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ORIGINAL PAPER

Citrate, a Ubiquitous Key Metabolite with Regulatory Function in the CNS

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Abstract Citrate is key constituent of the tricarboxylic acid (TCA) cycle, serves as substrate for fatty acid and sterol biosynthesis, and functions as a key regulator of intermediary energy metabolism. Ursula Sonnewald had initiated studies using for the first time both proton- and ^{13}C-NMR to investigate metabolic processes in cultured neurons and astrocytes resulting in the important observation that citrate was specifically synthesized in and released from astrocytes in large amounts which is in keeping with the high concentration found in the CSF. The aim of this review is to highlight the possible roles of citrate in physiological and pathophysiological processes in the CNS. An interesting feature of citrate is its ability to chelate Ca^{2+}, Mg^{2+} and $Zn^{2+}$and thereby playing a pivotal role as an endogenous modulator of glutamate receptors and in particular the NMDA subtypes of these receptors in the CNS. Besides its presence in cerebrospinal fluid (CSF) citrate is also found in high amounts in prostate fluid reaching concentrations as high as 180 mM and here Zn^{2+} seems also to play an important role, which makes prostate cells interesting for comparison of features of citrate and Zn^{2+} between these cells and cells in the CNS.

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Introduction

Citrate is key constituent of the tricarboxylic acid (TCA) cycle, serves as substrate for fatty acid and sterol biosynthesis, and functions as a key regulator of intermediary energy metabolism [1, 2]. In addition, citrate is an excellent chelating agent [2], binding divalent cations like Ca^{2+}, Zn^{2+}, Fe^{2+} and Mg^{2+} all of which are essential for normal health and development. Studies performed in recent years have indicated that citrate may also play an important role in other key biological processes being part of inflammation, cancer, insulin secretion, histone acetylation, and non-alcoholic fatty acid liver [3]. In the central nervous system (CNS) there is insufficient knowledge about specific CNS processes in which citrate may play a prominent role. Using both in vivo and in vitro techniques it has been reported that citrate may have a functional role in neurotransmitter synthesis and release [4–6] as well as in pathophysiological processes [3, 7, 8] where the level of citrate is a potential harbinger. It is of note that at a time where the use of NMR spectroscopy technology was being pioneered to study brain metabolism of ^{13}C-labeled substrates such as glucose and acetate [25], Ursula Sonnewald had initiated studies using for the first time this technology based on both proton- and ^{13}C-NMR to investigate metabolic processes in cultured neurons and astrocytes [6, 9]. This resulted in the important observation that citrate was specifically synthesized in and released from astrocytes in large amounts [6]. The synthetic capacity for citrate in astrocytes had also been reported in the preceding work from the group of Herman Bachelard cited above [10] but they did not show this

very large capacity for release noted by Sonnewald et al. [6], a finding of importance for the demonstrations of a regulatory role of citrate as an extracellular chelating agent [5].

The aim of this review is to highlight the possible roles of citrate on physiological and pathophysiological processes in the CNS.

Citrate Synthesis

Citrate is synthesised as part of the TCA cycle in the mitochondria via condensation of acetyl CoA and oxaloacetate catalysed by citrate synthase (for details, see Schousboe et al. [11]). Citrate is isomerised to isocitrate by aconitase, an enzyme inhibited by fluorocitrate and fluoroacetate [2, 12], key inhibitors to probe astrocytic metabolism [12–14]. Besides being the major source of cellular ATP production after complete oxidation in the TCA cycle, citrate is transported to the extra-mitochondrial milieu by the citrate carrier situated in the inner mitochondrial membrane [15, 16] and which has been shown to have a widespread expression throughout the rat brain [15]. In the cytosol citrate serves as substrate for fatty acid and sterol synthesis by formation of acetyl-CoA and oxaloacetate catalysed by citrate lyase. Acetyl-CoA is then available for malonyl-CoA formation by acetyl-CoA-carboxylase. Malonyl-CoA represents a regulator of the citrate carrier [17]. In addition, cytosolic citrate exerts positive as well as negative allosteric modulations of enzymes involved in glycolysis, gluconeogenesis and fatty acid synthesis [1]. In this context it shall be mentioned that the brain contains the gluconeogenic and lipogenic key enzymes needed for de novo synthesis of glycogen, lipids and cholesterol from 2 to 3 carbon precursors [18–20]. However, these features seem mainly to be associated with astrocytes [18–20]. Since citrate and other TCA cycle intermediates are transported out of the mitochondria it is necessary to replenish these intermediates in order for the TCA cycle to remain operative. The major anaplerotic pathway for this in the brain is the carboxylation of pyruvate to form oxaloacetate catalysed by pyruvate carboxylase a process taking place in astrocytes and oligodendrocytes but not in neurons [21–24]. Citrate as well as other TCA cycle constituents has been found to be released from astrocytes to the extracellular space [25, 26] which is in agreement with the existence of a sodium dependent citrate transporter in astrocytes [27]. The above summarizes key metabolic processes in which citrate is involved as a regulatory agent in neurons and astrocytes. The different features will be discussed in more depth and put into context throughout this review.

Citrate Uptake and Release

It was shown using ^{13}C NMR spectroscopy on media from cultured neurons and astrocytes incubated with ^{13}C-labeled glucose or acetate, that only astrocytes were able to perform a net synthesis of citrate and to release it to the incubation medium [6, 28, 29]. A similar observation using ^{13}C NMR was made in brain slices [10]. This study demonstrated that citrate was more highly labelled from acetate than from glucose suggesting involvement of the astrocytic metabolic pool. As mentioned above, this study could not distinguish if the citrate was localised in the extra- or intra-cellular space. The metabolic features of citrate in both neurons and astrocytes were further characterised under different metabolic conditions [25] and a summary of key data are displayed in Table 1. The intracellular content of citrate was essentially the same in neurons and astrocytes whereas the release of citrate per hour was 12 times higher than the intracellular content in astrocytes indicating a considerable net synthesis and release to the incubation medium against a concentration gradient. This may be contradictory to the activity of CS in granule cells being slightly higher compared to astrocytes. It should, however, be kept in mind that the presence of PC is obligatory for net synthesis and therefore release of citrate and that granule cells are devoid of this enzyme (see above). Moreover, no saturation kinetics for citrate uptake could be demonstrated either by Westergaard et al. [25] or Metshitsuka and Aremu [30] and the CO_2 production from citrate was found to be low compared to other substrates such as glucose or glutamate [25]. Citrate release from astrocytes was found to be dependent on the concentration of bicarbonate in the incubation medium and exhibited saturation kinetics with an apparent K_m of

Table 1 Metabolic features of citrate in cultured cerebellar astrocytes and granule cells

	CS activity (nmol/min × mg)	Citrate release (nmol/h × mg)	Citrate content (nmol/mg)	CO_2 production (nmol/h × mg)
Cerebellar astrocytes	123.2	71.8	6.2	1.8
Cerebellar granula cells	178.6	4.1	4.7	0.8

Data are from [25] and further descriptions of methods can be found here. Citrate synthase (CS) activity was determined in cell extracts; citrate release was measured in cells incubated in bicarbonate buffered saline containing 3 mM glucose and cellular citrate content was measured in cell extracts. CO_2 production was monitored by the use of [^{14}C]citrate

2 mM. The rate of citrate release corresponds to the activity of PC [21] and the rate of CO_2 fixation in astrocytes [31] underlining the importance of PC as the rate limiting step in de novo synthesis of citrate since this enzyme requires bicarbonate as substrate. The inability of astrocytes to take up citrate has led to the suggestion that citrate could play a supportive role as energy substrate for neurons especially under hypoglycemic conditions [30]. As shown in Table 1, the results by Westergaard et al. [25] do not support this notion as can be seen by the low CO_2 production from neurons. However, under the assumption that CS activity is high in the intact astrocytes it seems evident that citrate is also a precursor for other metabolites since only 1% of the citrate produced from CS appears to be released. Citrate release can be blocked by the presence of aluminium in the culture medium probably by entering astrocytes to form an aluminium–citrate complex in the cytosol which is not transported out of the cells [30].

Functional Roles of Citrate in CNS

The ability of astrocytes to produce large amounts of citrate may be reflected in the relatively high concentration found (0.4 mM) in cerebrospinal fluid (CSF) similar to the level of glutamine [32–34]. The functional importance of such high concentrations of citrate in CSF and the large release of citrate from astrocytes is, however, not fully understood.

Analysis of CSF metabolites can provide information regarding their involvement in neurologic and psychiatric diseases. So far no correlation between citrate levels and levels of key metabolites (e.g. valine, glutamine, pyruvate and lactate) found in the CSF has been found in neurological diseases [33, 34] although, a positive correlation between CSF glutamine and Mg^{2+} concentrations has been reported in depressed patients compared to controls [33]. The above mentioned characteristics of citrate and the lacking ability of citrate to sustain transmitter release in glutamatergic neurons [25] and in vivo [14] do not support that citrate plays any important role in supplying neurons with the carbon skeleton for synthesis of neurotransmitter glutamate and GABA. However, a negative correlation between the concentration of citrate and glutamine has been shown both in vitro and in vivo by the use of fluorocitrate. Inhibition of the glial TCA cycle caused elevation of citrate and a decrease in glutamine leading to impairments of GABA synthesise [14] thus supporting the glutamate–glutamine cycle in the brain in vivo.

It thus appears more attractive to suggest a role of citrate as a chelator of divalent cations such as Ca^{2+}, Mg^{2+} and Zn^{2+}. It is therefore conceivable to believe that citrate might influence the excitable state of neurons via regulation of the free extracellular concentrations of these ions thereby playing a pivotal role as a endogenous modulator of glutamate receptors and in particular the NMDA subtypes of these receptors in the CNS [35].

It has been found that fluorocitrate or fluoroacetate, by inhibition of citrate degradation [36] increases the citrate level in brain [37] and citrate release from astrocytes [13, 38], a condition leading to seizures [39]. Simultaneous intracerebroventricular injection of Ca^{2+} abolishes the effect of fluorocitrate, indicating that the extracellular free Ca^{2+} concentration is pivotal in this context [39]. However, while the role of Ca^{2+} in neuro-excitability is well known and described, there is only an emerging growing recognition of the role of Zn^{2+} in the brain. Zinc ions are present in high amounts in the CNS and especially in the CA-3 region of the hippocampus where it has been estimated to be as high as 100–300 μM and in the brain in general in the range of 1–20 μM (cf. [40]). Also endogenous Zn^{2+} can be released from hippocampus during neuronal activity [39–41]. Zn^{2+} has been found to be taken up by presynaptic vesicles by the zinc transporter-3 (ZnT3), which is expressed e.g. in various nervous tissue and testes [42] and regulated by hormones, fatty acids, glucose and zinc-chelation [42]. Analysis of the PC12 cell lines expressing ZnT3, vesicular glutamate transporter-1 (Vglut-1) or both showed that vesicular zinc uptake was increased by Vglut1 expression. Conversely, production of ZnT3 increased the vesicular uptake of glutamate in a zinc-dependent fashion suggesting a coupling of ZnT3 and Vglut-1 transport mechanisms regulating neurotransmitter content in secretory vesicles [41]. Zn^{2+} is co-released with glutamate during brain activity [43–45] and has been shown to attenuate the NMDA-induced neurotoxicity [35, 46, 47]. For a more detailed view of the role of Zn^{2+} as a modulator of voltage- and ligand-gated ion channels, see [40, 48].

Studies of the function of Zn^{2+} in the brain have revealed that the biochemical action requires careful regulation of its concentration in order to ensure proper balance between normal physiological functions and pathological consequences [49]. In addition to its inhibitory action on NMDA receptors, Zn^{2+} has been shown to potentiate non-NMDA induced responses [50, 51] and non-NMDA induced neuronal cell death [52]. Taken together, these observations indicate that Zn^{2+} could play a major role as an endogenous modulator of the NMDA receptor in the CNS and citrate as a chelator of Zn^{2+} and other divalent cations could play a key part of this equation. As an example of regulation of Zn^{2+} and a physiological outcome, chelation of zinc in the extracellular area of the spinal cord, using EDTA or dipicolinic acid, inhibits the antinociceptive effect of capsaicin in adult mice suggesting zinc to play a role in pain [53].

Citrate and the NMDA Receptor

Formation constants (log K) for Ca–EDTA and Zn–EDTA complexes have been reported to be in average 11 and 16 and the corresponding constants for Ca–Citrate and Zn–Citrate 4.6 and 6, respectively, suggesting that both citrate and EDTA have a preference for Zn^{2+} over Ca^{2+} and Mg^{2+}. For details see [5, 25].

The influence of citrate on Zn^{2+}-mediated inhibition of the NMDA receptors under Mg^{2+} free conditions has been investigated using cerebellar granule cells and the *Xenopus* oocyte expression system [5]. In granule cells zinc inhibited in a dose-dependent fashion the evoked release of [^{3}H]D-aspartate with an IC_{50} value in the 5 µM range. Both EDTA and citrate were able to abolish the inhibitory action of Zn^{2+} in these cells with EDTA being most effective (Table 2). That endogenous Zn^{2+} can be released from brain tissue during neuronal activity has been shown to occur in hippocampus [43–45]. The results obtained from the studies of neurotransmitter release in granule cells are compatible with the results obtained from *Xenopus* oocytes injected with mRNA from cerebellum (Table 3). No attenuation of Zn^{2+} on glutamate + MK-801 (uncompetitive antagonist of the NMDA receptor [54]) and kainate induced responses were observed. These results clearly indicate that EDTA and citrate, by a chelating action can influence the attenuation of NMDA receptor activity produced by Zn^{2+} in a reversible manner. It is therefore attractive to suggest a hitherto unknown regulatory function of citrate and in particular astrocytes, since citrate is synthesized and released exclusively from these cells [25].

Table 3 Effects of zinc and citrate on excitatory amino acid induced currents in *Xenopus* oocytes injected with mRNA from mouse cerebellum

1.5 mM Ca^{2+}	% of control
300 µM NMDA (control)	100
300 µM NMDA + 50 µM Zn^{2+}	38
300 µM NMDA + 50 µM Zn^{2+} + 1.0 mM Citrate	160
100 µM KA (control)	100
100 µM KA + 50 µM Zn^{2+}	97
100 µM KA + 50 µM Zn^{2+} + 1.0 mM Citrate	103

Table summarizes experiments performed in *Xenopus* oocytes and shows the effect of 50 µM Zn^{2+} or 50 µM Zn^{2+} + 1.0 mM citrate on currents induced by 300 µM NMDA and 10 µM glycine or 100 µM kainate (KA) in oocytes injected with mRNA from mouse cerebellum. Results are expressed as percent of control (100%, no added Zn^{2+} and citrate) [5]

Role of Citrate Outside the CNS

Citrate is clearly an important key intermediary metabolite in cellular energy metabolism. In addition to that, recent studies have indicated that citrate may also play an important role in other key biological processes as part of inflammation, cancer, insulin secretion, histone acetylation, and non-alcoholic fatty acid liver (see [3, 8]). Besides its presence in CSF, citrate is also found in high amounts in prostate fluid reaching concentrations as high as 180 mM [55]. In order to maintain this high net production of citrate, the replenishment of the TCA cycle is mainly taking place by acetyl-CoA coming from glycolysis and oxaloacetate coming from transamination of aspartate [3]. This is opposite to the brain, where PC is responsible for production of oxaloacetate [11]. Another interesting feature is that the prostate cells to maintain this high net production of citrate are taking up high amounts of Zn^{2+}, which inhibits aconitase and thereby further oxidation of citrate in the TCA-cycle. The ZnT1 is responsible for the accumulation of Zn^{2+} [56] and has been reported to be as high as 3000–5000 nmol/g compared to 200–400 nmol/g in other tissues. Opposite the normal prostate cells, the level of citrate is significantly reduced in prostate cancer since the prostate cancer cells are much more oxidative and energy demanding compared to normal prostate cells. For review of the role of zinc in normal prostate and prostate cancer, see [57]. The high concentration of citrate found in the CSF and prostate fluid as well as Zn^{2+} seems also to play an important role both places, making prostate cells interesting for comparison of features of citrate and Zn^{2+} in cells and the CNS cells.

Table 2 Impact of calcium and zinc on transmitter release from cerebellar granule cells

	Control	EDTA (100 µM)	Citrate (1.0 mM)
[3H]D-aspartate release as % of value with no Zn^{2+} added i.e. 100%	18%	110%	67%
Free [Ca^{2+}]	1500 µM	1450 µM	581 µM
Free [Zn^{2+}]	50 µM	2.3 nM	2 µM

Impact of free [Ca^{2+}] and [Zn^{2+}] on the release of [^{3}H]D-aspartate from cerebellar granule cells after stimulation with 100 µM NMDA and 10 µM glycine under Mg^{2+} free conditions without EDTA and Citrate (control) and in the presence of 1 mM citrate or 100 µM EDTA. Data recalculated from [5]

Conclusion

The pioneering work of Ursula Sonnewald and her co-workers using both proton- and ^{13}C-NMR-spectroscopy to investigate metabolic processes in cultured neurons and astrocytes has given important new insight and knowledge not only about the localisation and concentrations of key CNS metabolites involved in energy metabolism and neurotransmission, but also about which metabolic pathways are involved in the biosynthesis of these metabolites as well as on neuronal–astrocytic interaction [6, 9, 11]. This review clearly demonstrates how NMR-spectroscopy was used to show that citrate was specifically synthesized in and released from astrocytes and how, based on the ^{13}C-labelling patterns of citrate, it could be shown that PC was involved in the biosynthesis. This finding and the use of other advanced techniques have led to the suggestion of a hitherto new and unknown regulatory function of citrate playing a pivotal role as a endogenous modulator of glutamate receptors and in particular NMDA receptors in CNS [5]. While this role of citrate should be further explored studies performed in recent years have indicated that citrate may also play an important role in other key biological processes being part of inflammation, cancer, insulin secretion, histone acetylation and non-alcoholic fatty acid liver as well in prostate fluid [3, 8].This should inspire new experiments designed to further understand the role of citrate in the CNS.

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