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Opioid mediated activity and expression of mu and delta opioid receptors in isolated human term non-labouring myometrium

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ABSTRACT

The existence of opioid receptors in mammalian myometrial tissue is now widely accepted. Previously enkephalin degrading enzymes have been shown to be elevated in pregnant rat uterus and a met-enkephalin analogue has been shown to alter spontaneous contractility of rat myometrium. Here we have undertaken studies to determine the effects of met-enkephalin on *in vitro* human myometrial contractility and investigate the expression of opioid receptors in pregnant myometrium. Myometrial biopsies were taken from women undergoing elective caesarean delivery at term. Organ bath experiments were used to investigate the effect of the met-enkephalin analogue [D-Ala 2, D-met 5] enkephalin (DAMEA) on spontaneous contractility. A confocal immunofluorescent technique and real time PCR were used to determine the expression of protein and mRNA, respectively for two opioid receptor subtypes, mu and delta. DAMEA had a concentration dependent inhibitory effect on contractile activity (1×10^{-7} M–1×10^{-4} M; 54% reduction in contractile activity, $P<0.001$ at 1×10^{-4} M concentration). Mu and delta opioid receptor protein sub-types and their respective mRNA were identified in all tissues sampled. This is the first report of opioid receptor expression and of an opioid mediated uterorelaxant action in term human non-labouring myometrium *in vitro*.

1. Introduction

The endogenous opioid system consists of a family of structurally related small endogenous peptides acting through multiple opioid receptors (mu, delta and kappa) as neurotransmitters, autocrine or paracrine factors and hormones. The original endogenous opioid peptide families are enkephalins, dynorphins and endorphins. Most of the knowledge that has been accumulated about the expression, release and function of the endogenous peptides are derived from studies of the central and peripheral nervous systems and the neuroendocrine system. Here they have a role in regulating a wide variety of physiological functions, which include but are not limited to nociception, the control of respiration, thermoregulation, the immune response and hormone secretion (Bodnar, 2008).

The existence of both endogenous peptides and opioid receptors in non-neural peripheral sites in animal (Tang et al., 1982; Zhu and Pintar, 1998; Barron, 2000; Denning et al., 2008) and human tissues (Sastry et al., 1980; Belisle et al., 1988; Agirregoitia et al., 2006; Rittner et al., 2008), is now widely accepted. These include placental (Sastry et al., 1980; Belisle et al., 1988) and uterine tissue (Zhu and Pintar, 1998) where they have been implicated in the regulation of female reproduction (Sastry et al., 1980; Belisle et al., 1988; Ahmed et al., 1989; Cemerikic et al., 1991; Zhu and Pintar, 1998). In mouse uterus, all three opioid receptor genes and endogenous opioid peptide precursors have been detected after implantation of the embryo with their expression pattern pointing toward a role in adaptation to pregnancy and in the regulation of uterine motility (Sastry et al., 1980). Binding sites for an opioid receptor antagonist have been identified in rat uterine membranes and are subject to down-regulation during gestation (Baraldi et al., 1985).

Met-enkephalin is a member of the enkephalin family of endogenous peptides. DAMEA ([D-Ala-2, D-met 5] enkephalin), an analogue of met-enkephalin significantly increases the duration of spontaneous contractions in rat myometrium in late gestation (Adjroud, 1985).

There is evidence that the pregnant rat uterus contains higher concentrations of enkephalinase, an enkephalin degrading enzyme, than a non-pregnant uterus, and that enkephalinase levels decrease the day before parturition commences (Ottlecz et al., 1991). Enkephalinase therefore, may act as a regulator of uterine motility by controlling levels of met-enkephalin in pregnancy.

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Opioid receptors are coupled to inhibitory G proteins. Opioid receptor activation leads to decreased cyclic adenosine monophosphate (cAMP) formation and modification of ion channel activities resulting in reduced calcium influx and stimulation of potassium efflux (Zollner, 2006). These mechanisms suggest that opioids could directly affect the contractile response of human myometrium through the modulation of myocyte calcium fluxes and in decreasing cAMP generation, which at term promotes uterine contractility (Lopez Bernal, 2003) and decreased progesterone responsiveness (Smith, 2007).

The effect of met-enkephalin on contractile parameters of human term myometrium has not previously been explored. Furthermore it is not known whether opioid receptors exist in human pregnant myometrium. The aim of this study was twofold. Firstly to investigate the effect of the met-enkephalin analogue DAMEA on spontaneous human pregnant myometrial contractility and secondly to systematically investigate the presence of mu and delta opioid receptors by immunohistochemical and PCR analysis in term non-labouring human myometrium.

2. Materials and methods

2.1. Subjects and preparation of tissues

All biopsy specimens of human pregnant myometrial tissue were obtained from women undergoing elective lower segment caesarean delivery. Ethical approval for the study was obtained from the Research Ethics Committee of the Coombe Women and Infants University Hospital and all patients gave written consent. Criteria for inclusion consisted of a singleton gestation between 38 and 40 weeks who were not in labour. Exclusion criteria included (1) a history of intravenous opioid drug abuse; (2) history of a chronic pain condition or history of regular opioid analgesia intake; (3) the use of prostaglandins to induce labour; (4) history of ruptured membranes or (5) a diagnosis of human immunodeficiency virus, hepatitis B or C or pre-existing diseases such as diabetes mellitus/pre-eclampsia or renal disease. Indications for caesarean delivery included breech presentation and prior caesarean section. All patients received antacid prophylaxis with 30 ml of 0.3 M sodium citrate and 400 mg cimetidine orally, prior to spinal anaesthesia with 2.0–2.4 ml 0.5% hyperbaric bupivacaine, with 20–25 μg of intrathecal fentanyl and 100–150 μg of intrathecal morphine. Table 1 shows the demographic data for all the patients used in this study.

The myometrial biopsy was excised from the midline of the upper margin of the lower uterine segment incision (inner myometrial layer) following delivery of the baby and placenta. All specimens were rinsed in Ringer's lactate solution ensuring all traces of blood were removed and that the specimen was free of placental tissue. For tissue bath experiments the biopsies were placed in a sterile container and refrigerated at 4 °C until used, which was within 2–12 h of collection. For immunoflouresence studies each specimen was embedded with Tissue-Tek O.C.T Compound (Sakura Finetek, Alphen van den Rijn, Netherlands) before being snap frozen in liquid nitrogen and subsequently stored at −80 °C. For mRNA studies each specimen was placed in RNAlater (Ambion, Austin, TX) and refrigerated at 4 °C for between 1 and 3 days before the RNA extraction process.

Table 1
Demographic details of all patients recruited into the contractility, IHC immunohistochemisrty and PCR polymersase chain reaction experimental protocol. Each *n* represents a different patient.

	Water bath	IHC	PCR
Maternal age (years)			
20–25	0	0	1
26–35	6	2	1
>35	2	3	2
Ethnicity (%)			
Caucasian	100	100	100
Parity			
Nulliparous	1	1	1
Multiparous	7	4	3
Indications for caesarean section			
Breech presentation	1	1	1
Previous caesarean section	7	4	3
Total number (*n*)	8	5	4

2.2. Contractile analysis

Uterine biopsies were dissected into at least 4 longitudinal muscle strips 12 mm × 5 mm × 1 mm under a naked eye, ensuring fibrous tissue, serosa or blood vessels were not included. Isometric tension recordings were obtained from an eight-chamber organ bath (either 10/15 ml, water jacketed) system (Myobath, World Precision Instruments Inc. Sarasota, Florida). The organ baths contained Krebs–Henseleit physiological salt solution (NaCl 118 mmol/l, D-glucose 11.1 mmol/l, $NaHCO_3$ 24.9 mmol/l, $MgSO_4$ 1.2 mmol/l, KCl 4.7 mmol/l, KH_2PO_4 1.2 mmol/l, and $CaCl_2$ 2.5 mmol/l, pH 7.4) and were aerated with a gas mixture of 95% oxygen and 5% CO_2 and maintained at 36.7 °C. A resting tension of 1 g was initially applied, and subsequently re-applied as necessary over the first 30–40 min until a steady tension was achieved as previously described (Fanning et al., 2008). During this equilibration period, the Krebs solution was changed every 10 min. When spontaneous contractions became regular (within 60–90 min) experiments were carried out as follows: (1) a 30 min control period followed by cumulatively increasing concentrations of DAMEA (Sigma-Aldrich, St. Louis, MO) in 1 log molar increases every 30 min from 1×10^{-8} M to 1×10^{-4} M ($n=7$) and (2) a 30 min control period followed by the addition of naloxone (1×10^{-5} M; Sigma-Aldrich; Yoo et al., 2001) and then 30 min later by cumulatively increasing concentrations of DAMEA in 1 log molar increases every 30 min from 1×10^{-8} M to 1×10^{-4} M ($n=7$). A time matched control strip, exposed only to Krebs solution was run in parallel from each separate *n* to ensure tissue viability for the duration of the experiment. Strips exposed to DAMEA were also pretreated once off with the peripheral enkephalinase inhibitor thiorphan (1×10^{-4} M; Adjroud, 1985) for 30 min prior to the addition of DAMEA (Sigma-Aldrich) to inhibit tissue enkephalinases. Prior to these experiments, cumulative concentration–effect curves were determined in myometrial biopsies from other women for both naloxone (1×10^{-9}–1×10^{-4} M, $n=3$), thiorphan (1×10^{-7}–1×10^{-4} M, $n=7$) and alcohol (diluent for thiorphan) (0.1% w/v $n=3$), which established that these agents had no effect on myometrial contractility. For each experiment Krebs–Henseleit solution was prepared freshly each day. All stock solutions of drugs were prepared according to the supplier's instructions. Thiorphan (1×10^{-2} M) was diluted in alcohol to a final bath concentration of 0.1% w/v. DAMEA (1×10^{-2} M) and naloxone (1×10^{-1} M) were diluted in distilled water. The organ baths and tubing were siliconised. All stock solutions were stored at −20 °C. Drugs were diluted further immediately before each experiment from the stock solution using Krebs solution. Following completion of the experiment the weight of each muscle strip was recorded to ensure weight and size equality.

2.3. RNA extraction, reverse transcription and polymerase chain reaction

RNA was isolated from myometrial biopsies using TRIzol solution (Invitrogen, Carlsbad, CA) according to manufacturers' instructions. A QiagenTissueLyser II (Qiagen, Germantown, MD) was used to disrupt biopsies using acid washed glass beads for two 30 s periods at a frequency of 30 Hz. All RNA samples were