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ORIGINAL ARTICLE

Citicoline (CDP-choline) increases Sirtuin1 expression concomitant to neuroprotection in experimental stroke

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Abstract

CDP-choline has shown neuroprotective effects in cerebral ischemia. In humans, although a recent trial International Citicoline Trial on Acute Stroke (ICTUS) has shown that global recovery is similar in CDP-choline and placebo groups, CDP-choline was shown to be more beneficial in some patients, such as those with moderate stroke severity and not treated with t-PA. Several mechanisms have been proposed to explain the beneficial actions of CDP-choline. We have now studied the participation of Sirtuin1 (SIRT1) in the neuroprotective actions of CDP-choline. Fischer rats and *Sirt1*$^{-/-}$ mice were subjected to permanent focal ischemia. CDP-choline (0.2 or 2 g/kg), sirtinol (a SIRT1 inhibitor; 10 mg/kg), and resveratrol (a SIRT1 activator; 2.5 mg/kg) were administered intraperitoneally. Brains were removed 24 and 48 h after ischemia for western blot analysis and infarct volume determination. Treatment with CDP-choline increased SIRT1 protein levels in brain concomitantly to neuroprotection. Treatment with sirtinol blocked the reduction in infarct volume caused by CDP-choline, whereas resveratrol elicited a strong synergistic neuroprotective effect with CDP-choline. CDP-choline failed to reduce infarct volume in *Sirt1*$^{-/-}$ mice. Our present results demonstrate a robust effect of CDP-choline like SIRT1 activator by up-regulating its expression. Our findings suggest that therapeutic strategies to activate SIRT1 may be useful in the treatment of stroke.

Keywords: CDP-choline, citicoline, neuroprotection, SIRT1, stroke.

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Sirtuin1 (SIRT1; silent information regulator 1) is a member of the sirtuin family of class III histone deacetylases implicated in a wide range of cellular functions (Haigis and Sinclair 2010). SIRT1 has been studied for its role in caloric restriction, the prevention of aging-related diseases, and the maintenance of metabolic homeostasis. The identification of SIRT1 activators is an active field of investigation because they are thought to be potentially beneficial not only for diseases related to metabolism but also for neurodegenerative diseases such as Alzheimer's disease (Bonda *et al.* 2011), Parkinson's disease (Donmez *et al.* 2012), and Huntington's disease (Pasinetti *et al.* 2011). Regarding stroke, although there is no direct evidence on the effects of SIRT1 on this pathology, there are several reports that support its beneficial role in this setting (Della-Morte *et al.* 2009; Zhu *et al.* 2010; Vang *et al.* 2011; Wang *et al.* 2011).

CDP-choline (Citicoline) is an intermediate in the biosynthesis of phosphatidylcholine, which has shown neuroprotective effects in a variety of CNS injury models including cerebral ischemia (Kakihana *et al.* 1988; Aronowski *et al.* 1996; Andersen *et al.* 1999; Adibhatla and Hatcher 2005;

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Abbreviations used: ICTUS, International Citicoline Trial on Acute Stroke; IH, infarcted hemisphere; MCAO, middle cerebral artery occlusion; RSV, resveratrol; SIRT1, silent information regulator 1; TTC, 2,3,5-triphenyltetrazolium chloride.

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Davalos and Secades 2011). In this context, previous work from several groups including ours has shown that CDP-choline is neuroprotective by decreasing extracellular glutamate levels and neurorestorative by promoting synaptic plasticity and functional recovery in experimental stroke models (Hurtado *et al.* 2005, 2007, 2008, 2011; Diederich *et al.* 2012).

CDP-choline has been also extensively studied in clinical trials. In a pooled analysis with individual patient data of randomised clinical trials, oral CDP-choline was associated with some evidence of efficacy in patients with moderate-to-severe acute ischemic stroke (Davalos *et al.* 2002) which it was not confirmed in a recent clinical trial (ICTUS) (Davalos *et al.* 2012). Although ICTUS has shown that global recovery is similar in CDP-choline and placebo groups, CDP-choline was more beneficial than placebo for some patients, as those with moderate stroke severity or not treated with t-PA (Davalos *et al.* 2012).

The beneficial properties of CDP-choline in different settings suggest the involvement of unknown, pleiotropic effects of this drug. An unexplored possibility is that these pleiotropic actions of CDP-choline might involve SIRT1. Therefore, we have embarked in a new study to search for novel mechanisms of action of CDP-choline involving SIRT1, in an attempt to identify new possible targets of its action in experimental stroke.

Methods

Animals

Experiments were performed on male Fischer rats weighing 250–300 g, and on *Sirt1*$^{-/-}$ mice. *Sirt1* heterozygous male mice (McBurney *et al.* 2003), kindly provided by Prof. Michael W. McBurney, were crossed to obtain gene-deficient (*Sirt1*$^{-/-}$) and wild-type (WT) animals from the same littermates. All procedures were carried out in accordance with the European Communities Council Directive (86/609/EEC) and reviewed by the Ethics Committees on Animal Welfare of University Complutense. A special effort was made to reduce the number of animals used in the study and to provide them with the most comfortable conditions possible.

Permanent focal cerebral ischemia model in rat

All experiments have been performed and quantified in a randomized manner by investigators blinded to treatment groups for the prevention of bias. Rats were anesthetized with isoflurane 1.5–2% in a mixture of 70% nitrogen/30% oxygen, and body temperature was maintained at physiological levels with a heating pad during the surgical procedure and anesthesia recovery. The femoral artery was cannulated for continuous monitoring of arterial pressure and blood sampling for analysis of pH, gases, and glucose. The studied physiological variables were not significantly different between groups of animals before, during or after middle cerebral artery occlusion (MCAO) (data not shown). Permanent focal cerebral ischemia was induced as described previously (Hurtado *et al.* 2008). Briefly, for the common carotid artery ligature, a midline ventral cervical incision was made, and the common carotid artery was isolated and permanently occluded with a silk ligature. For the MCA occlusion, a 1-cm incision perpendicular to the line connecting the lateral canthus of the left eye and the external auditory canal was made to expose and retract the temporalis muscle. A 2-mm burr hole was drilled and the MCA was exposed by cutting and retracting the dura. The MCA was elevated and cauterized (MCAO). Rats in which the MCA was exposed but not occluded served as sham-operated controls (SHAM). Following surgery, subjects were returned to their cages and allowed free access to water and food. The body temperature of animals was monitored throughout the experiment and was maintained at 37.5 ± 0.5°C using a heating pad. All experimental protocols adhered to the guidelines of the Animal Welfare Committee of the Universidad Complutense (EU directives 86/609/CEE, 2003/65/CE and RD 1201/2005). A special effort was made to reduce the number of animals used in this study and to provide them with the most comfortable conditions possible. The survival rate of the animals until the end of the experiment was 90%.

Permanent focal cerebral ischemia in *Sirt1*$^{-/-}$ mice

Middle cerebral artery (MCA) was exposed and occluded permanently by ligature as previously described (Sobrado *et al.* 2009). Briefly, a small craniectomy was made over the trunk of the MCA and above the rhinal fissure, and the artery was transitorily ligated with a 9–0 suture just after its bifurcation into the frontal and parietal branches producing a permanent MCA occlusion (pMCAO). Complete interruption of the blood flow was confirmed under an operating microscope. Sham-operated animals were subjected to anesthesia and the surgical procedure, but the occlusion of the arteries was omitted. Following surgery, subjects were returned to their cages and allowed free access to water and food. The survival rate of the animals until the end of the experiment was 90% and this rate was the same in all groups of animals. CDP-choline 2 g/kg was administered 10 min after the occlusion.

Experimental groups in rats

CDP-choline (0.2 g/kg or 2 g/kg), sirtinol (10 mg/kg), and resveratrol (RSV; 2.5 mg/kg) were administered in rats by intraperitoneal (i.p.) injection. Three sets of experiments were established. In the first set, five groups of animals were used for western blot analysis; all of them were killed 24 h after surgery: (i) naïve animals (naïve; $n = 4$); (ii) sham-operated animals (sham; $n = 4$); (iii) sham + CDP-choline 2 g/kg administered 4 h after operation (sham + CDP-choline; $n = 4$); (iv) permanent MCAO + an i.p. injection of saline 4 h after the occlusion (MCAO ipsi and MCAO contra -non-infarcted size-; $n = 4$); (v) permanent MCAO + an i.p. injection of CDP-choline 2 g/kg 4 h after the occlusion, (MCAO + CDP-choline; $n = 4$). In a second set of experiments, aimed at the pharmacological study of the role of SIRT1 in CDP-choline-induced neuroprotection, four groups were used for determination of infarct size 48 h after the occlusion: (i) MCAO + saline 4 h after occlusion ($n = 6$); (ii) MCAO + CDP-choline 2 g/kg administered 4 h after occlusion ($n = 6$); (iii) MCAO + the SIRT1 inhibitor, sirtinol 10 mg/kg administered 10 min, 24 h, and 48 h after occlusion ($n = 6$); (iv) MCAO + sirtinol 10 mg/kg administered 10 min, 24 h, and 48 h after MCAO + CDP-choline 2 g/kg 4 h after occlusion ($n = 6$). Finally, in a third set of experiments aimed at the study of a synergistic effect between CDP-choline and

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SIRT1 activators, four groups were used for determination of infarct volume 48 h after MCAO: (i) MCAO + saline 10 min and 3 h after occlusion ($n = 8$). (ii) MCAO + CDP-choline (0.2 g/kg 10 min after occlusion; $n = 6$); (iii) MCAO + the SIRT1 activator RSV (2.5 mg/kg 3 h after occlusion; $n = 7$); (iv) MCAO + CDP-choline (0.2 g/kg CDP-choline 10 min after occlusion) + RSV (2.5 mg/kg RSV 3 h after occlusion; $n = 6$).

All the groups were performed and quantified in a randomized fashion by investigators blinded to treatment groups. Animals were allocated by randomization (coin toss) to the different groups.

Determination of infarct size

Two days after MCAO, animals were killed to assess infarct outcome. Brain was removed and cut into 2 mm thick coronal brain slices (Brain Matrix, WPI, UK) and stained with 2,3,5-triphenyltetrazolium chloride (1% TTC in 0.1 M phosphate buffer). Infarct volumes in% of infarcted hemisphere (IH) were calculated as described (Sobrado *et al.* 2009).

Primary culture of pure cortical neurons

Pure cortical neuronal cultures were performed as described (Romera *et al.* 2004). Studies were performed in 9–10 days *in vitro*, time at which the cultures consisted of 94 ± 6% neurons, as determined by flow cytometry (Romera *et al.* 2004). In some experiments, CDP-choline 100 μM was included during 24 h and SIRT1 protein and activity were evaluated.

Isolation of neuronal nuclear extracts

Cultured rat cortical neurons were collected 24 h after treatment with vehicle or 100 μM CDP-choline in phosphate-buffered saline (10^6 cells/mL). The nuclear extracts were prepared as described (Hurtado *et al.* 2012).

Western blotting

Untreated or 100 μM CDP-choline-treated neurons and tissue collected from the peri-infarct brain area of rats killed 24 h after MCAO ($n = 4$ for each group) were prepared as described (Ramadori *et al.* 2008). For the detection of proteins, commercially available antibody was used for Sirt1 (1 : 100; Santa Cruz Biotechnology, Inc., Dallas, TX, USA). Sp1 antibody (Merck Millipore, Madrid, Spain) (1 : 1000) and β-actin antibody (Sigma-Aldrich, St. Louis, MO, USA) (1 : 1000) were used as loading control for nuclear and cytoplasm expression.

Measurement of SIRT1 enzymatic activity

To measure SIRT1 activity, cultured cortical neurons were collected 24 hours after vehicle or 100 μM CDP-choline treatment, and the total, nuclear, and cytosolic extract were analyzed using a SIRT1 fluorescent activity assay kit (Enzo Life Sciences AG, Lausen, Switzerland) based on Fluor–de-Lys-SIRT1 substrate peptide.

Isolation of rat peripheral blood mononuclear cells

Blood samples were collected 24 h from rats treated with 2 g/kg CDP-choline and centrifuged with 2 mL of anticoagulant-treated blood and an equal volume of phosphate-buffered saline (PBS, pH 7.6). Ficoll-Paque PREMIUM (GE Healthcare Europe GmbH, Barcelona, Spain) was added to the centrifuge tube and carefully layered on the diluted blood samples on Ficoll-Paque Premium. The sample was centrifuged at 400 *g* for 20 min at 20°C and the layer of mononuclear cells was transferred to a sterile centrifuge tube. The mononuclear cells were suspended in a homogenized buffer and mixed with the electrophoresis sample buffer for western blot analysis.

Statistical analysis

Results are expressed by mean ± SD of the indicated number of experiments. Comparisons between animal groups were performed using unpaired Student's *t*-test or one-way ANOVA with the Bonferroni *post hoc* test for multiple comparisons. Results were considered significant at $p < 0.05$.

Results

SIRT1 levels in rat brain. Effect of CDP-choline

First, we studied SIRT1 protein levels in brains from naïve and sham rats, as well as in both contralesional and ipsilesional hemispheres after exposure to MCAO. As shown in Fig. 1a, exposure to MCAO caused an increase in protein levels of SIRT1.

We then studied the effect of CDP-choline on SIRT1 expression in sham, and ischemic rat brain. As shown in Fig. 1b, CDP-choline caused an increase in SIRT1 protein expression in both brains from both sham and MCAO-exposed animals.

Effect of CDP-choline on infarct size after MCAO in rat. Effect of the SIRT1 inhibitor sirtinol

As previously demonstrated (Hurtado *et al.* 2008), administration of CDP-choline (2 g/kg) 4 h after the occlusion reduced infarct size determined 48 h after the occlusion (Fig. 2a). To study the role of SIRT1 in CDP-choline-induced neuroprotection, the effects of the SIRT1 inhibitor sirtinol were studied in this setting. Sirtinol (10 mg/kg) abolished CDP-choline-induced neuroprotective effect (Fig. 2a), strongly suggesting that SIRT1 is involved in the actions of CDP-choline in experimental stroke.

Synergistic neuroprotective effect of CDP-choline and the SIRT1 activator resveratrol in rats exposed to MCAO

As CDP-choline-induced increase in SIRT1 protein expression was involved in its neuroprotective effect, we hypothesized that this drug might display synergistic effects with other compounds that increase SIRT1 enzymatic activity. Therefore, we studied the effect of the association of subeffective doses of CDP-choline (200 mg/kg) and the SIRT1 activator resveratrol (2.5 mg/kg). Our data show that this association has a remarkable synergistic effect, demonstrated as a 60% reduction in infarct volume after MCAO (Fig. 2b), thus supporting that these drugs share a common target.

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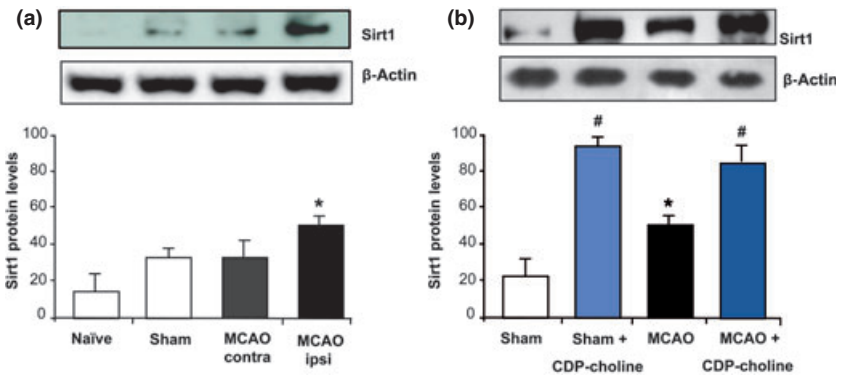

Fig. 1 Silent information regulator 1 (SIRT1) levels in rat brain are increased after middle cerebral artery occlusion (MCAO). Effect of CDP-choline. (a) Representative immunoblot shows the expression of SIRT1 protein in naïve, sham, MCAO contralateral/non-infarcted side and MCAO ipsilateral/infarcted side. Equal loading protein was confirmed by measuring β-actin. Bottom: Densitometric analysis of SIRT1 protein. Data are means ± SD; $^{*}p < 0.05$ versus sham; (b) Representative immunoblot shows the expression of SIRT1 protein in peri-infarct cortical tissue of sham and MCAO-exposed animals, and the effect of CDP-choline treatment. Equal loading protein was confirmed by measuring β-actin. Bottom: Densitometric analysis of SIRT1 protein. Data are means ± SD; $^{*}p < 0.05$ versus sham; $^{\#}p < 0.05$ versus sham or MCAO.

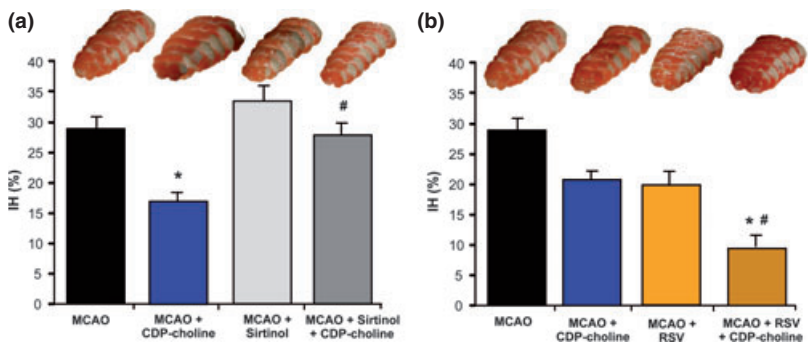

Fig. 2 Effect of CDP-choline, sirtinol, and resveratrol on infarct volume after middle cerebral artery occlusion (MCAO) in rats. (a) The protective effect of CDP-choline (2 g/Kg) is inhibited by the Silent information regulator 1 (SIRT1) inhibitor, sirtinol (10 mg/Kg). $^{*}p < 0.05$ versus MCAO and $^{\#}p < 0.05$ versus MCAO + CDP-choline. (b) Synergistic effect of subeffective doses of CDP-choline (200 mg/Kg) and of the SIRT1 activator, resveratrol (RSV; 2.5 mg/Kg). Rats were subjected to 48 h of permanent MCAO and% of infarcted hemisphere (IH) was quantified (see Materials and Methods) from 2,3,5-triphenyltetrazolium chloride (TTC)-stained serial coronal sections. $^{*}p < 0.05$ versus MCAO and $^{\#}p < 0.05$ versus MCAO + CDP-choline or MCAO + RSV.

Effect of CDP-choline on infarct size in *Sirt1*$^{-/-}$ mice after MCAO

To confirm that CDP-choline acts through SIRT1, we have tested the effect of this drug on infarct volume in *Sirt1*$^{-/-}$ mice after MCAO. In agreement with the important role of SIRT1 in CDP-choline effects, our data show that CDP-choline fails to reduce infarct volume in *Sirt1*$^{-/-}$ mice (Fig. 3).

Effect of CDP-choline on SIRT1 protein and activity in nuclear and cytosolic fractions from cultured rat cortical neurons

Our experiments using extracts obtained from cultured neurons show that SIRT1 is mainly localized in neuronal nuclei, with a minor expression in cytosol (Fig. 4a). Treatment with CDP-choline (100 μM) increased SIRT1 levels in these cells, being this increase localized mainly in the nuclear fraction (Fig. 4a).

We next determined whether the increased expression correlated with an increase in SIRT1 enzymatic activity in total, nuclear, and cytosolic extracts. Our results demonstrate that, in agreement with SIRT1 expression levels, CDP-choline significantly increased SIRT1 activity in total and nuclear extracts of these cells (Fig. 4b).

Effect of CDP-choline on SIRT1 levels in mononuclear cells

Finally, we examined whether CDP-choline might affect SIRT1 protein expression not only in rat brain and cultured neurons but also in other cells, such as peripheral blood mononuclear cells. Our results show that protein expression levels of SIRT1 were significantly higher in circulating

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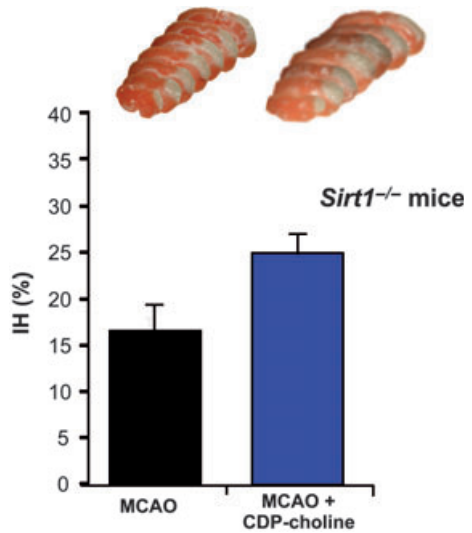

Fig. 3 Effect of CDP-choline on infarct size in *Sirt1*$^{-/-}$ mice after middle cerebral artery occlusion (MCAO). Mice were subjected to 48 h of permanent MCAO and% of infarcted hemisphere (IH) was quantified (see Materials and Methods) from 2,3,5-triphenyltetrazolium chloride (TTC)-stained serial coronal sections. Data are means ± SD, $n = 6$.

mononuclear cells from CDP-choline-treated rats, when compared with control rats (Fig. 4c).

Discussion

We have demonstrated that CDP-choline increases SIRT1 protein expression in rat brain, cultured neurons and in circulating blood mononuclear cells, an effect that is involved in the neuroprotective actions of this drug.

The first suggestion of the involvement of SIRT1 in the effects of CDP-choline derived from our findings showing that sirtinol, a specific inhibitor of SIRT1 that does not affect class I or class II HDACs (Grozinger *et al.* 2001), abolishes the neuroprotective effect of CDP-choline at a concentration previously shown to be effective (Grozinger *et al.* 2001), without having any effect on infarct volume in the absence of CDP-choline. In addition, we demonstrate that CDP-choline increases SIRT1 protein levels in rat brain from both sham and MCAO-exposed animals concomitant to neuroprotection, as well as in rat cultured cortical neurons and in circulating blood mononuclear cells. Moreover, this increased protein expression was functional as it was associated with an increased enzymatic activity. Whereas there are several reports that support the beneficial role of increasing SIRT1 activity (Della-Morte *et al.* 2009; Zhu *et al.* 2010; Vang *et al.* 2011; Wang *et al.* 2011), to our knowledge this is the first report on a drug able to modulate SIRT1 at the expression level, an action that could mediate neuroprotective actions in experimental stroke. Interestingly, we have also found that exposure to MCAO increases SIRT1 protein levels, in agreement with the results described in mice (Hernández-Jiménez *et al.*, 2013), suggesting that this protein may play a role as a stress response aimed at endogenous neuroprotection. In addition, the results showing SIRT1 protein expression in a peripheral population such as blood mononuclear cells and its modulation with CDP-choline suggest the possible utility of this parameter as a biomarker of SIRT1 status that could correlate with stroke severity/outcome, an important issue that remains to be ascertained in future studies.

To establish the role of the up-regulation of SIRT1 protein levels in CDP-choline-induced neuroprotection, we have studied the effects of this drug after pharmacological inhibition (using the SIRT1 inhibitor sirtinol in rats) or genetic deletion (using *Sirt1*$^{-/-}$ mice) of SIRT1. Our results show that CDP-choline-induced reduction in infarct volume is totally abolished in the absence of SIRT1, thus demonstrating that this enzyme is crucial for the neuroprotective effects of CDP-choline. In this sense, we have also shown that *Sirt1*$^{-/-}$ mice display larger infarct volumes than their wild-type counterparts after being subjected to ischemia, also pointing to an important neuroprotective action of endogenous SIRT1 in stroke (Hernández-Jiménez *et al.* 2013). Although our present data do not demonstrate a statistically significant effect of sirtinol alone on infarct volume, further studies using higher doses are required to clarify this issue.

Sirtuins, a NAD^+-dependent deacetylases family, have recently emerged as proteins involved in metabolism, aging, longevity, resistance to oxidative stress, and in genome maintenance (Lavu *et al.* 2008; Milne and Denu 2008; Donmez 2012). Sirtuins modulate gene expression depending on the energetic state of the cell, acting as energy sensors from NAD^+ levels, by deacetylating histones, transcription factors, and co-regulators. Specifically, SIRT1 is a novel emerging therapeutic target involved in the regulation of metabolism, senescence, and cancer, and it has crucial roles in stress-responsive signaling pathways (Kwon and Ott 2008). Several experimental approaches suggest its beneficial effects in several disorders, including metabolic (type 2 diabetes, mitochondrial myopathies), cardiovascular and inflammatory diseases, cancer, and neurodegenerative diseases (as Alzheimer's disease) (Donmez 2012). According to this, increased protein expression and/or activity of SIRT1 have been shown to slow down *in vitro* neuronal death as well as *in vivo* neurodegeneration (Kwon and Ott 2008). Recent data from our laboratory (Hernández-Jiménez *et al.* 2013) together with our present data now demonstrate that SIRT1 also plays a neuroprotective role in stroke and that therapeutic strategies that increase its protein expression, such as CDP-choline, may be considered for the treatment of this important pathology.

As commented above, to our knowledge, CDP-choline is the first drug able to increase SIRT1 activity by inducing an

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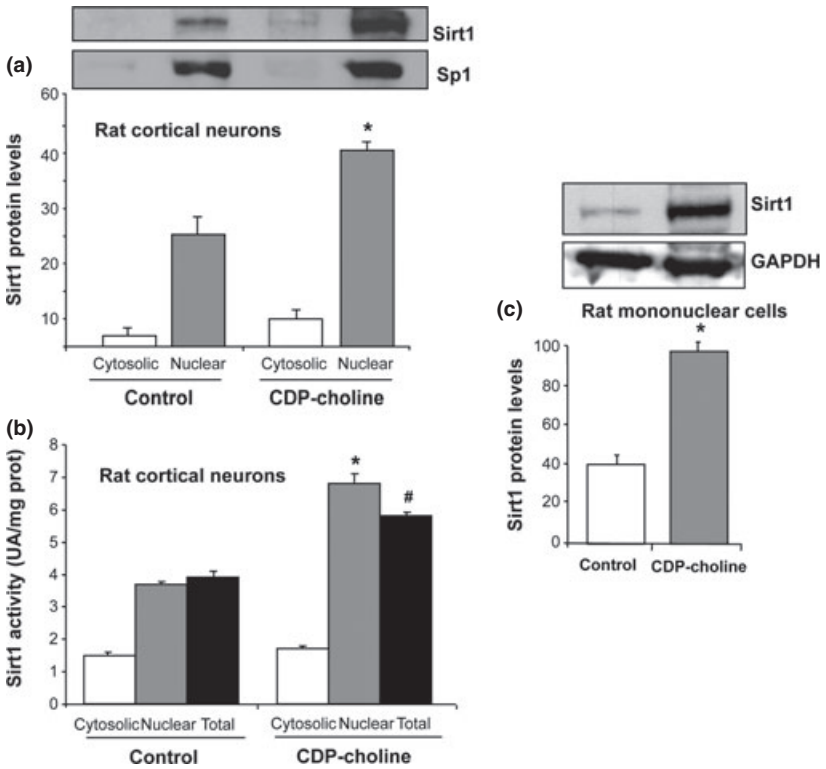

Fig. 4 Effect of CDP-choline on Silent information regulator 1 (SIRT1) in rat cultured cortical neurons and mononuclear cells. (a) Effect of CDP-choline on SIRT1 protein levels in rat cortical neurons. Representative immunoblot showing expression of SIRT1 in cytosolic and nuclear extracts (see Methods) in control and after CDP-choline treatment. Equal loading protein was confirmed by measuring Sp1. Densitometric analysis of SIRT1 protein. Data are mean ± SD, $^{*}p < 0.05$ versus nuclear control. (b) Effect of CDP-choline on SIRT1 enzymatic activity in rat cortical neurons. SIRT1 enzyme activity was measured in total, nuclear and cytosolic extract obtained 24 h after CDP-choline treatment. Data are mean ± SD, $^{*}p < 0.05$ versus nuclear control, $^{\#}p < 0.05$ versus total control. (c) Effect of CDP-choline on SIRT1 protein levels in rat mononuclear cells. Representative immunoblot showing expression of SIRT1 in mononuclear cells (see Methods). Equal protein loading was confirmed by measuring GAPDH. Densitometric analysis of SIRT1 protein from three independent experiments. Data are mean ± SD, $^{*}p < 0.05$ versus Control.

increase in its protein expression similar to the effects caused by stimuli such as calorie restriction, starvation, or DNA damage, among others (Kwon and Ott 2008). SIRT1 expression might be regulated at a transcriptional level, but also by increasing the stability of its transcript (Kwon and Ott 2008). This might be the case with CDP-choline, since this drug has been shown to decrease oxidative stress (Hurtado *et al.* 2011), a condition that decreases *Sirt1* mRNA stability (Abdelmohsen *et al.* 2007). However, further studies are required to unravel the mechanisms by which CDP-choline increases SIRT1 protein expression level and nuclear translocation.

SIRT1 studies have elicited significant interest in the development of drugs able to activate this pathway. In this context, several molecules have been described as acting, directly or indirectly, by increasing SIRT1 deacetylase enzymatic activity. This is the case of the natural polyphenol resveratrol, known as a promoter of protective effects against cardiovascular diseases. Specifically, in cerebral ischemia, resveratrol has demonstrated neuroprotective effects in animal models (Della-Morte *et al.* 2009; Vang *et al.* 2011), which seem to be mediated, at least partly, by SIRT1 activation (Della-Morte *et al.* 2009). It is not clear, however, if this is a direct effect on SIRT1 (Borra *et al.* 2005); in addition, other mechanisms of action different of SIRT1 activation have been reported to account for the effects of resveratrol (Vang *et al.* 2011).

In any case, theoretically, a combination of CDP-choline with SIRT1 activators might be an attractive and effective drug combination to increase SIRT1 activity in different settings. Our data demonstrate for the first time that, indeed, CDP-choline and the SIRT1 activator resveratrol have a potent synergistic effect leading up to 60% reductions of the infarct volume after MCAO, when used together at doses individually subeffective of these drugs. These data open new possibilities for the use of this drug association, able to yield maximal efficacy at concentrations devoid of adverse side effects.

A remaining issue regards to the targets of SIRT1 that may lead to the final neuroprotective effects of CDP-choline in cerebral ischemia. SIRT1 is an NAD+-dependent deacety-

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lase, but not only deacetylates histones as H1, H3, and H4, also deacetylates many non-histone proteins including p53, FOXO, LXRα, Ku70, p300, Rb, E2F1, NF-κB, p73, and PGC-1α. Its substrates are involved in the regulation of cellular metabolism, energy expenditure, inflammation, and cell stress response (Lavu *et al.* 2008). In this context, we have recently shown a significant increase in p53 and NFkB (p65) acetylation after inhibition/deletion of SIRT1 (Hernández-Jiménez *et al.* 2013). Other target is UCP2, which results inhibited, an effect that causes increased ATP production and therefore is beneficial in diabetes (Bordone *et al.* 2006) and stroke models (Della-Morte *et al.* 2009). Interestingly, we described that CDP-choline delays ischemia-induced ATP fall in experimental stroke models (Hurtado *et al.* 2005), an effect in which SIRT1-induced UCP2 decreased expression might be involved.

Also, further studies are required to establish whether SIRT1 and its regulation by CDP-choline may also serve to improve long-term outcomes, assessed in the chronic phase of stroke.

The effects proposed to explain some of the neuroprotective actions of CDP-choline have been thoroughly reviewed and include prevention of fatty acid release, stimulation of phosphatidylcholine synthesis, preservation of cardiolipin and sphingomyelin levels, increase in glutathione synthesis and glutathione reductase activity, restoration of Na^+/K^+-ATPase activity, and anti-apoptotic effects (Hurtado *et al.* 2011). Our findings, that demonstrate a novel way to increase SIRT1 activity, in this case by increasing its expression with CDP-choline, constitute a therapeutical approach which may explain CDP-choline neuroprotective effects in stroke, but which may also be useful for several pathologies ranging from neurological disorders (stroke, Alzheimer's disease, among others), diabetes, cardiovascular diseases, cancer, and diseases of aging. In addition, the potent synergy between CDP-choline and SIRT1 activators such as resveratrol opens a new way of treatment of these diseases.

Conclusions

Our present results demonstrate for the first time a robust effect of CDP-choline like SIRT1 activator by up-regulating its protein levels. Our findings suggest that therapeutic strategies to activate SIRT1 in the brain may be useful in the treatment of stroke.

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