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.

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 [\(\text{PaCO}_2\)], 38–42 mmHg) with 2.0 vol% isoflurane in nitrous oxide and oxygen (inspiratory oxygen fraction [\(\text{FiO}_2\)], 0.33). Catheres 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 study.

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.
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 µg · kg⁻¹ · h⁻¹) and nitrous oxide in oxygen (FiO₂ = 0.33). Animals in group 2 (n = 32) received 0.8–1.2 mg · kg⁻¹ · min⁻¹ intravenous propofol sufficient to induce burst suppression on the electroencephalogram and oxygen in air (FiO₂ = 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 infused over 15 min. Vecuronium was given as a neuromuscular blockade, to prevent the animals from surviving 1, 3, 7, or 28 days according to a predefined random list. After the equilibration period, 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 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 cosinophilic 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 (FL393), 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 (Vectorshield® 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% H₂O₂ 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 antiactivate 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;
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 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 positive cells. 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 hemisphere 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

### Table 1. Physiologic Variables

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Baseline</th>
<th>Ischemia</th>
<th>Reperfusion</th>
<th>Recovery</th>
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<tbody>
<tr>
<td>MAP, mmHg</td>
<td>Control 130 ± 12</td>
<td>40 ± 1†</td>
<td>126 ± 10</td>
<td>119 ± 12</td>
</tr>
<tr>
<td></td>
<td>Propofol 100 ± 13*</td>
<td>40 ± 1†</td>
<td>111 ± 16</td>
<td>100 ± 20</td>
</tr>
<tr>
<td>PaO₂, mmHg</td>
<td>Control 139 ± 19</td>
<td>143 ± 20</td>
<td>142 ± 21</td>
<td>125 ± 30</td>
</tr>
<tr>
<td></td>
<td>Propofol 153 ± 23</td>
<td>143 ± 36</td>
<td>136 ± 30</td>
<td>124 ± 33</td>
</tr>
<tr>
<td>PaCO₂, mmHg</td>
<td>Control 37 ± 4</td>
<td>40 ± 5</td>
<td>40 ± 4</td>
<td>40 ± 9</td>
</tr>
<tr>
<td></td>
<td>Propofol 38 ± 4</td>
<td>40 ± 4</td>
<td>41 ± 5</td>
<td>42 ± 4</td>
</tr>
<tr>
<td>Hemoglobin, g/dl</td>
<td>Control 13.3 ± 1.1</td>
<td>9.8 ± 1.6</td>
<td>12.8 ± 1.0</td>
<td>12.7 ± 1.0</td>
</tr>
<tr>
<td></td>
<td>Propofol 12.2 ± 1.0</td>
<td>9.1 ± 1.0</td>
<td>11.3 ± 0.9</td>
<td>11.1 ± 1.0</td>
</tr>
<tr>
<td>Glucose, mg/dl</td>
<td>Control 64 ± 9</td>
<td>55 ± 9</td>
<td>67 ± 9</td>
<td>85 ± 19</td>
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<tr>
<td></td>
<td>Propofol 66 ± 9</td>
<td>59 ± 11</td>
<td>56 ± 9</td>
<td>56 ± 8</td>
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</tbody>
</table>

Mean arterial blood pressure (MAP), arterial blood gas tensions (PaO₂ and PaCO₂), 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.
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 caspase-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. 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 interfere 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. 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-
Cytochrome C activates the cytosolic caspases and thereby induces the apoptotic cell death. Caspases are constitutively expressed in the cytoplasm as pro-caspases. 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.
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. 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 nonschismic hemisphere of 40–60% 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 nonschismic hemisphere, 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. 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.

References


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