

Constitutive expression of heat shock proteins hsp25 and hsp70 in the rat oviduct during neonatal development, the oestrous cycle and early pregnancy

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Certain heat shock proteins are regulated by steroid hormones and are associated with oestrogen receptor function in reproductive tissues, indicating that these proteins have a role during implantation, decidualization and placentation. In the present study, the expression of hsp25, hsp70 and oestrogen receptor α were examined by immunohistochemistry in oviducts from rats during neonatal development, the oestrous cycle and during early pregnancy. Oestrogen receptor α was the first protein observed in the neonatal oviduct, and its expression preceded that of hsp70 and hsp25. Although these heat shock proteins have been associated with the oestrogen receptor, this study showed that during early development of the oviduct, the receptor protein was not associated with the concomitant expression of hsp25 and hsp70. However, these heat shock proteins were expressed when oviductal cells became differentiated. In the adult oviduct, hsp70 was more abundant than hsp25, moreover, there were no significant modifications in expression of hsp25 during the oestrous cycle. In contrast, the expression of hsp70 was significantly higher in epithelial cells during dioestrus, when the maximum amount of oestrogen receptor α was also observed. Therefore, the present study shows that hsp70, but not hsp25, is an oviductal protein modulated by the oestrous cycle and that it is a protein marker for specific phases of the oestrous cycle. In addition, hsp70 was more responsive to the hormonal changes in the infundibulum and ampullar regions of the oviduct. During early pregnancy, hsp25 expression was downregulated (unlike in the endometrium), whereas hsp70 was relatively abundant in the oviduct. hsp70 was observed in all functional segments of the oviduct during pregnancy, indicating that in the oviduct, this protein is modulated by oestrogens and progesterone and possibly by other pregnancy-related hormones.

Introduction

Heat shock proteins are detected in almost all types of organism and cell; they are involved mainly (acting as molecular chaperones) in protein folding and unfolding and participate in a number of vital processes (Ciocca *et al.*, 1993; Hendrick and Hartl, 1993; Jakob and Buchner, 1994). hsp25, a member of the small heat shock protein family, varies significantly depending on development, differentiation and the physiological or pathological status of the organs examined (Ciocca *et al.*, 1993; Welsh and Gaestel, 1998). hsp25, like other heat shock proteins, is regulated transcriptionally by heat shock transcription factors (binding to heat shock responsive elements) (Ciocca *et al.*, 1993; Mathew and Morimoto, 1998), but it is also regulated by oestrogens (the promoter region contains an oestrogen-

responsive half-palindromic motif) (Ehrnsperger *et al.*, 1997). Expression of hsp27 (human homologue of rat hsp25) is relatively high in several oestrogen target organs: in the endometrium, hsp27 shows significant changes in cellular content and localization during the different phases of the menstrual cycle (Ciocca *et al.*, 1983). In contrast, in the rat endometrium, hsp25 is either absent or expressed in low concentrations during the oestrous cycle. However, during pregnancy, expression of hsp25 is high in the rat endometrium at implantation: in endothelial cells of the endometrial vessels and in the luminal epithelium of the antimesometrial region (implantation site) (Ciocca *et al.*, 1996). In addition, hsp25 was also observed in predecidual and decidual cells and in other types of placental cell (Ciocca *et al.*, 1996). Overall, these data indicate that hsp25 is involved in implantation, decidualization and placentation.

hsp25 in the rat oviduct was examined to investigate further the possible implications of hsp25 in the biology of reproduction. The rat oviduct produces many factors that

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influence fertilization and early cleavage-stage embryonic development.

The role of hsp70 in the oviduct was also investigated. hsp70 is involved in steroidogenesis as well as in the assembly and trafficking of steroid receptors (Pratt and Scherrer, 1994; Khanna *et al.*, 1995; Liu and Stocco, 1997). hsp70 binds to nuclear oestrogen receptors acting as a co-activator, modulating oestrogen receptor α activity in breast cancer cells (Hurd *et al.*, 2000). In addition, there is evidence that heat shock transcription factors and the expression of hsp70 are under oestrogenic regulation (Tang *et al.*, 1995; Yang *et al.*, 1995). hsp70 is present in both constitutive and inducible forms and the transcriptional control of these proteins has been studied in early embryos (Chandolia *et al.*, 1999). Therefore, it was of interest to investigate the role of hsp70 in the oviduct.

The specific objectives of the present study were: (i) to identify hsp25 and hsp70 in the development of the oviduct; (ii) to compare and study hsp25 and hsp70 during the oestrous cycle and early pregnancy; and (iii) to examine hsp25 and hsp70 association with oestrogen receptor α regulation.

Materials and Methods

Animals and tissue preparation

Oviducts were obtained from Wistar rats at days 4, 7 and 10 of neonatal development ($n = 5$ per group); from virgin adult cyclic rats during proestrus, oestrus and dioestrus ($n = 3$ per group); and from rats on days 3, 5, 6 and 9 of pregnancy ($n = 3$ per group). Rats were kept in a light- and temperature-controlled environment and had free access to water and rat chow. For pregnant rats, the day on which spermatozoa were present in the vagina was considered day 1 of pregnancy. Rats were killed by decapitation and tissues (oviduct and uterus) were removed immediately. All protocols were performed according to the Institutional Rules for Animal Care and Use. The different segments of the oviduct and the uterine tissues were fixed by immersion in Bouin's fixative for 48 h. The tissues were processed for embedding in paraffin wax (TissuePrep2, Fisher Scientific, Fair Lawn, NJ). As a positive control, a solid tumour was used. This was obtained by implanting human breast cancer MCF-7 cells (containing hsp27, hsp70 and oestrogen receptors) into the flank of an athymic nude mouse.

Western blot analysis

Western blot analysis was performed on tissue homogenates prepared as described by Vargas-Roig *et al.* (1997). The protein lysate (20–40 μg per lane) was subjected to electrophoresis on 12.5% SDS-polyacrylamide gels (w/v) and transferred to nitrocellulose membranes. One lane was loaded with a positive control (breast cancer cytosol containing hsp25 and hsp70). Molecular mass markers (Rainbow Protein Markers, Amersham) were: myosin (200 kDa), phosphorylase b (97.4 kDa), BSA (69 kDa), ovalbumin (46 kDa), carbonic anhydrase (30 kDa), trypsin

inhibitor (21.5 kDa) and lysozyme (14.3 kDa). The filters were blocked with 5% BSA in PBS and 0.05% Tween 20 (v/v) and incubated with the antibodies against hsp25 and hsp70: (i) rabbit polyclonal antibody against recombinant hybrid hsp25 and hsp27 protein, which recognizes the N-terminal part of murine hsp25 (Engel *et al.*, 1991), used at a dilution of 1:2000 (in 0.02 mol NaPO_4H_2 l^{-1} , 0.15 mol NaCl l^{-1} , 0.04% sodium azide (w/v), 1% BSA, pH 7.6); and (ii) mouse monoclonal antibody BRM22 against the constitutive and inducible forms of hsp70 (Sigma Chemical Co., St Louis, MO), used at a dilution of 1:2000. After incubation overnight at 4°C, the filters were incubated with a biotinylated swine antibody to rabbit immunoglobulins to detect hsp25 or with a biotinylated rabbit antibody to mouse immunoglobulins to detect hsp70. Both secondary antibodies were purchased from Dako (Glostrup) and were used at a dilution of 1:4000 for 60 min. The filters were incubated with peroxidase-labelled streptavidin–biotin complex at a dilution of 1:6000 for 45 min to detect the proteins immunoenzymatically. The bands were developed using chemiluminescence reagents according to the manufacturer's instructions (Dupont NEN, Boston, MA) and the light emitted was captured on autoradiography film (Sigma). Each step of this method required washing procedures with $1 \times$ PBS with 0.05% Tween 20 (v/v) and solutions were mixed by shaking at room temperature.

Immunohistochemistry

Paraffin wax serial sections (5 μm) were mounted on slides coated with 3-aminopropyltriethoxy-silane (Sigma). The slides were used for routine haematoxylin and eosin staining and for immunohistochemistry. Immunostaining was performed as described by Vargas-Roig *et al.*, (1997) using a sensitive peroxidase-labelled streptavidin–biotin detection system (Dako Corporation, Santa Barbara, CA). Primary antibodies used to detect hsp25 and hsp70 were those described in the western blot analysis. For immunohistochemistry, a mouse monoclonal antibody (1D5) raised against the N-terminal domain (A/B region) of recombinant human oestrogen receptor α protein (Dako) was added. This antibody shows crossreactivity to rat oestrogen receptor and was used at a dilution of 1:50. The specificity of these antibodies has been tested by the suppliers and by this laboratory using western blot analysis. All antibodies were incubated with the tissue sections at 4°C overnight in humidifying chambers. In the negative control slides (absence of immunostaining), the tissue sections were incubated with dilution buffer without the primary antibodies. An antigen retrieval protocol with a microwave oven was used for detecting oestrogen receptor α (Shi *et al.*, 1995). Diaminobenzidine (DAB) (2 mg ml^{-1}) and hydrogen peroxide (0.001%) (v/v) was used as chromogen substrate and the slides were counterstained with methyl green (0.5%) (w/v) for 2 min or with toluidine blue 1% (w/v) in dH_2O for 15 s at 80–90°C. The time for all DAB reactions for immunohistochemical detection of hsp25 and hsp70 and oestrogen receptor α was constant (10 min), thus the tissue was subsequently used for quantitative analysis.

Quantitative analysis of immunostaining and statistical analysis

For hsp25, hsp70 and oestrogen receptor immunostaining, the percentage of immunostained cells and the intensity of staining (weak, moderate or strong) was evaluated under a $\times 100$ oil immersion objective. An average of 6300 cells was counted per group. In the stromal cells, the distribution of oestrogen receptors in small cells was relatively homogeneous; therefore, oestrogen receptors were quantified by using an image analyser. Approximately 200 000 nuclei were counted per slide using a Leitz microscope and a video camera (Newvicom Dage MTT) which transmits image data to an AT386 computer equipped with a UIC software (Image I). The images were obtained using a $\times 25$ objective. Standardized control slides were used to yield comparable conditions for reading the slides. The statistical analyses performed were: one-way ANOVA followed by Duncan's test, two-way ANOVA followed by Scheffé's test and Student's *t* test.

Results

Neonatal oviduct

On day 4, immunostaining for hsp25 was absent in the oviduct, and staining for hsp70 was negative or very weak and limited to the epithelium. Oestrogen receptors were observed in epithelial cells (nuclei) with weak to moderate intensity (Fig. 1c). Oestrogen receptors were also observed with weak intensity in muscle and stromal cells. On day 7, weak to moderate immunostaining for hsp25 was observed in the oviductal epithelium (in cell clusters) in the ampullar region (Fig. 1a), but no hsp25 immunostaining was observed in the infundibulum and isthmus regions. hsp70 was expressed in the ampullar and infundibular regions (Fig. 1b). Oestrogen receptors were expressed in epithelial and stromal cells in all regions of the oviduct. On day 10, hsp25 was observed in epithelial cells of the ampullar region, but was not present in the infundibulum and isthmus regions. hsp70 was observed in the ampulla and infundibulum; epithelial cells in these regions showed hsp70 in the apical region of the cells, sometimes in the form of tiny apical protrusions. Mesothelial cells showed strong hsp70 immunostaining. On day 7, the percentage of hsp70 stained cells in the epithelium in the ampulla was $(33.9 \pm 3.8)\%$, which increased to $(45.5 \pm 1.5)\%$ on day 10 ($P < 0.05$). Similar percentages of oestrogen receptor-positive cells were detected on day 7 and day 10.

The concentration of hsp25 and hsp70 on day 7 and day 10 of neonatal development, as revealed by western blot analysis, is presented (Fig. 2).

Adult oviduct

Immunostaining for hsp25 was observed in the cytoplasm of epithelial cells in the ampulla, infundibulum and isthmus, in which the protein appeared in cell clusters (Fig. 1d). hsp25

immunostaining was also observed in muscle cells, but the intensity of staining was weak. Western blot analyses were not carried out on adult rat oviducts because hsp25, like hsp70, was observed in muscle cells, which are numerous in the oviduct. Protein expression was subsequently evaluated more specifically by immunohistochemistry. The percentages of hsp25-immunoreactive cells displaying moderate and strong immunostaining in the three regions of the oviduct are shown (Fig. 3a). The percentage of hsp25-positive cells tended to increase during oestrus and dioestrus, mainly in the isthmus, but this result did not reach statistical significance.

Immunostaining for hsp70 was abundant in the adult oviduct (Fig. 1e). This protein was moderately to strongly labelled in the cytoplasm of epithelial cells in the three oviductal regions examined. The infundibular and ampullar regions of the oviduct showed more hsp70-positive cells than did the isthmus at pro-oestrus, oestrus and dioestrus (Fig. 3b). A significant increase in the percentage of hsp70-positive cells was recorded at dioestrus in the infundibulum and ampullar regions compared with that in the isthmus ($P < 0.05$). Stromal cells and muscle cells were weakly positive for hsp70.

Oestrogen receptors appeared in the nuclei of epithelial cells; the percentage of oestrogen receptor-immunoreactive cells during proestrus, oestrus and dioestrus is shown (Fig. 4a). During the immunostaining protocol to reveal oestrogen receptors (antigen retrieval with a microwave oven), on several occasions small oviductal regions (mainly the infundibulum) did not remain attached to the glass slide, which made it difficult to determine the immunostaining in each oviductal region. A significant increase in the percentage of oestrogen receptor-positive cells was observed during dioestrus ($P < 0.001$). Oestrogen receptors were also observed in the stroma of the oviduct and the percentage of oestrogen receptor-positive cells tended to be higher during dioestrus (Fig. 4b), but this result did not reach statistical significance.

Pregnancy

The percentage of hsp25-positive cells in all regions of the oviduct of pregnant rats was significantly lower compared with that found in non-pregnant rats ($P < 0.05$) (Figs 3a and 5a). However, the percentage of hsp25-positive cells did not remain constant during early pregnancy; a significant increase was recorded in the ampullar region on day 5 of pregnancy compared with the other days of pregnancy. Moreover, the percentage of hsp25-positive cells tended to increase in the isthmus on day 6 of pregnancy but this result was not significant. During pregnancy, the lowest percentage of hsp25-positive cells was observed in the infundibulum, and there was a constant decrease as pregnancy advanced. Immunostaining for hsp25 in the ampulla of a rat at day 9 of pregnancy is shown (Fig. 1f).

During pregnancy, the percentage of hsp70-immunostained cells was higher than the percentage of hsp25-positive cells ($P < 0.005$). In addition, the percentage of hsp70-positive cells during pregnancy was higher than that

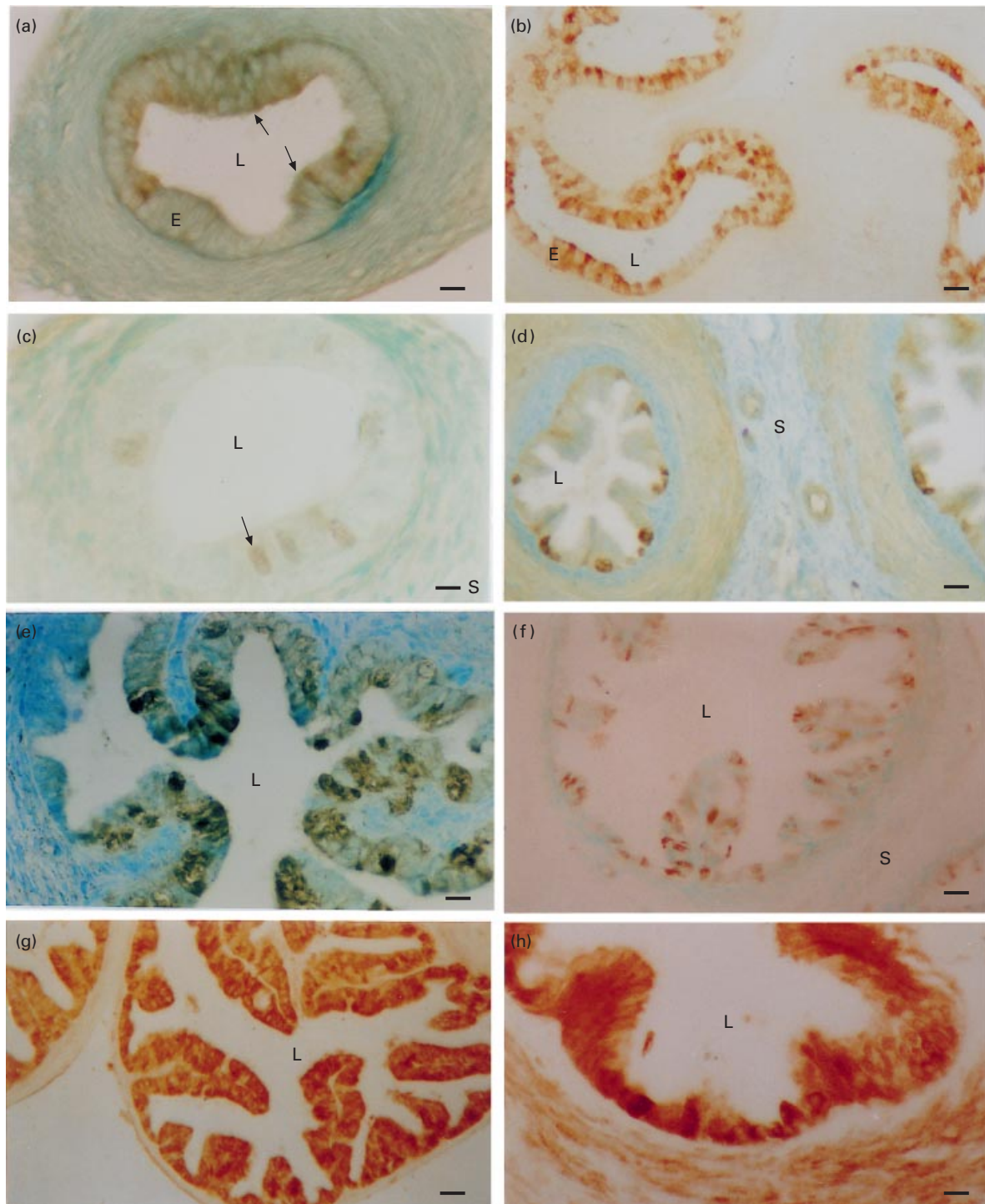


Fig. 1. Expression of hsp25, hsp70 and oestrogen receptor α in the rat oviduct as revealed by immunohistochemistry. (a) Postnatal day 7 oviduct showing weak hsp25 immunostaining in a few epithelial cells (arrows); (b) postnatal day 7 oviduct showing numerous epithelial cells expressing hsp70; (c) postnatal day 4 oviduct showing oestrogen receptor-positive nuclei in epithelial cells (arrow); (d) adult oviduct from an oestrous rat (ampulla) showing hsp25 expression in epithelial cell clusters, weak protein expression can also be seen in muscle cells; (e) adult oviduct from a proestrous rat (ampulla) showing hsp70 expression in numerous epithelial cells; (f) oviduct from a rat at 9 days of pregnancy (ampulla) showing weak hsp25 immunostaining in epithelial cells; (g) oviduct from a rat at 3 days of pregnancy (ampulla) showing strong hsp70 staining in the epithelium; and (h) oviduct shown in (g) but showing hsp70 expression in the isthmic region. E: epithelium; L: lumen; S: stroma. Scale bars represent 20 μ m (a,e,h), 10 μ m (c) and 50 μ m (b,d,f,g).

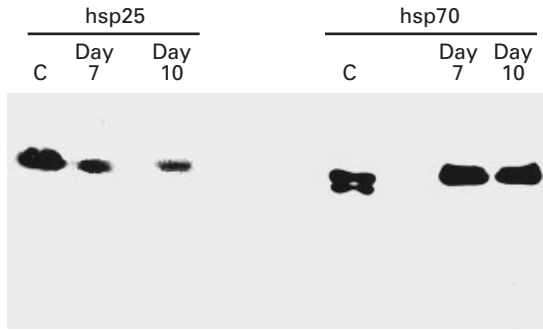


Fig. 2. Western blot analysis of postnatal day 7 and day 10 rat oviducts showing bands corresponding to hsp25 and hsp70. Note that the hsp70 content was higher than that of hsp25. C: control breast cancer cytosol. Note the two overlapping bands in the control lane for hsp70, corresponding to hsc70 (upper) and hsp70 (lower).

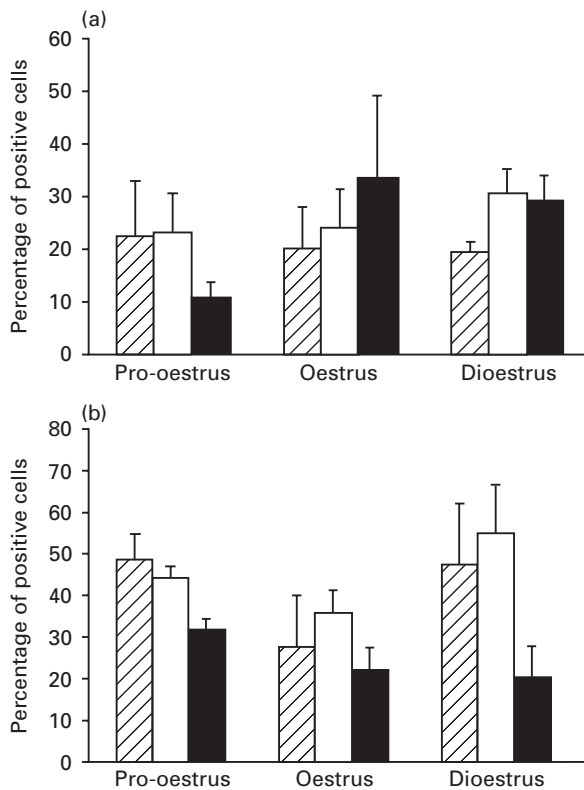


Fig. 3. Relative percentage of epithelial cells in the adult rat oviduct displaying (a) hsp25 and (b) hsp70 immunostaining. ▨, infundibulum; □, ampulla; ■, isthmus. No significant changes were observed throughout the oestrous cycle in the percentage of hsp25-positive cells, whereas in dioestrus the percentage of hsp70-positive cells was higher in the infundibulum and ampulla than in the isthmus ($P < 0.05$).

observed in the oviduct of non-pregnant rats (Figs 3b and 5b). Statistical analysis revealed that the concentration of hsp70 was significantly higher ($P < 0.05$) in the isthmus during pregnancy, compared with that during the oestrous cycle. The percentage of hsp70-positive cells in the isthmus reached similar values to those observed in the ampullar and infundibular regions (a higher percentage was recorded on

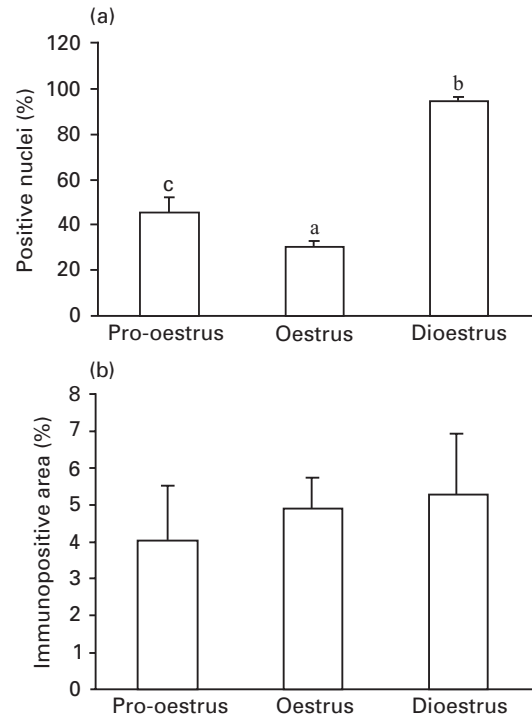


Fig. 4. Expression of oestrogen receptors in the adult rat oviduct throughout the oestrous cycle. In the epithelium (a), a significantly higher percentage of oestrogen receptor-positive cells was observed during dioestrus ($P < 0.001$, b versus a) and pro-oestrus ($P < 0.05$, c versus a). In the stroma (b), the percentage of oestrogen receptor-positive cells was also greater during dioestrus, but this result was not significant.

day 3 of pregnancy, $P < 0.05$). There was no significant difference in the percentage of hsp70-immunostained cells in the different oviductal regions as pregnancy advanced. hsp70 immunostaining in the ampulla and isthmus during day 3 of pregnancy is shown (Fig. 1g,h).

During pregnancy, oestrogen receptor-positive cells were abundant in the epithelium and stroma (Fig. 6a,b); however, in the epithelium a higher percentage of oestrogen receptor-positive cells was observed in the isthmus ($P < 0.05$).

Discussion

This study shows that during postnatal development of the oviduct, the expression of oestrogen receptor α precedes the expression of hsp25 and hsp70 (the antibody used detected both cognate hsc70 and inducible hsp70). Oestrogen receptor α -positive cells were observed on day 4, but more clearly on day 7, whereas staining for hsp25 was absent or very weak on these days. Moreover, the expression of hsp25 was restricted to a few epithelial cell clusters in the ampullar region. The current study also revealed that hsp70 was expressed earlier and was more abundant than hsp25, and that it was also present in the ampulla and infundibulum. The isthmus region remained negative for hsp25 and hsp70 until day 10 of postnatal development. Therefore, although

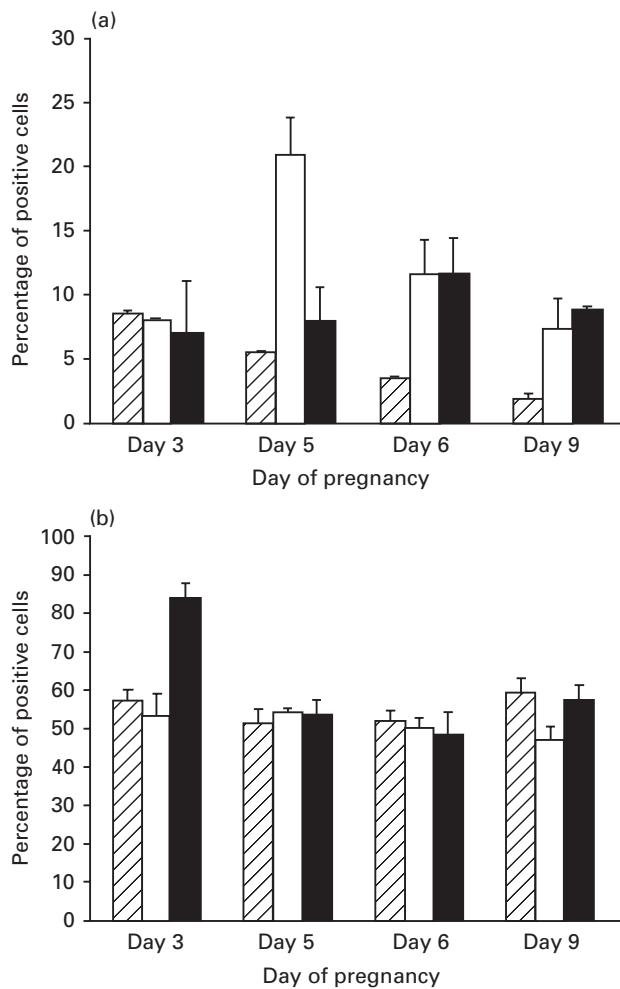


Fig. 5. Oviducts from early pregnant rats showing the relative percentage of epithelial cells with (a) hsp25 and (b) hsp70 immunostaining. ▨, infundibulum; □, ampulla; ■, isthmus. A significantly higher percentage of hsp25-positive cells was recorded in the ampulla on days 5, 6 and 9 of pregnancy ($P < 0.05$, compared with day 3). In contrast, the percentage of hsp70-positive cells remained constant; moreover, on day 3, a higher percentage of hsp70-positive cells was noted in the isthmus ($P < 0.05$).

hsp70 is involved in oestrogen receptor assembly and trafficking (Pratt and Scherrer, 1994; Liu and Stocco, 1997), findings from the present study indicate that during early development the receptor protein is not escorted by the hsp27 and hsp70 molecular chaperones. Li (1994) demonstrated that the proliferative activity in the developing mouse oviduct precedes the expression of oestrogen receptors. Results from the current study indicate that hsp25 and hsp70 are not necessary for cell proliferation in the developing oviduct. This is consistent with a study by Vargas-Roig *et al.* (1997) in which expression of hsp27 (the human counterpart of hsp25) was inversely correlated with cell proliferation. The current study demonstrates that the expression of the heat shock proteins is initiated when oviductal cells become differentiated.

In the adult oviduct a greater percentage of hsp70-positive cells was observed than of hsp25-positive cells. Moreover,

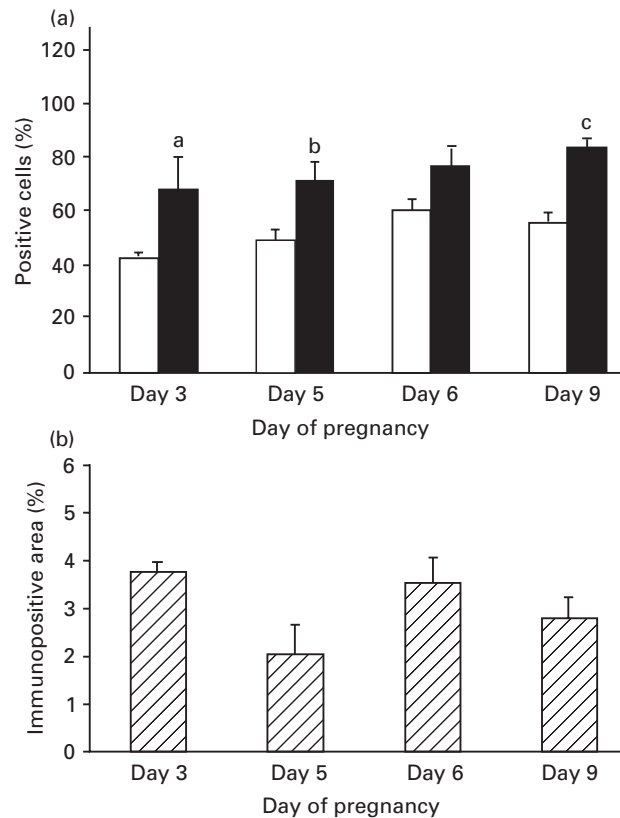


Fig. 6. Percentages of oestrogen receptor-positive cells during early pregnancy in rats in (a) the epithelium (□, ampulla; ■, isthmus) and (b) the stroma (▨). In the stroma, the percentage was evaluated per area of tissue quantitated by the image analyser.

the percentage of hsp25-immunoreactive cells did not change significantly during the oestrous cycle. In contrast, the percentage of hsp70-positive cells tended to decrease during oestrus and to increase during dioestrus, as was also observed for the percentage of oestrogen receptor-positive cells. Horvat *et al.* (1992) reported that the expression of proteins varied during the oestrous cycle as revealed by SDS-PAGE; these proteins were expressed at high concentrations during dioestrus. Thus, the results of the current study indicate that hsp70 (but not hsp25) is an oviductal protein that is modulated during the oestrous cycle: the number of hsp70-positive cells varied during the oestrous cycle. In addition, different regions of the adult oviduct showed different degrees of sensitivity, and hsp70 was more responsive to the hormonal changes in the infundibulum and ampullar regions.

During early pregnancy the percentage of hsp25-positive cells in the oviduct was low, which is in contrast to other studies in the rat endometrium during early pregnancy (Ciocca *et al.*, 1996). Ciocca *et al.* (1996) reported that, in the endometrium during early pregnancy, hsp25 was abundant in epithelial cells of the antimesometrial region, whereas in the present study in the oviduct on day 3 of pregnancy, hsp25 was observed in very few cells. This finding supports a specific role of hsp25 during blastocyst implantation in the

endometrium. In contrast, in the oviduct, a greater number of hsp70-positive cells were found during early pregnancy than in non-pregnant rats during the oestrous cycle. This finding indicates that during pregnancy, in the oviductal cells, hsp70 is modulated by oestrogens and progesterone and perhaps by other hormones that appear during pregnancy. During the oestrous cycle, there was a low concentration of hsp70 in the isthmus; however, during early pregnancy a high percentage of hsp70-positive cells was found in different functional segments of the oviduct (including the isthmus). During early pregnancy there are numerous hsp70-positive cells and few hsp25-positive cells in the isthmus in contrast to the situation in the endometrium (Ciocca *et al.*, 1996; M. L. Mariani, unpublished). It is speculated that hsp70 protects the oviduct from blastocyst implantation, whereas hsp25 facilitates implantation in the endometrium. However, the specific roles of these heat shock proteins in the oviduct and endometrium during early pregnancy remain to be elucidated.

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