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Homocysteine-Induced Brain Lipid Peroxidation: Effects of NMDA Receptor Blockade, Antioxidant Treatment, and Nitric Oxide Synthase Inhibition

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The effect of homocysteine (HCY) on lipid peroxidation (LP), a current mechanism of oxidative neurotoxicity, was investigated in rat brain synaptosomes. LP was assessed by measuring the amount of thiobarbituric acid-reactive substances (TBARS) formed from synaptosomal fractions following HCY treatment. Increasing HCY concentrations (5-1000 µM) enhanced the TBARS formation in brain synaptosomes in a concentration-dependent manner. When compared at equimolar concentrations (100 µM), the oxidative potency of HCY was lower than that of the oxidant ferrous sulfate, similar to that produced by glutamate (Glu) and the mitochondrial toxin 3-nitropropionic acid, and higher than that of the Glu agonists, kainate and quinolinate. The *N*-methyl-D-aspartate receptor (NMDAr) antagonist dizocilpine (MK-801) completely blocked the HCY-induced LP at concentrations from 5 to 1000 µM, whereas the well-known antioxidant *N*-acetylcysteine (NAC) was less effective, but still protective against the HCY oxidative toxicity at higher concentrations (400 and 1000 µM). Three nitric oxide synthase (NOS) inhibitors, 7-nitroindazole (7-NI), *N*ω-nitro-L-arginine (L-NARG) and *N*ω-nitro-L-arginine methyl ester (L-NAME), were also tested on HCY-induced LP at increasing concentrations. Both nonspecific NOS inhibitors (L-NARG and L-NAME) decreased more effectively the HCY-induced LP than did the selective neuronal NOS inhibitor, 7-NI. These results show that submillimolar concentrations of HCY can induce oxidative injury to nerve terminals, and this effect involves NMDAr stimulation, NOS activation, and associated free radicals formation.

Keywords: Homocysteine; Lipid peroxidation; Nitric oxide synthase; NOS inhibitors; NMDA receptor; MK-801; *N*-acetylcysteine; Free radicals

INTRODUCTION

Lipid peroxidation (LP), a common expression of oxidative stress, is a deleterious process affecting membrane lipids as the result of free radicals attack (Nakazawa *et al.*, 1996). A cell membrane whose phospholipids undergo peroxidation becomes rigid, loses channel function, and fails to preserve its own integrity. The disruption of membrane permeability or its fragmentation leads to Ca^{2+} influx and irreversible cell destruction (Nakazawa *et al.*, 1996). LP is particularly active in isolated nerve terminals presenting *N*-methyl-D-aspartate receptors (NMDAr) in their membrane surfaces (Šťastný *et al.,* 1999). Since several excitatory agents such as glutamate (Glu) and its agonists evoke oxidative stress in brain tissue after NMDAr stimulation and increased Ca^{2+} influx, LP has been involved in NMDAr-mediated excitotoxic injury (Coyle and Puttfarcken, 1993; Santamaría *et al.,* 2001).

Homocysteine (HCY), a naturally occurring amino acid containing a reactive sulfhydryl group (-*SH*), is related with the pathologic condition known as hyperhomocysteinemia, when its levels (and its disulfide, homocystine) are increased in blood (Jacobsen, 2000). Hyperhomocysteinemia is a vascular human disorder caused by deficiencies of key enzymes controlling HCY levels (McCully, 1969), and it has been associated with arteriosclerosis and stroke. The toxicity exerted by HCY on endothelial vascular cells has been attributed to its

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capability to produce highly cytotoxic reactive oxygen species (Jacobsen, 2000), while, in nerve tissue - particularly in cultured cortical neurons and cerebellar granule cells -, HCY toxicity has been related with NMDAr activation (Kim and Pae, 1996; Lipton *et al.,* 1997) and its consequent oxidative damage (Kim and Pae, 1996).

Conversely, nitric oxide (NO), a highly-diffusible molecule with physiological activity in the brain, is known to be a cellular messenger (Moncada *et al.,* 1989; Bredt and Snyder, 1990; Garthwaite, 1991), also exerting neurotoxic effects, depending either on its redox status (Lipton *et al.*, 1993; Snyder, 1993) or its concentrations (Kashii *et al.*, 1996). Consequently, NO has been currently related with pathological events such as apopotosis, oxidative stress and cell damage (Rubbo *et al.*, 1994), involving the Ca^{2+}-calmodulin- (Ca^{2+}/CAM-) dependent activation of constitutive nitric oxide synthase (cNOS) after NMDAr activation (Garthwaite *et al.*, 1989; Kiedrowski *et al.*, 1992). The role of NO as a potential factor to induce toxicity has been reported in several models, including excitotoxic conditions produced by glutamate agonists, such as quinolinic acid (Santamaría *et al.*, 1997; Pérez-Severiano *et al.*, 1998; Santamaría *et al.*, 1999), as well as in neurotoxic events, such as those produced by methamphetamine (Imam *et al.*, 1999), and these effects are likely to be mediated by the formation of peroxynitrite (Noack *et al.*, 1998; Imam *et al.*, 1999), a highly toxic molecule produced by interaction of NO with superoxide anion. Although it has been recently reported that the sulfhydryl group of HCY may react with NO, producing HCY nitrosation, and this effect may ameliorate HCY-induced neurotoxicity and calcium responses in primary cultures of rat cortical neurons, the precise role of NO on HCY neurotoxicity remains to be elucidated in light of the evidence mentioned above suggesting a possible neurotoxic role of NO. Therefore, in order to provide further information on the mechanisms of toxicity elicited by HCY in brain tissue, in this work we tested the effects of NMDAr blockade by dizocilpine (MK-801) (Santamaría and Ríos, 1993; Šťastný *et al.*, 1999), as well as NOS inhibition by either a selective neuronal NOS inhibitor 7-nitroindazole (7-NI), or the nonspecific NOS inhibitors *N*ω-nitro-L-arginine (L-NARG) and *N*ω-nitro-L-arginine methyl ester (L-NAME), on HCY-induced LP in brain tissue. In addition, since HCY toxicity has been associated with free radical formation, we also tested the effect of *N*-acetylcysteine (NAC) as an antioxidant and precursor of glutathione (GSH) (Pocernich *et al.*, 2001). For these purposes, brain synaptosomes represent a useful tool because this biological preparation behaves as metabolically autonomous minicells.

METHODS

Reagents

MK-801 was obtained from RBI (Natick, MA, USA). All other reagents, including HCY, were obtained from Sigma Chemical Co. (St. Louis, MO, USA) or Merck (Mexico). Deionized water (Milli R/Q System, Millipore) was used for preparation of all solutions.

Isolation of Brain Synaptosomes

Adult male Wistar rats (250-300 g) were housed 3 per cage under controlled standardized laboratory conditions, with food and water *ad libitum*. Animals were sacrificed by decapitation and their brains removed on ice. Animal sacrifice was carried out in accordance with the institutional guidance and general recommendations on the use of animals for scientific purposes, avoiding unnecessary pain. Crude synaptosomal P_2 fractions were isolated from whole brains, according to a previous report (Cotman and Matthews, 1971). All reagents employed throughout the study were prepared in deionized water.

Treatments and Assay of Lipid Peroxidation

Lipid peroxidation was estimated in isolated synaptosomes by the assay of thiobarbituric acid-reactive substances (TBA-RS) formation, according to a modified method (Santamaría *et al.*, 2001) from an original report (Ríos and Santamaría, 1991). Briefly, the synaptosomal fractions (970 µl aliquots) were incubated in a 40 mM Tris plus 10 mM HEPES buffer (pH 7.4) containing a subpathological concentration of glycine (10 µM) to evoke stimulatory activity of HCY on the NMDAr (Lipton *et al.*, 1997). Incubation was done at 37°C for 60 min in the presence of increasing concentrations of either HCY, MK-801, NAC, 7-NI, L-NARG and L-NAME (all 5-1000 µM), or their combinations (final volumes adjusted to 1000 µl). An additional set of experiments was performed to compare the effect of HCY with the oxidative potency evoked by other agents, and included the incubation of synatosomes in the presence of equimolar concentrations (100 µM) of either HCY, glutamate (Glu), different Glu agonists such as quinolinate (QUIN) and kainate (KA), the mitochondrial toxin 3-nitropropionic acid (3-NPA), or a well-known peroxidant agent (iron as $FeSO_4$). After incubation, samples were mixed with 2 ml of the TBA reagent (0.375 g thiobarbituric acid + 15 g trichloroacetic acid + 2.5 ml HCl). The reaction was controlled by addition of 10 µl desferrioxamine (1.5 mM) and 10 µl butylated hydroxytoluene (3.75% w/v) to the mixture. Final solutions were heated in a boiling water bath for 30 min and centrifuged at 3000*g* for 15 min. Optical density was measured in supernatants at

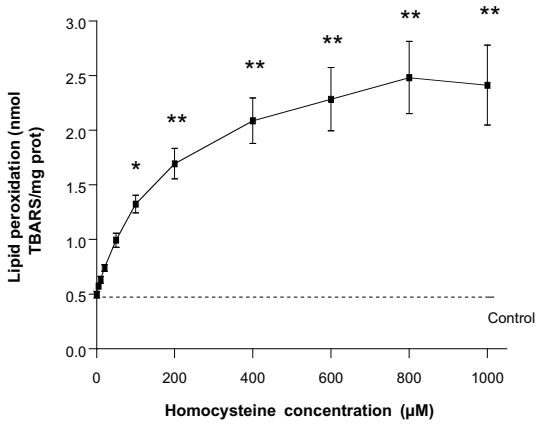

FIGURE 1 Concentration-response effect of homocysteine (HCY) on lipid peroxidation assessed by the formation of thiobarbituric acid-reactive substances (TBARS) in rat brain synaptosomal P_2 fractions. Each bar represents the mean ± S.E.M. from 6 experiments. *p <0.05 and **p <0.01 indicate statistical differences from control (without HCY); one-way ANOVA followed by Dunnett's test.

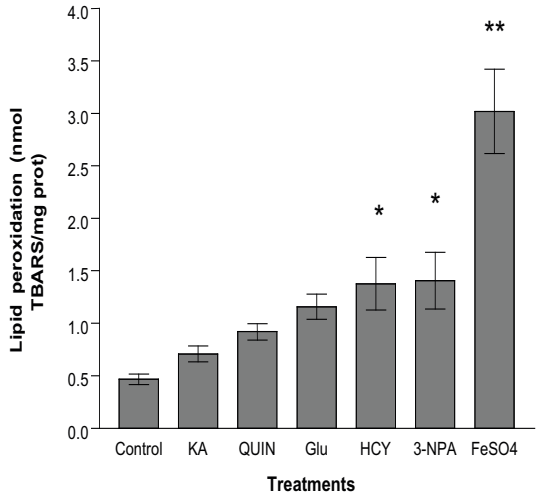

FIGURE 2 Comparative effects of homocysteine (HCY), kainate (KA), quinolinate (QUIN), glutamate (Glu), 3-nitropropionic acid (3-NPA), and ferrous sulfate (FeSO4) on lipid peroxidation in rat brain synaptosomes. Equimolar concentrations (100 μM) of each agent were added to synaptosomes in incubation. Each bar represents the mean ± S.E.M. from 5 experiments. *p <0.05 and **p <0.01 indicate statistical differences from control (without HCY); one-way ANOVA followed by Dunnett's test.

532 nm. Results were expressed as nmol of TBARS formed per mg of protein.

Statistical analysis

Data were analyzed by one-way ANOVA followed by Dunnett's test for comparisons against control. Values of p <0.05 and p <0.01 were considered statistically significant.

RESULTS

Concentration-Dependent Increase in TBARS by HCY

Incubation of rat brain synaptosomes in the presence of increasing concentrations of HCY resulted in a concentration-dependent enhancement of TBARS formation (FIG. 1), attaining significant levels at concentrations between 100 and 1000 μM (167% to 396%, respectively, as compared to the control value). Since 100 μM was the minimal concentration of HCY needed to attain a significant effect on TBARS formation, we characterized the oxidative properties of HCY under the proposed experimental conditions using this particular concentration.

Comparative Effects of HCY vs Other Toxins on Lipid Peroxidation

The oxidative potency of HCY in synaptosomal fractions was also compared with different agents at equimolar concentrations. Among these agents, we included excitotoxic molecules such as Glu and its agonists KA and QUIN, the mitochondrial toxin 3-NPA, and the well-known oxidant $FeSO_4$. HCY-induced TBARS formation was lower than that produced by $FeSO_4$ (-54%), similar to that exhibited by 3-NPA and Glu (-2% and 15%, respectively), and higher than that produced by the Glu agonists, QUIN and KA (50% and 94%, respectively) (FIG. 2).

Effects of MK-801 and NAC on HCY-Induced Lipid Peroxidation

In order to test whether the HCY-induced lipid peroxidation is mediated by NMDAr stimulation and / or by free radical-mediated oxidative stress, further experiments were performed in the presence of increasing concentrations of the NMDAr antagonist MK-801, and the antioxidant agent NAC, respectively. Both agents decreased the HCY-induced TBARS formation in a concentration-dependent manner, although MK-801 was more effective than NAC in preventing lipid peroxidation (FIG. 3). MK-801 produced a significant attenuation of HCY-induced lipid peroxidation even at the lowest concentrations tested (-37% for 5 μM, -45% for 10

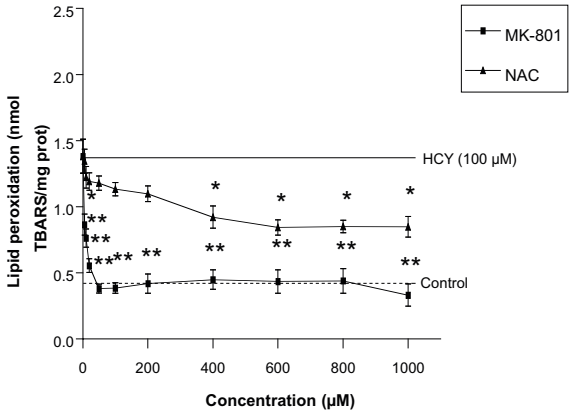

FIGURE 3 Concentration-response effects of dizocilpine (MK-801) and *N*-acetylcysteine (NAC) on homocysteine (HCY)-induced thiobarbituric acid-reactive substances (TBARS) formation in rat brain synaptosomal P_2 fractions. MK-801 and NAC were added independently to synaptosomes in incubation exposed to HCY (100 µM). Mean ± S.E.M. from 6-14 experiments are represented. $^{*}p < 0.05$ and $^{**}p < 0.01$ indicate statistical differences from control (with HCY alone); one-way ANOVA followed by Dunnett's test.

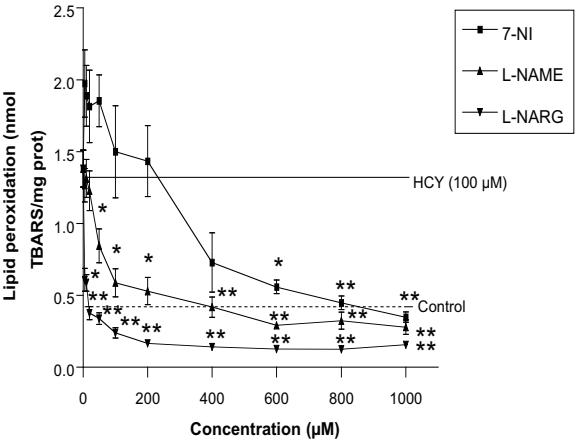

FIGURE 4 Concentration-response effects of 7-nitroindazole (7-NI), Nω-nitro-L-arginine (L-NARG) and Nω-nitro-L-arginine methyl ester (L-NAME) on homocysteine (HCY)-induced thiobarbituric acid-reactive substances (TBARS) formation in rat brain synaptosomal P_2 fractions. All nitric oxide synthase inhibitors were added independently to synaptosomes in incubation exposed to HCY (100 µM). Mean ± S.E.M. from 7-14 experiments are represented. $^{*}p < 0.05$ and $^{**}p < 0.01$ indicate statistical differences from control (with HCY alone); one-way ANOVA followed by Dunnett's test.

µM, and -60% for 20 µM, as compared to HCY alone), resulting in complete prevention at concentrations between 50 to 1000 µM, whereas NAC produced significant attenuation of HCY-induced TBARS formation at concentrations between 400 and 1000 µM (-33% for 400 µM, -39% for 600 µM, -38% for 800 µM, and -39% for 1000 µM, all vs HCY alone).

Effects of 7-NI, L-NARG and L-NAME on HCY-Induced Lipid Peroxidation

The effects of increasing concentrations of the NOS inhibitors 7-NI, L-NARG and L-NAME on HCY-induced LP are shown in Fig. 4. Addition of L-NARG plus HCY to synaptosomal fractions in incubation resulted in a significant reduction of TBARS formation at all concentrations tested (5-1000 µM, -56% to -89% vs HCY alone, respectively), while L-NAME produced a significative protective effect against HCY-induced TBARS formation from 50 µM and up to 1.0 mM(-39% to -80% vs HCY alone, respectively). 7-NI significantly decreased HCY-induced LP at concentrations between 600 and 1000 µM (-60% to -75% vs HCY, respectively). The order of efficacy against HCY-induced LP among the NOS inhibitors was as follows: 7-NI<L-NAME<L-NARG.

DISCUSSION

General Findings

This report describes the peroxidative action induced by HCY in brain synaptosomes. Its major findings were: a) the concentration-dependent peroxidative effect of HCY in synaptosomes found at micromolar concentrations; b) the oxidative potency exhibited by HCY, which was similar to that of Glu, but higher than other Glu agonists; c) the concentration-dependent protective action of the NMDAr antagonist MK-801 against HCY-induced oxidative damage, which was more effective that than exhibited by the antioxidant and glutathione precursor NAC; and d) the protective effects that three different NOS inhibitors exerted on the HCY-induced lipid peroxidation. Taken together, these findings lead us to support previous observations on a toxic nature of HCY on its own, either as a Glu receptor stimulant (probably acting as a Glu agonist at the NMDAr (Lipton *et al.*, 1997), as a modulator at the NMDAr, or at metabotropic Glu receptors (Folbergrová *et al.*, 1997)), as a pro-oxidant molecule, or a combination of both, given the higher oxidative potency exhibited by HCY in comparison with some other Glu agonists.

HCY, Oxidative stress and NMDA Receptor

In the nervous system although HCY has been typically related with excitatory events, such as increased intracellular Ca^{2+} concentrations in cultured cortical neurons after NMDAr activation (Lipton *et al.*, 1997; Kim, 1999), and seizures as an expression of excitotoxicity (Folbergrová *et al.*, 1997), most of the attention of HCY toxicity has been focused on diseases such as hyperhomocysteinemia, affecting vascular cells. In such pathologic processes, the hypothesis of an active role of oxidative stress as the key toxic insult of HCY is strongly supported (Jacobsen, 2000), since elevated levels of HCY have been reported to produce lipid peroxidation, which seems to be the main risk factor for vascular injury under a considerable number of experimental and clinical conditions (Jones *et al.*, 1994; Jacobsen, 2000), involving iron-catalyzed peroxidative processes, such as that observed for low-density lipoprotein (Hirano *et al.*, 1994). In this study, the concept of oxidative stress induced by HCY is supported by observations of stimulated lipid peroxidation at submillimolar concentrations, considerable lower than those producing lactate dehydrogenase leakage and cell death in cerebellar granule cells (Kim and Pae, 1996). However, the preferential susceptibility of this effect to MK-801 instead of NAC suggests that the lipoperoxidative action of HCY is more likely due to excitotoxic events first related to NMDA receptors activation with associated free radical production (Lipton, *et al.*, 1997), as MK-801, a well-known NMDAr antagonist (Santamaría and Ríos, 1993; Šťastný *et al.*, 1999), completely blocked the HCY oxidative toxicity at low concentrations. Therefore, it seems that the HCY-induced lipid peroxidation which is prevented by NAC, could be the result of free radical formation after excitotoxic events related with NMDAr activation. Moreover, a similar concentration of HCY (100 μM) used by others to produce damage to endothelial cells via the formation of hydrogen peroxide (H_2O_2) (Starkebaum and Harlam, 1986; Wall *et al.*, 1980), was also the minimal concentration needed to produce lipid peroxidation in our experiments, suggesting that the toxic mechanisms exerted by HCY both on endothelial and nerve cells could be partially sharing a similar component: excessive NMDAr stimulation and associated oxidative stress. This hypothesis is supported by evidence showing that NMDA-binding sites are also well-characterized in cerebral capillary membranes (Koenig *et al.*, 1992), and thus, the alterations produced by HCY in cerebrovascular disease might be the result of a cascade of toxic events following NMDAr activation and associated oxidative stress. Whether HCY is directly acting as an NMDAr agonist, or its excitatory actions are related with its interaction with NMDAr modulatory sites as suggested by Folbergrová and coworkers (1997), its a question deserving further investigation. Nevertheless, it cannot be ignored that NAC, at concentrations from 400 to 1000 μM, also proved to be protective against HCY-induced lipid peroxidation, suggesting that at least a fraction of the oxidative action of HCY is sensitive to antioxidant treatment. In fact, although the protective action of NAC on HCY-induced lipid peroxidation was less effective than that exhibited by MK-801, it represents a relevant physiological finding in light of its antioxidant properties. NAC has been shown to be an important antioxidant molecule (Halliwell, 1991; Pocernich *et al.*, 2001), also exhibiting antiapoptotic (Deigner *et al.*, 2000; Kannan and Jain, 2000) and neuroprotective properties (Fontaine *et al.*, 2000; Martínez *et al.*, 2000). NAC mainly acts as a thiol reductant and GSH precursor (Pocernich *et al.*, 2001), directly scavenging hydroxyl radicals mainly formed from H_2O_2, and there is evidence that H_2O_2 is a reactive oxygen species commonly formed by HCY (Wall *et al.*, 1980; Starkebaum and Harlam, 1986; Kim and Pae, 1996). Thus, it is likely that NAC could be reducing hydroxyl radicals generated from HCY-mediated H_2O_2 formation. Nevertheless, further studies are needed to support these considerations.

NOS Inhibition and HCY-Induced Lipid Peroxidation

Kim (1999) has recently suggested that NO attenuates HCY-induced neurotoxicity in primary cultures of rat cortical neurons, ameliorating the adverse properties of HCY via *S*-nitrosylation. Since it is known that NO reacts with thiol groups to form *S*-nitrosothiols (Byler *et al.*, 1983), and *S*-nitrosothiols may exert both cytoprotective and antithrombotic properties against HCY toxicity in vascular endothelial cells (Stamler *et al.*, 1993), it seems that NO might initially represent a protective factor against HCY neurotoxicity. However, our results suggest the opposite situation, given that inhibition of NO synthesis in synaptosomal preparations by three different NOS inhibitors resulted in a significant attenuation of HCY-induced LP. Possible explanations of our findings are related to recent observations published by D'Emilia and Lipton (1999), suggesting that HCY-induced neurotoxicity is dependent on the ratio of *S*-nitrosohomocysteine (SNHCY) to HCY or other thiols: it has been demonstrated that nitrosothiols, such as nitrosohomocysteine, may undergo homolytic cleavage to produce NO and subsequent neurotoxicity via the reaction with superoxide anion (O_2^-) to form a highly reactive and toxic molecule, peroxynitrite ($ONOO^-$) (Lipton *et al.*, 1993). In addition, these authors found that micromolar concentrations of nitrosothiols, such as SNHCY, generate detectable amounts of NO, but in the presence of excess thiol, such as HCY, no free NO was available to form

$OONO^-$. In light of these findings, we hypothesize that, under the experimental conditions we employed in this study (submillimolar concentrations of HCY in the presence of a subpathologic glycine concentration (10 μM) sufficient to evoke HCY-induced NMDAr activation), HCY may follow two toxic pathways: a) micromolar levels of HCY might produce NO via direct NMDAr activation and subsequent Ca^{2+}/CAM-mediated NOS-induced NO formation available for $OONO^-$ formation after its reaction with O_2^-, a mechanism currently described for conditions of NMDAr overactivation (Garthwaite *et al.*, 1989); or b) HCY might be reacting with NO generated from different sources to eventually form SNHCY by nitrosylation (Kim, 1999) at low levels, which in turn, in the absence of high concentrations of thiol groups (D'Emilia and Lipton, 1999), could be releasing NO to produce $OONO^-$ and further oxidative stress. Both of these mechanisms, alone or in combination, support a protective role of NOS inhibitors against HCY-induced LP. In fact, these considerations may also serve as an additional explanation for the protective effect of NAC observed in this work, since NAC represents an effective source of thiols (Pocernich *et al.*, 2001), potentially available for modulation of nitrosothiol:thiol balance.

Moreover, we have previously reported that the use of nonspecific NOS inhibitors, such as L-NARG (Santamaría *et al.* 1997; Santamaría *et al.*, 1999) and L-NAME (Pérez-Severiano *et al.*, 1998), represents a consistent protective factor to reduce QUIN-induced NMDAr-mediated neurotoxicity and oxidative stress in different rat brain preparations. Furthermore, since 7-NI, a well-known selective neuronal NOS inhibitor previously reported as effective against neurotoxic insults such as those produced by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridinium ion (MPP^+) (O'Byrne and Tipton, 2002), exhibited less potent antioxidant effect against HCY-induced LP as compared with L-NARG and L-NAME in our study, it is likely that both neuronal and endothelial forms of NOS are relevant to control NO levels and further HCY-induced oxidative damage.

In conclusion, the precise role of NO in HCY-induced neurotoxicity and oxidative stress depends on several factors, including glycine levels and NMDAr activation, triggering of Ca^{2+}/CAM-dependendent NOS activation and NO availability, rate of *S*-nitrosylation and *S*-nitrosothiol:thiol balance, and finally, peroxynitrite formation. Thus, this paradigm still remains to be elucidated in further investigations.

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