Neuroprotective, Anesthetic, and Cardiovascular Effects of the NMDA Antagonist, CNS 5161A, in Isoflurane-anesthetized Lambs


Background: N-methyl-D-aspartate (NMDA) receptor antagonists are neuroprotective in animal models of cerebral ischemia, but adverse cardiovascular and neurobehavioral effects have precluded their clinical use. The authors present the neuroprotective, anesthetic, and cardiovascular effects of a novel NMDA antagonist, CNS 5161A.

Methods: Lambs, 4.0-6.5 kg, were anesthetized with isoflurane, intubated, and ventilated and had thermodilution catheters placed in the pulmonary artery and 20-g catheters placed in the femoral artery. The minimum alveolar concentration (MAC) of isoflurane was determined using the "bracketing technique." CNS 5161A was given as a bolus and then as an infusion at three doses. Cardiovascular measurements were determined every 15 min. Other lambs (n = 25) were subjected to cardiopulmonary bypass (CPB) with hypothermic circulatory arrest (HCA) for 120 min. Eighteen received CNS 5161A, and seven received saline vehicle. One hour after CPB, brains were perfusion-fixed and removed for in situ hybridization and immunohistochemistry analysis in half of the animals. The other half survived 48 h before their brains were examined for neuronal degeneration.

Results: Isoflurane at MAC significantly decreased blood pressure, heart rate, cardiac output, and systemic vascular resistance by 30-48% (n = 16; P < 0.05). CNS 5161A (n = 12) had no significant cardiovascular effects. All concentrations of CNS 5161A caused a significant reduction (21-29%) of the MAC of isoflurane (n = 12; P < 0.05). CNS 5161A, at serum concentrations greater than 25 ng/ml, completely inhibited c-fos mRNA and c-FOS protein expression in hippocampal neurons after 120 min of HCA, attenuated neuronal degeneration, and improved functional outcome by 47% (P < 0.05).

Conclusions: CNS 5161A at neuroprotective concentrations before CPB-HCA significantly reduces the MAC of isoflurane without cardiovascular effects. (Key words: Cardiopulmonary bypass; Immunohistochemistry; in situ hybridization; minimum alveolar concentration; N-methyl-D-aspartate.)

Both the competitive and noncompetitive N-methyl-D-aspartate (NMDA) receptor antagonists are neuroprotective in animal models of cerebral ischemia and cardiopulmonary bypass (CPB).1-5 Neuronal damage that occurs after ischemic brain injury is believed to result, in part, from the excessive release of the excitatory neurotransmitter glutamate and its actions at NMDA and non-NMDA receptors. Under pathologic conditions, including hypoxia, seizures, ischemia, and neurodegenerative diseases, the excessive intracellular accumulation of cations, particularly calcium, is a key step in the development of neuronal injury.6-8 NMDA antagonists either directly or indirectly decrease the intracellular accumulation of calcium and the subsequent activation of signaling pathways that result in neuronal death.9

NMDA receptor antagonists may potentiate volatile anesthetics and have analgesic properties.10-15 However, many of these drugs have adverse hemodynamic effects, precluding their use at neuroprotective doses. For example, the noncompetitive NMDA antagonist, ketamine, can increase heart rate and blood pressure and decrease myocardial performance.16,17 Cerestat, another noncompetitive, neuroprotective NMDA antagonist, increases mean arterial blood pressure.18 whereas other
NMDA antagonists cause profound hypotension. NMDA antagonists may eventually be used in patients with an increased risk of cerebral ischemia, such as those undergoing CPB, hypothermic circulatory arrest (HCA), vascular surgery, or neurosurgery. Glutamate-mediated excitotoxic injury to neurons from CPB with HCA has been confirmed in animal models. CPB with HCA significantly increases the release of glutamate and other excitatory neurotransmitters in the brain. In high-risk patients, the pharmacologic properties, hemodynamic effects, and anesthetic interactions of these drugs are important. Recent improvements in the design of these molecules have resulted in drugs with greater specificity at the NMDA receptor, short half-lives with more desirable pharmacokinetics, and less effects at other receptors within and outside the central nervous system. Here we present the neuroprotective and cardiovascular effects and changes in the minimum alveolar concentration (MAC) of isoflurane of a novel, short-acting, noncompetitive NMDA receptor antagonist, CNS 5161A.

Methods

All experiments were conducted in accordance with the guide for the care and use of laboratory animals (US Department of Health and Human Services, Public Health Service, National Institutes of Health, Bethesda, MD). The Animal Research Committee of the Cleveland Clinic Foundation approved the study (protocol no. 5652). Lambs (n = 40) aged 10–21 days and weighing 4.0–6.5 kg were used for these experiments.

Anesthesia was induced using isoflurane in 100% oxygen by facemask. Each lamb was orotracheally intubated during deep isoflurane anesthesia. Muscle relaxants were not used at any time during these experiments. The concentration of isoflurane was reduced to 2–3% in 100% oxygen delivered at 3 l/min while the invasive monitors were placed. Monitoring included five-lead electrocardiogram, pulse oximetry, and rectal and nasopharyngeal temperatures. The lungs were mechanically ventilated to maintain normocapnia at an arterial carbon dioxide partial pressure of 35–45 mmHg. End-tidal concentrations of isoflurane were measured with a Datex Monitor 222 (Datex Co., Helsinki, Finland). The ventilator settings were not changed during the experiments.

A percutaneous 20-gauge catheter was inserted into a femoral artery for continuous monitoring of arterial blood pressure and for obtaining blood samples to measure arterial blood gases and serum concentrations of CNS 5161A. Another percutaneous 20-gauge catheter was placed in a foreleg vein to infuse 5% dextrose at 4 ml·kg⁻¹·h⁻¹ throughout the experiment. A 5-French percutaneous thermodilution catheter was inserted through the external jugular vein into the pulmonary artery to measure cardiac output and hemodynamic parameters (Transcope, Marquette Electronics, Milwaukee, WI). The animals were ventilated with 2–3% isoflurane in 100% oxygen for approximately 2 h before proceeding.

Determination of Minimum Alveolar Concentration

In the first set of experiments, baseline MAC of isoflurane was determined using the bracketing technique of Quasha et al. The animal was stimulated with a hemostat applied to the ear and clamped for 60 s. Any purposeful muscular movement of the head or extremities constituted a positive response. Nonpurposeful movement, such as shivering, twitching, or chewing, was disregarded. The isoflurane concentration was then adjusted by 10% (upward after a positive response, downward after a negative one) and maintained for 15 min at that concentration before repeated application of the hemostat. MAC was defined as the average of the lowest concentration at which a negative response occurred and the highest concentration at which a positive response was observed. After establishing the MAC of isoflurane, animals received CNS 5161A (provided by Cambridge Neuroscience, Inc., Cambridge, MA) in three doses: a 0.3-mg/kg bolus dose followed by a 0.6 mg·kg⁻¹·h⁻¹ infusion (n = 4); a 0.6-mg/kg bolus dose followed by a 1.2-mg·kg⁻¹·h⁻¹ infusion (n = 4); or a 1.2-mg/kg bolus dose followed by a 2.0-mg·kg⁻¹·h⁻¹ infusion (n = 4). These doses were selected based on the experiments described below that determined the neuroprotective doses required for CPB and HCA. A control group (n = 3) received saline vehicle. Blood samples to measure serum concentrations of CNS 5161A were obtained at 30-min intervals throughout the experiment. Serum concentrations of CNS 5161A were determined by high-performance liquid chromatography.

The MAC of isoflurane was again determined as previously described. Hemodynamic parameters were measured at 15-min intervals throughout the experiment. After determining MAC, isoflurane was discontinued while the CNS 5161A infusion was continued another hour. Hemodynamic values and end-tidal isoflurane concentrations were recorded every 15 min. The CNS 5161A infusion was then discontinued and the measurements repeated until the animal was awake.

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Cardiopulmonary Bypass and Hypothermic Circulatory Arrest

Twenty lambs were anesthetized by mask with isoflurane and monitored and instrumented as previously described. Temporalis muscle, esophageal, and rectal thermistors monitored temperature. Temperature was maintained at 38°C before and after CPB with a heating blanket. In these experiments, lactated Ringer’s solution was given intravenously at 4 ml·kg⁻¹·min⁻¹. Solutions containing glucose were avoided because of reports that hyperglycemia may exacerbate the effect of ischemia on neurons.²² CPB was achieved as previously described.³ Briefly, through a median sternotomy, the right atrium and ascending aorta were cannulated with 20-French venous and 10-French arterial catheters (Bard USCI, Tewksbury, MA), respectively. The CPB circuit included the Capiox SX-10 membrane oxygenator (Terumo, Japan), Capiox pediatric arterial line filter (Terumo, Japan), and Stockert roller pump (Muenchen, Germany). The pump prime consisted of 500 ml fresh whole sheep blood, 25 mEq sodium bicarbonate, 1,500 U heparin, 250 ml chilled 4% sodium paraformaldehyde. The brains were returned to their mothers and then every 35 min, volume of distribution at steady state of 163 ml/kg, and clearance of 163 ml/kg. In 10 animals, serum samples to measure CNS 5161A concentrations were obtained throughout the experiment: before drug, on CPB immediately after HCA, and at 30-min intervals until the end of the experiment.

After initiating CPB, the animals were cooled by surface and core cooling to 16°C. The duc tus arteriosus was also tied. When all temperatures were 16°C, the CPB pump was turned off, the head was packed in ice, and HCA was maintained for 120 min. From previous experiments, we determined that 2 h of HCA is needed to produce detectable neuronal death in the hippocampus in this animal model.³ After HCA, CPB was reinitiated and the animals were rewarmed and weaned from CPB at 38°C. No inotropes other than calcium chloride were used.

One hour after terminating CPB, half of the animals from each group were killed with KCl bolus. Their brains were perfusion-fixed with 1 l of iced normal saline followed by 1 1 of chilled 4% paraformaldehyde. The brains were removed for in situ hybridization and immunohistochemistry analyses (described below). The remaining animals were weaned from mechanical ventilation, extubated, and allowed to recover for 48 h. Surviving animals received intercostal nerve blocks that were placed under direct vision by the surgeons with 0.5% bupivacaine before the chest was closed. Chest tubes were placed during closure and removed before extubation. The surviving animals underwent neurologic examinations every hour after extubation until they were returned to their mothers and then every 8 h thereafter. A scale of functional recovery to assess the time to extubation, standing, bleating, and nursing was used in all groups.³,4 After 2 days, the surviving animals were again anesthetized by mask with isoflurane before being killed with KCl and their brains perfused with paraformaldehyde. Brains were removed and imbedded in paraffin, and 5-μm coronal sections were cut and stained with hematoxylin and eosin. The sections were examined and photographed using a light microscope. A blinded observer counted the number of dead neurons within specific regions of the hippocampal formation. The dead neurons were counted in the same regions of the hippocampal formation as were analyzed by in situ immunohistochemistry.

In Situ Hybridization and Immunohistochemistry Analyses

Brains from the acute experiments were cut into 12-μm coronal sections for in situ hybridization and immunohistochemistry as previously described.³,4,23 Briefly, to assay for messenger RNA, 12-μm coronal sections were cut and thaw-mounted onto gelatin-coated slides. Plasmids containing either sense or antisense reading frames were transcribed using SP6 polymerase.₂₄ Sections were hybridized at 55°C overnight, treated with RNAase to eliminate nonspecifically bound probe, and stringently washed at 55°C in 0.1 X standard saline citrate. Slides were apposed to x-ray film for 48 days, the surviving animals

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Table 1. Physiologic Values Measured during Determination of MAC Experiments

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>CNS 5161A 0.3 mg/kg</th>
<th>CNS 5161A 0.6 mg/kg</th>
<th>CNS 5161A 1.2 mg/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arterial pH</td>
<td>7.45 ± 0.07</td>
<td>7.40 ± 0.09</td>
<td>7.51 ± 0.04</td>
<td>7.52 ± 0.05</td>
</tr>
<tr>
<td>Pao2 (mm Hg)</td>
<td>37 ± 10</td>
<td>38 ± 1</td>
<td>33 ± 2</td>
<td>43 ± 1</td>
</tr>
<tr>
<td>Paco2 (mm Hg)</td>
<td>510 ± 41</td>
<td>486 ± 51</td>
<td>522 ± 102</td>
<td>542 ± 28</td>
</tr>
<tr>
<td>HCO3¬ (mM)</td>
<td>27 ± 2</td>
<td>26 ± 2</td>
<td>27 ± 4</td>
<td>32 ± 1</td>
</tr>
<tr>
<td>Body temperature (°C)</td>
<td>37.7 ± 0.1</td>
<td>38.0 ± 0.1</td>
<td>38.2 ± 0.1</td>
<td>38.0 ± 0.1</td>
</tr>
<tr>
<td>Na+ (mM)</td>
<td>141 ± 2</td>
<td>142 ± 1</td>
<td>141 ± 1</td>
<td>140 ± 5</td>
</tr>
<tr>
<td>K+ (mM)</td>
<td>3.9 ± 0.4</td>
<td>4.2 ± 0.5</td>
<td>4.0 ± 0.4</td>
<td>3.7 ± 0.1</td>
</tr>
<tr>
<td>Ca2+ (mM)</td>
<td>1.3 ± 0.2</td>
<td>1.3 ± 0.09</td>
<td>1.3 ± 0.08</td>
<td>1.4 ± 0.04</td>
</tr>
</tbody>
</table>

No significant changes were observed after CNS 5161A was administered.

Pao2 = arterial carbon dioxide tension; Paco2 = arterial oxygen tension.
MAC = minimum alveolar concentration.

Specific regions of the hippocampal formation and then examined under dark-field illumination at ×20 magnification to quantify the c-Fos-encoding mRNA.

Immunohistochemistry analyses were performed to visualize and quantitate intranuclear translated c-Fos protein. The 12-μm coronal sections were pretreated with 0.1% H2O2 in methanol for 20 min. Slide-mounted sections were incubated with primary antibody for c-Fos protein (Santa Cruz, CA). The sections were incubated with biotinylated goat antirabbit serum and processed by the avidin-biotin-peroxidase method (Vector, Burlingame, CA) using diaminobenzidine as the peroxidase substrate. This procedure was modified to include cadmium intensification of the reaction. Mounted and coverslipped tissue sections were examined under a microscope at ×20 magnification. The finding of intranuclear black-reaction product indicated the presence of immobilized antigen. A blinded observer quantified the number of neurons with intranuclear c-Fos immunoreactivity within specific regions of the hippocampal formation. Two 20× fields were counted for each region and averaged. Brain tissue was included from a control group (n = 3) that received general anesthesia with 2% isoflurane for 3 h but did not have CPB.

Neuronal Injury

Animals from the survivor groups were assessed using a modification of the ovine behavioral scale previously described.3.4.25 The neurologic deficit scoring consisted of five major components, including level of consciousness, motor and sensory function, nursing, and vocalization. A score of 10 was given for normal function and 0 for no function. Final neurologic scores were agreed upon by at least two members of the surgical team.

All values are expressed as the mean ± SD. Immuno-

histochemical data were evaluated for the treatment groups using analysis of variance on the ranks. Study groups were compared using Sigma Plot (Jandel Scientific, San Rafael, CA). Correlation analyses were performed with the Spearman rank order test. A P value < 0.05 was considered statistically significant.

Results

Determination of the Minimum Alveolar Concentration of Isoflurane

Arterial blood gases, electrolytes, nasopharyngeal temperature, and oxygen saturation remained constant throughout the experiments (table 1).

The MAC of isoflurane in lambs was 2.5 ± 0.2%. At MAC, isoflurane significantly (P < 0.05) decreased mean arterial pressure, heart rate, cardiac output, and systemic vascular resistance (n = 12; fig. 1). The addition of CNS 5161A had no significant effect on these parameters, central venous pressure, stroke volume, pulmonary vascular resistance, pulmonary arterial pressure, or pulmonary capillary wedge pressure (n = 12; fig. 1).

All concentrations of CNS 5161A caused a significant (n = 12; P < 0.05) reduction (21-29%) in the MAC of isoflurane. Although 1.2 mg/kg of 5161A appeared to cause a greater decrease in the MAC of isoflurane than the 0.3-mg/kg infusion, this was not statistically significant. The effects on MAC of CNS 5161A achieved steady state by 30 min and did not change thereafter with time of infusion. Serum concentrations of CNS 5161A at MAC were 61.7 ± 9.2 ng/ml, 116.6 ± 16.4 ng/ml, and 237.1 ± 27.8 ng/ml for 0.3 mg/kg, 0.6 mg/kg, and 1.2 mg/kg, respectively.
Cardiac Index, MAP, Heart Rate, and Systemic Vascular Resistance (SVR) were recorded every 15 min before and after administration of CNS 5161A. Data are presented at minimum alveolar concentration and as mean ± SD. Isoflurane at minimum alveolar concentration significantly decreased all hemodynamic parameters compared with CNS 5161A alone (P < 0.01). There was no significant difference in any of the hemodynamic parameters with CNS 5161A alone versus control (animal awake). Baseline values in awake animals were as follows: MAP, 101.5 ± 8 mmHg; HR, 164.8 ± 9 beats/min; CI, 6.6 ± 1.1 · min⁻¹ · m⁻²; and SVR, 4,795 ± 578 dyn · s⁻¹ · cm⁻⁵.

Cardiopulmonary Bypass and Hypothermic Circulatory Arrest Experiments

All animals survived the CPB and HCA experiments. Animals that did not have CPB or HCA (n = 3) but had general anesthesia with isoflurane for 2 h did not have any cfos mRNA expression above background or c-Fos immunoreactivity in any neurons of the hippocampal formation or cortex. CPB with 120 min of HCA in vehicle-treated animals (group 1; n = 3) induced massive expression of cfos mRNA in neurons throughout the hippocampal formation and cortex (data not shown). c-Fos immunoreactivity was also significantly expressed in all regions of the hippocampal formation (fig. 2) after 120 min of HCA.

CNS 5161A, when given as a bolus dose in group 2 (n = 5), did not significantly decrease cfos mRNA expression or the number of c-Fos-positive nuclei from that observed in the vehicle-treated animals in group 1 (fig. 2). In group 3 (n = 5), however, CNS 5161A completely inhibited cfos mRNA and c-Fos immunoreactivity after 120 min of HCA in all regions of the hippocampal formation.

Two days after CPB and 2 h of HCA, significantly greater neuronal necrosis was observed in the cortex and CA1 and CA3 neurons of animals in groups 1 (n = 4) and 2 (n = 4) than of those in group 3 (n = 4; P < 0.05; fig. 3). Necrosis was not observed in the dentate gyrus in any group.

Animals in group 3 (n = 4; P < 0.05) had significantly higher behavioral scores 24 h after HCA than those in groups 1 (n = 4) or 2 (n = 4). There was no statistical difference in scores between the saline vehicle-treated animals and those in group 2. This difference was not significant among the groups after 48 h (fig. 4).

The serum concentrations of CNS 5161A were highest in group 3 and always exceeded 25 ng/ml before the onset of HCA (table 2). Group 2 animals had significantly lower concentrations of CNS 5161A before the onset of HCA (table 2) but not during rewarming or after CPB (data not shown). During rewarming after HCA, after the second bolus dose of CNS 5161A in group 2, serum concentrations averaged 126.8 ± 34.4 ng/ml at the end of rewarming. Serum concentrations of CNS 5161A before the onset of HCA correlated negatively (r = -0.87; P = 0.001) with the number of c-Fos-positive nuclei and dead neurons in the CA1 region of the hippocampus.
Fig. 4. Functional behavioral score after cardiopulmonary bypass (CPB) and 2 h of hypothermic circulatory arrest. Group 3: CNS 5161A 0.6 mg/kg bolus and 1.0 mg·kg⁻¹·h⁻¹, significantly improved neurologic outcome 24 h after surgery (n = 5; *P < 0.05 for groups 1 and 2 vs group 3). There was no difference between groups at 48 h.

<table>
<thead>
<tr>
<th>Group</th>
<th>Functional Score</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>3.5</td>
</tr>
<tr>
<td>2</td>
<td>4.0</td>
</tr>
<tr>
<td>3</td>
<td>6.0</td>
</tr>
</tbody>
</table>

**Discussion**

In this study we demonstrate the effects of a novel noncompetitive NMDA receptor antagonist, CNS 5161A, on the reduction of the MAC of isoflurane in lambs. CNS 5161A caused a significant reduction of the MAC of isoflurane at all three doses studied without changing blood pressure, heart rate, vascular resistance, or cardiac output. At these same serum concentrations, CNS 5161A is neuroprotective after CPB and 120 min of HCA. The effects of isoflurane on heart rate, cardiac output, mean arterial pressure, and vascular resistance in lambs observed in these experiments is similar to the cardiovascular depressive effects of inhalational anesthetics in mammalian infants reported by other investigators.  

Isoflurane anesthesia in infants is usually associated with a 30-40% decrease in heart rate and mean arterial pressure.

The magnitude of the reduction in MAC by CNS 5161A is comparable to other competitive and noncompetitive NMDA receptor antagonists.  

**Table 2. Serum Concentrations of CNS 5161A before the Onset of HCA for 120 Min and the Number of c-fos-positive Nuclei in the Regions of the Hippocampal Formation**

<table>
<thead>
<tr>
<th>5161A, ng/ml</th>
<th>CA1</th>
<th>CA3</th>
<th>CA4</th>
<th>Dentate gyrus</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.5 (Group 2)</td>
<td>18</td>
<td>3</td>
<td>8</td>
<td>49</td>
</tr>
<tr>
<td>16.4 (Group 2)</td>
<td>13</td>
<td>6</td>
<td>6</td>
<td>46</td>
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<tr>
<td>18.0 (Group 2)</td>
<td>15</td>
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<td>6</td>
<td>57</td>
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<td>19.6 (Group 2)</td>
<td>11</td>
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<td>4</td>
<td>31</td>
</tr>
<tr>
<td>27.6 (Group 2)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>71.8 (Group 3)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>124.2 (Group 3)</td>
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<td>0</td>
<td>0</td>
<td>7</td>
</tr>
<tr>
<td>140.0 (Group 3)</td>
<td>0</td>
<td>0</td>
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<tr>
<td>201.6 (Group 3)</td>
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<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>207.3 (Group 3)</td>
<td>0</td>
<td>0</td>
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</table>

Spearman rank order correlation for CNS 5161A concentrations and the number of c-fos-positive nuclei in CA1 is $-0.87; P = 0.001$.  

In conclusion, the noncompetitive NMDA antagonist, CNS 5161A, reduces the MAC of isoflurane without affecting cardiac hemodynamics and is neuroprotective when given preemptively in an animal model of CPB and HCA.
References

7. Choi DW: Calcium-mediated neurotoxicity: Relationship to specific channel types and role in ischemic damage. Trends Neurosci 1988; 11:465–9

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