Poster Sessions CP13: Hypoxia, Ischemia and Oxidative Stress

CP13-01

Changes of brain nitric oxide production in experimental cerebral ischemia

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In order to study the role of nitric oxide (NO) in ischemic brain injury. Global cerebral ischemia was established in SD rats by modified Pulsinelli's method. The activities of constitutive nitric oxide synthase (cNOS), inducible NOS (iNOS), neuronal NOS (nNOS), nitrite (NO_2) and cyclic GMP in cerebral cortex, hippocampus, striatum and cerebellum at different time intervals were measured by radioimmunoassy, NADPH-d histochemistry and fluorometry methods. The results showed that the activities of cNOS increased at 5 min in four regions and decreased in cortex, hippocampus and striatum at 60 min, in cerebellum at 15 min iNOS increased in cortex and striatum at 15 min, in hippocampus and cerebellum at 10 min, and persisted to 60 min. The expression of nNOS increased after 5 min ischemia in cortex, striatum and hippocampus, and return to normal at 30–60 min. The NO_2 and cGMP also increased after 5–15 min ischemia and returned to normal after 30–60 min ischemia. These results indicated that the NO participated in the pathogenesis of cerebral ischemia injury and different types of NOS play different role in the cerebral ischemia injuries. Selected specific NOS inhibitors to decreased the excessive production of NO at early stage may help to decrease the ischemic injury.

CP13-02

Neuroprotection of mild hypothermia beginning at different time intervals on cerebral ischemia/reperfusion injury

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The effects of mild hypothermia beginning at different time intervals on cerebral ischemia was studied in SD rats which were divided into sham-operated, normothermia (37–38°C) ischemia and mild hypothermia (31–32°C) group. The latter was subdivided into: 240 min hypothermia, 30 min normothermia plus 210 min hypothermia, 60 min normothermia plus 180 min hypothermia and 90 min normothermia plus 150 min hypothermia. Global cerebral ischemia was induced by modified 4-vessel occlusion model. 240 min reperfusion following 20 min cerebral ischemia. Results showed that in normothermia group, the amount of SOD, GSH-Px, GSH, ATP, K^+ were lower and MDA, lactate, water content, Ca^{++} were higher than those in sham-operated group. Mild hypothermia beginning immediately within 60 min delayed the consumption of SOD, GSH-Px, GSH, ATP and decreased the accumulation of MDA, lactate, water content and Ca^{++} as compared with normothermia group. Mild hypothermia beginning immediately within 30 min significantly decreased the elevation of Na^+, Ca^{++} and increased K^+ in brain tissue when compared with normothermia. Mild hypothermia beginning immediately at 90 min has little effect on the content of SOD, GSH-Px, GSH, MDA, lactate, ATP, water content and Na^+, K^+, Ca^{++} as compared with the normothermia group. Mild brain hypothermia beginning immediately after ischemia delay consumption of endogenous antioxidant enzyme and energy metabolism, decrease accumulation of lactate and lipid peroxidation and the development of brain edema, which is involved in the mechanism of cerebral protection by mild hypothermia. Mild hypothermia limits ischemia injury beginning immediately within 30 or 60 min, but lost its function when beginning at 90 min following ischemia.

CP13-03

Novel mediators of ischemic preconditioning induced neuroprotection

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A brief ischemic episode induces protection against a subsequent severe ischemic insult. This phenomenon, known as ischemic preconditioning (PC) might be mediated by the activation of several genes of the second messenger signalling pathways. A 60-min transient middle cerebral artery occlusion (MCAO) in adult, SHR rats results in an infarct encompassing cerebral cortex and striatum by 24 h reperfusion. Present study observed that a 10-min PC, 3 days before the 60 min transient MCAO, results in a 30% reduction in the infarct volume. To understand the molecular mechanisms of the PC induced neuroprotection, we analyzed the mRNA expression profiles of rat cerebral cortex at 6, 24 and 72 h after a 10-min transient MCAO by using Affymetrix GeneChip and real-time PCR. Expression of the members of TGF-β signalling (TGF-β1, *p* 38 MAP kinase, Smad 1 and Smad 7) is observed to be up-regulated after PC. This pathway plays an essential role in tissue repair and immune homeostasis, but not hitherto implicated in PC induced neuroprotection. TGF-β1 enhances Smad transcriptional activity through activation of P38 gene expression to induce neuroprotection. Consistent with previous studies, expression of several neuroprotective genes including HSP70, HSP27, HO-1, MT-1/MT-2 and IL-6, were also observed to be up-regulated after PC. These studies shows that TGF-β1/p38 MAP-kinase signalling pathway is a promising target for developing drugs to limit ischemic neuronal damage.

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CP13-04

Superoxide activates synaptic particulate nitric oxide synthase: implications for superoxide signalling and neurotoxicity

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Mounting evidence supports the view that nitric oxide may protect against superoxide-induced oxidative damage by mechanisms involving nitrosylation, direct scavenging of oxygen radicals including superoxide, and up-regulation of antioxidant enzymes. However, the ability of neurons to respond to superoxide by increasing nitric oxide synthesis is unknown. We report here that superoxide, added as potassium superoxide (KO_2), produced a rapid activation (up to 12-fold) of nitric oxide synthase (NOS), measured by the conversion of [3H]arginine to [3H]citrulline, and nitrite production in a synaptic particulate fraction from rat brain cerebral cortex. Stimulation of NOS by KO_2 required the presence of ATP, which by itself inhibited basal NOS activity. Decayed KO_2 or authentic hydrogen peroxide did not increase NOS activity and, importantly, activation of NOS by KO_2 was blocked by addition of superoxide dismutase. In addition, stimulation of NOS activity by KO_2 was abolished by the NOS inhibitor N-monomethyl-L-arginine and EGTA but only partially reduced by the neuronal NOS inhibitor 7-nitroindazole. These findings are the first to demonstrate directly that superoxide activates a synaptic NOS isoform. The nature of the particulate fraction, the experimental conditions, and the low sensitivity to 7-nitroindazole strongly suggest that superoxide activated mitochondrial NOS (mtNOS). We propose that activation of the particulate NOS by superoxide reflects a novel mitochondrial response that may contribute to neuronal oxidant defenses although chronic activation of NOS under conditions of superoxide generation may produce toxic quantities of peroxynitrite and other reactive nitrogen oxides.

CP13-05

PARP-1 activation causes mitochondrial dysfunction

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When activated by DNA damage, PARP1 consumes NAD^+ to form ADP-ribose polymers on acceptor proteins. Excessive PARP1 activation during oxidative DNA damage is known to cause NAD+ depletion and cell death, but the intervening steps are not well understood. Sustained inhibition of the NAD+-dependent step in glycolysis is a potential consequence of NAD+ depletion. If this occurs, it should be possible to rescue cells from PARP1-mediated cell death by supplying alternative substrates for the mitochondrial tricarboxylic acid cycle. PARP1 was activated in cortical astrocyte and astrocyte-neuron cocultures with the DNA alkylating agent, N-methyl-N′-nitro-N-nitrosoguanidine (MNNG). Studies using the 2-deoxy-glucose method confirmed a sustained reduction in glycolytic flux, and studies with the JC-1 and TMRM indicate mitochondrial membrane depolarization. The addition of 1 mM of a-ketoglutarate or 1 mM pyruvate after washout of MNNG reduced cell death from 60 to 80% to near control levels, while addition of PARP inhibitors or excess glucose after MNNG washout had negligible effect. Cytoprotective effects remained significant with substrate delivery delayed up to 3 h after MNNG washout. The findings suggest that impaired glycolytic flux is a major factor in PARP1-mediated cell death, and suggest that supply of alternative mitochondrial substrates may be a promising strategy for delayed treatment of PARP1-mediated cell death in ischemia and other disorders.

CP13-06

Oxidative modification of mitochondrial proteins and cell death in Parkinson's disease

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Oxidative stress is one of the cell death mechanisms in neurodegenerative disorders, such as Parkinson's disease (PD) and Alzheimer's disease. Most of reactive oxygen species (ROS) generate in mitochondria through oxidative phosphorylation, and a part of them are not scavenged by antioxidative system and react with bioactive molecules. Recently, alpha-synuclein containing nitrotyrosine, a marker for oxidative modification by peroxynitrite, was identified in Lewy body. In addition, inhibitors of mitochondrial respiratory chain were reported to induce formation of Lewy body-like inclusion *in vivo* and *in vitro*. In this paper it was examined whether ROS and reactive nitrogen species (RNS) generated in mitochondria oxidize mitochondrial respiratory enzymes and induce the formation of inclusion body and cell death in PD. Human neuroblastoma SH-SY5Y cells were treated with a peroxynitrite donor, SIN-1, or an inhibitor of complex I, rotenone. After the treatment, proteins modified with toxic aldehydes, 4-hydroxynonenal and acrolein, and containing nitrotyrosine were analyzed by immunoblotting. Particularly in mitochondrial fraction, the oxidized protein was characterized by two-dimensional immunoblotting. Most of the oxidized proteins were detected in subunits proteins of complex I. These results indicate that mitochondrial complex I is a main target of oxidative stress in dopamine neurons and its dysfunction may be involved in the death mechanism in neurodegenerative disorders.

CP13-07

Induction of tissue transglutaminase in response to ischemic brain injury

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Tissue transglutaminase (tTG) is a member of the transglutaminase family that catalyzes the post-translational modification of proteins by inserting isopeptide bond within or between polypeptide chains in a Ca++ dependent manner. Although tTG is known to be involved in cell growth, apoptosis and neurodegeneration, the role of tTG in traumatic and ischemic brain injury (TBI and IBI) is largely unknown. Previously, we showed that tTG was up-regulated at mRNA and protein levels after TBI in rats. Although TBI and IBI can result in some common pathological sequelae, there are many important differences. Thus, we hypothesized that tTG message and protein is up-regulated after transient middle cerebral artery occlusion (MCAO), and that the magnitude and temporal expression differ from TBI. tTG protein and mRNA of ipsi-cortex and hippocampus from rats of 0, 1, 3, 5 and 7 days after MCAO were quantified by Western blot and real time PCR with tissues from similar contra-lateral areas as controls. Results showed that tTG mRNA in ipsi-cortex increased steadily from 1 to 5 days (1.5–10 fold of control level) after MCAO and dropped to control level by 7 days. In ipsi-hippocampus, tTG mRNA increased ~2 fold of control at 1 day after MCAO and gradually returned to control level by 7 days. tTG protein levels for both ipsi-cortex and ipsi-hippocampus increased steadily during the experiment with a more significant increase in cortex (~5.5 fold in ipsi-cortex vs. 2 fold in ipsi-hippocampus). Levels of tTG mRNA and protein of control tissues were unchanged. These results indicate that tTG after MCAO in cortex and hippocampus is up-regulated in a similar pattern to those in TBI brains. Studies of functional significance of tTG after ischemia and TBI are underway.

CP13-08

Role of oxidative stress in the ammonia-induced mitochondrial permeability transition in cultured astrocytes

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Ammonia is the principal neurotoxin implicated in the pathogenesis of hepatic encephalopathy (HE). The mechanisms of ammonia toxicity are unclear, but appear to involve oxidative stress and mitochondrial dysfunction. Astrocytes appear to be cell mainly affected. We have previously shown that ammonia increases the production of reactive oxygen species (ROS), and that it also induces the mitochondrial permeability transition (MPT) in cultured astrocytes. Since ROS is a factor commonly implicated in the induction of the MPT, we investigated the role of oxidative stress in the induction of the MPT by ammonia. We therefore examined the effects of various antioxidants on the induction by ammonia of the MPT in cultured astrocytes. Astrocytes were subjected to NH_4Cl (5 mM) treatment for two days alone or with various antioxidants. The MPT was assessed by 2-deoxyglucose (2-DG) permeability into mitochondria, and by changes in the mitochondrial membrane potential ($\Delta\Psi_m$) by confocal microscopy using the potentiometric dye JC-1. Astrocytes treated with ammonia significantly increased 2-DG permeability (90%, $p < 0.01$) and concomitantly dissipated the $\Delta\Psi_m$ (60%, $p < 0.01$), consistent with the induction of the MPT. The antioxidants superoxide dismutase (by 50%, $p < 0.05$), vitamin E (by 80%, $p < 0.01$), and the spin trapping agent PBN (by 80%, $p < 0.01$), all significantly blocked the increase in 2-DG permeability and the dissipation of the $\Delta\Psi_m$ produced by ammonia. These data demonstrate the induction of the MPT by ammonia and suggest the involvement of ROS in this process. Increased oxidative stress and subsequent induction of the MPT by ammonia in astrocytes may contribute to the pathogenesis of HE.

CP13-09

Acidification-induced changes in Cx43 protein–protein interactions

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Astrocytic gap junctions close in response to low intracellular pH, a process that may occur during ischemia. We propose that pH gating of Connexin43 (Cx43) involves binding of the carboxyl terminal domain (Cx43CT) to a pore-affiliated 'receptor' and, possibly, interaction of Cx43 with other molecular partners. We studied the role of intra– and intermolecular interactions in Cx43 pH gating. Peptides were made corresponding to intracellular regions of Cx43. Using Surface Plasmon resonance (SPR) we found pH dependent binding of the second half of the cytoplasmic loop to Cx43CT (Kd = 80 and 225 mM, pH 6.5 and 7.4, respectively). Binding was inhibited by preincubation with a synthetic peptide from region 346–362 of Cx43CT and by an antibody to this epitope, suggesting the binding site lies partially within this region. Nuclear magnetic resonance (NMR) studies showed formation of alpha helices in response to low pH in amino acid sequences VEMHL (aa 123–127) and IEEHGK (aa 139–143) in the loop, and a helical region within the suggested Cx43CT binding site. In addition, we found that acidification of astrocytes *in vitro* led to dissociation of Cx43 from its scaffold protein ZO-1. SPR showed that this was not due to pH dependence of Cx43-ZO-1 interaction (Kd = 2.23 mM at pH 7.4, 1.71 mM at pH 6.5) but to a strong pH–dependent interaction of Cx43 with c-Src (Kd = 0.63 mM at pH 7.4, 0.04 mM at pH 6.5) which caused ZO-1 to dissociate from Cx43CT. Separate experiments showed activation of astrocytic src at low pH. These changes in the composition of the Cx43 Nexus may play a major role in limiting tissue damage during brain ischemia.
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CP13-10

Sphingolipids in rat model of transient focal cerebral ischemia: implication for stroke injury

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Lipids are essential for signal transduction in response to trauma leading to neurodegeneration. Ceramide is an important mediator of apoptosis and cell proliferation. We studied the involvement of ceramide/sphingomyelin pathway in rat brain (stroke model) after 45 min ischemia followed by 24-h reperfusion. Ischemia was performed through occlusion of right middle cerebral artery (MCA). The level of ceramide was found increased (70–100% in ischemic side of brain v/s contralateral side of brain). Sphingomyelin levels were also decreased by 20–25% in ischemic brain v/s contralateral side of brain. Increase in ceramide and decrease in sphingomyelin were in good agreement with observed apoptotic cell loss (TUNEL assay) and decrease in the level of cardiolipin (a mitochondrian specific phospholipids) in affected ischemic brain. N-acetyl cysteine (NAC), a therapeutic agent recognized as potent antioxidant provided protective effect. Pretreatment with NAC before ischemia reduced the infarct volume size, suppressed apoptosis, restored cardiolipin level and decreased the levels of free fatty acids. However, NAC did not normalize the ceramide level. These interesting observations raise a question about the role of ceramide and its relationship with apoptosis and oxidative stress in rat brain ischemia.
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CP13-11

Inhibition of peroxisomal functions and production of excessive oxidative stress by psychosine in rat C6 glial cells

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The primary defect of abnormality in the metabolism of psychosine in Krabbe disease results in the loss of oligodendrocytes, increased oxidative stress, inflammatory reactions and profound demyelination. To understand the basis of toxicity of psychosine in the CNS of Krabbe disease, we examined the effects of psychosine on the peroxisomal β-oxidation of very long chain fatty acids (VLCFA), plasmalogen biosynthesis and on the production of reactive oxygen/nitrogen species. Rat C6 glial cells were treated with psychosine (2–10 mM) in absence and presence of cytokines (LPS + IFN-γ, or TNF-α + IL-1β) for 24 and 72 h. Psychosine inhibited peroxisomal b-oxidation of VLCFA (30–40% decrease compared to control) and induced the accumulation of VLCFA measured as levels of 26 : 0 (100% increase compared to control). Furthermore, psychosine treatment decreased the levels of plasmalogens (35–50% decrease compared to control). Plasmalogens levels were also drastically low in Krabbe brain than normal control brain. Psychosine also potentiated the known cytokine-induced inhibition of peroxisomal β-oxidation, accumulation of VLCFA and NO production. We also measured overall oxidative stress using dichlorofluorescin diacetate in psychosine-treated cells and found 1–2 fold increase in oxidative stress production. Both psychosine-mediated loss of peroxisomal functions as well as ROS production could be inhibited by antioxidant, N-acetylcysteine. Our results suggest that inhibition of peroxisomal functions and increased free radical production by psychosine may be responsible, at least in part, for the loss of oligodendrocytes and break down of myelin in Krabbe brain.
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CP13-12

Diazepam prevents changes in intracellular Cl^- and Ca^{2+} and restores neuronal activity after ischemia *in vitro*

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We have shown that diazepam (DZ) protects hippocampal neurons when administered early after transient cerebral ischemia. To examine mechanisms of DZ action, in the absence of systemic effects, we used an *in vitro* model of ischemia [oxygen-glucose deprivation (OGD)] in the adult hippocampal slice. We are interested in determining if DZ can prevent upstream events such as loss of ionic homeostasis and its consequences on neuronal transmission. We measured the effect of OGD on changes in intracellular Cl^- and Ca^{2+}. Application of DZ after OGD prevented the increase in intracellular Cl^- in area CA1 pyramidal neurons, measured by optical imaging with the Cl^- sensitive fluorescent probe, MEQ. The increase in intracellular Cl^- after OGD led to a reduction in GABA responses 2 h later, measured in the presence of the GABA agonist, muscimol. When DZ was added for 1 h after OGD, muscimol responses were restored completely. To measure changes in intracellular Ca^{2+} after OGD, we bath-loaded adult hippocampal slices with calcium green-1 AM. OGD increased intracellular Ca^{2+} in CA1 pyramidal neurons. The addition of DZ prior to OGD reduced the increase in intracellular Ca^{2+} by approximately 50%. To determine the effect of DZ on neuronal transmission within the hippocampus, we measured field responses in area CA1 pyramidal neurons. Area CA1 population spikes were suppressed by OGD and they did not recover 1 h later. When DZ was included in the reoxygenation buffer for only 30 min, it restored the population spike amplitude to within 85% of that in normal slices. Thus, after OGD, DZ restores ionic homeostasis, preserves GABA responses and restores neuronal activity within the hippocampus.
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CP13-13

Nitric oxide – mediated activation of ERK and JNK during hypoxia in neuronal nuclei of newborn pigs

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The extracellular signal-regulated kinase (ERK) and c-Jun N-Terminal kinase (JNK) phosphorylate antiapoptotic proteins thereby may regulate hypoxia-induced programmed cell death. The present study tests the hypotheses that hypoxia activates ERK and JNK in neuronal nuclei of newborn pigs and the hypoxa-induced activation of ERK and JNK is mediated by nitric oxide (NO). Activated ERK and JNK were assessed by determining phosphorylated ERK and JNK by immunoblotting in 6 normoxic (Nx), 10 hypoxic (Hx) and 5 N-nitro-L-arginine (a NOS inhibitor)-treated (40 mg/kg) hypoxic (NNLA-HX) 3–5-day-old-piglets. Animals were exposed to an FiO_2 of 0.05–0.07 for 60 min Neuronal nuclei were isolated and nuclear proteins were immunoblotted with antiphosphorylated ERK and JNK. Protein bands were detected, analyzed and expressed as (OD × mm^2). Tissue hypoxia was documented by ATP levels. ATP levels were 4.45 ± 0.57, 1.16 ± 0.33 and 1.10 ± 0.25 μM/g br in Nx, Hx ($p < 0.001$ vs. Nx) and NNLA-Hx ($p < 0.001$ vs. Nx) groups, respectively. Phosphorylated ERK density was 170.5 ± 53.7 in Nx vs. 419.6 ± 63.9 in the Hx group ($p < 0.001$) and 270.0 ± 28.7 in the NNLA-Hx group ($p < 0.002$ vs. Hx). Density of phosphorylated JNK was 172.8 ± 42.8 in the Nx vs. 364.6 ± 60.1 in the Hx ($p < 0.002$ vs. Nx) and 254.8 ± 24.8 in the NNLA-Hx group ($p < 0.002$ vs. Hx). We data demonstrate increased activation of ERK and JNK following hypoxia. NNLA,a NOS inhibitor, decreased the hypoxia-induced activation of ERK and JNK. We conclude that the hypoxia-induced activation of ERK and JNK is NO-mediated.

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CP13-14

Effect of neuronal NOS inhibitor, 7-nitro-indazole, on cytosolic caspase 9 activity *in vitro*

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Previous studies have shown that nitric oxide synthase (NOS) inhibitors prevent or reduce the hypoxia-induced neuronal injury. The present study tests the hypothesis that neuronal NOS inhibitor, 7-nitro-indazole (7-NI) will inhibit the cysteine protease, caspase 9, the initiator of programmed cell death. The effect of 7 NI on caspase 9 activity was determined in the cytosolic fraction of the cerebral cortex of normoxic (Nx, $n = 4$) and hypoxic (Hx, $n = 4$) newborn piglets. The animals were exposed to either 21% (Nx) or 7% (Hx) oxygen for 60 min. Cerebral tissue hypoxia was documented by the ATP and phosphocreatine levels. Cytosols were prepared and caspase 9 activity determined using a specific fluorogenic substrate (-Ac-Leu-Glu-His-Asp-AFC) for caspase 9 and monitored at 400 nm excitation and 505 nm emission wavelength. Caspase 9 activity in Nx group was 629 ± 49 as compared to 960 ± 105 pmoles AFC/min/mg protein ($p < 0.002$, 50.2% increase) in the Hx group. In presence of 10, 50 and 100 μM 7-NI, caspase 9 activity decreased to 459 ± 41, 252 ± 36 and 256 ± 29 pmoles/min/mg protein, respectively, in Nx group. In Hx group, the activity decreased to 578 ± 49, 402 ± 37, 393 ± 35 pmoles/min/mg protein, respectively. The data demonstrate that 7-NI inhibits caspase 9 in both the Nx and Hx groups. It is concluded that 7-NI is an inhibitor of caspase 9. It is proposed that 7 NI administration will be neuroprotective due to its properties as inhibitor of nNOS as well as of caspase 9 and prevent the hypoxia-induced neuronal death.

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CP13-15

The glucose paradox of cerebral ischemia – a possible resolution

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The glucose paradox of cerebral ischemia, the aggravation of delayed neuronal damage by preischemic hyperglycemia, is a well-documented phenomenon. Recently, we demonstrated that corticosterone (CT) plasma levels were significantly elevated within 30 min after glucose administration (i.p.) only to return to baseline levels by 120 min. In this study we examined the postulate that preischemic glucose loading aggravates the postischemic outcome via the induction of a short-lived, massive elevation in CT plasma levels. Hence, we tested the ability of the CT synthesis inhibitor metyrapone (MT) and the glucocorticoid receptor antagonist, mifepristone (RU486), to attenuate the aggravated delayed neuronal damage in a rat model of cardiac-arrest-induced transient global cerebral ischemia (TGI). Seven different groups of 24-h-fasted rats were used and were administered several treatments prior to TGI that were aimed at either inhibiting CT synthesis (MT, 100 mg/kg, i.p.) or at blocking the brain glucocorticoid receptors (RU486, 40 mg/kg, i.p.). Plasma glucose and CT levels were measured 2 min pre-TGI. Neuronal damage was assessed 7 days post-TGI in hippocampal slices using electrophysiological means. Plasma glucose levels were not reduced in MT- or RU486-treated rats. Both MT and RU 486 prevented preischemic hyperglycemia-aggravated ischemic damage, the former presumably via inhibition of CT synthesis, the latter by glucocorticoid receptor antagonism. The present data strongly suggest that preischemic hyperglycemia-aggravated neuronal damage, i.e. the glucose paradox, results from glucose-induced CT release, rather than intensification of lactic acidosis in the ischemic brain.