Redistribution of aquaporins 1 and 5 in the rat uterus is dependent on progesterone: a study with light and electron microscopy

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Abstract

During early pregnancy in the rat there is a dramatic reduction in luminal fluid which is associated with uterine receptivity for blastocyst implantation. This study investigates the presence and distributional changes of several members of the aquaporin (AQP) family in the rat uterus in response to hormonal regime. An increase in apical AQP5 protein expression was found in response to progesterone alone or in combination with oestrogen, which is similar to that seen at the time of implantation. AQP1 was found in endothelial cells of the endometrium and in the inner circular layer of smooth muscle, with maximal protein expression seen after three doses of progesterone plus 8 hr of oestrogen treatment. These results, for the first time, show that the up-regulation of AQP5 in the apical plasma membrane of uterine epithelial cells and AQP1 in the inner circular layer of myometrium, is dependent on progesterone. Furthermore, unlike during normal pregnancy, there is no differential gradient of AQP5 expression between mesometrial and antimesometrial poles of the progesterone treated uterus. Hence it is suggested that the differential gradient of AQP5 is dependent on the presence of a blastocyst, in addition to the appropriate hormonal environment.

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Introduction

Water transport across an epithelial barrier can occur by transcellular or paracellular means. The paracellular pathway of fluid transport between cells is mainly regulated by the tight junction complex (Claude & Goodenough 1973). Transcellular fluid transport across cells can occur by diffusion alone or through specialised transporters, known as aquaporins (AQP). AQP are present in a variety of epithelia and exhibit a greater capacity for water movement when compared with simple diffusion alone (Agre *et al.* 2002). AQP1, 4 and 5 are water selective and therefore are members of the classical AQP family (Agre *et al.* 1998).

AQP1, a 28 kDa protein initially isolated from red blood cells (Agre *et al.* 1987, Denker *et al.* 1988), has been localised to endothelial cells (Nielsen *et al.* 1993) as well as the inner circular layer of uterine smooth muscle (Lindsay & Murphy 2004a). Furthermore, it was found that AQP1 was most concentrated within the mesometrial myometrium at the time of implantation in the rat (Lindsay & Murphy 2004a). AQP4 is widely distributed in various tissue types, such as the kidney (Yamamoto & Sasaki 1998, van Hoek *et al.* 2000), respiratory tract (Song *et al.* 2001) and brain (Nielsen *et al.* 1997b) but was absent from the pregnant rat

uterus (Lindsay & Murphy 2004b). AQP5 has mainly been localised in apical plasma membranes of various secretory glands including salivary glands and submucosal glands of the respiratory tract as well as type I pneumocytes (Nielsen *et al.* 1997a). AQP5 was found to be present on the apical plasma membrane of uterine epithelial cells at the time of implantation in the rat, with the greatest concentration of channels found in mesometrial uterine epithelial cells (Lindsay & Murphy 2004b).

At the time of implantation in the rat, as a component of the complex alterations in uterine epithelial cells (Julian *et al.* 2005), there is a dramatic reduction in the volume of uterine luminal fluid, with the uterine lumen closing down resulting in close apposition between the uterine epithelium and trophoblastic cells of the implanting blastocyst (Enders & Schlafke 1967). Freeze fracture studies demonstrated an increase in depth of the tight junctions and more branching of the tight junction strands at this time (Murphy 2000). Recent evidence has shown that AQP5 is redistributed to the apical plasma membrane of uterine luminal epithelial cells on days 6 and 7 of pregnancy (Lindsay & Murphy 2004b). Hence it is likely that at the time of implantation in the rat, uterine fluid is transported from the lumen into the endometrial stroma by

AQP channels present within the apical plasma membrane of luminal epithelial cells.

These uterine changes of early pregnancy are driven by ovarian steroid hormones. Oestradiol causes stromal oedema, beginning at the antimesometrial pole of the uterus while after several days of oestrogen, the uterine lumen is distended and full of fluid (Ljungkvist 1971a). Progesterone alone, or in combination with oestrogen results in closing down of the uterine lumen (Ljungkvist 1971b). Studies investigating tight junctions in luminal epithelial cells of the rat found that oestrogen led to 'leaky' tight junctions between these cells, while progesterone led to the formation of 'tight' tight junctions (Murphy *et al.* 1981). There is however no accepted explanation of this well established phenomenon of stromal oedema nor the regulation of uterine fluid volume by ovarian hormones.

Previous studies using immature rats indicate that AQP1 mRNA is up-regulated in response to administration of oestradiol (Li *et al.* 1997). Furthermore, the expression of AQP1 was found in the inner circular layer of myometrium in control and progesterone treated animals (Richard *et al.* 2003). Another study found AQP1 protein within both layers of the myometrium in all treatment groups with a slight increase in oestradiol treated mice and a slight decrease in the progesterone treated group, when compared with the control group (Jablonski *et al.* 2003). Animals treated with oestradiol or progesterone followed by oestradiol showed a biphasic response with expression initially in the inner circular layer of myometrium and 24 hrs later, a shift of AQP1 mRNA expression to the uterine stromal endothelial cells (Richard *et al.* 2003).

AQP2 was found to be absent from the pregnant mouse uterus (Richard *et al.* 2003). However, AQP2 was shown to be strongly regulated by oestrogen, with protein present in luminal and glandular epithelium as well as in the myometrium (Jablonski *et al.* 2003). AQP4 mRNA was not found in ovariectomised mice treated with either oil, oestradiol, progesterone or a combination of progesterone followed by oestradiol (Jablonski *et al.* 2003, Richard *et al.* 2003). In uterine luminal epithelial cells AQP5 mRNA was up-regulated after exposure of ovariectomised mice to progesterone followed by a nidatory dose of oestradiol (Richard *et al.* 2003), but was absent in the control or oestrogen treated mouse uterus (Jablonski *et al.* 2003).

It is thus evident that AQP channels are likely to be important in the implantation process of rodents during normal pregnancy but to date the hormonal control of this phenomenon is unknown. Previous studies have highlighted differences in AQP expression, particularly AQP5, between rats and mice at the time of implantation (Lindsay & Murphy 2004b). Hence the aim of this current study is to investigate the hormonal regulation of AQP1, AQP4 and AQP5 protein expression in the ovariectomised and hormone treated rat model using light and electron microscopy techniques.

Materials and Methods

Animals

Twenty-five adult, female, virgin Wistar rats were used in this study and housed at 21 °C with a 12 h light:darkness cycle and given food and water *ad libitum*. Animals 12–14 weeks old were bilaterally ovariectomised using a ventral approach under isoflurane anaesthesia and allowed to recover for at least 4 weeks. Rats were randomly divided into five groups each with five animals and treated with different hormonal regimes. All hormonal doses were within the normal physiological range (Ljungkvist 1971a, b). Hormones were dissolved in benzyl alcohol (Sigma, St Louis, MO, USA) and diluted in peanut oil to achieve levels as described below. Subcutaneous injections were given in the scruff of the neck. Animals in the control group were injected with 0.1 ml peanut oil alone for three consecutive days. Animals in group 2 were injected with 0.5 µg 17-β-oestradiol (Sigma) in 0.1 ml peanut oil for three consecutive days. Group 3 animals were injected for three consecutive days with 5 mg progesterone (Sigma) in 0.2 ml peanut oil. All animals in these three groups were killed 24 h after the last injection with an intraperitoneal injection of sodium pentobarbitone (Nembutal, Merial Australia, Parramatta, NSW, Australia). Animals in the remaining groups were injected with 5 mg progesterone for 2 days and 0.5 µg 17-β-oestradiol as well as 5 mg progesterone on day 3 on opposite sides of the neck. Half of these animals were killed 8 h after the last injection with the remainder killed 24 h after the last injection. Uterine horns were excised from the euthanased animals and randomly assigned for either light (LM) or electron microscope (EM) studies.

Light microscopy

Uterine tissue was immediately placed in ice-cold 0.1 M phosphate buffer (pH 7.4), cut into 5 mm blocks and immersed in OCT compound (Tissue Tek, Tarrance, CA, USA). Blocks were then immersed in supercooled isopentane (BDH Laboratory Supplies, Poole, England) before being stored in liquid nitrogen. Two blocks per animal were cut using a CM3050 cryostat (Leica, Heerbrugg, Switzerland) and 8 µm thick sections were placed onto gelatin-coated slides. After briefly air drying, sections were fixed in acetone at –20 °C for at least 1 h. Remaining steps were carried out at room temperature. After briefly air drying, sections were incubated in a pre-blocking solution of PBS containing 1% BSA (Sigma). This solution was also used as diluent for all primary and secondary antibodies. Sections were then incubated in the primary antibody solution for at least 3 h.

Primary antibodies used in this study were directed against unique sequences of rat AQP1, AQP4 or AQP5 (Alpha Diagnostics International, San Antonio, TX, USA) and were used at concentrations of 2.5, 20 and 3.3 µg/ml respectively. These members of the AQP family were

chosen as they have previously been shown to be present in the pregnant and hormonally treated mouse uterus (Richard *et al.* 2003). Sections were washed in PBS and incubated for 1 h in FITC conjugated goat-anti-rabbit secondary antibodies at a concentration of 7.5 µg/ml. From this point onwards sections were kept in the dark to prevent quenching of the fluorescent signal. Sections were then washed in PBS, mounted with Vectashield (Vector, Burlingame, CA, USA), cover slipped and visualised within 1 h of completing this protocol with a Diaplan microscope (Leica). Digital micrographs were taken using a Leica DC200 camera and micrographs were produced using Photoshop software (Adobe Systems, San Jose, CA, USA). Final magnifications were calculated using a stage micrometer.

Electron microscopy

The uterine horns randomly selected for electron microscopy (EM) processing were immediately fixed in freshly prepared 4% formaldehyde solution for 40 mins during which time 5 mm and finally 1 mm rings were cut. Tissue was then washed in 0.1 M phosphate buffer (pH 7.4) and from this point onwards all incubation steps were carried out at 4°C. Tissue was then dehydrated in graded alcohol, infiltrated with LR white resin (Sigma) containing 0.5% benzoin ethyl ether (Fluka, Buchs, Switzerland) embedded in gelatin capsules with fresh resin and finally polymerised with u.v. light.

An ultracut UCT ultramicrotome (Leica) was used to cut two blocks per animal. Silver-gold ultrathin sections were collected onto nickel grids and air dried. Prior to immunolabeling, sections were rehydrated and non specific staining was blocked by incubation in PBS containing 0.05 M glycine followed by PBS containing 1% BSA. Sections were incubated overnight at 4°C in solutions containing anti-AQP1 or anti-AQP5 antibodies. After washing in PBS containing 1% BSA, sections were incubated for 2 h at room temperature in a solution of ultrasmall gold conjugated goat-anti-rabbit secondary antibodies (Aurion, Wageningen, Netherlands) at a concentration of 0.6–0.8 µg/ml. Further washing was followed by post fixation in 2% glutaraldehyde in PBS, washed again and silver enhanced (Aurion) for 30–35 mins. Sections were washed in distilled water, air dried and viewed unstained using a JEM-1010 electron microscope operating at 80 kV (Jeol, Tokyo, Japan).

Controls

Negative controls, in which the primary antibody was omitted, were run in parallel with all experimental runs. Four randomly selected sections for each animal in light microscopy (LM) studies and at least 2 randomly selected grids per animal in EM studies were processed as negative controls to detect any non specific binding of the secondary antibody.

Skeletal muscle sections were run in parallel with anti-AQP4 immunofluorescence studies as a positive control.

Non-immune controls were carried out in LM and EM experiments by replacement of primary antibodies with normal purified rabbit IgGs (Sigma). In addition, randomly selected grids were processed without incubation with primary or secondary antibodies in order to detect any non-specific silver enhancement.

Results

Light microscopy

AQP1 immunofluorescent staining is seen in endometrial blood vessels in all treatment groups, with a variation in AQP1 staining of the myometrium between treatment groups. An increase in AQP1 is seen in the inner circular layer of myometrium in ovariectomised animals treated with progesterone alone (Fig. 1B) or in combination with oestrogen (Fig. 1D and F) when compared with animals treated with oestrogen alone (Fig. 1A). There is an increase in mesometrial compared with antimesometrial AQP1 staining, in the inner circular layer of myometrium, in animals treated with progesterone, in combination with 8 h of oestrogen (Fig. 1C, antimesometrial; 1D, mesometrial) and 24 h of oestrogen (Fig. 1E, antimesometrial; 1F, mesometrial). There is an increase in AQP1 immunofluorescence in the mesometrial inner circular layer of ovariectomised animals treated with progesterone and 8 h of oestrogen (Fig. 1D) compared with other treatment groups.

There is no AQP4 staining in the uterus from any treatment group. Oestrogen (Fig. 2C) and progesterone in combination with oestrogen (Fig. 2D) results are shown.

AQP5 immunofluorescent staining is seen in the luminal epithelial cells from all treatment groups examined, except for the oestrogen treated group (Fig. 3B). In control animals (Fig. 3A), there is apical AQP5 staining. In ovariectomised animals treated with either progesterone alone or in combination with oestrogen there is apical AQP5 staining with no difference between mesometrial (Fig. 3D, progesterone; 3F, progesterone in combination with oestrogen) and antimesometrial (Fig. 3C, progesterone; 3E, progesterone in combination with oestrogen) luminal epithelial cells.

Controls were carried out with all experimental sections and no staining was observed. Negative controls of myometrium (Fig. 2E) and luminal epithelium (Fig. 2F) from ovariectomised animals treated with progesterone in combination with oestrogen are shown.

Electron microscopy

Immunogold localisation of AQP1 molecules in myometrium from ovariectomised animal treated with progesterone in combination with oestrogen revealed cytoplasmic localisation in longitudinal muscle cells (Fig. 4A). In

Figure 1 Immunofluorescent images of the rat myometrium showing AQP1 staining. AQP1 is present in the inner circular layer of myometrium (m) in ovariectomised animals treated with oestrogen (A), progesterone alone (B) or in combination with oestrogen (C–F). Immunofluorescent staining is increased in the inner circular myometrium at the mesometrial pole in all animals (A, oestrogen; B, progesterone; D, progesterone and 8 h oestrogen treatment; F, progesterone and 24 h oestrogen treatment). The antimesometrial myometrium shows very little immunofluorescent staining in progesterone primed animals treated with either 8 (C) or 24 h (E) of oestrogen. There is an increase in immunofluorescent staining of the inner circular layer of muscle at the mesometrial pole in progesterone primed animals with 8 h of oestrogen when compared with other treatment groups. Staining of endometrial blood vessels is seen in all treatment groups (arrow). Scale bar, 50 μ m.

circular muscle cells, AQP1 was localised to the plasma membrane (Fig. 4B and C).

AQP5 immunogold labelling was seen in all treatment groups. In ovariectomised control animals (Fig. 5A) and those treated with oestrogen (Fig. 5B), there was mainly cytoplasmic immunogold localisation, with very little staining of the apical plasma membrane. However, in ovariectomised animals treated with progesterone alone (Fig. 5C) or in combination with oestrogen (Figures 5D

and E) there was an increase in immunogold particles associated with the apical plasma membrane. There was no difference in immunogold labelling between luminal epithelial cells from mesometrial and antimesometrial poles of the uterus (not shown).

All controls carried out showed no staining. Progesterone in combination with oestrogen is shown as an example (Fig. 4D, smooth muscle cells from inner circular layer of myometrium; Fig. 5F, luminal epithelium).

Figure 2 Light micrographs of the uterus from ovariectomised animals treated with oestrogen (A&C), or progesterone in combination with oestrogen (B, D, E and F). AQP1 immunofluorescent staining is seen in endometrial blood vessels (A and B) but is absent from the uterine luminal epithelium (e). There is no immunofluorescent staining of the uterine stroma or luminal epithelium (e) with anti-AQP4 antibodies (C and D). Negative control images of myometrium (m) and luminal epithelium (e) showing no immunofluorescent staining. Scale bar, 50 μ m.

Discussion

This study shows for the first time that the increase in apical AQP5 protein expression in rat luminal epithelial cells is controlled by progesterone. It also demonstrates that AQP1 protein levels increased in the inner circular layer of myometrium in the ovariectomised rat treated with progesterone and 8 h oestrogen.

The increase in AQP5 expression in the apical plasma membrane of luminal epithelial cells of the ovariectomised rat treated with progesterone alone or in

combination with oestrogen is consistent with mRNA results in ovariectomised mice treated with progesterone in combination with oestrogen (Richard *et al.* 2003). However in the rat it appears that progesterone is the minimum requirement to cause such a change, and not progesterone followed by a nidatory oestrogen surge, as is the case in mice (Richard *et al.* 2003). During normal pregnancy in the rat, a similar increase was seen at the time of implantation however, ovariectomy and hormone administration did not lead to the differential concentration of AQP5 staining seen at the time of implantation.

Figure 3 Light micrographs demonstrating AQP5 staining of the rat uterus from control ovariectomised animals treated with oil alone (A), and those treated with oestrogen (B), progesterone alone (C and D) or in combination with oestrogen (E and F). Apical AQP5 immunofluorescent staining is seen in the uterine epithelium from control animals (A). Very little immunofluorescence is seen in the luminal epithelium (e) from the uterus treated with oestrogen (B). AQP5 immunofluorescent staining is seen in the apical part of luminal epithelial cells from ovariectomised animals treated with progesterone alone (C and D) or in combination with oestrogen (E and F). There is no difference in apical AQP5 staining of luminal epithelial cells from mesometrial and antimesometrial poles of the uterus in animals treated with progesterone alone (C, antimesometrial; D, mesometrial) or in combination with oestrogen (E, antimesometrial; F, mesometrial). Scale bar, 50 μ m.

The fact that the differential concentration of AQP5 staining between mesometrial and antimesometrial poles of the uterine lumen does not exist in ovariectomised animals under any of the hormonal regimes studies, as it does during normal pregnancy (Lindsay & Murphy 2004b), suggests that this is due to some effect of the blastocyst, perhaps paracrine signals from the blastocyst to the uterine epithelium (Roberts *et al.* 1989).

The presence of AQP5 proteins in the apical plasma membrane of luminal epithelial cells in response to progesterone, as first demonstrated in light and electron microscopy experiments in the current study, provides a possible mechanism for the reabsorption of luminal fluid seen in the progesterone treated uterus. Since fluid can travel via paracellular or transcellular pathways, and the tight junctions in the progesterone treated uterus are relatively

Figure 4 Electron micrographs showing the distribution of AQP1 staining in outer longitudinal (A) and inner circular muscle cells (B–C). There is diffuse gold staining of the outer longitudinal muscle cells (A), while plasma membrane staining (arrows) is seen in muscle cells from the inner circular layer of myometrium (B–C). Negative control (D) showing the absence of gold particles in muscle cells from the inner circular layer.

'tight' (Murphy *et al.* 1981), fluid may be transported by transcellular means through AQP channels, particularly apical AQP5 channels as are up-regulated in the progesterone treated uterus.

The movement of fluid across an epithelial barrier through AQP channels is a passive process, following osmotic gradients set up by various other solute transporters. In this context, it has been demonstrated that the cystic fibrosis transmembrane conductance regulator (CFTR) and epithelial sodium channel (ENaC) play an important role in the movement of chloride and sodium ions across the endometrial epithelium (Chan *et al.* 2002). CFTR in the luminal epithelium leads to the secretion of chloride ions into the uterine lumen and the movement of fluid into the lumen during proestrus and early oestrus (Chan *et al.* 2002). Oestrogen leads to up-regulation of CFTR in the uterine epithelium (Rochwerger & Buchwald 1993, Rowlands *et al.* 2001), and progesterone, alone or in combination with oestrogen, leads to a down-regulation of CFTR expression (Mularoni *et al.* 1995). Hence during oestrogen stimulation when chloride ions are secreted into the uterine lumen and there are leaky tight junctions (Murphy *et al.* 1981), fluid most likely travels into the uterine lumen via paracellular mechanisms. However, during progesterone stimulation where there is a decrease in chloride ion secretion (Mularoni *et al.* 1995) and an increase in sodium ion

secretion into the lumen (Nordenvall *et al.* 1989), there is an osmotic gradient favoring the movement of water from the lumen to the endometrial stroma. This osmotic gradient, in addition to 'tight' tight junctions seen at this time (Murphy *et al.* 1981), suggests that fluid movement is via the transcellular pathway involving apical AQP5 channels.

The increase in AQP1 water channels in blood vessels of the endometrial stroma in response to various hormonal regimes is a common feature seen in several organs (Nielsen *et al.* 1993, Borgnia *et al.* 1999, King *et al.* 2000) including the uterus (Lindsay & Murphy 2004b). Oestrogen causes increases in vascular permeability and stromal oedema (Ljungkvist 1971a, Anderson *et al.* 1972) and thus the presence of AQP1 molecules in the endometrial stromal blood vessels provides a mechanism for the formation of this stromal oedema.

The presence of AQP1 in the myometrium has previously been demonstrated in the pregnant rat uterus, where there was an increase in the inner circular layer of myometrium and a further increase in this layer of myometrium from the mesometrial compared with the antimesometrial pole (Lindsay & Murphy 2004a). A similar result was seen in the present work with an increase in AQP1 staining in the inner circular layer of myometrium in response to progesterone alone or in combination with oestrogen.

Figure 5 Electron micrographs showing the distribution of AQP5 in the apical portion of luminal epithelial cells from control animals (A), and ovariectomised animals treated with oestrogen (B), progesterone (C) or progesterone in combination with oestrogen (D and E). In control and oestrogen treated animals, there are immunogold particles in the uterine epithelial cell cytoplasm, with very little plasma membrane staining. An increase in immunogold particles associated with the apical plasma membrane is seen in animals treated with progesterone alone or in combination with oestrogen. A higher magnification image (E) shows the immunogold particles associated with the apical plasma membrane. Negative control electron micrograph of luminal epithelial cell from an ovariectomised animal treated with progesterone in combination with oestrogen showing no immunogold staining (F).

Results from this study show that maximal AQP1 staining occurred after 8 h of oestrogen stimulation to progesterone primed rats, when compared with other hormonal regimes. Similarly, in mice AQP1 protein expression in the myometrium increased in response to oestrogen, and decreased in progesterone treated animals (Richard *et al.* 2003). The difference in response of rat myometrial AQP1 localisation after 8 and 24 h of oestrogen treatment to a

progesterone-primed rat is consistent with the biphasic response to oestrogen seen in the ovariectomised and progesterone-primed mouse uterus, where maximal AQP1 mRNA expression was found one hour after oestrogen administration (Richard *et al.* 2003). The time difference between these two studies most likely consists of slight differences in timing between mouse and rat implantation, and the delay in translocation of mRNA into protein.

Hence, the maximal expression of AQP1 observed in the inner circular layer of myometrium 8 h after oestrogen administration may represent another aspect of the biphasic response of oestrogen in the progesterone-primed rat.

AQP1 in the inner circular layer of myometrium, seen in the present study, may allow the movement of water from the myometrium into the endometrial stroma, particularly after 8 h of oestrogen treatment. The decrease in myometrial AQP1 immunofluorescence seen after 24 h of oestrogen treatment provides further evidence that stromal oedema, which is part of the early response to oestrogen (Anderson *et al.* 1972), is dependent on AQP1 in the myometrium.

We also observed a differential concentration of AQP1 staining between the mesometrial and antimesometrial pole of the uterus. This suggests that the influence of the asymmetrical AQP1 myometrial expression on blastocyst positioning, as described during early pregnancy (Lindsay & Murphy 2004b), is not dependent on signaling or the presence of the blastocyst, but is rather an intrinsic effect of the hormonal milieu. It also seems that this could lead to swelling of the uterine myometrium and closing of the lumen, which would explain the appearance of the closed lumen seen in the progesterone treated uterus (Ljungkvist 1971b, Png & Murphy 2000).

In summary, this is the first study to show that ovarian hormones change the distribution of AQP molecules. Thus, the hormones which regulate implantation are involved in the redistribution of AQP5 to the apical plasma membrane of the luminal epithelial cells and an increase in AQP1 staining of the myometrium. These are the conditions of uterine receptivity, and the present study adds to our understanding of uterine fluid transport and the hormonal mechanisms regulating the switch from paracellular transport mechanisms, controlled by the tight junction complex (Murphy 2000) to transcellular transport by regulation of AQP expression. It also adds to the events which constitute the plasma membrane transformation (Murphy 2000).

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