DOI 10.1007/s00702-005-0384-3
J Neural Transm (2006) 113: 855–868

Journal of Neural Transmission
Printed in Austria

Neuroprotection of Cerebrolysin in tissue culture models of brain ischemia: post lesion application indicates a wide therapeutic window

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Received May 12, 2005; accepted September 10, 2005
Published online December 14, 2005;

Summary. All attempts to reduce neuronal damage after acute brain ischemia by the use of neuroprotective compounds have failed to prove efficacy in clinical trials so far. One of the main reasons might be the relatively narrow time window for intervention. In this study 2 different tissue culture models of ischemia, excitotoxic lesion by the use of glutamate and oxygen–glucose deprivation (OGD), were used to investigate the effects of delayed application of Cerebrolysin (Cere) on neuronal survival. This drug consists of low molecular weight peptides with neuroprotective and neurotrophic properties similar to naturally occurring growth factors. After both types of lesion, acute as well as delayed treatment with Cere resulted in a dose dependent and significant rescue of neurons. In the model of excitotoxic cell death significant drug effects were found even when the treatment started with a delay of 96 hours after addition of glutamate. In the OGD model pronounced effects were found after 48 hours delay of treatment, and even after 72 hours a small but significant rescue of neurons was detected. The neuroprotective effects of a single addition of Cerebrolysin to the culture medium resulted in significant protection until end of the experiments which was up to 2 weeks after the initial lesion. A shift of the efficacious dosages from low to high concentrations indicates that most likely active compounds are used up, indicating that multiple dosing might even increase the effect size. In conclusion the results indicate that Cere displays a relatively wide therapeutic time window which might be explained by a combination of acute neuroprotective properties and neurotrophic efficacy.

Keywords: Cerebrolysin, ischemia, primary cultures, neurons, apoptosis, necrosis.

Introduction

Millions of people worldwide are suffering from acute brain ischemia every year. Ischemic stroke is the third leading cause of death and it is the main reason for severe disability in adults. The dramatic consequences for the patients, losing basic activities of daily living often resulting in the need of lifelong intensive health care, are demanding the development of efficacious treatment

procedures. So far only thrombolytic therapy within the first 3 hours after onset of the stroke symptoms resulted in a significant therapeutic effect (Baker, 2005). The potential side effects of this type of treatment are restricting the number of patients for thrombolytic therapy to approximately 5 to 10% of all stroke cases (Grotta et al., 2001; Schellinger et al., 2004; Wardlaw et al., 2003). In addition it must be considered that not in all the treated patients a successful reperfusion is achieved. Therefore the interest of research was focused on neuroprotective treatment to prevent ongoing cell death which continues after days up to weeks after the acute event (Adams, 2001; Devuyst et al., 2001; Sun et al., 1999).

Based on the pathophysiological cascade of brain ischemia there was substantial hope in the effects of different types of glutamate receptor antagonists especially fostered by extremely positive data from animal experiments showing highly significant reduction of infarct volume (Ahmed et al., 2000; Katsuta et al., 1995; Prass et al., 1998). In spite of the promising pre-clinical data none of the compounds so far could show significant therapeutic effects in clinical trials (Calabresi et al., 2003; Liebeskind et al., 2001). The same conclusion has to be made for different types of calcium channel blockers (Kobayashi et al., 1998). It seems obvious that animal models of stroke have a rather low predictive value for the clinical development of neuroprotective drugs (Corbett et al., 1998; deLeciñana et al., 2001). The lack of efficacy of neuroprotective treatment in the clinical situation cannot only be explained by a relatively narrow time window for therapeutic interventions. New data support that neuronal cell death is continuing for days and weeks after stroke (Adams, 2001) with a significant shift from acute necrotic cell death to apoptosis (Back, 1998). Therefore also new compounds blocking apoptosis, inhibiting inducing and executing caspases and drugs modulating anti-apoptotic factors are under investigation (Ashwell, 2001; Holcik et al., 2001; Mouw et al., 2002; Waldmeier, 2003; Wiessner et al., 2000). But still no positive clinical data are available. Beside the relatively short time window also the fact that the infarcted area as well as the surrounding penumbra cannot be reached by therapeutic relevant drug dosages might be a factor. Studies in combination of thrombolytic and neuroprotective therapy addressing this problem have been performed only recently (Zhang et al., 2002) and the first data are published, but again, without pronounced efficacy.

The increasing knowledge about brain plasticity and the potency of pluripotent stem cells is even suggesting the transplantation of autologous or heterologous stem cells for stroke treatment, or the development of compounds inducing their proliferation and differentiation. Again, at least data in animal experiments are promising and are even indicating that stem cells are migrating to the lesion areas and are integrating into the neuronal circuits forming functional synapses. However, the application of the methodology is far away from clinical routine (Abe, 2000; Shimazaki, 2003).

Another option is the use of naturally occurring growth factors or compounds stimulating the expression of these factors. Some of them like the basic fibroblast growth factors (FGF-2) or the activity dependent neurotrophic factor and its derivatives showed promising effects in animal studies, anyhow, data from clinical trials with FGF-2 have not brought conclusive data (Abe, 2000; Bethel et al., 1997; Cuevas, 1997; Ferrer et al., 1998, 2001; Jin et al., 2000; Lee et al., 1991; Unoki et al., 1994). One of the biggest problems for the use of growth factors is the mode of application because they are not or only poorly penetrating the blood brain barrier. On the other hand due to their properties they might have both, an acute neuroprotective effect and an influence on rehabilitation by inducing neuronal sprouting and re-establishment of brain con-

nectivity, or addressing stem cell proliferation and differentiation.

Cerebrolysin (Cere) is a drug produced by standardized biotechnological procedures consisting of low molecular weight peptides and amino acids. It has been used since years for the clinical treatment of different neurological disorders including dementia and sequels of stroke or brain trauma. Different publications indicate that this drug has growth factor like activities and is promoting neuronal survival and sprouting similar to the naturally occurring nerve growth factor (NGF) (Satou et al., 2000; Windisch et al., 1993). In different lesion models addition of Cere to the tissue culture media resulted in a significant increase in neuronal viability (Gutmann et al., 2002; Hutter-Paier et al., 1996). All of the published data derived from experiments with pre-treatment of the neurons with the drug for a couple of days before the lesion was performed. Anyhow, the findings indicate protection against growth factor withdrawal, glutamate excitotoxicity, chronic calcium overload and chronic oxidative stress due to treatment of the tissue cultures with iron–citrate (Hutter-Paier et al., 1996). More detailed investigations about the possible mechanism of action indicated a modulation of the activity of both calpain isoforms, the one which is activated by low, and the other which is activated by high calcium concentrations (Wronski et al., 2000). Data from enzyme assays are in accordance with the fact that Cere is protecting neurons against loss of microtubuli associated protein-2 (MAP-2), because this cytoskeletal protein is the preferred substrate for calpains and is usually degraded rapidly after cell lesions (Hutter-Paier et al., 2000). But the results suggest that in addition the drug is inducing the expression of MAP-2, what is in accordance with the published neurotrophic activity.

Several in vivo experiments using different animal models of ischemic stroke have been published. After bilateral carotid artery-occlusion in combination with decreased blood pressure pre-treatment with Cere resulted in a significant decrease of acute mortality within the first 8 hours after induction of ischemia (Schwab et al., 1997), which was explained by the significant prevention of edema formation especially in areas of the brain stem. The same authors reported that Cere treatment in a model of middle cerebral artery (MCA) occlusion in the rat resulted in significant protection of MAP-2 (Schwab et al., 1998). In the ischemic hemisphere of saline controls there was an almost complete loss of MAP-2 immunoreactivity whereas in Cere treated rats the immunoreactivity in ipsi- and contralateral hemisphere were almost identical. These data are supporting the initial findings from the tissue culture experiments.

It was demonstrated that in a rat model of bilateral carotid artery-occlusion and sodium cyanide intoxication intraperitoneal and intracerebroventricular application of Cere are normalizing stroke dependent behavioral deficits already four days after lesion to the level of performance usually measured in controls (Gschanes et al., 1997). In a gerbil model of carotid artery-occlusion/re-perfusion it is reported that Cere is preventing the formation of different radical species (Sugita et al., 1993). All these findings indicate already the possible usefulness of this drug for treatment of brain ischemia, but recently published data demonstrating that Cere can induce the proliferation of brain stem cells and their differentiation into neurons in rats, point to another useful mechanism of action (Tatebayashi et al., 2003). So it can be speculated that Cere due to a multifactorial influence on neurons mediating acute neuroprotection as well as supporting neuronal survival by trophic stimulation can be an alternative way of treatment of stroke patients. Anyhow, so far only limited knowledge about the relationship between onset of treatment and efficacy was available (Ladurner, 2001). Therefore tissue culture experiments have been designed to

assess the effects of delayed Cere treatment on neuronal survival. Based on pathomechanisms of stroke two different models were utilized in this study. At first excitotoxic lesion was induced by addition of glutamate to tissue cultures of chicken cortical neurons, because protective data after pretreatment with Cere have been published already. As a second model the widely used oxygen glucose deprivation (OGD) model was studied. The main target was to collect data about the effects of delayed treatment on survival of lesioned neurons.

Material and methods

Primary culture of chicken telencephalon neurons

Primary neuronal cultures from 8 days old Lohman brown chicken embryo telencephalons were prepared as described previousely (Pettmann et al., 1979), whereas the cells were maintained in Dulbecco's minimum essential medium (DMEM, Cambrex) supplemented with 5% (v/v) Nu serum (Becton Dickinson), 2 mM glutamine (Bio Whittaker) and 0.1 mg/ml gentamycine (Cambrex). The experiments were performed in 96 well plates.

Glutamate lesion

The lesion with L-glutamate was performed on the third day in vitro by adding 10 µl of a glutamate solution to each well (except unlesioned controls) to reach a final concentration of 1 mM. The toxic substance remained with the cells for 24 hours. Before, during and after the lesion the cells were maintained in an incubator at 37°C, 95% humidity and 5% CO_2.

Treatment and evaluation schedule of glutamate lesioned samples

Cere was added to the samples on day five in vitro (24 hrs time window) or on day eight in vitro (96 hrs time window). Measurement of viability was performed on day 7, 9, 11 and 14 (24 hrs time window) or on day 9, 11, 14 and 17 in vitro (96 hrs time window).

Oxygen glucose deprivation (OGD)

On the 3rd day in vitro the medium was replaced by glucose-free DMEM and the plates – without lids – were placed in the OGD-chamber, which had been cleaned with 70% EtOH and checked for leakage before every use. On the lower level two Petri dishes filled with water are placed to ensure sufficient humidity during the lesion period. After closing the chamber is flushed with a gas mixture containing 95% N_2 and 5% CO_2 at a flow rate of 25 l per minute and closed hermetically. Subsequently the chamber is put in an incubator at 37°C. 45 minutes later the chamber is flushed again for 2 minutes at the same flow rate to ensure an atmosphere completely free of oxygen. Now the chamber is placed in the incubator again to rest at 37°C for the remaining lesion period. The entire lesion period was 24 hrs.

Maintenance of the cells during the recovery period (after OGD)

After the lesions the media were replaced by EMEM with 1 g glucose/l, 2% FCS, 0.01% gentamycin-sulfate and 2 mM L-glutamine. Until the end of the experiment the plates were kept in an incubator at 37°C, 95% humidity and 5% CO_2.

Treatment and evaluation schedule of OGD treated samples

In the first set of experiments, OGD was performed on the third day in vitro whereas the MTT test was performed 24 hours after Cere addition. Cere was added on day 3 (together with lesion onset), 4, 5 or 6 in vitro.

In the second set of experiments Cere was added directly after the lesion (day 4 in vitro). The evaluations were done on days 8, 10, 12 or 15 in vitro.

Statistics

Statistica 99′, Statsoft Inc., USA was used for data processing and statistical analyses. The Newman-Keuls test was performed to determine significance levels and a probability of error lower than 5% was considered as significant.

Results

After a 24 hours exposure to L-glutamate approximately 2 third of the neurons have been killed 4 days later. The application of Cere 24 hours after the lesion showed a significant increase in the number of viable neurons ($p < 0.05$) when viability was measured on the 4th day after lesion (Fig. 1a). The neuroprotection is dose-dependent, almost displaying double the number of viable cells in case of 80 µl of Cere per ml of tissue

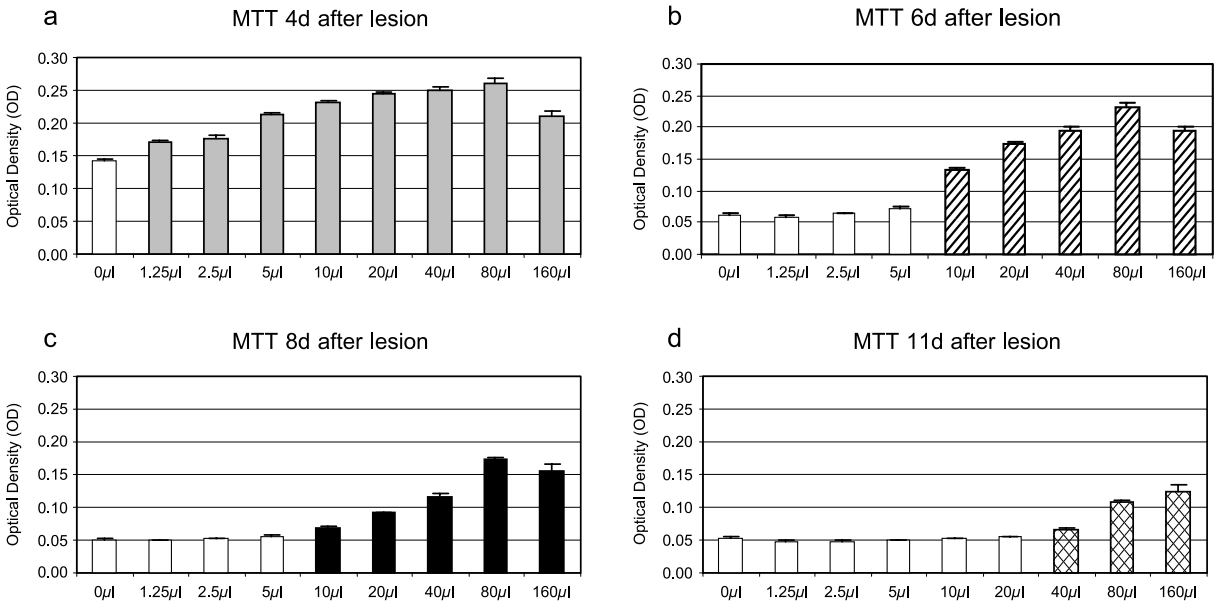

Fig. 1. Dose dependent protective effects of Cere application 24 hours after exposure of cortical neurons in culture to L-Glutamate. Cell viability was measured by means of the MTT assay 4 days (**a**), 6 days (**b**), 8 days (**c**) and 11 days (**d**) after lesion respectively. Values shown mean ± SEM. Number of experiments n = 18. Grey, hatched, black and angular chequered columns represent samples significantly different from controls

culture medium ($p<0.001$). 6 days after the lesion there is a highly significant reduction in living neurons ($p<0.001$) in the control wells, but also at that time point Cere still exhibits a significant neuroprotection ($p<0.05$) with a dosage of 10 µl per ml, showing again a peak effect at 80 µl per ml ($p<0.0001$, practically preventing further cell death with this dosage (Fig. 1b). Evaluating neuronal viability 8 days after lesion shows that there is a further small, but significant neuronal loss in the controls ($p<0.05$), but Cere again shows the dose dependent neuroprotection, with a significant increase in viable neurons in the dose range between 10 and 160 µl per ml (Fig. 1c). The last assessment of viability was done 11 days after lesion, showing that there is no further decline in the number of living neurons, and Cere still displays a significant protection with the dosages 40, 80 and 160 µl per ml ($p<0.01$), where the highest dosage increases viability almost 3 fold compared to controls (Fig. 1d). In a second series of experiments the treatment with Cere started 96 hours after the lesion. The first assessment of cell viability was performed 6 days after the lesion, 2 days after the treatment with the drug. 10 µl per ml of tissue culture medium produced a significant ($p<0.05$) increase in viability, and the protective effect was again dose dependent with highest rescue in 80 and 160 µl per ml treated wells (Fig. 2a). Evaluation of the living neurons 8 days after lesion shows that there is still a significant continuing cell death ($p<0.01$), and the Cere effects are completely maintained as shown at the earlier assessment time point (Fig. 2b). Further evaluations of cell viability were performed 11 and 14 days after lesion, showing that there is still a small but significant decline in the number of living neurons, but at both time points Cere shows a clear ($p<0.05$ to $p<0.001$) neuroprotection in the dose range between 5 and 160 µl, in case of the evaluation 14 days after lesion between 10 and 160 µl per ml of tissue culture medium (Fig. 2c and d). In both of the high-

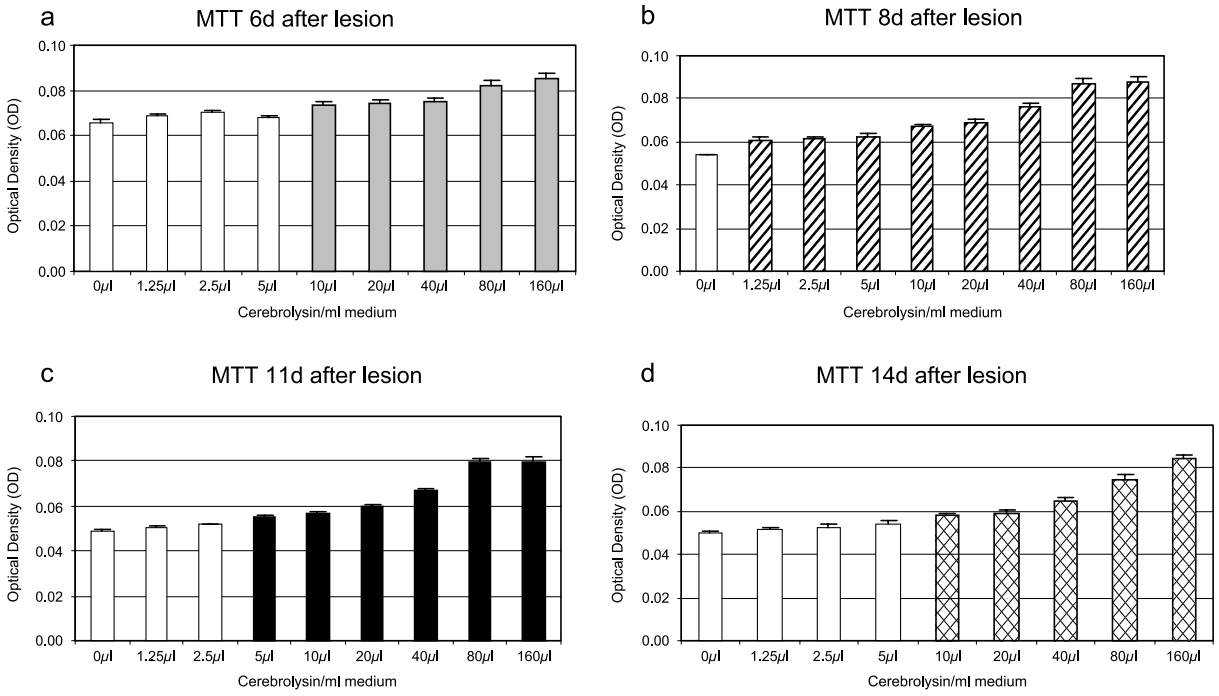

Fig. 2. Dose dependent protective effects of Cere application 96 hours after L-glutamate lesion of cortical neurons in culture. Cell viability was measured by means of the MTT assay 6 days (**a**), 8 days (**b**), 11 days (**c**) and 14 days (**d**) after lesion respectively. Values shown mean ± SEM. Number of experiments n = 18. Grey, hatched, black and angular chequered columns represent samples significantly different from controls

est dosages (80 µl and 160 µl) the cell counts do almost not change over the whole experimental period. In the lower dosage groups there is ongoing loss of neurons, but still a detectable neuroprotective effect.

In a further series of experiments the OGD model was used to simulate brain ischemia. Also this lesion leads to a significant reduction in the number of viable neurons under control conditions. In contrast to the glutamate lesion there is no further cell death between the assessment 24 hours and 72 hours after end of the lesion (Fig. 3a to d). Addition of Cere immediately after lesion, 24, 48 and 72 hours later resulted always in a significant neuroprotection, indicating an open therapeutic window for Cere in this model of at least 72 hours. Anyhow, the most pronounced effects were detected when the treatment started immediately with reoxygenation, showing a significantly increased ($p < 0.05$) number of neurons already at the dosage of 20 µl per ml and reaching a maximum effect at 40 µl per ml ($p < 0.001$). Similar efficacy was shown for the dosages of 80 and 160 µl per ml (Fig. 3a). A delayed treatment starting 24 hours after end of the OGD protected even better, and the effective dose range was identical to the early treatment experiment (Fig. 3b). A delay of drug application for 48 hours resulted in lower efficacy and first significant effects could be achieved only with a dosage of 80 or 160 µl per ml ($p > 0.001$, Fig. 3c). When the treatment started with a delay of 72 hours the effects were weak and a slight protection was achieved in the dose range between 20 and 160 µl. A significant cell rescue was found only at 160 µl per ml ($p > 0.05$; Fig. 3d). To get more information about the time course of Cere activity after lesion again OGD was performed and all wells were treated with the drug immediately after end of the 24 hours hypoxic period, and the assess-

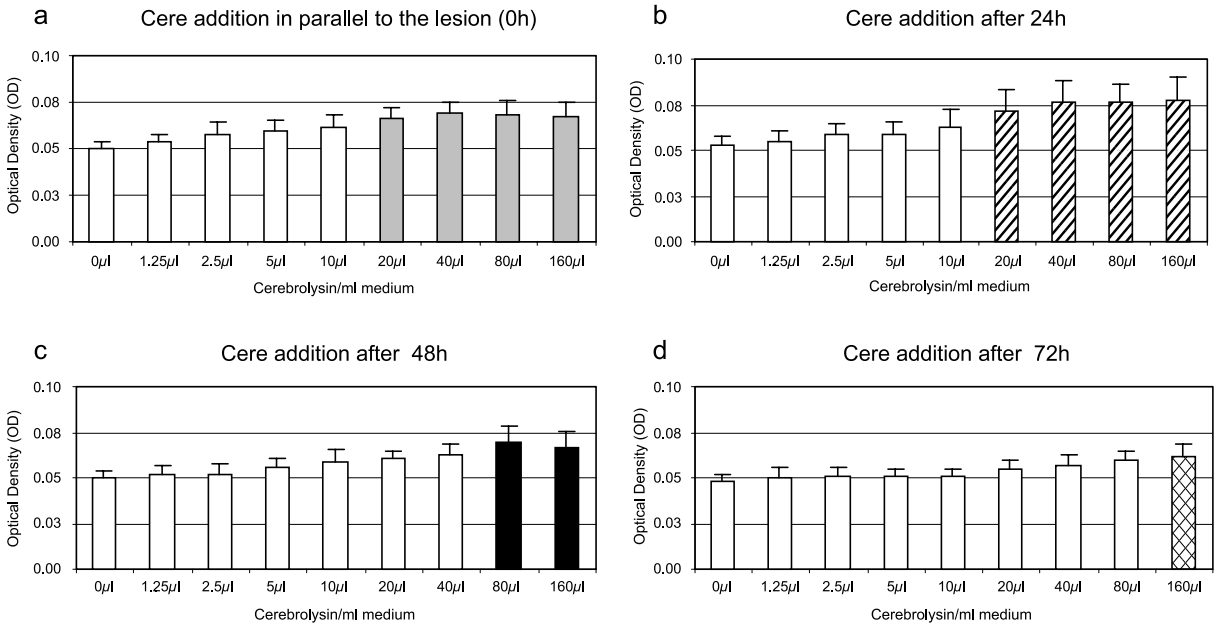

Fig. 3. Dose and time dependent effects of Cere in cultures of cortical neurons after oxygen glucose deprivation. Cere was applied together with onset of the lesion (**a**), with a delay of 24 hours (**b**), 48 hours (**c**) or 72 hours (**d**) after lesion respectively. Viability of neurons was assessed with the MTT-assay 24 hours after addition of the drug. Values shown mean ± SEM. Number of experiments n = 18. Grey, hatched, black and angular chequered columns represent samples significantly different from controls

ment of viability was performed 4, 6, 8 and 11 days later. In these experiments a similar correlation between time point of assessment and neuronal viability as in the glutamate experiments could be detected, showing a significant ongoing cell death between day 4 and day 11. When treatment effects were measured 4 days after lesion a significant protection was shown at 5 and 10 µl per ml, with a peak efficacy at 5 micro liters per ml (Fig. 4a). Higher dosages showed inconclusive data, not significantly influencing neuronal viability. The dosage of 160 µl even significantly decreased the number of living neurons ($p < 0.001$). 6 days after OGD the significant protection by the drug was still maintained in the dose range between 5 and 160 µl per ml ($p < 0.05$ to $p < 0.001$, Fig. 4b). Determination of cell viability 8 days after lesion resulted in a significant prevention of cell loss by Cere in the dose range between 5 and 160 µl per ml ($p < 0.05$ to $p < 0.001$, Fig. 4c). At the latest assessment time, 11 days after OGD, significant protection could be shown in the dose range of 40 to 160 µl per ml ($p < 0.05$ to $p < 0.01$, Fig. 4d). In summary all the data demonstrate that Cere displays clear neuroprotective potency even if treatment is initiated with a delay of up to 96 hours after onset of the lesion. The ongoing cell death after ischemic events advocates for an early initiation of neuroprotective treatment to rescue a maximum of cells.

Discussion

Neuroprotective treatment of ischemic stroke should help to minimize the loss of neurons, to keep the volume of brain damage as low as possible (Fisher et al., 2003). At the moment it is considered as an important therapeutic step to achieve reperfusion of the infarcted brain areas within a narrow time window which is resulting in a significantly better clinical outcome (Abboud et al., 2004). But the overall effects of early thrombolysis

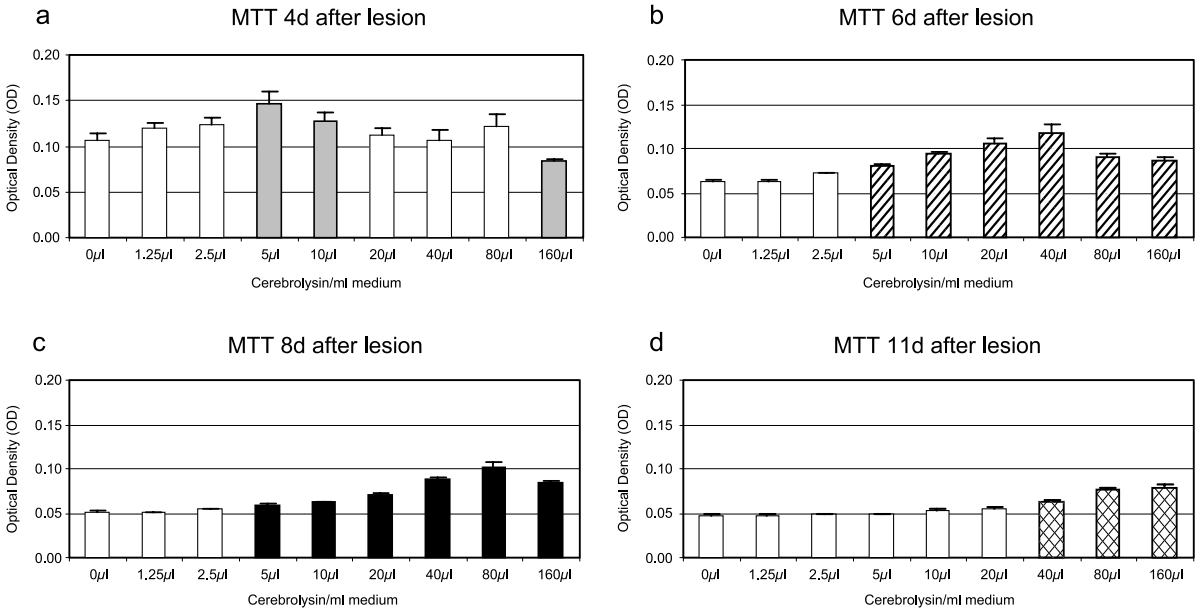

Fig. 4. Protective effects of Cere in tissue cultures of cortical neurons after oxygen glucose deprivation. The drug was added to the cultures immediately after end of the 24 hours lesion period and the assessment of viability was performed using MTT assay 4 days (**a**), 6 days (**b**), 8 days (**c**) or 11 days (**d**) after lesion respectively. Values shown mean ± SEM. Number of experiments n = 18. Grey, hatched, black and angular chequered columns represent samples significantly different from controls

are still not satisfying. Also a considerable number of patients do not qualify for this type of therapy (Svoboda, 2005). Therefore additional benefit was expected from neuroprotective drugs. Different compounds that showed promising effects in experimental models of ischemia unfortunately failed in clinical studies (Goldstein, 2004; Wahlgren et al., 2004).

The investigated drug Cere represents growth factor like properties and is able to maintain normal neuronal function, to induce sprouting reactions and is able to protect against different lesions in vitro and in vivo (Windisch et al., 1998). The compound showed significant and dose dependent protection in several tissue culture models of ischemia like sodium-cyanate intoxication, lesion with iodoacetate or excitotoxic damage using glutamate or NMDA (Gutmann et al., 2002; Hutter-Paier et al., 1996, 1998). But all of these experiments have been performed with pretreatment of the neuronal cultures for several days before the ischemia was initiated. In those studies cell viability was increased, and also a protection of neuritic outgrowth (Hartbauer et al., 2001b) has been shown which was explained by the prevention of MAP-2 loss (Hutter-Paier et al., 2000). This was explained by the growth factor like properties and a possible increase in protein synthesis (Piswanger et al., 1990). Of course additional effects like modulation of calpain activity has been reported (Wronski et al., 2000), so that the overall efficacy most likely results from direct inhibition of mechanisms related to necrotic as well as to apoptotic cell death and the possible induction of protective proteins (Hartbauer et al., 2001a).

But so far the presented series of experiments are the first to show clear protective effects of Cere when drug application started with delay after onset of the ischemic event. It has been proposed that during the first 24 hours after ischemia necrotic cell death

is prevailing, and then there is a continuous shift to apoptotic cell death, which can continue in vivo for days and may be even weeks (Back et al., 2004; Hu et al., 2002). In the glutamate model a 24 hour delayed application displays significant protection in all dosages if viability is measured 4 days later. With increase of the time period between lesion and viability measurement neuroprotective activity shifts to higher concentrations, but until 6 days the most efficacious dosages prevent further neuronal loss compared to the control treated groups. It is remarkable that even at day 11 post lesion the cell survival in the highest dosage group is tripled compared to the control. These data are indicating that Cere is active during the apoptotic phase of delayed neuronal cell death, but it also seems that some of the active compounds are consumed over time, which would explain the shift of efficacious dosages from low to high. Alternatively it can be speculated that different constituents of this complex drug are active in changing phases of lesion development. Delay of Cere application for 96 hours after L-glutamate lesion still shows a persisting neuroprotection. The overall neuronal viability measured is already much lower indicating that a significant proportion of cells were lost between day 4 and day 6 after lesion. Under control conditions ongoing degeneration can be detected until day 8, whereas even low dosages of Cere seem to prevent this progressive cell death at least to a certain degree. High dosages are completely blocking cell loss until 2 weeks after lesion. Again, a shift of efficacy like in the early application experiment was observed. Such long-term protection was not reported with any other compound. Also no experiments with such a long delay of application of a potentially neuroprotective drug have been published so far. Most of the experiments report efficacy only until 24 or 48 hours after lesion. Some studies show that NMDA antagonists are efficacious only in case of pre-treatment or direct application together with the lesion (Kaku et al., 1991; Rootwelt et al., 1998), but there are also studies demonstrating that a delayed application of compounds like MK-801 is able to rescue neurons subjected to glutamate excitotoxicity (Vornov et al., 1994). Reports about bilobalide, constituents of gingko biloba extracts which are believed to protect against oxidative stress demonstrate protection only in case of pre-incubation or if given directly with the lesion (Bastianetto et al., 2000; Chandrasekaran et al., 2003). No long term effects in vitro have been reported. In the glutamate lesion assay protection against free radicals seems to be an efficacious strategy because Ebselene, a radical scavenger, is achieving an almost complete protection for up to 48 hours (Porciúncula et al., 2001). This is in accordance with data about vitamin E and vitamin C effects in the glutamate model (Ciani et al., 1996). There are no reports about successful delayed application of radical scavengers. Also sigma ligands achieved reduction of neuronal damage in some ischemia models, but in rat neuronal cultures no effect was seen after NMDA excitotoxicity (Lesage et al., 1995; Nakazawa et al., 1998). To explain the effects of Cere data from studies with other growth factors might be helpful. TGF-β1 is reported to stabilize calcium homeostasis after ischemic lesion in vitro (Krieglstein, 1997), and there are also several reports about activity depending neurotrophic factor (ADNF) and derivatives demonstrating neuroprotection in tissue cultures (Glazner et al., 1999; Gozes et al., 2000; Guo et al., 2000). Because Cere contains various low molecular weight peptides there is also the possibility that effects of known neuropeptides are mimicked. For example that of VIP, which displays neurotrophic activity via induction of ADNF (Shoge et al., 1998; Sigalov et al., 2000), or that of different opioid peptides which can rescue from neuronal death via interaction with delta opiod receptors (Zhang et al., 2000).

But in the current study also a second, more complete model of ischemia, the oxygen–glucose deprivation was used. Again, Cere rescued neurons in a dose dependent way when applied either directly with the lesion or with delay of up to 72 hours. Addition of Cere 24 hours after OGD increases neuronal viability by approximately 50%, and similar efficacy is demonstrated after 48 and 72 hours respectively, but again there is a shift to higher dosages to see significant effects. In a second set of experiments Cere was added to the cultures immediately after end of the 24 hours of OGD and then neuronal viability was measured 4 days, 6 days, 8 days and 11 days after lesion. Again, significant protection can be seen at every time point of measurement with the already known dose shift but even at higher drug concentrations there is a further loss of viable neurons over time, but compared to controls the number of surviving neurons is increased approximately by 50%. It seems that the neuroprotection in the OGD lesion is less than after glutamate, but this can be explained by the fact that excitotoxicity displays only one aspect of ischemia, whereas OGD results in a complete break down of energy metabolism similar to in vivo conditions of brain ischemia. Also other compounds have been investigated in this model, but different cell types, culture conditions or measurements of drug efficacy are reported. Inhibitors of poly (ADP-ribose) polymerase are protective in vitro in case of pre-treatment, but the same compound (PJ34) shows also efficacy in a rat model of MCA (middle cerebral artery) occlusion/reperfusion when applied after onset of ischemia (Abdelkarim et al., 2001). MAO-B inhibitors like selegiline or rasagiline protect PC12 cells or SHSY5Y cells against OGD. The proposed mechanism of action is mediated by induction of bcl-2, an anti-apoptotic factor and activation of protein-kinase C and by downregulating the proapoptotic FAS and Bax protein families (Abu-Raya et al., 2002; Youdim et al., 2005). The induction of anti-oxidative genes has been shown to protect against the effects of hypoxia/ischemia and it might be part of the activity of different growth factors (Guo et al., 2000). But also in the OGD model most of the investigated compounds achieve significant neuroprotection only in case of pre-treatment. Relatively few compounds are reported to protect in a delayed treatment schedule like for example isoquinolinone derivatives in mouse cortical neuron cultures also influencing PARP (Chiarugi et al., 2003). Anyhow, no one has investigated neuronal viability over time periods of up to 2 weeks like it was done in the present study. The previously mentioned MAO-B inhibitors did not show neuroprotection 2 to 3 days after anoxic/hypoglycemic lesion, but they significantly protected in the first 24 hours (Ekblom et al., 1998). Again, there are several reports about growth factors and growth factor related compounds displaying anti-ischemic efficacy (Guo et al., 2000; Kusumoto et al., 1997). Their mechanisms of action cannot be explained in a satisfying way so far, but there are at least a few reported attempts. Vascular endothelial growth factor seems to protect via Akt and NFkappaB pathway and it involves suppression of cell-death pathways mediated by caspase-3 (Jin et al., 2001). TGF-β1 as mentioned above stabilizes intracellular calcium homeostasis. Insulin and insulin like growth factors seem to have an influence on density of neuronal GABA-A receptors (Mielke et al., 2005). Nerve growth factor (NGF) derived peptides seem to induce transcription factors which are stimulating expression of neuroprotective proteins (Guo et al., 2000). NGF and BDNF related peptides can reverse the increase of ischemia dependent intracellular calcium to normal and they induce an up to 60 fold increase in the activity of the transcription factor AP-1 (Shashoua et al., 2003). The vasoactive intestinal polypeptide, acting via induction of ADNF, seems to activate cAMP dependent pathways and protein-

kinase A (Sigalov et al., 2000). Several of these mechanisms might also help to explain the observed activity of Cere. In both investigated models of ischemia the inhibition of calpain by Cere as well as the effect on MAP-2 can be part of the protective mechanism (Hutter-Paier et al., 2000; Wronski et al., 2000). So far it is not known if Cere is also able to induce the expression of anti-apoptotic factors. But this drug showed activity in different in vivo models of ischemia. Reported effects were decrease in overall acute mortality, in vivo prevention of MAP-2 loss (Schwab et al., 1997, 1998), prevention of free radical formation (Sugita et al., 1993) and finally also accelerated and more complete recovery from functional deficits (Gschanes et al., 1997). Due to the complex composition of the drug most likely different synergistic effects contribute to the protection, but activity observed after delayed Cere addition might be also achieved by direct neurotrophic stimulation, like induction of sprouting and re-establishment of neuronal circuits. Such effects could significantly contribute to improvement of rehabilitation after stroke. At the moment it is difficult to make any conclusions from experimental studies about potential clinical efficacy, because of their relatively low predictive value. But for Cere first clinical data are already available indicating therapeutic efficacy (Barolin et al., 1996; Ladurner et al., 2005).

Acknowledgement

This work was funded by EBEWE Pharma, Unterach, Austria.

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