Neurochemical Research, Vol. 30, No. 8, August 2005 (© 2005), pp. 1067–1073
DOI: 10.1007/s11064-005-7201-0

Citicoline Inhibits MAP Kinase Signalling Pathways after Focal Cerebral Ischaemia

J. Krupinski,[1,2] M. Slevin,[3,4] and L. Badimon[1]

(Accepted July 5, 2005)

The link between membrane phospholipids and different intracellular signal transduction pathways affected by cerebral ischaemia is unclear. CDP-choline, a major neuronal membrane lipid precursor and its intracellular target proteins and transcription factors were studied to further understand its role in ischaemic stroke. Cerebral ischaemia was produced by distal, permanent occlusion of the middle cerebral artery (MCAO) in the rat. Animals receiving 500 mg/kg of CDP-choline in 0.5 ml of 0.9% saline, intraperitoneally, 24 h and 1 h before MCAO and 23 h after MCAO demonstrated a notable reduction in the phosphorylation of MAP-kinase family members, ERK1/2 and MEK1/2, as well as Elk-1 transcription factor, compared with control animals treated with 0.5 ml of 0.9% saline. Immunohistochemistry showed a particular reduction in immunoreactivity in glia. The effects of CDP-choline on intracellular mechanisms of signal transduction, suggests that this molecule may play a key role in recovery after ischaemic stroke.

KEY WORDS: CDP-choline; cerebral ischaemia; MAP kinase; signal transduction.

INTRODUCTION

Cytokines released following ischaemic brain stroke induce various important intermediates of intracellular signal transduction. The link between cytokines produced during the initial phase of stroke, early gene expression and subsequent extension of the ischaemic penumbra points to MAP kinase family of proteins as important target molecules. Shortly after ischaemia, multiple cytokines activate the ras pathway via G proteins-coupled mechanisms. Ras has several effectors, of which the serine/threonine kinase Raf and its downstream MAP-ERK kinase (MEK)/early response kinase (ERK) cascade are well established (1). Following activation, ERKs translocate to the nucleus and then phosphorylate and activate several transcription factors (*c-fos*, *c-jun* and *egr−1* genes) (2). Simultaneously, another related group of stress-activated protein kinases (SAPKs) p38 and c-Jun N terminal kinase (JNK) are activated (3). Phosphorylation of Thr and Tyr residues of p38 results in MAPK-activated protein 2-induced activation of transcription factor 2, whereas phosphorylation of JNK results in c-Jun induction (4). Other major subsets of cytokines may utilize receptors with no direct tyrosine kinase activity i.e. STAT−1. The STATs form dimers, translocate into nucleus, bind to response elements in target genes and activate transcription. The Ets family of transcription factors like Elk−1 and Ets−1 are phosphorylated and bind to various motifs of DNA (5).

[1] Cardiovascular Research Center, IIBB/CSIC-HSCSP-UAB, St.pau hospital Barcelona, Spain.
[2] Servicio de Neurologia, Hospital Universitari de Bellvitge, CSUB, ĹHospitalet de LLobregat, Barcelona, Spain.
[3] Department of Biological Sciences, Manchester Metropolitan University, Manchester, UK.
[4] Address reprint requests to: Dr. M Slevin, Department of Biological Sciences, John Dalton Building, Manchester Metropolitan University, Manchester M1 5GD, UK. Tel.: +0161-2471172; Fax: +0161-2476365; E-mail: m.a.slevin@mmu.ac.uk

0364-3190/05/0800–1067/0 © 2005 Springer Science+Business Media, Inc.

Cerebral ischemia alters the balance between phospholipids, resulting in a decrease of phospholipid content and loss of integrity of biological membranes (6). CDP-choline, crucial in the brain for the formation of phosphatidylcholine, is a phospholipids precursor essential for the maintenance of intracellular and extracellular membranes (7). CDP-choline plays an important role in the generation of phospholipids, which are involved in membrane formation and repair and contributes to formation of nucleic acids, proteins and intercellular messangers (8). Although clinical trials have not clearly demonstrated the beneficial effects of citicoline in reducing ischaemia-induced brain damage, phase II and III clinical trials demonstrated significant improvement of neurological, functional and global outcomes in citicoline treated patients (500 and 2000 mg/d; within 24 h of stroke) compared with placebo 12 weeks after stroke onset (9). In a second study, treatment with citicoline 500 mg showed significant benefits in a subgroup of patients with moderate to sever strokes in terms of functional recovery (10). In a third trial, 2000 mg of citicoline did not demonstrate significant differences in the primary end point (11). However, an individual patient data pooling analysis of clinical trials showed that treatment with oral citicoline within 24 h after onset in patients with moderate to severe stroke increased the probability of complete recovery at 3 months (12).

Recent evidence has suggested that the mechanisms through which citicoline elicits neuroprotection, may involve modulation of the activity of different signal transduction pathways. Therefore, we examined the ability of CDP-choline to modulate the phosphorylation of stroke-associated known signaling intermediates using an animal model of focal cerebral ichaemia.

EXPERIMENTAL PROCEDURE

Animals and Surgical Procedures. All stroke experiments were performed on female Sprague-Dawley rats weighting 230–270 g, as they do appear to suffer less during ischemic/traumatic insults, perhaps due to higher endogenous steroid levels reducing free radical damage. This model was chosen for purpose, as our intention was to produce small cerebral infarction. The animals were housed at 21°C with free access to food and water. Animal welfare was conducted according to the regulations of the Real Decreto 223:1998, which makes recommendations similar to those of the NIH Report Public Health Service Policy of the Care and Handling of Laboratory Animals. Cerebral ischemia was produced using a modified method of Tamura et al. (13) with distal, permanent occlusion of the middle cerebral artery by electrocautery. The animal mortality in this model is very low (i.e. in our group of animals there was no mortality). Anesthesia was induced by 4% isoflurane in O_2/N_2O mixture at a standard concentration as in normal air, and maintained at 1–2% isoflurane in O_2/N_2O during the procedure. Rectal temperature was maintained at 37°C with a controlled heating pad during the surgery and MCAO. Animals were randomized into four groups: (A) Animals treated with CDP-choline, 500 mg/kg in 0.5 ml of 0.9% saline intraperitoneally (i.p.), 24 h and 1 h prior to MCAO, and 24 h after MCAO: (B) Animals treated with citicoline, 500 mg/kg in 0.5 ml of 0.9% saline i.p., within 30 min after MCAO, and followed by one dose at 23 h; and, (C) MCAO-subjected rats, treated with 0.5 ml of 0.9% saline, i.e. at the same time-points as for group A and B, respectivelly, (D) sham-operated animals that were used as controls. MCA was exposed, but not electrocoagulated, and 0.5 ml of 0.9% saline was administered i.p. We did not follow changes in blood pressure and blood gas parameters as previous studies have demonstrated that CDP-choline does not affect these parameters. The animals were killed at 12 and 24 h after MCAO ($n = 3$ per group and time-period, total of 24 animals). Animals killed at 12 h after MCAO did not receive the second dose programmed at 23 h after MCAO. In this model of focal ischemia, the infarcted lesion is limited to neocortical areas leaving broad area of penumbra.

Antibodies (Abs). All antibodies used recognize active, i.e. phosphorylated forms of the corresponding signal transduction molecules and were dually phosphorylated. They were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA): p-ERK1/2 (sc−7383), p-Elk1 (sc−8406), p-STAT−1 (sc−7988), p-JNK (sc−6254), p-c-Jun (sc−822), and p-MEK1/2 (sc−219). Anti α-actin (Sigma, UK), was used as a protein loading control.

Immunohistochemistry. For morphological studies and immunohistochemistry, the rats were perfused through the heart, under deep anesthesia, with phosphate buffer saline (PBS) followed by 4% paraformaldehyde in PBS. Immediately afterwards, the brains were removed from the skull and fixed with the same fixative solution for 24 h at 4°C. Fixed brains were cryoprotected and stored at −70°C. Serial sections, 50 μm thick, were cut with a cryostate. For morphological studies, the sections were stained with haematoxylin and eosin. In this model, penumbra is easilly identified as an adjacent tissue to the ischemic core. This was verified based on morphology of neurons according to classification by Eke et al. (14). The avidin–biotin-peroxidase method (ABC kit, Vectastain, Vector) was used for immunohistochemistry. After blocking endogenous peroxidase, the sections were incubated with normal serum and then at 4°C overnight with one of the primary antibodies. Next, sections were incubated for 1 h with biotinylated anti-goat IgG or anti-mouse antibodies diluted 1:100, and finally with ABC at a dilution of 1:100 for 1 h at room temperature. The peroxidase reaction was visualised with 0.05% diaminobenzidine (DAB) and 0.001% hydrogen peroxide. The specificity of the immunoreaction was tested by preincubation of the antibodies with the corresponding antigenic peptides prior to immunohistochemistry. Immunostaining was abolished in these sections.

Western Blotting. For protein studies, we used 2 × 2 mm cortical samples, comprising the infarct core and penumbra as well as the corresponding area in the contralateral hemisphere. After dissecting the infarcted area, the penumbra was defined as the tissue adjacent to the infarcted core. All samples were processed in parallel. Samples were homogenised with 300 μl of ice-cold homogenisation buffer containing 1% sodium deoxycholate, 1% Triton X−100, 100 μM EDTA, and 200 μM PMSF. Samples were

centrifuged at 14,000 rpm for 5 min and stored in aliquots at −20°C. The protein concentration of each sample was determined with BSA assay (Biorad). SDS-PAGE (10%) was carried out using a mini-protean system (Biorad). 20 μg of protein was loaded in each lane with loading buffer containing 0.125 M Tris, pH 6.8, 20% glycerol, 10% mercaptoethanol, 4% SDS, and 0.002% bromophenol blue. Samples were heated at 97.5°C for 5 min prior to gel electrophoresis. Proteins were transferred to PVDF membranes (Immobilon) using an electrophoretic chamber system (Trans-blot, Bio-Rad) at 40 mA for 1 h. After blocking with Tween−20 TBS (TTBS) containing 7.5% skimmed milk for 30 min, they were incubated with the primary antibody in TTBS containing 7.5% skimmed milk, at 4°C overnight. After washing, the membranes were incubated with the corresponding secondary antibody labelled with horseradish peroxidase at a dilution of 1:1000 for 1 h at room temperature, washed again, and developed with the Super Signal kit (Amersham). Control of protein content in each lane was evaluated by the staining duplicate gels with Coomasie blue and staining membranes with antibodies to α-actin. Semi-quantification of protein band intensity was carried out with Bio-Rad Gel Doc 1000 scanner. Experiments were repeated three times, and a representative example is shown.

RESULTS

Western Blotting

Inhibition of MAP Kinase Phosphorylation by CDP-choline

Our previous studies using this animal model of focal cerebral ischaemia demonstrated that phosphorylated forms of the MAP kinase family were significantly up-regulated in infarcted core and penumbra, whilst total protein expression was not altered (15). In this study, the protective effect of CDP-choline was apparent 12 h after stroke, in animals treated before and following MCAO (group A). Changes in group B were not significant and are not presented here. In group A, p-ERK−1/2 was decreased in both infarcted and penumbral tissue (0.6–0.1-fold), when compared with contralateral areas. In contrast, placebo controlled animals (group C) demonstrated a notable increase of p-ERK1/2 in infarcted core and penumbra (1.5–2.0-fold) (Fig. 1a). At 24 h, an observed decrease of p-ERK1/2 was seen in infarcted core for p-ERK1 and in penumbra for p-ERK2 (data not shown). The upstream activator of ERK1/2, p-MEK1/2 was also modified by CDP-choline. Infarcted core and penumbra expression was similar to contralateral areas (0.8–1.2-fold) in CDP-choline treated animals, but notably increased in animals from group C (2.5–3.0; Fig. 1b). CDP-choline did not reduce the level of phosphorylation of JNK1/2 in stroke affected tissue (data not shown).

Modulation of Expression of Target Genes

We studied various members of gene families involved in direct up-regulation of gene expression. Expression of p-c-jun, and p-STAT−1, was not affected by CDP-choline treatment in infarcted and penumbral tissue following MCAO (data not included). Elk−1, one of the ets transcription factor family participating in DNA binding activity via phosphorylation, increased in both infarcted and penumbra areas (1.5–2.4-fold). This phosphorylation was significantly down-regulated by CDP-choline (0.5–0.4-fold) (Fig. 1c).

In each case, tissue from the contralateral hemisphere of group A rats was compared with that from sham operated rats (group D), and phosphorylated protein expression found to be comparable (Fig. 1a–c).

Immunolocalization of Phosphorylated Proteins after Ishaemia

p-ERK1/2. In the sham operated rats, there was basal expression of MAPK in the II and III cortical layers, entorhinal cortex and ventro-medial thalamus. There was a strong expression in the polymorphic neurones in the hilus of the hippocampus, but not in the mossy fibers or pyramidal cells. There was some immunohistochemistry around the blood vessels and no staining in the corpus callosum. At 12 h after experimental ischaemia, there was a notable increase in the immunohistochemistry in the ipsilateral hemisphere (Fig. 2a). Neurones and most of the glial cells in all the layers were strongly stained at the vicinity of the infarction. There was also staining in the ipsilateral corpus callosum and striatum (Fig. 2b). Weak staining in the contralateral hemisphere was similar to that in the sham-operated rats. In the hippocampus, there was strong staining in neurones of the hilus. At 24 h, staining was strong but mainly in the glia and not in the neurones. Staining was significantly increased throughout the corpus callosum. In the hippocampus mossy fibers showed intense immunoreactivity. Treatment with CDP-choline significantly reduced immunohistochemistry, especially at 12 h post-ischaemia. This was visible in all the cortical layers, striatum and corpus callosum (Fig. 2c). ERK1/2 expression dramatically disappeared from the glia and was mainly limited to neurones (Fig. 2d). Morphology of neurons positive for ERK1/2 was more preserved when com-

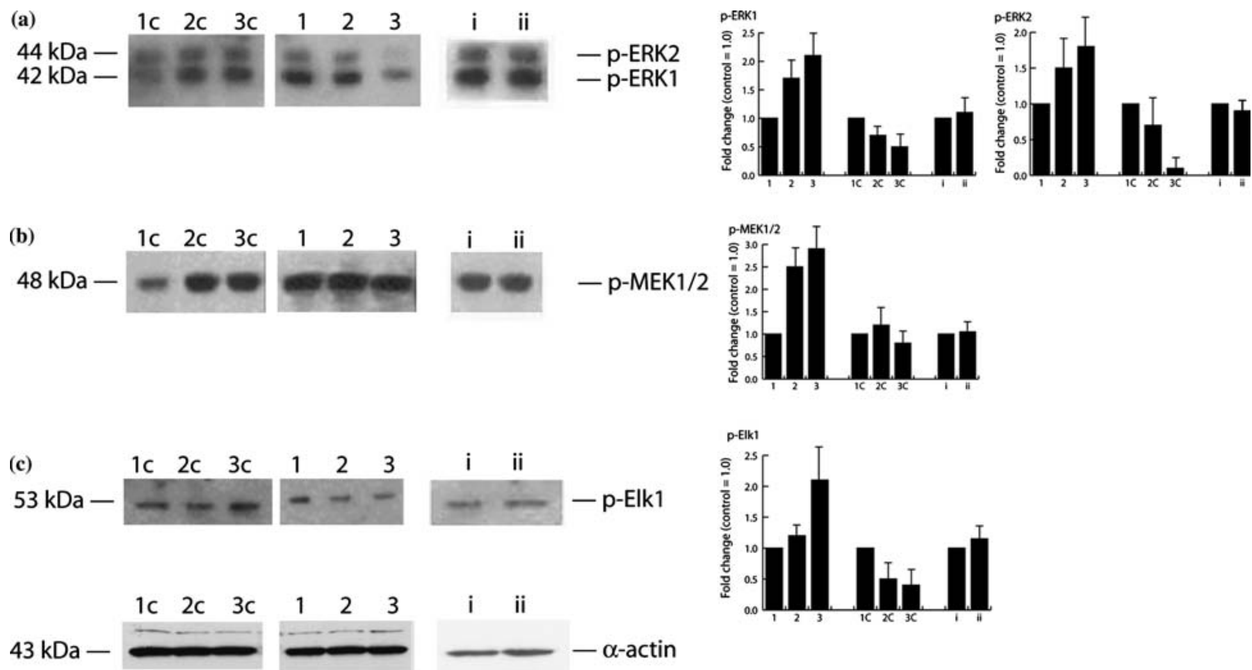

Fig. 1. Western immunoblot analysis of brain extracts taken 12 h following focal ischaemia; animals treated as Group A (lane 1, contalateral hemisphere, lane 2, surrounding penumbra and lane 3, infarcted core) and control group C (1c through 3c, respectively; IC-infarcted core; P-penumbra, C-contralateral). Lanes i and ii show a comparison between normal contralateral tissue from group A and sham operated tissue from group D, respectively. All graphics are a semiquantitative representation of western immunoblots and are normalized to corresponding contralateral areas (for lanes i and ii, group D expression is compared with the average from group A). Top panel (a) phosphorylation of ERK1/2, (b) MEK1/2 and (c) Elk−1 after focal cerebral ischaemia. Lower panel shows protein loading control stained with α-actin. The bar graphs illustrate the average fold changes from three experiments ± range, compared with the normal contralateral hemisphere. Note that the expression of phosphorylated protein between group A and group C is not comparable since the gels were developed separately.

pared with the placebo group. Expression of ERK1/2 was present in neuronal cytoplasm and in neurites. This was in contrast to the group treated with placebo, where staining was mainly limited to the cytoplasm and severely damaged neuritis (compare upper panels in Fig. 2b and d).

p-MEK1/2. At 12 h, staining was limited strictly to the perinfarcted areas and mainly single neurones were stained, in deeper layers some staining was present in the pericallosal neurones. At 24 h, there was mainly staining in the neurones, often in all the layers in the infarcted and penumbra tissue, and only in a few astroglial cells. There was also staining in the corpus callosum and ethorhinal cortex. Treatment with CDP-choline significantly reduced staining, which was comparable to p-ERK1/2 in group A of animals (data not shown).

p-STAT−1. With p-stat−1, there was strong staining in the infarcted neurones, also some glial cells at 12 h. Neurones around the infarct and in the c.callosum stained more intensively than with p-MEK−1. At 24 h, more staining was also seen in the hilus of the hippocampus, but less than with the others antibodies. Treatment with CDP-choline did not notably alter immunolocalization of p-STAT−1, which was comparable in all studied groups.

p-Elk−1. At 12 h and 24 h after ischaemia, p-Elk−1 expression dramatically increased in both neurones and glia and not only in the infarcted areas, but also in the far penumbra and corpus callosum. In group A, rats treated with CDP-choline, this immunolabelling decreased in all areas and was mainly limited to the neuronal population (data not included).

p-JNK/p-c-Jun. Immunoreactivity with both antibodies was identical. This molecule was very selectively expressed in the infracted, but not contralateral hemisphere. Only some neurones expressed c-jun and there was no expression in glia. There was no change in expression or localization of these phosphorylated proteins following treatment with CDP-choline.

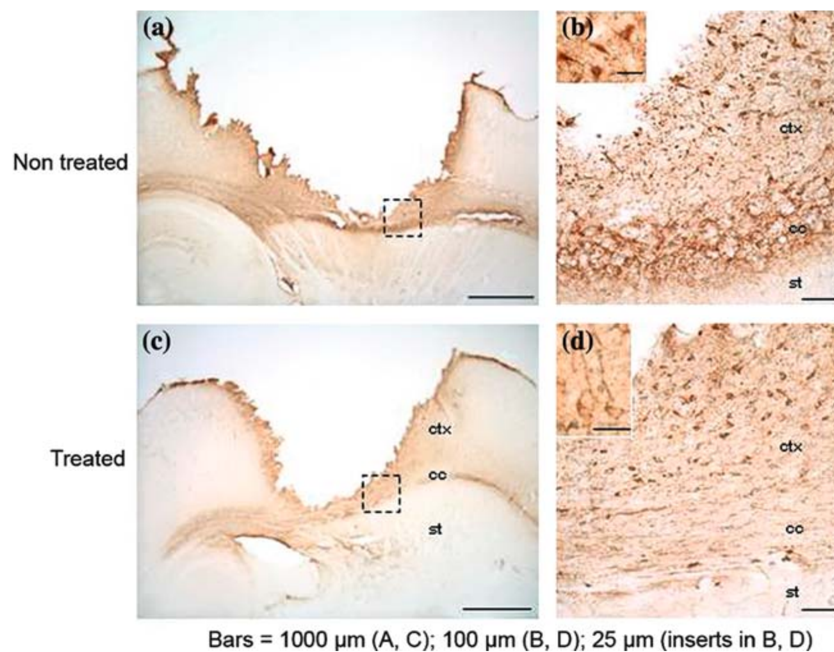

Fig. 2. (a) p-ERK1/2 was expressed in surrounding ischaemic lesions in both grey and white matter and extended to the adjacent corpus callosum and striatum. Both neurones and glia were strongly stained. The up-regulation was seen 12 h after ischaemia and later decreased (not shown). (b) As shown in the upper panel, neurones were severely affected (type III, IV damage according to Eke et al. (10). (c) Treatment with CDP-choline before and after inducing ischaemia (group A animals) reduced expression of p-ERK1/2 in all areas. White matter and striatum immunohistochemistry were mostly affected. (d) The reduction in p-ERK1/2 expression was mainly due to lower immunohistochemistry in the glia. Furthermore, neuronal expression was present in surviving neurones (type I, II damage according to Eke et al. (10)? (upper panel d). Ctx-cortex, cc-corpus callosum, st-striatum; bars 1000 μM (a, c), 100 μM (b, d), 25 μM (inserts in b, d).

DISCUSSION

The effects of CDP-choline given *in vivo* have been frequently reported in the literature. However, the neuroprotective mechanism has not been clearly identified, and its potential in stroke treatment might still be fully recognized (16). One of the main reasons for that may be due to the fact that strong evidence for the incorporation of CDP-choline into the brain has not been shown. However partial in direct mechanisms through which it acts have recently been elucidated. Experimental studies in a rat model of MCAO and also primary cultured cortical neurones exposed to oxygen–glucose deprivation, demonstrated the neuroprotective effects of citicoline may be due to decreased glutamate release after ischaemia (17). Sobrado et al. (18) showed that reduced neuronal apoptosis was associated with an increase in Bcl−2 expression in citicoline treated rats exposed to MCAO. Previous studies have demonstrated that citicoline treatment reduced arachidonic acid release after ischaemia-perfusion injury in a gerbil model of stroke (19). Pharmokinetic data has shown that brain uptake of citicoline metabolites occurs within 30 min, and they can remain biologically active for around 3 h after administration. Therefore, increased presence of citicoline may stimulate phosphatidylcholine synthesis and reduce the availability of hydrolysis products phospolipase C and diacylglycerol. Neuronal protection afforded by citicoline may be related to inhibition of protein kinase C activation, which lies above Ras in the MAP kinase signalling cascade. Our results demonstrate that membrane stabilization with CDP-choline significantly alters protein phosphorylation of various signal transduction intermediates. In this animal model of focal cerebral ischaemia, at least two proteins of the MAP kinase family i.e. p-ERK1/2 and p-MEK1/2 were significantly down-regulated in the grey matter of infarcted and penumbra tissue. This inhibition was more evident at 12 h than 24 h post-infarction. The MAP kinase family of kinases participates in the stress response mechanisms in many cell types mediated by cytokine-receptor interaction following IL−1, TNF or caspase activation (20,21). Furthermore, the agonist stimulation of a variety of cell receptors results in the release of

specific lipid moeities from the nuclear membranes, supporting the hypothesis of an important role of the lipids in nuclear signal transduction (22). In cardiac myocytes, it was observed that adrenergic stimulation induced signal transduction activation with subsequent increase in CDP-choline (23). In humans, the regulation of the MAP kinases is important during early evolution of the infarction (15,24,25). After experimental cerebral ischaemia, MAP kinases participate in the inflammatory microglia/macrophage response (26). In this study, as shown by immunohistochemistry, CDP-choline mainly reduced MAP kinase expression in glia while most of the neuronal expression was maintained. Our finding that CDP-choline reduces the expression of phosphorylated ERK1/2 and MEK1/2, suggest that CDP-choline may have a protective anti-inflammatory effect. This could promote neuronal survival and by supressing glial activation may reduce scarring after ischaemia. Glutamate neurotoxicity, a key event following ischaemic stroke leads to MAP kinase activation. MEK inhibitors reduce phosphorylation of ERKs and protect against synaptic mediated toxicity (27). Phosphorylation of ERKs is associated with induction of early response genes and hyperphosphorylation of Elk−1 and c-AMP/calcium-responsive element-binding protein (28). Inhibition of glial p38 MAP kinase expression *in vitro* results in attenuation of induction of c-fos, c-jun mRNAs (23).

While the existence of a phosphatidylinositol cycle has been reported in cellular nuclei, little attention has been given to the metabolism of phosphatidylcholine in nuclear signaling. In neuroblastoma, there is evidence of the existence of a phosphatidylcholine cycle in these nuclei (22). As reported in this study, downstream cellular mechanisms involving transcriptional factors, in particular Elk−1 were affected by CDP-choline. Neuroprotective agents like clomethiazole, attenuate c-fos, c-jun, and AP−1 activation through inhibition of p38 MAP kinase (29), suggesting a possible therapeutic use for CDP-choline.

CONCLUSION

This study demonstrates that the effects of CDP-choline in ischaemic stroke may go beyond its action on membrane synthesis and acetylcholine rescue. CDP-choline seems to have an inhibitory action on signal transduction molecules, which could have neuroprotective properties. There are various reports on the beneficial effect of CDP-choline in clinical stroke and in experimental studies (30,31). Further studies are required in order to elucidate the role of this major neuronal membrane lipid precursor in the development of stroke.

ACKNOWLEDGMENTS

This study was partially funded by Grupo Ferrer Internacional SA, Fundación Investigación Cardiovascular, Catalana-Occidente, FIS99–0907, SAF2000–0174

REFERENCES

1. Runden, E., Seglen, P., Haug, F., Ottersen, O., Wieloch, T., Shamloo, M., and Laake, J. H. 1998. Regional selective neuronal degeneration after protein phosphatase inhibition in hippocampal slice cultures: evidence for a MAP kinase-dependent mechanism. J. Neurosci. 18:7296–7305.
2. Yuan, J. 1997. Transducing signals of life and death. Curr. Opin. Cell. Biol. 9:247–251.
3. Kyriakis, J. and Avruch, J. 1996. Protein kinase cascades activated by stress and inflammatory cytokines. Bioessays 18:567–577.
4. Robinson, M. and Cobb, M. 1997. Mitogen-activated protein kinase pathways. Curr. Opin. Cell. Biol. 9:180–186.
5. Schindler, C. and Darnell, J. 1995. Transcriptional responses to polypeptide ligands: the JAK-STAT pathway. Ann. Rev. Biochem. 64:621–651.
6. Aronowski, J., Strong, R., and Grotta, J. 1996. Citicoline for treatment of experimental focal ischaemia: histologic and behavioral outcome. Neurol. Res. 18:570–574.
7. Trovarelli, G., deMedio, G., Dorman, R., Piccinin, G., Horrocks, L., and Porcellati, G. 1981. Effects of cytidine diphosphate choline (CDP-choline) on ischaemia-induced alteration of brain lipid in the gerbil. Neurochem. Res. 6: 821–833.
8. Weiss, G. 1995. Minireview, metabolism and action of CDP-choline as an endogenous compound and administered exogenously as citicoline. Life Sci. 56:637–660.
9. Clark, W. M., Warach, S. J., Pettigrew, L. C., Gammans, R. E., and Sabounjian, L. A. 1997. A randomized dose–response trial of citicoline in acute ischemic stroke patients. Citicoline stroke study group. Neurology 49:1234–1235.
10. Clark, W. M., Williams, B. J., Selzer, K. A., Zweifler, R. M., Sabounjian, L. A., and Gammans, R. E. 1999. A randomized efficiacy trial of citicoline in patients with acute ischaemic stroke. Stroke 30:2592–2597.
11. Clarke, W. M., Wechsler, L. R., Sabounjian, L. A., and Schwiderski, U. E. 2001. A phase III randomized trial of 2000 mg citicoline in acute ischaemic stroke patients. Citicoline stroke study group. Neurology 57:1595–1602.
12. Davalos, A., Castillo, J., Alvarez-Sabin, J., Secades, J. J., Mercadal, J., Lopez, S., Cobo, E., Warach, S., Sherman, D.,

Clark, W. M., and Lozano, R. 2002. Oral citicoline in acute ischemic stroke: an individual patient data pooling analysis of clinical trials. Stroke 33:2850–2857.

13. Tamura, A., Graham, D., McCulloch, J., and Teasdale, G. 1981. Focal cerebral ischaemia in the rat: description of technique and early neuropathological consequences following middle cerebral artery occlusion. J. Cereb. Blood Flow Metab. 1:53–60.
14. Eke, A., Conger, K., Anderson, M., and Garcia, J. 1990. Histologic assessment of neurons in rat models of cerebral ischemia. Stroke 21:299–304.
15. Krupinski, J., Slevin, M., Marti, E., Catena, E., Rubio, F., and Gaffney, J. 2003. Time-course phosphorylation of the MAP kinase group of signaling proteins and related molecules following middle cerebral artery occlusion in the rat. Neuropath. App. Neurobiol. 29:144–158.
16. Adibhatla, R. M. and Hatcher, J. F. 2002. Citicoline mechanisms and clinical efficacy in cerebral ischemia. Neurosci. Res. 70:133–139.
17. Hurtado, O., Moro, M. A., Cardenas, A., Sanchez, V., Fernandez-Tome, P., Leza, J. C., Lorenzo, P., Secades, J. J., Lozano, R., Davalos, A., Castillo, J., and Lizasoain, I. 2005. Neuroprotection afforded by prior citicoline administration in experimental brain ischaemia: effects on glutamate transport. Nerobiol. Dis. 18:336–345.
18. Sobrado, M., Lopez, M. G., Carcellar, F., Garcia, A. G., and Roda, J. M. 2003. Combined nimodipine and citicoline reduce infarct size, attenuate apoptosis and increase bcl−2 expression after focal cerebral ischaemia. Neuroscience 118:107–113.
19. Rao, A. M., Hatcher, J. F., and Dempsey, R. J. 2001. Does CDP-choline modulate phospholipase activities after transient forebrain ischaemia? Brain Res. 893:268–272.
20. Asahi, M., Hoshimaru, M., Uemura, Y., Tokime, T., Kojima, M., Ohtsuka, T., Matsuura, N., Aoki, T., Shibahara, K., and Kikuchi, H. 1997. Expression of interleukin−1β converting enzyme gene family and bcl−2 gene family in the rat brain following permanent occlusion of the middle cerebral artery. J.Cereb. Blood Flow. Metab 17:11–18.
21. Mallampalli, R., Ryan, A., Salome, R., and Jackowski, S. 2000. Tumor necrosis factor-alpha inhibits expression of CTP-phosphocholine cytidylyltransferase. J. Biol. Chem. 31:9699–9708.
22. Antony, P., Kanfer, J. N., and Freysz, L. 2000. Phosphatidylcholine metabolism in nuclei of phorbol ester-activated LA-N−1 neuroblastoma cells. Neurochem. Res. 25: 1073–1082.
23. Meister, A., Kleuser, B., Ollert, M., and Gercken, G. 1996. Betaine generation in cardiac myocytes after adrenergic activation of phosphatidylcholine hydrolysis. J. Mol.Cell Cardiol. 28:1109–1118.
24. Slevin, M., Krupinski, J., Slowik, A., Gaffney, J., and Szczudlik, A. 2000. Activation of MAP kinase (ERK−1/ERK−2), tyrosine kinase and VEGF in the human brain following acute ischaemic stroke. Neuroreport 11:2759–2764.
25. Irving, E., Barone, F., Reith, A., Hadingham, S., and Parsons, A. 2000. Differential activation of MAPK/ERK and p38/SAPK in neurones and glia following focal cerebral ischaemia in the rat. Brain Res. Mol. Brain Res. 77:65–75.
26. Walton, K., DiRocco, R., Bartlett, B., Koury, E., Marcy, V., Jarvis, B., Schaefer, E. M., and Bhat, R. V. 1998. Activation of p38MAPK in microglia after ischemia. J. Neurochem. 70:1764–1767.
27. Skaper, S., Facci, L., and Strijbos, P. 2001. Neuronal protein kinase signalling cascades and excitotoxic cell death. Ann. NY Acad. Sci. 939:11–22.
28. Zhau, G., Bao, Z., and Dixon, J. 1995. Components of new human protein kiase signal transduction pathway. J. Biol. Chem. 270:12665–12669.
29. Simi, A., Ingelman-Sundberg, M., and Tindberg, N. 2000. Neuroprotective agent clomethizole attenuates c-fos, c-jun, and AP−1 activation through inhibition of p38 MAP kinase. J. Cereb. Blood Flow Metab. 20:1077–1088.
30. Clark, W., Warach, S., Pettigrew, L., Gammans, R., and Sabounjian, L. A. 1997. Randomized dose–response trial of citicoline in acute ischaemic stroke patients. Neurology 49:671–678.
31. Schabitz, W., Weber, J., Takano, K., Sandage, B., Locke, K., and Fisher, M. 1996. The effects of prolonged treatment with citicoline in temporary experimental focal ischaemia. J. Neurol. Sci. 138:21–25.