Expression and regulation of the fatty acid amide hydrolase gene in the rat uterus during the estrous cycle and peri-implantation period

A.Z.Xiao1,2, Y.G.Zhao1 and E.K.Duan1,3

1The State Key Laboratory of Reproductive Biology, Institute of Zoology, Chinese Academy of Sciences, Beijing, 100080 and 2Department of Physiology, Jinzhou Medical College, Jinzhou, 121001, China
3To whom correspondence should be addressed. E-mail: duane@panda.ioz.ac.cn

Fatty acid amide hydrolase (FAAH) is involved in embryo development and implantation. Sex hormones down-modulate FAAH activity in the mouse uterus. However, the regulation of the FAAH gene in the uterus is unknown. Our results showed that FAAH mRNA is localized to uterine epithelial cells and circular myometrium during the estrous cycle. In ovariectomized rats, estradiol (E2) plus progesterone (P4) increased FAAH levels in both epithelial cells and circular myometrium. Interestingly, during the implantation period, FAAH mRNA was detected not only in epithelial cells and circular myometrium, but also in the primary decidual zone surrounding the implanting embryo on day 6 and in whole decidualized stromal cells on day 7. Its levels in the stromal cells were markedly higher at the implantation sites than at the inter-implantation sites on days 6 and 7. When implantation was delayed and then induced by E2 or E2 plus P4, FAAH mRNA levels were significantly increased in subepithelial stromal cells and circular myometrium, indicating that blastocyst activation and initiation of implantation in rats requires higher expression of the FAAH gene in subepithelial stromal cells and circular myometrium. In conclusion, the expression of FAAH mRNA is different in the non-pregnant and pregnant rat uterus and sex hormones up-regulate FAAH gene expression.

Key words: estrous cycle/FAAH gene/implantation/steroid hormones/uterus

Introduction

Anandamide (N-arachidonoylthanolamine, ANA) is an endogenous ligand for both the brain-type (CB1-R) and spleen-type (CB2-R) cannabinoid receptors. It is isolated from brain and peripheral tissues (Devane et al., 1992; Mechoulam et al., 1998), and exerts a wide spectrum of central and peripheral effects (Calignano et al., 1998; Meng et al., 1998; Murillo-Rodriguez et al., 1998). The peri-implantation mouse uterus contains high levels of ANA. The levels are highest during the non-receptive phase in pseudopregnant uterus and at the inter-implantation sites, and lowest at the sites of embryo implantation (Schmid et al., 1997). Low levels (7 nmol/l) of ANA accelerate blastocyst trophoblast differentiation and outgrowth, while inhibition of trophoblast differentiation is observed with a higher dose (28 nmol/l) of ANA in vitro (Wang et al., 1999). ANA also inhibits 2-cell embryo development to the blastocyst stage and reduces the rate of zona hatching of blastocysts in vitro. A synthetic cannabinoid (CP 55 940) prevents implantation. All these effects are mediated via CB1-R in embryos and uterus (Paria et al., 1995, 1998; Wang et al., 1999; Maccarrone et al., 2000a).

The effectiveness of cannabinoids depends on their metabolism and turnover by the target organ. Fatty acid amide hydrolase (FAAH) can catalyse ANA hydrolysis to arachidonate and ethanolamine (Di Marzo et al., 1994; Cravatt et al., 1996; Giang and Cravatt, 1997). During human gestation, FAAH is the only critical event controlling ANA level or action (Maccarrone et al., 2002). FAAH expression and activity is detected in mouse and human uterine epithelium during the peri-implantation and non-pregnant period, and in preimplantation and implanting embryos (Paria et al., 1996, 1999; Maccarrone et al., 2000a). FAAH activity is higher at the implantation sites and lower at the inter-implantation sites in the mouse uterus (Paria et al., 1996). Sex steroids, progesterone (P2) and estrogen down-modulate mouse uterine FAAH activity (Maccarrone et al., 2000a), but the regulation of the FAAH gene and its expression in rat uterus are not known. In this investigation, we used in-situ hybridization and densitometric analysis to examine the expression of FAAH mRNA in rat uterus during the estrous cycle and peri-implantation period, and study its regulation by ovarian steroid hormones.

Materials and methods

Chemicals and reagents

HindIII, XhoI, Yeast tRNA, P4 and 17β-estradiol (E2) were purchased from Sigma. DNase free RNase and EcoRI were the product of Promega. λDNA/HindIII markers and pGEM-3Zf (+)/HaeIII markers were purchased from Pharmacia. X-Gal, digoxigenin RNA labelling kit (SP6/T7), anti-digoxigenin-alkaline phosphatase Fab fragments, 5-bromo-4-chloro-3-indolyl phosphate (BCIP), nitro blue tetrazolium (NBT) chloride and blocking reagent were from Boehringer Mannheim. Salmon sperm DNA was from Gibco.

Animal and tissue preparation

Sprague-Dawley female rats (220–250 g) were supplied by the Experimental Animal Center of the Institute of Zoology, Chinese Academy of Sciences.
They were housed with free chow and water and 12:12 h light-dark cycle for 1 week. The stages of the estrous cycle were identified by vaginal smear. The uterine horns were removed at different stages of the estrous cycle for in-situ hybridization (n = 3 animals per stage).

To examine the effects of ovarian steroid hormones on FAAH gene expression in the non-pregnant rat uterus, rats were ovariectomized without regard to the stage of the estrous cycle. These rats rested for 2 weeks before receiving any treatment. The ovariectomized rats were divided into four groups and respectively injected with sesame oil (0.1 ml/rat), E2 (0.1 mg/rat), P4 (0.4 mg/rat), or E2 (0.1 mg/rat) plus P4 (0.4 mg/rat) (Feng et al., 1998). Steroids were dissolved in sesame oil and injected s.c. with the same volume. These rats were killed at 24 h after injection. Uterine horns were collected and tested (n = 3 animals per group).

Virgin female rats (220–250 g) were mated with fertile males of the same strain. The morning of finding sperm was designated as day 1 of pregnancy. The rats on days 1–5 of pregnancy were killed at 17:00–18:00 and embryos were recovered on days 2–5 from the reproductive tract to confirm pregnancy. The rats on days 6 and 7 of pregnancy were also killed at 17:00–18:00. Implantation sites were visualized with i.v. injections of Trypan Blue dye solution (0.1% in saline, 0.1 ml/rat) on day 6. Implantation sites were distinct on day 7 and blue dye injection was not required. Uterine horns of the rats on days 1–7 of pregnancy (n = 3 animals per day) were excised, and uterine horns on days 6 and 7 were separated into the implantation and interimplantation sites.

To examine the effects of ovarian steroid hormones on FAAH gene expression in the pregnant rat uterus, a delayed implantation rat model was established. Rats were ovariectomized at 8:00–9:00 on day 4 of pregnancy. These ovariectomized rats were divided into two groups at random. In the first group, rats were injected s.c. with sesame oil (0.1 ml/rat) at 9:00 every day (days 5–7 of pregnancy) and this was designated as the control group. These rats were killed at 9:00 on day 8 (n = 3 animals), and their uteri were collected for in-situ hybridization. To maintain the implantation delay, in the second group rats were injected with P4 (2 mg/rat) at 9:00 on days 5 and 6 of pregnancy. These rats were divided into three groups at random, and treated respectively with P4 (2 mg/rat), E2 (0.1 mg/rat), or E2 (0.1 mg/rat) plus P4 (2 mg/rat) on day 7, and killed at 9:00 on day 8 (n = 3 animals per group). The uteri of rats with implantation sites were collected for in-situ hybridization. Steroids were dissolved in sesame oil and injected s.c. with the same volume.

Hybridization probes
The FAAH plasmid was kindly provided by Dr S.K.Dey (Department of Molecular and Integrative Physiology, Ralph L. Smith Research Center, University of Kansas Medical Center, Kansas City, Kansas 66160–7338). The plasmid was linearized with restriction enzymes XhoI and HindIII as a template for transcription in vitro. The antisense digoxigenin-labelled cRNA probe was generated using T7 RNA polymerase, and the sense digoxigenin-labelled cRNA probe was generated using SP6 RNA polymerase.

In-situ hybridization
The method for in-situ hybridization was performed according to a previous report (Zhao et al., 1999, 2002). Briefly, frozen uterine sections (10 µm) were mounted onto poly-L-lysine coated slides. When required, frozen specimens were cut serially to detect the sites of the embryo. All the slides were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 15 min at 4°C. Following prehybridization, the slides were hybridized with digoxigenin-labelled antisense or sense (control) cRNA probes for 18 h at 60°C. After washing with 6×standard saline citrate (SSC), the slides were incubated with RNase A (20 µg/ml) at 37°C for 30 min, then washed with 6×SSC and 2×SSC. After blocking with 0.5% blocking reagents in Tris-buffered saline (TBS, 100 mmol/l Tris, 150 mmol/l NaCl, pH 7.5) for 30 min and washing with TBS for 10 min ×3, the slides were covered with anti-DIG-AP antibody (1:400) for 2 h. Following washing with TBS, the slides were stained with NBT and BCIP at 37°C for 6 h. The positive signals were visualized and photographed under a microscope. All experiments were repeated three times with similar results.

Densiometric and statistical analysis
Photographs were scanned by the Personal Densitometer SI scanning instrument (Molecular Dynamics). Three to five areas with positive signals were selected for quantitation by the ImageQuaNT image analysis program (Molecular Dynamics). Three areas with negative signals (without positive signals) on each photograph were also selected for quantitation. After running the program, the signal intensity and area for each selected area were obtained and expressed as the optical density (O.D.) value. The O.D. value for each selected area was divided by its area, giving the O.D. value/pixel. The average O.D. value/pixel of three negative signal areas was the background value (ObjAverage). Separate ObjAverages were taken for each photograph. The final signal value for each positive signal area was determined by subtracting its ObjAverage from the O.D. value/pixel of the each positive signal area. Statistical analysis was performed with Scheffe’s correction of analysis of variance for multiple comparisons. Data represents the mean ± SD from the three experiments, and P < 0.05 was considered significant.

Results

Expression of FAAH mRNA in rat uterus during the estrous cycle
The signals for FAAH mRNA were localized to luminal and glandular epithelial cells and circular myometrium, and were absent in stroma cells during the estrous cycle (Figure 1A–D). The signals in luminal epithelial cells and circular myometrium were strongest at estrus, strong at diestrus, and weaker at proestrus and metaestrus. The levels of this mRNA in luminal epithelial cells were significantly higher at estrus than at proestrus (P < 0.01) or metaestrus (P < 0.01), but there was no significant difference (P > 0.05) between levels at estrus and diestrus. There also were no significant differences (P > 0.05) of this mRNA in circular myometrium among the four stages of the estrous cycle (Figure 2). Sections hybridized with sense probe showed very weak signals (Figure 1E).

Effects of ovarian steroid hormones on uterine FAAH mRNA expression in ovariectomized rats
Only weak signals for FAAH mRNA were detected in epithelial cells and circular myometrium, and signals for this mRNA were absent in stroma cells in ovariectomized rats (Figure 3A). As compared with the control or P4 treatments, an injection of E2 significantly increased (P < 0.01) the levels of this mRNA in epithelial cells, but not in circular myometrium (P > 0.05; Figure 3B and Figure 4). P4 treatment did not affect the levels of FAAH mRNA in epithelial cells or circular myometrium (P > 0.05; Figure 3C and Figure 4). However, E2 plus P4 significantly increased the levels of this mRNA in epithelial cells as compared with control (P < 0.01), E2 (P < 0.05) or P4 treatment groups (P < 0.01) and significantly increased the levels in circular myometrium as compared with the control (P < 0.05) or E2 treatment groups (P < 0.01; Figure 3D and Figure 4). Sections hybridized with sense probe showed very weak signals (Figure 3E).

Expression of FAAH mRNA in the peri-implantation rat uterus
FAAH mRNA was evidently present in uterine epithelial cells and circular myometrium on days 1 and 2 of pregnancy (Figure 5A,B). Strong signals for this mRNA were also detected in the subepithelium stromal cells on days 3 and 4, but the signals clearly decreased on day 5 (Figure 5C–E). Interestingly, with the progression of implantation, FAAH mRNA was strongly localized in the decidualized stromal cells (primary decidual zone) around the implanting embryo on day 6 (Figure 5F) and in all decidualized stromal cells (primary and secondary decidual zone) at the implantation sites on day 7 (Figure 5I). In addition, weak signals for this mRNA were present in the implanting embryo blood vessels (Figure 5G,J) and in circular myometrium at the implantation sites on days 6 and 7 (Figure 5F,J), and in the epithelial cells, subepithelium stromal cells and circular
Expression and regulation of FAAH gene in rat uterus

Figure 1. In-situ hybridization of FAAH mRNA in rat uterus during the estrous cycle. Dark colour indicates the positive signal of mRNA accumulation. (A) Proestrus; (B) estrus; (C) metaestrus; (D) diestrus; (E) estrus sense. le = luminal epithelium; s = stroma; ge = glandular epithelium; cm = circular myometrium; lm = longitudinal myometrium. All sections were on the same slide except (E). These experiments were repeated three times with similar results. The photographs are shown at 40× original magnification.

Figure 2. Densitometric analysis of FAAH mRNA expression in rat uterus during the estrous cycle. le = luminal epithelium; m = circular myometrium. Each value represents the mean ± SD from three experiments. *P < 0.01.

Effects of ovarian steroid hormones on uterine FAAH mRNA expression in delayed implantation rats

As shown in Figure 7A,C, signals for FAAH mRNA were detected in uterine epithelial cells, subepithelial stromal cells and circular myometrium in the control and P₄ treatment groups. As compared with the control group, E₂ and E₂ plus P₄ not only significantly increased the levels of this mRNA in stromal cells (P < 0.05) and circular myometrium (P < 0.05), but also in epithelial cells (P < 0.01 and P < 0.05 respectively; Figure 7B,D and Figure 8). P₄ alone also significantly increased the levels of this mRNA in stromal cells (P < 0.05) and in epithelial cells (P < 0.05), but not in circular myometrium (P > 0.05; Figure 7B,D and Figure 8). Interestingly, as compared with the P₄ treatment group, E₂ and E₂ plus P₄ significantly increased the levels of FAAH mRNA in uterine subepithelial stromal cells (P < 0.05) and circular myometrium (P < 0.05), but not in the epithelial cells (Figure 7B,D and Figure 8). Sections hybridized with sense probe only showed very weak signals (Figure 7E).

Discussion

This investigation demonstrated that FAAH mRNA was expressed in rat uterine luminal and glandular epithelial cells and in circular myometrium, but not in stromal cells during the estrous cycle. The levels of this mRNA in the uterine luminal epithelial cells were significantly higher at estrus than at proestrus or metaestrus. FAAH activity and content have been detected in luminal and glandular epithelia of the non-pregnant mouse uterus (Maccarrone et al., 2000a). Human uterine epithelial cells have also shown appreciable FAAH

myometrium at the inter-implantation sites on days 6 and 7 (Figure 5H,K). The levels of this mRNA in the stromal cells were significantly higher (P < 0.01) at the implantation sites than at the inter-implantation sites on day 6 and 7 (Figure 6). Sections hybridized with the sense probe showed very weak signals (Figure 5L).
activity (Maccarrone et al., 2000a). Rat uterine endometrium undergoes a cyclical change during the estrous cycle, which is regulated by ovarian steroid hormones. Both E$_2$ and P$_4$ in blood reach their highest levels at the end of proestrus. They decline at estrus, and maintain a lower level at metaestrus. P$_4$ reaches a second high level at the end of metaestrus (Smith et al., 1975; Parr and Parr, 1989). Therefore, the expression of FAAH mRNA in rat uterus during the estrous cycle might be regulated by ovarian steroid hormones.

Indeed, we found that in ovariectomized rats, as compared with control or P$_4$ treatment groups, E$_2$ significantly increased the levels of FAAH mRNA in uterine epithelial cells, but not in circular myometrium. Although P$_4$ had no effect on FAAH mRNA expression, when compared with control or E$_2$ treatment groups, E$_2$ plus P$_4$ significantly increased its levels both in epithelial cells and in circular myometrium. These results suggest that ovarian steroid hormones can up-regulate the expression of FAAH mRNA in epithelial cells and circular myometrium in the non-pregnant rat uterus.

In this study, we used ovariectomy-induced delayed implantation as a tool to understand the relative roles played by the embryo and ovarian steroid hormones in modulating the expression of FAAH mRNA in the pregnant rat uterus. Delayed implantation is a phenomenon that occurs when a pregnant female mouse or rat is ovariectomized before implantation; the preparation of the uterus for implantation is arrested and the blastocyst remains in a state of suspended development (Flint et al., 1980). Delayed implantation can be maintained by continued P$_4$ treatment, or terminated by an injection of E$_2$ with blastocyst activation and initiation of implantation. E$_2$ administration is rapidly followed by uterine changes that make the uterus receptive to the implanting blastocyst (Yoshinaga and Adams, 1966; Paria et al., 1993). This investigation demonstrated that, as compared with the control group, P$_4$ significantly increased the levels of FAAH mRNA in epithelial cells and subepithelial stromal cells, but not in circular myometrium, while E$_2$ and E$_2$ plus P$_4$ significantly increased the levels of mRNA in epithelial cells, subepithelial stromal cells and circular myometrium. The results suggest that in the presence of an embryo, ovarian steroid hormones can up-regulate the expression of FAAH mRNA not only in epithelial cells and circular myometrium,
Expression and regulation of FAAH gene in rat uterus

Figure 5. In-situ hybridization of FAAH mRNA in rat uterus during early pregnancy. Dark colour indicates the positive signal of mRNA accumulation. (A) Day 1; (B) day 2; (C) day 3; (D) day 4; (E) day 5; (F) day 6 implantation site; (G) day 6 implantation site (at 200×); (H) day 6 inter-implantation site; (I) day 7 implantation site; (J) day 7 implantation site (at 200×); (K) day 7 inter-implantation site; (L) day 2 sense. le = luminal epithelium; s = stroma; ge = glandular epithelium; cm = circular myometrium; lm = longitudinal myometrium; bv = blood vessel. Sections (A)–(D) or (E)–(J) were on the same slide except (L). These experiments were repeated three times with similar results. The photomicrographs are shown at 40× original magnification except (G) and (K).

Figure 6. Densitometric analysis of FAAH mRNA in uterine stromal cells in inter-implantation and implantation sites on day 6 and 7 of pregnancy. non-im = inter-implantation site; im = implantation site. Each value represents the mean ± SD from three experiments. *P < 0.01.

During the peri-implantation period, FAAH mRNA was present not only in uterine epithelial cells and in circular myometrium, but also in stromal cells, especially in the primary decidual zone surrounding the implanting embryo on day 6 and in whole decidualized stromal cells (including primary and secondary decidual zones) at the implantation sites on day 7. The expression of FAAH mRNA in decidualized stromal cells was significantly higher at the implantation sites as compared with the inter-implantation sites in rat uterus, consistent with the reported higher activity of FAAH at the implantation sites and lower activity of FAAH at the inter-implantation sites in mouse uterus (Paria et al., 1996). This may explain the low level of ANA at the implantation sites and high level of ANA at the inter-implantation sites in the mouse uterus (Schmid et al., 1997).
**Figure 7.** In-situ hybridization of FAAH mRNA in rat uterus after termination of delayed implantation by E₂ or E₂ plus P₄. Dark colour indicates the positive signal of mRNA accumulation. (A) Control; (B) E₂; (C) P₄; (D) E₂ + P₄; (E) E₂ + P₄ sense. le = luminal epithelium; s = stroma; ge = glandular epithelium; cm = circular myometrium; lm = longitudinal myometrium. All sections were on the same slide except (E). These experiments were repeated three times with similar results. The photographs are shown at 40× original magnification.

**Figure 8.** Densitometric analysis of effects of ovarian steroid hormones on FAAH mRNA expression in delayed implantation rat uterus. le = luminal epithelium; s = stroma; m = circular myometrium. Each value represents the mean ± SD from three experiments. *P < 0.05; **P < 0.01.
et al. have reported that the FAAH genes and proteins in rat, mouse and human have high homology (Giang et al., 1997). FAAH mRNA was localized to the epithelia of the non-pregnant rat uterus, consistent with the localization of FAAH expression and activity to human and mouse uterine epithelial cells (Paria et al., 1999; Maccarrone et al., 2000a). Ovarian steroid hormones up-modulate the expression of the FAAH gene in rat uterus. P4 also stimulates the activity of FAAH in human lymphocytes (Maccarrone, et al., 2001). Therefore, the observations reported here about the FAAH gene in the rat uterus may also hold true for human and mouse counterparts. Low levels of cannabinoid agonists accelerate trophoblast differentiation and outgrowth, while higher doses of these agonists inhibit trophoblast differentiation and implantation in mouse (Wang et al., 1999).

Therefore, lower levels of ANA, which result from the higher expression of the FAAH gene and activity at the implantation sites, might help trophoblast differentiation and outgrowth at the implantation sites. Higher levels of ANA, which resulted from lower FAAH gene activity at the inter-implantation sites, could be responsible for the inhibition of trophoblast proliferation at these regions (Paria and Dey, 2000). Recently, Maccarrone et al. reported that low FAAH activity, protein content and mRNA concentration in peripheral lymphocytes and subsequent increased ANA levels in blood cause early pregnancy failure in humans (Maccarrone et al., 2000b, 2002). Peripheral lymphocytes have a critical role in embryo implantation and successful pregnancy in human beings (Piccinni et al., 1998). It is possible that the implanting embryo can down-modulate the local or lymphocyte levels of ANA by inducing higher expression of the FAAH gene at the implantation sites in rat, mouse and human, and this may be important for successful pregnancy in these species.

Although the expression of FAAH mRNA in rat uterine circular myometrium and blood vessels was observed, the significance is not clear. Because FAAH can convert ANA to arachidonic acid and ethanolamine, and arachidonic acid can be converted to vasodilatory eicosanoids such as prostacyclin or the epoxyeicosatrienoic acids, leading to vasorelaxation (Pratt et al., 1998), the expression of the FAAH gene in rat uterine circular myometrium and blood vessels during pregnancy might be associated with smooth muscle vasorelaxation. The uterine circular myometrium and blood vessel vasorelaxation might provide a safe environment and rich nutrition for embryo implantation.

In summary, the present results show that expression of the FAAH gene is different in the non-pregnant and pregnant rat uterus. Ovarian steroid hormones up-regulate expression of the FAAH gene in the rat uterus. Blastocyst activation and initiation of implantation in the rat appears to require higher expression of the FAAH gene in stromal cells and circular myometrium.

Acknowledgements
We thank Professor S.K.Dey (Department of Molecular and Integrative Physiology, Ralph L-Smith Research Center, University of Kansas Medical Center, Kansas City, Kansas 66160-7338) for providing us with the FAAH plasmid. We thank Professor Zeng Guoqing for helpful discussions and assistance. We also thank Mr Charlie Greenwell, Dr Hyun I.Park and Douglas R.Hurst (Department of Chemistry, Florida State University, Tallahassee, FL 32306-1390) for their kind help in revising this paper. This work was supported by grants from Special Funds for Major State Basic Research Project, grant no. G1999505903; the ‘Climbing Project of China’, grant no. 970211019-3; and 100 Scientist Program, grant no. B1997.

References


Submitted on March 12, 2001; resubmitted on December 4, 2001; accepted on March 31, 2002.