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Peroxynitrite Anion Stimulates Arginine Release from Cultured Rat Astrocytes

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Abstract: The biosynthesis of the physiological messenger nitric oxide (•NO) in neuronal cells is thought to depend on a glial-derived supply of the •NO synthase substrate arginine. To expand our knowledge of the mechanism responsible for this glial–neuronal interaction, we studied the possible roles of peroxynitrite anion ($ONOO^-$), superoxide anion ($O_2^{\bullet-}$), •NO, and H_2O_2 in L-[^{3}H]arginine release in cultured rat astrocytes. After 5 min of incubation at 37°C, initial concentrations of 0.05–2 m*M* $ONOO^-$ stimulated the release of arginine from astrocytes in a concentration-dependent way; this effect was maximum from 1 m*M* $ONOO^-$ and proved to be ~400% as compared with control cells. $ONOO^-$-mediated arginine release was prevented by arginine transport inhibitors, such as L-lysine and N^G-monomethyl-L-arginine, suggesting an involvement of the arginine transporter in the effect of $ONOO^-$. In situ xanthine/xanthine oxidase-generated $O_2^{\bullet-}$ (20 nmol/min) stimulated arginine release to a similar extent to that found with 0.1 m*M* $ONOO^-$, but this effect was not prevented by arginine transport inhibitors. •NO donors, such as sodium nitroprusside, *S*-nitroso-*N*-acetylpenicillamine, or 1-[2-(2-aminoethyl)-*N*-(2-ammonioethyl)amino]diazen-1-ium-1,2-diolate, and H_2O_2 did not significantly modify arginine release. As limited arginine availability for neuronal •NO synthase activity may be neurotoxic due to $ONOO^-$ formation, our results suggest that $ONOO^-$-mediated arginine release from astrocytes may contribute to replenishing neuronal arginine, hence avoiding further generation of $ONOO^-$ within these cells. **Key Words:** Arginine—Release—Nitric oxide—Peroxynitrite—Superoxide—Astrocytes.
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The CNS physiological messenger nitric oxide (•NO) (Palmer et al., 1987; Garthwaite et al., 1988) is biosynthesized from arginine through the •NO synthase (NOS)-catalyzed reaction (Bredt and Snyder, 1989). All isoforms of NOS [neuronal (nNOS), endothelial, and inducible] are present in CNS cells (Murphy and Grzybicki, 1996), and stimulation of glutamate receptors in neurones or in astrocytes leads to a Ca^{2+}-dependent activation of nNOS and/or endothelial NOS activity (Garthwaite et al., 1988; Agulló and Garcia, 1992). However, whereas under optimal substrate and cofactor conditions •NO is thought to be the major product of nNOS activity, an intracellular deficiency in arginine availability impairs glutamate receptor-dependent •NO formation in neurones (Culcasi et al., 1994).

Within the brain, free arginine is located predominantly in astrocytes (Aoki et al., 1991; Pow, 1994; Kharazia et al., 1997), which have the y^+ cationic amino acid-carrier system for arginine plasma membrane transport (Schmidlin and Wiesinger, 1994). Neurones are able to take this amino acid up, because they have the same carrier system (Westergaard et al., 1993). However, unlike in astrocytes, the arginine content in neuronal cells is very low and is limiting for nNOS activity (Garthwaite et al., 1989; Culcasi et al., 1994). Consistent with this, Grima et al. (1998) have demonstrated recently that the rate of nNOS activity in neurones depends on the supply of arginine from glial cells. Furthermore, glial-derived arginine is increased upon activation of ionotropic glutamate (non-NMDA) receptors (Grima et al., 1997), although the precise nature of the molecules involved in the intracellular signalling cascade leading to arginine release is not known.

Previous studies have demonstrated that purified nNOS activity in the absence of arginine (Pou et al., 1992) or arginine-depleted cultured neurones subject to glutamate (NMDA and non-NMDA) receptor activation

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Abbreviations used: CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; DETA/NO, 1-[2-(2-aminoethyl)-*N*-(2-ammonioethyl)amino]diazen-1-ium-1,2-diolate; DMEM, Dulbecco's modified Eagle medium; NAME, N^G-nitro-L-arginine methyl ester; NMMA, N^G-monomethyl-L-arginine; nNOS, neuronal nitric oxide synthase; •NO, nitric oxide; NOS, nitric oxide synthase; $O_2^{\bullet-}$, superoxide anion; $ONOO^-$, peroxynitrite anion; SNAP, *S*-nitroso-*N*-acetylpenicillamine; SNP, sodium nitroprusside.

(Culcasi et al., 1994) generate superoxide anion ($O_2^{\cdot-}$). However, under suboptimal arginine concentrations, H_2O_2 (probably derived from $O_2^{\cdot-}$) and $^{\cdot}NO$ are generated simultaneously by brain NOS activity (Heinzel et al., 1992). Under these conditions, both free radicals ($O_2^{\cdot-}$ and $^{\cdot}NO$) are thought to react together to form peroxynitrite anion ($ONOO^-$) (Beckman et al., 1990), an unstable compound that has been shown to be toxic for neurones, but not for astrocytes (Bolaños et al., 1995).

As arginine deficiency shifts glutamate-mediated neuronal $^{\cdot}NO$ biosynthesis towards the production of $ONOO^-$ and/or $O_2^{\cdot-}$, we wondered whether under certain conditions these molecules might have an important physiological role in the sense of their acting as signalling molecules to facilitate astrocytic arginine release, i.e., preventing sustained $ONOO^-/O_2^{\cdot-}$ formation by neurones following activation of nNOS. Here we address the issue of whether these neurotoxic anions are responsible for the stimulation of arginine release from astrocytes. We show that $ONOO^-$ and $O_2^{\cdot-}$, but not $^{\cdot}NO$ donors or H_2O_2, stimulate arginine release in cultured rat astrocytes. Moreover, whereas the effect of $O_2^{\cdot-}$ was unspecific, the effect of $ONOO^-$ appeared to be mediated by arginine transporter activation. We suggest that $ONOO^-$ formation may be a mechanism contributing to the replenishment of intracellular arginine, hence avoiding further generation of $ONOO^-$ in neuronal cells.

MATERIALS AND METHODS

Materials

$ONOO^-$ was synthesized and quantified spectrophotometrically ($\varepsilon_{320} = 1{,}670\ M^{-1} \times cm^{-1}$) as previously described (Hughes and Nicklin, 1968). Alkaline stock solutions, with an approximate $ONOO^-$ concentration of 0.3–0.4 *M*, were stable at −70°C for at least 3–4 months. Synthesized $ONOO^-$ had a half-life of ≈1.7 s at pH 7.4 (37°C) in Hanks' buffer (5.26 m*M* KCl, 0.43 m*M* KH_2PO_4, 132.4 m*M* NaCl, 4.09 m*M* $NaHCO_3$, 0.33 m*M* Na_2HPO_4, 5.44 m*M* glucose, 2 m*M* $CaCl_2$, and 20 m*M* HEPES, pH 7.4). Dulbecco's modified Eagle medium (DMEM) and fetal calf serum were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Plastic tissue culture dishes were purchased from Nunc (Denmark). L-[2,3,4,5-^{3}H]Arginine (63 Ci/mmol), L-[U-^{14}C]serine (166 mCi/mmol), L-[U-^{14}C]glutamic acid (150 mCi/mmol), and L-[2,3,4,5-^{3}H]citrulline (65 Ci/mmol) were from Amersham International (Buckinghamshire, U.K.). Sodium nitroprusside (SNP), *S*-nitroso-*N*-acetylpenicillamine (SNAP), and 1-[2-(2-aminoethyl)-*N*-(2-ammonioethyl)amino]diazen-1-ium-1,2-diolate (DETA/NO) were from Alexis Corp. (San Diego, CA, U.S.A.). Xanthine oxidase was obtained from Boehringer Mannheim (Germany). Xanthine, N^G-monomethyl-L-arginine (NMMA), N^G-nitro-L-arginine methyl ester (NAME), L-arginine, L-citrulline, L-ornithine, and L-lysine were obtained from Sigma. TLC silica gel plates were from Merck (Darmstadt, Germany). 6-Cyano-7-nitroquinoxaline-2,3-dione (CNQX) was purchased from Research Biochemicals Inc. (Natick, MA, U.S.A.).

Animals

Albino Wistar rats fed ad libitum on stock laboratory diet were used for the experiments. Rats were maintained at 23°C with a 12-h light/dark cycle. Virgin females weighing 210–250 g were caged overnight with males, and conception was confirmed the next morning by the presence of spermatozoa in vaginal smears.

Cell culture

Astroglia-rich primary cultures derived from neonatal 1-day-old rats were prepared as previously described (Bolaños et al., 1997). Cell suspensions were plated in DMEM supplemented with 10% (vol/vol) fetal calf serum at a density of 1.25×10^5 cells/cm^2 in 150-cm^2 flasks. Cells were maintained in a humidified incubator under an atmosphere of 5% CO_2/95% air at 37°C with a medium change twice a week. After 12–14 days astrocytes reached confluence, and cells were collected by trypsinization, reseeded in DMEM plus 10% (vol/vol) fetal calf serum at a density of 2.5×10^5 cells/cm^2 in six-well plates, and used after 24 h.

Amino acid release experiments

Release experiments were carried out essentially as previously described (Ohkuma et al., 1995). In brief, 24 h after reseeding the culture medium was removed and cells were washed once with prewarmed (≈37°C) Hanks' buffer. Astrocytes were then incubated in 1 ml of fresh Hanks' buffer containing 1 μCi/ml L-[2,3,4,5-^{3}H]arginine, L-[2,3,4,5-^{3}H]-citrulline, L-[U-^{14}C]serine, or L-[U-^{14}C]glutamic acid at 37°C. After 30 min, the buffer was discarded and cells were washed three times with warm Hanks' buffer to remove excess extracellular radioactivity. Cells were incubated further in warm Hanks' buffer (containing the inhibitors when appropriate) for 10 min, the buffer was aspirated, and cells were again incubated in Hanks' buffer (containing the inhibitors when appropriate) for a further 20 min with a buffer change every 5 min. Liquid scintillation analyses of the samples collected every 5 min showed that radioactivity release after minute 20 was constant (basal release). At minute 25, $ONOO^-$ was added to the cells in Hanks' buffer (containing the inhibitors when appropriate) at final concentrations ranging between 0.05 and 2 m*M*; cells were incubated for a further 5 min and the radioactivity released was counted. The maximum volume of stock $ONOO^-$ solution added to Hanks' buffer was 2 μl, giving a final buffer pH between 7.3 and 7.4. Control cells were treated in the same way, except that the $ONOO^-$ solution was previously degraded in Hanks' buffer at 37°C for 15 min before being added to the cells. Despite this, previous experiments had shown that amino acid release from astrocytes treated with Hanks' buffer lacking "degraded" $ONOO^-$ was identical to that obtained with "degraded" $ONOO^-$. In some cases, an aliquot of the buffer was subjected to TLC analysis for identification of the radioactive sample, as described below. The release of amino acids was expressed as percentages of release obtained in each treatment as compared with the controls (degraded $ONOO^-$-treated cells), which were conferred arbitrarily a value of 100%. In experiments focused on inhibiting arginine transporter, all incubations (except the first 30-min L-[^{3}H]arginine loading incubation) were carried out as described above in the presence of L-lysine or NMMA at the indicated concentrations. For the selective inhibition of NOS activity, all incubations (except the first 30-min L-[^{3}H]arginine loading incubation) were carried out as described above in the presence of NAME at the indicated concentrations. Finally, for the inhibition of non-NMDA subtype glutamate receptor, all incubations (except the first 30-min L-[^{3}H]arginine loading incubation) were carried out as described above in the presence of 30 μ*M* CNQX.

In some experiments, the effect of NO, $O_2^{\cdot-}$, or H_2O_2 on L-arginine release was also studied. In these cases, the protocol used was identical to the one described above except that, instead of $ONOO^-$, fresh solutions (final concentrations) of SNP (1 m*M*), SNAP (0.1 or 1 m*M*), DETA/NO (1 m*M*), H_2O_2 (1 m*M*), or a mixture of xanthine (400 μM) plus xanthine oxidase (20 U/ml) were added. The radioactivity released was quantified after 5 min of incubation at 37°C. The generation of $O_2^{\cdot-}$ from the xanthine/xanthine oxidase system was previously determined spectrophotometrically in Hanks' buffer at 37°C by the superoxide dismutase-inhibitable reduction of cytochrome *c* (ε_{550} = 19,200 M^{-1} cm^{-1}) (McCord and Fridovich, 1969), proving to be 20 nmol/min/ml.

TLC analysis

In a series of L-[2,3,4,5-^{3}H]arginine-release experiments, TLC analysis of the buffer obtained after $ONOO^-$ treatment was carried out as previously described (Olken and Marletta, 1993). This was achieved by loading 6 μl of the radioactive samples on silica gel TLC plates, running these for 80 min at room temperature in chloroform/methanol/ammonium chloride (2:2:1, by volume) as the mobile phase. Standard solutions of L-arginine, L-citrulline, and L-ornithine in Hanks' buffer were run individually in parallel and as internal standards. Plates were developed with ninhydrin, and the amino acids were identified by comparison with the R_f values of the standards (0.23, 0.65, and 0.43 for L-arginine, L-citrulline, and L-ornithine, respectively). Silica gel was scraped off the plates from the corresponding stains, and the radioactivity present in each amino acid was counted and expressed as the percentage of total radioactivity loaded.

Statistical analysis

Data are expressed as mean ± SEM values for the number of culture preparations indicated in the figure legends. Statistical significance for the comparison of two groups was evaluated using Student's *t* test. $p < 0.05$ was considered significant.

RESULTS

$ONOO^-$ stimulates arginine release in a concentration-dependent fashion

The effect of $ONOO^-$ on arginine release from astrocytes was assessed by exposing cells previously loaded with L-[^{3}H]arginine to $ONOO^-$ or to degraded $ONOO^-$ (control cells) at 37°C for 5 min. The radioactivity released by the cells into the incubation medium was measured by liquid scintillation counting. Arginine release was expressed as the percentage of radioactivity (counts per minute) found in the medium as compared with that found in control cells, which was given a value of 100%. Under these conditions, the exposure of astrocytes to $ONOO^-$ concentration-dependently (0.05–2 m*M*) stimulated arginine release. This effect was maximal from 1 m*M* $ONOO^-$ and proved to be ~400% as compared with the corresponding controls (Fig. 1).

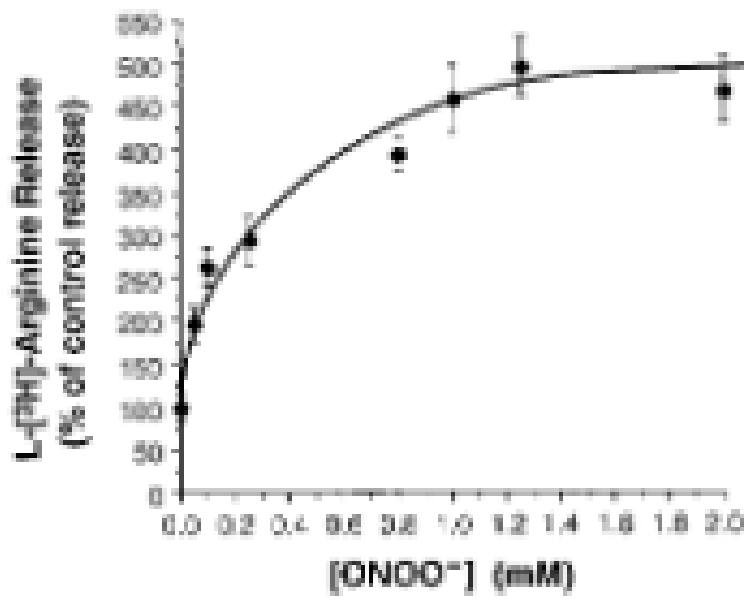

FIG. 1. Effect of $ONOO^-$ on arginine release from rat cultured astrocytes. Astrocytes were loaded with 1 μCi/ml L-[2,3,4,5-^{3}H]arginine for 30 min at 37°C. After washing, cells were incubated with Hanks' buffer for 10 min, the buffer was aspirated, and cells were again incubated in Hanks' buffer for a further 20 min with a buffer change every 5 min, until the basal radioactivity released was constant. At minute 25, $ONOO^-$ (0.05–2 m*M*) or degraded $ONOO^-$ (control cells) was added in Hanks' buffer; cells were incubated for a further 5 min, and the radioactivity released was quantified as described in Materials and Methods. The stimulated release of arginine in the presence of $ONOO^-$ was expressed as a percentage as compared with that in control cells (100% value). Values are means ± SEM from four to seven separate experiments. Control release was 1,184 ± 61 cpm/10^6 cells.

TLC analysis of the radioactive samples identifies L-arginine as the major amino acid released

Once inside the cell, arginine can be transformed readily into ornithine through arginase activity (Nakamura et al., 1990) or into citrulline through basal NOS activity (Agulló and Garcia, 1992). Accordingly, we assessed the relative presence of radioactive arginine, ornithine, and citrulline in the extracellular buffer after $ONOO^-$ stimulation. TLC analysis revealed that although most of the radioactivity was due to arginine (59.0 ± 0.5%), a considerable proportion (27.0 ± 3.5%) was due to ornithine. The radioactivity due to citrulline was lower (14.0 ± 2.8%). The recovery of radioactivity was ~100%.

Xanthine/xanthine oxidase-generated $O_2^{\cdot-}$, but not NO donors or H_2O_2, can also stimulate arginine release

We investigated the possible effect of $O_2^{\cdot-}$, NO, and H_2O_2 on arginine release by astrocytes. $O_2^{\cdot-}$ was generated by the xanthine/xanthine oxidase system, which under our experimental conditions yielded a constant $O_2^{\cdot-}$ production of 20 nmol/min in 1 ml of the incubation buffer, hence generating an estimated amount of $O_2^{\cdot-}$ of 0.1 mmol in the 5-min incubation period. As shown in Fig. 2, exposure of astrocytes to the $O_2^{\cdot-}$-generating system increased arginine release at a rate comparable to that found with 0.1 m*M* $ONOO^-$. In contrast, the presence of the NO donors SNAP (0.1 or 1 m*M*), SNP (1 m*M*), or DETA/NO (1 m*M*) only very weakly stimulated arginine release when compared with the effect achieved with $ONOO^-$ (Fig. 2). Exposure of astrocytes to 1 m*M* H_2O_2 did not affect arginine release (Fig. 2).

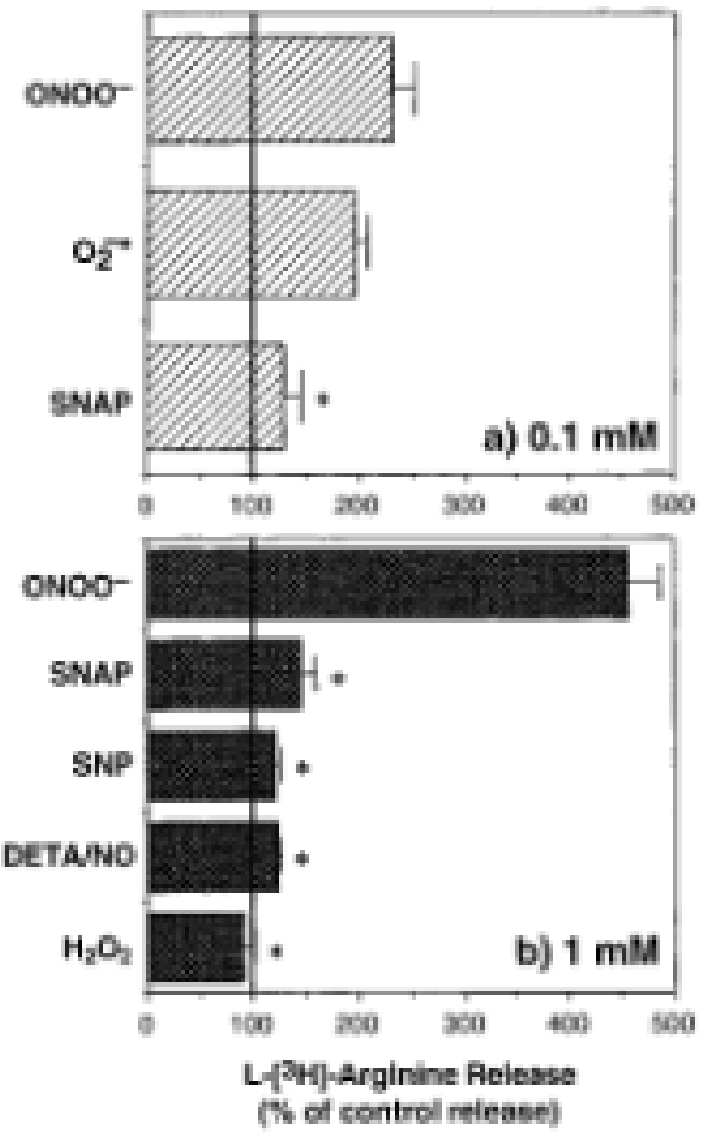

FIG. 2. Effect of $ONOO^-$, $O_2^{\cdot-}$, $^{\cdot}NO$ donors (SNAP, SNP, and DETA/NO), and H_2O_2 on arginine release from rat cultured astrocytes. Astrocytes were loaded with 1 μCi/ml L-[2,3,4,5-^{3}H]arginine for 30 min at 37°C. After washing, cells were incubated with Hanks' buffer for 10 min, the buffer was aspirated, and cells were again incubated in Hanks' buffer for a further 20 min with a buffer change every 5 min, until the basal radioactivity released was constant. At minute 25, $ONOO^-$ (0.1 or 1 mM), xanthine/xanthine oxidase-generated $O_2^{\cdot-}$ (20 nmol/min), SNAP (0.1 or 1 mM), SNP (1 mM), DETA/NO (1 mM), or H_2O_2 (1 mM) was added in Hanks' buffer; cells were incubated for a further 5 min, and the radioactivity released was quantified as described in Materials and Methods. The stimulated release of arginine in the presence of $O_2^{\cdot-}$, $^{\cdot}NO$ donors, or $ONOO^-$ was expressed as a percentage as compared with that in control cells (100% value). Values are means ± SEM from five separate experiments. Control release values ranged between 955 ± 85 and 1,140 ± 109 cpm/10^6 cells. *$p < 0.05$ versus $ONOO^-$.

Inhibition of the y^+ transport system prevents $ONOO^-$-mediated, but not $O_2^{\cdot-}$-mediated, arginine release

Arginine is transported through the y^+ carrier system for basic amino acids, which has been shown to be present in rat cultured astrocytes (Schmidlin and Wiesinger, 1994). To ascertain the involvement of this transporter in $ONOO^-$-mediated arginine release after L-[^{3}H]arginine loading, cells were preincubated further in the presence of increasing concentrations (0.1–5 m*M*) of L-lysine, an amino acid that strongly inhibits the y^+ arginine carrier system in cultured astrocytes (Schmidlin and Wiesinger, 1994). As shown in Fig. 3, the presence of L-lysine prevented $ONOO^-$-mediated arginine release in a concentration-dependent fashion. To ascertain whether the effect of $O_2^{\cdot-}$ was mediated through arginine transporter activation, the arginine transporter inhibitor L-lysine (1 m*M*) was included in the $O_2^{\cdot-}$ experiments as described in Materials and Methods. As shown in Fig. 4, L-lysine did not prevent $O_2^{\cdot-}$-mediated arginine release but did prevent $ONOO^-$ (0.1 m*M*)-mediated arginine release. Furthermore, the arginine transporter inhibitor NMMA (1 m*M*) prevented $ONOO^-$-mediated, but not $O_2^{\cdot-}$-mediated, arginine release (Fig. 4). Moreover, the effect of NMMA on $ONOO^-$-mediated arginine release does not seem to be due to a possible inhibition of NOS activity, because the NOS inhibitor NAME, which does not affect the arginine transporter (Schmidlin and Wiesinger, 1994), did not prevent $ONOO^-$-mediated arginine release (Fig. 4).

$ONOO^-$ mainly affects the release of arginine and, to a lesser extent, of citrulline, serine, or glutamate

To investigate the relative specificity of $ONOO^-$ in stimulating arginine release, we studied the effect of $ONOO^-$ on the release of other amino acids that use carrier systems different from the y^+ system. Thus, we tested the release of serine and citrulline as neutral amino acid representatives (using the L system) and glutamic acid as an acidic amino acid representative (Christensen, 1990; Wiesinger and Schmidlin, 1993; Schmidlin and Wiesinger, 1994). Table 1 shows that $ONOO^-$-mediated release of arginine was 3.0-, 2.5-, and 3.1-fold higher than that of serine, glutamate, and citrulline, respectively. The presence of the non-NMDA receptor inhibitor CNQX did not prevent $ONOO^-$-mediated arginine release (1 m*M* $ONOO^-$, 458 ± 38%, n = 4; 1 m*M* $ONOO^-$ + 30 μ*M* CNQX, 414 ± 25%, n = 4).

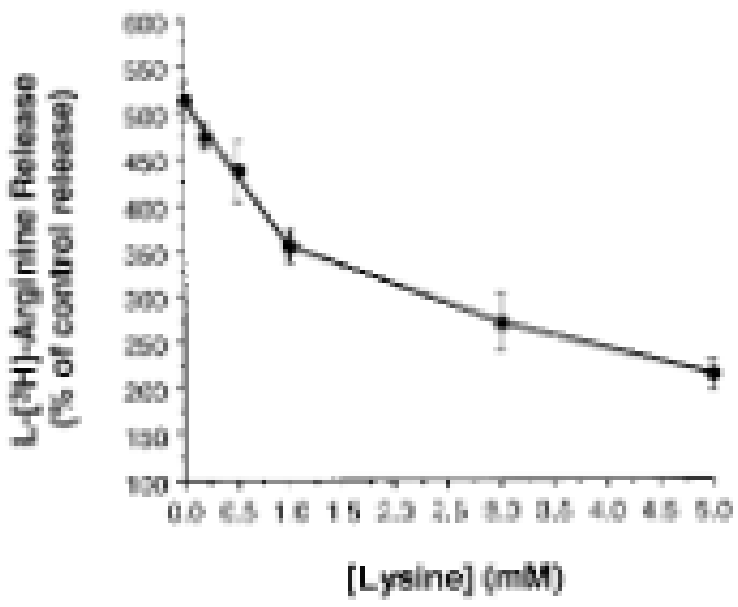

FIG. 3. Effect of L-lysine on $ONOO^-$ (1 m*M*)-induced arginine release from rat cultured astrocytes. Astrocytes were loaded with 1 μCi/ml L-[2,3,4,5-^{3}H]arginine for 30 min at 37°C. After washing, cells were incubated with Hanks' buffer containing L-lysine (0.1–5 m*M*) for 10 min, the buffer was aspirated, and cells were again incubated in Hanks' buffer (containing L-lysine) for a further 20 min with a buffer change every 5 min, until the basal radioactivity released was constant. At minute 25, $ONOO^-$ (1 m*M*) or degraded $ONOO^-$ (control cells) was added in Hanks' buffer (containing L-lysine); cells were incubated for a further 5 min, and the radioactivity released was quantified as described in Materials and Methods. The stimulated release of arginine in the presence of $ONOO^-$ was expressed as a percentage as compared with that in control cells (100% value). Values are means ± SEM from four separate experiments. Control release was 1,086 ± 31 cpm/10^6 cells.

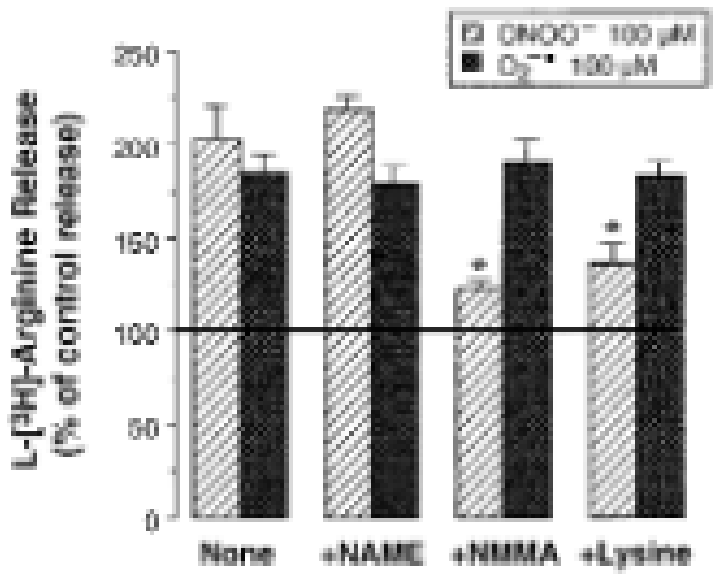

FIG. 4. Effect of L-lysine, NMMA, and NAME on $ONOO^-$ and $O_2^{\cdot-}$-mediated arginine release from rat cultured astrocytes. Astrocytes were loaded with 1 μCi/ml L-[2,3,4,5-^{3}H]arginine for 30 min at 37°C. After washing, cells were incubated with Hanks' buffer containing L-lysine (1 m*M*), NMMA (1 m*M*), or NAME (1 m*M*) for 10 min, the buffer was aspirated, and cells were again incubated in Hanks' buffer containing the inhibitors for a further 20 min with a buffer change every 5 min, until the basal radioactivity released was constant. At minute 25, $ONOO^-$ (0.1 m*M*) or xanthine/xanthine oxidase-generated $O_2^{\cdot-}$ (20 nmol/min) was added in Hanks' buffer containing the inhibitors; cells were incubated for a further 5 min, and the radioactivity released was quantified as described in Materials and Methods. The stimulated release of arginine was expressed as a percentage as compared with that in control cells (100% value). Values are means ± SEM from four separate experiments. Control release values ranged between 980 ± 35 and 1,202 ± 53 cpm/10^6 cells. *$p < 0.05$ versus $ONOO^-$.

DISCUSSION

In the present work, we show that exogenous $ONOO^-$ significantly stimulates arginine release from astrocytes in primary culture. This effect was concentration-dependent and was already detectable at 50 μ*M* $ONOO^-$, reaching maximum at 1 m*M* $ONOO^-$. These concentrations seem to be high in comparison with those used in other studies on cultured neuronal cells (Ohkuma et al., 1995). However, our previous studies had shown that, unlike neurones, astrocytes are highly resistant to $ONOO^-$ (Bolaños et al., 1995). Thus, addition of $ONOO^-$ at up to 2 m*M* to astrocytes does not cause cell death, as judged by lactate dehydrogenase release and mitochondrial respiratory function (Bolaños et al., 1995). In contrast, low $ONOO^-$ concentrations (10–100 μ*M*) are damaging for cultured neurones as judged by the same parameters (Lipton et al., 1993; Bolaños et al., 1995). Previous studies (Ischiropoulos et al., 1992) estimated that activated murine macrophages may generate $ONOO^-$ up to 0.11 nmol/10^6 cells/min, resulting in an approximate local rate of $ONOO^-$ synthesis of ~0.5–1.0 mmol/min. Taken together, these results suggest that neuronal $ONOO^-$ formation at amounts comparable to those used in the present study is not damaging to neighbouring astrocytes.

Previous reports have demonstrated that glutamate activates arginine release by acting on the non-NMDA receptor (Grima et al., 1997, 1998). As $ONOO^-$ causes glutamate accumulation, a possible activation of the non-NMDA receptor and subsequent arginine release may have occurred under our conditions. However, this did not seem to be the case, because the inhibition of the non-NMDA receptor with CNQX did not prevent $ONOO^-$-mediated arginine release. It should be mentioned that although the release of arginine was the highest found for the compounds tested (arginine, serine, glutamate, and citrulline), $ONOO^-$ may induce the release of several amino acids in a nonspecific manner. In any case, incubation of the cells with L-lysine, one of the most potent inhibitors of arginine transport in glial cells (Schmidlin and Wiesinger, 1994), prevented $ONOO^-$-mediated arginine release in a concentration-dependent way. This result suggests that the effect of $ONOO^-$ on arginine release is mediated by the y^+ arginine transporter.

It is well known that $ONOO^-$ causes protein sulfhydryl nitrosylation and lipid peroxidation (Radi et al., 1991*a*,*b*). Whether $ONOO^-$-stimulated arginine release is due to a possible modification in the y^+ arginine transporter remains to be elucidated. It is interesting that the $^{\cdot}NO$-mediated increase in GABA release from neurones is mediated by $ONOO^-$ (Ohkuma et al., 1995), although no mechanism to account for such an effect was suggested. Under our experimental conditions, $^{\cdot}NO$ donors were unable to stimulate arginine release, even at relatively high concentrations (1 m*M*). This lack of effect may be due to a low in situ $ONOO^-$ formation from added $^{\cdot}NO$ and cell-derived $O_2^{\cdot-}$. Alternatively, the effect of $ONOO^-$ on arginine release could be mediated by extracellular interaction with membrane components. In contrast, exposure of astrocytes to $O_2^{\cdot-}$ stimulated arginine release to a similar extent to that induced by 0.1 m*M* $ONOO^-$. This effect cannot be due to a possible superoxide dismutase-mediated H_2O_2 generation from $O_2^{\cdot-}$, because exposure of cells to up to 1 m*M* H_2O_2 did not

TABLE 1. *Effect of $ONOO^-$ on the release of several amino acids from rat cultured astrocytes*

Amino acid	Release (% of control)
L-Arginine	428 ± 29
L-Serine	145 ± 16*
L-Glutamate	174 ± 17*
L-Citrulline	136 ± 11*

Astrocytes were loaded with 1 μCi/ml L-[2,3,4,5,6-^{3}H]arginine, L-[U-^{14}C]serine, L-[U-^{14}C]glutamate, or L-[2,3,4,5,6-^{3}H]citrulline for 30 min at 37°C. After washing, cells were incubated with Hanks' buffer for 10 min, the buffer was aspirated, and cells were again incubated in Hanks' buffer for a further 20 min with a buffer change every 5 min, until the basal radioactivity released was constant. At minute 25, $ONOO^-$ (1 m*M*) or degraded $ONOO^-$ (control cells) was added in Hanks' buffer; cells were incubated for a further 5 min, and the radioactivity released was measured as described in Materials and Methods. $ONOO^-$-stimulated amino acid release was expressed as a percentage as compared with that of control cells. Values are means ± SEM from four separate experiments. The control release values (in cpm/10^6 cells) were 1,230 ± 59 for L-arginine, 1,319 ± 38 for L-serine, 1,351 ± 121 for L-glutamate, and 345 ± 10 for L-citrulline.

*$p < 0.01$, *$p < 0.001$ versus $ONOO^-$-stimulated L-arginine release.

alter arginine release. Furthermore, $O_2^{\cdot-}$-mediated stimulation of arginine release was not prevented by arginine transporter inhibitors, suggesting that $O_2^{\cdot-}$ does not activate the arginine transporter, but possibly causes a generalized plasma membrane disruption. Thus, whereas the exact molecular mechanism responsible for increased arginine release by $ONOO^-$ has not been elucidated in this study, it appears that a modification of the arginine transporter, possibly through $ONOO^-$-mediated protein nitrosylation, cannot be ruled out.

In conclusion, our results show that $ONOO^-$ stimulates arginine release, possibly as either a direct or indirect effect on the arginine transporter in cultured rat astrocytes. When the NOS substrate arginine becomes a limiting factor for NO synthesis (Culcasi et al., 1994), or in the absence of sufficient superoxide dismutase activity (Schmidt et al., 1996), glutamate-receptor stimulation actively produces $ONOO^-$ in neurones. As this compound is highly lipid-soluble (Beckman et al., 1990; Radi et al., 1991*a*), it is able to diffuse from neurones to astrocytes, hence representing an intercellular signal designed to activate arginine release from neighbouring astrocytes, which work as an arginine reservoir (Aoki et al., 1991; Pow, 1994; Kharazia et al., 1997). In contrast, ionotropic non-NMDA glutamate receptor activation stimulates arginine release in both astrocytes and brain slices (Grima et al., 1997, 1998). In this sense, it should be noted that glutamate (NMDA and non-NMDA)-receptor stimulation activates NOS activity in astrocytes (Agulló and García, 1992), as well as in neurones (Garthwaite et al., 1988; Frandsen et al., 1989; Gunasekar et al., 1995). Whether the reported glutamate-mediated increase in arginine release (Grima et al., 1997, 1998) is brought about by endogenous $ONOO^-$ production by neurones and/or astrocytes is not known, but the results reported in the present study are compatible with such a mechanism as an explanation for arginine release activation. As neurones are able to take up this amino acid efficiently from the extracellular fluid (Westergaard et al., 1993), a replenishment of cytosolic arginine could facilitate the formation of physiological NO, instead of neurotoxic $ONOO^-$ by neurones. If so, this neuronal–glial interaction could help to prevent the propagation of neuronal death upon excess glutamate-receptor activation.

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