Cycloheximide rapidly inhibits cortical COX activity and COX-dependent pial arteriolar dilation in piglets

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Domoki, Ferenc, James V. Perciaccante, Roland Veltkamp, Greg Robins, Ferenc Bari, Thomas M. Louis, and David W. Busija. Cycloheximide rapidly inhibits cortical COX activity and COX-dependent pial arteriolar dilation in piglets. Am. J. Physiol. 277 (Heart Circ. Physiol. 46): H1113-H1118. 1999.—We have previously shown that cycloheximide (CHX) preserved neuronal function after global cerebral ischemia in piglets, in a manner similar to indomethacin. To elucidate the mechanism of this protection, we tested the hypothesis that CHX would inhibit cyclooxygenase (COX) activity in the piglet cerebral cortex and vasculature. Pial arteriolar responses to hypercapnia, arterial hypotension, and sodium nitroprusside (SNP) were determined before and 20 min after treatment with CHX (0.3–1 mg/kg iv) using a closed cranial window and intravital microscopy. We also determined baseline and arachidonic acid (AA)-stimulated cortical PGF2, and 6-keto-PGF1a production before and 20–60 min after CHX (1 mg/kg iv) treatment, using ELISA kits. CHX did not affect baseline diameters (100 μm) but significantly decreased arteriolar dilation to COX-dependent stimuli, such as hypercapnia and hypotension, but not to COX-independent SNP. In the 1 mg/kg CHX-treated group, increases in vascular diameters were reduced from 22 ± 2 to 10 ± 2%, from 49 ± 5 to 31 ± 3% (means ± SE, 5 and 10% CO2, respectively, n = 8), from 12 ± 3 to 3 ± 1%, and from 26 ± 5 to 2 ± 2% (25 and 40% decreases in blood pressure, respectively, n = 6). CHX also inhibited conversion of exogenous AA to both PGF2 and 6-keto-PGF1a; for example, 20 min after CHX treatment 10 μg/ml AA-stimulated PGF2a concentrations in the artificial cerebrospinal fluid decreased from 14.28 ± 3.04 to 5.90 ± 1.26 ng/ml (n = 9). Thus CHX rapidly decreases COX activity in the piglet cerebral cortex. This result may explain in part the preservation of neuronal function of CHX in cerebral ischemia.

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Body temperature was maintained at 37–38°C by a water-circulating heating pad. The head of the piglet was fixed in a stereotactic frame. The scalp was incised and removed along with the connective tissue over the calvaria. A circular (diameter 19 mm) craniotomy was made in the left parietal bone. The dura was cut and reflected over the skull. A stainless steel cranial window with three needle ports was placed into the craniotomy, sealed with bone wax, and cemented with cyanoacrylate ester and dental acrylic.

The closed window was filled with artificial cerebrospinal fluid (aCSF), warmed to 37°C, and equilibrated with 6% O2 and 6.5% CO2 in balance N2 to give a pH of 7.33, Pco2 of 46 mmHg, and Pco2 of 43 mmHg. The aCSF consisted of (in mM) the following: 132 NaCl, 2.9 KCl, 1.2 CaCl2, 1.4 MgCl2, 24.6 NaHCO3, 6.7 urea, and 3.7 glucose. Diameters of pial arterioles were measured using a microscope (Wild M36, Switzerland) equipped with a video camera (Panasonic, Japan) and a video microscaler (IV-550, For-A-Co. Newton, MA). After surgery, the cranial window was gently perfused with aCSF until a stable baseline was obtained. At the end of the experiments, the animals were killed with an intravenous injection of pentobarbital.

Measurement of pial arteriolar responses. Instrumented piglets (n = 22) were divided into three groups: 1) vehicle-treated control, 2) animals treated with 0.3 mg/kg CHX, and 3) animals treated with 1 mg/kg CHX. We examined the responses of cerebral arterioles to arterial hypercapnia, arterial hypotension, and sodium nitroprusside (SNP) before and 20 min after drug treatment. Hypercapnia was elicited by artificially ventilating the animal with a gas mixture containing (5% or 10% CO2, 21% O2, balance N2). Arterial hypotension was induced by withdrawing venous blood to yield a decrease in mean arterial pressure (MAP), respectively. After the measurements the heparinized blood was reinfused. SNP (10⁻⁵ mol/l) dissolved in aCSF was administered topically through the injection ports of the cranial window. Each dose of AA was applied to the brain surface for 10 min and then the cranial window was gently flushed and the effluent aCSF (~300 µl) was collected and frozen. AA was applied at 1-h intervals twice before and then 20 min and 1 h after CHX (1 mg/kg iv) treatment. Typically, we applied AA three times in each animal. Because the data obtained from these animals did not differ significantly, we combined these data as shown in Table 1. From the aCSF samples we determined concentrations of PGF2α and 6-keto-PGF1α using ELISA kits (Oxford Biomedical Research, Oxford, MI).

Statistics. Data are expressed as means ± SE. Data were analyzed using repeated measures ANOVA, and one-way ANOVA was used for differences between treatment groups. Pairwise comparisons were made using the Student-Newman-Keuls test where appropriate.

**RESULTS**

Arterial blood pressure was within normal limits (Table 1) and did not change significantly during hypercapnia. CHX administration caused a transient increase (5–15 mmHg) in MAP, but blood pressure values returned to baseline within 10 min of CHX administration.

Graded hypercapnia induced by ventilating the animals with gas mixtures containing either 5 or 10% CO2 resulted in a concentration-dependent increase in pial arteriolar diameters in accordance with the elevated Pco2 levels in all groups of animals (Table 1). In the vehicle-treated control group, repeated exposure to high Pco2 levels elicited essentially identical vasodilation in pial arterioles. However, in the groups intravenously injected with either 0.3 or 1.0 mg/kg CHX, different stimuli the window was flushed with aCSF, and the arteriolar diameters returned to baseline values. CHX (0.3–1 mg/kg) was dissolved in 3 ml of saline and injected intravenously. Twenty minutes after treatment, challenges of hypercapnia, arterial hypotension, and SNP were repeated according to the procedure described above.

**Table 1. Effect of CHX treatment on pial arteriolar dilation to arterial hypercapnia and hypotension**

<table>
<thead>
<tr>
<th>Diameter, µm</th>
<th>Pco2, mmHg</th>
<th>% Increase</th>
<th>Pco2, mmHg</th>
<th>% Increase</th>
<th>Pco2, mmHg</th>
<th>% Increase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td>Before</td>
<td>After</td>
<td>Before</td>
<td>After</td>
<td>Before</td>
<td>After</td>
</tr>
<tr>
<td>Control</td>
<td>5</td>
<td>112 ± 11</td>
<td>113 ± 11</td>
<td>38 ± 1</td>
<td>41 ± 1</td>
<td>27 ± 5</td>
</tr>
<tr>
<td>CHX 0.3 mg/kg</td>
<td>6</td>
<td>102 ± 2</td>
<td>107 ± 5</td>
<td>33 ± 2</td>
<td>34 ± 2</td>
<td>26 ± 5</td>
</tr>
<tr>
<td>CHX 1 mg/kg</td>
<td>8</td>
<td>93 ± 3</td>
<td>94 ± 5</td>
<td>34 ± 2</td>
<td>29 ± 2</td>
<td>22 ± 2</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Diameter, µm</th>
<th>MAP, mmHg</th>
<th>% Increase</th>
<th>MAP, mmHg</th>
<th>% Increase</th>
<th>MAP, mmHg</th>
<th>% Increase</th>
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<tr>
<td>Baseline</td>
<td>Before</td>
<td>After</td>
<td>Before</td>
<td>After</td>
<td>Before</td>
<td>After</td>
</tr>
<tr>
<td>Control</td>
<td>5</td>
<td>108 ± 10</td>
<td>109 ± 8</td>
<td>71 ± 1</td>
<td>71 ± 1</td>
<td>20 ± 4</td>
</tr>
<tr>
<td>CHX 0.3 mg/kg</td>
<td>6</td>
<td>104 ± 3</td>
<td>106 ± 6</td>
<td>75 ± 5</td>
<td>72 ± 6</td>
<td>10 ± 1</td>
</tr>
<tr>
<td>CHX 1 mg/kg</td>
<td>6</td>
<td>96 ± 6</td>
<td>104 ± 12</td>
<td>63 ± 5</td>
<td>59 ± 6</td>
<td>12 ± 3</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = no. of piglets. CHX, cyclohexamide; MAP, mean arterial pressure. *P < 0.05, significantly different from respective value before treatment.
vasodilation was significantly reduced to either level of hypercapnia (Table 1, Fig. 1). The attenuation of the response was larger in the group treated with the higher dose of CHX, especially at the lower level of hypercapnia (Fig. 1).

Graded arterial hypotension induced by venous blood withdrawal resulted in a dose-dependent increase in pial arteriolar diameters in accordance with decreased arterial blood pressure levels (Table 1). In the vehicle-treated control group pial arteriolar responses in response to stimulation by arterial hypotension were unaltered (Fig. 1). The vasodilatory response to arterial hypotension was also largely retained in the group treated with 0.3 mg/kg CHX. However, arteriolar responsiveness to arterial hypotension was severely reduced in the animals treated with 1 mg/kg CHX (Table 1, Fig. 1).

SNP (n = 7) induced dose-dependent pial arteriolar vasodilation that was unaffected by treatment with 1 mg/kg CHX. Baseline arteriolar diameters were not significantly different before and after CHX treatment (112 ± 6 vs. 118 ± 7 µm, respectively), and the percent changes were 21 ± 4 vs. 19 ± 4% at 10−9 mol/l and 38 ± 5 vs. 37 ± 5% at 10−8 mol/l.

Topical application of 1, 10, and 20 µg/ml AA onto the brain surface elicited a dose-dependent increase in the aCSF concentrations of PGF2α and 6-keto-PGF1α (Figs. 2 and 3). Repeated application of AA resulted in similar changes in aCSF PG levels. CHX (1 mg/kg iv) attenuated baseline as well as AA-stimulated PGF2α levels as early as 20 min after CHX administration (Fig. 2). The inhibition of PGF2α synthesis lasted at least as long as 1 h after CHX administration. Similarly, AA-stimulated 6-keto-PGF1α levels were also significantly reduced 20 min after administration of CHX (Fig. 3).

DISCUSSION

The major new finding in this study is that in vivo CHX rapidly inhibits COX activity in the piglet cerebral cortex and vasculature. More specifically, cerebrovascular reactivity to COX-dependent, vasodilatory stimuli is diminished within 20 min of CHX administration. Similarly, cerebral cortical PG-synthesizing capacity is largely reduced shortly after CHX treatment.

The most likely mechanism of how CHX inhibits COX activity in our experimental model is through its inhibitory effect on protein translation. Although we did not determine the effect of 0.3–1 mg/kg CHX on cortical protein synthesis, similar doses of CHX have been shown previously to be effective in rats (26), and we are unaware of any data suggesting a species difference in response to CHX. Another possible mechanism would be a nonspecific interaction of CHX with COX. However, CHX has been shown not to influence activity of purified COX in vitro (12). Additionally, in the present study, the intact vasodilatory response to the nitric oxide donor SNP suggests that CHX did not have nonspecific inhibitory effects on vascular smooth muscle function. In contrast, CHX did inhibit baseline and AA-stimulated PGE2 and prostacyclin production in brain, spleen, and muscles slices from rats. This effect of CHX and other protein synthesis inhibitors was found to be proportional to their effect on general protein synthesis inhibition (12). In this study, however, we cannot exclude the remote possibility that CHX could affect other proteins as well that may modulate COX activity. But the potent, rapid decrease in COX activity after CHX treatment may be explained by the biochemical characteristics of COX. COX is thought to be rapidly inactivated by self-produced superoxide anions, such that in an active system, COX...
has a half-life not more than 5–10 min (11). This suggests that maintaining active COX levels would require continuous de novo enzyme synthesis and may explain the rapid attenuation of COX-dependent vascular responses and PG synthesis after CHX administration in the piglet cerebral cortex observed in the present study. Unfortunately, immunoblotting of COX after CHX treatment would probably not yield additional support for this theory. Because COX is rapidly inactivated and degraded, functional and immunoreactive COX levels are not equivalent.

Previous evidence suggests that the PGs required for arteriolar vasodilation to hypercapnia and arterial hypotension are synthesized in the vascular endothelium (18, 20). In the present study, CHX treatment likely affected vascular endothelial cells shown by the attenuation of vascular responses to hypercapnia and arterial hypotension. We also found that 6-keto-PGF$_1$$_\alpha$ levels were reduced in the aCSF after CHX treatment, indicating decreased prostacyclin synthesis, further confirming the effect of CHX on cerebrovascular endothelial cells. In contrast, the reduced baseline and AA-stimulated PGF$_2$$_\alpha$ levels may represent a more general inhibitory effect of CHX on COX synthesis in both neural and vascular cells.

In our previous study we demonstrated that CHX employed a dose-dependent protective effect on the NMDA-induced vasodilation (28). NMDA-induced vaso-

![Fig. 2. Effect of CHX treatment on conversion of exogenous arachidonic acid (AA) to PGF$_2$$_\alpha$. Topical application of 1–20 µg/ml AA resulted in concentration-dependent, reproducible increases in PGF$_2$$_\alpha$ concentrations in the aCSF (control 1 and control 2). In contrast, 20 min after CHX (1 mg/kg iv) treatment, baseline and AA-stimulated PGF$_2$$_\alpha$ levels were significantly decreased compared with control values. At 1 h after CHX treatment we obtained similar results, and there was a trend toward further inhibition of PGF$_2$$_\alpha$ synthesis. *Significantly different from respective control values (P < 0.05).](http://ajpheart.physiology.org/)

![Fig. 3. Effect of CHX treatment on conversion of exogenous AA to 6-keto-PGF$_1$$_\alpha$. Topical application of 1–20 µg/ml AA resulted in concentration-dependent, reproducible increases in aCSF concentrations of 6-keto-PGF$_1$$_\alpha$ (control 1 and control 2). In contrast, 20 min after CHX (1 mg/kg iv) treatment, AA-stimulated 6-keto-PGF$_1$$_\alpha$ levels were significantly decreased compared with control values. At 1 h after CHX treatment we obtained similar results, and there was a trend toward further inhibition of 6-keto-PGF$_1$$_\alpha$ synthesis. *Significantly different from respective control values (P < 0.05).](http://ajpheart.physiology.org/)
dilation is a complex sequence involving the activation of neuronal NMDA receptors, activation of neuronal nitric oxide synthase (nNOS), and pial arterial dilation by nitric oxide (13, 22). This response was used as a bioassay to assess the functional integrity of the neuronal-vascular axis after ischemic stress. This response is attenuated by ischemia. The effect of ischemic stress likely affects the events before nNOS activation because nNOS levels and activity as well as vascular dilation to SNP were shown to be unaltered after ischemia (5). COX is an ample source of oxygen radicals in the early reperfusion period and plays a significant role in attenuating the NMDA vascular sequence after ischemia. After ischemic stress, NMDA-induced vasodilation has been shown to be preserved by pretreatment with COX inhibitors and oxygen radical scavengers, clearly indicating the involvement of COX (3–5). In our previous study, 0.3–1 mg/kg CHX was given 15 min before 10 min of global cerebral ischemia. Our present results support the concept that active COX levels could have been reduced before the initiation of ischemia. Thus the acute protective effect of CHX on the NMDA-induced vasodilation may be largely mediated via the inhibition of COX synthesis. We (23, 24) have shown similar results in cultured astroglial cells from piglets and fetal lambs.

CHX was also shown to employ long-term neuroprotective effects in different experimental ischemia models. Pretreatment with CHX was shown to reduce delayed neuronal death after transient focal ischemia (10, 21), and CHX also ameliorated cerebral infarction caused by reperfusion injury after reversible focal ischemia in rats (2). CHX has also been demonstrated to protect CA1 hippocampal neurons after transient global ischemia (14, 25). The mechanism of neuroprotection by CHX in the above-cited studies is not fully understood, but different possible mechanisms have been proposed, including CHX-induced hypothermia, inhibition of apoptosis, and suppression of the postischemic induction of a “noxious/killer protein.” However, our present data reveal that pretreatment with protein synthesis inhibitors can result in not only inhibiting the appearance of a noxious/killer protein after ischemia but also in the rapid disappearance of a potentially harmful albeit continuously expressed protein: COX.

At least two distinct isoforms of COX exist (COX-1, COX-2)(9). Originally COX-1 was considered the constitutively expressed isoform, and COX-2 was designated as the inducible isoform. However, in the brain and cerebral blood vessels of newborn pigs, COX-2 but not COX-1 has been identified as the major constitutively expressed isoform (7, 27). COX-2 is an immediate early gene and is readily inducible by a wide variety of stimuli, including ischemic stress in piglet cerebral cortex and blood vessels (6, 8). There is a substantial increase in porcine cortical COX-2 mRNA levels as early as 2 h after ischemic stress, and COX-2 immuno-reactivity is also increased within 8 h of cerebral ischemia. However, the short half-life and extremely rapid turnover rate of the COX enzyme may conceal an even more dramatic change in COX expression after ischemic stress. The increased expression of COX-2 may participate in the brain pathology after ischemic stress by increasing the production of oxygen radicals and inflammatory prostanoids. This is an interesting possibility, because overall protein synthesis is assumed to be inhibited even by short periods of cerebral ischemia and reperfusion (16). However, translation of some other immediate early gene mRNAs including heat shock proteins and protooncogenes appears to be increased rapidly after ischemic stress, in contrast to the generally depressed protein synthesis (15). It is quite conceivable that after cerebral ischemia, 1 mg/kg CHX has a greater inhibition on COX synthesis than we have shown with the approaches used in our present study.

In conclusion, to our knowledge we demonstrated for the first time that CHX rapidly inhibits COX activity in vivo in the cerebral cortex, as shown by the attenuation of COX-dependent pial arteriolar responses and decreased cortical metabolism of exogenous AA. This effect of CHX may be responsible for the previously reported early protective effect on neuronal and cerebrovascular function after cerebral ischemia.

This research was supported by National Heart, Lung, and Blood Institute Grants HL-30260, HL-46558, and HL-50587 and in part by T-026295 Országos Tudományos Kutatási Alap from the Hungarian Science Foundation.

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Received 11 March 1999; accepted in final form 22 April 1999.

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