Effects of Nω-nitro-L-arginine and L-arginine on quinolinic acid-induced lipid peroxidation

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Abstract

The effects of a nitric oxide synthase inhibitor, Nω-nitro-L-arginine (L-NARG), and a nitric oxide precursor, L-arginine (L-ARG), on the lipid peroxidation induced by quinolinic acid (QUIN, an NMDA receptor agonist), were both tested in synaptosomal fractions from whole rat brain. Baseline of lipid peroxidation was found at 2.43 ± 0.24 fluorescence units/mg protein or 14.27 ± 1.24 nmoles of TBARS/mg protein (100%). QUIN (100 μM)-induced lipid peroxidation in synaptosomes (256% and 166% vs. control, as measured by lipid fluorescent products and thiobarbituric acid-reactive substances, respectively) was inhibited by concentrations of 10, 40, 100, 200 and 400 μM of L-NARG (74%, 58%, 56%, 48% and 48% vs. quinolinate value, respectively). Coincubation of synaptosomes with QUIN plus L-ARG (100 μM), which alone resulted a potent pro-oxidant (277% vs. control), increased the lipoperoxidative effect induced by QUIN alone in 120% (290% vs. control). Synaptosomes simultaneously exposed to QUIN (100 μM) plus L-ARG (100 μM) plus L-NARG (200 μM) showed levels of lipid peroxidation similar to those of quinolinate alone. These findings suggest that nitric oxide may contribute to the oxidative damage induced in vitro by QUIN. © 1997 Elsevier Science Ireland Ltd.

Keywords: Oxidative stress; Nitric oxide; NMDA receptor; Quinolinate neurotoxicity; Nω-nitro-L-arginine; L-arginine; Synaptosomal fractions

1. Introduction

Quinolinic acid (2,3-pyridine dicarboxylic acid, QUIN), an endogenous tryptophan metabolite from the kynurenine pathway, is also a potent...
neurotoxin. Its excitotoxic action is mediated by overactivation of glutamate receptors, as QUIN is a selective NMDA subtype of glutamate receptor agonist (Stone, 1993). QUIN has been commonly employed to reproduce some of the pathological consequences of Huntington’s disease (Schwarcz et al., 1984), and more recently, Heyes et al. (1992) described an involvement of QUIN in inflammatory and infectious human brain disorders as AIDS-dementia complex. Enhanced cytosolic Ca\(^{2+}\) concentrations, ATP exhaustion, circling behavior and GABA depletion (Foster et al., 1983; Schwarcz et al., 1984; Susel et al., 1989) are all important features observed as a result of QUIN-induced neurotoxicity, as well as increased oxidative stress and lipid peroxidation (Ríos and Santamaría, 1991; Santamaría and Ríos, 1993; Stípek et al., 1997).

On the other hand, nitric oxide (NO), a novel neuronal modulator, has been postulated to play a role as a retrograde messenger in the central nervous system, showing both regulatory and neurotoxic effects, depending on its redox status (Lipton et al., 1993; Synder, 1993) and on its concentrations (Kashii et al., 1996). NO is involved in several physiological processes such as vascular relaxation, long-term potentiation, learning and memory (Moncada et al., 1991); while, under pathological conditions, NO may promote apoptosis, oxidative stress and cell damage (Rubbo et al., 1994).

An increased production of NO mediated by a Ca\(^{2+}\)-calmodulin-dependent activation of the constitutive nitric oxide synthase (cNOS) has been demonstrated after stimulation of NMDA receptors (Garthwaite et al., 1989; Kiedrowski et al., 1992). In order to elucidate whether nitric oxide is able to modify the quinolinate-induced oxidative stress in vitro, a NOS inhibitor, \(\text{N}_\omega\)-nitro-L-arginine, and the NO precursor, L-arginine, were both employed to evaluate the generation of lipid peroxidation in QUIN-exposed rat brain synaptosomes, as measured by two current indexes of oxidative stress, the assay of lipid fluorescent products (LFP) and the assay of thiobarbituric acid-reactive substances (TBARS).

2. Materials and methods

2.1. Animals

Male Wistar bred-in-house rats (250–300 g) were used throughout the experiments. Animals were housed in acrylic box cages and provided with Rodent Chow (Purina Chow) and water ad libitum. Animals were maintained under conditions of constant temperature (25 ± 3°C), humidity (50 ± 10%) and lighting (12:12 light:dark cycle). Rats were killed by decapitation and their brains were then removed for tissue homogenization.

2.2. Chemicals

Deionized water (Milli R/Q System, Millipore) was used for preparation of all solutions. QUIN, 2-amino-5-phosphonovaleric acid (APV), Folin and Ciocalteu’s phenol reagent, quinine, 2-thiobarbituric acid (TBA), ferrous sulfate (FeSO\(_4\)), copper sulfate (CuSO\(_4\)) and sucrose were purchased from Sigma Chemical (St. Louis, MO). L-arginine (L-ARG) and \(\text{N}_\omega\)-nitro-L-arginine (L-NARG) were purchased from RBI. All other chemicals were obtained from E. Merck (Mexico).

2.3. Isolation of synaptosomal-enriched fractions

Synaptosomal fractions were obtained according to a modification (Ríos and Guzmán-Méndez, 1990) of the procedure reported by Löschler and coworkers (Löschler et al., 1985). Animals were killed by decapitation and their brains rapidly removed and briefly stored at −5°C in isotonic saline solution (0.9%, pH 7.4). Pooled brains (without cerebellum) from two animals were gently homogenized in a 0.32 M sucrose solution with a Teflon pestle-glass homogenizer (eight up-and-down strokes). Homogenates were then centrifuged at 1500 g for 10 min and the supernatants centrifuged again at 11 000 g for 20 min. The final supernatants were discarded and the pellets resuspended in 5 ml of sucrose (0.32 M). Resuspended preparations were gently layered onto 20 ml of a 0.8-M sucrose solution in polycarbonate tubes and centrifuged at 10 800 g for 27 min at 4°C in a
swinging bucket rotor. After removal of the first layer, the interface was recovered and diluted in 30 ml of a sucrose solution (0.32 M). A final centrifugation was performed at 24,000 g for 15 min. Pellets obtained at the end of this procedure were finally resuspended in 9 ml of a non-calcium-free isotonic saline solution and represented the synaptosomal fraction.

2.4. Treatment of brain synaptosomes

Volumes of 970 µl of the resuspended synaptosomal aliquots (final volume adjusted to 1 ml with deionized water) were exposed to equal volumes of 10 µl of either isotonic saline (pH 7.4) to reach final concentrations of QUIN (100 µM), APV (250 µM), L-ARG (100 µM), L-NARG (10, 40, 100, 200 and 400 µM) or some combinations of them. The concentrations of L-ARG and L-NARG used here were selected according to previous reports (Kiedrowski et al., 1992; Dawson et al., 1993; Vigé et al., 1993). Aliquots were then gently vortexed, incubated in a shaking water bath at 37°C for 1 h, and assayed either for the content of lipid fluorescent products or the thiobarbituric acid-reactive substances, as described below.

2.5. Assay of lipid fluorescent products (LFP)

Formation of lipid-soluble fluorescent products was measured by the technique described by Triggs and Willmore (1984) and modified for in vitro studies (Rios and Santamaria, 1991). After incubation, synaptosomal-enriched 1 ml aliquots were added with 3 ml of a chloroform-methanol mixture (2:1, v:v). Tubes were capped, gently vortexed for 15 s and placed on ice for 30 min. The aqueous phase was discarded and 800 µl of the chloroform layer were transferred into a quartz cuvette and mixed with 100 µl of methanol. The fluorescent signals of the samples were measured at 370 and 430 nm of excitation and emission wavelengths, respectively. The sensitivity of the spectrophotometer was adjusted just before measurement of samples, to 140 fluorescence units, with a quinine standard solution (0.1 µg/ml) prepared in 0.05 M of sulfuric acid. Final results were expressed as fluorescence units per mg of protein or as a percent of lipid peroxidation versus control values.

In order to assess the quality of the results of LFP monitoring, additional aliquots containing the synaptosomes were incubated under the same conditions in the presence of a well-known peroxidant, ferrous sulfate (2 µM).

2.6. Assay of thiobarbituric acid-reactive substances (TBARS)

Production of thiobarbituric acid-reactive substances was measured according to the technique previously described for in vitro studies (Rios and Santamaria, 1991). One ml aliquots containing the incubated synaptosomes were added with 2 ml of the TBA reagent (0.375 g of TBA + 15 g of trichloroacetic acid + 2.5 ml of concentrated HCl in 100 ml of water) and the final solution (3 ml total volume) was heated in a boiling water bath for 30 min. After ice-cooled, samples were centrifuged at 3000 g for 15 min, the absorbance was measured in the respective supernatants by spectrophotometry at 532 nm. Concentrations of TBARS were calculated by interpolation in a standard curve of periodic oxidation of 2-deoxy-D-ribose. Final results were expressed as nmoles of TBARS per mg of protein.

2.7. Protein measurement

Content of protein in tissue samples from brain synaptosomes was measured by the Folin and Ciocalteu’s phenol reagent (Lowry et al., 1951). Results of lipid peroxidation were corrected by protein content in each sample.

2.8. Statistical analysis

All data were analyzed employing a one-way ANOVA, followed by Tukey’s test for multiple comparisons (Steel and Torrie, 1980). Values of $P < 0.05$ were considered of statistical significance.
3. Results

3.1. Effect of iron on synaptosomal lipid peroxidation

Measurement of lipid peroxidation (LP) was first assessed by exposure of synaptosomal fractions to 2 μM of ferrous sulfate (FeSO₄), a well-known lipid peroxidant (Triggs and Willmore, 1984). LP induced by iron was enhanced by 581% (P < 0.05), as compared to control values (data not shown).

3.2. Effect of APV on quinolinate-induced lipid peroxidation

Fig. 1 shows the effect of APV on QUIN-induced LP. APV, a well-known competitive NMDA receptor antagonist, was first employed in our system in order to assess an involvement of NMDA receptors activation on LP mediated by QUIN, as previously reported (Lipton et al., 1993; Stıpek et al., 1997). Basal values of LP (100%) were found to be 2.43 ± 0.24 F.U./mg prot (n = 8; Fig. 1) or 14.27 ± 1.24 nmol TBARS/mg prot (n = 4; Table 1). Incubation of brain synaptosomes in the presence of QUIN resulted in a significant increase of LP, as compared to control values (256% as fluorescent products in Fig. 1 or 166% as TBA-reactive substances in Table 1). APV alone had no effect on basal LP (110% vs. control), whereas co-incubation of synaptosomes with QUIN plus APV completely inhibited the QUIN-induced LP (109% vs. control).

3.3. Effect of L-NARG on quinolinate-induced lipid peroxidation

Fig. 2 shows the effect of increasing concentrations of L-NARG, a NOS inhibitor, on QUIN-induced LP, as measured by the assay of LFP. Coadministration of QUIN plus L-NARG at concentrations of 10, 40, 100, 200 and 400 μM, decreased the lipoperoxidative effect produced by QUIN alone (74%, 58%, 56%, 48% and 48% vs. QUIN, respectively), showing concentration-dependency mainly among the lower concentrations (10 and 40 μM). The same concentrations of L-NARG, administered alone, did not produced significant changes on LP, as compared to control values (data not shown).

The effect of L-NARG (200 μM) on QUIN-induced LP was also tested by the assay of TBARS (Table 1). L-NARG alone had no effect on basal LP in the incubated synaptosomes, whereas coadministered with QUIN, L-NARG inhibited the oxidative action produced by QUIN (76% as compared to QUIN values).
Table 1
Lipid peroxidation in quinolinate- and L-NARG-treated synaptosomes

<table>
<thead>
<tr>
<th>Treatment</th>
<th>nmol TBARS/mg prot (mean ± S.E.M.)</th>
<th>% vs. Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>14.27 ± 1.24</td>
<td>100</td>
</tr>
<tr>
<td>QUIN (100 μM)</td>
<td>23.65 ± 1.30*</td>
<td>166</td>
</tr>
<tr>
<td>L-NARG (200 μM)</td>
<td>12.01 ± 0.70</td>
<td>84</td>
</tr>
<tr>
<td>QUIN + L-NARG</td>
<td>18.09 ± 0.87</td>
<td>127</td>
</tr>
</tbody>
</table>

Synaptosomal fractions were incubated for 1 h at 37°C in the presence of the different treatments; n = 4 experiments per group; *P < 0.05, significantly different from control value.

3.4. Effect of L-ARG on quinolinate-induced lipid peroxidation

Fig. 3 presents data (percentage versus control values) of the effect of L-ARG, the precursor of NO, as well as L-NARG at a concentration of 200 μM, on QUIN-induced LP. QUIN, coadministered with 100 μM L-ARG, slightly enhanced the pro-oxidant effect of QUIN alone by 120% (290% vs. control values). L-ARG alone significantly promoted the basal LP (277% vs. control). As shown in Fig. 2, QUIN, coadministered with L-NARG, significantly prevented the QUIN-induced LP, whereas L-NARG alone had no effect (Fig. 3). Synaptosomes coincubated with L-ARG (100 μM) + L-NARG (200 μM) showed no significant increase in LP values as compared to the control (103%, data not shown in Fig. 3). The effect of the same combined treatment (L-ARG + L-NARG) resulted to be significantly lower as compared to L-ARG alone (23% vs. L-ARG, P < 0.05). Finally, when synaptosomes were incubated in the presence of QUIN + L-ARG + L-NARG, lipid peroxidation was significantly increased by 233% as compared to the control values, but below the level of QUIN alone (91% vs. QUIN).

4. Discussion

In accordance with previous reports (Rios and Santamaria, 1991; Santamaria and Rios, 1993), we observed that quinolinic acid augmented the oxidative damage produced in vitro by the enhancement of lipid peroxidation, as measured both by LPS and TBARS assays. Such an effect is thought to be mediated by the selective activation of NMDA receptors, since an NMDA antagonist, APV, was able to completely abolish the quinolinate-mediated lipid peroxidation in rat brain synaptosomes. The same protective effect of NMDA receptor antagonists, such as kynurenic...
which seems to be partially related to the ability of quinolinate to form complexes with iron. It has also been reported that nitric oxide can cause a release of iron from ferritin, producing cell death (Reif and Simmons, 1990), suggesting that nitric oxide might contribute to the quinolinate-induced neurotoxicity by this mechanism.

In this work, we also found that the inhibition of nitric oxide synthase by L-NARG was able to inhibit lipid peroxidation after incubation of synaptosomes with quinolinate, suggesting that blockade of NO synthesis constitutes an important factor to prevent the production of reactive oxygen species and the further oxidative cell damage. In addition, L-ARG, the substrate of NOS which forms citrulline and NO, significantly increased the basal levels of lipid peroxidation, but it did not significantly increase the QUIN-induced peroxidative effect. The lack of summation of the peroxidative effects of L-ARG and QUIN suggests that both are acting through the same mechanism: enhanced formation of NO by NOS activation. However, this consideration remains to be elucidated. Interestingly, the coadministration of L-ARG plus L-NARG to synaptosomes, completely prevented the peroxidative effect induced by L-ARG alone, suggesting that the peroxidative effect of L-ARG is dependent on the production of NO by NOS. From these findings, it is not surprising to observe a partial protective effect of L-NARG on lipid peroxidation produced by QUIN + L-ARG, since L-NARG, in the ratio (2L-NARG:1L-ARG) employed for this purpose, has been shown to suppress the toxic action of L-ARG (Nakamura et al., 1995), reducing the peroxidation to the same level of QUIN alone, as observed in this study.

Although several reports suggested both modulatory and neuroprotective effects of NO in the CNS, such as regulation of mitochondrial respiration (Brown, 1995), reduction of brain damage in focal ischemia (Zhang et al., 1994), antioxidant actions (Kanner et al., 1991), blocked calcium influx in NADPH diaphorase-containing cortical neurons (Ikeda et al., 1993) and inactivation of the NMDA receptors by reaction with sulfhydryl groups at the redox modulatory site by formation of S-nitrosothiols and the consequent generation of disulfide bonds (Lei et al., 1992); other groups
have described alternative neurotoxic events mediated by NO, involving excessive stimulation of neurons by glutamate, besides of calcium influx and Ca\(^{2+}\)-calmodulin dependent NOS activation, protection against NMDA-induced neurotoxicity by inhibition of NOS and the participation of superoxide anions in the neurotoxicity elicited by NMDA (Dawson et al., 1993). Moreover, it has been demonstrated that increased levels of hydroxyl radicals are associated to NOS activation after NMDA receptors overstimulated (Hogg et al., 1992; Hammer et al., 1993).

Some other authors have shown that the stimulation of NMDA receptors by glutamate resulted in the activation of NOS, and a further production of NO (Kiedrowski et al., 1992). Nakamura et al. (1995) tested the effect of different drugs either related to the inhibition or the activation of NOS, on quinolinate-induced convulsions in mice. They found that L-ARGIN, either alone or in combination with 5,6,7,8-tetrahydrobiopterine (a cofactor of NOS), potentiated the clonic and tonic convulsions induced by QUIN, whereas N\(^{G}\)-monomethyl-L-arginine, a competitive NOS inhibitor, decreased the toxic action of QUIN.

Further studies testing other nitric oxide donors, as well as other NOS inhibitors, are needed to clarify the nature of nitric oxide-derived species involved in this pattern of toxicity. The relevance of this work can be also related to their implications on those neurotoxic mechanisms involved in the action of quinolinic acid as an endogenous metabolite in the CNS, which has been demonstrated to play a role in human neurological disorders (Heyes et al., 1992).

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References


