Adrenomedullin increases ciliary beat frequency and decreases muscular contraction in the rat oviduct

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Abstract

Our laboratory previously showed that oviduct produced the greatest amount of adrenomedullin (ADM) in the rat female reproductive tract. The aim of this study is to investigate the changes in ADM levels resulting from the contact between the sperm and the oviduct and the possible roles of ADM in ciliary beating and oviductal contractility. Oviducts from Sprague–Dawley rats removed at pre- and post-ovulatory stages were cut open longitudinally and treated with ADM and/or receptor blockers before ciliary beat frequency (CBF) was measured. The effects of sperm on ADM production and CBF in the oviduct were also determined. The contraction of the oviduct after treatment with ADM and receptor antagonists was measured using the organ-bath technique. The results showed that ADM increased the CBF in rat oviduct and this stimulating effect was blocked by the calcitonin-gene-related peptide (CGRP) receptor antagonist, hCGRP8–37. CBF was lower in post-ovulatory than pre-ovulatory oviducts. The presence of sperm in the oviduct increased both the ADM level and CBF. ADM treatment was shown to inhibit the contractility of the oviduct by lowering the basal tone and decreasing the contraction amplitude. The ADM receptor antagonist, hADM22–52, was effective in counteracting the relaxation effect of ADM in the oviduct. All in all, these results indicate that ADM may play a crucial role in transporting the gametes/embryos by regulating ciliary beating and muscular contraction.

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Introduction

The oviduct is the part of the female reproductive tract that provides maternal communication with gametes and embryos and is crucial for providing the appropriate environment for the maturation of sperm, for fertilization and for the transport of ova/embryos to the site of implantation. The interaction of gametes/embryos with the oviduct may activate the long- and short-range mechanisms to regulate oviductal function and embryonic development (Fazeli & Pewsey 2008). The contact of sperm, oocyte, and embryos with the oviduct induces different gene transcription activity in this part of the female reproductive tract for the successful implantation in the uterus (Lee et al. 2002, Bauersachs et al. 2003).

The sperm stays at the isthmus part of the oviduct and is gradually released from the sperm reservoir to the ampullary region to meet the ovum for fertilization (Smith & Yanagimachi 1990). After ovulation, the ovum, which is picked up by the cilia of the fimbria, is transported to the ampulla to be fertilized (Lyons et al. 2006). After fertilization, the timely transportation of the embryo to the uterus is needed for successful implantation. This transport relies on the ciliary activity and contraction of the tubal muscles. Ciliary action plays a dominant role during this process of tubal transfer since reduced fertility is known to be associated with the decrease in ciliary beat frequency (CBF), as is found in immotile cilia syndrome or endometriosis (McComb et al. 1986, Lyons et al. 2002).

The upregulation of adrenomedullin (ADM) expression caused by the entry of sperm into the oviduct suggested that ADM may play a role in the female tract (Fazeli et al. 2004). As a member of the calcitonin family of peptides, ADM can bind to the calcitonin-gene-related peptide (CGRP) and ADM receptors formed by the calcitonin receptor-like receptor (CALCRL) and receptor activity modifying proteins (RAMP1, RAMP2, and RAMP3). Coexpression of CALCRL and RAMP1 leads to formation of a CGRP receptor while coexpression of CALCRL with RAMP2 results in an ADM1 receptor, which binds with ADM. Coexpression of CALCRL with RAMP3 produces an ADM2 receptor, which binds with CGRP with a greater affinity than ADM (Hay et al. 2004).
hCGRP$_{8-37}$ and hADM$_{22-52}$ are the receptor antagonists for CGRP and ADM1 receptors while hCGRP$_{8-37}$ is more potent than hADM$_{22-52}$ at ADM2 receptors (Coppock et al. 1999). We have demonstrated a high expression of ADM peptide and mRNA (Li et al. 2008) and an action of ADM on ciliary beat (Chiu et al. 2010) in the rat oviduct. This suggests that ADM is a potential factor to affect oviductal function. This study of ADM in CBF and muscle contraction in rat oviduct has helped to a better understanding of its oviductal function in the reproductive process.

Results

Effect of ADM on CBF in rat oviduct

In oviducts from both the pre- and post-ovulatory rats, addition of ADM (100 nM) stimulated the CBF up to 16\% ($P<0.05$) as shown in Fig. 1. This increase was reversed by the addition of the CGRP receptor blocker, hCGRP$_{8-37}$ ($P<0.05$) but not by the addition of the ADM receptor blocker, hADM$_{22-52}$. The CBF tended to be lower in the post-ovulatory than in the pre-ovulatory oviduct.

Effects of sperm on ADM and CBF in the rat oviduct

Both the Adm gene expression and ADM peptide content increased in the oviduct cultured in direct contact (DC) with sperm ($P<0.05$; Fig. 2A and B). A decrease in ADM secretion into the medium was observed in oviductal cultures with/without DC with sperm (Fig. 2C). However, CBF increased in the oviduct with or without DC with sperm ($P<0.05$; Fig. 2D).

Effect of ADM relaxed the oviduct contraction

The tracings of rat oviducts treated with ADM, ADM receptor blocker, and CGRP receptor blocker are shown in Fig. 3A. Treatment of oviducts with ADM decreased the basal tone and amplitude by 16.90 ± 1.86 and 57.57 ± 7.29\% ($P<0.05$; Fig. 3B and C) respectively, but did not alter the contraction frequency (Fig. 3D). hADM$_{22-52}$ almost completely inhibited the relaxation effect of ADM; hCGRP$_{8-37}$ was much less effective in

Figure 1 The effect of ADM on ciliary beat frequency (CBF) in the rat oviduct. Addition of ADM (100 nM) in the culture medium significantly increased CBF in oviduct cilia (*$P<0.05$, ADM 100 nM versus control). The stimulating effect was blocked by the antagonist, hCGRP$_{8-37}$ (1 mM) (**$P<0.01$, hCGRP$_{8-37}$ + ADM 100 nM versus ADM 100 nM). CBF was lower in post-ovulatory than pre-ovulatory oviduct. One-way ANOVA followed by the Newman–Keuls test. Data are presented as mean±s.e.m. of five repeated experiments.

Figure 2 The effect of coculturing rat oviduct with spermatozoa in direct contact (DC) and no direct contact (NDC) on the expression of Adm mRNA (A), ADM peptide in the oviductal tissue (B), secreted ADM peptide into the culture medium (C), and ciliary beat frequency (CBF; D). A total of five oviductal strips from each rat were used to measure the CBF; four rats were involved in each control/experimental group. Sperm in direct contact with oviduct significantly increased Adm mRNA (A) and ADM peptide content (B), **$P<0.01$ DC versus control. A decrease in ADM secretion into the culture medium was observed in the NDC and the DC groups (C), *$P<0.05$, NDC/DC versus control. CBF increased in both the NDC and DC groups (D), *$P<0.05$ NDC/DC versus control. Data are presented as mean±s.e.m. of four repeated experiments and analyzed by the Newman–Keuls post comparison.

Effect of ADM relaxed the oviduct contraction

The tracings of rat oviducts treated with ADM, ADM receptor blocker, and CGRP receptor blocker are shown in Fig. 3A. Treatment of oviducts with ADM decreased the basal tone and amplitude by 16.90 ± 1.86 and 57.57 ± 7.29\% ($P<0.05$; Fig. 3B and C) respectively, but did not alter the contraction frequency (Fig. 3D). hADM$_{22-52}$ almost completely inhibited the relaxation effect of ADM; hCGRP$_{8-37}$ was much less effective in
which the basal tone and amplitude still decreased by 12.02 ± 1.68 and 27.25 ± 5.05% after ADM treatment (P<0.05 versus control and ADM; Fig. 3B and C) respectively.

Discussion

In this study, we have demonstrated for the first time the actions of ADM in the rat oviduct on increasing the ciliary beat and inhibiting oviductal contraction.

The CBF increased with ADM treatment in oviducts from both the pre- and post-ovulatory rats, and the ADM-stimulated beating frequency tended to be lower in post-ovulatory stage compared with pre-ovulatory oviducts. Similar observation in our laboratory was found in human oviduct incubated with estradiol and high level of progesterone (Li et al. 2010). This finding is consistent with the reported inhibitory effect of high progesterone levels on ciliary beating and suggests that treatment of women with high levels of progesterone in ovulation-induction cycles could be the cause of tubal pregnancy (Paltieli et al. 2000). The use of specific CGRP and ADM receptor blockers showed that this stimulatory effect of ADM was mediated by a CGRP receptor.

The interaction of gametes/embryo with the oviductal epithelial cells changes the microenvironment within the oviduct and triggers a change of the protein secretion profile for the subsequent reproductive events (Fazeli 2008). Coculture with sperm has been shown to increase CBF in primary culture of human oviductal epithelial cells (Morales et al. 1996). In an in vivo study in mice, the expression of ADM and prostaglandin was reported to be upregulated after the arrival of sperm in the oviduct (Fazeli et al. 2004). A similar report showed that motile spermatozoa increased the secretion of prostaglandin and vasoactive peptides from the bovine oviduct in culture (Kodithuwakku et al. 2002). These findings suggest that sperm may increase CBF via the release of ADM and other substances – a suggestion that is upheld by our results in this study. Oviducts cultured in DC with sperm showed an increase in ADM peptide and Adm mRNA levels (Fig. 2A and B) yet sperm separated from the oviduct could still increase CBF to a smaller extent (Fig. 2D). This discrepancy may be explained by the secretion from the sperm of other chemicals that can also stimulate CBF. The lower ADM levels in the culture media of oviductal–sperm coculture compared with oviductal culture could be due to the increase in binding of secreted ADM to the oviduct.

Apart from ciliary beat, contraction is another important function of the oviduct in the transportation of the gametes and embryos. A previous report on the rat showed that endothelin-2 induced oviductal contraction via an endothelin subtype A receptor (Al-Alem et al. 2007). In this study, we were able to show for the first

Figure 3 (A) Representative tracings of muscular contraction in the rat oviduct after treatment with ADM and receptor blockers hADM22–52/ hCGRP8–37. Lowered basal tone and amplitude were observed after treatment with ADM (a). After a pretreatment with hADM22–52 (5 µM) (b) and hCGRP8–37 (5 µM) (c) 1.5 h in the oviduct, the relaxation effect of ADM was inhibited. The basal tone (B) and amplitude (C) were decreased after treatment with ADM (*P<0.05, ADM versus control, paired t-test) and hCGRP8–37 (#P<0.05, hCGRP8–37 + ADM versus control and hCGRP8–37, the Kruskal–Wallis test). Treatment with hADM22–52 receptor blocker was effective in inhibiting the relaxation in the basal tone and amplitude change. There was no significant difference in the frequency (D) after the ADM and ADM receptor antagonist treatments. Data are presented as mean ± S.E.M.
time that ADM inhibited contractility by reducing the basal tone and the amplitude by 16.9 and 57.0% respectively of the control. This effect was mostly mediated by the ADM receptor. These opposite effects of ADM and endothelin (ET) on muscle contracture have already been reported in the vasculature (Stangl et al. 2001) and in the testis (Santiemma et al. 2001). Endothelin was a 21 amino acid vasoconstricting peptides produced primarily in the endothelium and plays a key role in vascular homeostasis.

It is known that sperm increases oviductal ADM and this may increase sperm motility to increase the chance of fertilization of the ovum (Fazeli et al. 2004). We have indeed found that ADM regulates sperm motility and oviductal ciliary beat via a cAMP/protein kinase A and nitric oxide pathway (Chiu et al. 2010). This study demonstrated that the contact of sperm with oviductal epithelium stimulated the production of ADM, which increases the ciliary beat in the epithelial cells to transport the ovum to the labyrinth luminal folding of the ampullary region of the oviduct for fertilization to occur. The upregulated level of ADM in the tissue also relaxed the oviduct, hence reducing the peristaltic contraction of oviduct for the transportation of ovum/embryo toward the uterus. It is pertinent to note that the effects of ADM on ciliary beating and inhibition of oviductal contraction are mediated by two different receptors, i.e. CGRP receptor for ciliary beating and ADM receptor for oviductal contraction. The use of two receptors is important for an integrated fine control, as the two processes can now be independent of each other (both spatially and temporally). Previous report showed that explants of human oviduct treated with prostaglandin F2α (PGF2α) and prostaglandin E2 (PGE2) increased the contractility whereas PGE1, progesterone, levonorgestrel, mifepristone, oxytocin, and human chorionic gonadotropin (hCG) decreased the contraction (Wanggren et al. 2008). Results in this study would add ADM to the list of oviduct relaxants. It was reported that nearly 50% of all tubal pregnancy developed from a fertilized egg to result in tubal implantation. In summary, our present findings illustrated the crucial role of ADM in gamete–oviduct interaction and fertility by regulating the contraction and ciliary beating of the oviduct.

**Conclusion**

Sperm in contact with the oviduct increased ADM expression. ADM increased the ciliary beat and decreased oviductal contraction to facilitate the transport of the gametes/embryos.

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**Materials and Methods**

**Animals**

Female Sprague–Dawley rats were obtained from the Laboratory Animal Unit, Faculty of Medicine, the University of Hong Kong. Immature rats primed with equine chorionic gonadotropin (eCG) and hCG were used for functional studies. Adult rats at pre- and post-ovulatory stages, as determined by vaginal smear, were used for the study on sperm and oviduct interaction. The rats were killed with an overdose of sodium pentobarbitone. All procedures were approved by the Committee on the Use of Live Animals for Teaching and Research, the University of Hong Kong, and were carried out in accordance with the Guide for the Care and Use of Laboratory Animals (the National Academy of Science).

**Study on the effect of ADM on CBF**

SD rats of 4–5 weeks of age were injected with 15 IU eCG, followed by 20 IU hCG 48 h later to simulate the post-ovulatory state. Oviduct was cut open longitudinally with a pair of fine scissors under the microscope, rinsed to remove the content and blood. Small 1–2 mm strips of oviductal tissue were collected into microfuge tubes filled with DMEM/F12 medium (Invitrogen). The oviduct strip was treated with ADM (0, 10, 100 nM) for 1 h with or without pretreatment with 1 μM of a receptor antagonist, hADM22–52 or hCGRP8–37 (Phoenix Pharmaceuticals, Inc., Burlingame, CA, USA) for 1.5 h at 37 °C. CBF measurement was carried out at the Department of Medicine, Queen Mary Hospital, according to the method reported by Tsang et al. (2000). The oviductal strips were incubated with the epithelial surface facing sideways on a 37 °C warm plate for 10 min before measurement of CBF under a Leica DM LB phase contrast microscope (Leica Microsystems, Wetzlar, Germany) at ×400. The frequency was conveyed to a multipurpose vehicle (MPV)-COMBI photo-multiplier (Leica) and a custom-made digital converter, which translated the signal into CBF (Hz). From each strip, five to seven readings were taken randomly and five strips were measured for each group. Each control/experimental group consisted of five rats.

**Study on the effect of sperm–oviduct interaction on ADM and CBF in the rat oviduct**

Sperm were collected from the epididymis of the male SD rat (12 weeks) into DMEM/F12 medium. The motile sperm was isolated by using the swim-up technique. The sperm in 1.5 ml DMEM/F12 medium were collected in centrifuge tubes and incubated at an angle of 45 °C for 15 min at 37 °C in 5% CO2 atmosphere. The top layer containing the sperm was washed with DMEM/F12 medium. After being adjusted to a concentration of 1 × 106/ml, the sperm were incubated in the capacitation medium (94.6 mM NaCl, 4.78 mM KCl, 1.71 mM CaCl2, 1.19 mM KH2PO4, 1.19 mM MgSO4, 25.07 mM K2HPO4, 21.58 M sodium lactate, 0.5 mM sodium pyruvate, 5.56 mM glucose, 4.0 mg/ml BSA, 50 μg gentamicin, and 75 μg penicillin) at 37 °C for 5 h to complete capacitation. The oviducts from the pre- or post-ovulatory SD rats (10 weeks)
were cut open longitudinally. Oviductal strips were cocultured with 2 x 10⁵ capacitated sperm, either placed inside an insert (Micellin culture plate insert, Millipore Corporation, Billerica, MA, USA; not directly in contact, NDC) or outside the insert (DC). Oviductal strips cultured alone served as controls. All cultures were incubated in DMEM/F12 medium at 37 °C, 100% humidity, 5% CO_2/95% air for 24 h. Some of the oviductal strips were then harvested for the determination of Adm mRNA and ADM levels. Other strips with similar treatments were used for CBF measurement, as detailed earlier. A total of five oviductal strips from each rat were used to measure the CBF; four rats were involved in each control/experimental group.

**Effect of ADM on in vitro contractility by an organ-bath technique**

The female SD rats (23–25 days) were treated with 15 IU eCG to stimulate follicular development, followed by 20 IU hCG 48 h later to induce ovulation. Oviducts from post-ovulatory rats were isolated and rinsed in Kreb’s solution (118 mM NaCl, 4.8 mM KCl, 1 mM MgSO_4, 1.15 mM NaH₂PO₄, 15 mM NaHCO₃, 10.5 mM glucose) immediately. The oviduct was carefully dissected from the connective tissue and the unwound oviduct was tied, via silk threads, to tissue holder electrodes in a 10 ml organ bath and attached to a force transducer coupled to a graph recorder. After equilibration, the oviduct was preincubated with or without hADM₂₂₋₅₂ (5 μM) or hCGRP₈₋₃₇ (5 μM) for 1.5 h, and then ADM (500 nM) was added into the bath. The basal tone, amplitude, and frequency of the tracing were calculated as percentage of the control. Each control/experimental group consisted of five rats.

**Determination of Adm mRNA level by real-time RT-PCR**

The oviduct cultured without sperm is the control group, and the oviducts cultured with/without DC with sperm are the NDC and DC experimental groups. The oviduct tissue was put into Trizol reagent (Invitrogen) immediately. The tissue was homogenized in 1 ml Trizol reagent using a polytron. After the addition of 200 μl chloroform and centrifugation at 12 000 g for 15 min, the clear upper layer was transferred to a new tube. Then, 500 μl isopropanol was added and the mixture was centrifuged at 12 000 g for 10 min. The RNA pellet was washed with 70% ethanol in diethyl pyrocarbonate (DEPC) H₂O and dissolved in 30 μl DEPC H₂O. RNA concentration was quantified by the GeneQuant II RNA/DNA calculator (Pharmacia Biotech). RT was performed using the High Capacity cDNA RT kit (Applied Biosystem, Foster City, CA, USA). The condition was as follows: 25 °C for 10 min, 37 °C for 2 h, and 85 °C for 5 s. In total, 2 μg total RNA was used for PCR in a total volume of 20 μl. The concentration of MultiScribe Reverse Transcriptase was 2.5 U/μl. The primer sequences, product length, and accession number are shown in Table 1. Real-time PCR for was conducted under the following condition: 95 °C for 10 min for denaturing, followed by 40 cycles of 95 °C for 30 s, 57 °C for 30 s, and extension for 10 min. The reaction volume was 20 μl and the cDNA template was 2 μl. The IQ SYBR Green Supermix (2 X reaction buffer with dNTPs, 6 mM MgCl₂, iTaq DNA polymerase, SYBR Green I, fluorescein, and stabilizers) was used for PCR (Bio-Rad Laboratories). The primer concentration was 100 nM. The MyIQ 2 color real-time PCR detection system was used to perform real-time PCR. Standard curves for each primer pair were prepared using serial dilutions of cDNA to determine the PCR efficiency. Six reference genes were used for normalization following the method reported by Bustin et al. (2009). The relative quantities of Adm cDNA in each sample were calculated using the 2^ΔΔCt method, where Ct is the threshold cycle, and were expressed as their fold changes relative to the control group. Melt curve analysis for each primer set revealed only one peak for each product. qPCR results were analyzed using the Bio-Rad iQ5-standard edition, version 2.1.94.617 (Bio-Rad Laboratories).

**Measurement of ADM peptide level**

The oviducal tissue was homogenized in 2 M acetic acid on ice, followed by boiling for 10 min. A 50 μl was taken out for protein assay and the remaining homogenate was centrifuged at 15 000 g for 20 min at 4 °C. The supernatant was lyophilized overnight and stored at −20 °C until assay. The ADM level was measured using the ADM EIA assay kit (Phoenix Pharmaceuticals, Inc.). After boiling, 50 μl homogenate or BSA standard with 950 μl NaOH for 10 min to solubilize the proteins, 50 μl of resulting solution was mixed with 2 ml protein assay reagent (Bio-Rad Laboratories). After 10 min incubation, the absorbance was measured at 595 nm wavelength (Analytical Instruments, LLC, Golden Valley, MN, USA). The ADM level in the rat oviduct tissue was expressed as pg/mg protein.

**Statistical analysis**

Results are presented as mean ± S.E.M. One-way ANOVA was used to compare the CBF in each group. The relative expression of Adm and ADM contents in the control, NDC, and DC groups was compared using one-way ANOVA, followed by the
Newman–Keuls post comparison. The basal tone, amplitude, and frequency were presented as the percentage of control; control and ADM were compared by the paired t-test and the groups of control–receptor antagonists–ADM were compared by the Kruskal–Wallis test. Statistical significance was taken at the P<0.05 level.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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