Intravenously and Iontophoretically Administered Naloxone Reverses Ischemic Changes in Rat Hippocampus

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Forty rats under urethane anesthesia were subjected to cerebral ischemia by ligation of the right carotid, the right plus the left carotid, or the right carotid plus two vertebral arteries. Ischemia caused three types of changes in the field potential of the right hippocampal CA1 region evoked by fimbrial stimulation: 1) completely reversible deterioration (57% and 16% of the rats with unilateral and bilateral carotid artery ligation, respectively), 2) moderate deterioration (37% and 24% of the rats with unilateral and bilateral carotid artery ligation) and 3) irreversible loss of the evoked activity (6% and 60% of the rats with unilateral and bilateral carotid artery ligation and all the rats subjected to three-vessel occlusion). Naloxone improved the moderate deterioration in 10 of 11 rats (1-3 mg/kg i.v.) and in 15 of 16 (50-150 nA) iontophoretic applications, but naloxone did not restore the lost evoked activity. Intravenous morphine (10 mg/kg) aggravated the ischemic changes, and this effect was reversed by naloxone, while iontophoretic administration of morphine caused only excitation. These findings suggest that naloxone has a favorable effect on cerebral ischemia not severe enough to cause transmission failure. The reversal of ischemic changes by iontophoretic naloxone indicates that its site of action is at the neuronal or microcirculatory level. (Stroke 1989;20:1059-1064)

Since the original report by Baskin and Hosobuchi that naloxone might reverse the effects of cerebral ischemia, the controversy about the role of naloxone and the opiates in cerebral ischemia has not been resolved despite numerous clinical and experimental studies. However, many anecdotal reports indicate that naloxone can cause a dramatic but usually temporary recovery of neurologic function. This effect, which can be reproduced by repeated injections, is not compatible with any known natural course of cerebral ischemic events and thus strongly suggests that naloxone may exert a favorable action on cerebral ischemia. This unique action of naloxone therefore deserves further research to evaluate its potential therapeutic role and to understand better the pathophysiology of cerebral ischemia.

**Materials and Methods**

We subjected 40 male Swiss albino rats weighing 200–300 g to anesthesia obtained by 1.2–1.5 g/kg i.p. urethane. Both carotid arteries were exposed following a ventral median surgical incision in the neck. The carotid arteries were separated from accompanying vagal and sympathetic nerves and suspended with 0 surgical silk sutures. To obtain arterial occlusion, using these sutures we pulled the carotid arteries into a glass tube and fixed the sutures by a clamp. The clamp was released and the glass tube was pulled back to restore blood flow. The vertebral arteries were cauterized using the method described by Pulsinelli and Brierley.

After completion of surgery on the neck, we placed the rats in a stereotactic apparatus. The rectal temperature was monitored and maintained at 37–38°C with a radiant lamp. The heart rate was monitored by an electrocardiographic amplifier and a ratemeter. Stimulating and recording electrodes were lowered through burr holes in the skull to the appropriate sites, and the exposed surfaces of the cortex were subsequently covered with agar (3% wt:vol in physiological saline). Two parallel tungsten electrodes (tip separation 1.0 mm) were used for stimulation of the fimbria/commissure. The tips of the electrodes were lowered to a region with the following coordinates: anterior (A) 6.5, lateral (L) 0.5, height (H) 7.0 (according to the atlas of Albe-Fessard et al). Single-barrelled or multibarrelled...
glass microelectrodes were used for recording the extracellular field potential and for microiontophoretic administration of drugs. The recording barrel was routinely filled with 3 M NaCl; when multibarreled electrodes were used for iontophoretic applications, the other barrels were filled with naloxone HCl (Sigma Chemical Co., St. Louis, Missouri; 40 mM in 0.15 M saline at pH 6.0), morphine HCl (Verenidge, 40 mM in 0.15 M saline at pH 6.0), and saline (0.15 M at pH 6.0). The current applied for iontophoresis of drugs was automatically balanced with a current having the same magnitude but opposite polarity and was passed through the saline-containing barrel.

Positioning of the recording electrode in the CA1 region of the right hippocampus was guided by stereotactic coordinates (A 4.0, L 2.0–2.5, H–2) and by observation of the characteristic field response evoked by fimbrial/commissural stimulation. Upon completion of the experiment, we perfused the rats with 10% formalin through a cannula in the left ventricle and removed their brains. The position of the stimulating electrodes was confirmed by minimal bleeding in the track and that of the recording electrode by the spot stained with pontamine blue (which had been iontophoresed through the recording channel after termination of recording in the CA1).

We administered the drugs systemically via a polyethylene catheter inserted into the jugular vein. Naloxone and morphine were prepared in physiological saline solutions at a 0.1 ml volume, which provides a dose of 1 mg/kg for a rat weighing 250 g.

Results
Stimulation of fimbrial/commissural fibers evokes a characteristic field potential in the CA1 region of the hippocampus; this potential consists of a posi-

Figure 1. Upper row: Rapid and irreversible deterioration of field potential recorded from CA1 region of right hippocampus upon ligation of ipsilateral carotid artery (RCA) in rat whose vertebral arteries had been cauterized. Lower row: In another rat ligation of both carotid arteries (R+LCA) (vertebral arteries patent) caused similar response. Neither naloxone (NLX) (D and H) nor restitution of carotid artery blood flow was effective in restoring field potential. Record H: 10 minutes after restitution of blood flow in both carotid arteries and after NLX had been applied iontophoretically for the last 5 minutes.

Figure 2. Upper row: Reversible ischemic changes in field potential recorded from CA1 region of right hippocampus of rat. Although interruption of ipsilateral carotid artery (RCA) blood flow continued, field potential recovered in 3 minutes (D). Lower row: Upon ligation of contralateral carotid artery (R+LCA), field potential rapidly deteriorated (F), then partially recovered (G), and stabilized (H) after 3rd minute of ligation. Records in lower row were obtained from same rat at different spot in CA1 region.
CONTROL

RCA LIGATION

10 min

NLX 70 nA

A

B

C

D

E

F

FIGURE 3. Field potential recorded from CA1 region of right hippocampus of rat rapidly deteriorated and stabilized 3 minutes after ligation of ipsilateral carotid artery (RCA). Record B: Field potential recorded during 10th minute of ischemia, just before naloxone (NLX) injection. NLX restored field potential to great extent (C and D).

tive wave interrupted by a negative spike when recorded extracellularly within the pyramidal cell layer (Figures 1–5). The initial positive wave coincides with excitatory postsynaptic currents, the negative spike with the synchronous firing of pyramidal cells, and the later part of the positive wave with recurrent inhibitory postsynaptic currents.29 Following interruption of the blood supply, the negative population spike rapidly disappeared (in 14±1.5 seconds, mean±SEM) and the amplitude of positive wave decreased (Figures 2B and 4B). Some rats deteriorated further, and the field response was transformed into a slow negative wave followed by a small positive wave (Figures IF, 2F, 3B, and 4C), and finally an isoelectric line appeared (Figure 1, D, G, and H).

The severity of the deterioration in electrical activity increased with the number of arteries ligated and was quite variable among rats. We obtained the most consistent findings in five rats, the vertebral arteries of which had been cauterized. Although normal field potentials could be recorded from these rats after cauterization, field responses rapidly and irreversibly disappeared following ligation of the ipsilateral carotid artery (Figure 1, A–D). The irreversible loss of the field potential was also observed in two of 35 rats after ligation of the ipsilateral carotid artery (the vertebral arteries intact) and in 15 of 25 rats in which both carotid arteries were ligated (Figure 1, E–H). Restitution of blood flow after 10 minutes did not restore the evoked activity in these rats (Figure 1H). We designated this kind of reaction to ischemia as a profound ischemic response. In another group of rats, deterioration of the field response was less pronounced and was completely reversible within 10 minutes despite the continued interruption of carotid blood flow (Figure 2, A–D). We observed this second type of reaction (a reversible ischemic response) in 20 of 35 rats in which the ipsilateral carotid arteries were ligated and in four of 25 rats in which both carotid arteries were clamped. In the rest of the rats subjected to ischemia, deterioration of the field response reached a maximum at around 1 minute and then partially recovered and remained stable after the third minute of ischemia (Figure 2, E–H, Figure 3B, Figure 4, B and C). Thirteen of 35 rats with ipsilateral carotid

FIGURE 4. Population spike recorded from CA1 region of right hippocampus of rat disappeared within 12 seconds following interruption of ipsilateral carotid artery (RCA) blood flow (B). Ischemic changes reached maximum and stabilized within 2.5 minutes. Record C: Response at 10th minute of ischemia. Iontophoresis of naloxone (NLX) within pyramidal layer restored field potential almost to normal (D and E). Effect of NLX in this trial was partially reversible (F).

FIGURE 5. Ligation of ipsilateral carotid artery (RCA) and morphine infusion 40 minutes after ligation led to ischemic changes in field potential recorded from CA1 region of right hippocampus of rat that spontaneously recovered within 2 minutes (A). Contralateral carotid artery was also ligated (R+LCA), and stable ischemic response was produced (B). Ischemic response was reversed by iontophoretically applied naloxone (NLX) (C), but field potential continued to deteriorate after iontophoresis (D and E). Intravenous NLX was partially effective in restoring greatly deteriorated field potential in Record E (F).
arteries ligated and six of 25 rats in which both carotid arteries were ligated showed this third type of reaction (a moderate ischemic response). Table 1 illustrates the distribution of the three types of responses to ischemia among the rats subjected to ligation of different cerebral arteries. Differences between the groups were evaluated by the McNemar test for analyzing frequency data and were found to be significant \((p<0.01)\).

Before the naloxone injections, we gave 0.1 ml of physiological saline intravenously and observed the field response for 3 minutes. We repeated the saline injections three times in eight rats and followed them for 10 minutes. In both protocols, we detected no effect that could be ascribed to the saline injections. Thus, the changes following naloxone injection were safely attributed to the drug itself.

Naloxone did not have any consistent effect on the profound ischemic response. Once the field potential disappeared, even repeated injections of naloxone up to a cumulative dose of 3 mg/kg were unable to restore the response (Figure 1D). In 11 rats showing the moderate ischemic response, 1 mg/kg naloxone was given during the 10th minute of interruption of carotid blood flow, after the field response had been stabilized. Three rats were followed for an additional 10 minutes before naloxone injection to make sure that a 10-minute observation was adequate, and no spontaneous recovery was observed. Naloxone (1 mg/kg) reversed the ischemic changes in seven of the 11 rats (Figure 3), an effect that was evident within the first minute of injection. Recovery of the field response was almost complete, with the reappearance of the population spike, but the spike had a lower amplitude (20–90%) and was slightly delayed (0.4–0.9 msec). The effect of naloxone lasted during the 30–60 minute follow-up. Escalating the dose of naloxone to 2 mg/kg in the rats in which the first injection was ineffective increased the number of responders to nine, and an additional injection of 1 mg/kg naloxone increased the number of responders to 10 (91%). Naloxone (1–3 mg/kg) did not appreciably modify the field response in four control rats who were not subjected to vessel occlusion or in the group exhibiting the reversible ischemic response.

Iontophoretically administered naloxone, including long (5-minute) applications of high amounts (150 nA), was also found to have no effect on the profound ischemic response (Figure 1H). As with systemic administration, naloxone iontophoresis reversed the moderate ischemic response in 15 of 16 trials (Figure 4). The effect was evident within the first or second minute of application and required currents usually >70 nA (50–150 nA). Upon cessation of iontophoresis, the recovered field potential deteriorated within 2–5 minutes, but restitution of naloxone iontophoresis readily restored the deteriorated field potential. In some sites, the recovered field potential persisted after ceasing naloxone iontophoresis, although penetrations into neighboring sites of the CA1 region demonstrated that ischemia continued. Iontophoresis of naloxone in the neighboring ischemic sites also restored the deteriorated field potential. Our evidence concerning the reproducibility of naloxone action is strengthened by showing that the iontophoresis of identical currents of saline having the same pH as the naloxone solution did not affect the field potential.

A total dose of 10 mg/kg morphine was given by slow infusion in three rats in which a reversible ischemic response was observed upon ligation of the ipsilateral carotid artery. The field potential deteriorated within 1 minute of morphine infusion, but this effect was reversible within 3 minutes after cessation of the drug. Following interruption of the contralateral carotid artery blood flow as well, the field potential severely deteriorated and no subsequent spontaneous recovery was observed (Figure 5). Both iontophoretic and intravenous administration of naloxone (1 mg/kg) restored the field potential (Figure 5). Contrary to rats that were not given morphine, additional doses of 1 mg/kg naloxone was required every 10 minutes to maintain the field potential in these three morphine-treated rats. However, naloxone did not prevent further deterioration after the third injection, and finally an isoelectric line appeared. Additional injections or iontophoresis of naloxone and even restitution of blood flow in both carotid arteries were ineffective in restoring the field potential.

Iontophoresis of morphine in eight rats showing the reversible ischemic response did not lead to
deterioration of the field potential, but these rats exhibited a powerful excitatory action of morphine.

Discussion

The failure of naloxone to reverse ischemia in some experimental and clinical studies is not surprising if one considers the heterogeneity of ischemic neuronal damage. It is likely that to demonstrate the beneficial effect of naloxone, neuronal damage must not have progressed to an irreversible stage and the functional reserve of at least some neurons must be close to the threshold for regaining electrical activity. Indeed, our finding that naloxone reverses the moderate ischemic response but not profound ischemic changes is compatible with the above view and suggests that naloxone could improve ischemic changes at a level before the failure of synaptic transmission.30-32

Development of ischemia in the present model is an unexpected finding if one considers the well-known resistance to ischemia of rats subjected to ligation of only the carotid arteries.33-35 In fact, our initial intention was to use the four-vessel occlusion model.27 However, interruption of carotid artery blood flow following cauterization of vertebral arteries consistently led to irreversible loss of evoked activity. This sensitivity may have arisen from aggravation of ischemia by reduced systemic blood pressure due to urethane anesthesia26 and by the sensitivity of the electrophysiological method used as well as the vulnerability of the hippocampus to ischemia.37,38 Location of the hippocampal area in the watershed between the carotid and vertebrobasilar territories39 may have been a significant factor in making the CA1 region susceptible to the hemodynamic changes created in the present model.

Several previous articles reporting a beneficial effect of naloxone on cerebral ischemia studied the concomitant changes in cerebral blood flow,20 blood pressure, and cardiac output.6,9-11,14,20,23 and found no significant change in these parameters. Accordingly, naloxone was thought to act at the neuronal or microcirculatory level. Our data showing that iontophoretically applied naloxone can reverse ischemia-induced electrophysiological changes as well as does systemically administered naloxone agree with these reports and clearly demonstrate that naloxone can exert its anti-ischemic action without any contribution of cardiovascular factors or of the other regions of the brain. However, the data from iontophoretic experiments do not differentiate whether naloxone acts at the neuronal level or on the microenvironment of the neuron including capillaries and glial cells.

Is the anti-ischemic action of naloxone opiate-specific?40 It has been reported that morphine exacerbated neurologic dysfunction in two patients who showed a favorable response to naloxone.1 This observation has been confirmed in several experimental studies, and the action of morphine has been reported to be opiate-specific and independent of its effects on the cardiovascular and respiratory systems.14,20,26 We also reproduced the unfavorable action of morphine on ischemia and showed this effect to be reversible by both intravenously and iontophoretically applied naloxone. The involvement of opiates at the cellular level could have been demonstrated convincingly by iontophoretic administration. Unfortunately, the well-known powerful excitatory action of iontophoretically administered morphine in the hippocampus41,42 complicated the interpretation of changes in field potential. Yet it was clear that iontophoresis of large amounts of morphine (up to 200 nA) did not lead to a significant deterioration of the field potential. For example, a moderate ischemic response never progressed to an electrical silence after morphine iontophoresis.

In conclusion, our findings show that naloxone can reverse electrophysiologic effects of acute ischemia not severe enough to lead to total failure of evoked activity. The site of the anti-ischemic action of naloxone is probably at the neuronal level because local application of naloxone onto ischemic neurons was found to be as effective as its systemic administration. Although we found that intravenously applied morphine had an unfavorable effect on ischemia that was reversible by both systemically and iontophoretically applied naloxone, the opiate-specificity of naloxone’s action could not be demonstrated unequivocally at the neuronal level, possibly due to the powerful excitatory action of iontophoretically applied morphine in the hippocampus.

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