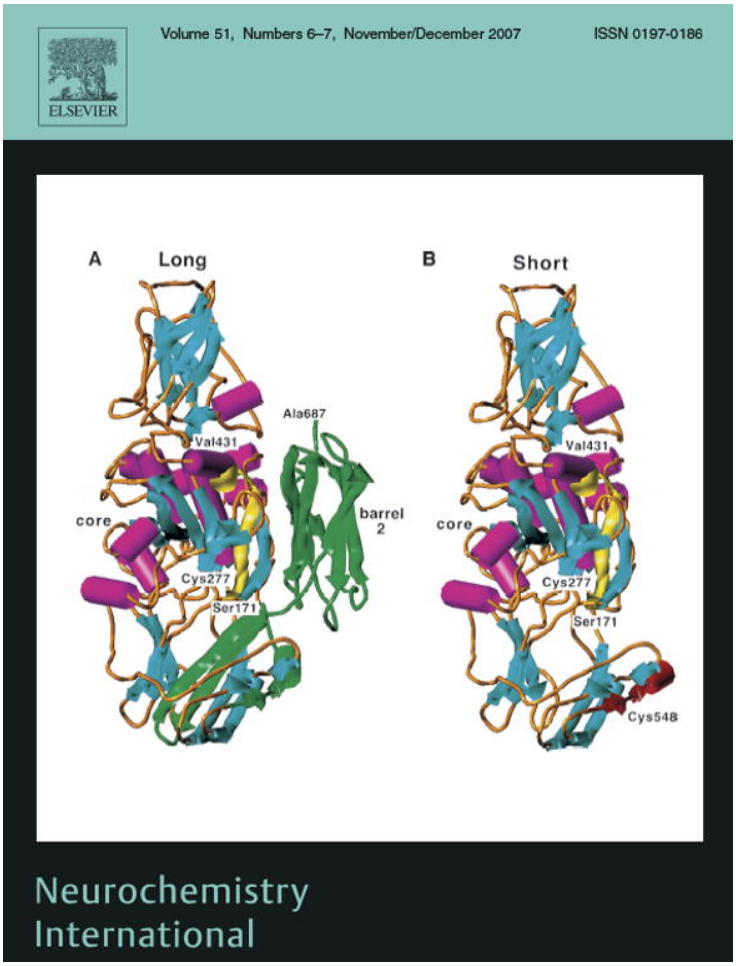

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Effect of nitroxyl on the hamster retinal nitridergic pathway

Daniel A. Sáenz [a], Sara E. Bari [b], Ezequiel Salido [a], Mónica Chianelli [a], Ruth E. Rosenstein [a,*]

[a] *Laboratorio de Neuroquímica Retiniana y Oftalmología Experimental, Departamento de Bioquímica Humana, Facultad de Medicina, Universidad de Buenos Aires, CEFyBO, CONICET, Buenos Aires, Argentina*

[b] *Departamento de Química Inorgánica, Analítica y Química Física/INQUIMAE, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Buenos Aires, Argentina*

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Abstract

There is a growing body of evidence on the role of nitric oxide (NO) in retinal physiology. Recently, interest has developed in the functional role of an alternative redox form of NO, namely nitroxyl (HNO/NO^-), because it is formed by a number of diverse biochemical reactions. The aim of the present report was to comparatively analyze the effect of HNO and NO on the retinal nitridergic pathway in the golden hamster. For this purpose, sodium trioxodinitrate (Angeli's salt) and diethylammonium (*Z*)-1-(*N*,*N*-diethylamino)diazen-1-ium-1,2-diolate (DEA/NO) were used as HNO and NO releasers, respectively. Angeli's salt and DEA/NO significantly decreased nitric oxide synthase activity. In addition, Angeli's salt (but not DEA/NO) significantly decreased L-arginine uptake. DEA/NO significantly increased cGMP accumulation at low micromolar concentrations, while Angeli's salt affected this parameter with a threshold concentration of 200 μM. Although Angeli's salt and DEA/NO significantly diminished reduced glutathione and protein thiol levels in a similar way, DEA/NO was significantly more effective than AS in increasing *S*-nitrosothiol levels. None of these compounds increased retinal lipid peroxidation. These results suggest that HNO could regulate the hamster retinal nitridergic pathway by acting through a mechanism that only partly overlaps with that involved in NO response.

Keywords: Nitroxyl; Nitric oxide; Retina

1. Introductory statement

Nitric oxide (NO), a highly reactive short-lived radical species, plays an important role as an inter- and intracellular messenger in the central nervous system. It is generated by a family of enzymes called NO synthases (NOS) that oxidizes L-arginine to L-citrulline producing NO.

NO activates a soluble guanylyl cyclase, and thereby it increases cGMP levels. Unlike other neural structures in which the role of cGMP is not completely understood, it is well known that this nucleotide plays a critical role in the retinal phototransduction mechanism (O'Brien, 1982). Numerous studies strongly support that NO is involved in several processes of the vertebrate retina. For example, NO influences light responses of amphibian rods (Noll et al., 1994), it activates a retinal soluble guanylyl cyclase, and modulates a Ca^{2+} channel and a voltage-independent conductance in the inner segment of photoreceptors (Kurenny et al., 1994). Effects of NO were also recorded on hemi-gap junction channels in horizontal cells, on cyclic GMP-gated channels in retinal ganglion cells (Ahmad et al., 1994), and in ON-bipolar cells (Nawy and Jahr, 1990). NO has also been involved in retinal pathology. In this sense, it was demonstrated that hyperglycemia increases NO production in retinal cells, and that aminoguanidine (a selective inhibitor of the inducible isoform of NOS), inhibits the development of retinopathy in diabetic animals (Du et al., 2002). In addition to the well-characterized binding of NO to the heme iron of soluble guanylyl cyclase, a principal target of cellular NO is the thiol group of cysteines in peptides and proteins. *S*-nitrosylation conveys a large part of the ubiquitous regulatory influence of NO on cellular signal transduction (Hess et al., 2005). In previous reports, we have characterized NOS activity (Llomovatte et al., 1997) and

* Corresponding author at: Departamento de Bioquímica Humana, Facultad de Medicina, UBA, Paraguay 2155, 5°P, 1121 Buenos Aires, Argentina. Tel.: +54 11 4508 3672x37; fax: +54 11 4508 3672x31.
E-mail address: ruthr@fmed.uba.ar (R.E. Rosenstein).

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L-arginine transport system in the hamster retina (Sáenz et al., 2002) and we showed that both parameters are regulated by the photic information.

Recently, interest has developed in the functional role of an alternative redox form of NO, namely nitroxyl (HNO/NO^-), because it is formed by a number of diverse biological reactions, such as the decomposition of *S*-nitrosothiols in the presence of thiols (Arnelle and Stamler, 1995). Moreover, several authors have suggested that NOS produces nitroxyl rather than NO under certain conditions, particularly at low cofactor concentrations (Schmidt et al., 1996; Adak et al., 2000), although this hypothesis has been challenged (Xia and Zweier, 1997). Nitroxyl is the one electron reduction product of NO. The uncertainty on physicochemical relevant parameters (redox potential, pK_a) during the past decades has led to misinterpretation of the distinct effects of NO and HNO/NO^-. It is currently accepted that HNO to NO conversion does not take place in every tissue (Feelisch, 2003). In fact, orthogonal effects for both species on postischemic myocardial injury (Wink et al., 2003), and on cardiac inotropic and lusitropic action on failing hearts (Paolocci et al., 2003) have been described. Distinct chemical reactivity of both redox partners against hemeproteins (Miranda et al., 2003a), thiol compounds (Wong et al., 1998), and in the formation of *N*-nitroso derivatives (Peyrot et al., 2006), is supposed to explain the molecular basis of their differential biological actions. In addition, while NO actuates via a soluble guanylyl cyclase activation, it was suggested that nitroxyl activates a calcitonin gene-related peptide that ends on cAMP accumulation (Feelisch, 2003). This means that even parallel effects cannot be simply explained by just the intracellular interconversion of both species. In this sense, we showed that although similar responses were provoked by NO and HNO on human platelets, their mechanisms of action were different in some aspects (Bermejo et al., 2005).

At physiological pH, nitroxyl exists largely in its protonated form, HNO, which can readily cross cell membranes. Although nitroxyl is a highly unstable species, the introduction of a number of nitroxyl donors such as Angeli's salt (AS, sodium trioxodinitrate) (Bonner and Ravid, 1975; Miranda et al., 2005), has facilitated the elucidation of its biological actions. Indeed, considerable amount of work strongly support that AS is a nitroxyl releasing compound but not a NO donor, which releases the respective nitrogen oxide at high rate under biological conditions ($t_{1/2,\ 37\ ^\circ C}$ = 2.8 min) (Hughes and Cammack, 1999).

Despite the potentially significant role of NO in retinal development, physiology, and pathology (for review, see Cudeiro and Rivadulla, 1999), the biological effects of NO redox-related species remain unknown at retinal level. The present investigation was purposely designed to discriminate the actions of HNO and NO on several retinal parameters, particularly on the nitridergic pathway. In the present study DEA/NO was used as a NO donor since it decomposes with a half-time close to that of AS ($t_{1/2}$ at 37 °C and pH 7.4 = 2.1 min) (Maragos et al., 1991).

2. Experimental procedures

2.1. Materials

2.1.1. Reagents and drugs

Diethylammonium (Z)-1-(*N*,*N*-diethylamino)diazen-1-ium-1,2-diolate (DEA/NO) was obtained from Cayman Chemical (Ann Arbor, MI, USA), 3-isobutyl-1-methylxanthine (IBMX), reduced glutathione, malondialdehyde bis-dimethyl acetal (MDA), 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB), dimethyl sulfoxide (DMSO), and L-arginine were obtained from Sigma Chemical Co. (St. Louis, MO, USA), whereas Dowex was from Bio-Rad Laboratories (Richmond, CA, USA). L-[^{3}H]-arginine (specific activity 53.4 Ci/mmol, purity greater than 97%), L-[^{3}H]-glutamine (specific activity 51 Ci/mmol), and L-[^{14}C]-leucine (specific activity 339 mCi/mmol) were purchased from New England Nuclear Corp. (Boston, MA, USA), while 2,3-diaminonaphthalene (DAN) was from Invitrogen (Eugene, OR, USA). The rabbit anti-cyclic GMP antiserum was from Chemicon International Inc. (Temecula, CA, USA).

2.1.2. AS and DEA/NO solutions

AS was synthesized as previously described (Hughes and Cammack, 1999). In brief, hydroxylamine hydrochloride (3.04 g in absolute ethanol) was added to a solution of sodium ethoxide (9 g in absolute ethanol) at room temperature. A precipitate of sodium chloride was filtered off. Ethyl nitrate (3.98 g in ethanol) was then added to the filtered solution at room temperature. A fine precipitate of AS was slowly formed. The solution was stored in a refrigerator until precipitation was complete. The product was then filtered off and washed with ethanol. To avoid decomposition of AS or DEA/NO, freshly prepared solutions were kept on ice in the dark until added to retinas or synaptosomal fractions, usually no more than 5 min later.

2.2. Animals

Male golden hamsters (average weight 120 ± 20 g), derived from a stock supplied by Charles River Breeding Laboratories (Wilmington, MA, USA), were kept under a 14 h light: 10 h dark lighting schedule (lights on at 06.00 h), with free access to food and water. Animals were sacrificed at midday by decapitation, eyes were enucleated, vitreous removed, and the retinas dissected and processed as described below for each protocol. All animal use procedures were in strict accordance with the NIH Guide for Care and Use of Laboratory Animals.

2.3. NOS activity assessment

Each retina was homogenized in 100 μl of buffer solution containing 0.32 M sucrose and 0.1 mM EDTA (adjusted to pH 7.4 with Tris base). Homogenates were preincubated in the presence or absence of AS or DEA/NO (10–500 μM) for 30 min at 37 °C. In another set of experiments, homogenates were preincubated with AS or DEA/NO (both 200 μM) for 5 or 15 min. Then, NOS activity was assessed in reaction mixtures that contained 50 μl of the enzyme source and 50 μl of a buffer stock solution (final concentrations: 10 mM HEPES, 3 mM $CaCl_2$, 1 mM NADPH, 5 μM FAD, 1 mM β-mercaptoethanol, L-[^{3}H]-arginine (5 μCi/ml), and 1 μM L-arginine, with or without 1 mM dithiothreitol (DTT). After incubation at 37 °C for 30 min, the reaction was stopped by adding 200 μl of stop buffer (50 mM HEPES, 10 mM EDTA, and 10 mM EGTA, pH 5.5) and cooling the tubes for 5 min. The solution was mixed with 600 μl of resin Dowex AG50W-X8 (Na^+ form) to remove L-arginine, and centrifuged at 10,000 × *g* for 5 min. L-[^{3}H]-citrulline in the supernatant was quantified by liquid scintillation counting. Non-enzymatic conversion of L-[^{3}H]-arginine to L-[^{3}H]-citrulline was tested by adding buffer instead of the enzyme source or a heat-inactivated enzyme solution.

2.4. Assessment of L-[^{3}H]-arginine, L-[^{3}H]-glutamine, and L-[^{14}C]-leucine uptake

L-Arginine uptake was examined in a crude synaptosomal fraction of hamster retinas as previously described (Sáenz et al., 2002). Retinas were

homogenized (1:9, w/v) in 0.32 M sucrose containing 1 mM $MgCl_2$, and centrifuged at 900 × *g* for 10 min at 4 °C. Nuclei-free homogenates were further centrifuged at 30,000 × *g* for 20 min. The pellet was immediately resuspended in buffer Tris–HCl, and aliquots (100–300 μg protein/100 μl) were preincubated for 30 min in the presence or absence of AS or DEA/NO, before the addition of L-[^{3}H]-arginine. At the end of the preincubation period, 100 μl of L-[^{3}H]-arginine (10 μM, 800,000–1,000,000 dpm/tube, specific activity 53.4 Ci/mmol) was added. After 10 min, L-[^{3}H]-arginine uptake was terminated by adding 4 ml of ice-cold Tris–HCl buffer. The mixture was immediately poured onto Whatmann GF/B filters under vacuum. The filters were washed twice with 4 ml-aliquots of ice-cold buffer and the radioactivity on the filters was counted in a liquid scintillation counter. Non-specific uptake of L-[^{3}H]-arginine into synaptosomes was assessed by adding an excess of L-arginine (10 mM). A similar protocol was followed in order to assess the effect of AS or DEA/NO on L-[^{3}H]-glutamine (10 μM, 500,000–800,000 dpm/tube,) and L-[^{14}C]-leucine uptake (10 μM, 1,000,000–2,000,000 dpm/tube). In these cases, non-specific uptake into synaptosomes was assessed in the presence of an excess of L-glutamine or L-leucine (10 mM), respectively.

2.5. Cyclic GMP levels assessment

Retinal cell suspensions obtained by mechanical disruption (100 μl, 250–350 μg protein/tube) were incubated for 30 min at 37 °C in buffer Tris–HCl with 0.5 mM IBMX, in the presence or absence of AS or DEA/NO. Cell suspensions were centrifuged at 800 × *g* for 5 min and the pellets were resuspended in 0.4 ml of water and boiled for 2 min. The content of cGMP in the golden hamster retina was assessed as previously described (Sáenz et al., 2002). Briefly, the suspensions were centrifuged at 5000 × *g* for 5 min at 4 °C. Cyclic GMP content was measured in the supernatants by RIA after acetylation. For this purpose, aliquots of samples or standards were acetylated with acetic anhydride/triethylamine. The acetylated samples and the standard curve were mixed with [^{125}I]-cyclic GMP (15,000–20,000 dpm, specific activity 140 mCi/mmol) and a rabbit antiserum (Chemicon International Inc.) diluted 1:150 and incubated overnight at 4 °C. The antibody complex was precipitated with ethanol at 4 °C using 2% bovine serum albumin as a carrier, centrifuged at 2000 × *g* for 30 min, and separated by aspirating supernatants. The radioactivity was measured in a gamma counter. The range of the standard curves was 10–5000 fmol of cGMP.

2.6. Assessment of retinal-reduced glutathione levels (GSH)

GSH levels in retinal tissue were assessed as described by Beutler et al. (1963). Each retina was homogenized in 120 μl of 50 mM potassium phosphate buffer, pH 7.4. Homogenates were incubated in the presence or absence of AS or DEA/NO for 30 min at 37 °C. Then, 100 μl of these samples were mixed with 25 μl of 50% trichloro-acetic acid (TCA) plus 1 mM EDTA. After 5 min at 4 °C, samples were centrifuged at 13,200 × *g* for 2 min. Aliquots of the supernatants were mixed with 800 μl of 0.25 mg/ml DTNB diluted in 0.5 M potassium phosphate buffer. Absorbance was recorded at 412 nm. The range of the standard curves of reduced glutathione was 0.50–50 nmol.

2.7. Determination of protein reduced thiol groups

Retinal protein reduced thiol levels were assessed as described by Dalle-Donne et al. (2005). Each retina was homogenized in 300 μl of 50 mM potassium phosphate buffer, pH 7.4. Homogenates were incubated in the presence or absence of AS or DEA/NO for 30 min at 37 °C. Then, proteins were precipitated by adding 10% (w/v, final concentration) TCA plus 1 mM EDTA and centrifuged (15,000 × *g* for 5 min). Pellets were resuspended with a glass rod in 1 ml of 1.5% (w/v, final concentration) TCA and then pelleted by centrifugation (15,000 × *g* for 5 min); this step was repeated three times. Finally, the protein pellet was resuspended in 0.2 M sodium phosphate buffer, pH 7.4, containing 1% (w/v, final concentration) SDS, being gently stirred with a glass rod until proteins were dissolved. Protein reduced thiol groups were then measured spectrophotometrically at 412 nm with 5,5-dithio-bis(2-nitrobenzoic acid).

2.8. Determination of retinal S-nitrosothiol levels (RSNO)

Total *S*-nitrosothiol levels in retinal tissue were assessed as described by Wink et al. (1999a). Each retina was homogenized in 200 μl of 50 mM potassium phosphate buffer, pH 7.4. Homogenates were incubated in the presence or absence of AS or DEA/NO for 30 min at 37 °C. Then, 100 μl of these samples were mixed with 5 μl of 50 mM *N*-ethylmaleimide for 10 min. The solution was mixed with 900 μl of 250 μM DAN solution in phosphate-buffered saline and 10 μl DMSO or 20 mM of $HgCl_2$ in DMSO. After 1 h of incubation at room temperature in darkness, samples were centrifuged at 15,000 × *g* for 10 min. The levels of *S*-nitrosothiol in the supernatants were quantified by spectrofluorometry. The absorbance was measured at an emission wavelength 450 nm using an excitation wavelength of 375 nm with a Jasco FP 770 fluorescence spectrophotometer (Japan Spectroscopic Co. Ltd., Tokyo, Japan). The range of the standard curve of *S*-nitrosoglutathione was 0.10–10 nmol and GSNO was prepared freshly daily as reported Wink et al. (1999a). Results were expressed as nmol *S*-nitrosothiol per mg protein.

2.9. Measurement of thiobarbituric acid reactive substances (TBARS) levels

Retinal TBARS levels were assessed as previously described (Sacca et al., 2003). Retinal cell suspensions obtained by mechanical disruption (300 μl, 500–700 μg protein/tube) were preincubated in buffer Tris–HCl for 30 min at 37 °C with or without AS or DEA/NO. Cell suspensions were centrifuged at 800 × *g* for 5 min. Pellets were resuspended in 300 μl total volume of cold 15 mM potassium phosphate buffer pH 7.4 plus 60 mM KCl, 75 μl 10% SDS and 1.4 ml 0.8 g% thiobarbituric acid dissolved in 10% acetic acid (pH 3.5). This solution was heated to 100 °C for 60 min. After cooling (10 min in ice-water bath), the flocculent precipitate was removed by centrifugation at 3200 × *g* for 10 min. After addition of 1.0 ml water and 5.0 ml of *n*-butanol-pyridine mixture (15:1, v/v), the mixture was vigorously shaken and centrifuged at 2000 × *g* for 15 min. The absorbance of the organic layer was measured at an emission wavelength 553 nm by using an excitation wavelength of 515 nm with a Jasco FP 770 fluorescence spectrophotometer (Japan Spectroscopic Co. Ltd., Tokyo, Japan). The range of the standard curve of malondialdehyde bis-dimethyl acetal (MDA) was 10–2000 pmol. Results are expressed as nanomoles MDA per milligram protein. No interference of AS or DEA/NO with the detection method used was observed (data not shown).

Protein content was determined by the method of Lowry et al. (1951), using bovine serum albumin as the standard.

Statistical analysis of results was made by a two-way analysis of variance (ANOVA) followed by a Dannett's test or Tukey's, as stated.

3. Results

3.1. Effect of HNO and NO on retinal NOS activity

Fig. 1 depicts the effect of AS and DEA/NO on retinal NOS activity. Both compounds significantly diminished the conversion of L-arginine to L-citrulline, with a threshold concentration of 200 μM. No additive effect was observed when maximal concentrations (500 μM) of AS and DEA/NO were added together (data not shown). When 1 mM DTT was added to incubation mixture, no changes in the inhibitory effect AS or DEA/NO (both 500 μM) on NOS activity were observed. When a solution of 500 μM AS was allowed to be decomposed at 37 °C (pH 7.4) for 60 min, and then added to retinal homogenates, or when homogenates were preincubated in the presence of an equivalent concentration of sodium nitrite ($NaNO_2$), no changes in NOS activity were observed (data not shown). Both donors were also effective in inhibiting NOS activity at 5 and 15 min of preincubation, as shown in Table 1.

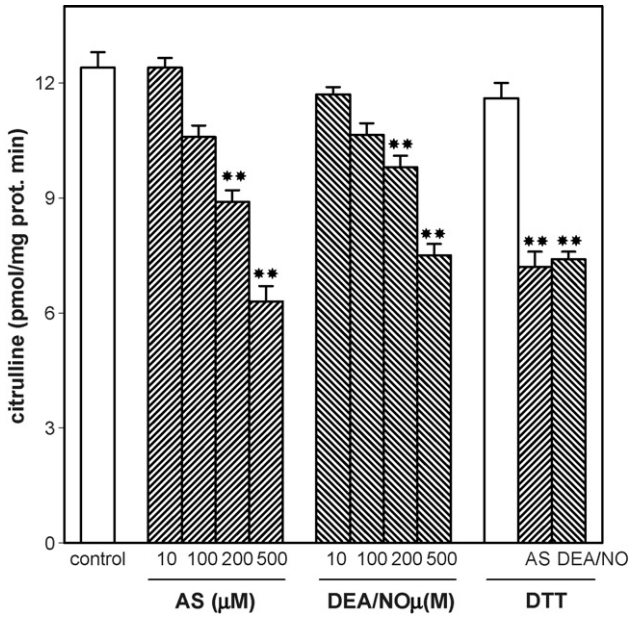

Fig. 1. Effect of AS and DEA/NO on retinal NOS activity. Both donors significantly decreased this parameter with a threshold concentration of 200 μM. The effect of AS and DEA/NO (both 500 μM) was not modified in the presence of 1 mM DTT. Data are means ± S.E. (n = 12–18), $^{**}p < 0.01$ vs. control, by Tukey's test.

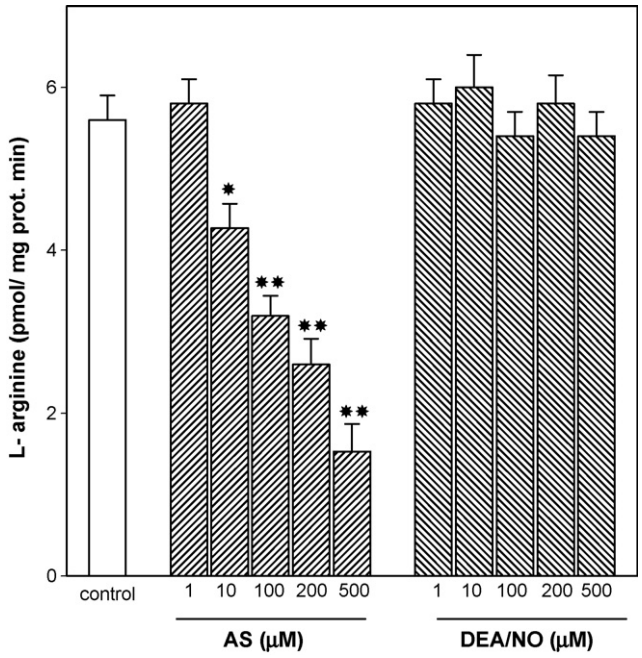

Fig. 2. Effect of AS and DEA/NO on L-arginine uptake in the golden hamster retina. Retinal synaptosomal fractions were incubated with 10 μM L-[^{3}H]-arginine in the presence of various concentrations of AS or DEA/NO (1–500 μM). The effect of AS on L-arginine influx was already evident at 10 μM, whereas DEA/NO was ineffective at any concentrations tested. Shown are means ± S.E. (n = 15 retinas/group), $^{*}p < 0.05$, $^{**}p < 0.01$, by Dunnett's test.

3.2. Effect of HNO and NO on retinal L-arginine, L-glutamine, and L-leucine uptake and cGMP accumulation

Besides NOS activity, another limiting step in the regulation of NO synthesis is the availability of the precursor L-arginine. Therefore, the effect of AS or DEA/NO on L-arginine uptake was examined (Fig. 2). Only AS (but not DEA/NO), significantly and dose-dependently reduced L-arginine influx. The effect of AS on L-arginine influx was already evident at 10 μM, whereas DEA/NO was ineffective at any concentrations tested. Also in this case, decomposed AS (60 min at 37 °C) or the addition of 500 μM $NaNO_2$, did not affect L-arginine uptake (data not shown). No changes in L-[^{3}H]-glutamine and L-[^{3}H]-leucine uptake were observed in the presence of AS or DEA/NO (100 μM), as shown in Table 2.

Fig. 3 shows the effect of AS and DEA/NO on retinal cGMP accumulation in the presence of IBMX. While a 1 μM concentration of DEA/NO already increased this parameter, a concentration of 200 μM was needed for AS to provoke a significant increase in cGMP accumulation.

3.3. Effect of HNO and NO on retinal thiol, S-nitrosothiol levels and lipid peroxidation

AS and DEA/NO significantly decreased protein reduced thiols levels, being the threshold concentration 100 and 200 μM, respectively (Fig. 4). As shown in Fig. 5, both compounds were almost equally effective in decreasing retinal-reduced glutathione levels (GSH). In this case, a similar threshold was observed for AS and DEA/NO.

Fig. 6 shows the effect of AS and DEA/NO on retinal S-nitrosothiol level. While a 200 μM concentration of DEA/NO already increased this parameter, a concentration of 500 μM was needed for AS to provoke a significant increase in S-nitrosothiol level. Fig. 7 shows that AS and DEA/NO did not affect retinal TBARS levels (an index of lipid peroxidation).

Table 1
Effect of HNO and NO on retinal NOS activity citrulline

	5 min (pmol/ [mg protein·min])	15 min (pmol/ [mg protein·min])
Control	12.8 ± 0.5	12.4 ± 0.3
AS	8.2 ± 0.4**	8.6 ± 0.4**
DEA/NO	9.4 ± 0.5**	9.2 ± 0.4**

The effect of AS and DEA/NO (200 μM) on retinal NOS activity was assessed after 5 or 15 min of preincubation. Both compounds provoked a significant decrease of this parameter at both intervals. Shown are means ± S.E. (n = 10 retinas/group), $^{**}p < 0.01$, by Dunnett's test.

Table 2
Effect of HNO and NO on L-glutamine and L-leucine uptake

	L-Glutamine (pmol/ [mg protein·min])	L-Leucine (pmol/ [mg protein·min])
Control	6.9 ± 0.5	17.5 ± 0.9
AS	7.3 ± 0.3	18.6 ± 0.6
DEA/NO	6.3 ± 0.5	18.9 ± 0.9

L-Glutamine and L-leucine uptake was assessed in a retinal synaptosomal fraction, as described in Section 2. No changes in these parameters were observed in the presence of AS or DEA/NO (100 μM). Shown are means ± S.E. S.E. (n = 10 retinas/group).

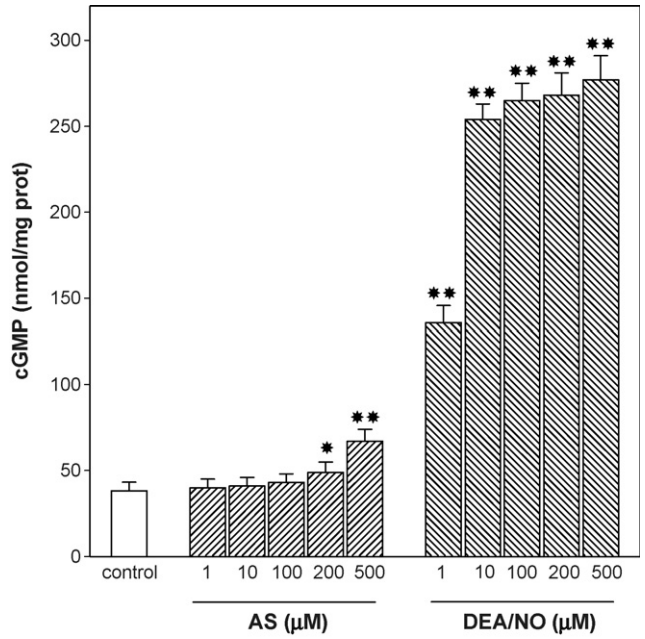

Fig. 3. Effect of AS and DEA/NO on retinal cGMP accumulation. The retinas were excised and processed as described in Section 2, with or without AS or DEA/NO, in the presence of IBMX. AS significantly increased this parameter with a threshold concentration of 200 μM, whereas 1 μM DEA/NO was already effective in increasing cGMP levels. Shown are means ± S.E. (n = 15 retinas/group), $^{*}p < 0.05$, $^{**}p < 0.01$, by Dunnett's test.

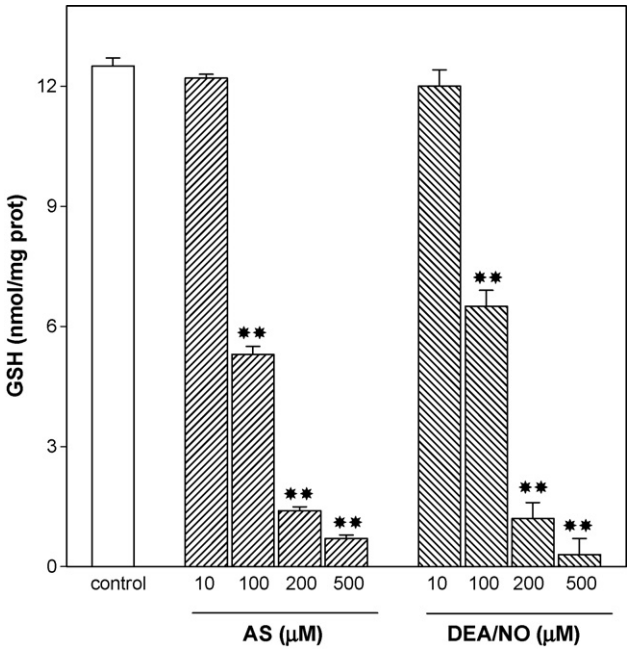

Fig. 5. Effect of AS and DEA/NO on GSH levels in golden hamster retina. Both donors significantly decreased this parameter in a similar fashion. Data are means ± S.E. (n = 10), $^{**}p < 0.01$ by Dunnett's test.

4. Discussion

The foregoing results indicate that HNO and NO regulate the hamster retinal nitridergic pathway. Both AS and DEA/NO, but not $NaNO_2$ decreased retinal NOS activity. Several mechanisms might modulate NOS activity, including protein phosphorylation (Dawson et al., 1993), acylation (Sessa, 1994), and subcellular localization (Michel et al., 1993), among others. The present results support that hamster retinal NOS activity may be regulated through a feedback mechanism by which excessively generated NO can regulate the amount of

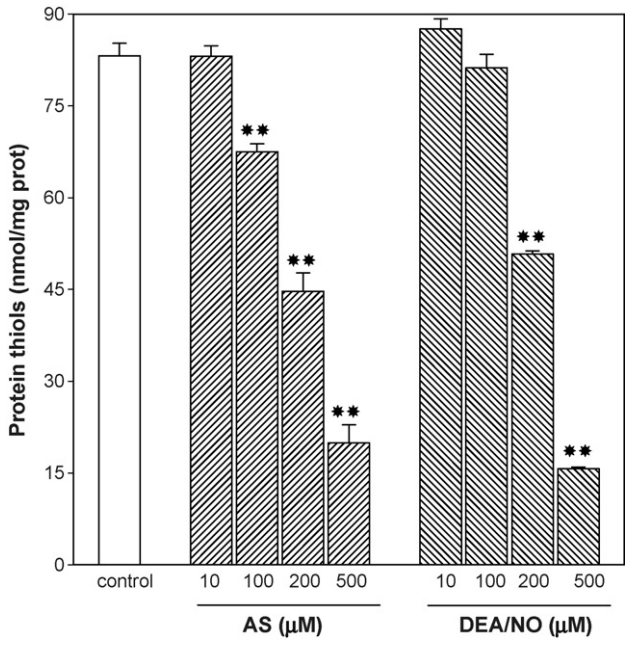

Fig. 4. Effect of AS and DEA/NO on protein reduced thiol levels. Both donors significantly decreased this parameter with a threshold concentration of 100 and 200 μM, respectively. Shown are means ± S.E. (n = 15 retinas/group), $^{**}p < 0.01$, by Dunnett's test.

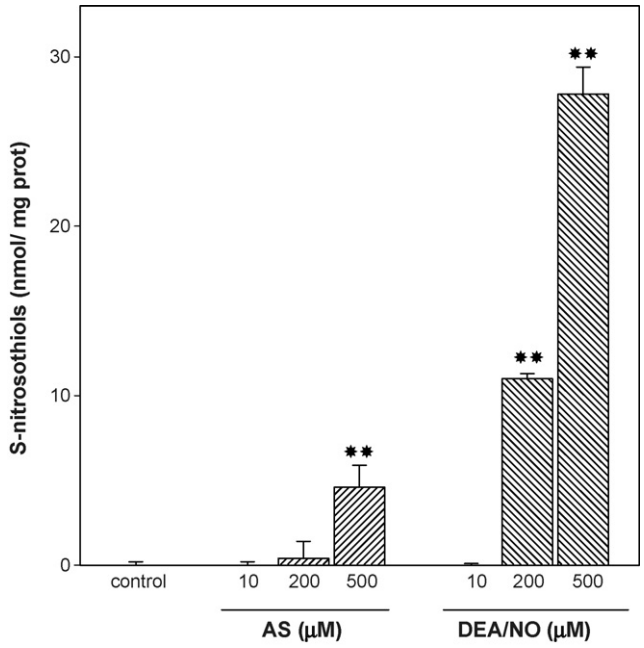

Fig. 6. Effect of AS and DEA/NO on retinal S-nitrosothiol levels. While a 200 μM concentration of DEA/NO already increased this parameter, a concentration of 500 μM was needed for AS to provoke a significant increase in S-nitrosothiol levels. Data are means ± S.E. (n = 12–18), $^{**}p < 0.01$ vs. control, by Tukey's test.

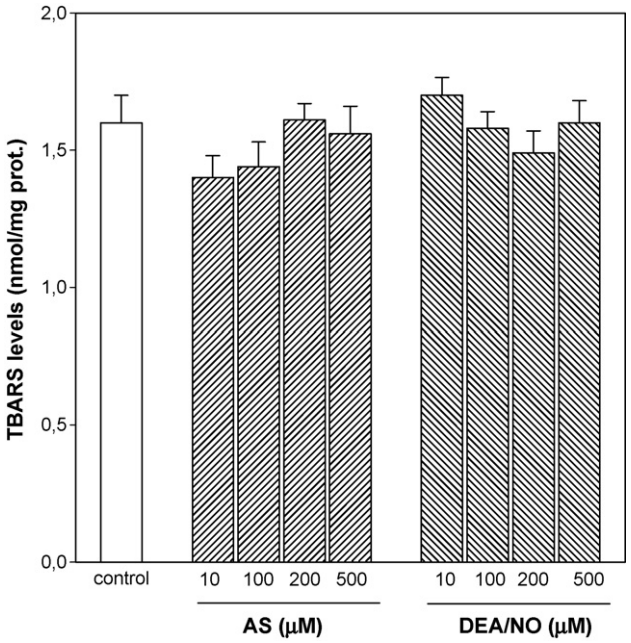

Fig. 7. Effect of AS and DEA/NO on TBARS levels in golden hamster retina. None of these compounds affected retinal lipid peroxidation at any concentration tested. Data are means ± S.E. ($n = 10$).

subsequent NO synthesis. In agreement, it was shown that similar concentrations of NO donors diminish NOS activity in several tissues (Rogers and Ignarro, 1992; Griscavage et al., 1994). Our results indicate that AS also inhibited this enzymatic activity. Since HNO may be synthesized during the NOS-catalyzed oxidation of L-arginine under certain cofactor conditions (Adak et al., 2000), the inhibition of NOS by HNO may be another pathway of NOS auto inhibition. Kotsonis et al. showed that low concentrations of HNO and NO decrease the activity of neuronal NOS (nNOS or NOS-1) purified from pig cerebellum (Kotsonis et al., 1999). We extend this conclusion by showing that both compounds were effective even in retinal homogenates. As tissue homogenates are mixtures containing varied concentrations of possible targets for HNO and NO, it is not surprising that higher concentrations of both donors were required for retinal NOS inhibition in our assay conditions. Although this effect was considerably fast, being already evident after 5 min of preincubation, the molecular mechanism(s) by which NO and HNO decreased retinal NOS activity is yet to be fully understood. When DEA/NO and AS were added together at a dose causing individual maximum reduction, NOS activity decreased in a similar extent to that provoked by each one of this compounds alone, suggesting the existence of a same target for HNO and NO in the inhibition of NOS activity. It was demonstrated that all NOS isoforms bind and require GSH to protect essential NOS protein thiols (Hofmann and Schmidt, 1995), and that *S*-nitrosylation by NO donors inhibits both endothelial NOS (eNOS or NOS-3) (Erwin et al., 2005) and inducible NOS (iNOS or NOS-2) (Mitchell et al., 2005) activity. Thus, since both AS and DEA/NO decreased retinal GSH and protein reduced thiol levels, it seems likely the participation of thiol groups in NOS inhibition induced by DEA/NO and AS. On the other hand, participation of the heme iron cannot be disregarded, as direct interaction of NO and HNO with the heme iron of hemeproteins (Bazylinski and Hollocher, 1985; Miranda et al., 2003a) and heme model compounds (Bari et al., 2003) has been demonstrated.

The availability of the precursor L-arginine, which depends on the presence of a specific uptake system for this amino acid, is one of the limiting steps in the regulation of NO synthesis. We have demonstrated that the y^+ is the predominant system of L-[^{3}H]-arginine uptake in the hamster retina (Sáenz et al., 2002). The present results indicate that HNO significantly decreased retinal L-arginine but not L-glutamine or L-leucine influx. This effect of AS is affiliated specifically to HNO, since the same concentration of nitrite (the other final breakdown product of AS), or decomposed AS did not cause similar effects. In contrast to this result, it was recently shown that 3 mM but not 300 μM AS significantly increases L-arginine uptake in neuronal cells expressing tyrosine hydroxylase (Bae et al., 2005). Although there is no ready explanation for this discrepancy, it seems possible that the difference in the biological system studied could account for the different result.

The sulfhydryl reagent *N*-ethylmaleimide (NEM) was shown to inhibit the activity of L-arginine transporter y^+ system in several systems (Devés and Boyd, 1998). However, although both AS and DEA/NO were able to react with thiol groups in the retina, only HNO significantly reduced retinal arginine influx, suggesting that differential mechanisms should account for this effect. The differential effect of HNO and NO on L-arginine uptake can be attributed to the demonstrated formation of diffusible oxidized products of HNO that are reactive towards protein thiols in the hydrophobic protein environment of the membranes where L-arginine transporters are located. Conversely, the major oxidation product of NO, N_2O_3, is rapidly hydrolyzed to nitrite, which is neither effective as a thiol nitrosating agent nor at crossing membranes (Espey et al., 2002; Lopez et al., 2007). Furthermore, a differential chemical behavior of HNO and NO towards thiol groups can also account for the different effect on L-arginine influx.

As the assay of NOS activity involves the incubation of retinal homogenates in the presence of controlled concentrations of L-arginine, the diminished NOS activity observed in the presence of AS cannot be attributed to a decrease in L-arginine uptake. In fact, AS but not DEA/NO decreased L-arginine influx in the golden hamster retina, although both compounds decreased NOS activity. In addition, while the effect of AS on NOS activity was evident at 200 μM, lower concentrations of the nitroxyl donor were required to significantly reduce L-arginine uptake. Purified NOS from different sources has been reported to have a low half-saturating L-arginine concentration (EC_{50} ~10 μM). Since high levels of intracellular L-arginine ranging from 0.1 to 1 mM have been measured in many systems (Block et al., 1995; Mc Donald et al., 1997), it was expectable that endogenous L-arginine would support maximal activation of NOS. However, a number of *in vivo* and *in vitro* studies indicate that NO production under physiological conditions can be increased by extracellular arginine despite saturating intracellular L-arginine concentrations. This has been termed

"the arginine paradox" (Kurz and Harrison, 1997). One possible explanation could be that intracellular L-arginine is sequestered in one or more pools that are poorly, if at all, accessible to NOS, whereas extracellular L-arginine transported into the cells is preferentially delivered to NO biosynthesis (Kurz and Harrison, 1997). Assuming this hypothesis, the inhibition of L-arginine uptake by AS could be another pathway of NO biosynthesis inhibition by HNO *in vivo*.

The effects of HNO were formerly explained by its conversion to NO. This hypothesis would have AS reproducing all the effects elicited by NO. The fact that only AS (but not DEA/NO) significantly inhibited L-arginine uptake supports that the effects of HNO cannot be merely attributable to its conversion to NO, at least at retinal level.

Although the activation of a soluble guanylyl cyclase by NO is a very well-known fact, a similar effect for HNO is a matter of active debate. A number of effects of AS were shown to be cGMP independent (Miranda et al., 2003b), and some authors reported that NO is the only nitrogen monoxide redox form capable of directly activating soluble guanylyl cyclase (Dierks and Burstyn, 1996). In contrast, it was demonstrated that HNO elicits vasorelaxation in rabbit aorta and bovine intrapulmonary artery (Fukuto et al., 1992) and it enhances human neutrophils migration (Vanuffelen et al., 1998) through a guanylyl cyclase-dependent pathway. Furthermore, the addition of a phosphodiesterase inhibitor enhances, whereas guanylyl cyclase inhibitors almost abolished relaxation of urethral smooth muscle induced by AS (Costa et al., 2001). The present results indicate that in the hamster retina, both DEA/NO and AS significantly increased cGMP accumulation. However, in comparison with DEA/NO, a relatively small (although significant) increase in cGMP levels was observed in the presence of AS.

Besides its canonical signaling role involving cGMP, NO is also believed to signal through a guanylyl cyclase-independent mechanism mediated by the interaction of reactive nitrogen oxides with reduced cysteines of proteins, that can change protein function (reviewed by Stamler et al., 2001; Martinez-Ruiz and Lamas, 2004). In agreement with other reports (Wink and Mitchell, 1998; Erwin et al., 2005; Hess et al., 2005), present results indicate that both NO and HNO are able to react with retinal-free and protein-bound thiols. Strategically located thiol-residues can become inactivated by *S*-nitrosylation or by formation of sulfinamide derivatives (Wong et al., 1998), thus compromising the enzyme's functions. Over the past decade, the number of reported substrates for *S*-nitrosylation has grown to well over a hundred (Stamler et al., 2001), which is consistent with the ubiquity of regulatory and/or active-site thiols across protein classes. Although both HNO and NO significantly decreased protein thiol and reduced glutathione levels, DEA/NO was more effective in increasing *S*-nitrosothiol levels than AS. This result suggests that HNO could trigger chemical modifications different from *S*-nitrosylations (e.g. formation of sulfinamides or disulfides).

It was reported that NO and NO-related species could have either pro- or antioxidative properties, which are highly condition-dependent (Wink et al., 1999b). Many pro-oxidative properties of NO result from its reaction with superoxide, and the subsequent production of peroxynitrite, a powerful oxidant (Blough and Zafiriou, 1985), while it was recently suggested that the reaction of HNO with oxygen forms an oxidant with a distinct chemical profile compared to $ONOO^-$ (Miranda et al., 2001, 2005). In contrast to our results, it was shown that low concentrations (less than 100 μM) of AS increase lipid peroxidation in brain homogenates and that sodium nitroprusside stimulates formation of TBARS in rat retinal homogenates (Osborne and Wood, 2004). However, the redox environment could affect the pro-oxidative effects of AS. In fact, AS does not increase the formation of fluorescent products of lipid peroxidation in substantia nigra (Vaananen et al., 2003).

It remains unknown whether the HNO signaling observed in the present study can occur endogenously. This deficit is caused largely by the current lack of an assay for detecting nitroxyl *in vivo* directly or even indirectly, although ongoing efforts may provide methods to obtain such data in the future. Several studies (Adak et al., 2000; Xia and Zweier, 1997) have shown that HNO can be synthesized by NOS isoforms *in vitro*, so there is reason to suspect that *in vivo* synthesis may indeed occur. Regardless of whether endogenous synthesis is ultimately confirmed, the present results suggest that HNO could regulate the hamster retinal nitridergic pathway by acting through a mechanism that only partly overlaps with that involved in NO response. In particular, present results indicate that both compounds inhibit its own synthesis by decreasing NOS activity and in the case of HNO also by reducing L-arginine availability.

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