Molecular Human Reproduction vol 2 no.8 pp 549–554, 1996

Cytokine induction of heat shock protein in human granulosa–luteal cells

Alexis H.Kim[1], Amit Khanna, Raymond F.Aten, David L.Olive and Harold R.Behrman

Sections of Reproductive Endocrinology and Infertility and Reproductive Biology, Departments of Obstetrics and Gynecology, Biology and Pharmacology, Yale University School of Medicine, 333 Cedar Street, New Haven, CT 06520, USA

[1]To whom correspondence should be addressed at: 540 University Avenue, Suite 200, Palo Alto, CA 94301, USA

The infiltration of leukocytes is a characteristic feature of luteolysis in humans. Leukocytes are known to generate physiological inducers of cell stress such as cytokines which have been implicated as mediators of functional luteal regression. In cells exposed to stress, a response characterized by an increase in heat shock protein (HSP) synthesis occurs. Recently, the induction of HSP-70 in rat luteal cells has been shown to inhibit luteinizing hormone (LH) and cAMP-sensitive progesterone production, possibly by interfering with the translocation of cholesterol to the mitochondrial cytochrome $P450_{scc}$. We therefore investigated whether HSP-70 is induced in human granulosa–luteal cells and its relationship to steroidogenesis. [^{35}S]Methionine labelling showed an increase in a 70 kDa protein after heat treatment which was demonstrated to be HSP-70 by Western analysis using monoclonal antibodies against the constitutive and inducible forms of HSP-70. Induction of HSP-70 in human granulosa–luteal cells was also seen with interferon (IFN)γ (10 ng/ml), tumour necrosis factor (TNF)-α (100 ng/ml) and a combination of IFNγ/TNF-α (10/50 ng/ml). Interleukin-1β (IL-1β) (30 ng/ml) showed minimal induction of HSP-70 above control values. An increase in activated heat shock factor, which binds to the heat shock transcriptional control element, was detected after heat shock, IFN/TNF, and IFN treatment. Coincident with the induction of HSP-70 by heat shock was the inhibition of progesterone production compared with non-shocked granulosa–luteal cells. Heat shock inhibition of progesterone synthesis was partially reversed by the cell- and mitochondria-permeant cholesterol analogue, 22R-hydroxycholesterol. Cell viability was unaffected by heat treatment. White blood cell-depleted granulosa–luteal cell cultures treated with IFN demonstrated a significant reduction in progesterone production. Treatment with IFN/TNF, TNF, and IL-1 also decreased progesterone secretion, although statistical significance was not achieved. These findings provide evidence that a stress response occurs in human granulosa–luteal cells in response to heat and cytokines. The inhibition of gonadotrophin-sensitive steroidogenesis coincident with the induction of HSP-70 synthesis by physiological agents which are present in the corpus luteum implicates HSP-70 as a potential mediator of luteolysis in the human.

Key words: cytokines/granulosa–luteal cells/heat shock protein/luteal regression/progesterone

Introduction

In organisms exposed to stress, responses occur which activate protective mechanisms to ensure cell survival. One such stress response is an inducible genomic response resulting in the production of heat shock proteins (HSP) (Lindquist and Craig, 1988). Once activated, heat shock transcription factor (HSF) binds to heat shock elements (HSE) located in the promoter region of heat shock genes (Morimoto, 1993), initiating transcription of an intron-less mRNA with subsequent HSP synthesis. In some instances, however, activation of HSF does not result in HSP synthesis (Jurivich *et al.*, 1992) and, rarely, HSP production occurs without HSF activation (Simon *et al.*, 1988). Although these proteins are referred to as 'heat shock', a variety of conditions in addition to heat stress induce their synthesis such as environmental stresses, pathophysiological, disease, and inflammatory states, and normal cell growth and differentiation (Morimoto *et al.*, 1992). Among the key constitutive functions attributed to HSPs are binding to nascent polypeptides in order to stabilize an unfolded conformation, the transport of these proteins to appropriate organelles within the cell, and assisting in the completion of folding to the final tertiary structure (Chirico *et al.*, 1988; Beckmann *et al.*, 1990). After a cellular insult, the protective effect of HSPs against irreversible damage probably results from the binding of denatured and misfolded proteins, thus enabling HSPs to unfold and correctly refold abnormal polypeptide structures or possibly target them for lysosomal degradation (Pelham, 1986; Chiang *et al.*, 1989).

Recently, in the rat, the induction of HSP-70 by heat shock was shown to exhibit a specific inhibition of luteinizing hormone (LH) and cAMP-sensitive progesterone production (Khanna *et al.*, 1994). Although the exact mechanism of steroidogenesis inhibition is unknown, HSP-70 appears to interfere with cholesterol translocation into the mitochondria. This is supported by the observations that an increase in HSP-70 production did not affect LH-stimulated cAMP concentra-

© European Society for Human Reproduction and Embryology

tions and the inhibition of steroidogenesis was fully reversed by 22R-hydroxycholesterol, a cell- and mitochondria-permeant analogue of cholesterol. Moreover, blocking HSP-70 synthesis with the transcription inhibitor actinomycin-D (Khanna *et al.*, 1994) and with an antisense oligodeoxynucleotide (Khanna *et al.*, 1995a) partially reverses the inhibition of progesterone synthesis. Subsequently, physiological agents known to inhibit LH-dependent steroidogenesis such as prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) and tumour necrosis factor-α (TNF-α) were shown to induce HSP-70 production in rat luteal cells with simultaneous inhibition of progesterone synthesis (Khanna *et al.*, 1995b). In the ewe, $PGF_{2\alpha}$-induced luteal regression was also associated with accumulation of HSP-70 in luteal cells (McPherson *et al.*, 1993).

A characteristic feature of corpus luteum regression in the human is the infiltration of leukocytes (Adams and Hertig, 1969). Cytokines such as TNF-α are generated by leukocytes and can function as physiological inducers of cell stress (Khanna *et al.*, 1995b). Cytokines have also been shown to reduce progesterone synthesis (Fukuoka *et al.*, 1992; Wang *et al.*, 1992; Best *et al.*, 1994), thus suggesting a permissive role in functional luteal regression in humans. The heat shock response has not been examined in the human ovary with respect to its role in potentially modulating steroidogenesis. Since luteal regression exhibits elements of a stress response and data from rat studies implicate HSP-70 as a mediator of luteolysis, we assessed whether cytokines are able to induce HSP-70 in human granulosa–luteal cells and whether HSP induction might influence steroidogenesis.

Materials and methods

Hormones, drugs, and reagents

22R-hydroxycholesterol (22-OH cholesterol) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Interferon (IFN)-γ was purchased from Becton Dickinson (Bedford, MA, USA). Interleukin (IL)-1β and TNF-α were purchased from R&D Systems (Minneapolis, MN, USA). Percoll was purchased from Sigma Chemical Co. Penicillin and streptomycin were purchased from Gibco-BRL (Grand Island, NY, USA). L-[^{35}S]Methionine and [^{32}P] ATP for labelling were purchased from Amersham (Arlington Heights, IL, USA). The protein HSC-70 (constitutive HSP-70) and the antibody against the constitutive and inducible forms of HSP-70 (HSP-73 and HSP-72, respectively) were obtained from StressGen Biotechnology Corp. (Sidney, Canada). Anti-CD45 magnetic immunobeads were obtained from Immunotech Inc. (Westbrook, ME, USA).

Isolation of human granulosa–luteal cells

Human granulosa–luteal cells were obtained from infertility patients attending an in-vitro fertilization (IVF) programme. Ovarian hyperstimulation was performed by using a gonadotrophin-releasing hormone (GnRH) analogue (Lupron; TAP Pharmaceuticals, Deerfield, IL, USA) and purified follicle stimulating hormone (FSH, Metrodin; Serono Laboratories, Norwell, MA, USA) or human menopausal gonadotrophin (Pergonal; Serono Laboratories) with follicular aspiration occurring 36 h after the administration of human chorionic gonadotrophin (HCG) (10 000 IU, i.m.). After removal of the cumulus–oocyte complex, the granulosa–luteal cells from all follicles of each patient were combined and washed twice with minimum essential medium (MEM 320–1380, Gibco). The suspension of granulosa–luteal cells was filtered through a 150 μm diameter nylon mesh (Tetko Inc., Elmsford, NY, USA), layered over an equal volume of 45% Percoll, and centrifuged at 200 *g* for 30 min to pellet the red blood cells. The granulosa–luteal cells collected at the interface were washed once with MEM. In a few experiments, cells from two different patients were pooled to obtain a sufficient number of cells for Western analysis.

The granulosa–luteal cells were further processed in order to remove contaminating white blood cells using anti-CD45 magnetic immunobeads, as previously described (Best *et al.*, 1994). This technique was reported to reduce the total population of white blood cells to 4% and the population of macrophages to <1%. Cell count was performed with a haemacytometer with assessment of viability (75–85%) by Trypan Blue dye exclusion.

Functional studies

In all heat shock experiments, granulosa–luteal cells were suspended in 3.0 ml McCoy's 5A medium (Gibco) supplemented with 0.1% bovine serum albumin (BSA) in the presence of 5% carbon dioxide and 95% air and allowed to incubate for 3 h (37°C) in order to minimize any stress effects from the isolation procedure. After the recovery period, half of the cells were stressed for 10 min at 42°C. The non-shocked and heat-shocked cells were then allowed to incubate for another 2 h prior to treatment with 10 μg/ml 22-OH cholesterol or 10 IU/ml HCG. After 1 h incubation, cell count for viability was performed. The media were heat-treated (90°C, 10 min) and stored (−20°C) for later analysis of progesterone concentrations.

In all cytokine experiments, granulosa–luteal cells were incubated (37°C) in 2.5 ml McCoy's 5A medium supplemented with 0.1% BSA, 100 IU/ml penicillin, and 100 μg/ml streptomycin. After a 3 h recovery period, this medium was replaced with an equal aliquot of fresh medium. The granulosa–luteal cells were then divided into equal volumes for treatment for 24 h (37°C) with IFN/TNF (10 ng/ml; 50 ng/ml), IFN (10 ng/ml), TNF (100 ng/ml), or IL-1β (30 ng/ml). The media were heat-treated and stored.

Hormone assays

Progesterone concentrations in the media were determined by radioimmunoassay (RIA) as previously described (Behrman *et al.*, 1980).

Radioactive labelling of cells

After a 3 h post-isolation recovery period, the granulosa–luteal cells were heat-treated (42°C, 10 min) and then allowed to incubate (37°C) for an additional 3 h. They were then labelled for 1 h with 20 μCi/ml L-[^{35}S]methionine in McCoy's 5A medium. Cells pelleted by centrifugation were lysed with 2.3% sodium dodecyl sulphate (SDS) and subsequently analysed by SDS–polyacrylamide gel electrophoresis (PAGE) under reducing conditions. The cell lysates were mixed with an SDS-loading buffer and heated, prior to loading on a 4% stacking and 10% running gel. Electrophoresis was performed for 2 h at 100 V using a standard running buffer. Preparation of the gel for autoradiography was accomplished with 2,5-diphenyloxazole (PPO) and dimethyl sulphoxide (DMSO) as previously described (Bonner and Laskey, 1974).

Western analysis

After treatment with either heat shock, IFN/TNF (10 ng/ml, 50 ng/ml), IFN (10 ng/ml), TNF (50 ng/ml), or IL-1β (30 ng/ml), cells were allowed to incubate for 6 h at 37°C and were then collected by centrifugation (300 *g*; 10 min). Cell pellets were stored at −20°C. Thawed cells were homogenized on ice in a solution containing 20 mM HEPES (pH 7.5), 400 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 20% glycerol, 0.2 mM dithiothreitol (DTT), 0.5 mM phenyl-

methylsulphonylfluoride (PMSF), and 0.5 mM leupeptin Homogenates were centrifuged at 140 000 *g* (4°C, 30 min) to pellet cellular debris. Total cellular protein concentration in the supernatant was determined by the Bradford method (Bradford, 1976), using the Bio-Rad Protein Assay reagent (Bio-Rad Laboratories, Hercules, CA, USA). For each SDS–PAGE gel (4% stacking, 10% running), the same amount of total protein (60 μg or 90 μg) was mixed with a denaturing loading buffer (SDS, bromphenol blue, mercapto-ethanol) and applied to each lane. Fractionation of protein was performed by electrophoresis (100 V, 90 min) using a standard running buffer. Protein transfer to a nitrocellulose membrane was accomplished overnight at 50 mA (4°C) using a buffer (pH 11.0) containing 10 mM 3-[cyclohexylamino]-1-propanesulphonic acid (C-APS) and 10% (v/v) methanol. The membrane was then blocked for 2 h with a blocking buffer supplied by the Rad-Free system (Schleicher and Schuell, Keene, NH, USA). Subsequently, the membrane was incubated with a primary antibody against HSP-72 and 73 (1:1333 dilution) for 1 h, washed, and incubated with an alkaline phosphatase-secondary antibody conjugate (Rad-Free system) for 30 min. After a second wash, the membrane was placed on a chemiluminescent sheet (Rad-Free system) and exposed to Kodak XAR film (Eastman Kodak Co., Rochester, NY, USA) at 37°C.

HSE binding assay

After treatment with either heat shock, IFN/TNF, IFN, or TNF, granulosa–luteal cells were allowed to incubate for 1 h at 37°C and were collected by centrifugation at 300 *g* for 10 min. Isolation of total cellular protein and determination of protein concentration was performed as described above. Total protein (20 μg) was incubated (30 min, 25°C) with 20 000 c.p.m. [^{32}P]ATP end-labelled double-stranded HSE (Goldenberg *et al.*, 1988) oligonucleotide probe and 2 μg poly (dI-dC) in a binding buffer [12 mM HEPES (pH 7.9), 12% glycerol, 60 mM KCl, 2 mM $MgCl_2$, 0.12 mM EDTA, 0.3 mM DTT, and 0.3 mM PMSF] (Baler *et al.*, 1992) whose volume was adjusted according to the final volume of the reaction mixture. The mixture was then fractionated on a non-denaturing 4.5% polyacrylamide gel using TGE buffer (40 mM Tris, 270 mM glycine, and 2 mM EDTA, pH 8.0). A 200-fold molar excess of unlabelled probe was incubated with the protein mixture for 15 min prior to the addition of the labelled probe in order to determine non-specific binding.

Statistical anlysis

Each treatment was repeated at least three times in separate experiments. For the functional studies, each treatment was replicated three times within an experiment. Statistical significance ($P < 0.05$) was evaluated using a randomized block analysis of variance or two-way analysis of variance followed by the Bonferroni procedure for multiple pairwise comparisons.

Results

HSP-70 is induced by heat shock, IFN/TNF, IFN, and TNF

The [^{35}S]methionine pulse-labelled protein pattern revealed the synthesis of a 70-kDa protein in the cells treated by heat shock (Figure 1). In order to determine whether this 70-kDa protein was a member of the HSP family, Western analysis was performed using an antibody specific for both the inducible and constitutive forms of HSP-70. Figure 2 shows a representative Western blot in which HSP-70 is identified and induced in granulosa–luteal cells treated by heat shock with a small amount of HSP-70 detected in the control cells. Granulosa–luteal cells treated with physiological inducers of cell stress which have been previously demonstrated to impair progesterone synthesis also demonstrated HSP-70 induction after 6 h (Figure 3). Cytokine treatment of cells with the combination of IFN/TNF as well as IFN and TNF alone increased HSP-70 values above control (Figure 4). Treatment with IL-1β, however, did not appreciably increase HSP-70 levels. Densitometry

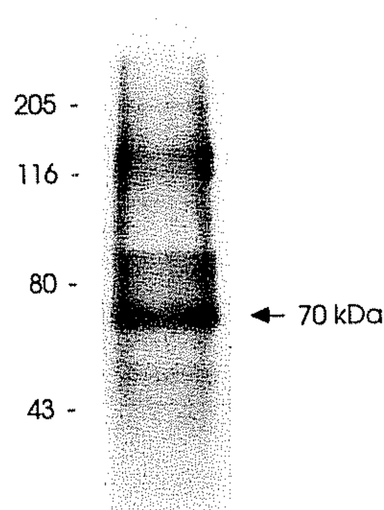

Figure 1. Detection of a 70 kDa protein in human granulosa–luteal cells. After a 3 h post-isolation recovery period, cells were heat shocked for 10 min at 42°C. Cells were labelled with [^{35}S]methionine following a 3 h post-treatment period and the cell lysates analysed by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE).

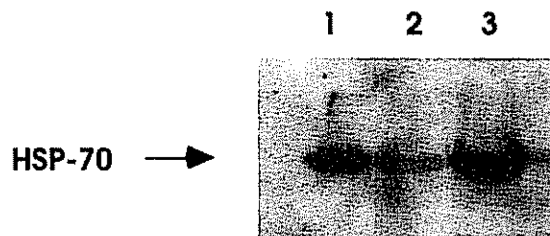

Figure 2. Induction of heat shock protein-70 (HSP-70) by heat shock. A representative Western analysis of protein extracts from human granulosa–luteal cells using a primary antibody specific for the constitutive and inducible forms of HSP-70. Lane 1, 1 μg constitutive HSP-70 (HSC-70); lane 2, control; lane 3, heat shock (42°C, 10 min).

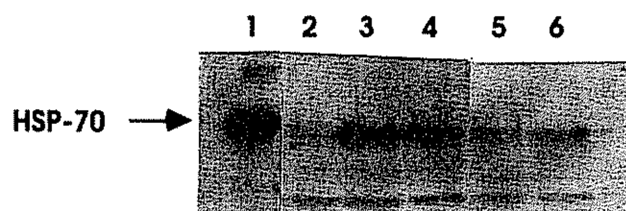

Figure 3. Induction of heat shock protein-70 (HSP-70) by cytokines. A representative Western analysis of protein extracts from human granulosa–luteal cells treated with interferon (IFN)γ/tumour necrosis factor (TNF)-α (lane 3), IFNγ (lane 4), TNF-α (lane 5) or interleukin (IL)-1β (lane 6). Lane 1 represents a positive control (1 μg constitutive HSC-70) and lane 2 represents untreated cells.

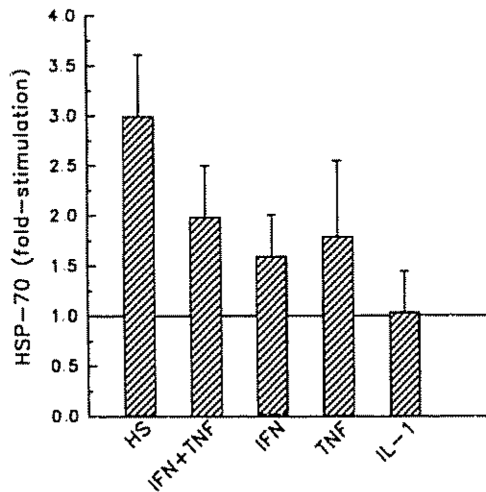

Figure 4. Effect of cytokines on heat shock protein-70 (HSP-70) induction. Autoradiographs of Western blots were quantified by densitometry. The densitometry results (integrated optical density) for each treatment were normalized to the control value and expressed as fold-stimulation above control values (1.0 after normalization). Represented above are the mean ± SEM of at least three separate experiments

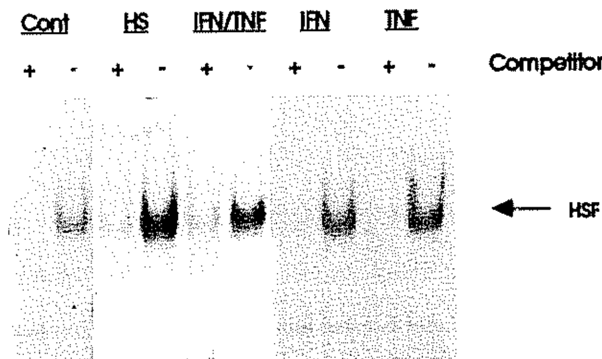

Figure 5. Activation of heat shock transcription factor (HSF) binding by heat shock or cytokines. Granulosa–luteal cells were treated with heat shock, interferon (IFN)γ/tumour necrosis factor (TNF)-α, IFNγ, or TNF-α. After 1 h, protein was extracted from the cells and incubated with a labelled heat shock element (HSE) oligonucleotide probe to determine HSF binding. The specificity of binding was determined by preincubating the protein extracts with a 200-fold molar excess of unlabelled competitor HSE oligonucleotide (+ competitor).

readings (integrated optical density), shown in Figure 4, represent the mean values of at least three separate experiments, expressed as fold-stimulation above control values.

HSP-70 induction occurs through the activation of HSF

After treatment of granulosa–luteal cells with either heat shock or the cytokines which induced HSP-70 synthesis, HSF activation was assessed 1 h later by determining the presence or absence of a labelled high molecular weight complex formed by binding to HSE. HSF activation was observed in all the treatment groups (heat shock, IFN/TNF, IFN, and TNF) and, to a lesser extent, in the control group (Figure 5). Specificity of the activated DNA-binding ability of HSF was demonstrated

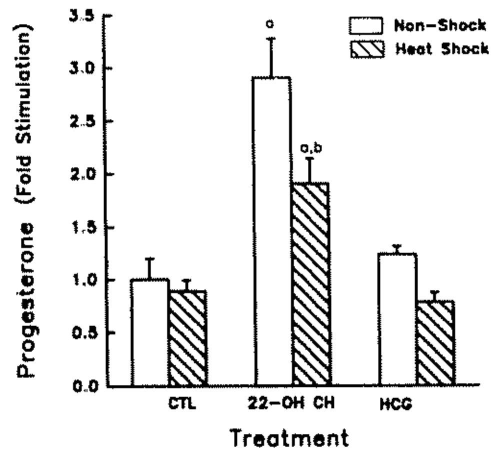

Figure 6. Effect of heat shock on progesterone production in human granulosa–luteal cells. For the heat shock group, cells were allowed to recover for 2 h after heat treatment and then incubated with 22-OH cholesterol (22-OH CH) or human chorionic gonadotrophin (HCG) for 1 h. Data represented are the mean ± SEM of progesterone concentrations normalized to the control, non-shocked group (CTL) (39.6 ± 9.1 ng/10^5 cells) of at least three separate experiments each performed in triplicate. a, $P < 0.05$ vs respective control; b, $P < 0.05$ within a treatment group

by the disappearance of the high molecular weight complex due to competition from unlabelled HSE oligonucleotides.

Effect of heat shock or cytokines on progesterone synthesis

The heat shock paradigm used previously to induce HSP-70 production also interferes with the ability of granulosa–luteal cells to produce progesterone. Cell count and viability pre- and post-heat shock was unchanged, indicating the unlikelihood of non-specific hyperthermic cell death as a cause of decreased progesterone production. In Figure 6, comparison of progesterone production between all of the heat-shocked versus non-shocked cells revealed a significant increase in progesterone output in the non-shocked group ($P < 0.05$). Basal progesterone production (mean ± SEM; ng/10^5 cells) was 39.6 ± 9.1. HCG increased progesterone secretion by 1.3-fold (NS) with complete inhibition of HCG stimulation by heat shock treatment. Addition of 22-OH cholesterol partially reversed the effects of heat treatment ($P < 0.01$), but the level of stimulation was significantly less than non-shocked cells (1.9-fold and 2.8-fold above control respectively; $P < 0.05$). Basal production of progesterone was essentially unchanged by heat shock (0.9-fold; NS).

Figure 7 shows the effects of various cytokines on progesterone production expressed as a percentage of control values. The basal production of progesterone in the control group was 224.1 ± 48.8 (mean ± SEM; ng/10^5 cells). IFN treatment resulted in the greatest inhibition of progesterone secretion to 86% of control values ($P < 0.05$). Although the remainder of the treatment groups were not statistically significantly different from the control values, all of the treatments resulted in a decrease of progesterone production with IL-1 exhibiting the least amount of inhibition. Cell count and viability remained unchanged after all of the treatments.

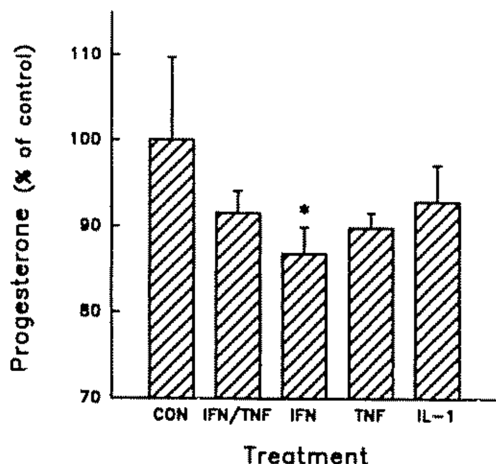

Figure 7. Effect of cytokines on progesterone production in human granulosa–luteal cells Following a 3 h post-isolation recovery period, cells were incubated with interfereon (IFN)γ/tumour necrosis factor (TNF)-α, IFNγ, TNF-α, or interleukin (IL)-1β for 24 h. Progesterone values are expressed as a percentage of the control (CON) value (224.1 ± 48 8 ng/10^5 cells). Values represent the mean ± SEM for at least three separate experiments each performed in triplicate. *P <0.05 versus control.

Discussion

The present studies show that the cellular stress protein, HSP-70, is induced in human granulosa–luteal cells by physiological inducers of cell stress. The cytokines, IFNγ and TNF-α, induce HSP-70 synthesis alone and in combination by the activation of HSF binding to HSE. The inhibition of progesterone synthesis by cytokines is consistent with previous reports which show that they inhibit steroidogenesis in humans (Fukuoka *et al.*, 1992; Wang *et al.*, 1992; Best *et al.*, 1994). The decrease in progesterone production subsequent to the induction of HSP-70 implicates the involvement of a stress response in the inhibition of progesterone production.

Although non-specific cytotoxic effects of the cytokines were unlikely since cell viability was unaffected, other methods of inhibition of steroidogenesis, in addition to the proposed stress protein mechanism, are probably also involved. In the rat, HSP-70 induction was shown to interfere with post-cAMP events, most likely involving cholesterol transport to the inner mitochondrial membrane (Khanna *et al.*, 1995a). Since cytokines such as IFNγ inhibit both cAMP accumulation and stimulation of progesterone secretion in response to a cAMP analogue in humans (Fukuoka *et al.*, 1992), activation of HSP-70 may represent only one of several different effects these agents produce in human granulosa–luteal cells.

In contrast to the rat model, heat treatment appears to impair steroidogenesis by other mechanisms in addition to the inhibition of cholesterol transport in humans. Although these studies do not specifically address the localization of the sites of action, evidence from the only partial reversal of the heat-induced inhibition of progesterone by 22-OH cholesterol indicates a functioning but perhaps impaired mitochondrial cytochrome $P450_{scc}$ complex and/or cytosolic 3β-OH steroid dehydrogenase (3β-HSD). This species difference is also apparent in hydrogen peroxide (H_2O_2)-mediated inhibition of progesterone synthesis. In humans, H_2O_2 decreases progesterone synthesis at the level of both $P450_{scc}$ and 3β-HSD (Endo *et al.*, 1993), whereas in the rat, inhibition occurs primarily at the post-cAMP site of cholesterol transport to the $P450_{scc}$ (Behrman and Aten, 1991).

As in previous studies on the effects of cytokines on progesterone production (Fukuoka *et al*, 1992; Wang *et al*, 1992; Best *et al.*, 1994), IFNγ treatment in the present study reduced progesterone synthesis but not as dramatically. The other cytokine treatment groups also showed a trend toward the reduction of progesterone synthesis, although statistical significance was not achieved despite removal of contaminating white blood cells. One possibility for this finding is that the control group of cells were also exposed to a stressor (stress of cell isolation, periovulatory cytokines, blood from the retrieval, or infection/inflammation in the patient), thus reducing the amount of progesterone produced and the ability to discern a significant difference between control and treatment groups. The presence of HSP-70 (part of which may be constitutive HSP-70) in the control group supports this possibility. This may also explain the discrepancy between previous studies and the present results in which significantly smaller reductions in progesterone production were observed. The protocol used in the previous studies (Fukuoka *et al.*, 1992; Best *et al.*, 1994) involved at least an overnight incubation prior to cytokine treatment which may have allowed the cells to recover from any exposure to stress, thus permitting a greater response to be observed between control and treatment groups.

Speculation as to the mechanism of HSP-70 interference with progesterone synthesis is based upon the unique characteristics of HSP-70 function. Recently, it has been proposed that a steroidogenic acute regulatory protein (StAR) is essential for the transfer of cholesterol across the mitochondrial membrane to the $P450_{scc}$ (Stocco and Soderman, 1991; Lin *et al.*, 1995). In view of the ability of HSP-70 to bind to proteins and act as an ATP-dependent unfoldase (Zimmermann *et al.*, 1988) during mitochondrial protein import (Deshaies *et al.*, 1988), HSP-70 may sequester or unfold the rapidly turning over StAR and block cholesterol transport. Conversely, by binding to nascent polypeptides and assisting in the folding to their final tertiary structure (Chirico *et al.*, 1988; Pelham, 1986), HSP-70 may stabilize proteins involved in the inhibition of progesterone synthesis. HSP-70 also binds to cytoskeletal elements (Margulis and Welsh, 1991; Tsang, 1993) which may disrupt the actin network and interfere with cholesterol translocation. Another possible explanation is the acute inhibition of protein synthesis during HSP induction (Lindquist, 1981) which would block the synthesis of proteins necessary for steroidogenesis (Garren *et al.*, 1965).

We have shown that physiological agents such as cytokines which inhibit progesterone production induce a stress response in human granulosa–luteal cells characterized by the formation of HSP-70. The possibility of cytokine-mediated induction of stress proteins in the human ovary may provide insight into the mechanisms of abnormal ovarian function in inflammatory states such as endometriosis and infection. Although the exact role of stress protein induction in the human corpus luteum remains to be elucidated, it is interesting to speculate that since functional luteolysis exhibits elements of a stress

response, HSP-70 may be a mediator of this response which results in functional luteal regression

Acknowledgements

We wish to thank Thomas R Kolodecik for his excellent technical advice and support

References

Adams. E C and Hertig, A T (1969) Studies on the human corpus luteum I Observation on the ultrastructure of development and regression of the luteal cells during the menstrual cycle *Am J Cell Biol*. **41**, 696–715

Baler, R, Welch, W J and Voellmy. R (1992) Heat shock gene regulation by nascent polypeptides and denatured proteins: hsp70 as a potential autoregulatory factor *J Cell Biol*. **117**, 1151–1159

Beckmann, R P. Mizzen, L A and Welch. W J (1990) Interaction of hsp 70 with newly synthesized proteins: implications for protein folding and assembly *Science*, **248**, 850–854

Behrman. H R and Aten. R F (1991) Evidence that hydrogen peroxide blocks hormone-sensitive cholesterol transport into mitochondria of rat luteal cells *Endocrinology*, **128**, 2958–2966

Behrman, H R, Preston. S L and Hall, A K (1980) Cellular mechanism of the antigonadotropin action of LHRH in the corpus luteum *Endocrinology*, **107**, 656–664

Best. C L, Pudney. J, Anderson, D J and Hill, J A (1994) Modulation of human granulosa cell steroid production *in vitro* by tumor necrosis factor alpha: implications of white blood cells in culture *Obstet Gynecol*, **84**, 121–127

Bonner, W M and Laskey. R A (1974) A film detection method for tritium-labelled proteins and nucleic acids in polyacrylamide gels *Eur. J Biochem*, **46**, 83–88

Bradford, M M (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding *Anal Biochem*. **72**, 248–254

Chiang, H.-L. Terlecky. S R, Plant, C P and Dice, J F (1989) A role for a 70-kilodalton heat shock protein in lysosomal degradation of intracellular proteins *Science*. **246**, 382–385

Chirico, W J, Waters, M G and Biobel. G (1988) 70K heat shock-related proteins stimulate protein translocation into microsomes *Nature*, **332**, 805–809

Deshaies. R J, Koch. B D, Werner-Washburne, M *et al* (1988) A subfamily of stress proteins facilitates translocation of secretory and mitochondrial precursor polypeptides *Nature*. **332**, 800–805

Endo. T, Aten. R F, Leykin, L and Behrman, H R (1993) Hydrogen peroxide evokes antisteroidogenic and antigonadotropic actions in human granulosa luteal cells *J Clin Endocrinol Metab*., **76**, 337–342

Fukuoka. M, Yasuda, K., Emi. N *et al* (1992) Cytokine modulation of progesterone and estradiol secretion in cultures of luteinized human granulosa cell *J. Clin Endocrinol Metab*., **75**, 254–258

Garren, L D. Ney, R L and Davis. W W (1965) Studies on the role of protein synthesis in the regulation of corticosterone production by adrenocorticotropic hormone *in vivo Proc Natl Acad Sci USA*. **53**, 1443–50

Goldenberg, C J. Luo, Y. Fenna, M. *et al* (1988) Purified human factor activates heat shock promoter in a HeLa cell-free transcription system *J Biol Chem*. **263**, 19734–19739

Jurivich. D A, Sistonen, L, Kroes. R A and Morimoto. R I (1992) Effect of sodium salicylate on the human heat shock response *Science*. **255**, 1243–1245

Khanna, A, Aten. R F and Behrman. H R (1994) Heat shock protein induction blocks hormone-sensitive steroidogenesis in rat luteal cells *Steroids*, **59**, 4–9

Khanna. A. Aten. R F and Behrman, H R (1995a) Heat shock protein-70 mediates luteal regression in the rat *Mol Endocrinol*, **9**, 1431–1440

Khanna, A, Aten, R F and Behrman. H.R (1995b) Physiological and pharmacological inhibitors of luteinizing hormone-dependent steroidogenesis induce heat shock protein-70 in rat luteal cells *Endocrinology*. **136**, 1775–1781

Lin, D, Sugawara. T, Strauss. J F *et al* (1995) Role of steroidogenic acute regulatory protein in adrenal and gonadal steroidogenesis *Science*. **267**, 1828–1831

Lindquist, S (1981) Regulation of protein synthesis during heat shock *Nature*, **293**, 311–314

Lindquist. S and Craig, E A (1988) The heat-shock proteins *Ann Rev Genet*. **22**, 631–677

Margulis, B A and Welsh. M (1991) Isolation of hsp70-binding proteins from bovine muscle *Biochem Biophys Res Commun*, **178**, 1–7

McPherson. L A, Van Kirk, E A and Murdoch, W J (1993) Localization of stress protein-70 in ovine corpora lutea during prostaglandin-induced luteolysis *Prostaglandins*, **46**, 433–440

Morimoto, R I (1993) Cells in stress: transcriptional activation of heat shock genes *Science*. **259**, 1409–1410

Morimoto, R I. Sarge. K D and Abravaya, K (1992) Transcriptional regulation of heat shock genes *J Biol Chem*, **267**, 21987–21990

Pelham. H R B (1986) Speculations on the functions of the major heat shock and glucose-regulated proteins *Cell*, **46**, 959–961

Simon. M C., Fisch, T M. Benecke, B J *et al*. (1988) Definition of multiple, functionally distinct TATA elements, one of which is a target in the hsp70 promoter for E1A regulation *Cell*. **52**, 723–729

Stocco, D.M and Soderman, T C (1991) The 30-kDa mitochondrial proteins induced by hormone stimulation in MA-10 mouse Leydig tumor cells are processed from larger precursors *J Biol Chem*, **266**, 19731–19738

Tsang, T C (1993) New model for 70 kDa heat-shock proteins' potential mechanism of function *FEBS Lett*, **323**, 1–3

Wang, H Z, Lu. S H, Han, X J *et al* (1992) Inhibitory effect of interferon and tumor necrosis factor on human luteal function *in vitro Fertil Steril*. **58**, 941–945

Zimmermann, R, Sagstetter. M, Lewis. M J and Pelham. H R B (1988) Seventy-kilodalton heat shock proteins and an additional component from reticulocyte lysate stimulate import of M13 procoat protein into microsomes *EMBO J*, **7**, 2875–2880

Received on February 23. 1996: accepted on June 20. 1996