Neuroscience Letters, 132 (1991) 141–145

ADONIS 0304394091006266

NSL 08142

Anoxia-resistant turtle brain maintains ascorbic acid content in vitro

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(Received 26 April 1991; Revised version received 22 July 1991; Accepted 25 July 1991)

Key words: Ascorbate; Vitamin C; Turtle; Cerebellum; Anoxia; Tissue compartmentalization

The isolated turtle brain maintains intra- and extracellular concentrations of ascorbate when incubated in ascorbate-free physiological saline for as long as 24 h. After incubation for 1 h, total tissue content of ascorbate in the turtle cerebellum was the same as in unincubated controls. After 20–24 h, tissue ascorbate content remained at 65% of control levels, while extracellular ascorbate concentration, measured with carbon fiber voltammetric microelectrodes, was 56% of the initial value. For an intermediate incubation period of 6 h, reduced ascorbate content was maintained at about 80% of control levels, regardless of whether incubation was under normal conditions or in the absence of glucose or oxygen. By contrast, only 4% of the ascorbate content of guinea pig brain slices remained after a 6 h incubation. Maintenance of high levels of ascorbate by the anoxia-resistant turtle brain could be an important factor in the amelioration of oxidative injury in this tissue. Inclusion of ascorbate in media used for in vitro studies of mammalian brain tissue is recommended.

Ascorbic acid is found at millimolar levels in all tissues of the body, including the brain [14, 25, 31]. This water soluble vitamin is fully dissociated as the anion, ascorbate, at physiological pH. In the brain, ascorbate is actively taken up from the circulation into cerebrospinal fluid [19, 38] against a 10-fold concentration gradient, then further concentrated into brain cells [39, 42]. Brain and spinal cord have among the highest concentrations of ascorbate in the body [12, 14]. The turnover rate in brain is only 2% per hour [39], which minimizes loss during ascorbate deficiency [16]. Paradoxically, when slices of mammalian brain are studied in vitro in ascorbate-free media, ascorbate is rapidly lost from the tissue [16, 18, 24, 35]. Indeed, total tissue content is decreased by 75% after incubation for only 30 min [35]. Interestingly, extracellular ascorbate concentration ($[Asc]_o$) is homeostatically maintained at normal in vivo levels (200–300 μM) during this period [35]. Normal tissue content can be maintained when ascorbate is included in the incubation medium at this usual extracellular concentration [24].

That the brain retains ascorbate so tenaciously in vivo supports the hypothesis that this vitamin has a critical role in brain function. One likely role is to help to maintain the reducing environment of the tissue and limit reactivity of free radical intermediates generated by normal aerobic metabolism [2]. Ascorbate, as a free radical scavenger, can neutralize peroxyl and hydroxyl radicals, as well as superoxide anion and singlet oxygen [2, 3, 27]. Such reactive species formed during reperfusion of the brain following stroke or ischemia [6, 10] have been proposed to initiate lipid peroxidation and other oxidative chain reactions leading to damage of brain vasculature and to cell death [9, 17]. Increased release and utilization of ascorbate, in fact, have been demonstrated using the middle cerebral artery occlusion (MCAO) stroke model [9, 13]; $[Asc]_o$ remains elevated during MCAO [13], while the total ascorbate content of the tissue falls continually during the occlusion [9]. Consequently, the effectiveness of ascorbate as a protective agent during reperfusion may be compromised if tissue ascorbate stores are depleted in the absence of replenishment from the blood supply.

In contrast to the susceptibility of mammalian brain tissue to anoxic damage, the brains of pond turtles are strikingly resistant to anoxic injury [15, 22]. Because of this property, isolated regions of turtle brain have been used successfully as in vitro preparations in recent years (for review, see ref. 15). In a previous study of ascorbate release in the isolated turtle cerebellum, we were surprised to find that stimulated increases in $[Asc]_o$ were sustained for many hours in vitro [31]. This suggested that tissue ascorbate levels might be maintained better

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by turtle brain tissue in vitro than by rat or guinea pig tissue. In the present study, we have examined total tissue content and resting $[Asc]_o$ in the isolated turtle cerebellum to evaluate ascorbate compartmentalization during prolonged incubations. In addition, we have compared the ratio of ascorbate to that of its oxidation product, dehydroascorbate (DHA) during incubation under hypoxia and ischemic conditions.

Turtles (*Pseudemys scripta* or *Chrysemys picta*) of either sex were rapidly decapitated, the cranium opened and the entire cerebellum dissected from the brainstem [20, 31]. Tissue to be used for control measurements of ascorbate content was immediately frozen on dry ice and stored at −70°C until analysis. For measurements of $[Asc]_o$, the cerebellum (6 animals) was transferred to a recording chamber [31] in which it was continually superfused with physiological saline of the following composition (in mM): NaCl 97, KCl 5, $NaHCO_3$ 40, glucose 10 or 20, $CaCl_2$ 3.5 and $MgCl_2$ 3.5, bubbled with 95% O_2/5% CO_2. Depth profiles of $[Asc]_o$ were recorded during the first 6 h of incubation, then again after 20–24 h in vitro. In separate experiments to determine ascorbate content after prolonged incubation, cerebella were hemisected and transferred to individual vials [32] that were continuously bubbled with 95% O_2/5% CO_2. After an average incubation time of 1 h, one hemisphere from each cerebellum was removed from solution, adherent solution was gently blotted from the tissue and the tissue frozen for total ascorbate analysis. The remaining tissues were incubated for 22 h longer, then frozen. In separate experiments to study the effect of hypoxia and glucose-free solutions on ascorbate and DHA, cerebella were incubated for an intermediate time of 6 h in individual vials that were bubbled with 95% O_2/5% CO_2 or 95% N_2/5% CO_2. Glucose-free media had the same composition as the normal medium, except for glucose content. All incubations were at room temperature, 22–25°C.

In one experiment, the ascorbate and DHA content of guinea pig brainstem slices that were incubated for 6 h were determined for comparison with the turtle brain results. The guinea pig slices were prepared as described elsewhere [21]. Guinea pig physiological saline had the following composition (in mM): NaCl 124, KCl 5, $NaHCO_3$ 25.8, KH_2PO_4 1.2, $CaCl_2$ 2.75, $MgCl_2$ 3.5 and glucose 10, bubbled with 95% O_2/5% CO_2. Incubation was at room temperature in individual vials as above. The ascorbate content determined for the guinea pig slices was probably slightly lower than endogenous levels, because the slices were cut while submerged in physiological saline, which contained no external ascorbate.

Ascorbate content was analysed by two different methods. To determine total tissue ascorbate only, the ascorbic acid oxidase method [34] was used exactly as described earlier [31]. For comparison of ascorbate and DHA content, an HPLC method was used [5]. After chromatographic separation, ascorbate was monitored at an electrochemical detector, whereas DHA was monitored at a tandem UV detector [5]. The data for tissue ascorbate and DHA content are presented as μmol per g tissue sample wet weight.

Extracellular ascorbate concentration was determined using voltammetric microelectrodes constructed from 35 μm carbon fibers [26, 31] (Avco, Specialty Materials Division, Lowell, MA). The voltammetric technique used was chronamperometry [26, 31] with current sampling at the end of a 100 ms duration voltage step (from −0.2 V to +0.45 V vs Ag/AgCl) applied from a two-electrode potentiostat [26] at 2 s intervals. Post calibration factors were used for all calculations of $[Asc]_o$ [31]. Previous studies [31] demonstrated that greater than 90% of the voltammetric response in the isolated turtle cerebellum was from $[Asc]_o$. The residual response after local injection of ascorbic acid oxidase was shown to be primarily from uric acid [31]. Other reducing agents, like glutathione, were not detectable at the potential used.

$[Asc]_o$ was sampled at 100 μm steps throughout the cerebellum; the baseline concentration 30 s after lowering the electrode to a new site was considered to represent resting $[Asc]_o$. Because of the observed gradient of $[Asc]_o$, with highest $[Asc]_o$ near the center of the tissue and decreasing levels near the superfused surfaces [31], the overall $[Asc]_o$ for the cerebellum was estimated from the average $[Asc]_o$ obtained from depth profiles obtained during incubation periods of 1–6 h and 20–24 h.

In marked contrast to earlier results with mammalian tissue [16, 18, 24, 35], the total ascorbate content of turtle brain after incubation for 1 h in ascorbate-free media was indistinguishable from that of unincubated controls (Table I). Furthermore, after 20–24 h in vitro, tissue ascorbate content was reduced by only 35% from control values (Table I). As discussed above, an $[Asc]_o$ gradient occurs in the turtle cerebellum in vitro [31]. This gradient, consistent with loss of ascorbate from the tissue, is maintained throughout incubation, although it became less steep with time. The average $[Asc]_o$ was reduced by 44% after 20–24 h (Table I) from the average $[Asc]_o$ after 1–6 h incubations. The average $[Asc]_o$ at 500 μm from the dorsal surface was 427 ± 59 μM ($n = 10$) during 1–6 h incubations, decreasing by 49% to 218 ± 21 μM ($n = 11$) after 20–24 h. The relative constancy of $[Asc]_o$ through periods of prolonged incubation demonstrated that turtle brain has a mechanism for $[Asc]_o$ homeostasis, as does mammalian brain [35].

Intracellular ascorbate concentration $[Asc]_i$ was calculated using the following expression:

$$0.25 \cdot [Asc]_o + 0.6 \cdot [Asc]_i = [\text{Total tissue Asc}].$$

TABLE I

ASCORBATE COMPARTMENTALIZATION IS MAINTAINED IN THE ISOLATED TURTLE CEREBELLUM IN ASCORBATE-FREE MEDIA

Values are mean ± S.D.; ascorbate content is given per g tissue wet weight.
*, from Rice & Nicholson [31].

Incubation period	Ascorbate content (μmol/g)	Ascorbate % control	$[Asc]_o$ (μM)	$[Asc]_i$ (μM)	$[Asc]_i$:$[Asc]_o$
Control	*1.81 ± 0.09 (n = 10)	-	-	-	
1–6 h	1.83 ± 0.23 (n = 4)	101	242 ± 51 (n = 103)	2950	12
20–24 h	1.18 ± 0.15 (n = 4)	65	136 ± 23 (n = 124)	1910	14

The extracellular compartment of the turtle cerebellum is about 25% of cerebellar volume, averaged from the extracellular volume fraction of the granular layer and molecular layer of the cerebellum (Rice, Okada and Nicholson, unpublished data), while the intracellular compartment can be estimated to be about 60%, with the remainder comprised of blood vessels and brain solids [8]. The ratio of $[Asc]_i$ to $[Asc]_o$ in turtle brain in vitro remained constant for 24 h (Table I), indicating the stability of ascorbate compartmentalization in the absence of an external supply.

Total tissue content of ascorbate and DHA were determined for turtle cerebella incubated with or without glucose for 6 h under normoxic or anoxic conditions. These data were compared with ascorbate and DHA in guinea pig brain slices incubated under normoxic conditions for the same period (Table II). The most striking feature of these results was that after 6 h of incubation, turtle brain ascorbate content was still 80% of control, while guinea pig content was only 4%. The ascorbate content of the turtle cerebellum was maintained at similar levels under normoxic conditions without glucose and under anoxic conditions with glucose present (Table II). The tissue did not survive anoxia without glucose, which is consistent with earlier reports [15]. This was reflected in the nearly complete loss of total ascorbate (ascorbate + DHA) from the tissue.

The ratio of Asc:DHA, typically about 10:1 in turtle brain tissue, generally reflected incubation conditions, and as such, might prove to be a useful index of utilization of ascorbate. This ratio (Table II) was smaller under aerobic than anaerobic conditions, and further de-

TABLE II

EFFECT OF IN VITRO ANOXIA AND ISCHEMIA ON TISSUE ASCORBATE AND DHA

Values are mean ± S.D. (n = 3); incubation period was 6 h; ascorbate and DHA content are given per g tissue wet weight.

Incubation conditions	Ascorbate content (μmol/g)	Ascorbate % control	DHA content (μmol/g)	Asc:DHA
Turtle cerebellum				
Control	1.7 ± 0.4	-	0.24 ± 0.12	7
O_2 with 10 mM glucose	1.4 ± 0.8	82	0.14 ± 0.10	10
O_2 no glucose	1.2 ± 0.3	70	0.13 ± 0.11	9
Control	4.1 ± 0.6	-	0.35 ± 0.13	12
N_2 with 10 mM glucose	3.1 ± 0.8	76	0.29 ± 0.06	11
N_2 no glucose	0.2 ± 0.1	5	0.06 ± 0.05	3
Guinea pig brainstem slice				
Control	1.40 ± 0.7	-	0.34 ± 0.16	4
O_2 with 10 mM glucose	0.06 ± 0.01	4	0.06 ± 0.05	1

creased in zero glucose. That anaerobic conditions did not simply protect Asc from oxidation is indicated by the low ratio of Asc:DHAA in the slices that did not survive zero glucose without O_2. In guinea pig slices with aerobic incubation, the ratio was unity.

The tissue content of ascorbate in turtle brain reported here and elsewhere [31] is comparable to that of mammalian [14, 25], including human [28], brain. Variation in control levels of ascorbate in the turtle cerebellum in the present study (Table II) apparently reflected real differences in tissue content between animals obtained from the supplier at different times. Seasonal variation in ascorbate content in various frog tissues has been reported [36]. Because the animals provided by our supplier are collected from various sites, depending on seasonal availability, individual histories were not known for the animals used in the present report. By coincidence, however, data from the control and incubated samples included in Table I and those in the O_2 incubation portion of Table II were obtained in the latter half of April, although in different years, while the higher values in Table II were obtained in March, the $[Asc]_0$ measurements were recorded over several months of the winter and spring. It should be noted, however, that the basic results were not affected by variations in absolute ascorbate content, because each study had its own control group.

The ability of turtle brain to maintain intra- and extracellular levels of ascorbate during incubation in ascorbate-free media suggests that ascorbate is being sequestered much more strongly than in mammalian brain cells, or may even be synthesized in the turtle brain. It is attractive to suggest that this sequestered ascorbate might be an important factor in the maintenance of turtle brain cell viability, as the following discussion elaborates. Conversely, if ascorbate loss from mammalian tissue in vitro were the result of cell death, maintenance of normal tissue ascorbate levels in turtle brain might be the result of superior turtle brain cell viability. Nonetheless, the continuous availability of this antioxidant to turtle brain cells would further protect them from oxidative damage.

Indeed, the maintenance of high ascorbate levels in the turtle is reminiscent of the high ascorbate content and anoxic resistance of neonatal mammals, compared to adults of the same species [1, 37], which points toward the role of antioxidants as protective agents under normal aerobic, as well as pathological conditions. A widely proposed mechanism of ischemic cell death involves the excitotoxic action of glutamate. Such a mechanism may be linked to the production of free radicals, since Ca^{2+} entry from excitatory amino acid receptor activation [33] can initiate second messenger processes known to produce reactive intermediates. Significantly, Wilson and Kriegstein [41] recently reported that turtle cortical cells are much less susceptible to glutamate-induced neurotoxicity than are mammalian brain cells. This may reflect the protective action of maintained levels of ascorbate in the turtle cells.

In keeping with the notion that ascorbate plays a vital role in brain function, it is important to note that ascorbate is rapidly lost from slices of mammalian brain tissue during incubations in ascorbate-free media [35], with nearly complete removal of this antioxidant after several hours (Table II). While it is clear that brain slices remain physiologically functional for prolonged periods in vitro, we recently found that including ascorbate and thiourea in physiological incubation media reduced edema in slices of rat neostriatum [32]. In addition, ascorbate has been implicated as a neuromodulator in a variety of neurotransmitter systems, especially the dopaminergic system of the striatum [7, 11, 30, 40]. Ascorbate interactions with glutamate neurotransmission [4, 29] are also becoming increasingly well-defined, including the recent observation that ascorbate can modulate *N*-methyl-D-aspartate (NMDA) receptor activity [23]. The retention of ascorbate by the anoxia-resistant turtle brain and the possible actions of ascorbate as a neuromodulator suggest that this vitamin should be included in incubation media used for studies of mammalian brain preparations in vitro.

We gratefully acknowledge support from NIH Grants NS-28480, NS-13742, NS-08740 and BRSG S07 RR05399 -29 and NSF Grant CHE-8607096. J.C. was an NIH predoctoral trainee under PHS Grant GM-07775. The authors thank Drs. C. Nicholson and R.N. Adams for their support and input and Dr. C.S. Leonard for providing guinea pig brain slices.

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