Heat shock proteins in human endometrium throughout the menstrual cycle

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Human endometrium is a steroid-sensitive tissue and there is evidence that supports the viewpoint that heat shock proteins (HSP) are implicated in the regulation of steroid function. Therefore, in this study we examined the expression of various members of the heat shock family of proteins in the steroid-responsive human endometrium. Western blot analysis revealed that the expression of HSP90 showed minimal changes throughout the menstrual cycle. When normalized to the amount of HSP90, the expression of HSP27, HSP60 and the constitutive form of heat shock protein 70 (HSC70) increased progressively during the late proliferative and early secretory phases, and diminished in the mid- to late secretory and menstrual phases. In contrast, the inducible form of heat shock protein 70 (HSP70) did not undergo these changes. The cellular and subcellular localizations of these proteins were examined in human endometria by immunohistochemical staining. With the exception of HSP70, which was found primarily in the epithelial cells, the immunoreactivity for other heat shock proteins was found in both the stroma and the epithelium. Immunoreactivity for HSP27 was found in the lymphoid aggregates within endometrial stroma, and both HSP27 and HSP90 were found in endothelial cells. The immunoreactive heat shock proteins were found in the nuclei and/or cytoplasm of cells. However, no consistent nuclear versus cytoplasmic staining emerged, and such localization was irrespective of the site, the cell type or the phase of the menstrual cycle. Our findings show that endometrium has a full complement of heat shock proteins. The menstrual cycle-dependent changes in the amounts of heat shock protein suggest regulation by steroid hormones.

Key words: endometrium/heat shock protein/human/menstrual cycle/stress/uterus

Introduction

Human endometrium is a tissue that, in response to steroid hormones, undergoes a series of exquisitely controlled phases which characterize the menstrual cycle. These changes are induced by binding of the steroid hormones to steroid receptors and the subsequent regulation of transcription of the target genes in this tissue. Evidence exists which suggests that the function of the steroid hormone receptors is regulated by a group of constitutively synthesized heat shock proteins (HSP). These are expressed at relatively low levels under normal conditions but are inducible by a number of signals, including steroid hormones and cytokines (Jaattela and Wissing, 1992; Baniahmad and Ming-Jer, 1993). The HSP70 family is the most conserved group of proteins within the heat shock protein superfamily (Hunt and Morimoto, 1985). Other members of this family include the nucleolar HSP110 (Subjeck et al., 1983), the highly conserved HSP90 family (Bardwell and Craig, 1987), mitochondrial HSP60 (Jindal et al., 1989), collagen-binding HSP47 (Hirayoshi et al., 1991) and the heterogeneous family of small heat shock proteins with molecular weights ranging from 16 to 40 kDa (Lindquist and Craig, 1988). Of these, in humans only HSP27/HSP28 has been identified thus far (Arrigo and Welch, 1987; McGuire et al., 1989). The HSP60 family consists of abundant proteins that are constitutively synthesized and moderately stress-inducible. The HSP70 family comprises several proteins that are localized in distinct cellular compartments. Constitutively synthesized protein, designated HSC70 and found in the cytosol and nuclei, is moderately stress-inducible. On the other hand, under normal conditions, the most strictly inducible member of this family of proteins is not found in most species except primates in the cytosol and nuclei of cells. The HSP90 family of proteins is abundant and comprises constitutively synthesized cytosolic proteins which are only moderately stress-inducible. The term 'molecular chaperone' is applied to proteins which prevent incorrect interactions and which participate in assembly of the proteins without being part of the final protein structure (Ellis, 1987; Craig et al., 1994). Considerable evidence suggests that members of the heat shock protein family act as molecular chaperones, e.g. HSP60 and HSP70 participate in the folding and unfolding of cellular proteins (Jaattela and Wissing, 1992). HSP90 interacts with steroid receptors, tubulin, actin and several protein kinases, and prevents the aggregation of citrate synthase and casein kinase II in vitro. Non-liganded forms of the steroid hormone receptors (aporeceptor) exist as complexes associated with various members of the heat shock proteins, such as HSP90, HSP70 and HSP56 (p59), a 40 kDa cyclophilin-related protein and an uncharacterized 22 kDa protein species (Reinor et al., 1990; Bagchi et al., 1991; Baniahmad and Ming-ker, 1993; Ratajczak et al., 1993). Modulation of the important functions of these receptors, including the prevention of DNA binding
and optimization of the transcriptional activity, has been attributed to their interaction with HSP90.

In view of the important role of heat shock proteins in various biological responses, including those related to the steroid hormones, here we have examined the relative abundance of various members of the heat shock protein family in human endometrium throughout the menstrual cycle. In addition, the cellular and subcellular distributions of heat shock proteins were examined in normal human endometria.

Materials and methods

Reagents

Mouse monoclonal antibodies against human HSP27 (clone G3.1), HSP60 (clone LK-1), a mouse monoclonal antibody that recognizes both the constitutive (HSC70) and inducible (HSP70) forms of heat shock protein 70 (clone N27F3-4) and a rat monoclonal antibody to HSP90 (clone 16F1) were obtained from Stressgen Biotechnology Inc. (British Columbia, Canada). These antibodies have been characterized previously (Ciocca et al., 1982; Lai et al., 1984; Riabowol et al., 1988; Boog et al., 1992). The avidin and biotinylated peroxidase (Vectastain kit, PK 4002) and biotinylated horse anti-mouse and goat anti-rat antibodies were obtained from Vector Laboratories (Burlingame, CA, USA).

Processing of endometria

Normal endometria were obtained from patients aged 21–43 years. Hysterectomy specimens were removed for abnormalities other than endometrial in origin, including uterine leiomyomas and cervical or ovarian lesions (n = 21). These tissues were obtained in accordance with the rules and regulations of the institution and after approval of the institutional review board. Patients had a history of regular menstrual cycles and had not used an intrauterine device or taken any hormones for at least 6 months prior to hysterectomy. In addition, they did not have a history of abnormal bleedings. Each hysterectomy specimen was transferred immediately to the laboratory. Endometrial blocks (0.5 cm) fixed in 10% (v/v) buffered formalin and embedded in paraffin were used for dating the endometria using established criteria (Noyes et al., 1955). Identical blocks were placed in OCT embedding agent (Miles Laboratories, Naperville, IL, USA), snap frozen in a mixture of methylpropane and dry ice, and kept at −70°C prior to use. Fragments of endometrium were also snap frozen for the Western blot analysis, as described below. Several endometria from each phase of the menstrual cycle were used for immunostaining or Western blot analysis to confirm the reproducibility of the findings. The endometria were dated respectively to the early proliferative (n = 2), mid-proliferative (n = 3), late proliferative (n = 3), early secretory (n = 3), mid-secretory (n = 4), late secretory (n = 3) and menstrual (n = 3) phases. These endometria showed normal morphology.

Immunostaining procedure

Immunostaining was performed according to the avidin–biotin–peroxidase complex procedure (Hsu et al., 1981). Cryostat sections were fixed in formalin, incubated sequentially with 2% normal serum, primary antibody, appropriate biotinylated secondary antibody and avidin–biotin–peroxidase complex, and developed in a mixture of

Figure 1. Western blot analysis of heat shock proteins in human endometrium throughout the menstrual cycle. Endometrial proteins were extracted as indicated in the text. Equal amounts (30 μg) of protein were subjected to sodium dodecyl sulphate–polyacrylamide gel electrophoresis in a 12% gel. Equal loading was confirmed by Ponceau S staining of the blots, as described in the text. The demonstrated data were confirmed by using an additional panel of endometria, as indicated in the text. (A) Blot stained for HSP27 and HSC70/HSP70. (B) Blot stained for HSP60 and HSP90. The HSP27, HSC70, HSP70, HSP60 and HSP90 are shown by arrows. Other bands detected may be related to the presence of degradation products of these proteins or of proteins with similar immunoreactivities. (C) The relative optical densities of the immunoreactive bands were quantitated by laser scanning densitometry. Three independent measurements were made for each band, and the means of such measurements were calculated. The relative optical densities of the bands immunoreactive for HSP27, HSP60, HSC70 and HSP70 were normalized to the relative optical densities of the corresponding immunoreactive bands of HSP90 and expressed as the relative optical density ratio. The molecular weight markers are shown in kDa. MP = mid-proliferative; LP = late proliferative; POD = postovulatory day; M = menstrual.
Figure 2. Immunohistochemical localization of HSP27 in human endometrium. (A) Immunoreactivity for HSP27 is detectable in the cytoplasm as well as the nuclei of the glandular cells in a mid-proliferative endometrium. HSP27 is relatively less abundant in the cytoplasm of the cells in the stroma (original magnification ×260). (B) Immunoreactivity for HSP27 is intense in the endometrial glands, endothelial cells and cells within a lymphoid aggregate (large arrowhead) in a late proliferative endometrium (original magnification ×260). (C) Immunoreactivity for HSP27 is diminished in the endometrial gland (arrowheads) in a postovulatory day 2 endometrium. However, there is a relative increase in the immunoreactivity of HSP27 in the stroma. Intense staining of HSP27 is seen in the surface epithelial layer to the right of the figure (original magnification ×260). (D) The nuclear immunoreactivity for HSP27 is intense in the glandular cells in this menstrual endometrium. The surrounding stroma shows little staining (original magnification ×260). No counterstain.

3,3′-diaminobenzidine tetrahydrochloride (DAB)–H₂O₂. Controls consisted of the substitution of primary antibody with phosphate-buffered saline (0.1 mol/l, pH 7.4) or isotype-specific irrelevant antibody at the same protein concentration as the primary antibody.

Western blotting
Sodium dodecyl sulphate (SDS)-solubilized lysates of endometria were prepared. The amount of protein in these lysates was quantitated by a method modified from that described by Lowry (Bensadoun and Weinstein, 1976; Peterson, 1977). Lysates were boiled for 5 min and subjected to SDS–polyacrylamide gel electrophoresis (PAGE) in a 12% gel. Thirty micrograms of protein was loaded into each lane. Separated proteins were transferred to nitrocellulose membranes. Equal loading in each lane was confirmed by staining the blots with Ponceau S. The staining was performed with a solution of 0.2% Ponceau S for 1 min. Prior to immunostaining, the blots were washed twice in distilled water (5 min) and then twice with TBST (20 mmol/l Tris, pH 7.6, 0.9% NaCl, 0.1% Tween 20; 10 min). Blots were pre-blocked in Blotto [1 mmol/l glycine, 5% (w/v) non-fat milk powder, 1% ovalbumin and 5% bovine calf serum] and washed twice in TBST for 5 min each at 25°C. Blots were incubated sequentially for 2 h with TBST containing 2% milk powder and primary antibody, followed by a 1 h incubation with secondary antibody and then a 1 h incubation with avidin–biotin complex. Each incubation was followed by two washes in TBST. Blots were developed in a mixture of DAB–H₂O₂. The relative optical density of the immunoreactive bands was obtained by laser scanning densitometry using SigmaGel analysis software (Jandel Scientific Software, San Rafael, CA, USA).

Results
Analysis of the expression of heat shock proteins by Western blotting during the menstrual cycle
To see whether the relative amounts of protein from the heat shock protein family change during the menstrual cycle, the endometrium-derived proteins were subjected to a Western blot analysis. Equal loading of each lane was confirmed by Ponceau S staining. A distinct and prominent band migrating at ~27 kDa characterized endometrial HSP27 (Figure 1A). However, in addition to a prominent band with mobility at ~60 kDa, some other bands were detected when the blots were probed with monoclonal antibody to HSP60 (Figure 1B). Based on the immunoreactivity with the monoclonal antibody to heat shock proteins 70, two bands were detected. The faster-migrating heat-inducible form of heat shock protein 70, also called HSP68, HSP70 or HSP72 (Gunther and Walter, 1994), is referred to here as HSP70. This band could be distinguished easily from the slower-migrating constitutive form of this protein, which is also called HSC70, HSP72 or HSP73 (Gunther and Walter, 1994) and is referred to here as HSC70 (Figure 1A). Endometrial HSP90 consisted of a single and
well-defined band at ~90 kDa (Figure 1B). Bands identified as heat shock proteins, based on their immunoreactivity with the primary antibodies and their expected molecular weights, were not identified in a Western blot analysis when the primary antibodies were omitted (data not shown).

The relative amount of HSP90, as determined by laser scanning densitometry, was consistent throughout the menstrual cycle and was reduced minimally during the late secretory and menstrual phases (Figure 1B). Therefore, the relative optical densities of the immunoreactive bands of other members of the heat shock protein family were normalized to the relative optical densities of the bands immunoreactive for HSP90. Once normalized, the amount of HSP27 and HSP60 exhibited a sharp increase during the late proliferative/early secretory phase of the menstrual cycle (Figure 1C). A smaller peak of increase was detectable only for HSC70 and not the inducible form of this heat shock protein (Figure 1C). This sharp increase diminished progressively during the mid- to late secretory and menstrual phases, reaching values obtained during the early to mid-proliferative phases (Figure 1C).

Cellular, subcellular and phase-specific distribution of heat shock proteins in human endometria

To localize the cellular and subcellular sites of the heat shock proteins, endometrial sections were immunostained using monoclonal antibodies to heat shock proteins. The immunoreactivity for HSP27 was found in various cells, including the epithelial, stromal, endothelial and lymphoid cells in human endometrium (Figure 2A–D). However, during the proliferative phase the immunoreactivity for this protein was by far greater in the endometrial epithelial cells compared with other cell types (Figure 2A and B). HSP27 was localized to both the nuclei as well as the cytoplasm of endometrial cells (Figure 2A–D). As early as on day 2 of the secretory phase, the immunoreactivity of this protein in endometrial epithelium was diminished whereas the immunoreactivity in the stromal cells was increased (Figure 2C). The immunoreactivity of this protein in the endometrial epithelium increased in the late secretory phase and persisted during the menstrual phase (Figure 2D).

The immunoreactivity for HSP60 was confined primarily to the endometrial epithelium during the entire menstrual cycle (Figure 3A). Some of the immunoreactivity was seen in the cytoplasm of cells in the form of small aggregates, which is presumably caused by the localization of this protein in the mitochondria (Figure 3A). Except for a decrease in the intensity of staining for HSP60 in the endometrial glands during the menstrual phase, there was no detectable change in the immunoreactivity of HSP60 during the menstrual cycle (Figure 3B).

The immunoreactivity of heat shock proteins 70 was found primarily in the endometrial epithelium throughout the entire menstrual cycle (Figure 4A–D). Cytoplasmic staining was seen and a small number of cells exhibited nuclear staining (Figure 4A–D). Both glandular (Figure 4A–C) and surface epithelia showed staining. Stromal cells, lymphoid cells in the stroma, and endothelial cells failed to exhibit detectable immunoreactivity for heat shock protein 70 (Figure 4A–D). With the exception of a decrease during the menstrual phase, the expression of HSP70 in human endometrium did not undergo changes detectable by immunohistochemical staining throughout the menstrual cycle.

HSP90 was found in both the stromal and the epithelial cells of human endometrium throughout the menstrual cycle (Figure 5A–D). During the entire menstrual cycle, in the endometrial epithelium as well as the surrounding stroma, the nuclear staining was more pronounced than the cytoplasmic staining (Figure 5A–D). Nuclear staining was also detectable in the endothelial cells of particularly large vessels (Figure 5B). Not all the epithelial or stromal cells exhibited nuclear staining — some cells had a weak to undetectable level of immunoreactive HSP90 (Figure 5A–D). The immunohistochemical staining failed to reveal significant changes in the immunoreactivity of HSP90 throughout the menstrual cycle.

Omission of the primary antibodies and their substitution with phosphate-buffered saline or isotype-specific irrelevant antibody at the same protein concentration as the primary antibodies failed to reveal any specific staining in any of the sections. In these sections, the reaction product was confined to those cells with peroxidase or pseudoperoxidase activity.
Figure 4. Immunohistochemical localization of heat shock proteins 70 in human endometrium. (A) The immunoreactivity for heat shock proteins 70 is confined primarily to the endometrial glands in this mid-proliferative phase endometrium. Little, if any, immunoreactivity is observed in the stroma (original magnification ×260). (B) Some cells in a late proliferative phase endometrium exhibit nuclear staining (arrowheads), whereas the reaction in the majority of cells is cytoplasmic (original magnification ×260). (C) The immunoreactive HSP70 is found only in the endometrial gland in this mid-secretory phase endometrium (original magnification ×260). (D) The endometrial glands are the primary site of immunoreactive heat shock proteins 70 in this menstrual phase endometrium (original magnification ×260). No counterstain.

Discussion

In this report we demonstrated that HSP27, HSP60, HSC70, HSP70 and HSP90 were expressed in normal human endometria. These findings show that human endometrium has the full complement of proteins essential for the actions of oestrogen and progesterone receptors. During the early secretory phase, once normalized to the amount of HSP90, Western blot analysis showed a progressive increase in the relative abundance of HSP27, HSP60 and HSC70 but not HSP70 or HSP90. Whether this change in the levels of expression of the heat shock proteins is related to changes in the systemic concentrations of steroid hormones or to the impact of other endometrial signals remains to be determined. The rise in the relative abundance of HSP27 in the late proliferative and early secretory phases, as judged by Western blot analysis, was associated with reduced staining of the epithelium and a coincident rise in the staining of the endometrial stroma for HSP27. However, by and large, immunohistochemical localization failed to reveal significant changes in the expression of the heat shock proteins throughout the proliferative and secretory phases. Nevertheless, consistent with a previous report (Padwick et al., 1994), the reduced expression of HSP27 in endometrial glands was observed, in particular during the menstrual phase, presumably because of a loss of protein from the stroma. A similar reduced expression in the staining intensity of endometrial glands for HSP60 was observed during the menstrual phase.

The expression of HSP90, HSP70 and HSP27 has been reported previously in human endometria (Nip et al., 1994; Koshiyama et al., 1995). As reported here, Koshiyama et al. (1995) reported that both HSP70 and HSP90 were expressed in human endometrium throughout the menstrual cycle. The expression of HSP70 was also found to be stronger in the epithelium than the stroma, whereas HSP90 was present in both the epithelium and stroma. However, in that report there is no indication of the intracellular distribution of these proteins. In addition, although the expression of HSP70 was found in the basalis, it was reported to be weak in the functionalis during the proliferative phase. The expression of HSP90 was also found to be weak during the secretory phase (Koshiyama et al., 1995). The underlying basis for these differences remains to be determined but may be related to differences in the processing of the tissues, such as the use of a weak fixative, 1.0% paraformaldehyde, and subsequent treatment of the tissue sections with 0.3% hydrogen peroxide (Koshiyama et al., 1995). In the study by Nip et al. (1994) the two forms of the heat shock protein could not be resolved.
Figure 5. Immunohistochemical localization of HSP90 in human endometrium. (A) The immunoreactivity for HSP90 is found in both the endometrial glands and the stroma in this early proliferative phase endometrium. The staining is localized primarily to the nuclei of cells (original magnification ×260). (B) A nuclear immunoreactivity for HSP90 is seen in the endothelial cells (arrowheads) in this mid-proliferative phase endometrium (original magnification ×260). (C) Immunoreactivity for HSP90 is seen in the nuclei of the endometrial glands in a late secretory phase endometrium (original magnification ×260). (D) Endometrial glands have retained the nuclear immunoreactivity for HSP90 in this menstrual phase endometrium. Immunoreactivity for HSP90 is relatively low in the stroma (original magnification ×260). No counterstain.

by Western blot analysis. In our report, the two forms of heat shock protein were resolved and the progressive increase in the relative amount of HSP70 was more pronounced for the constitutive rather than the inducible form of this protein. The subject population in the study by Nip et al. (1994) consisted of women who were either infertile or had haemorrhaged, which did not allow for an evaluation of the heat shock proteins in normal menstrual cycles. However, based on the Western blot analysis, it was suggested that the relative amount of HSP70 was increased in the infertile group compared with those with haemorrhage (Nip et al., 1994).

The function of the heat shock proteins in human endometrium, particularly those of the heat shock protein 70 family, may be to limit the extent of cytotoxic damage by cytokines or apoptosis. Leukocytes produce high levels of reactive oxygen species as well as cytokines, both with the ability to regulate the expression of heat shock proteins (Jacquire-Sarlin et al., 1994). Because leukocytes accumulate progressively in human endometrium during the secretory phase (Tabibzadeh et al., 1986; Kamat and Isaacson, 1987; Bulmer et al., 1991), and the amount of endometrial tumour necrosis factor (TNF-α) increases progressively during the secretory/menstrual phase (Tabibzadeh et al., 1994, 1995), the function of heat shock proteins in human endometrium may be to protect cells from the side-effects of leukocyte accumulation or cytokine release. The presence of cytokines such as interleukin (IL)-1 and TNF-α leads to the activation of phospholipase A₂, the generation of lipid mediators of inflammation and a rapid rise in the concentration of mitochondrial reactive oxygen radicals (Jacquire-Sarlin et al., 1994). On the other hand, heat shock proteins intervene with the DNA strand breaks and lipid peroxidation imposed by the reactive oxygen species, and protect mitochondrial structure and function (Jacquire-Sarlin et al., 1994). It has been demonstrated that cells transfected with HSP70 are protected from cytotoxic damage by TNF-α (Jaattela, 1993). Furthermore, the overexpression of HSP70 and not HSP27, or the inhibition of endogenous HSP70 synthesis by the expression of antisense HSP70 RNA, did not change the ability of the cells to bind TNF or to internalize and degrade the receptor-bound ligand. The TNF-α-induced activation of NF-κ B-like transcription factors was also unaffected by altered concentrations of HSP70, suggesting that resistance to TNF-α, conferred on the cells by the overexpression of HSP70, was not mediated by changes in either the TNF receptors or the regulation of transcription of genes whose expression is regulated by NF-κ B-like transcription factors. Protection against TNF-α seems to correlate best with the activation of arachidonic acid metabolism (Jaattela, 1993). Based on these studies, it has been proposed that resistance against TNF-α endowed by HSP70 is mediated by the reduced activation of phospholipase A₂. In addition, some studies show that heat shock or chemical stress, which induce a heat shock
protein response, concomitantly inhibits IL-1β and TNF-α production at the transcriptional level (Hall, 1994). When mouse peritoneal macrophages stimulated with lipopolysaccharide (LPS) were heated at 45°C for 12 min, a reciprocal increase in the expression of HSP70 and a decrease in the production of IL-1 and TNF-α were observed (Snyder et al., 1992). In line with these findings, heat shock prevented LPS-induced TNF-α synthesis by rat mononuclear phagocytes (Fouqueray et al., 1992). The inference from these and similar studies is that the role of HSP70 may be to oppose the action of TNF-α in human endometrium and to limit the extent of cytotoxic damage by this cytokine. The number of apoptotic cells is low during the proliferative phase in endometrium. However, the number of these cells increases progressively, particularly in the endometrial epithelium, reaching a peak during the menstrual phase (Tabibzadeh et al., 1994). Because HSP70 can protect cells from apoptosis (Wei et al., 1994), one function of HSC70/HSP70 may be to inhibit excessive apoptosis in human endometrium.

In conclusion, we have identified that the major members of the heat shock protein family are expressed in human endometrium. The expression of several members of the heat shock protein family increases in the secretory phase. Heat shock proteins are expressed in both the stroma and the endometrial epithelial compartments, and are found in the cytoplasm or nuclei of cells. The heat shock proteins may be part of the repertoire which regulates steroid-mediated responses in human endometrium and affords protection against signals with cytotoxic and apoptosis-inducing effects in this tissue.

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