Accumulation of Noradrenaline and Its Oxidation Products by Cultured Rodent Astrocytes

John X. Wilson¹ and Greame A. R. Wilson¹

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The accumulation of [3H]noradrenaline ([3H]NA) and its oxidation products was studied in primary cultures of cerebral astrocytes. Astroglial accumulation of radiolabeled catecholamine ([3H] NA and oxidation products) was enhanced by manganese or iron, but it was inhibited by unlabeled NA, dopamine or ascorbate. Tissue:medium ratios of radioactivity increased as extracellular [3H]NA was oxidized. When extracellular oxidation was prevented by ascorbate, as confirmed by high performance liquid chromatography with electrochemical detection, either ouabain pretreatment or nominally Na⁺-free incubation medium inhibited approximately one-half of specific [3H]NA accumulation by rat (but not mouse) astrocytes. These observations suggest that neurological responses to trace metals and ascorbate may arise from the effects of these agents on the clearance of extracellular catecholamines. Astrocytes can accumulate oxidation products of NA more rapidly than they take up NA itself, but ascorbate at physiological concentrations prevents the oxidation process in extracellular fluid. Furthermore, in the presence of ascorbate, Na⁺-dependent transport mediates a significant component of NA accumulation in rat astrocytes.

KEY WORDS: Catecholamine transport; catecholamine oxidation; ascorbate; astrocytes; rat brain; mouse brain.

INTRODUCTION

Termination of the neurobiological actions of catecholamines in brain is associated with binding and uptake of the transmitters and their metabolites by cells. Brain astrocytes are located around synapses and at the periphery of capillaries, so their uptake systems likely are important for regulating the neural environment. The presence in astrocytes of a system for catecholamine clearance was suggested by the observation of high-affinity, Na⁺-dependent [3H]noradrenaline ([3H]NA) uptake in rat astroglial cultures (1-4). However, other workers failed to confirm this observation (5,6). A possible problem in carrying out [3H]NA uptake experiments arises from the tendency of the catecholamine to oxidize in aqueous solutions at physiological pH. NA oxidation products polymerize to form neuromelanin (7-9), which has been observed to accumulate within cerebral astrocytes in situ (10,11) and in primary culture (12).

Ascorbate is stored in brain within neurosecretory granules and secreted with catecholamines, with the result that its concentration in extracellular fluid can rise to about 500 μM when catecholaminergic neurotransmission is stimulated (13). The vitamin may modulate neurotransmission since it has been found to alter catecholamine-mediated behaviors (14-16). In preliminary experiments, we found that ascorbate inhibits accumulation (binding and uptake) of radioactivity by mouse astroglial cultures incubated with [3H]NA (17-19). This finding was consistent with in situ studies of rat and guinea pig, which showed that turnover rates for brain NA vary inversely with changes in ascorbate concentration (20,21). We hypothesized that this physiological

¹ Department of Physiology, The University of Western Ontario, London, Ontario, Canada N6A 5C1.
effect of ascorbate might be attributable to its antioxidant properties. However, our observation that ascorbate and unlabeled NA inhibit the same component of radiotracer accumulation when mouse astrocytes are incubated with [3H]NA (19) appeared inconsistent with a report that ascorbate does not affect the Na\(^+\)-dependent component of [3H]NA uptake in rat astrocytes (4). Therefore, the present study investigates the mechanism by which ascorbate alters catecholamine clearance in astroglial cultures. Our results indicate that physiological concentrations of ascorbate prevent NA oxidation and that, in the presence of this antioxidant, a significant component of NA uptake by rat astroglial cultures is Na\(^+\)-dependent.

EXPERIMENTAL PROCEDURE

Materials. L-[7-3H(N)]NA (14-20 Ci/mmol) and D-[14C(U)]sorbitol (253 Ci/mmol) were purchased from Dupont Canada; horse serum, from Gibco Canada; fetal bovine serum, from Gibco Canada and Bocknek Laboratories; Nu-serum, from Collaborative Research; ferrous sulfate, from J.T. Baker Chemical Company; L-ascorbate, from J.T. Baker Chemical Company and Sigma Chemical Company; adrenochrome, dibutyryl cyclic AMP, 3,4-dihydroxybenzylamine hydrobromide, 3,4-dihydroxyphenylmandelic aldehyde, dopamine hydrochloride, ethylenediaminetetraacetic acid (EDTA) disodium salt, reduced glutathione, D-isoascorbate, manganese chloride, N-methyl-D-glucamine (NMG), L-NA hydrochloride, pargyline, tropolone and vanillylmandelic acid also were obtained from Sigma.

Cell Culture. Mouse L-2 fibroblasts (23) were cultured, as previously described (24), in Eagles minimum essential medium containing 8% Nu-serum plus 2% heat-inactivated fetal bovine serum. Primary cultures of astroglial cells were prepared from the neopallium of neonatal Swiss CD-1 mice and Sprague-Dawley rats according to the procedures of Hertz et al. (25). The glial cultures were incubated at 37°C, 95%/5% mixture of atmosphere and carbon dioxide) in 60 mm petri dishes containing modified Eagle’s minimum essential medium (22) with 20% serum. Horse serum was used for all mouse astroglial cultures, while either horse or fetal bovine serum was used for rat cultures. The culture medium was changed twice weekly and the serum concentration was reduced to 10% after 1 week. The cellular composition of representative astroglial cultures was confirmed by immunostaining for glial fibrillary acidic protein (24). Additionally, we observed that treatment with 0.25 mM dibutyryl cyclic AMP changed the flat polygonal astrocytes to process-bearing stellate astrocytes and increased GFAP mRNA levels (26). Unless stated otherwise in the text, polygonal astrocytes were grown for 4 weeks in culture before being used for experiments. Stellate cells were exposed to 0.25 mM dibutyryl cyclic AMP during weeks 3 and 4 in culture.

NA Analysis. The concentration of NA in the extracellular medium was determined by high performance liquid chromatography with electrochemical detection (HPLC-ED). 3,4-Dihydroxybenzylamine (1 μM) was added as an internal standard. Eluate fractions were collected for scintillation counting and the elution volumes of amines were determined with a Waters M460 amperometric detector. Retention times were: 3,4-dihydroxyphenylmandelic aldehyde, 1.3 min; vanillylmandelic acid, 1.4 min; adrenochrome, 1.8 min; NA, 3.0 min; 3,4-dihydroxybenzylamine, 5.6 min. The NA concentration of each sample was calculated by interpolation on a standard curve using a computer algorithm.

[3H]Catecholamine oxidation products were quantified as radioactivity eluted from the HPLC column during 0-2 min.

Measurement of Radiotracer Accumulation. Growth medium was aspirated and replaced by buffered salt solution (incubation medium). This incubation medium was made with Type 1 water (Barnstead Nanopure II) and contained (in mM): NaCl 122; KCl, 5.4; NaH2PO4, 1; glucose, 10; MgSO4, 0.8; Hepes, 20; NaHCO3, 10; CaCl2, 1.8 (pH 7.3). [3H]NA was included at a specific activity of 0.5 μCi/ml. In those cases where [3H]NA oxidation was to be prevented, the catecholamine was dissolved in incubation medium containing ≥ 10 μM reductant. The final concentration of total NA in the medium was achieved by adding unlabeled catecholamine. In some experiments, [3C]sorbitol (0.5 μCi/ml medium) was used as an extracellular marker. Cell cultures were incubated at 37°C in the buffered salt solution, then rinsed seven times with ice-cold sucrose solution and harvested. A 100 μl aliquot of the cell suspension was saved for protein determination (27) and the remainder was taken for scintillation counting. Tissue:medium ratios were calculated based upon an internal volume of cultured cerebral astrocytes of 4 μl/mg protein (28). In experiments where catecholamine oxidation was prevented, uptake rates were computed based on the specific activity of [3H]NA in the medium and expressed as pmol NA/g protein/min.

Statistics. Results are presented as the mean ± SEM of n number of independent experiments with duplicate or triplicate determinations in each. Half-maximal inhibitory concentrations (IC50) were determined by probit analysis. Differences between mean values were evaluated using paired or pooled t-tests and P < 0.05 was considered significant.

RESULTS

Unlabeled NA (Figure 1) and ascorbate (Figure 2) each inhibited accumulation of radioactivity in mouse

![Fig. 1. Inhibition by unlabeled noradrenaline (NA) and L-ascorbate (Asc) of radiotracer accumulation by mouse astroglial cultures incubated with [3H]NA. Polygonal astrocytes, after 4 weeks of culture in growth medium supplemented with horse serum, were incubated (10 min, 37°C) with or without 10 μM L-ascorbate in buffered salt solution containing 0.04 μM [3H]NA, 2 nM [3C]sorbitol and the indicated concentrations of unlabeled NA. Plotted are means ± SE of triplicate determinations. Error bars were omitted where smaller than the symbol.](image-url)
astrogial cultures incubated with [3H]NA. Half-maximal inhibitory concentrations (IC50) were 1 μM NA (n = 6) and 0.1 μM ascorbate (n = 8). There were no marked effects on cell integrity since [14C]sorbitol space was not changed by either NA or ascorbate (Figure 1). Astrocytes cultured for 2, 3 or 4 weeks all showed similar effects of ascorbate. Polygonal and stellate astrocytes were compared after 4 weeks in culture and were found not to differ with respect to rate of accumulation of radiolabeled catecholamine ([3H]NA and oxidation products) or sensitivity to ascorbate (data not shown).

L-Ascorbate, D-isoascorbate, and reduced glutathione all had similar concentration-dependent inhibitory effects on mouse astroglial [3H]catecholamine accumulation (Figure 2). Unlabeled dopamine also was an effective inhibitor and at a concentration of 100 μM it decreased the tissue:medium ratio of [3H]catecholamine to the same extent as did L-ascorbate (Figure 2).

The metal chelating agent, EDTA, slowed astrogial [3H]catecholamine accumulation (Figure 3). In contrast, omission of Ca2⁺ or Mg2⁺ from the incubation medium did not affect astroglial accumulation of radiolabeled catecholamine (data not shown). However, manganese and iron stimulated [3H]catecholamine accumulation and their effects could be diminished by ascorbate. Tissue:medium ratios of [3H]NA in mouse polygonal astrocytes at the end of 10 min incubations were: control, 1.3 ± 0.2; 10 μM ascorbate, 0.33 ± 0.05; 100 μM manganese, 2.6 ± 0.6; 10 μM ascorbate and 100 μM manganese, 0.33 ± 0.05; 100 μM iron, 11.7 ± 2.2; 10 μM ascorbate and 100 μM iron, 4.4 ± 0.2 (n = 3). Figure 3 shows that the inhibitory effect of ascorbate on manganese-stimulated accumulation of radiotracer increased with increasing concentration of the vitamin up to 10 μM.

To determine if the effect of ascorbate was exclusive to astrocytes, comparisons were made between mouse astrogial and L-2 cell cultures (Table I). During incubation with either cell type, significant amounts of [3H]NA were oxidized in the nominally ascorbate-free medium. In contrast, ascorbate (10 μM) preserved almost all of the external [3H]NA, limiting the concentration of [3H]catecholamine oxidation product to 2% of the total radiotracer in the medium. Retention times of the oxidation products in the HPLC system (< 2.0 min) were shorter than that of NA itself (3.0 min) and similar to those of 3,4-dihydroxymandelic aldehyde (1.3 min), vanillylmandelic acid (1.4 min) and adenochrome (1.8 min). Tissue:medium ratios of radiotracer in mouse astrocytes exceeded those in L-cells, but in both cell types radiotracer accumulation was decreased by approximately one-half when ascorbate was present to diminish [3H]NA oxidation (Table I).

Rat astrocytes incubated with [3H]NA also accumulated radiotracer by mechanisms that were sensitive to unlabeled NA and ascorbate. However, while in mouse astrocytes all of the radiotracer accumulation that could be inhibited by unlabeled NA could also be blocked by ascorbate (Figure 1), in rat astrocytes there was a spe-
Table I. Effect of Ascorbate on [3H]Noradrenaline Oxidation and Cellular Accumulation in Mouse Astroglial and L-2 Cell Cultures

|                      | Astrocytes | L-2 cells |
|----------------------|------------|-----------|
|                      | Control    | Ascorbate | Control    | Ascorbate |
| Medium Noradrenaline (μM) | 0.40 ± 0.08 | 0.98 ± 0.02* | 0.61 ± 0.03 | 1.06 ± 0.12* |
| Medium [3H]Catecholamine Oxidation Product (%) | 22.0 ± 4.9 | 2.3 ± 0.7* | 18.4 ± 2.5 | 1.7 ± 0.3* |
| Tissue:Medium Ratio of [3H] | 1.7 ± 0.4 | 0.85 ± 0.13* | 0.35 ± 0.05 | 0.20 ± 0.05* |

*Cultures of mouse polygonal astrocytes or L-2 cells were incubated for 10 min with [3H]noradrenaline (NA, nominally 1 μM). Ascorbate (10 μM) was present in the medium where indicated. The concentrations of total (radiolabeled and unlabeled) NA and [3H]catecholamine oxidation product in the medium at the end of the incubation were determined by HPLC with electrochemical detection and liquid scintillation counting. [3H]Catecholamine oxidation product is expressed as a percentage of total radiotracer in the medium. Data are the means ± SE of 3 independent experiments with triplicate replications in each. * P < 0.05 compared to control value.

The observation that unlabeled catecholamines and ascorbate inhibit accumulation of radiotracer in mouse astrocytes incubated with [3H]NA (Figures 1 and 2) may be explained by the redox properties of these chemicals. Ascorbate can prevent catecholamine oxidation by competing for oxidizing agents (29). When ascorbate and catecholamines are added to physiological salt solution, the sequence of addition determines the role played by the vitamin. Ascorbate acts as an antioxidant only if added to the solution before the catecholamine (29). The antioxidant property of ascorbate evidently underlies its inhibitory effect on astroglial [3H]catecholamine accu-
Fig. 4. Na⁺-dependence of [³H]noradrenaline ([³H]NA) uptake by rat astrocytes cultured in medium supplemented with fetal bovine serum. The effect of elevated intracellular Na⁺ concentration was determined by preincubating astroglial cultures for 2 h in serum-free medium with either ouabain (OUA, 0.5 mM) or drug vehicle (VEH), prior to the 1 min [³H]NA uptake incubation (1 min, 37°C, 0.04 μM [³H]NA and 10 μM ascorbate in medium). The effect of external Na⁺ was determined by measuring astroglial uptake in either control (CON) medium that contained 138 mM Na⁺ or nominally Na⁺-free medium in which N-methyl-D-glucamine (NMG) was substituted for Na⁺. The top panel shows initial rates of total uptake and the bottom panel shows specific uptake. Nonsaturable uptake was the accumulation of [³H]NA in the presence of excess (1 mM) unlabeled NA and was subtracted from the total uptake to determine specific uptake. Plotted are means ± SE of 4 experiments. *P < 0.05 compared to control values.

Fig. 5. Na⁺-dependence of [³H]noradrenaline ([³H]NA) uptake by rat astrocytes after culturing in medium supplemented with horse serum. The effect of elevated intracellular Na⁺ concentration was determined by preincubating astroglial cultures for 2 h in serum-free medium with either ouabain (OUA, 0.5 mM) or drug vehicle (VEH), prior to the 1 min [³H]NA uptake incubation (1 min, 37°C, 0.04 μM [³H]NA and 10 μM ascorbate in medium). The effect of external Na⁺ was determined by measuring astroglial uptake in either control (CON) medium that contained 138 mM Na⁺ or nominally Na⁺-free medium in which N-methyl-D-glucamine (NMG) was substituted for Na⁺. The top panel shows initial rates of total uptake and the bottom panel shows specific uptake. Nonsaturable uptake was the accumulation of [³H]NA in the presence of excess (1 mM) unlabeled NA and was subtracted from the total uptake to determine specific uptake. Plotted are means ± SE of 4 experiments. *P < 0.05 compared to control values.

mulation, since the IC₅₀ for the vitamin was identical to that of two other reductants, namely D-isoascorbate and glutathione (Figure 2). This antioxidant role may be of physiological importance in vivo since vitamin C deficiency enhances the turnover of brain NA (21) and elevated concentrations of ascorbate increase brain NA content (20).

The chelating agent EDTA has been shown previously to inhibit catecholamine oxidation initiated by some trace metals (9,30). Therefore, the fact that EDTA slowed [³H]catecholamine accumulation in astroglial cultures to the same extent as did ascorbate (Figure 3) implicates metal-catalyzed oxidation reactions in the accumulation process.

Manganese stimulated radiotracer accumulation by astroglial cultures incubated with [³H]NA (Figure 3). This observation is consistent with reports that manganese catalyzes the oxidation of catecholamines in vitro (30) and accelerates catecholamine turnover rates in the brains of adult rodents (32,33). The finding that manganese-stimulated catecholamine accumulation is opposed by ascorbate (Figure 3) may explain why steady state concentrations of NA in the brain are less affected by manganese in neonatal than in adult rats (34–36).
Thus, cerebral concentrations of ascorbate which are much higher in neonates than in adults (37) may defend the developing nervous system against manganese toxicity. Like manganese, iron can catalyze catecholamine oxidation (29). This catalytic property may have pathological significance because catecholaminergic dysfunction in Parkinson's disease is associated with elevated total iron and Fe$^{3+}$ contents in some brain regions (31). The present experiments found that iron stimulated accumulation of radiotracer by astrocytes incubated with $[3H]$NA. Ascorbate, which reduces ferric (Fe$^{3+}$) iron to the ferrous (Fe$^{2+}$) state (38) and prevents $[3H]$NA oxidation, inhibited the stimulatory effect of iron on radiotracer accumulation. Thus, the effects of ascorbate, EDTA, manganese and iron all indicate that astrocytes accumulate oxidation products of NA more rapidly than they take up NA itself.

L-2 fibroblasts also accumulated $[3H]$catecholamine oxidation products, but higher tissue:medium ratios were achieved in astrocytes (Table I). This difference suggests that astrocytes may be specialized to scavenge oxidized catecholamines. NA oxidation yields degradation products that are toxic to brain cells (39). The initial semiquinone and quinone products react to form noradrenochrome, which subsequently polymerizes to neuromelanin (7–9). Neuromelanin accumulates into cytoplasmic granules within astrocytes both in culture (12) and in situ (10,11). The avidity of astrocytes for catecholamine oxidation products may protect neurotransmission pathways. For example, the effect of manganese on $[3H]$catecholamine accumulation is stimulatory in astrocytes (Figure 3) but inhibitory in brain synaptosomes (40).

The evidence reviewed above indicates that cellular transport of authentic $[3H]$NA is best studied with the native reductant ascorbate present to prevent oxidation of the radiotracer. It is not clear in other studies of astroglial $[3H]$NA uptake (1–6) to what extent oxidation might have occurred. Particular experimental conditions can greatly influence results. For example, short incubation periods, such as 1 min in the present experiments, mitigate catecholamine oxidation at physiological pH and temperature. Additionally, high concentrations of antioxidants must be present from the moment that catecholamines are put into physiological saline (29), as was done in the studies that discovered Na$^{+}$-dependent $[3H]$NA uptake in rat astrocytes (Dr. H. Kimelberg, personal communication).

It is not clear why specific NA uptake was not detectable in mouse astroglial cultures (Figure 1). In contrast to NA transport, rates of specific, Na$^{+}$-dependent uptake of ascorbate are actually greater in mouse than in rat astrocytes grown under identical conditions in medium supplemented with horse serum (41).

Kimelberg and colleagues reported that removal of external sodium inhibited approximately 60% of total $[3H]$NA uptake by rat astrocytes incubated with 1 mM ascorbate and 0.1 $\mu$M $[3H]$NA for 60 min (3). However, investigations in other laboratories failed to confirm the presence of Na$^{+}$-dependent catecholamine transport in astrocytes (5,6). Unlike the present experiments, tests of Na$^{+}$-dependent catecholamine transport were carried out after preincubation of the cultures with pargyline and tropoline (100 $\mu$M), to inhibit monoamine oxidase and catechol-O-methyltransferase, respectively (1,6). In the present experiments, pargyline and tropoline did not increase initial rates of astroglial $[3H]$NA accumulation, perhaps because of a relatively short (1 min) incubation period during the $[3H]$NA transport assay, a period that was selected in order to ensure that oxidation remained negligible. Another methodological difference involves ascorbate concentrations, which were lower in the present experiments than the supraphysiological (1 mM) concentration employed by others (1,3).

In rat astrocytes incubated with ascorbate but without pargyline and tropoline, approximately one-half of the initial rate of total $[3H]$NA accumulation was sensitive to temperature and unlabeled NA (Figures 4 and 5). In turn, approximately one-half of this specific $[3H]$NA uptake was sensitive to ouabain pretreatment or incubation in nominally Na$^{+}$-free medium. This Na$^{+}$-dependent component was present in both of the rat astroglial models investigated, namely, those cultured in fetal bovine serum (Figure 4) or horse serum (Figure 5). Furthermore, the presence of Na$^{+}$-dependent $[3H]$NA uptake did not depend on a unique method of preparing astroglial cultures, since our culturing procedure (22,25) differed from the one previously used to show Na$^{+}$-dependence (1–3). It may be concluded that, in the presence of physiological concentrations of ascorbate, Na$^{+}$-dependent transport mediates a significant component of NA uptake by rat astrocytes.

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