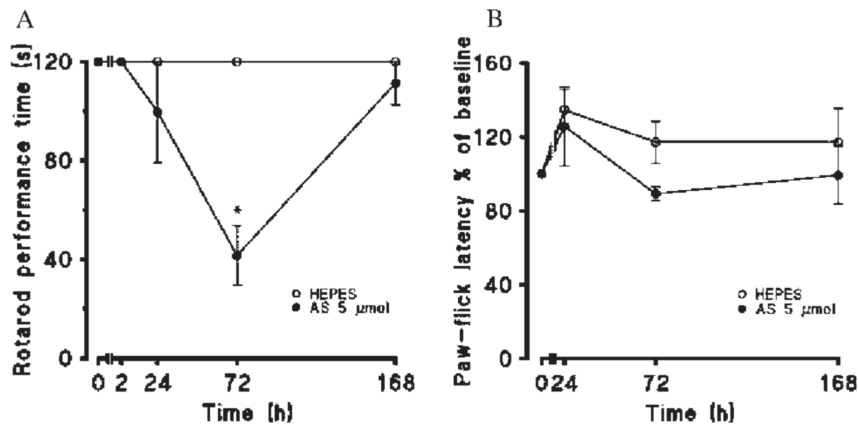

FIGURE 2 The behavioural effects of a single 5 μmol intrathecal dose of Angeli's salt (AS) on rat motor and sensory functions at various time points after treatment. (A) The effects of AS (•) or HEPES control solution (pH 6.1) (□) on motor functions were measured by using rotarod test. The rotarod performance was measured five times from each rat and best result was recorded. Cut-off time was 120 s. (B) The effects of AS (•) or HEPES control solution (□) on paw-flick latency time were measured. The paw-flick latency time expressed as a percent of baseline value. Mean ± SEM values of four rats are shown. *$p < 0.05$ compared to respective control group.

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TABLE II Comparison of the locomotor activity after intrathecal delivery of fresh Angeli's salt (AS), breakdown products of AS, sulphononoate and papanonoate in adult rat (mean ± SEM)

Treatment	24 h
NaOH	1323 ± 108
HEPES	1526 ± 152
AS fresh (10 μmol)	616 ± 166*
AS exhausted (10 μmol)	1499 ± 113
Sulphononoate (5 μmol)	1441 ± 152
Papanonoate (5 μmol)	1033 ± 101

One day after intrathecal delivery of fresh AS (10 μmol in 1 M HEPES; pH 6.1), 96 h old HNO exhausted old AS solution (in 1 M HEPES), HEPES buffer, sulphononoate (5 μmol) and papanonoate (5 μmol) rats were placed in dark boxes and spontaneous locomotor activity was recorded. Sulphononoate and papanonoate, which release nitrous oxide and NO, respectively, were given in 10 mM NaOH. Mean + SEM values of total locomotor activity counts for four animals during a 20 min observation period is shown. *$p < 0.05$ AS compared to all other treatment groups.

treatment and control groups. In line with our single dose study repeated administration of AS did not affect paw-flick latency during treatment period. However, there was also an increase in the paw-flick latency time in three out of eight rats starting from day 7 after the last of the five AS doses (Fig. 4B).

The Effect of MK-801 on the Behavioural Effects of AS

The effect of an NMDA receptor blocker MK-801 was tested on the neurotoxicity induced by AS. Treatment with MK-801 (100 nmol) alone caused only temporary motor deficiency lasting approximately 1 h after which animals recovered with no further signs of neurotoxicity. In the AS (5 μmol i.t.) group the rotarod performance time fell to 85.8 ± 22.9 s ($n = 4$) and in the MK-801 pre-treated group the rotarod performance time fell to 58.0 ± 24.0 s ($n = 4$) when measured 72 h after the AS delivery. Both groups recovered to full 120 s performance time by the day 14 after the delivery of AS. There was no change in the paw-flick or tail-flick latencies in either group (data not shown).

Biochemical and Histological Effects of Intrathecal Delivery of AS

Effects of AS on the Expression of GFAP, FGF-2, Laminin-1 and γ1-laminin and Nitrotyrosine

AS (5 μmol, i.t.) induced injury was further studied by measuring the expression of GFAP, FGF-2, laminin-1 and γ1-laminin, markers previously used to evaluate an experimental mechanical injury in the rat spinal cord.[35] A time point of 7 days after the intrathecal drug delivery was chosen for the study. Reactive gliosis reflected by an increase in expression of GFAP in both grey and white matters of the spinal cord were detected after HEPES (Fig. 5A) and more markedly after AS treatment (Fig. 5B). In addition, AS induced increased GFAP expression in reactive astrocytes in the proximity of the motor neurons in the ventral horn (Fig. 5B). FGF-2, a growth factor showing trauma-related changes after injury[35] was not expressed in motor neurons in the normal adult rat spinal cord (Fig. 5C). Increased immunoreactivity against FGF-2 antibody was seen following both HEPES and AS treatment in the motor neurons (Fig. 5D,E). In addition, AS treatment induced glial expression of FGF-2 (Fig. 5E) not seen in normal (Fig. 5C) or HEPES-treated grey matter (Fig. 5D).

Motor neurons of the normal spinal cord do not express laminin-1 or γ1-laminin.[35] Therefore, the expression of these proteins was further studied after intrathecal administration of AS

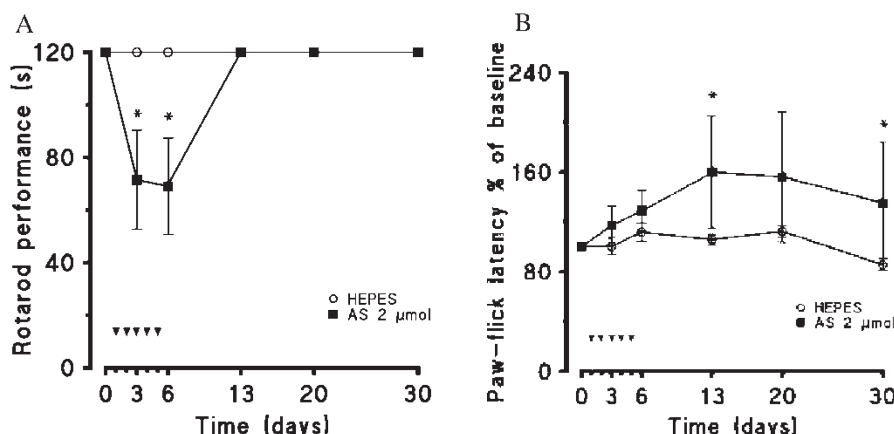

FIGURE 4 The effects of 5 times repeated daily 2 μmol intrathecal dose of Angeli's salt (AS) or control solution on rat motor and sensory functions. The measurements were done immediately prior the daily drug injection schedule. (A) The effects of AS (■) or HEPES control solution (pH 6.1;○) on motor functions were measured by using rotarod test. The rotarod performance was measured 5 times from each rat and best result was recorded. Cut-off time was 120 s. (B) The effects of AS salt (■) or HEPES control solution (○) on paw-flick latency time were measured and expressed as percent of baseline value for mean of three measurements on both hind paws. Mean ± SEM values of 8 rats are shown. *$p < 0.05$ for treatment group vs. control group.

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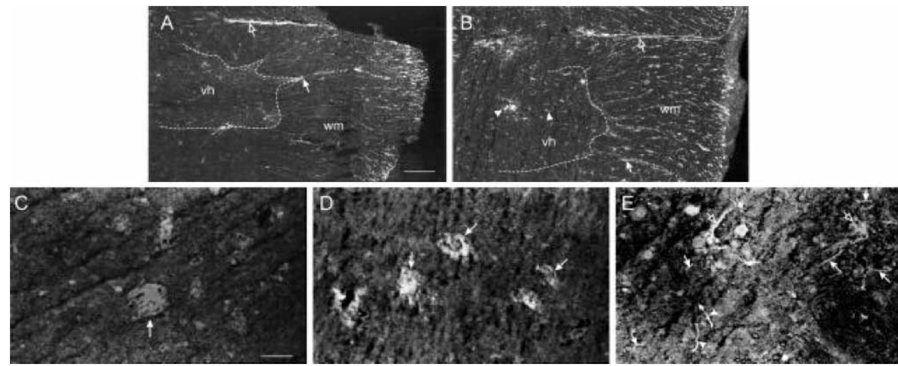

FIGURE 5 Expression of glial fibrillary acidic protein (GFAP) (A,B) and fibroblast growth factor (FGF-2) (C–E) in adult rat spinal cord of a normal rat (C) or following the intrathecal administration of HEPES (1 M; pH 6.1) (A,D) or Angeli's salt (AS) (5 μmol freshly prepared in 1 M HEPES) (B,E). The hatched line in A&B indicate the approximate border of the grey and white matter areas. Scale bar = 150 μm (A,B) and 40 μm (C–E). In A, a low magnification photograph of the right ventral horn (vh) and ventral white matter (wm) of the HEPES-treated rat spinal cord demonstrates GFAP-immunoreactivity that is particularly strong in the ventral roots (arrow) and in the white matter surrounding the median fissure (open arrow). In B, a clear increase in expression of GFAP after exposure to AS is evident in both white (wm) and grey (vh) matters. In the ventral horn (vh) the arrow heads point to reactive astrocytes present close to motor neurons. The open arrow indicates the position of the median fissure and an arrow indicates the glial elements of the white matter (wm). In C, motor neurons in the normal, uninjected spinal cord show only autofluorescence (arrow) and are negative for FGF-2. In D, The intrathecal injection of HEPES alone is sufficient to induce expression of FGF-2 in motor neurons (arrows). In E, intrathecal administration of AS also induces FGF-2 in motor neurons (open arrows). In addition, reactive astrocytes in the spinal cord express FGF-2 in their fibers (arrows and arrow heads).

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(5 μmol). Laminin-1 was present only in capillary basement membranes (Fig. 6A) after the injection of HEPES control solution. However, in the spinal cord exposed to AS-treatment also the motor neurons expressed laminin-1 (Fig. 6B). AS also induced a marked increase in expression of γ1-laminin immunoreactivity in motor neurons as compared to HEPES-treated specimens (Compare Fig. 6C and D). In addition, AS induced glial expression of γ1-laminin in the spinal cord (Fig. 6D).

In the HEPES-treated spinal cord, motor neurons and glial fibres around them showed little immunoreactivity for nitrotyrosine (Fig. 7B), whereas after AS-treatment motor neurons and glial fibres around them showed intense immunoreactivity for nitrotyrosine (Fig. 7A). No neuronal immunoreactivity for nitrotyrosine was apparent in the dorsal

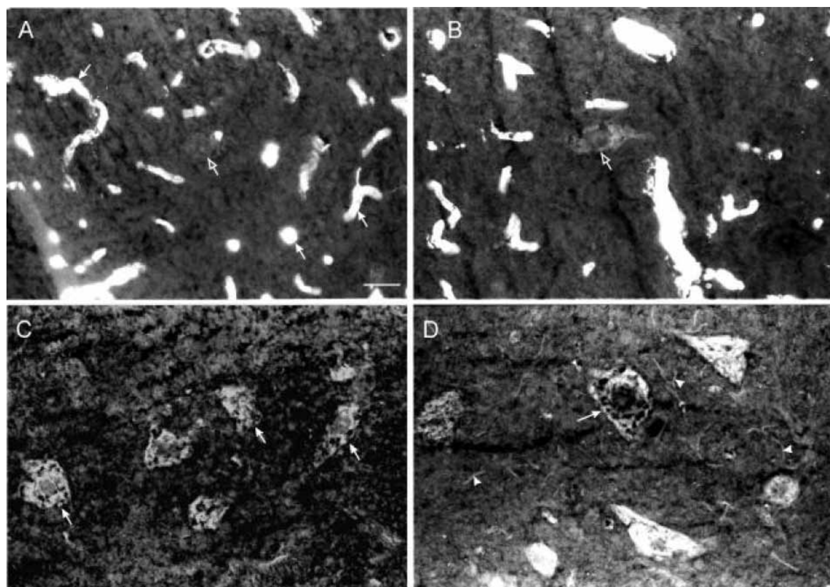

FIGURE 6 Expression of laminin-1 (A,B) and γ1-laminin (C,D) after intrathecal administration of either 1 M HEPES (pH 6.1) alone (A,C) or Angeli's salt (AS) (5 μmol freshly prepared in 1 M HEPES) (B,D). Scale bar = 40 μm. In A, laminin-1 was present only in capillary structures (arrows). The motor neuron in the middle of the microscopic field (open arrow) shows no expression of laminin-1. In B, after exposure to AS, motor neuron in the center (open arrow) is immunoreactive for laminin-1. In C, motor neurons of the ventral horn show moderate expression of γ1-laminin (arrows). In D, motor neurons show increased expression of γ1-laminin (arrow). Furthermore, glial fibers (arrow heads) in the ventral horn show immunoreactivity for γ1-laminin.

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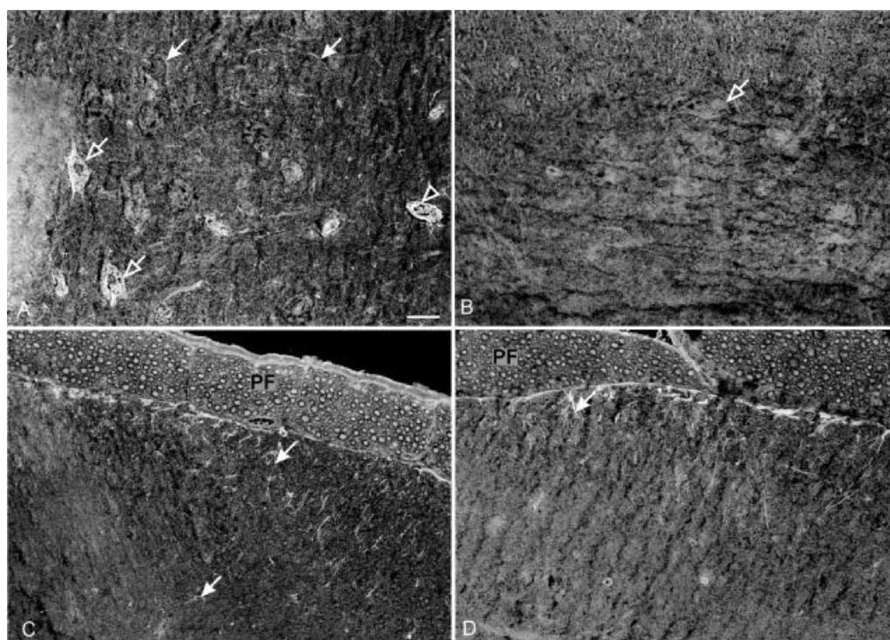

FIGURE 7 Expression of nitrotyrosine in rat spinal cord after intrathecal administration of either Angeli's salt (AS) (5 μmol; freshly prepared in 1 M HEPES)(A,C) or in 1 M HEPES (pH 6.1) alone (B,D). Scale bar = 40 μm. In A, motor neurons (open arrows) and glial fibers (arrows) around them show distinct immunoreactivity for nitrotyrosine after intrathecal administration of AS. Note the intense nitrotyrosine-immunoreactivity of the blood vessels (open arrow head). In B, motor neurons (open arrow) in the ventral horn of the HEPES-treated spinal cord show little nitrotyrosine immunoreactivity. Note that printing of this figure is lighter than in A to allow visualising the low background fluorescence of the tissue. In C, glial fibers of the dorsal spinal cord of AS treated animals show intense immunoreactivity for nitrotyrosine (arrows). The peripheral nerves (PF) also are immunoreactive for nitrotyrosine. In D, the glial fibers of HEPES-treated spinal cord show moderate nitrotyrosine immunoreactivity (open arrow). However, the intensity is lower than in C and the fibers are visible due to lighter printing of the photograph (compare backgrounds in C and D).

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parts of the spinal cord (not shown). Glial immunoreactivity was present in the dorsal spinal cord near the surface in the white matter facing the meningeal membranes (Fig. 7C and D). AS treatment increased the intensity of glial nitrotyrosine immunoreactivity (Compare Fig. 7C and D).

Effect of AS on Protein Carbonyl Formation

The amount of protein carbonyl groups was determined by the sensitive ELISA method to analyse the association of protein oxidation with the injury. Protein carbonyl residues were studied on spinal cord tissue samples taken both 3 days and 7 days after the delivery of either 1 or 5 μmol dose of AS or HEPES control solution. In this study however, we found no changes in the protein carbonyl quantity (Table III).

TABLE III Protein carbonyl groups in a spinal cord tissue sample assayed by an ELISA method following intrathecal delivery of Angeli's salt (AS) or HEPES

Treatment	Time (days)	
HEPES	3	1.13 ± 0.13
AS 1 μmol	3	1.23 ± 0.26
AS 5 μmol	3	1.27 ± 0.19
HEPES	7	1.42 ± 0.12
AS 5 μmol	7	1.95 ± 0.33

Tissue samples were taken either 3 or 7 days after intrathecal delivery of AS (1 or 5 μmol in 1 M HEPES) or HEPES control solution (pH 6.1) (mean ± SEM for $n = 2$ samples).

DISCUSSION

The purpose of these experiments was to compare the toxicity of HNO and NO at the spinal level. Intrathecal delivery of AS, a HNO releasing compound, but not papanonoate, a NO donor caused decreased motor performance. However, AS did not affect significantly sensory functions. The effect may be due to the release of HNO because neither nitrous oxide releasing sulphononoate, nor old AS solution had any effect on the rotarod test. Immunohistochemical examination revealed that AS caused gliosis and increased expression of lesion markers, such as laminin-1 and γ1-laminin in the motor neurons of the spinal cord. Furthermore, we were able to visualise increased expression of nitrotyrosine in motor neurons in AS treated animals, which further supports the hypothesis that formation of HNO in the spinal cord could lead into progressive loss of motor function.

The AS induced dose-dependent decrease of motor performance on the rotarod test was seen

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3–7 days after the AS delivery. Immediately following the intrathecal delivery of any of the tested compounds there was a marked irritation with occasional seizures possibly reflecting an immediate reaction to the parent compound structure or the physical properties of the injected solutions. After this initial reaction the animals were normal and no loss of motor function was seen until up to 24 h after the drug delivery. After this time point the motor performance deteriorated with higher doses of AS. Because the half-life of AS is only 2–3 min under physiological conditions, HNO apparently does not cause immediate dysfunction of the motor neurons but rather initiates a cascade of events leading to decreased motor function. The rotarod performance recovered over time in those animals having mild paralysis. Because we could not follow those animals having the most extensive paralysis more than 1 week due to ethical reasons, it remains unknown if the higher degree of paralysis is irreversible. We have previously shown that AS causes neurotoxicity and cell death after local intranigral administration demonstrating that AS may cause irreversible injury.[13] The finding that partial disruption of nerve function is reversible is not abnormal but represents a rather common scenario in various neuropathies such as those associated with toxic effects of nitrous oxide or other toxic substances.[36,37] Also familial ALS patients can obviously resist the abnormal function of the mutated SOD enzyme to some extent because usually the disease does not manifest until the middle age. Moreover, similarly as in ALS, AS caused motor neuron dysfunction but did not affect the sensory system.

Based on our hypothesis a mutation in the SOD gene might lead into continuous generation of HNO, which is toxic in our acute model. To further study this hypothesis continuous infusion of AS would have been an ideal method. However, the markedly short half-life of AS even in 10 mM NaOH at 37°C prohibits the use of continuous minipump systems. To compromise this problem we delivered a 2 μmol bolus dose AS on five consecutive days. In this experiment, we found that the motor performance declined already after two doses. The decline was of same magnitude during the whole treatment regimen but was recovered few days after the last injection. During the period of motor deficiency there were no marked changes in response to sensory stimulus, similarly as following an acute 5 μmol dose of AS. Technical reasons and increased AS induced irritation made it impossible to continue bolus injections for a longer time period. These results support the hypothesis that continuous formation of HNO at the spinal level might cause permanent motor deficiency.

AS has been used extensively as a source of HNO in previous studies and may be the best compound to study the effects of HNO even tough its reactions may not be solely mediated by the released HNO and it has been suggested to react directly with biomolecules.[38] To address these points we studied the effects of papanonoate and sulphononoate, which have structural similarities and/or might release some of the same products as AS. In addition we studied the effect of old, degraded, AS solution, which contains the stable breakdown products of AS. However, only fresh solution of AS had inhibitory effect on motor performance, supporting the hypothesis that the effects are mediated by the release of HNO, but not NO, nitrous oxide or stable breakdown products of AS. Because HEPES buffer may modify the effects of AS[38] it is important to note that AS was even more toxic to motor neurons (data not shown) when it was administered in NaOH (10 mM) solution. These results support the hypothesis that HNO is far more toxic to motor neurons than NO.

In line with these results we have recently shown that intranigral infusion of AS but not sulphononoate or NO_2^- is toxic to nigral neurons.[13] HNO has been shown to induce oxidative stress leading into double strand breaks in DNA chains[12] as well as to deplete intracellular glutathione[11] thus potentially rendering cells susceptible to further attacks by oxidative species.

Increase in nitrotyrosine immunoreactivity and protein oxidation has been found in spinal cord samples of ALS patients.[39,40] AS derived oxidants have been shown to introduce nitrotyrosine residues into proteins[41] and carbonyl residues into BSA *in vitro* (Väänänen, unpublished result). AS treatment induced remarkable increase of nitrotyrosine immunoreactivity, especially in motor neurons in ventral horn located less than 1 mm from the intrathecal liquor space. It is important to note that in dorsal parts of the spinal cord no neuronal nitrotyrosine immunoreactivity was seen. However, it remains unknown to us if the increased immunoreactivity is caused by HNO, or its derived peroxynitrite type reaction product.[10] Also interestingly motor neurons show increased nitrotyrosine immunoreactivity but sensory nerves are spared from similar effect this being in agreement with the behavioural data.

We could not show increase of protein carbonyl residues in the present study. However, protein oxidation was measured in a larger tissue sample and therefore, we cannot definitively exclude that there might have been an increase of the protein carbonyl groups in a smaller area similarly as was shown in nitrotyrosine immunoreactivity.

Delivery of AS into the intrathecal liquor space immediately cranial of the lumbar enlargement did

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not cause extensive unspecific destruction, such as necrosis, of the spinal cord tissue. However, AS induced molecular changes in expression of GFAP, FGF-2, laminin-1 and γ1-laminin and the effects were closely related to those previously shown after an experimental mechanical spinal cord injury.[35] After exposure to AS, reactive gliosis as indicated by an increase in expression of reactive GFAP-positive astrocytes was apparent in both grey and white matters especially around the motor neurons. Induction of γ1-laminin and FGF-2 positive reactive astrocytes was a further sign of gliosis and in line with previous work on trauma related changes in adult rat spinal cord.[35] In addition, AS induced expression of laminin-1 in motor neurons, not detected in motor neurons of the normal spinal cord.[35] Gamma-1-laminin was already expressed in motor neurons after HEPES-treatment, but its expression was considerably increased by AS treatment, which is consistent with trauma-induced induction of γ1-laminin in the adult rat spinal cord.[35] Thus, AS induced not only motor dysfunction but also injury-related changes in the expression of laminins in the spinal motor neurons.

Even though the cascade of intracytoplasmic events leading to increase in expression of laminins, FGF-2 and GFAP after exposure to AS are not presently understood, it is probable that some of these changes represent a defence mechanism to protect spinal cord against the oxidative damage induced by HNO derived reactive species. This can be concluded as rapid induction and prolonged expression of γ1-laminin has been detected in spinal cord injuries.[35] Furthermore, the neurite outgrowth domain of γ1-laminin is known to promote neuronal survival and axon growth of both rodent and human CNS-neurons.[30,42–44]

Insertion of the polyethylene tube and injection of HEPES to subarachnoidal space were enough to induce a moderate changes in expression of GFAP, FGF-2 and γ1-laminin. This is in line with earlier observations that mere insertion of intrathecal cannula caused tissue changes.[29] However, increased expression of GFAP in astrocytes as well as γ1-laminin in neurons, were more pronounced in rats, which received AS in comparison to the HEPES control (Figs. 5 and 6). Thus, both molecular and behavioural changes induced by AS may indeed be specific and not due to spinal shock or toxic effects to nerve roots.

Increased activity of glutaminergic system has been suggested to play a role in the pathogenesis of ALS.[45,46] In addition, HNO has been proposed to modulate NMDA receptor function.[47,48] We therefore studied the effect of MK-801 pre-treatment on the behavioural effects of AS. In our experiment MK-801 pre-treatment did not provide protection against the toxicity of AS or augment it either. Therefore, the toxicity is not probably due to NMDA receptor mediated excitotoxicity, which has been suggested to mediate the toxicity of large (100 nmol) intrathecal doses of excitatory amino acids in similar models.[49]

In conclusion, we have described for the first time that AS causes a dose dependent loss of motor performance with only minor changes in response to sensory stimulus. The results obtained from immunohistochemical analysis suggest that the observed behavioural abnormalities in our model are not caused by overall destruction of the spinal cord tissue or massive unspecific oxidative/nitrosative attack but rather due to the more targeted injury in motor neurons. These results might have significance under such pathological conditions potentially leading to increased presence of HNO close to the motor neurons.

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