Site and mechanism of action of dynorphin A-(1–13) and N-methyl-D-aspartate on ACTH release in fetal sheep

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Dynorphin A-(1–13) and N-methyl-D-aspartate receptor agonists have been shown to be potential secretagogues of ACTH release in the fetal hypothalamo-pituitary-adrenal (HPA) axis. However, their site of action and mechanism of action remain to be determined. To determine the site of action and mechanism of Dyn A-(1–13) and NMDA on the fetal HPA axis, we conducted this study. Pregnant ewes were anesthetized with sodium pentobarbital, and the fetal pituitary was surgically disconnected under sterile conditions. ACTH release was measured using a competitive radioreceptor assay. NMDA and Dyn A-(1–13) elicit ACTH release in fetal sheep before 135 days of gestation, but both are effective secretagogues after 135 days of gestation. This study was designed to determine the site of action of Dyn A-(1–13) and NMDA on the fetal HPA axis. Previous studies have shown that antagonists to the well known ACTH secretagogues corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP) do not alter the fetal response to Dyn A-(1–13) or NMDA. This is also true of the effects of Dyn A on the fetal HPA axis, where ACTH release was not blocked by naloxone but was completely blocked by MK-801, a noncompetetive NMDA receptor antagonist. Furthermore, the developmental profiles of ACTH response to Dyn A-(1–13) and NMDA are similar; neither is able to elicit ACTH release in fetal sheep before 135 days of gestation, but both are effective secretagogues after this time.

Although Dyn A exhibits high affinity for the k-opioid receptor, many of its actions cannot be reversed by the opioid receptor antagonist naloxone and are thought to be mediated via nonopioid mechanisms. There is evidence to suggest that N-methyl-D-aspartate (NMDA) receptors might be involved in these nonopioid actions of Dyn A (for review see Ref. 26). This is also true of the effects of Dyn A on the fetal HPA axis, where ACTH release was not blocked by naloxone but was completely blocked by MK-801, a noncompetetive NMDA receptor antagonist. Furthermore, the developmental profiles of ACTH response to Dyn A-(1–13) and NMDA are similar; neither is able to elicit ACTH release in fetal sheep before 135 days of gestation, but both are effective secretagogues after this time.

This study was designed to determine the site of action of Dyn A-(1–13) and NMDA on the fetal HPA axis. Previous studies have shown that antagonists to the well known ACTH secretagogues corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP) do not alter the fetal response to Dyn A-(1–13) or NMDA. In pregnancy, the placenta is a potential site of action for Dyn A to release ACTH. ACTH is synthesized in the human placenta, and its release is regulated locally by a placental CRH-like peptide (25). The possibility that Dyn A-(1–13) and NMDA might cause release of ACTH from the placenta was suggested by the new finding presented here that the newborn lamb does not respond to either Dyn A-(1–13) or NMDA. To determine whether release of ACTH occurred from the fetal pituitary or from an extrapituitary site such as the placenta, we studied fetuses that were either intact or in which the pituitary had been removed. On finding that ACTH release was from the pituitary, we studied fetuses in which the pituitary and hypothalamus had been surgically disconnected. Our results show clearly that both Dyn A-(1–13) and NMDA release ACTH by actions within the fetal hypothalamus.
Although our results showed that ACTH release was not from the placenta, the placenta might still be important for the actions of Dyn A-(1–13) and NMDA in the fetus. Prostaglandins (PG) are released from the placenta into both maternal and fetal blood, and prostanoids have been implicated in the regulation of ACTH release. Indomethacin, a nonspecific inhibitor of prostaglandin synthases-1 and -2, inhibited the ACTH response to fever, swimming exercise, bacterial endotoxin, and interleukin-1 in rats (8, 21, 35, 37, 38). Infusion of PGE₂ has been shown to elicit dose-dependent increases in ACTH in rats and fetal sheep (6, 15, 36, 38). We have therefore investigated the effect of indomethacin on Dyn A-(1–13) and NMDA release of ACTH in the fetal lamb.

Methods

Animal Preparation

The hypophysectomy (HX) and hypothalamo-pituitary disconnection (HPD) experiments were conducted at Monash University in accordance with the Australian Code of Practice for the Care and Use of Animals for Experimental Purposes and were approved by the Monash University Committee for Ethics in Animal Experimentation. Aseptic surgery was performed on 12 pregnant Border-Leicester × Merino ewes at 115–119 days of pregnancy (term 147 days). Surgery was performed under general anesthesia induced with 1 g of thiopentone sodium (iv) and maintained with 1.5% halothane administered by intermittent positive-pressure ventilation. At the time of surgery, four fetuses underwent HPD (3), four fetuses underwent HX (20), and four animals underwent a sham surgical procedure in which the pituitary was left intact and remained connected to the hypothalamus (INTACT). Vascular catheters, filled with heparin-saline, were inserted into the jugular vein and carotid artery of all of these fetuses; the maternal jugular vein was also catheterized. The fetal catheters were exteriorized via an incision in the ewe’s flank. After surgery, the animals were returned to individual metabolic cages and were allowed 14 days to recover before the experiments began. The ewes were fed once daily, and water was available ad libitum.

All other experiments were carried out at Weill Medical College of Cornell University. Guidelines approved by the Institution for the Care and Use of Animals were followed for all surgical procedures and experimental protocols. Aseptic surgery was carried out in pregnant sheep at 115–120 days of gestation under epidural lidocaine anesthesia supplemented with intravenous pentobarbital sodium. Indwelling catheters were placed in the fetal distal aorta and inferior vena cava as described previously (28). All animals were then allowed to deliver spontaneously at term, and fetuses were then studied again as lambs at 3–10 days of age.

Experimental Protocols

ACTH responses in fetal and postnatal lambs. Dyn A-(1–13) (0.5 mg/kg), NMDA (4 mg/kg), or U-50488H [trans-(±)-3,4-dichloro-N-methyl-[2-(1-pyrrolidinyl)cyclohexyl]benzene-acetamide], a selective κ-opioid agonist, 1 mg/kg, was administered intravenously to three fetuses at 135–142 days of gestation and then to the same animals as lambs at 3–10 days of age. For animals that received more than one drug, ≥2 days were allowed between studies. Blood samples (2 ml) were taken at −5, 5, 15, 30, 45, and 60 min after drug administration.

ACTH and cortisol responses in INTACT, HX, and HPD fetal lambs. Dyn A-(1–13) (0.5 mg/kg), NMDA (4 mg/kg), or U-50488H (1 mg/kg) was given intravenously to INTACT, HX, and HPD fetuses at 135–142 days of gestation (n = 4 each group) in a fully orthogonal design with a minimum of 1 day between successive experiments. The saline experiments were conducted at 142 days of gestation. Samples (3 ml) of fetal arterial blood were taken at −15, −5, 5, 10, 15, 30, 45, 60, and 120 min after drug or saline administration. At −146 days of gestation, the ewe and fetus were killed by an overdose of pentobarbital sodium given to the ewe. In all HPD animals, completeness of the hypothalamic-pituitary disconnection was confirmed by visual inspection at postmortem. The absence of pituitary tissue at postmortem confirmed the completeness of HX.

Fetal ACTH responses in the absence and presence of indomethacin. Intact fetuses at 135–142 days of gestation were pretreated with indomethacin (0.2 mg/kg) or an equivalent volume of saline 90 min before the administration of Dyn A-(1–13) (0.5 mg/kg), NMDA (4 mg/kg), or saline (n = 4 each treatment group). When several experiments were performed in the same animal, there was a minimum of 2 days between successive experiments. All studies were performed in random order. Blood samples (2 ml) were collected before and at −5, 5, 15, 30, 45, and 60 min after Dyn A-(1–13) or saline administration and at −5, 5, 30, 60, and 120 min after NMDA administration.

Assays

All blood samples were collected into chilled tubes containing EDTA (5.58 mg) immediately after collection, and they were then centrifuged for 10 min at 3,000 g at 4°C to recover the plasma. Aliquots of the plasma (500 μl) were placed in tubes containing aprotonin (5 × 10⁻⁸ trypsin inhibitor units). Concentrations of immunoreactive (ir)-ACTH and -cortisol were determined in duplicate using commercial radioimmunoassay kits (DiaSorin, Stillwater, MN), as described previously (33).

Data Analysis

All values are presented as means ± SE. A single-factor ANOVA with repeated measures (factor = time) was used to analyze the effects of the different drugs or saline on plasma hormone levels. Tukey’s test was used for post hoc analysis of significant differences.

Results

ACTH Responses in Fetal and Postnatal Lambs

Before drug or saline administration, plasma ir-ACTH concentrations were 40.8 ± 5.2 pg/ml in the fetuses and 123.7 ± 22.4 pg/ml in the postnatal lambs (n = 9). Figure 1 shows the peak changes in plasma ir-ACTH after intravenous administration of Dyn A-(1–13), NMDA, or U-50488H (n = 3 each drug). Maximal change in ir-ACTH was achieved 15 min after Dyn A-(1–13), and 30–60 min after NMDA and U-50488H. Administration of U-50488H elicited a similar ACTH response in both fetal and postnatal lambs, whereas Dyn A-(1–13) and NMDA had no significant effect on plasma ir-ACTH in the postnatal lambs.
ACTH and Cortisol Responses in HX, HPD, and INTACT Fetuses

In the INTACT fetuses, basal concentrations of ir-ACTH and ir-cortisol before drug administration were 33.0 ± 0.02 pg/ml and 12.1 ± 2.6 ng/ml, respectively, with no significant differences preceding infusion of the different drugs. In the HX fetuses, basal ir-ACTH and ir-cortisol levels were undetectable (<20 pg/ml and <2.1 ng/ml, respectively). In the HPD fetuses, plasma ir-ACTH levels (42.9 ± 4.3 pg/ml) were not significantly different from the levels in the INTACT fetuses, but ir-cortisol was undetectable. Administration of saline did not change ir-ACTH or ir-cortisol in any of the fetuses.

Effects of Dyn A-(1–13)

Within 5 min of Dyn A-(1–13) administration in INTACT fetuses, plasma ir-ACTH concentrations increased from 27.9 ± 2.9 to 263.5 ± 37.5 pg/ml (P < 0.05; Fig. 2A). Plasma ir-ACTH remained significantly elevated above basal levels until 45 min after Dyn A-(1–13) administration and declined to basal levels by 120 min. Dyn A-(1–13) also caused a significant increase of ir-cortisol in the INTACT fetuses from 10.5 ± 3.0 to 21.3 ± 2.7 ng/ml after 10 min (P < 0.05), and concentrations remained elevated for 60 min (Fig. 2B). In HX fetuses, ir-ACTH and ir-cortisol remained undetectable throughout the study period. In the HPD fetuses, plasma ir-ACTH did not change significantly after Dyn A-(1–13), and plasma ir-cortisol was undetectable at all times (Fig. 2A and B).

Effects of NMDA

In the INTACT fetuses, administration of NMDA resulted in an increase of plasma ir-ACTH concentrations from 29.8 ± 1.0 to 102.4 ± 26.6 pg/ml by 10 min (P < 0.05), and these concentrations remained significantly elevated until 60 min (Fig. 3A). Plasma ir-cortisol concentrations increased from 7.1 ± 2.9 to 22.1 ± 3.9 ng/ml by 10 min and remained significantly elevated for the rest of the study period (Fig. 3B). In the HX fetuses, both ir-ACTH and ir-cortisol were undetectable throughout the study period (Fig. 3). In contrast, in the HPD fetuses, NMDA administration did not change the plasma concentrations of ir-ACTH, and ir-cortisol was undetectable at all times.

Effects of U-50488H

In the INTACT fetuses, ir-ACTH concentrations increased from 31.4 ± 1.5 to 267.8 ± 38.8 pg/ml at 30 min after administration of the μ-opioid agonist; this increase was then sustained throughout the study period (Fig. 4A). There was a corresponding increase of plasma ir-cortisol in these fetuses, with concentrations increasing from 14.0 ± 4.8 to 27.5 ± 7.9 ng/ml at 10

Fig. 2. Plasma concentration of ir-ACTH (A) and ir-cortisol (B) in sham-operated (INTACT; ○), hypophysectomized (HX; □), and hypothalamo-pituitary-disconnected (HPD) fetuses (▲) after iv administration of Dyn A-(1–13) to fetal sheep. * Significant differences from concentrations at −15 and 0 min (P < 0.05). Dyn A-(1–13) was injected into the fetus at time 0.

Fig. 1. Maximal increases in plasma immunoreactive (ir)-ACTH in fetal sheep (open bars) and postnatal sheep (filled bars) after iv administration of dynorphin A (Dyn A)-(1–13) (0.5 mg/kg), N-methyl-D-aspartate (NMDA; 4 mg/kg), and [trans-(±)-3,4-dichloro-N-methyl-[2-(1-pyrollidinyl)-cyclohexyl]benzeneacetamide] (U-50488H; 1 mg/kg).

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Fetal ACTH Responses in the Absence and Presence of Indomethacin

Plasma ir-ACTH concentrations were similar before commencement of saline (29.0 ± 2.9 pg/ml; n = 4) or indomethacin infusion (30.8 ± 2.8 pg/ml; n = 4). Both Dyn A-(1–13) and NMDA elicited significant increases of plasma ir-ACTH concentrations by 15 min after administration (Fig. 5). Indomethacin pretreatment significantly attenuated the increase in ir-ACTH after Dyn A-(1–13) or NMDA administration (Fig. 5).

DISCUSSION

The results of this study clearly show that the source of ACTH released by Dyn A-(1–13), NMDA, and the κ-opioid agonist U-50488H is the pituitary. The HX fetus failed to respond to all three agents. Furthermore, these agents also failed to release ACTH in fetuses in which the hypothalamus and pituitary had been surgically disconnected. The pituitary function of HPD fetuses has been extensively characterized. The disconnected pituitary is capable of releasing the same amount of ACTH in response to CRH as intact fetuses (4, 24). Furthermore, the basal concentrations and secretory dynamics of ACTH do not differ between HPD and intact fetuses (9). Our findings indicate that the primary site of action of these agonists is within the fetal hypothalamus or at an unidentified suprahypothalamic site.

This study also showed that, unlike U-50488H, Dyn A-(1–13) and NMDA were ineffective as releasers of ACTH in the postnatal lamb. This led us to suggest...
that these agonists might be causing release of ACTH from the placenta, a possibility that arises from the observation that the placenta of several species contains both ACTH and a CRH-like peptide (25). Whereas ACTH release was completely abolished in HX fetuses, indicating that the placenta was not the site of action of Dyn A-(1–13) and NMDA, it remained possible that a factor released from the placenta was crucial for the actions of the agonists, thus explaining the loss of efficacy of these agents after birth. Prostaglandins were considered as possible modulators of the actions of Dyn A-(1–13) and NMDA, because both the constitutive and inducible activities of PG synthases are high in the placenta (14, 19), and PGE2 has been shown to modulate the HPA axis in fetal sheep (6, 15). Furthermore, the action of PGE2 in releasing ACTH was not observed in HPD fetuses (39), suggesting, as for the data presented here for Dyn A-(1–13) and NMDA, a site of action above the pituitary.

Our data show that indomethacin significantly attenuated the ACTH response to both Dyn A-(1–13) and NMDA, supporting a role for prostanoids in the release of ACTH. These studies, however, do not reveal the source or the identity of the particular prostanoid involved. Indomethacin can be expected to reduce prostanoid synthesis in the placenta as well as in the central nervous system. The lack of effect of NMDA and DYN A-(1–13) after birth would be consistent with an effect of a placentally derived prostanoid, of which the most likely candidate is PGE2. PGE2 is quantitatively the most important prostanoid in fetal plasma, and it is derived primarily from the placenta. PGE2 is also the most potent known ACTH-releasing factor in the fetus. However, studies in the rat have shown that multiple prostanoids (PGE1, -E2, and -F2α) may be involved in ACTH release (23). It is unlikely that Dyn A-(1–13) and NMDA caused sufficient release of prostanoids from the placenta to elicit ACTH by actions within the hypothalamus. Alternatively, PGE2 released from the placenta may serve as a permissive factor for the direct action of Dyn A-(1–13) on NMDA receptors in the hypothalamus. PGE2 has been reported to augment hyperalgesia elicited by NMDA in the spinal cord (18).

Our finding that Dyn A-(1–13) was acting at either the hypothalamus or a suprahypothalamic site was surprising, as it was not expected that a peptide of 13 amino acids would distribute to the brain so rapidly after intravenous administration. Peak ACTH levels were observed 5 min after Dyn A-(1–13) administration in the present studies. If Dyn A-(1–13) does reach the hypothalamus, it would be expected to act on μ-opioid receptors as well as on NMDA receptors. However, it was shown that naloxone does not attenuate the release of ACTH in the fetus in response to Dyn A-(1–13) (32). One possible explanation is that a degradative fragment of Dyn A-(1–13) is responsible for the action on the HPA axis. In vitro studies using human blood showed that Dyn A-(1–13) is truncated to Dyn A-(1–12) and Dyn A-(2–13) within 1 min, and then to Dyn A-(2–12), Dyn A-(3–12), and Dyn A-(4–12) within 2–3 min (11, 22). The NH2-terminal tyrosine is required for binding to opioid receptors. Dyn A-(2–13) is devoid of opioid actions but can elicit many of the nonopioid actions of Dyn A-(1–13) that involve the NMDA receptor, including hindlimb paralysis, barrel rolling, antinoception, decreases in spinal cord blood flow, and suppression of opioid tolerance and dependence (17, 29, 34). Although Dyn A-(1–13) degradation has not been determined in vivo, especially in the fetal sheep, it is possible that one or more of these degradative fragments may be the active peptide at the presumed hypothalamic (or suprahypothalamic) site in fetal sheep. We have previously shown that Dyn A-(2–13) elicits ACTH release in fetal sheep (32), and Dyn A-(2–13) may be degraded to even shorter fragments in vivo.

A hypothalamic site of action for NMDA is more easily understood. The NMDA receptor has been located on the hypothalamus of the fetal and adult rat (13), and there is a marked increase in the number of NMDA-binding sites in the hypothalamus of fetal sheep from ~135 days of gestation (2). In addition, NMDA elicits luteinizing hormone (LH) release in fetal

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**Fig. 5.** Plasma concentration of ir-ACTH after iv administration of Dyn A-(1–13) (A) or NMDA (B) to fetal sheep in the presence of saline (●, □) or indomethacin (○, △). *Significant differences from concentration at −5 min (P < 0.05). Saline or indomethacin (0.2 mg/kg) was given to the fetus 90 min before administration of Dyn A-(1–13) (0.5 mg/kg) or NMDA (4 mg/kg).
sheep (5, 7), but direct addition of NMDA to primary cultures of fetal sheep anterior pituitary cells failed to elicit the release of LH (5). NMDA also failed to cause the release of ACTH from murine anterior pituitary AtT-20 cells (10).

The interaction between Dyn A-(1–13) and NMDA receptors is not well understood. A high-affinity binding site for [125I]Dyn A-(2–17) has been shown for rat brain, where binding was modulated by ligands for the glutamate, glycine, polyamine, and channel sites of the NMDA receptor (30). It is noteworthy that these interactions were shown to occur at only relatively high concentrations of dynorphin. Our previous observation (27) that the release of ACTH by Dyn A-(1–13) in the fetus is blocked by MK-801 indicates that the peptide interacts with a site within the ion channel of the NMDA receptor. It is possible that dynorphin may stimulate the release of glutamate in the hypothalamus; this would readily explain why both competitive and noncompetitive NMDA antagonists have been reported to block the nonopioid actions of dynorphin peptides. Both Dyn A-(1–17) and Dyn A-(2–17) markedly increase extracellular levels of glutamate and aspartate in the rat hippocampus (12).

In summary, we have shown that Dyn A-(1–13) and NMDA elicit ACTH release in fetal, but not postnatal, sheep, a response that involves actions within the fetal central nervous system and that is modulated by prostaglandin synthesis. The exact mechanism by which Dyn A-(1–13) and NMDA induce ACTH release in the fetal sheep, and the loss of responsiveness after birth, remain to be determined.

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