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Influence of Propofol on Neuronal Damage and Apoptotic Factors after Incomplete Cerebral Ischemia and Reperfusion in Rats

A Long-term Observation

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Background: **Propofol reduces neuronal damage from cerebral ischemia when investigated for less than 8 postischemic days. This study investigates the long-term effects of propofol on neuronal damage and apoptosis-related proteins after cerebral ischemia and reperfusion.**

Methods: **Male Sprague-Dawley rats were randomly assigned as follows: group 1 (n = 32, control): fentanyl and nitrous oxide–oxygen; group 2 (n = 32, propofol): propofol and oxygen–air. Ischemia (45 min) was induced by carotid artery occlusion and hemorrhagic hypotension. Pericranial temperature and arterial blood gases were maintained constant. After 1, 3, 7, and 28 postischemic days, brains were removed, frozen, and sliced. Hippocampal eosinophilic cells were counted. The amount of apoptosis-related proteins Bax, p53, Bcl-2, and Mdm-2 and neurons positive for activated caspase-3 were analyzed.**

Results: **In propofol-anesthetized rats, no eosinophilic neurons were detected, whereas in control animals, 16–54% of hippocampal neurons were eosinophilic (days 1–28). In control animals, the concentration of Bax was 70–200% higher after cerebral ischemia compared with that in animals receiving propofol over time. Bcl-2 was 50% lower in control animals compared with propofol-anesthetized rats during the first 3 days. In both groups, a maximal 3% of the hippocampal neurons were positive for activated caspase-3.**

Conclusions: **These data show sustained neuroprotection with propofol. This relates to reduced eosinophilic and apoptotic injury. Activated caspase-3–dependent apoptotic pathways were not affected by propofol. This suggests the presence of activated caspase-3–independent apoptotic pathways.**

STUDIES in laboratory animals have shown that anesthetic agents reduce infarct size and improve neurologic outcome after transient focal and incomplete hemispheric ischemia.[1,2] Most of these studies have assessed histopathologic and neurologic outcome for a period of less than 8 days after injury. However, data in isoflurane-anesthetized rats subjected to middle cerebral artery occlusion indicate that this observation period is inadequate to assess long-term protective effects of anesthetic agents.[3] Therefore, it is possible that the general notion that anesthetics increase the tolerance of neurons in situations of low perfusion reflects a short-term improvement rather than sustained neuroprotection. It is also possible that the failure of isoflurane to provide sustained neuroprotection is a drug-specific phenomenon or reflects mechanisms involving apoptosis. The current study investigates the effects of propofol on histopathologic injury and key proteins of apoptotic cell death in a model of incomplete hemispheric ischemia for a period of 28 days.

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Materials and Methods

Preparation

The experimental protocol used in this study was approved by the Ethics Committee for Animal Experimentation of the state of Bavaria in Munich, Germany. Fasted male Sprague-Dawley rats weighing 400 ± 60 g were anesthetized in a bell jar saturated with isoflurane. Rats were then tracheally intubated and mechanically ventilated (arterial carbon dioxide tension [Pa_{CO_2}], 38–42 mmHg) with 2.0 vol% isoflurane in nitrous oxide and oxygen (inspiratory oxygen fraction [F_{IO_2}], 0.33). Catheters were inserted into the right femoral artery and vein for blood withdrawal, blood sampling, and blood pressure measurement. Two catheters were inserted into the right jugular vein for drug administration. A loose ligature was placed around the right common carotid artery for later clamping. Temperature sensors were inserted into the rectum and the right temporal muscle. Pericranial temperature was maintained constant at 37.5°C using a servocontrolled overhead heating lamp and a heating pad. On completion of the surgical preparation, all surgical incisions were infiltrated with 0.5% bupivacaine. Respiratory parameters, pericranial temperature, arterial blood gases, arterial pH, and plasma glucose concentration were monitored and maintained constant during the

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experiment. An electroencephalogram was recorded continuously using subdermal platinum needle electrodes placed over both hemispheres at the parietotemporal *versus* frontal cortex recording sites (AC/AD Strain Gage Amplifier, model P122; Grass Instruments Division, West Warwick, RI).

Cerebral Ischemia

At the end of the preparation, the background anesthetic was discontinued, and the animals were assigned by random lists to one of the following treatment groups: Animals in group 1 (n = 32, control group) were anesthetized with fentanyl (intravenous bolus: 10 μg/kg, intravenous infusion: 25 $\mu g \cdot kg^{-1} \cdot h^{-1}$) and nitrous oxide in oxygen (F_{IO_2} = 0.33). Animals in group 2 (n = 32) received 0.8–1.2 $mg \cdot kg^{-1} \cdot min^{-1}$ intravenous propofol sufficient to induce burst suppression on the electroencephalogram and oxygen in air (F_{IO_2} = 0.33). After an equilibration period of 45 min, cerebral ischemia was induced by clip-occlusion of the right common carotid artery in combination with hemorrhagic hypotension to a mean arterial blood pressure of 40 mmHg for 45 min. At the end of the ischemia, the clip was removed, and the withdrawn blood, which was kept at body temperature, was reinfused over 15 min. Vecuronium was given as a continuous infusion (0.1 $mg \cdot kg^{-1} \cdot min^{-1}$) to maintain neuromuscular blockade, to prevent the animals from breathing (Pa_{CO_2} control), and to avert a rigor of the animals that is caused by fentanyl. The physiologic variables were recorded at four time points: before hemorrhagic hypotension (baseline), at 45 min of cerebral ischemia (ischemia), 15 min after cerebral ischemia on reinfusion of the withdrawn blood (reperfusion), and 45 min after the end of reperfusion (recovery). After completion of cerebral ischemia, catheters were removed, and the wounds were closed. The animals were extubated and assigned to one of four time-control groups to survive 1, 3, 7, or 28 days according to a predefined random list. At the end of the observation period, brains were removed in deep anesthesia (halothane) and placed in tissue-freezing medium (Jung, Nussloch, Germany). Then the brains were frozen in methylbutan and later on dry ice before being stored at −70°C. The brains were cut into 7-μm slices and mounted on slides. Brains of eight untreated animals were prepared in an identical fashion and were used to measure the natural expression of the investigated proteins (group 3, nonischemic rats).

Hematoxylin–Eosin Staining

From each animal, two 7-μm coronal tissue sections were stained with hematoxylin and eosin. The absolute number of eosinophilic neurons was counted by a blinded investigator in the hippocampal areas CA1–3 of the ischemic hemisphere and compared to the total number of hippocampal neurons for each section. The hippocampus of the nonischemic hemisphere was also analyzed for eosinophilic neurons, but the total number of neurons was not counted. If neuronal damage was too severe (*e.g.*, formation of a scar after 28 days), hippocampal damage was assumed to be 100%.

Immunofluorescence

For immunofluorescence staining, the tissue sections were fixed in ethanol, followed by a washing period using phosphate-buffered saline (PBS) with 0.1% Tween® 20 (Fulka Chemie, Buchs, Switzerland). The brain sections were then incubated in blocking buffer (10% fetal calf serum in PBS with Tween® 20), followed by incubation with the first antibody (rabbit polyclonal antibodies; Santa Cruz Biotechnology, Santa Cruz, CA), Bax antibody (I-19 and P-19), p53 antibody (FL-393), Bcl-2 antibody (C21), and Mdm-2 antibody (C18). After incubation, the brain sections were washed and incubated with the second antibody (Alexa Fluor® 488 goat anti-rabbit IgG antibody; Molecular Probes, Leiden, Netherlands), followed by another washing period. The tissue sections were covered with mounting medium (Vectashield® H-1000; Vectorlabs, Burlingame, CA) and cover slips and were stored at 4°C. With every staining, a negative control was performed by omitting the first antibody to detect nonspecific fluorescence. As a positive control, tissue sections of rat liver were used because Bax, p53, Bcl-2, and Mdm-2 are constantly expressed in this tissue. Within the next 24 h, the immunofluorescence intensity of the proteins (two sections per protein) in the hippocampal regions CA1–3 (four images per hemisphere) was recorded with a confocal laser scanning microscope (LSM 410; Carl Zeiss, Jena, Germany) by an investigator blinded to treatment condition. The constant intensity of the laser light was controlled with a power meter. Images were evaluated with the KS400-software (Carl Zeiss Vision, Jena, Germany). With the help of the software, hippocampal neurons were marked. The mean intensity of immunofluorescence (gray levels) that is proportional to the concentration of the fluorescence marker and therefore proportional to the mean protein concentration was measured within these marked neurons. Then the background intensity was subtracted.

Double Staining for Activated Caspase-3 and NeuN

Frozen brain sections were fixed in 4% paraformaldehyde. After being washed in PBS, the tissue sections were quenched using 3% H_2O_2 in methanol at room temperature. Sections were washed again, and a blocking agent (70 μl; DAKO, Hamburg, Germany) was applied. Afterward, tissue sections were incubated with the first antibody diluted in blocking agent (purified rabbit antiactive caspase-3 monoclonal antibody, Clone C92-605; BD Pharmingen, San Jose, CA). After another washing period in PBS, sections were incubated for 30 min with the second antibody (Universal-LSAB TM Kit;

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Table 1. Physiologic Variables

	Treatment	Time: Baseline	Ischemia	Reperfusion	Recovery
MAP, mmHg	Control	130 ± 12	40 ± 1†	126 ± 10	119 ± 12
	Propofol	100 ± 13*	40 ± 1†	111 ± 16	100 ± 20
Pa_{O_2}, mmHg	Control	139 ± 19	143 ± 20	142 ± 21	125 ± 30
	Propofol	153 ± 23	143 ± 36	136 ± 30	124 ± 33
Pa_{CO_2}, mmHg	Control	37 ± 4	40 ± 5	40 ± 4	40 ± 9
	Propofol	38 ± 4	40 ± 4	41 ± 5	42 ± 4
Hemoglobin, g/dl	Control	13.3 ± 1.1	9.8 ± 1.6	12.8 ± 1.0	12.7 ± 1.0
	Propofol	12.2 ± 1.0	9.1 ± 1.0	11.3 ± 0.9	11.1 ± 1.0
Glucose, mg/dl	Control	64 ± 9	55 ± 9	67 ± 9	85 ± 19
	Propofol	66 ± 9	59 ± 11	56 ± 9	56 ± 8

Mean arterial blood pressure (MAP), arterial blood gas tensions (Pa_{O_2} and Pa_{CO_2}), hemoglobin concentration, and plasma glucose concentration during baseline, ischemia, reperfusion, and recovery (45 min after end of reperfusion). Data are presented as mean ± SD.

* $P < 0.05$ propofol *vs.* control. † Controlled parameter.

DAKO). After being washed in PBS, the brain sections were incubated with streptavidin-conjugated horseradish peroxidase (Universal-LSAB TM Kit) and washed again in PBS. The sections were stained with one drop of chromogen substrate (DAKO) and were then rinsed gently in distilled water. Sections were washed and then incubated with purified mouse anti-neuronal nuclei monoclonal antibody NeuN; Chemicon International, Temecula, CA). The sections were washed before the second antibody (biotinylated horse anti-mouse antibody; Vector Laboratories, Burlingame, CA) was applied. Then the sections were incubated with streptavidin-conjugated alkaline phosphatase (Vector Laboratories) and washed again. The Vector red alkaline phosphatase substrate kit (Vector Laboratories) was used to stain neurons red. Finally, brain sections were counterstained with Mayer's hematoxylin, dehydrated in ascending alcohol concentrations, and mounted with Roti Histokitt (Roth, Karlsruhe, Germany). Using a light microscope, cells that were double positive for activated caspase-3 and NeuN were counted in the hippocampus. Their number was compared to the total amount of hippocampal cells.

Statistical Analysis

Continuous variables are presented as mean ± SD. Bivariate comparison of continuous variables were performed *via t* test or if the normality assumption was not met by the Mann–Whitney U test. Inferential statistics between factors were assessed by analysis of variance models in the context of general linear model and *post hoc t* test using the Bonferroni adjustment. The Greenhouse-Geisser correction was applied if sphericity assumption was not met. In detail, the following models were performed:

Physiologic variables are analyzed *via* a full factorial repeated-measures model using four repetitions (baseline, ischemia, reperfusion, and recovery) as the within-subject factor and day (four levels: 1, 3, 7, and 28 days) and intervention group (two levels: control and propofol) as between-subject factors.

The concentration of apoptosis-related proteins (Bax, p53, Bcl-2, and Mdm-2) was recorded on ischemic and nonischemic hemispheres. A full factorial repeated-measures model was applied to the within-subject factor hemisphere and between-subject factors group (control, propofol, untreated) and day (1, 3, 7, and 28).

For the analyses of eosinophilic cells and activated caspase-3/NeuN–positive cells, a univariate analysis of variance model was conducted. Group (control, propofol, untreated) and day (1, 3, 7, and 28) are treated as between-subject factors.

Statistical analyses were performed using SPSS 11.5 (SPSS Inc., Chicago, IL). All tests were performed two sided on a 5% level of significance.

Results

The physiologic parameters mean arterial blood pressure, arterial blood gas tensions, hemoglobin concentration, and plasma glucose concentration are listed in table 1. There were no differences for control animals or for propofol-anesthetized animals between 1, 3, 7, and 28 days; therefore, these parameters were presented together for each treatment. Baseline mean arterial blood pressure was higher in control animals compared with propofol-anesthetized animals.

The number of eosinophilic neurons in relation to the total number of neurons in the ischemic hippocampus is shown in figure 1. Although no eosinophilic neurons were detected in animals anesthetized with propofol, 16–54% of the neurons in the hippocampus of control animals were eosinophilic over time. There was no injury in the nonischemic hemisphere of control animals or propofol-anesthetized rats.

Figure 2 shows the concentration of the apoptosis-regulating proteins Bax, p53, Bcl-2, and Mdm-2 in the ischemic hemisphere. The concentration of Bax was

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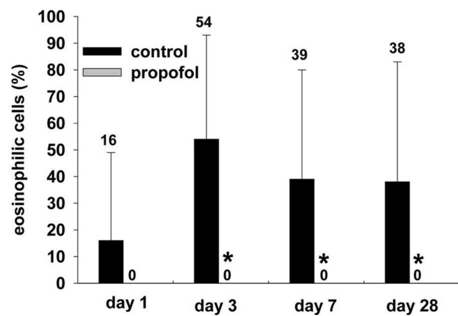

Fig. 1. Percentage of eosinophilic cells in the hippocampus of the ischemic hemisphere on days 1, 3, 7, and 28 after cerebral ischemia and reperfusion evaluated by hematoxylin and eosin staining. Data are presented as mean ± SD. * $P < 0.05$ *versus* control.

significantly higher in control animals compared with propofol-anesthetized or nonischemic animals on days 1, 3, and 7 (fig. 2A). An influence of the p53 protein could not be detected for different treatments over time (fig. 2B). The Bcl-2 concentration was lower in control animals compared with animals treated with propofol (during the first 3 days) and compared with nonischemic animals (at days 1, 3, and 28; fig. 2C). The concentration of Mdm-2 was lower in the control group compared with propofol-anesthetized animals after the first day and was higher after 28 days (fig. 2D).

Figure 3 shows the amount of hippocampal neurons (marker: NeuN) with a positive staining for activated capase-3. In both groups, 0.5–3.0% of all neurons were stained positive for activated caspase-3, with no difference between groups over time. In the nonischemic animals, no activated caspase-3–positive neurons were evident.

Discussion

In a model of incomplete cerebral ischemia and reperfusion, propofol reduces the number of eosinophilic neurons and favorably modulates apoptosis-regulating proteins. This neuroprotective effect is sustained and differs from transient neuroprotection seen with isoflurane.[3] Cerebral ischemia also induces active caspase-3, an additional marker of apoptotic cell death. However, propofol did not inhibit the expression of activated caspase-3. This suggests that over a long-term observation period, propofol can completely inhibit neuronal damage after cerebral ischemia and also interferes with caspase-3–independent apoptotic cell death.

Postischemic neurons develop cytoplasmic eosinophilia in hematoxylin and eosin preparations. This is due to increased production of basic proteins before the death of the cell.[4] There is a general notion that eosinophilia represents irreversible damage. In a light and transmission electron microscopic study in neurons exposed to hypoglycemic injury, eosinophilic neurons dis-

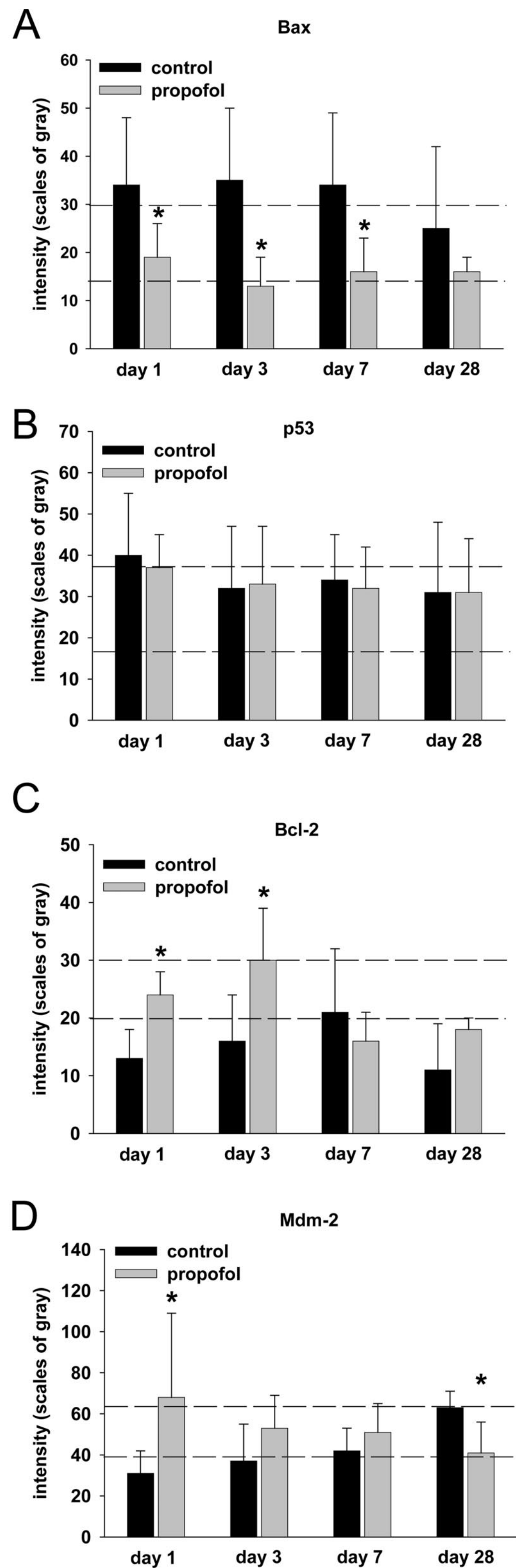

Fig. 2. Concentration of the proapoptotic proteins Bax (*A*) and p53 (*B*) and the antiapoptotic proteins Bcl-2 (*C*) and Mdm-2 (*D*) on days 1, 3, 7, and 28 after cerebral ischemia and reperfusion in the hippocampus of the ipsilateral hemisphere evaluated by immunofluorescence analysis. Data are presented as mean ± SD. * $P < 0.05$ *versus* control. The *dashed lines* show the normal range for protein concentrations in nonischemic rats. In the nonischemic hemisphere, similar results were found (data not shown).

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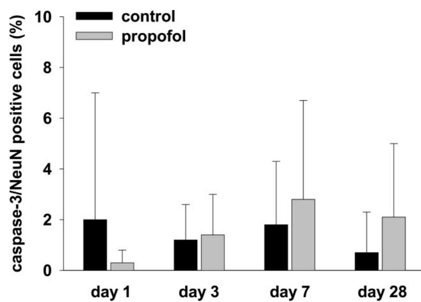

Fig. 3. Percentage of activated caspase-3– and NeuN–positive cells in the hippocampus of the ischemic hemisphere on days 1, 3, 7, and 28 after 45 min of cerebral ischemia evaluated by immunohistochemistry. Data are presented as mean ± SD.

played ultrastructural indicators of fatal cell injury, such as cytorrhexis and karyorrhexis.[5] Neurons exhibiting these electron-microscopic constellations underwent subsequent cytolysis. This justifies the use of eosinophilia as a morphologic endpoint of irreversible ischemic neuronal damage. In the current study, neuronal eosinophilia progressively increased with a peak at 3 days after ischemia and reperfusion. This temporal profile of neuronal damage is consistent with studies in rats after transient forebrain ischemia.[6–8] Anesthesia with propofol inhibited the ischemia-induced neuronal damage for the entire observation period of 28 days. This proves for the first time that neuroprotection previously observed with propofol[1,2] is not only a short-term rather than a sustained effect. The total suppression of neuronal damage by propofol might be related to the fact that, compared with other studies, a moderate form of cerebral ischemia was chosen to guarantee adequate survival of all animals for 1 month and to enable apoptotic cell death.

Apoptosis refers to programmed cell death. This endogenous program is essential in the modeling of organs during fetal life, enabling self-renewing processes, and eliminating infected or injured cells in adults. Superfluous cells die by apoptosis, and their death is initiated by a genetic program. When these dying cells have disintegrated into apoptotic bodies, they are phagocytosed by macrophages without inflammatory response. Apoptosis can be activated by an extrinsic (death receptor–mediated) or intrinsic (mitochondrial) pathway.[9] The intrinsic pathway is initiated by binding of the proapoptotic protein Bax to the permeabilization-related protein adenine nucleotide translocator, which is located in the mitochondrial membrane.[10] This increases the permeability of mitochondrial membranes and permits the release of cytochrome C from the mitochondria into the cytosol.[10,11] Rats subjected to permanent middle cerebral artery occlusion or transient incomplete cerebral ischemia developed increased concentrations of the death effector Bax.[12,13] The initial up-regulation of the proapoptotic protein Bax can be counteracted by the protein Bcl-2, which binds to the Bax protein.[14] Accordingly, transduction of Bcl-2 by a herpes simplex virus amplicon into the hippocampus of gerbils 24 h before transient global cerebral ischemia reduces hippocampal neuronal damage.[15] The concentration of Bax is up-regulated by the p53 protein, which normally controls cell cycle and DNA repair.[16,17] This p53 protein can be inactivated by the Mdm-2 protein, thereby inhibiting the intrinsic apoptotic pathway.[18] In the current study, cerebral ischemia increased the concentration of Bax and suppressed Bcl-2 formation compared with nonischemic animals. Propofol inhibited the ischemia-induced increase of the apoptosis-promoting protein Bax. In addition, propofol stimulated the synthesis of the apoptosis-inhibiting protein Bcl-2. This confirms previous observations where propofol prevented the up-regulation of Bax 4 h from cerebral ischemia.[19] The protein Bcl-2 reacts slower than Bax, because Bcl-2 was not decreased at 4 h[19] rather than 24 h after injury. In contrast to other studies, in the current preparation, the proapoptotic protein p53 was not involved in apoptotic mechanisms after cerebral ischemia.[20] In generation of hypothesis, this might be related to different mechanisms of neuronal trauma (various ischemia models, radiation, excitotoxic substances)[21] or regional differences (hippocampus, striatum, cortex). Furthermore, it is possible that the temporal window of p53 detection was not adequate to the dynamic or the half-life of the p53 protein. Surprisingly, the antiapoptotic protein Mdm-2 seems to act independent of p53, because Mdm-2 was increased on day 1 in propofol-anesthetized animals. In conclusion, propofol might avoid apoptotic cell death by influencing the equilibration of proapoptotic and antiapoptotic proteins in favor of apoptosis-inhibiting proteins.

Cytochrome C activates the cytosolic caspases and thereby induces the apoptotic cell death. Caspases are constitutively expressed in the cytoplasm as procaspases. After activation of caspase-9 by cytochrome C, procaspase-3 is cleaved. Caspase-3 belongs to the group of downstream terminators that destroy essential structural and vital proteins of the cell, leading to apoptotic bodies.[9] Because propofol seems to have antiapoptotic properties by mechanisms related to the formation of Bax and Bcl-2, a reduced amount of neurons that are positive for activated caspase-3 was expected in propofol-anesthetized animals. However, 0.5–3.0% of all neurons were positive for activated caspase-3 regardless of the presence of propofol. This might be explained by the existence of Bax– and Bcl-2–independent pathways for activation of caspase-3. For example, within the intrinsic apoptotic pathway, the enzyme caspase-2 can directly induce the release of cytochrome C from the mitochondria into the cytoplasm.[22] The extrinsic apoptotic pathways are mediated by death receptors (*e.g.*, Fas-receptor) independent of mitochondrial activation.[23] Both mechanisms (caspase-2 and Fas-receptors) result in the activation of caspase-3 independent of Bax and Bcl-2.

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However, these results do not disprove the key role of Bax and Bcl-2 in the execution of apoptosis. Both proteins regulate other key proteins, such as the apoptosis-inducing factor, another promoter of apoptosis independent of caspase-3 activation.[11,24] This emphasizes the problem that no reliable marker of apoptotic cell death exists, because of the different pathways leading to apoptotic cell death.

In this model of incomplete cerebral ischemia with reperfusion, changes in the concentration of apoptosis-regulating proteins are similar in the hippocampus of both hemispheres. This may be due to a decrease in cerebral blood flow in the nonischemic hemisphere of 40–60%[25] caused by the hemorrhagic hypotension during ischemia. Although the extent of cerebral blood flow reduction is above the threshold to induce neuronal necrosis in the nonischemic hemisphere,[26] it apparently induces apoptotic signals in a vulnerable brain region such as the hippocampus.

It has been shown that after 3 h of isoflurane anesthesia, the tolerance toward permanent middle cerebral artery occlusion in rats was increased even when ischemia was induced 24 h after isoflurane application.[27] An effect of preconditioning with isoflurane cannot be ruled out in the current study because isoflurane was given for 1 h during the preparation period. However, isoflurane anesthesia was used in both treatment groups. Even if there was an overall effect of preconditioning, it should not have influenced the comparability of both groups.

The current study shows for the first time that propofol inhibits neuronal damage after incomplete cerebral ischemia with reperfusion for at least 28 days after injury. Propofol also seems to possess antiapoptotic qualities by influencing apoptosis-regulating proteins after cerebral ischemia. Interestingly, the well-known caspase-3–dependent apoptotic pathway was not affected by propofol. Therefore, further caspase-3–independent apoptotic pathways should be investigated to better understand the antiapoptotic properties of propofol.

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