Activation of spinal opioid receptors contributes to hypotension after hemorrhage in conscious rats

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Ang, Kooi K., Robert J. McRitchie, Jane B. Minson, Ida J. Llewellyn-Smith, Paul M. Pilowsky, John P. Chalmers, and Leonard F. Arnolda. Activation of spinal opioid receptors contributes to hypotension after hemorrhage in conscious rats. Am. J. Physiol. 276 (Heart Circ. Physiol. 45): H1552–H1558, 1999.—Opioid receptors are activated during severe hemorrhage, resulting in sympathoinhibition and a profound fall in blood pressure. This study examined the location and subtypes of opioid receptors that might contribute to hypotension after hemorrhage. Intrathecal naloxone methiodide (100 nmol) abolished the fall in blood pressure after hemorrhage (1.5% of body wt; mean arterial pressure 122 ± 8 mmHg after naloxone methiodide vs. 46 ± 5 mmHg in controls, P < 0.001). Intracisternal naloxone methiodide was less effective than intrathecal naloxone methiodide, whereas intravenous naloxone methiodide, which does not cross the blood-brain barrier, did not alter the fall in blood pressure after hemorrhage. These results demonstrate that spinal opioid receptors contribute to hypotension after hemorrhage but do not exclude supraspinal effects. In separate experiments, the subtype-specific opioid antagonists ICI-174864 (δ-antagonist), norbinaltorphimine (nor-BNI; κ-antagonist), and H-D-Phe-Cys-Tyr-d-Trp-Orn-Thr-Pen-Thr-NH2 (CTOP; μ-antagonist) were each administered intrathecally to determine the minimum dose that would attenuate hypotension during severe hemorrhage. These antagonists were effective at similar doses (3 nmol for CTOP, 6 nmol for ICI-174864, and 10 nmol for nor-BNI), although the binding affinities of these three different agents for their target receptors varied >1600-fold. Comparisons of the minimum effective doses of these antagonists in relation to their binding affinities provides strong evidence for the participation of δ-receptors in mediating hypotension after hemorrhage. In contrast, the dose at which nor-BNI was effective suggests an effect at δ-receptors but not κ-receptors. The efficacy of CTOP, albeit at a high dose, also suggests an effect at μ-receptors.

sympathoinhibition; sympathetic nervous system; naloxone; enkephalin.

DURING PROGRESSIVE HEMORRHAGE in conscious rats, as in humans (2), an early sympathoexcitatory response that contributes to the maintenance of blood pressure is followed by sympathoinhibition and a profound fall in blood pressure. Sympathoexcitation, observed when blood loss is <25–35% of the total blood volume (24), is characterized by an elevation in total peripheral resistance and heart rate and a rise in renal sympathetic nerve activity so that the blood pressure falls little, if at all (24). When blood loss exceeds 25–35% of the total blood volume, there are decreases in sympathetic nerve activity (3, 21) and heart rate (9, 10, 17), dilatation in most vascular beds that are under sympathetic control (2), and a profound fall in arterial blood pressure. It is believed that cardiopulmonary afferents in the heart trigger this sympathoinhibitory response (9).

Naloxone restores blood pressure when given after severe hemorrhage (11, 14, 17), although it has little or no effect on arterial blood pressure when given to an unbled animal, suggesting that the activation of endogenous opioids during the compensatory phase of hypotensive hemorrhage impairs reflex vasconstrictor responses. It is likely that naloxone acts on central opioid receptors to reverse hypotension after hemorrhage, because intracerebroventricular administration of naloxone is effective at 1/100 of the dose required by intravenous administration (16). However, the central sites at which naloxone exerts this action have not been determined. There is indirect evidence pointing to a spinal site of action. Sympathetic preganglionic neurons (SPN) receive a heavy enkephalergic input (25), and the expression of preproenkephalin is increased in the spinal cord and brain stem after hemorrhage (12). Moreover, McAllen et al. (18) reported that hypotensive hemorrhage in conscious cats resulted in expression of the immediate-early gene c-fos, a marker of neuronal activation, in spinally projecting neurons of the rostral ventrolateral medulla (RVLM) but not in SPN. These RVLM neurons are believed to be the main source of excitatory inputs to SPN and have been shown to participate in the sympathetic activation elicited by hypotension (5). Thus the finding that bulbo spinal sympathoexcitatory pathways but not SPN are activated by hypotensive hemorrhage is consistent with an inhibitory effect at the level of the spinal cord rather than an inhibition of sympathoexcitatory RVLM neurons.

We hypothesized that activation of enkephalin inputs to SPN after severe hemorrhage contributes to sympathoinhibition and cardiovascular collapse and that, in consequence, intrathecal administration of an opioid receptor antagonist would be more effective in attenuating these effects than intravenously or intracisternally administration of an opioid receptor antagonist. Moreover, because enkephalins are likely to be the naturally occurring ligands at δ-opioid receptors (7), the blockade of δ-opioid receptors in the spinal cord would be more effective than blockade of either μ- or κ-opioid receptors in attenuating these effects. Conversely, an effect at κ-receptors might suggest that
dyorphins (7) mediate hypotension after hemorrhage, whereas an effect at µ-receptors might suggest activation of endorphins (1) or the recently discovered endomorphines (28).

METHODS

Ninety male Wistar-Kyoto rats (300–400 g) were obtained from the Flinders Medical Centre Animal House. Experiments were performed in conscious rats 9–12 h after catheter implantation (see below). All experimental procedures were approved by the Animal Welfare Committee at Flinders University of South Australia.

Vascular Catheters

Rats were anesthetized (pentobarbital sodium 60 mg/kg ip), and catheters were placed in the left common carotid artery [SV 8 (OD 0.50 mm PVC tube) attached to SV 45 (OD 0.96 mm); Dural Plastics] and in the right atrium (similar catheter with a 3-mm Silastic tip, OD 0.94 mm) via the left external jugular vein.

Intrathecal and Intracisternal Catheters

In 66 rats intrathecal catheters were also implanted. After incision of the atlantooccipital membrane, a stretched polyethylene tube (OD 0.80 mm, Dural Plastics SP 31) was inserted into the subarachnoid space via a small slit (1–2 mm) made along the midline of the membrane. The intrathecal catheter was threaded caudally for 8.5 cm so that the tip was positioned at the thoracolumbar junction. The free end of the catheter was externalized dorsally on the skull (13).

In 12 rats a short catheter was placed in the cisterna magna using the surgical approach used for intrathecal catheter placement.

Postoperative Procedures

After the surgical procedures were completed, povidone-iodine solution was applied to all incised areas after the wounds were sutured, and 5 ml of Ringer solution was injected subcutaneously. Animals were housed individually with water and food provided ad libitum. Rats appeared in good health and were moving freely at the time of study 9–12 h after catheter placement. Two rats with apparent neurologic defects after placement of intrathecal catheters were excluded from further studies.

Drugs and Vehicle Administration

All compounds were dissolved in artificial cerebrospinal fluid (aCSF: an aqueous solution of 1.4 mM CaCl₂, 1.0 mM MgCl₂, 2.6 mM KCl, and 128.6 mM NaCl, phosphate buffered to pH 7.2). For each intrathecal and intracisternal injection, 10 µl of solution was used. Aliquots (100–200 µl) of the antagonists were stored at −20°C. Serial dilutions from these stocks were made at the commencement of each experiment.

Naloxone Methiodide

In the intrathecal administration groups, arterial pressure and heart rate were recorded continuously throughout the experiment via the arterial catheter. Baseline arterial pressure and heart rate were averaged over the 5 min immediately preceding drug administration. Naloxone methiodide (Research Biochemicals International; 100 nmol in aCSF, n = 6) or aCSF alone (n = 6) was injected intrathecally; each injection was followed by a volume of aCSF equivalent to the dead space of the catheter. After 10 min, blood equivalent to 1.5% of body weight was withdrawn from the right atrial catheter. This hemorrhage was over a period of 5–10 min. Arterial pressure and heart rate were recorded for another 20 min before 10 µl of methylene blue were injected into the intrathecal catheter, followed by 10 µl of aCSF. In the intracisternal group, a similar protocol was followed, and 10 µl of naloxone methiodide (100 nmol in aCSF, n = 6) or aCSF alone (n = 6) were administered. In the intravenous administration group, 100 µl of naloxone methiodide solution (100 nmol in 0.9% saline, n = 6) or 0.9% saline (n = 6) were injected into the right atrium via the right atrial catheter. Each injection was followed by administration of 100 µl of 0.9% saline intravenously.

Opioid Receptor Subtype-Specific Antagonists

The protocol followed in experiments in groups receiving opioid receptor subtype-specific antagonists was similar to that applied to the group receiving intrathecal naloxone methiodide in the previous experiment. The 5-receptor antagonist ICI-174864 (Research Biochemicals International) (6, 8) and the µ-receptor antagonist H-D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH₂ (CTOP; Research Biochemicals International) (15) were administered in one of three doses (IC1-174864: 2 nmol, n = 4; 6 nmol, n = 6; or 20 nmol, n = 6; CTOP: 0.3 nmol, n = 5; 1 nmol, n = 5; or 3 nmol, n = 5) 10 min before hemorrhage. The κ-receptor antagonist norbinaltorphimine dihydrochloride (nor-BNI; Research Biochemicals International) (8, 23) was also administered in one of three doses (3 nmol, n = 6; 10 nmol, n = 6; or 30 nmol, n = 6) but was administered 1 h before hemorrhage. nor-BNI is reported to have a slow onset of action (26), and in preliminary experiments (n = 6) we demonstrated that a dose of 30 nmol administered 10 min before hemorrhage had no effect on blood pressure.

Autopsy

At the end of each experiment, rats were killed with an overdose of pentobarbital sodium. An autopsy was performed to confirm the location of the spinal and intracisternal catheters and to note the spread of methylene blue. In animals with intrathecal catheters, methylene blue was restricted to the spinal cord and was found to have spread ~2 cm above and below the tip of the catheter. In animals with intracisternal catheters, methylene blue was found to have spread over the surface of the brain and up to ~1–1.5 cm below the medulla. Three rats were excluded from this study because the intrathecal catheter had entered the spinal cord.

Statistical Analysis

Differences between groups were analyzed by repeated-measures analysis of variance (ANOVA) (22). In the experiments examining the effects of different routes of administration of naloxone methiodide, separate repeated-measures ANOVA were performed for naloxone methiodide versus vehicle at each site of drug administration. A significant time x treatment interaction or a significant treatment effect was taken as evidence of a significant difference between treatments. Because both intrathecal and intracisternal naloxone methiodide attenuated hypotension after hemorrhage, these two routes of administration were also compared using repeated-measures ANOVA. In the experiments in which subtype-specific antagonists were used, ninetreatment groups (3 doses of each of 3 different antagonists) were compared with a single control group using repeated-measures ANOVA. Simple contrasts were used to distinguish between vehicle-treated controls and rats receiving different doses of the three opioid receptor subtype antagonists (22). The data are pre-
RESULTS

Effect of Hemorrhage on Blood Pressure and Heart Rate After Administration of Naloxone Methiodide or Vehicle by Different Routes

Intrathecal administration of naloxone methiodide had no effect on resting blood pressure but prevented the dramatic fall in blood pressure after hemorrhage that was observed in rats that received vehicle only (Fig. 1; significant time × treatment interaction by repeated-measures ANOVA, F_{6,60} = 31.4, P < 0.001). Heart rate increased after hemorrhage in animals receiving intrathecal naloxone methiodide, but it decreased in vehicle-treated animals (Fig. 1; significant time × treatment interaction by repeated-measures ANOVA, F_{6,60} = 13.2, P < 0.001). Intracisternal naloxone methiodide slightly but significantly reduced the fall in blood pressure after hemorrhage (Fig. 2; significant time × treatment interaction by repeated-measures ANOVA, F_{6,60} = 2.9, P < 0.05). Heart rate decreased initially but increased after 5 min in the intracisternal naloxone methiodide-treated animals, whereas vehicle-treated animals showed a gradual fall in heart rate after hemorrhage (Fig. 2; significant time × treatment interaction by repeated-measures ANOVA, F_{6,60} = 4.1, P < 0.005). Intracisternal administration of naloxone methiodide was less effective than intrathecal administration of naloxone methiodide in preventing hypotension after hemorrhage (significant time × treatment interaction by repeated-measures ANOVA, F_{6,60} = 5.3, P < 0.001). In animals receiving naloxone methiodide or vehicle intravenously, both blood pressure and heart rate decreased, and the changes were similar after the two treatments (Fig. 3; neither significant time × treatment interaction nor significant treatment effect by repeated-measures ANOVA).

Effect of Hemorrhage on Blood Pressure After Opiate Receptor Subtype-Specific Antagonists

Blood pressure after hemorrhage in animals treated with various doses of subtype-specific opioid antago-
nists is shown in Fig. 4. Repeated-measures ANOVA revealed a significant time effect ($F_{6,270} = 170, P < 0.001$), significant time $\times$ treatment interaction ($F_{54,270} = 7.9, P < 0.001$), and significant treatment effects ($F_{9,45} = 24.3, P < 0.001$).

ICI-174864. Blood pressure after hemorrhage was significantly elevated in the rats receiving 20 or 6 nmol of ICI-174864 compared with that in the vehicle-treated controls ($P < 0.05$). At the lowest dose (2 nmol), ICI-174864 had no significant effect on blood pressure after hemorrhage compared with vehicle treatment.

nor-BNI. Blood pressure after hemorrhage was significantly elevated in the rats receiving 30 or 10 nmol of nor-BNI compared with that in the vehicle-treated controls ($P < 0.05$). At the lowest dose (3 nmol), nor-BNI had no significant effects on blood pressure after hemorrhage compared with vehicle treatment.

CTOP. Blood pressure after hemorrhage was significantly elevated in the rats receiving 3 nmol of CTOP compared with that in the vehicle-treated controls ($P < 0.05$). At the lower doses (1 and 0.3 nmol), CTOP had no significant effects on blood pressure after hemorrhage compared with vehicle treatment.

**DISCUSSION**

**Location of Opioid Receptors Active in Hemorrhage**

It is well established that activation of central opioid receptors leads to sympathoinhibition and contributes to hypotension during severe hemorrhage, because...
naloxone methiodide attenuates the fall in blood pressure after hemorrhage in conscious rats and is 100 times more potent when given intracerebroventricularly than intravenously (11, 16). However, the site of naloxone’s action has not been conclusively defined. Evans et al. (9) have shown that intracisternal naloxone blocks the hypotensive response to acute constriction of the thoracic inferior vena cava, a model that is claimed to simulate the response to hemorrhage. They postulated that the site of action of naloxone might be in the RVLM. RVLM neurons are believed to be the main source of excitatory inputs to SPN and have been shown to participate in the sympathetic activation elicited by hypotension (5), making the RVLM a promising candidate site for the hemorrhage-induced depressor effects of endogenous opioids. However, naloxone elicits similar pressor responses when injected into the RVLM of chloralose-anesthetized rabbits before and after hemorrhage (20). Thus opioid inputs to the RVLM appear to be tonically active and do not appear to be further activated by hemorrhage (20).

Several findings suggest a spinal site of action for naloxone. First, there is a heavy enkephalinergic input to the spinal cord (25), and hemorrhage increases expression of enkephalin in the brain stem and spinal cord (12). Second, the immediate-early gene c-fos, a marker of neuronal activation, is expressed in medullary sympathoexcitatory neurons during hypotensive hemorrhage, whereas SPN do not express c-fos and therefore are probably not activated (18).

The major finding of this study is that in conscious rats endogenous opioid peptides act on receptors in the spinal cord to reduce blood pressure after hemorrhage. To distinguish between peripheral, spinal, or supraspinal sites of action of naloxone during hypotensive hemorrhage, we administered naloxone methiodide, which does not cross the blood-brain barrier (19), either intravenously, intrathecally, or intracisternally. Intravenous administration of naloxone methiodide had no effect on blood pressure and heart rate responses to hemorrhage, consistent with the documented central site of action of naloxone (9, 16). In contrast, intrathecal naloxone methiodide virtually abolished the hypotension and prevented the bradycardia observed after hemorrhage in control animals, suggesting that intrathecal naloxone methiodide probably blocked the hemorrhage-induced fall in blood pressure by acting on spinal opioid receptors. Naloxone methiodide is unlikely to have diffused out of the spinal intrathecal space and blocked opioid receptors within the brain, because 1) methylene blue injections were contained within the spinal cord at autopsy and 2) intracisternally administered naloxone methiodide was less effective than intrathecally administered drug in preventing the fall in blood pressure after hemorrhage. Intermediate effects on blood pressure and heart rate after hemorrhage were observed with intracisternal naloxone methiodide. These effects may have been a result of blockade of opioid receptors in the brain stem or other brain sites or, alternatively, a consequence of diffusion of drug into the spinal cord.

Opioid Receptor Subtypes Active in Hemorrhage

To determine which opioid receptor subtypes mediated the responses observed with intrathecal naloxone methiodide, we used three doses of each of three subtype-selective opioid antagonists, with one-half log separation between doses. Each of the subtype-selective antagonists blocked the hemorrhage-induced fall in blood pressure at the high dose, and at least one of the lower doses of each antagonist had no effect. This pattern of response allowed us to determine the minimal effective dose for each antagonist. Our data could be interpreted as demonstrating a contributory role for each of the three receptor subtypes in mediating hemorrhage-induced hypotension. However, after careful examination of the affinity and selectivity of the antagonists for their target receptors, we believe our data supports the involvement of δ-opioid receptors and possibly μ-receptors, but not κ-receptors.

The δ-receptor antagonist ICI-174864 has a relatively low affinity for δ-receptors (inhibitory constant \( K_i = 98 \text{ nM} \)) as determined by displacement of the selective δ-receptor ligand [d-Pen²,d-Pen⁵]-enkephalin (DPDPE) from δ-receptors in the brain (8). As expected, we found that a relatively large dose was required to block δ-receptor-mediated effects using this antagonist. Evans et al. (10) also found that δ-opioid receptor antagonists prevented hypotension during simulated hemorrhage (inferior vena caval constriction) in the rabbit. Their conclusion that δ-receptors mediated hypotension after hemorrhage depended on a comparison of the observed and predicted minimum effective dose calculated from the observed minimum effective dose of naloxone and the relative affinity of naloxone and the subtype-specific antagonists for opioid receptor subtypes. In their experiments, ICI-174864 was effective at about the predicted dose.

nor-BNI has a much higher affinity for κ-receptors \( K_i = 0.06 \text{ nM} \) as judged by displacement of the specific κ-ligand U-69593 (8). Hence, if κ-receptors are involved in opioid effects after hemorrhage, nor-BNI should be effective in blocking cardiovascular changes at \( 1/1,600 \) of the dose at which ICI-174864 blocked δ-receptor-mediated effects. However, in our experiments, 3 nmol of nor-BNI (≈800 times the predicted minimum effective dose) did not alter hemorrhage-induced hypotension. This finding does not provide evidence for a role of κ-receptors. In fact, because nor-BNI displaces DPDPE from δ-receptors at a lower concentration \( K_i = 47.1 \text{ nM} \) than ICI-174864 \( K_i = 98 \text{ nM} \), the dose at which nor-BNI was effective in our experiments suggests that nor-BNI may be acting through δ- rather than κ-receptors (8). As in our study, the effects that Evans et al. (10) observed with nor-BNI were best explained by an effect at δ-receptors.

Displacement data are not available for the μ-receptor antagonist CTOP, but the \( K_i \) of CTOP for binding sites in the rat brain is 0.41 nM, as judged by the measurement of association and dissociation rates (15). The failure of the selective δ-ligand DPDPE or the μ-ligand U-69593 to displace CTOP implies that CTOP
does not bind with high affinity to either δ- or κ-receptors (15). If CTOP and ICI-174864 diffused to similar extents, CTOP should block μ-receptors at ~1/200 the dose at which ICI-174864 blocks δ-receptors. However, the minimum effective dose of CTOP in our experiments was 3 nmol compared with 6 nmol for ICI-174864. This dose of CTOP is higher than expected, but the affinity ratio δ/μ for CTOP is 32,500 (15), and this strong selectivity of the drug for μ-receptors over δ-receptors makes a μ-receptor-mediated effect the likely one. There is substantial evidence for interactions between δ- and μ-receptors, and the existence of a μ-δ-opioid receptor complex has been postulated (27). Actions at a μ-δ-opioid receptor complex might explain the efficacy of both δ- and μ-receptor antagonists to block hypotension after hemorrhage. At one of these sites δ-ligands bind much more strongly than μ-ligands (4), in keeping with the relatively high dose of CTOP (in relation to its Kᵦ) required to block hypotension after hemorrhage. Alternatively, CTOP might act to limit hypotension after hemorrhage at some site other than the spinal cord. For a given intrathecal dose, the concentration of CTOP at extraspinal opioid receptors could be significantly lower than at spinal receptors. In the experiments of Evans et al. (10), MR-2266 was used to lead to the prediction of similar minimum effective doses via μ and δ effects (see Table 5 of Ref. 10). Thus, whereas this drug was useful in excluding a κ-effect, a μ-effect could not be reliably excluded.

Our studies provide strong evidence that the spinal cord is a major site of action of opioids in hemorrhage-induced hypotension, although the response to intraspinally administered naloxone is consistent with additional supraspinal effects of endogenous opioids. We have also shown that, as in the rabbit, δ-opioid receptors are the primary mediators of the sympathoinhibitory responses contributing to cardiovascular collapse during severe hemorrhage in the rat. The efficacy of CTOP, albeit in a high dose, suggests that μ-receptors may also be involved. Our results have implications for the management of trauma accompanied by blood loss; the reluctance to administer opiate analgesics for fear of provoking cardiovascular collapse may be justified given our results suggesting μ- as well as δ-opioid receptor-mediated effects after hemorrhage. Similar mechanisms to those observed after hemorrhage may operate during fainting, or “vaso-vagal” or “neurocardiogenic” syncope, because the cardiovascular collapse elicited by hemorrhage is accompanied by all the features of a faint. If so, specific δ-opioid receptor antagonists may prove useful in the treatment of those who suffer recurrent syncopal episodes of this type.

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