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CDP-choline (citicoline) attenuates brain damage in a rat model of birth asphyxia

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To estimate protective potential of citicoline in a model of birth asphyxia, the drug was given to 7-day old rats subjected to permanent unilateral carotid artery occlusion and exposed for 65 min to a hypoxic gas mixture. Daily citicoline doses of 100 or 300 mg/kg, or vehicle, were injected intraperitoneally for 7 consecutive days beginning immediately after the end of the ischemic-hypoxic insult, and brain damage was assessed by gross morphology score and weight deficit two weeks after the insult. Caspase-3, α-fodrin, Bcl-2, and Hsp70 levels were assessed at 0, 1, and 24 h after the end of the hypoxic insult in another group of rat pups subjected to the same insult and given a single dose of 300 mg/kg of citicoline or the vehicle. Citicoline markedly reduced caspase-3 activation and Hsp70 expression 24 h after the insult, and dose-dependently attenuated brain damage. In the context of the well-known excellent safety profile of citicoline, these data suggest that clinical evaluation of the efficacy of the drug in human birth asphyxia may be warranted.

Key words: Bcl-2, birth hypoxia-ischemia, caspase-3, CDP-choline, heat shock protein 70, neuroprotection

List of abbreviations:
Bcl-2 – B-cell lymphoma protein 2
CDP-choline – cytidine-5'-diphosphocholine, citicoline
c.c.a. – common carotid artery
Hsp – heat shock protein
PD7 – postnatal day 7
PD21 – postnatal day 21

INTRODUCTION

Brain damage due to birth asphyxia is a leading cause of human neonate mortality as well as of cerebral palsy, epilepsy, learning disability and mental retardation in survivors (Vannucci 1997). Mechanisms of cell death triggered in the brain by perinatal hypoxia-ischemia are difficult to study in humans. However, the establishment of animal models believed to be clinically relevant such as the "Vannucci rat" (7-day old rat pup subjected to permanent unilateral common carotid artery (c.c.a.) occlusion and transient hypoxia (reviewed by Vannucci et al. 1999) enabled outlining several interrelated pathological processes which are likely involved in asphyxia-related CNS damage. Among them are: (1) activation of certain phospholipases leading to the breakdown of membrane phospholipids and build-up of platelet-activating factor in brain tissues, (2) disturbances of intracellular calcium homeostasis and activation of calpains, (3) activation of glutamate receptors, increased damage from free radicals, and (4) mitochondrial impairment leading directly to caspase-dependent and -independent apoptosis (Akisu et al. 1998, Peeters and van Bel 2001, Vexler and Ferriero 2001, Wang et al. 2001, Zhu et al. 2003, Hagberg 2004).

Ischemia/hypoxia-induced brain damage can be affected by induction of heat shock proteins (Hsp) such as Hsp70. These proteins act intracellularly as molecular chaperones and attenuate proteotoxic stress, and are usually considered neuroprotective and antiapoptotic (Yenari et al. 2005), both in adults and neonates (Ferriero et al. 1990, Matsumori et al. 2005). However, there is some indication that Hsp70 actually can promote brain

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cell apoptosis by acting as an extracellular proinflammatory cytokine (Asea et al. 2000). Interestingly, a recent study employing a rat model of unilateral hypoxic-ischemic brain damage similar to the "Vannucci rat" has shown that an increase in hypoxia-induced factor 1α (HIF-1α) protein and several other molecular changes occurred bilaterally, whereas neuronal damage and increase in Hsp70 expression took place only on the ipsilateral side (van den Tweel et al. 2006).

The brain exposed perinatally to hypoxia-ischemia becomes later the arena of secondary, delayed cell death wave. However, at least some of the asphyxia-affected neurons that, albeit dysfunctional, survive till the moment of reperfusion, could perhaps be rescued and have their functions restored (Vexler and Ferriero 2001). Still, no postnatal treatment has been offered that would benefit asphyxiated newborns. An intervention aimed at rescuing the surviving cells could involve inhibition of apoptosis-executing proteins. Indeed, systemic or intracerebroventricular administration of the cell-permeating broad spectrum caspase inhibitor BAF (BOC-aspartyl(O-methyl)-fluoromethyl-ketone) 3 h after neonatal hypoxia-ischemia resulted in a significant neuroprotection in rats (Cheng et al. 1998). Considering the leading role suggested for caspase-3 in the death of immature neurons (Hu et al. 2000, Wang et al. 2001), pharmacological prevention or attenuation of activation of the protease seems particularly promising. Another potentially useful intervention might be stimulation of expression of anti-apoptotic members of the Bcl-2 protein family. Actually, expression of human Bcl-xL protein in neonatal mice provided for resistance to brain hypoxia-ischemia (Parsadanian et al. 1998). Development of neonatal brain tolerance to hypoxia-ischemia by preconditioning with the noble gas xenon has been related to increased expression of Bcl-2 (Ma et al. 2006), and increased Bcl-2 expression may be involved in neuroprotective effect of xenon applied to neonatal rats hours after hypoxia-ischemia (Dingley et al. 2006).

Neuroprotective properties of CDP-choline (cytidine-5'-diphosphocholine, citicoline) have been known for over two decades. This non-xenobiotic, non-toxic compound can rescue brain cells in various experimental paradigms of hypoxia, ischemia and trauma in adult animals (Adibhatla and Hatcher 2002). Neuroprotection by citicoline may involve several mechanisms, including reinforcement of intracellular glutathione antioxidative system, attenuation of phospholipase A_2 activation and prevention of phospholipid degradation, enhancement of phospholipid synthesis, stabilization of cell membranes, and prevention of glutamate neurotoxicity (Adibhatla and Hatcher 2003, Mir et al. 2003). Importantly, some reports have suggested that citicoline prevents or attenuates increases in caspase-3 activity elicited in neurons by staurosporine (Barrachina et al. 2002) and ischemia (Krupinski et al. 2002). In yet another study citicoline given together with nimodipine increased Bcl-2 expression in brain cells and provided neuroprotection in adult brain focal ischemia (Sobrado et al. 2003), and a recent report showed that the drug enhanced Bcl-2 expression in rat retinal ganglion cells after partial optic nerve crush (Schuettauf et al. 2006).

Due to its propensity for beneficial modulation of the two proteins of key importance for survival of immature neurons, i.e. of caspase-3 and Bcl-2, citicoline might also counteract the development of perinatal asphyxia-related brain damage. However, to the best of our knowledge, no data are available regarding neuroprotective effect(s) of citicoline in newborns. There is a report showing that the drug suppresses elevation of ICAM-1 mRNA transcription after hypoxic-ischemic insult in neonatal rats (Miao et al. 2005), but no relation of this effect to brain morphology or functional outcome was investigated in that study. We were also unable to find data on the effects of citicoline on the expression of Hsp70 in the brain. Therefore we decided to assess neuroprotective effects of citicoline in the "Vannucci rat" model of perinatal brain hypoxia-ischemia, and to identify effects of this drug on brain expression or activation of caspase-3, α-fodrin, Bcl-2, and Hsp70.

METHODS

The reagents used were from Sigma/Aldrich unless specified otherwise and were of analytical grade.

Animals and treatments

Experiments were performed in accordance with the European Communities Council Directive of 24th November 1986 regarding the protection of animals used for experimental and other scientific purposes (86/609/EEC), and the study protocol has been approved by the 1st Local Ethical Commission in Warsaw. Locally

bred 7-day old (PD7) Wistar rat pups of both sexes (12–15 g body weight) were anesthetized by breathing halothane (4% v/v for induction and 1.5–2% v/v for maintenance) mixed with 37.5% nitrous oxide in oxygen. The left c.c.a. was isolated and double-ligated with 7-0 silk sutures, and cut between the ligatures. The wound was sutured and treated with lignocaine. After completion of the surgical procedure the pups were returned to their dams and allowed to recover for 1 h. Afterwards they were placed in a chamber ventilated with warm humidified air (35°C) at a flow rate of 3 L/min. After 30 min of breathing the air, hypoxia was introduced by switching to warm humidified 7.2–7.4% oxygen in nitrogen for 65 min. After completion of the hypoxia the pups were returned to their dams; the dams were housed singly at 20°C and 12/12 h light/dark cycle, and were allowed food and water *ad libitum*. Control pups were sham-operated (i.e. were anaesthetized and had had their left c.c.a. dissected, but not ligated), placed in the hypoxia chamber and ventilated with warmed humidified air only (see above) for 95 min and then returned to their dams.

Citicoline sodium salt, pharmaceutical grade (Kyowa, Japan) was always dissolved fresh in physiological saline to a concentration of 1 g/ml and injected intraperitoneally (i.p.) at a dose of 100 or 300 mg/kg. The first dose of the drug was given within 5 min after the end of hypoxia-ischemia; subsequent injections of the same drug doses were given once daily up to day 7 after the hypoxia. Control group pups received i.p. vehicle injections. The rationale for the dosing schedule was that, in the clinical setting of birth asphyxia, the treatment may be commenced immediately after the end of delivery which otherwise signals the end of asphyxia. On the other hand, according to some studies (Sun et al. 2004), the process which likely determines brain injury in the "Vannucci rat" model is active approximately for one week after the insult. In the experiments aimed at determination of levels in the brain of some proteins involved in modulation of apoptosis the animals received a single intraperitoneal injection of citicoline dose 300 mg/kg, or vehicle, 5 min after the end of hypoxia.

Assessment of brain damage

In the rat model of birth asphyxia damage is largely restricted to the ischemic hemisphere and is observed mostly in cerebral cortex, subcortical and periventricular white matter, striatum and hippocampus (Rice et al. 1981). Since, affected regions are well known, we evaluated brain damage using neuropathology score adopted for this model and by quantification of hemisphere weight deficit.

On day 14 post-treatment (PD21) the rats were sacrificed by decapitation. After removing the upper part of the skull the degree of brain damage was evaluated *in situ* using the semiquantitative gross morphology score (Yager et al. 1992) as modified by Bona et al. (1997). Briefly, normally looking brain was scored "0", brain showing mild atrophy of the left (ischemic) hemisphere was scored "1", brain showing deep atrophy of the hemisphere was scored "2", brain showing the presence of large cysts in the left hemisphere was scored "3", and brain showing only minor remains of the left hemisphere parasagittally was scored "4". Immediately after the evaluation of morphology the forebrain was separated and transsected sagittally; the two forebrain parts were weighted and the weight deficit (%) of the ischemic hemisphere relative to the contralateral hemisphere was calculated.

Determination of proteins' expression

In the model employed, maximum insult-related changes in the levels of some proteins that may modulate apoptosis in the brain (e.g. caspase-3 and Hsp70) occur 24 h post-insult (Hu et al. 2000, van den Tweel et al. 2006), whereas peak elevation of the other proteins (e.g. calpains) is observed 2–3 h post-insult (Blomgren et al. 1997). Therefore, the brains for studying expression of apoptosis-relevant proteins were collected by decapitation at 0, 1, or 24 h after hypoxia-ischemia. Forebrain was separated, cut sagittally and the two hemispheres were snap-frozen in liquid nitrogen and stored at −70°C till processed for analysis. Tissue samples were homogenized with a Tenbroeck tissue grinder (Wheaton) in 10 volumes of 20 mM Tris-HCl buffer pH 7.4 containing 150 mM NaCl, 1 mM EDTA, 1mM EGTA, 1% Triton X-100, 2.5 mM sodium orthovanadate, 1 μg/mL leupeptin and 1 mM Pefablock (Fluka), and centrifuged at 3 000 × g for 10 min. Supernatants were aliquoted, frozen and stored at −70°C until assessed for protein expression. Protein concentration in the supernatants was measured by the method of Bradford (1976).

One aliquot of each homogenate supernatant was used to assess Hsp70 content with the Human HSP70

ELISA DuoSet kit (R&D Systems). The other proteins were assessed semiquantitatively by Western blotting as follows: aliquots of brain homogenate supernatants were mixed with Laemmli buffer (Laemmli 1970), heated at 100°C for 5 min, subjected to SDS-PAGE together with prestained molecular weight standards (Broad range, Bio-Rad), and electrotransferred to nitrocellulose membranes (Amersham). The membranes were blocked with skimmed milk and incubated overnight with primary antibodies against caspase-3 (rabbit monoclonal; Cell Signaling Technology; dil. 1:1 000), α-fodrin (mouse monoclonal; Chemicon; dil. 1:2 000) or Bcl-2 (mouse monoclonal; BD Biosciences; dil. 1:500). Reaction of actin with the anti-actin mouse monoclonal antibody (Sigma; dil. 1:2 000) was used to check uniformity of protein loading. Finally the membranes were incubated with appropriate HRP-conjugated secondary antibody, i.e. anti-mouse ovine polyclonal antibody (Amersham; dil. 1:5 000) or anti-rabbit goat polyclonal antibody (Sigma; dil. 1:5 000). After incubation with the ECL substrate mixture (Amersham) the membranes were exposed to Hyperfilm (Amersham). After developing the film, protein bands were identified by their molecular weights. Band densities were quantified with a model GelExpert 4 densitometer (NucleoTech) relative to a chosen sample. Lysates of UV-treated HL60 cells (a gift from Dr. Anna Fiedorowicz, Nencki Institute of Experimental Biology, Warsaw, Poland) served as positive controls of apoptosis-related proteins.

Statistics

Significance of the treatment effects on the brain gross morphology score and on brain hemisphere weight deficits was evaluated by the Kruskal-Wallis non-parametric ANOVA followed by Mann-Whitney *U*-test and one-way parametric ANOVA followed by Student's *t* test, respectively. Significance of differences in Western blot protein band densities and Hsp70 contents assessed by ELISA were evaluated by Kruskal-Wallis non-parametric ANOVA followed by Mann-Whitney *U*-test when appropriate. $P<0.05$ was considered significant. All the statistical analyses were run using the Statistica for Windows v. 7.0 (StatSoft Inc., Tulsa, OK, USA) software package.

RESULTS

Efficacy of cerebroprotection

Vehicle-treated animals subjected to c.c.a. ligation and exposed to transient hypoxia developed severe brain damage in the ischemic hemisphere. Seven-day citicoline treatment dose-dependently and significantly attenuated weight deficit of the ischemic hemisphere and improved brain morphology 14 days after the hypoxia (Fig. 1). There was a positive correlation between the two effects across all the treatment groups (linear correlation coefficient $r=0.93$, $P<0.05$).

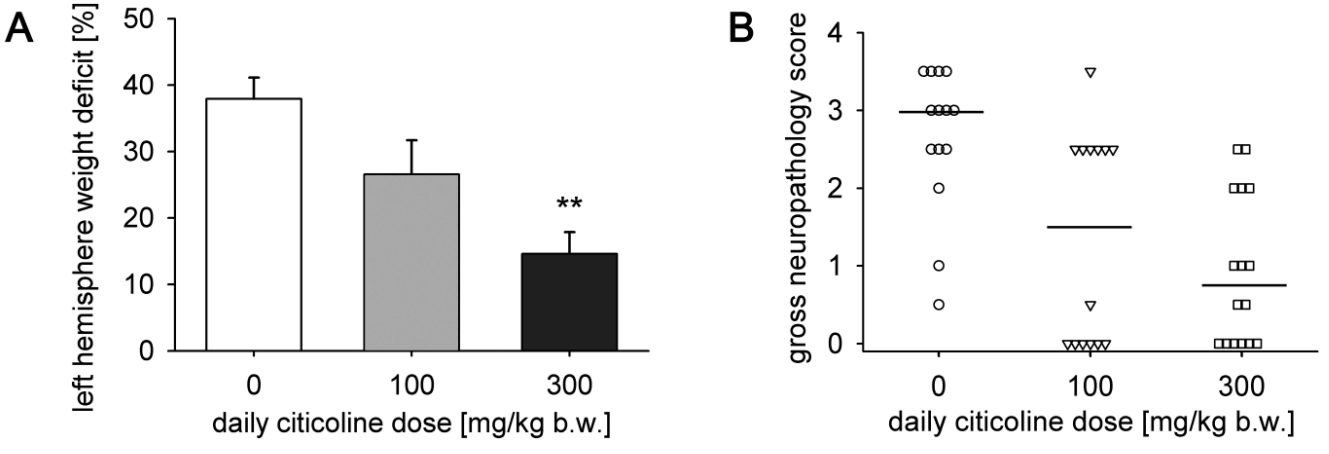

Fig. 1. Effects of citicoline treatment on brain damage assessed 14 days after hypoxia-ischemia (i.e., PD21). (A) percent ipsilateral weight deficit relative to the contralateral hemisphere (means ± SEM values). (B) the gross neuropathology scores. Solid horizontal lines represent median values. $^{**}P<0.01$, $n=14$ for dose 0 and 100 mg/kg, $n=16$ for dose 300 mg/kg.

Apoptosis-modulating proteins

In rats subjected to hypoxia-ischemia no activation of caspase-3 was observed 1 h post-treatment, but a massive elevation in the large subunit of active caspase-3 (19 kDa isoform) was found at 24 h in the left (ischemic) hemisphere. Citicoline (300 mg/kg b.w.) significantly attenuated the activation of caspase-3 24 h after the insult (Fig. 2). No significant effect of citicoline on procaspase-3 level was found in either the asphyxiated or the sham-operated rats (data not shown).

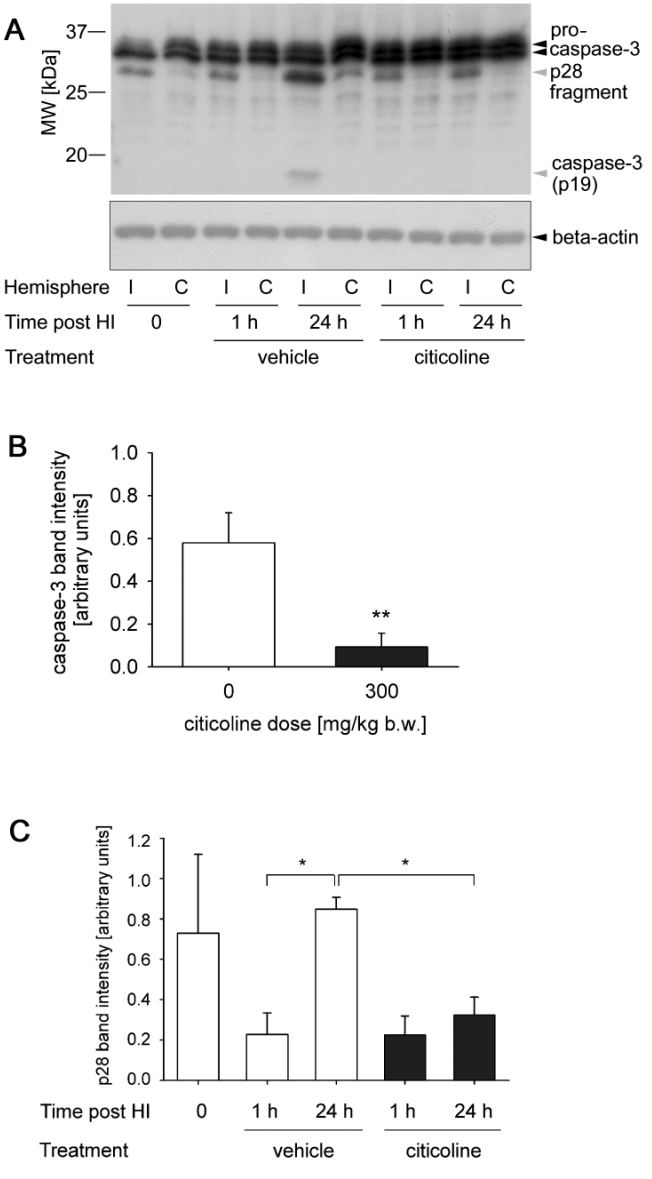

Fig. 2. The effects of citicoline treatment on the levels of procaspase-3 and its cleavage products at 0, 1, and 24 h after insult. (A) a representative blot. (B) citicoline affects caspase-3 level in ipsilateral hemispheres 24 h after treatment. (C) calpain-specific procaspase-3 breakdown product level declines in ipsilateral hemispheres 24 h after citicoline treatment. I – ischemic hemisphere; C – control (non-ischemic) hemisphere; **P*<0.05; ***P*<0.01, *n*=5 per each time point and dose. Data expressed as mean ± SEM.

To verify suppression of caspase-3 activity, the analysis of α-fodrin (brain α-spectrin) breakdown products was performed. Twenty-four hours after the ischemia-hypoxia insult caspase-3-specific breakdown product (120 kDa) level was barely detectable in the right (non-ischemic) hemispheres. In the left hemispheres the level of this breakdown product was significantly increased as compared to that in the sham-operated controls; citicoline treatment significantly attenuated the increase (Fig. 3). Hypoxia-ischemia also massively increased the level of calpain-specific procaspase-3 breakdown product (28 kDa) 1 h post-insult and this band was also visible 24 h after the insult. The citicoline treatment did not affect the level of this protein fragment 1 h post-insult, but significantly decreased it at 24 h (Fig. 2C). There was no significant effect of either the ischemia-hypoxia insult, or citicoline on Bcl-2 protein level (data not shown). Hsp70 was elevated 24 h after the insult only in the hemisphere ipsilateral to the ligated c.c.a.; citicoline treatment significantly attenuated this elevation (Fig. 4).

DISCUSSION

We showed in this study that intraperitoneal citicoline was cerebroprotective in a rat model of birth asphyxia. Repeated citicoline treatment dose-dependently diminished ischemic hemisphere weight deficit and improved brain gross morphology score assessed two weeks after ischemia-hypoxia insult. On a short-time scale, a single citicoline dose of 300 mg/kg given immediately after the insult reduced caspase-3 activation and Hsp70 overexpression 24 h post-insult.

Cerebroprotective/neuroprotective effects of citicoline have been described in various adult ischemia models (Grieb et al. 2001, Adibhatla and Hatcher 2002, Schuettauf et al. 2006), as well as in cultured neural cells (Mykita et al. 1986, Mir et al. 2003). As mentioned in the Introduction, the protection may involve several mechanisms. Recently it has been suggested that neuroprotective effect of citicoline on rat retinal ganglion cells following partial optic nerve crush involves increased Bcl-2 expression (Schuettauf et al.

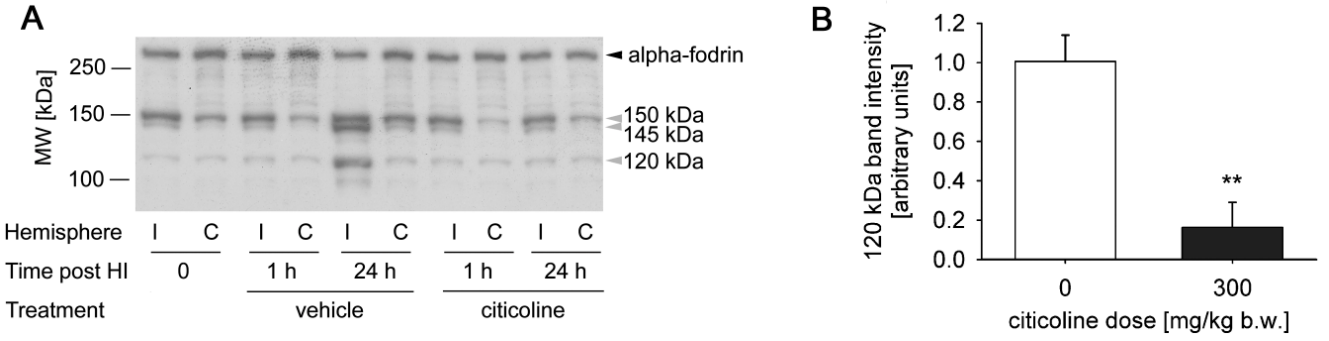

Fig. 3. The effects of citicoline treatment on the levels of α-fodrin and its breakdown products at 0, 1, and 24 h after insult. (A) a representative blot. (B) caspase-3-specific fodrin breakdown product (p120) level is significantly lower in ipsilateral hemispheres 24 h after citicoline treatment than in saline-treated controls. I – ischemic hemisphere; C – control (non-ischemic) hemisphere; $^{**}P<0.01$, $n=5$ per each time point and dose. Data are expressed as mean ± SEM.

2006). However, we found no significant change in the level of this protein in response to either hypoxia-ischemia or citicoline treatment. We have also found no effect of either hypoxia-ischemia or citicoline on the level of procaspase-3.

Our data suggest that citicoline may prevent caspase-3 activation by downregulating the calpain-specific procaspase-3 breakdown product. It is, therefore, possible that citicoline affects caspase activity indirectly by modulating calpain activity. Caspases and calpains are two systems of interrelated proteolytic enzymes. Decreased activity of caspase-3 results in decreased calpain activation because calpastatin (a calpain inhibitor) is a substrate of caspase-3 (Wang et al. 1998), whereas calpains may be activated by caspases (Wang 2000). It is also possible that citicoline does not affect caspases or calpains directly, but suppresses some cell death signals which activate both these protease families. According to some studies, the presence of calpain-specific procaspase-3 breakdown product is the indication of "pathological apoptosis" because this protein fragment does not appear during normal brain development despite the occurrence of developmental apoptosis (Blomgren et al. 2001). Citicoline might thus affect "pathological apoptosis" but not that occurring normally in the developing brain; this issue needs further studies.

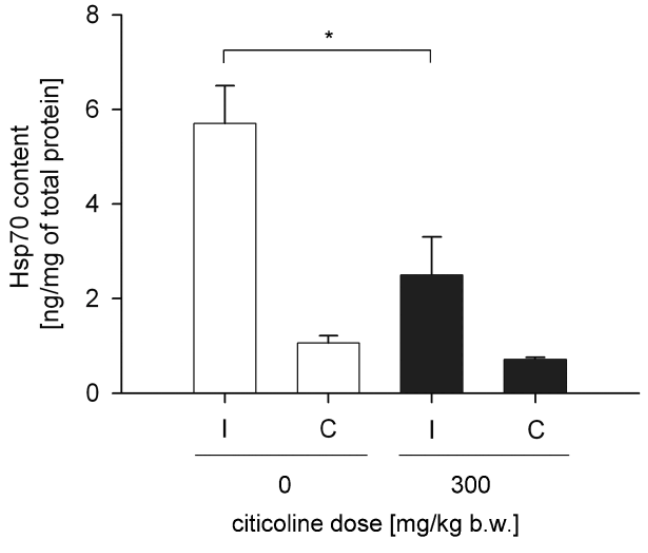

Fig. 4. The effects of citicoline treatment on the Hsp70 levels 24 h post-insult. Hsp70 level declines in ipsilateral hemispheres 24 h after citicoline treatment. I – ischemic hemisphere; C – control (non-ischemic) hemisphere; $^{*}P<0.05$, $n=5$ for each time point and dose. Data are expressed as mean ± SEM.

We have found that in the "Vannucci rat" model Hsp70 is induced only in the ischemic hemisphere. Similar observation has been recently reported by others (van den Tweel et al. 2006). Heat shock proteins are generally thought to protect cells against various insults (Latchman 2004), and inducers or coinducers of HSPs, e.g. teprenone and arimoclomol, are cerebroprotective in adults (Kieran et al. 2004, Yasuda et al. 2005). It was also shown that Hsp70 overexpression reduces hypoxic-ischemic brain injury in neonate mice (Matsumori et al. 2005) and infarction volume after focal cerebral ischemia is increased in *hsp70.1*-knockout mice (Lee et al. 2001). On the other hand, Hsp70 may act also as an extracellular proinflammatory and proapoptotic cytokine (Asea et al. 2000), and its overexpression may potentiate neuronal damage in some situations, e.g. after c.c.a. occlusion in adult mice (Olsson et al. 2004). In the present study citicoline attenuated Hsp70 overex-

pression induced by combined ischemia-hypoxia. This suggests that the cerebroprotective action of citicoline does not involve Hsp70 induction, but may result from suppression of Hsp70 overexpression.

Citicoline undoubtedly is an active neuroprotectant, but large quantities of the drug (0.3–1.0 g/kg/day, or 0.6–2.0 mmole/kg/day) are required to exert beneficial effects *in vivo*. However, in the aforementioned *in vitro* models neuroprotection was evident upon exposure of the cells to micromolar or lower concentrations of the drug. The difference may be related to the fact that the drug decomposes very slowly in neuronal cultures, whereas it is promptly hydrolysed and dephosphorylated to cytidine and choline after systemic administration. Since it has been shown that neither cytidine nor CMP are neuroprotective *in vitro* or *in vivo*, it is possible that neuroprotection is induced by unhydrolysed molecules of citicoline (Mykita et al. 1986, Grieb et al. 2001). Noteworthy, the early and sustained blood-brain barrier opening, which occurs in the PD7 rat subjected to brain ischemia (Benjelloun et al. 1999), may facilitate unhydrolyzed citicoline's entering the brain parenchyma.

A number of compounds applied immediately after the end of hypoxia has been found to offer a significant neuroprotection in the "Vannucci rat" model. However, only a few of them later have undergone preliminary clinical evaluation, and the results were discouraging. For example, trials with calcium channel blocker nicardipine and with magnesium sulphate had to be stopped because of dangerous cardiovascular side effects (Peeters and van Bel 2001). The key issue may thus be drug tolerance rather than efficacy. Compared to several other candidate neuroprotectants citicoline is unique in that it is not a xenobiotic and its toxicity is low. LD_{50} of intravenous citicoline is 4.6 g/kg and 4.15 g/kg in adult mice and rats, respectively (Grau et al. 1983). Interestingly, the drug is by almost 2 orders of magnitude less toxic than choline (Augt et al. 1983), which may indicate that choline released from citicoline upon hydrolysis is efficiently eliminated.

Citicoline was being given parenterally to human preterm infants as a means of increasing the synthesis of lung surfactant phospholipids to alleviate symptoms of respiratory distress syndrome. For the daily doses of 100–300 mg/kg no dangerous side effects have been reported (Colombo et al. 1976, Vallis i Soler et al. 1988).

CONCLUSION

Considering significant neuroprotective efficacy seen in the present study and excellent clinical safety profile of citicoline, its clinical evaluation as a cerebroprotectant in human perinatal asphyxia seems warranted.

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