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Original Contribution

# Neurotoxicity of nitroxyl: Insights into HNO and NO biochemical imbalance

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**Abstract**

Nitroxyl anion ($NO^-$), and/or its conjugate acid, HNO, may be formed in the cellular milieu by several routes under both physiological and pathophysiological conditions. Since experimental evidence suggests that certain reactive nitrogen oxide species can contribute significantly to cerebral ischemic injury, we investigated the neurotoxic potential of $HNO/NO^-$ using Angeli's salt (AS), a spontaneous $HNO/NO^-$-generating compound. Exposure to AS resulted in a time- and concentration-dependent increase in neural cell death that progressed markedly following the initial exposure. Coadministration of the donor with Tempol (1 mM), a one-electron oxidant that converts $NO^-$ to NO, prevented its toxic effect, as did the concomitant addition of Fe(III)TPPS. Media containing various chelators, catalase, Cu/Zn superoxide dismutase, or carboxy-PTIO did not ameliorate AS-mediated neurotoxicity, ruling out the involvement of transition metal complexes, $H_2O_2$, $O_2^-$, and NO, respectively. A concentration-dependent increase in supernatant protein 3-nitrotyrosine immunoreactivity was observed when cultures were exposed to AS under aerobic conditions, an effect lost in the absence of oxygen. A bell-shaped curve for augmented AS-mediated nitration was observed with increasing Fe(III)TPPS concentration, which contrasted with its linear effect on abating cytotoxicity. Finally, addition of glutamate receptor antagonists, MK-801 (10 μM) and CNQX (30 μM) to the cultures abrogated toxicity when given during, but not following, AS exposure; as did pretreatment with the exocytosis inhibitor, tetanus toxin (300 ng/ml). Taken together, our data suggest that under aerobic conditions, AS toxicity is initiated via $HNO/NO^-$ but progresses via secondary excitotoxicity.


*Keywords:* Protein nitration; Neurotoxicity; Excitotoxicity; Nitroxyl; Nitric oxide; Cell death; Free radicals

## Introduction

Reactive nitrogen species have been determined to contribute to the pathogenesis of numerous pathological conditions including the evolution of cerebral ischemic brain damage. Overactivation of the $Ca^{2+}$-dependent, constitutive, neuronal isoform of nitric oxide synthase (nNOS or NOS-1) contributes, in part, to the acute phase of tissue injury [1,2], while the inducible isoform of NOS (iNOS or NOS-2) is involved in postcerebral ischemic injury progression [3–6]. Although the free radical species (NO) is the best-known product of NOS, reports have suggested that nitroxyl ($HNO/NO^-$) may be the principle intermediate in the oxidation of arginine by NOS [7,8] and it is then converted to NO by suitable biological electron acceptors [7–9]. Thus, the form of NO that predominates may vary depending on cellular conditions. To wit, evidence suggests that $HNO/NO^-$ formation could be favored over NO production under ischemic conditions. For instance, formation of $HNO/NO^-$ is favored under

*Abbreviations:* nNOS or NOS-1, neuronal isoform of nitric oxide synthase; NO, nitric oxide; AS, Angeli's salt; FeTPPS, 5,10,15,20-tetrakis (4-sulfonatophenyl) prophyrinato ferric chloride; MK-801, dizocilpine maleate; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; Ara-C, cytosine arabinoside; MEM, modified Eagle's medium; MS, media stock; BSS, balanced salt solution; LDH, lactate dehydrogenase; TBS, Tris-buffered saline; DTPA, diethylenetriaminepentaacetic acid; TeNT, tetanus toxin; BSA, bovine serum albumin; RNOS, reactive nitrogen species.

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conditions of decoupled enzymatic activity [10–13]. Studies suggest nitrosothiols may generate $HNO/NO^-$ via reaction with thiols [14] and hetereolytic decomposition under acidic conditions [15] and intracellular acidosis ensues following cerebral ischemia [16]. In addition, the reduction of NO to $NO^-$ can be carried out by biological reducing agents. Such a conversion can be supported by cytochrome *c* [17], the release of which is also increased following cerebral ischemia [18,19]. Pertinently, $HNO/NO^-$ derived from Angeli's salt (3 μmol/kg) was shown to increase myocardial reperfusion injury in rabbits, while administration of an NO-generating compound prior to reperfusion was cardioprotective [20]. Coadministration of AS with ferricyanide, a one-electron oxidant that converts $NO^-$ to NO, completely blocked the injurious effects of AS and exerted significant cardioprotective effects [20]. In addition, cerebrovascular infusion of Angeli's salt (AS, 1.7 mmol/min) to rats causes a significant increase in blood brain barrier permeability [21]. Thus, it seems plausible that the beneficial effect of NOS inhibition on myocardial and, by inference, cerebral ischemic injury could be due to the prevention of $HNO/NO^-$ formation. Given this, we investigated the neurotoxic potential of $HNO/NO^-$ using AS as a synthetic source.

## Experimental procedures

### *Materials*

Angeli's salt was either purchased from Alexis (San Diego, CA) or was a kind gift from Prof. Jon Fukuto (UCLA). Tempol-H was synthesized at the NCI (Bethesda, MD). Superoxide dismutase (bovine erythrocytes), catalase (*Aspergillus niger*), FeTPPS [5,10,15,20-tetrakis (4-sulfonatophenyl) prophyrinato ferric chloride], tetanus toxin (*Clostridium tetani*), and desferroxiamine mesylate were obtained from Calbiochem (San Diego, CA). MK-801 (dizocilpine maleate) and CNQX (6-cyano-7-nitroquinoxaline-2,3-dione) were purchased from RBI (Natick, MA). Ara-C (cytosine arabinoside), EGTA, and DTPA (diethylenetriaminepentaacetic acid) were purchased from Sigma-Aldrich (St. Louis, MO). Tempol was purchased from Fluka (Milwaukee, WI). Carboxy-PTIO came from Biomol (Plymouth Meeting, PA). Anti-nitrotyrosine antibody (rabbit polyclonal) was purchased from United Biotechnology Incorporated (Mountainview, CA). Modified Eagle's medium (MEM, Earle's salts) was obtained from Mediatech (Herndon, VA). All sera were obtained from Hyclone (Logan, UT).

### *Cell culture*

Primary murine mixed cortical cell cultures were prepared from either CD1 or Swiss Webster mice (Charles River Laboratories, Wilmington, MA) as described in detail previously [22]. Experiments were performed on cortical cultures containing approximately 50% neurons and 50% astrocytes after 13–14 days in vitro.

### *Drug exposure*

Stock solutions of Angeli's salt (100–200 mM) are stable at pH > 9 and were therefore made in 0.1 N NaOH and diluted into a media stock (MS; see below) or a balanced salt solution (BSS; see below) immediately prior to addition to cultures. The pH of all solutions was adjusted to ≈7.4 using 0.1 N HCl. To achieve the desired experimental concentrations, 25 μl of an appropriate stock solution was added directly to each culture well (400 μl) followed by HCl (usually 25 μl; 0.1 N) to neutralize the pH (≈pH 7.4). In some experiments, AS was added directly to the culture medium and then immediately (<30 s later) washed into each culture well. AS has a reported half-life of ≈17 min at 25°C or ≈2.8 min at 37°C [23]. The yield of $HNO/NO^-$ from this compound cannot be determined directly although it has been reported that millimolar concentrations generate only micromolar steady-state levels [10,24–26].

Cultures were washed into a balanced salt solution or media stock ± donor and placed in a 37°C 6% $CO_2$-containing normoxic (21% $O_2$) incubator. For exposure under anaerobic conditions, cultures were placed in a chamber (Forma Scientific) containing a gas mixture of 5% $CO_2$, 10% $H_2$, 85% $N_2$ (<0.2%$O_2$) as described in detail previously [27]. Culture medium was replaced by thorough exchange with deoxygenated BSS ± donor and then placed in a 37°C incubator within the chamber. The BSS contained in mM: 116.4 NaCl, 5.4 KCl, 0.8 $MgSO_4$, 1 $NaH_2PO_4$, 26.2 $NaHCO_3$, 1.8 $CaCl_2$, 0.01 glycine, and 20 glucose. MS consisted of MEM (Earle's salt) supplemented with L-glutamine, glucose, and sodium bicarbonate to a final concentration of 2.0, 25.7, and 28.2 mM, respectively.

When using glutamate receptor antagonists, MK-801 was made as a 10 mM stock solution in $H_2O$ while CNQX was made as a 25 mM stock solution in 100% DMSO. They were either diluted to their final concentration in the experimental solution and washed into the culture well or added directly to the culture well. Experimental conditions contained identical concentrations of DMSO, which never exceeded 0.2%. For experiments involving tetanus toxin, cultures were pretreated with 300 ng/ml toxin (stock = 0.25 mg/ml in $H_2O$) ≈24 h prior to AS exposure to allow time for access to the presynaptic terminal. Successful cleavage of its target, synaptobrevin-2, was verified in each experiment as described [28].

### *Assessment of neural cell death*

In most cases, cell death was quantitatively assessed by the measurement of the amount of lactate dehydrogenase (LDH) released into the bathing medium by damaged or destroyed cells, as described [29,30]. Supernatant were