Regulation of GnRHI and GnRHII mRNA in the Human Ovary and the Role of these Two GnRH Forms in Apoptosis

By

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ABSTRACT

In humans, reproduction was generally believed to be controlled by only one form of GnRH called GnRHI. However, recently a second form of GnRH, analogous to chicken GnRHII, was discovered in several tissues including the human ovary. The regulation and function of GnRHI in the hypothalamus has been well studied. However, the function and regulation of GnRHI and particularly GnRHII in the ovary is less well understood. Since gonadal sex steroids are one of the main regulators of reproduction, in the present study we investigated the regulation of GnRHI and GnRHII mRNA expression by 17β-estradiol (E2) and progesterone (P4) in human granulosa luteal cells (hGLCs). Additionally, GnRHI and GnRHII have been shown to directly induce apoptosis in the ovaries of some vertebrates, and according to several studies, luteolysis might happen via apoptosis. Consequently, in the present study we also examined the ability of the two GnRH forms to induce apoptosis in hGLCs, in an attempt to define the putative roles of GnRHI and GnRHII in luteolysis.

The levels of the mRNA transcripts encoding the two GnRH forms were examined using semi-quantitative RT-PCR followed by southern blot analysis. DNA fragmentation was measured using cell death detection ELISA kit. With time in culture, GnRHI and GnRHII mRNA levels significantly increased by 120% and 210% at day 8 compared to day 1, respectively. The levels remained elevated until the termination of these experiments at day 10. A 24h dose dependent treatment of hGLCs with E2 (1-100 nM) resulted in a significant decrease and increase in mRNA expression of GnRHI and GnRHII, respectively. One nM of E2 decreased GnRHI mRNA levels by 55% and increased GnRHII mRNA levels by 294%. Time dependent treatment studies

demonstrated that E2 (1nM) significantly decreased GnRHI mRNA levels in a time dependent manner with maximal inhibition of 77% at 48h. In contrast, GnRHII mRNA levels significantly increased in a time dependent fashion, reaching a maximum level of 280% at 24h. Co-treatment of hGLCs with E2 and tamoxifen reversed the regulatory effects of E2 on the mRNA expression of GnRHI and GnRHII. Time and dose dependent treatment with RU486 did not affect GnRHI mRNA levels in hGLCs. In contrast, RU486 treatment significantly increased GnRHII mRNA levels in hGLCs in a time and dose dependent fashion, with a maximum increase being observed at 24h with a 10000nM of RU486. With time in culture, a significant increase (51%) was observed in the percentage of cells undergoing apoptosis at day 8 compared to day 1. The level of apoptosis in the cells remained elevated until the end of these studies at day 10. The present study also demonstrated that 10^3nM of GnRHI or GnRHII were capable of enhancing apoptosis in hGLCs after 12h.

In conclusion, the present study demonstrated that the expression of GnRHI and GnRHII at the transcriptional level is differently regulated by E2 and P4 in hGLCs, and the effect of E2 on mRNA expression of the two GnRH forms is exerted through the conventional estrogen receptors (ERs). GnRHI and GnRHII were also shown to be capable of inducing apoptosis in hGLCs. Therefore, the dynamic balance between E2 and P4 and the subsequent increase or decrease in GnRHI or GnRHII, may play a role in regulating the fate of the corpus luteum.

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List of Abbreviations

APAF-1	Apoptotic protease activating factor-1
Bax	Bcl-associated x gene
bFGF	Basic fibroblast growth factor
Bok	Bcl-2-related ovarian killer
bp	Base pairs
Bcl-2	B-cell lymphoma/leukemia-2
C	Celcius
CAD	Caspase-activated Dnase
cDNA	Complementary deoxyribonucleic acid
cGnRHII	Chicken gonadotropin-releasing hormoneII
DISC	Death-inducing signaling complex
dNTP	Deoxynucleoside triphosphate
EGF	Epidermal growth factor
ELISA	Enzyme-linked immunosorbant assay
ER	Estrogen receptor
ERE	Estrogen response element
FADD	Fas-associated death domain
FBS	Fetal bovine serum
Fas L	Fas ligand
FSH	Follicle stimulating hormone
g	Acceleration of gravity
GAP	GnRH associated peptide

GCs	Granulosa cells
GLCs	Granulosa luteal cells
Gly	Glycine
GnRH	Gonadotropin-releasing hormone
GnRHa	Gonadotropin-releasing hormone agonist
GnRH-R	Gonadotropin-releasing hormone receptor
h	Hour
hCG	Human chorionic gonadotropin
hGCs	Human granulosa cells
hGLCs	Human granulosa luteal cells
hOSE	Human ovarian surface epithelium
ICAD	Inhibitor of caspase-activated Dnase
IGF	Insulin-like growth factor
IVF	*In vtro* fertilization
LH	Luteinizing hormone
μ	Micro
M199	Medium 199
MC1-1	Myeloid cell leukemia-1
mGnRH	Mammalian gonadotropin-releasing hormone
ml	Milliliters
min	Minutes
MMP	Matrix metalloproteinase
mRNA	Messenger ribonucleic acid

n (as in nM)	Nano
OVCAR-3	Ovarian carcinoma
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
$PGF_{2\alpha}$	Prostaglandin $F_{2\alpha}$
PR	Progesterone receptor
RIA	Radioimmunoassay
ROS	Reactive oxygen species
RT-PCR	Reverse transcription polymerase chain reaction
SD	Standard deviation
SERMs	Selective estrogen receptor modulators
SR-B1	Scavenger receptor class B type 1
SRC-1	Steroid receptor coactivator 1
TGF	Transforming growth factor
TIMP	Tissue inhibitor of metalloproteinase
Txf	Tamoxifen

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1. INTRODUCTION

The hypothalamic-pituitary-gonadal axis plays a fundamental role in regulating reproduction in mammals. Gonadotropin-releasing hormone (GnRH), a key regulator of this hormonal cascade, is a decapeptide that is secreted from hypothalamus in a pulsatile manner and stimulates the synthesis and release of gonadotropins, follicle stimulating hormone (FSH) and luteinizing hormone (LH). Gonadotropins in turn, promote steroidogenesis and gametogenesis (Conn, 1994; Noar et al., 1995). The release of GnRH is regulated by pituitary gonadotropins, gonadal steroids (Figure 1) and GnRH itself (Kalra and Kalra, 1983).

In more than 80 vertebrate species, there are at least two GnRH forms present (Schalburg et al., 1999). Recent studies have demonstrated that in addition to the classical form of GnRH (GnRHI or GnRH), a second form of GnRH called chicken GnRHII (cGnRHII or GnRHII) is also expressed in human tissues (White et al., 1998). GnRHII is the most ubiquitous variant of GnRH (White et al., 1998).

In addition to the hypothalamus, the presence of GnRHI and GnRHII and their receptor has also been detected in extrapituitary tissues, including in the ovary (Dong et al., 1993, Kang et al., 2001c). The function of the two GnRH forms in the ovary is not very well understood. However, the detection of these two hormones and their receptor in the ovary, suggests that these hormones may work in an autocrine or paracrine manner in the ovarian tissues (Kang et al., 2000a).

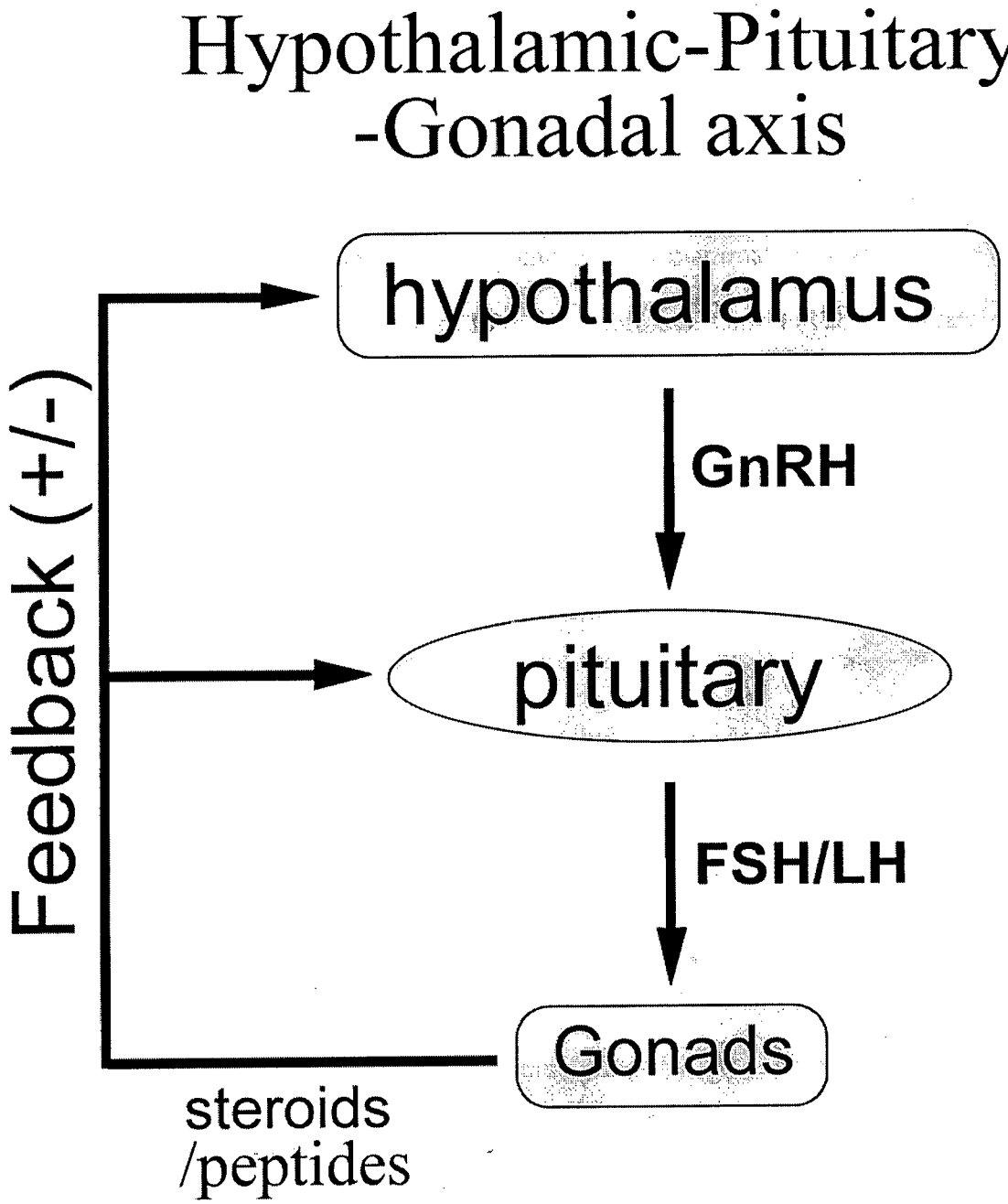

Figure 1. The hypothalamo-pituitary-gonadal axis. Synthesized in the hypothalamic neurons, GnRH stimulates the synthesis and release of LH and FSH in the pituitary. In the gonads, gonadotropins stimulate follicular development, spermatogenesis and steroidogenesis. In turn, gonadal steroids and peptide hormones regulate hypothalamic and pituitary function in both a positive and a negative feedback mechanism.

1.1 Structure and distribution of GnRHI and GnRHII

The gene for GnRH has been duplicated and structurally re-organized during evolution (King and Millar, 1995). As a result, so far 13 different forms of GnRH have been discovered in the animal kingdom (table 1). These different forms all consist of 10 amino acids in length. The amino acid sequence of different GnRH forms is highly conserved, indicating their important role in reproduction throughout evolution. They all have a pyroglutamyl-modified amino terminus and an amidated carboxy terminus. Amino acids in positions 1, 4, 9, and 10 are conserved between all different forms and amino acids 5-8 are the most variable ones. Multiple forms of GnRH with differential tissue distribution may exist in the same species (Carolsfield et al., 2000; Sherwood et al., 1997).

Previously only one kind of GnRH called mammalian GnRH (GnRHI) was known to control reproduction in humans. However, a second form of GnRH has recently been cloned from the human brain. This new form is analogous to chicken GnRHII (White et al., 1998). Administration of GnRHII to rhesus monkey during the luteal phase resulted in an up-regulation of LH, providing evidence for a biological function of GnRHII in primates (Lescheid et al., 1997).

Most of the neurons synthesizing GnRHI are localized in the preoptic area and adjacent sites in the rostral portion of the hypothalamus (Lescheid et al., 1997). GnRHI has also been detected in other regions of the central nervous system such as spinal cord, midbrain and amygdala (Lescheid et al., 1997). The expression of GnRHI and GnRHII mRNA has been indicated in extrapituitary tissues including the human ovary and endometrium. However, GnRHI expression levels are not high in non-neural tissues.

Surprisingly, in humans, GnRHII mRNA expression is much higher in tissues outside the brain including kidney, bone marrow, and prostate. Within the brain GnRHII is expressed more in the caudate nucleus and less in the hippocampus and amygdala (White et al., 1998). In the ovary GnRHI and GnRHII were localized to the granulosa cells (GCs) of follicles and corpus luteum (Clayton et al., 1992; Whitelaw et al., 1995; Kang et al., 2001). In the male, the Sertoli cells of the testis express GnRHI. In the placenta, GnRHI was detected in syncytiotrophoblast and cytotrophoblast (Seppala et al.,1980; Miyake et al., 1982). Radioimmunoassays determined that GnRHI levels in trophoblasts significantly increased during the first trimester of pregnancy and then remained constant for the rest of the pregnancy (Siler-Khodr and Khodr, 1978). GnRHI was also detected in cancers of the prostate (Qayum et al., 1990), breast (Harris et al., 1991), ovary (Irmer et al., 1995), and endometrium (Imai et al., 1994).

The genes encoding the two forms of GnRH have the same modular structure, comprising of four exons and three introns (Figure 2), suggesting that they have a common ancestral origin (White et al., 1998). The 1st exon encodes for the 5' untranslated region. The second exon encodes for a signal peptide of 21-23 amino acids long, the GnRH decapeptide, a proteolytic processing site, and part of a GnRH-associated peptide (GAP). The third exon and part of the fourth exon encode for the rest of the GnRH associated peptide. The rest of the exon 4 encodes for the 3' untranslated region (White et al., 1998). The gene for GnRHI in humans is located on chromosome 8 and the one for GnRHII is located on chromosome 20. The structure of the mRNA transcript from the two genes is very similar; however, the gene for GnRHI (5.1 kb) is much bigger than the gene for GnRHII (2.1 kb) (White et al., 1998). GnRH is derived from a

preprohormone that is comprised of a signal sequence, a GnRH decapeptide, a conserved proteolytic site and a GAP. The post-translational modification of this preprohormone gives rise to a mature GnRH peptide. GAP is 50% longer in GnRHII than in GnRHI (White et al., 1998). GAP was initially thought to have gonadotropin releasing and prolactin inhibiting properties in rat pituitary cells (Nikolics et al., 1985; Millar et al., 1986; Milton et al., 1986), but this was not supported by other data (Thomas et al., 1988). It is possible that GAP is involved in assisting GnRH to acquire the right conformation (Sherwood et al., 1993). Depending on the tissue, GnRHI and GnRHII transcripts have different lengths. This suggests different transcription initiation sites and different post-transcriptional modifications. Therefore, the regulation of transcription may be tissue specific (Urbanski et al., 2000, White et al., 1998).

Table 1: Primary Amino Acid Sequences of known GnRH Structures

GnRH	***1 2 3 4 5 6 7 8 9 10***
Mammal	p-Glu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2
Catfish	p-Glu-His-Trp-Ser-His-Gly-Leu-Asn-Pro-Gly-NH_2
Seabream	p-Glu-His-Trp-Ser-Tyr-Gly-Leu-Ser-Pro-Gly-NH_2
Chicken I	p-Glu-His-Trp-Ser-Tyr-Gly-Leu-Gln-Pro-Gly-NH_2
Chicken II	p-Glu-His-Trp-Ser-His-Gly-Trp-Tyr-Pro-Gly-NH_2
Dogfish	p-Glu-His-Trp-Ser-His-Gly-Trp-Leu-Pro-Gly-NH_2
Salmon	p-Glu-His-Trp-Ser-Tyr-Gly-Trp-Leu-Pro-Gly-NH_2
Guinea pig	p-Glu-Tyr-Trp-Ser-Tyr-Gly-Val-Arg-Pro-Gly-NH_2
Tunicate I	p-Glu-His-Trp-Ser-Asp-Tyr-Phe-Lys-Pro-Gly-NH_2
Tunicate II	p-Glu-His-Trp-Ser-Leu-Cys-His-Ala-Pro-Gly-NH_2
Lamprey I	p-Glu-His-Tyr-Ser-Leu-Glu-Trp-Lys-Pro-Gly-NH_2
Lamprey III	p-Glu-His-Trp-Ser-His-Asp-Trp-Lys-Pro-Gly-NH_2
Herring	p-Glu-His-Trp-Ser-His-Gly-Leu-Ser-Pro-Gly-NH_2

Adapted from Carolsfeld et al., 2000

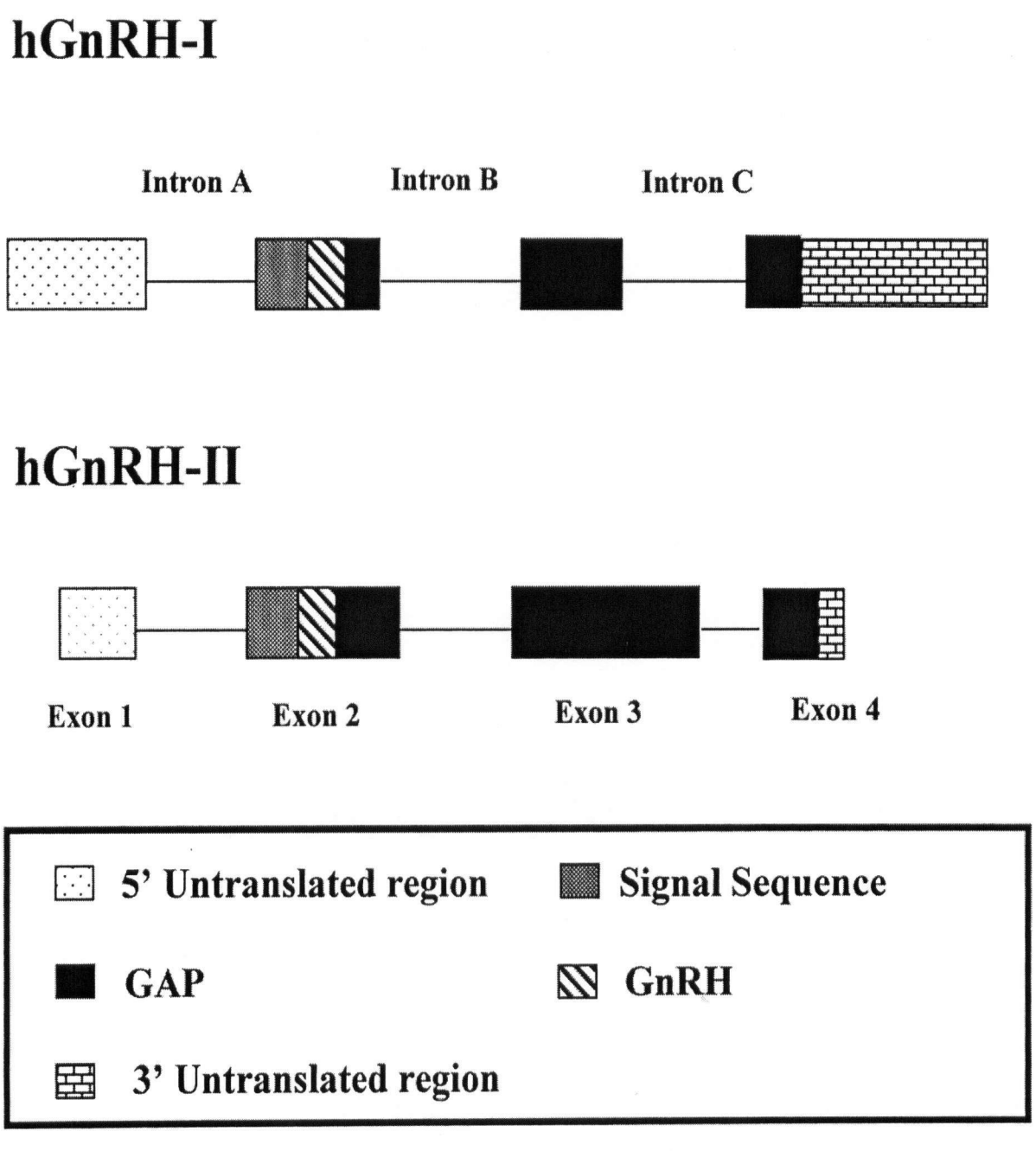

Figure 2. Schematic representation of the human GnRHI and GnRHII genes (not to scale). Adapted from White et al., 1998

1.2 Physiological roles of GnRHI and GnRHII

1.2.1 Roles in the central nervous system and pituitary

GnRHI is released from hypothalamus, binds to its receptor in pituitary and causes the release of FSH and LH from pituitary gland. FSH and LH are released into systemic circulation and bind to their receptors in the gonads, resulting in steroidogenesis and gametogenesis. In females, FSH stimulates follicular development; whereas, LH induces ovulation, formation of the corpus luteum and oocyte meiosis. In males, FSH stimulates spermatogenesis and LH stimulates androgen synthesis (Braden and Conn, 1993; Conn, 1994). GnRHII function is less well described. GnRHII peptide may be the result of an early gene duplication. It is conserved from fish to man and is widely distributed in the brain, suggesting important neuromodulatory functions such as regulating K+ channels in the sympathetic ganglion and stimulating sexual arousal. GnRHII is also proposed to be involved in controlling the reproductive behavior in some birds and mammals (Dellovade et al., 1995; Sakuma et al., 1980). Another possible function of GnRHII is specific stimulation of FSH release. GnRHI and GnRHII, in general, stimulate the release of both FSH and LH. However, recently some specific FSH-releasing factors such as cGnRHII and lamprey GnRHIII have been reported (Millar et al., 2001). A number of rams were treated with GnRHI and GnRHII respectively. Although GnRHI was more potent than GnRHII in releasing both FSH and LH, every individual ram exhibited a higher ratio of FSH to LH secretion when treated with GnRHII (Millar et al., 2000).

1.2.2 Intra-ovarian roles

In the ovary, depending on the stage of the menstrual cycle, GnRH seems to have different biological functions. During the follicular phase, the highest expression of GnRH receptor was demonstrated in atretic rat follicles and no GnRH receptor was detected in primordial follicles or oocytes (Whitelaw et al., 1995). Since GnRH was shown to induce apoptosis in cultured rat GCs, GnRH might be involved in the process of follicular atresia through the induction of apoptosis (Billig et al., 1994).

During the periovulatory period in the rat, GnRH may play a role in follicular rupture and oocyte maturation. This is probably done by the GnRH-induced increase in the transcription of certain genes such as prostaglandin endoperoxidase synthase II (Wong and Richards, 1992), plasminogen activator (Ny et al., 1987) and progesterone receptor (PR) (Natraj and Richards, 1993). GnRH is thought to be involved in luteolysis during the luteal phase. GnRH was demonstrated to stimulate the expression of matrix metalloproteinase 1 and 2 (MMP-1 and MMP-2) in the corpus luteum of rats. These enzymes are involved in degrading collagens and remodeling extracellular matrix leading to luteolysis (Goto et al., 1999). GnRH was also shown to induce apoptosis in the rat corpus luteum and in cultured human granulosa luteal cells (hGLCs) (Sirdaran et al., 1998; Zhao et al., 2000).

A particular agent may induce apoptosis in a given cell or tissue at one time during its life history but not at another. Whether a cell responds to such an agent by dying, may depend on interactive regulatory systems active in the cell at that time (Medh and Thompson, 2000). Cellular environment is also an important factor for determining the response of a cell or a tissue to an agent (Medh and Thompson, 2000). Therefore,

different hormonal levels in the ovary might be responsible for different functions of GnRH during various stages of menstrual cycle. For example, apoptotic effect of GnRH in the rat ovary decreased by concomitant treatment with FSH. Estradiol (E2) was also shown to inhibit the rat ovarian GC apoptosis (Billig et al., 1994). Consequently, GnRH may be an apoptosis-inducing agent in the ovary at one time and have other functions at other times.

Data regarding the regulatory effect of GnRH on steroidogenesis in the ovary is controversial. According to some groups GnRH inhibits steroidogenesis by suppressing FSH and LH receptors (Piquette et al., 1991; Tilly et al., 1992), P450 side-chain cleavage enzyme, steroidogenic acute regulatory protein, 3-β-hydroxy-steroid dehydrogenase (Sirdaran et al., 1999), and gonadotropin induced cAMP levels (Knecht et al., 1985; Richards 1994). Other groups showed that GnRHI and in one case GnRHII decreased the level of progesterone (P4) secretion in cultured hGLCs (Kang et al., 2001c; Tureck et al., 1982). However, some others demonstrated no (Casper et al., 1984) or a stimulatory effect (Olsson et al., 1990; Parinnaud et al., 1988) of GnRH on P4 production in hGLCs.

GnRHI and GnRHII were also shown to have an autocrine regulatory function in cultured human granulosa luteal cells. High concentrations of GnRHI (1nM and 100 nM), down regulate its own mRNA expression and a low concentration of GnRHI (0.01 nM), up regulates its mRNA expression (Kang et al., 2001c). However, both high and low concentrations of GnRHII down regulate its own mRNA expression (Kang et al., 2001c).

1.2.3 Roles in endometrium and placenta

GnRH was shown to improve the process of implantation (Raga et al., 1999). GnRH decreased the mRNA expression of tissue inhibitor of metalloproteinase (TIMP) in cultured human stromal endometrial cells. The balance between MMPs and their inhibitors is very important for the extensive tissue remodeling which is required for the process of implantation (Raga et al., 1999). The role of GnRH in implantation is further confirmed by the observations that women undergoing *in vitro* fertilization (IVF) have a much higher rate of pregnancy and implantation if they have received a GnRH agonist during the early stages of embryonic development and implantation (Gartner et al., 1997).

In the placenta, GnRH stimulates the synthesis and secretion of human chorionic gonadotropin (hCG). Studies using the GnRH receptor antagonists demonstrated that the stimulatory effect of GnRH on the synthesis of hCG is a receptor mediated event (Siler-Khodr et al., 1983, 1987; Currie et al., 1993). Like in the ovary, the co-expression of GnRH and its receptor in the human placenta suggests a possible autocrine/paracrine role for GnRH in this organ. The responsiveness of placenta to GnRH, seems to change according to variations in the mRNA expression of GnRH receptor in the placenta at different times of gestation. The GnRH receptor mRNA expression in the placenta is high at six weeks of gestation and reaches maximum at 9 weeks of gestation. GnRH receptor mRNA expression goes down in the second trimester and becomes undetectable in the term placenta. Accordingly, the GnRH stimulated hCG secretion in the placenta is high during the first trimester and undetectable at term (Currie et al., 1993; Siler-Khodr et al., 1983, 1987). hCG is required to maintain the vital steroidogenesis of the corpus

luteum until approximately the 9^{th} or 10^{th} week of gestation, by which time placental steroidogenesis is well established (Catt et al., 1975).

1.2.4 Roles in reproductive tumors

Synthetic analogues of GnRH have a therapeutic effect on sex steroid-dependent cancers such as breast, uterine endometrial, ovarian, and prostate cancers (Imai et al., 1994; Eidne et al., 1985; Bahk et al., 1998). The rationale for this treatment is that continuous administration of GnRH agonists causes inhibition of gonadotropin release from the pituitary and this reduces steroid production by the ovaries or testes (medical castration). Administration of analogues of GnRH initially stimulates the release of gonadotropins and sex steroids. This may temporarily worsen the disease. The use of GnRH antagonists immediately inhibits the function of pituitary and gonads which prevents the initial stimulatory effect on tumor growth (Schally et al., 1992). The first generation of antagonists were weak and the second generation had some unsafe side effects. However, a better third generation of GnRH antagonists are being used now (Karten, 1992). *In vitro* studies showed that GnRH might inhibit tumor cell growth by directly inducing apoptosis in the cells (Palyi et al., 1996; Szepeshazi et al., 1997).

1.3 Regulation of GnRHI and GnRHII

GnRH regulation is influenced by the interactions happening in the hypothalamo-pituitary gonadal axis. Factors controlling GnRH synthesis and secretion are either directly exerted on GnRH neurons or operate indirectly by modulating the neuronal systems that impinge upon GnRH neurons. These factors include GnRH itself, dopamine, catecholamines and neuropeptide Y (Sawyer, 1975). In the hypothamo-pituitary gonadal axis, GnRH is regulated via ultrashort, short and long feedback loops (Martinez et al., 1992). *In vivo* experiments showed that GnRH administration, through an ultrashort feedback, down regulated the secretion of hypothalamic GnRH in the rat (Valenca et al., 1987). In GT1-7 neuronal cell lines and also in the hGLCs, GnRH regulated its own expression in a biphasic manner (Krsamanovic et al., 1993; Peng et al., 1994). The short feedback loop indicates a negative feedback of pituitary hormones on their own secretion, presumably through inhibitory effects on GnRH in the hypothalamus. Long term and short term LH treatment in the rat suppressed LH and GnRH secretion (David et al., 1966; Corbin, 1996). LH also inhibited GnRH release in GT1-7 neuronal cell lines (Lei and Rao, 1994). The long feedback loop refers to the feedback effects of circulating levels of target gland hormones, and this occurs both in the hypothalamus and the pituitary. Contradictory results were reported regarding the regulatory effects of E2 on GnRH secretion. During the rat estrus cycle, the level of GnRH mRNA in the hypothalamus and E2 in the plasma were shown to be inversely related (Zoeller and Young, 1988). Administration of E2 for 7 days to ovariectomized rats increased the GnRH mRNA expression but E2 administration for 2 days decreased GnRH mRNA levels (Zoeller et al., 1988). Park et al. (1990) demonstrated that in the rat,

E2 increased GnRH gene expression and this increase resulted in gonadotropin surge before ovulation. The differences in GnRH regulation by E2 may be owing to methods used to measure GnRH secretion, dose and duration of treatments.

Experiments done on juvenile and adult castrated tilapia indicated no effect of E2 and P4 on hypothalamic GnRHII mRNA expression (Parhar et al., 2000; Soga et al., 1998; Parhar et al., 1998; Parhar et al., 1996). Similarly, steroids in chicken had no significant effect on the expression of GnRHII (Wilson et al., 1990). Taken together these observations suggest that GnRHII hypothalamic neurons may not be targets for the feedback effects of steroid hormones. In contrast, in the European eel, androgens in combination with E2 could decrease the expression of GnRHII (Montero et al., 1995). Furthermore, studies in the musk shrew showed that GnRHII was modified by ovulation, suggesting that ovarian steroids might modulate the release of GnRHII (Rissman and Li, 1998). The controversy between GnRHII regulation by steroids may result from species difference or different experimental conditions.

In the ovary, hCG did not affect the GnRHI mRNA expression in hGLCs (Peng et al., 1994). In contrast, Kang et al. (2001c) demonstrated the opposite results with hCG down regulating GnRHI mRNA expression and up regulating GnRHII mRNA expression in hGLCs. The controversy between the results was not explained in the second paper. In these experiments, hGLCs were obtained from IVF patients. The locus and cause of infertility in these patients is not known. Therefore, the changes in gene expression in response to exogenous factors may differ from patient to patient. This might have been the reason for controversial results. More studies showed that in hGLCs E2 down regulated GnRHI mRNA expression in a time and dose dependent manner (Nathwani et

al. 2000);whereas, Treatment of hGLCs with FSH resulted in a marked increase in GnRHII mRNA levels (Kang et al., 2001c).

1.4 Apoptosis

It is now widely accepted that apoptosis, a suicidal program of cell death, is of central importance for development and homeostasis (Steller, 1995). Apoptosis can be distinguished from necrosis, a passive form of cell death, both morphologically and biochemically. Necrosis is cell death resulting from injury or trauma and is accompanied by increased ion permeability of plasma membrane. The cells swell and the plasma membrane ruptures within minutes (osmotic lysis) (Walker et al., 1988; Wyllie et al., 1981). In contrast, apoptosis is an active orderly process which often requires the synthesis of new proteins. Apoptosis affects scattered single cells and involves cell shrinkage, chromatin condensation, nuclear fragmentation, cytoplasmic blabbing and cellular fragmentation into small apoptotic bodies. These apoptotic bodies are quickly phagocytosed and digested by neighboring cells or macrophages (Schwartzman and Cidlowski, 1993; Vinatier et al., 1996). The most striking feature of apoptosis is the activation of calcium/magnesium-dependent endonucleases, which specifically cleave DNA between regularly spaced nucleosomal units, resulting in the generation of DNA fragments in size multiples of 185-200 base pairs. These DNA fragments can be visualized as distinct ladder of DNA bands after agarose gel electrophoresis, and are markedly different from a smear pattern which results from the random breakdown of DNA that occurs during necrosis. In apoptosis, DNA degradation occurs several hours before plasma membrane breakdown (Schwartzman and Cidlowski, 1993; Vinatier et al., 1996).

Apoptosis is particularly important during metamorphosis, hormone induced atrophy, pathological conditions and embryonic development (Ishizuya-Oka and

Shimozava, 1992; Thompson, 1995). It is estimated that about 85% of embryonic neurons undergo apoptosis during development of the central nervous system (Barres et al., 1992; Oppenheim, 1991). Apoptosis is an important defense mechanism for removing unwanted and potentially dangerous cells such as, self reactive lymphocytes, cells that have been infected by viruses and tumor cells (Vaux et al., 1994; Williams, 1993). In adult tissues the occurrence of apoptosis can be divided into three different categories. Usually little apoptosis happens in the first group of tissues including heart, kidney and liver (Benedetti et al., 1988). The second group includes hematopoietic tissues, the epithelium lining intestinal crypts and spermatogonia in the testis. These tissues in contrast to the first group, exhibit constant cell turnover and have high rates of stem cell proliferation accompanied by massive apoptosis (Wyllie, 1987; Billig et al., 1995). In the third type of tissues such as in the ovary, a high rate of follicular cell apoptosis occurs during the reproductive life; however, there is no replacement of the lost cells (Hsueh et al., 1996). In the human ovary approximately 400,000 follicles exist at the onset of puberty, but only 400 follicles are ovulated during the female reproductive life. At the time of menopause, few follicles are found in the ovary. Therefore, more than 99% of human follicles undergo degeneration during the reproductive life and apoptosis has been shown to be the underlying mechanism for ovarian cell death (Hsueh et al., 1996).

Although the exact mechanism of apoptotic cell death is unknown, some of the processes involved appear to be highly conserved. Different signaling pathways, which are cell specific, ultimately converge to activate a common or similar apoptotic death program (Hsu and Hsueh, 1998).

1.4.1 Factors regulating apoptosis in the ovary

In the ovary, there are several specific regulators of apoptosis, including hormones, growth factors and cytokines (Chun and Hsueh, 1998). Lack or overexposure to some of the hormones may induce apoptosis by causing changes in the intracellular environment. For example, gonadotropins are known as follicle survival factors. Treatment of preovulatory follicles with FSH or LH prevented the spontaneous onset of apoptosis (Chun et al., 1994). GnRH, on the other hand, was suggested to play a physiological role associated with follicular atresia in the vertebrate ovary, possibly via stimulation of apoptosis (Kogo et al., 1995). GnRH receptor gene expression was indicated to be expressed to a greater extent in GCs of atretic follicles compared with preantral follicles in the rat (Kogo et al., 1995). In hypophysectomized, E2 treated rats, treatment with a GnRH agonist directly increased DNA fragmentation in the ovary (Billig et al., 1994). Concomitant treatment with FSH decreased ovarian apoptosis. Similarly, in isolated rat GCs, GnRH treatment increased DNA fragmentation, which was reduced with concomitant FSH treatment (Billig et al., 1994). Gonadal steroids are also efficient regulators of ovarian apoptotic cell death. Billig et al. (1993) demonstrated that when immature, hypophysectomized rats were treated with E2 for two days, followed by E2 removal, apoptotic DNA fragmentation increased in the ovary, occurring primarily in GCs. On the other hand, replacement of E2 completely prevented the ovarian weight loss and decreased apoptosis in GCs. P4 has also been shown to decrease apoptosis in cultured GCs (Peluso and Pappalardo, 1994, 1999).

Growth factors also play a role in regulating apoptosis (Luciano et al., 1994; Tilly et al., 1992). Intra-ovarian growth factors, including basic fibroblast growth factor

(bFGF) and epidermal growth factor (EGF) inhibited apoptosis by autocrine/paracrine mechanisms in preovulatory follicles of the rat (Luciano et al., 1994; Tilly et al., 1992). Insulin-like growth factor-1 (IGF-1) synergized with FSH and LH in stimulating E2 and P4 production by GCs, thereby functioning as a follicular survival factor (Chun et al., 1994). FSH was postulated to stimulate GCs to secrete IGF-1, which in turn stimulates theca cells to produce EGF and transforming growth factor α (TGFα), thereby exerting an antiapoptotic response (Hsueh et al., 1994). In contrast, expression of TGFβ in hormone-deprived follicles may promote apoptosis (Martimbeau and Tilly 1997).

In addition to growth factors, some locally produced cytokines may also have a role in regulating apoptosis in the ovary (Richards, 1994). Different cytokines are expressed in the normal ovary. For example, the interleukin-1 (Il-1) family were demonstrated to play an important role in the regulation of follicular differentiation (Chun et al., 1995). In cultured rat follicles, Il-1β suppressed follicle apoptosis in a dose dependent manner (Chun et al., 1995). In contrast, Il-6 was shown to induce apoptosis in cultured rat GCs (Gorospe and Spangelo, 1993).

1.4.2 Gene regulation of apoptosis in the ovary

Two important families of regulators of the apoptotic process are the caspase and the Bcl-2 families (Hengartner, 2000). To date 13 different types of caspases have been identified. Caspases are cytosolic proteases, and are synthesized as inactive precursors that must be cleaved by other caspases or autocatalytically in order to become activated. Triggering of apoptosis results in a cascade of caspase activation (Yuang, 1997; Nagata, 1997). More than100 substrates are known to be cleaved by caspases, including lamins,

causing nuclear shrinking and budding, and cytoskletal proteins, such as fordin and gelsolin, causing loss of overall cell shape (Hengartner, 2000). An important function of caspases is to activate caspase-activated Dnase (CAD), the endonuclease responsible for inter-nucleosomal DNA fragmentation, one of the most frequently used hallmarks of apoptosis (Yuang, 1997; Nagata, 1997). CAD and its inhibitory subunit, inhibitor of caspase-activated Dnase (ICAD), are constantly expressed in the cells. Caspase-mediated cleavage of the inhibitory subunit results in release and activation of the endonuclease (Yuang, 1997; Nagata, 1997). Different caspases including caspases 3, 8, and 9 are expressed in the ovary of various species including in the rat and mouse ovary (Boon and Tsang, 1998; Martikainen et al., 2001).

The Bcl-2 family of genes includes both apoptosis promoting (eg, Bax, Bok, and Bad) and apoptosis inhibiting (eg, Bcl-2 and Bcl-X_L, Mc1-1) members (Antonsson and Martinou, 2000). The Bcl-2 family members are located in the outer membranes of the mitochondria and can bind to each other in different pair wise conditions. In most cases the ratio of pro-apoptotic to anti-apoptotic Bcl-2 homologues within a cell determines whether the cell undergoes apoptosis or not (Gajewski and Thompson, 1996; Antonsson et al., 1997). The main function of the Bcl-2 family seems to be regulating the release of pro-apoptotic factors, particularly cytochrome C, from the mitochondria into the cytosol (Antonsson and Martinou, 2000). Many members of the Bcl-2 family have been isolated in the ovary, including Bad, Mcl-1, Bcl-X_L and Bok (Hsu and Hsueh, 2000). Another important gene involved in apoptosis and tumorigenesis is p53 (Bellamy, 1997). The p53 protein is an anti-proliferative transcription factor that is involved in several cellular functions related to genome stability, cell cycle and apoptosis (Bellamy, 1997). DNA

damage and growth factor withdrawal, among other adverse conditions, may lead to p53 dependent apoptosis in some cells (Bellamy, 1997). The p53 protein is present in the apoptotic nuclei of GCs from rat atretic follicles and its expression is suppressed following treatment with gonadotropins (Tilly et al., 1995).

1.4.3 Apoptotic signaling pathway in the ovary

In the follicular GCs, a range of hormones and locally produced factors regulate the decision to die, by way of their receptors (Andreu-Vieyra and Habibi, 2000). Examples of survival factors, as mentioned before, include FSH, LH, P4, E2, growth hormone, IGF-1, EGF, bFGF and insulin (Luciano et al., 1994; Tilly et al., 1992; Chun et al., 1994; Hsueh et al., 1994). DNA damage caused by, for example, irradiation, also initiates apoptosis via the protein p53 (Gottlieb and Oren, 1998). Execution of the apoptotic program converges in the mitochondria, in which pro- and anti-apoptotic members of the Bcl-2 family interact at the surface (Antonsson and Martinou, 2000). Excess pro-apoptotic activity causes release of a series of molecules from the mitochondria into the cytoplasm. One of the main molecules is cytochrome c, which associates with apoptotic protease-activating factor 1 (APAF-1) and pro-caspase 9 to form the apoptosome complex (Antonsson and Martinou, 2000; Scaffidi et al., 1998; Liu et al., 1996). This complex subsequently activates down stream caspases, such as caspase 3. Activation of caspase 3, leads to several events including exposure of phosphatidylserine on the cell surface (Svensson et al., 1999). Exposure of phosphatidylserine on the outer cell membrane, is a signaling mechanism that occurs in apoptotic cells. In the ovary, scavenger receptor class B type 1 (SR-B1) on theca cells

mediates recognition and binding of apoptotic granulosa cells expressing phosphatidylserine on the cell surface (Svensson et al., 1999). Another effect of caspase 3 activation is cleavage of ICAD resulting in release of CAD, the endonuclease responsible for inter-nucleosomal DNA fragmentation (Yuang 1997; Nagata 1997). Another pathway for apoptosis, is the death receptor pathway (Pru and Tilly, 2001). For example, a death ligand, called Fas ligand, binds to a death receptor (a cell surface receptor protein called Fas) to initiate intracellular signaling. Binding of ligand to the receptor causes receptor clustering and formation of a death-inducing signaling complex (DISC). This complex recruits multiple copies of pro-caspase 8, via the molecule Fas-associated death domain protein (FADD), resulting in activation of caspase 8 (Pru and Tilly, 2001). Activated caspase 8 subsequently activates other caspases, particularly caspase 3. Activation of caspase 3 leads to the events mentioned before (Pru and Tilly, 2001). The expression of APAF-1 has been described in the ovary of women undergoing IVF (Izawa et al., 1998). Fas expression has been shown in hGLCs (Quirk et al., 1995) and also in rat GCs undergoing atresia (Kim et al., 1999). Fas ligand has been demonstrated to be expressed in the ovary of different species including in GCs of atretic follicles from the mouse ovary (Guo et al., 1997).

1.5 RU486, Tamoxifen, Antide

RU486, also called mifepristone, has a similar structure to P4, but lacks the C19 methyl group and the 2-carbon side chain at C17 of P4 and has a conjugated C9-C10 double bond (Teutsch and Philibert 1994). The antiprogestin action of RU486 is mediated by the PR, a ligand-activated transcription factor with domains for DNA binding, hormone binding and transactivation (Teutsch and Philibert, 1994). The amino acid glycine at position 722, which is in the hormone binding domain of the human PR and at the comparable position of the PR of most other species, seems to be critical for RU486 binding and action (Benhamou et al., 1992). This was demonstrated by experiments in the chicken and the hamster, which are not sensitive to RU486 and have a PR with a cysteine rather than a glycine residue at this position. After substitution of this cysteine with glycine in the chicken PR, binding of RU486 and antagonistic action of this compound are observed (Benhamou et al., 1992).

RU486 competes with P4 for the PR. The affinity of RU486 for the PR is five times greater than that of the natural hormone (Teutsch and Philibert, 1994). In the absence of ligand, the PR is associated with heat-shock proteins. Association of PR with either P4 or RU486 induces different conformational changes in PR, resulting in dissociation of heat-shock proteins and dimerization of PR (Tsai and O'Malley, 1994). The activated receptor dimer binds to P4 response elements in the promoter region of P4-responsive genes. This binding, in the case of P4, will increase the transcription rate of these genes, leading to P4 effects (Tsai and O'Malley, 1994). In contrast, a receptor dimer complex that has been activated by RU486, after binding to P4 response elements, is not transcriptionally active because of an inhibitory function in the C-terminal region

of the hormone binding domain (Spitz et al., 1996). In the absence of hormone binding, the C-terminal region of the PR exerts an inhibitory effect on transcription. P4 induces a conformational change that overcomes the inherent inhibitory function within the carboxy tail of the receptor. Binding with RU486 produces a structural change that allows the inhibitory actions to be maintained. This is the basis for the P4 antagonistic action of RU486, underlying its abortifacient and contraceptive actions (Spitz et al., 1996).

Under certain circumstances, such as the absence of P4 in endometrium in postmenopausal women, RU486 may display P4 agonistic activity (Gravanis et al., 1985). One possible reason for RU486 to act as an agonist or antagonist may be related to the existence of two separate isoforms of PR (PR-A and PR-B) (Robbins and Spitz, 1996). Some *in vitro* transfection studies show that PR-B behaves as a partial agonist in the presence of RU486. However, when PR-A and PR-B are both present together, the antagonistic effect of PR-A can override the agonistic effect of PR-B. Therefore, depending on the ratio of the expression of the two PR isoforms in a tissue, RU486 may behave as an agonist or antagonist (Robbins and Spitz, 1996).

In ovariectomized E2-treated rats, P4 increased hypothalamic GnRH mRNA levels and RU486 blocked the P4 effect, an indication of the antagonistic effect of RU486 (Cho et al., 1994). In pregnant uterus, RU486 behaved as an abortifacient via its effect on the high concentration of PRs in the decidua. Blockage of these receptors by RU486 resulted in withdrawal of P4 support to the endometrium (Peyron et al., 1993). In cultured hGLCs, RU486 suppressed P4 production. This probably happened through a direct effect of RU486 on enzymes involved in P4 synthesis (Dimattina et al., 1986).

Tamoxifen was developed as a non-steroidal, orally active anti-estrogen, initially as a contraceptive agent, but was recognized as having potential benefit to treat hormone-responsive breast cancer patients (Harper and Walpole, 1966). It is presently known that tamoxifen is an antagonist in some target tissues, such as the breast and brain, and an agonist in others such as the bone, liver, uterus and the cardiovascular system (Grese and Dodge, 1998; Shang and Brown, 2002). In the ovary, tamoxifen was shown to act as an antagonist in cultured, rat GCs (Luciano and Peluso, 1995), bovine GCs (Uenoyama and Okuda, 1997), hGLCs (Nathwani et al., 2000), human ovarian carcinoma (OVCAR-3) cells and human ovarian surface epithelial (hOSE) cells (Kang et al., 2001a). Partial agonists, such as tamoxifen, are now better termed selective estrogen receptor modulators (SERMs) (Shang and Brown, 2002). Tamoxifen competes with E2 for binding to a site within the estrogen receptor (ER) ligand binding domain (Brzozowski et al. 1997). Tamoxifen or E2 work by binding to intracellular ERs and forming a complex (Shang and Brown, 2002). This complex can interact with target genes, either by binding directly to DNA response elements or through indirect tethering to other DNA binding transcription factors (Shang and Brown, 2002). Crystal structures of the ER bound to different ligands (E2, tamoxifen) reveal that ligands of different sizes and shapes induce different conformational states (Brzozowski et al. 1997). Depending on these states, co-regulator proteins are recruited to the receptor (Rosenfeld and Glass, 2001). Co-regulator proteins fall into two main functional classes. Co-activators that enhance receptor capacity to stimulate gene transcription and co-repressors which help receptors in turning off transcription of target genes (Rosenfeld and Glass, 2001). In terms of gene activation by nuclear receptors, a high level of co-activator promotes efficient gene activation,

whereas a high concentration of co-repressor acts to retard gene activation by receptors (Rosenfeld and Glass, 2001). Such co-regulator proteins may determine the tissue specific effects of mixed agonist/antagonist molecules such as tamoxifen (Shang and Brown, 2002). Tamoxifen may induce a receptor conformation intermediate to that induced by a pure agonist or pure antagonist. This conformation has an intermediate affinity for both co-activators and co-repressors (O'Mally and Strott, 1999). Therefore, if the cellular concentration of co-repressors is high, tamoxifen could act as an antagonist. In contrast, if the cellular concentration of co-activators is high, tamoxifen could act as an agonist (O'Mally and Strott, 1999). For example, the agonism of tamoxifen in the uterus might be because of the higher level of steroid receptor co-activator 1 (SRC1) in uterine cells than in mammary gland cells (Shang and Brown, 2002).

Antide is the decapeptide N-Ac-D-Nal(2), D-phe(pCl),Dpal(3),Ser,Lys(Nic),D-Lys(Nic),Leu,Lys(iPr),Pro,D-Ala-NH2 [Nal(2) represents 3-(2-naphthyl)alanine; Phe(p-Cl) represents 3-(-4chlorophenyl)alanine; Pal(3) represents 3-(3-pyridyl)alanine; Lys(Nic) represents N^{ε}-nicotinoyllysine; Lys-(iPr) represents N^{ε}-isopropyllysine] (Ljungqvist et al., 1988). Antide is an antagonist of GnRH which releases negligible histamine and has high anti-ovulatory activity (Ljungqvist et al., 1988). Antide was shown to be a GnRHI antagonist in pituitary cells (Ljungqvist et al., 1988), and in corpus luteum (Duffy et al., 2000) and a GnRHI and GnRHII antagonist in hOSE cells (Kang et al., 2000b), and OVCAR-3 cells (Kang et al., 2000a).

2. RATIONALE

GnRH agonists are used to treat many reproductive tract disorders such as endometriosis, fibroma, and endometrial cancer (Lefevre et al., 1991; Testart et al., 1993). GnRH agonists are also very commonly used in IVF programs (Ortmann et al., 2001). For example, exogenous administration of GnRH agonists is used to suppress endogenous gonadotropin secretion. This treatment, followed by exogenous administration of gonadotropins, results in ovulation induction in women (Ortmann et al., 2001). However, These treatment regimens are all based on the regulatory effects at the level of the hypothalamic-pituitary axis. Recently, GnRHI and GnRHII and their receptor have been discovered in different extrapituitary tissues, including the ovary. This observation suggests that the direct effects of GnRH agonists on follicle development and quality need to be re-examined (Lefevre et al., 1991; Testart et al., 1993). Therefore, a better understanding of the biological functions of the two GnRH forms as autocrine or paracrine factors in the ovary, may play an important role in the discernment of assisted reproductive technologies outcomes. As gonadal steroids are main regulators of reproductive functions, the present study examined the effects of E2 and P4 on GnRHI and GnRHII in hGLCs in an attempt to understand the biological functions that the two GnRH forms play in the human ovary. Furthermore, a number of reports have indicated that GnRHI and GnRHII can directly induce apoptosis in the ovaries of some mammalian and non-mammalian vertebrates (Imai et al., 2000; Billig et al., 1994; Yano et al., 1997; Andreu-Vieyra and Habibi, 2000). Since there is evidence that luteolysis might occur by apoptosis (Quirk et al., 1995), the present study also examined the direct effects of GnRHI and GnRHII on the incidence of apoptosis in

hGLCs, to define the putative roles of GnRHI and GnRHII in controlling the fate of the corpus luteum.

3. HYPOTHESIS

The levels of GnRHI and GnRHII mRNA produced by hGLCs, will change with time in culture and will be dynamically regulated by locally produced E2 and P4 during the luteal phase of the menstrual cycle. Furthermore, GnRHI and GnRHII will be capable of promoting apoptosis in hGLCs which may partly mediate the degradation of the corpus luteum.

4. OBJECTIVES

1. To examine GnRHI and GnRHII mRNA level, in hGLCs obtained from the same patient, at different times of culture.
2. To examine the ability of gonadal steroids, and anti-steroidal compounds to regulate GnRHI and GnRHII mRNA level in hGLCs obtained from the same patient.
3. To determine whether GnRHI and GnRHII promote apoptosis in hGLCs obtained from the same patient.

5. MATERIALS AND METHODS

5.1 Granulosa luteal cell culture and treatments with E2, tamoxifen, RU486, GnRHI, GnRHII, and antide

The use of hGLCs was approved by the Clinical Screening Committee for Research and Other Studies involving Human Subjects of the University of British Columbia. Follicular aspirates were collected during oocyte retrieval from women undergoing IVF at the University of British Columbia. The hGLCs were prepared according to methods described by Peng et al (1994) with some modifications. Briefly, the follicular aspirates were centrifuged at 1000 xg for 10 min and the supernatant was removed. The cells were resuspended in 6 ml of culture medium 199 (M199) (Gibco, Burlington, Ontario, Canada). Granulosa cells were then separated from red blood cells by centrifugation through equal volume of Ficoll Paque (Pharmacia Biotech, Morgan, Canada) at 1000 xg for 20 min. Cells at the interface were collected and washed once with M199. After brief centrifugation, the cell pellet was resuspended in M199 supplemented with10% fetal bovine serum (FBS), 100 U/ml penicillin G and 100 μg/ml streptomycin at a density of $1X10^5$ cells/ml. The cells were plated at a density of approximately $2X10^5$ cells per dish, in 35-mm culture dishes. The dishes were incubated at 37 °C under a water saturated atmosphere of 5% CO2 in air.

After 4 days of culture, the cells were transferred into phenol red free M199 supplemented with 2% charcoal stripped FBS for 24h. The cells were then serum starved for 4 hours and treated with the appropriate hormones (E2, tamoxifen, RU486, GnRHI, GnRHII, antide) in a dose and time dependent fashion. To examine the effects of E2 or RU486, in a dose dependent manner, on the expression of GnRHI and GnRHII mRNA in

cultured hGLCs, 5 day old cultured cells obtained from each patient were treated with different concentrations of E2 (0, 1, 10, 100nM) or RU486 (0, 0.01, 1, 100, 10000nM) for 24 hours. To examine the effects of E2 or RU486, in a time dependent manner, on the expression of GnRHI and GnRHII mRNA in cultured hGLCs, 5 day old cultured cells obtained from each patient were treated with 1nM of E2 or 10000nM of RU486 for 0, 6, 12, 24 and 48 hours. To determine whether the regulation of GnRHI and GnRHII by E2 is a receptor mediated process, 5 day old cultured hGLCs obtained from each patient were treated with 100nM of E2 in combination with varying doses of tamoxifen (0, 10, 100nM) for 24h. To examine changes in the level of apoptosis, and the regulation of GnRHI and GnRHII mRNA with time in culture, hGLCs were cultured for 1, 4, 8, 10 days with no treatment. To examine the time dependent effects of GnRHI or GnRHII on the induction of apoptosis, on day 5 of culture, hGLCs were treated with 10^3nM of GnRHI or GnRHII for 0,6,12,24,48 hours. Half of the culture dishes obtained from each patient were used for treatment with GnRHI and the other half were used for treatment with GnRHII. To examine the dose dependent effects of GnRHI or GnRHII on apoptosis, on day 5 of culture, hGLCs were treated for 12 hours with different doses (0, 10, 10^2, 10^3nM) of GnRHI or GnRHII. Some of the cells were also co-treated with antide plus GnRHI or antide plus GnRHII (10^3/10^3nM) and some were treated with antide (10^3nM) only. Half of the culture dishes obtained from each patient were used for treatment with GnRHI and the other half were used for treatment with GnRHII. In all time dependent studies, for each time point a control was included to take into account the changes during time in culture.

5.2 Total RNA extraction and first strand cDNA synthesis

All the cells, except the ones used for apoptosis studies, were subjected to RNA extraction. An Rnaid kit was used to extract total RNA from cultured hGLCs (Bio/Can Scientific, Mississauga, Canada) according to the manufacturer's protocol. Briefly, the cells were lysed and subjected to acid phenol extraction. RNA was purified from the aqueous phase on an RNA matrix and eluted into ribonuclease-free water. One μg of total RNA was reverse transcribed into first strand cDNA in a total volume of 15μl using the first strand cDNA synthesis kit (Amersham Pharmacia Biotech.). RNA concentration was determined at 260 nm using a spectrophotometer.

5.3 Semi-quantitative PCR and southern blot analysis

After completing total RNA extraction and first strand cDNA synthesis, the resultant products were subjected to PCR and southern blot analysis. In each trial, some of the 1st strand cDNA resulting from the cells obtained from each patient were subjected to PCR for GnRHI and some for GnRHII. PCR amplifications were carried out in 50 μl reactions containing 2 μl of cDNA, 2.5 units of Taq polymerase (Gibco-BRL Life Technologies, Burlington, Canada) and its buffer, 1.5mM MgCl2, 2mM dNTP and 50 pmol of forward and reverse primer. Primers for GnRHI were designed based on the published sequence for human hypothalamic GnRH. The forward and reverse primers were 5'-ATTCTACTGACTTGGTGCGTG-3' and 5'-GGAATATGTGCAACTTGGTGT-3', respectively. Forward and reverse primers for GnRHII were 5'-GCCCACCTTGGACCCTCAGAG-3' and 5'-CCAATAAAGTGTGAGGTTCTCCG-3' respectively (Nathwani et al. 2000). PCR amplification for GnRHI was carried out for 26

cycles with denaturing at 94°C for 60 sec, annealing at 53°C for 35 sec and extension at 72°C for 90 sec, followed by a final extension at 72°C for 15 min. PCR for GnRHII was carried out with denaturing for 1 min at 94°C, annealing for 65 sec at 62°C, extension for 90 sec at 72°C, and a final extension for 15 min at 72°C for 26 cycles (Nathwani et al. 2000). The size for GnRHII and GnRHI cDNAs were 327 and 380 bp respectively. Following electrophoresis, PCR products were transferred to a nylon membrane and fixed using UV irradiation. The blotted membranes were prehybridized for 3h at 42°C in prehybridization solution containing 50% formamide, 5x SSC, 0.1% N-lauroyl sarcosine, 0.02% SDS and 2% blocking solution. The prehybridized membranes were hybridized overnight at 42°C with digoxigenin-labled GnRHII or GnRHI cDNA probes. Membranes were then washed 2 times, 10 min each time, in ample 2x SSC and 0.1% SDS at room temperature and 2 times, 15 min each time, in 0.1x SSC and 0.1% SDS at 68°C, respectively. The hybridized membranes were detected with luminescence method (Boehringer Mannheim Co.) and exposed to Kodak Omat X-ray film. The specific bands were scanned and quantified using a computerized visual light densitometer (model 620, Biorad Laboratories, Richmond, CA). To standardize for the first strand cDNA synthesis efficiency, PCR for GAPDH was performed for 18 cycles. Primers for GAPDH were designed according to the published sequence (Ng et al., 1985).

5.4 Apoptosis assay

To quantify the promotion of apoptosis, DNA fragmentation was measured using the cell death detection ELISA kit (Roche Molecular Biochemicals) according to the manufacturer's protocol with some modifications. Briefly, the cells were harvested and centrifuged at 200 xg for 5 min. The supernatant was discarded and the pellet was diluted with culture medium to obtain a cell concentration of $1X10^4$ cells/ml. One ml of this dilution was centrifuged at 200 xg for 5 min. The cell pellet was resuspended in 500 µl of a lysis buffer (0.5mmol/L dithiothreitol, 1mmol/L ethylene glycol-tetra acetic acid, 1mmol/L sodium bicarbonate, 10 mmol/L N-[2-hydroxyethyl] piperazine-N-[2-ethanesulfonic acid], pH 7.9) for 30 min at 4°C. The supernatant containing fragmented DNA was then removed by centrifugation at 20,000 xg for 10 min. The supernatant was diluted 1:5 in the lysis buffer and overlaid in duplicate in microtiter plate modules already coated with antihistone antibody. The nucleosomes contained in the sample bound via their histone components to the immobilized antihistone antibody. An anti-DNA-peroxidase was then added to the microtitre plate modules. Anti-DNA-peroxidase reacts with the DNA part of the nucleosomes. Next, a substrate (2, 2'- azino-di-[3-ethylbenzthiazoline sulfonate]) for anti-DNA-peroxidase was added to the microtitre plate modules. The resultant photometric data were measured with an ELISA plate reader at 405nm against the control.

5.5 Statistical analysis

Each experiment was done with three different patient samples. The data were shown as mean +/- standard deviation (SD) and were represented as the percent change relative to the control. The raw data were analyzed by one-way analysis of variance

(ANOVA) followed by the Tukey's multiple comparison test before transformation to percentage (PRISM Graphed Version 2, Graphed Software, Inc., San Diego, USA).

$P \leq 0.05$ was considered statistically significant.

6. RESULTS

6.1 Validation of semi-quantitative RT-PCR for GAPDH, GnRHI, and GnRHII

Semi-quantitative RT-PCR was used to examine the mRNA levels of GnRHI and GnRHII in hGLCS. Southern blot analysis revealed a 372-bp product corresponding to GAPDH (Fig. 3), a 380 bp product corresponding to GnRHI (Fig. 4) and a 327 bp product (Fig. 5) corresponding to GnRHII. A linear relationship was found between the cycle numbers, 12 to 21, 23 to 32, 23 to 35, and optical density for GAPDH (Fig. 3), GnRHI (Fig. 4) and GnRHII (Fig. 5) respectively. As a result, 26 cycles for GnRHI and GnRHII and 18 cycles for GAPDH were chosen for quantification.

6.2 Changes in GnRHI and GnRHII mRNA expression with time in culture

For quantitative purposes, all time points were standardized to day 1 mRNA levels. There was a 120% ($P < 0.05$) and 195% ($P < 0.05$) increase in GnRHI mRNA levels on days 8 and 10 compared to day 1 cultures, respectively (Fig. 6). A similar trend was observed for GnRHII mRNA levels. There was a 210% ($P < 0.05$) and 220% ($P < 0.05$) increase in GnRHII mRNA levels on days 8 and 10 compared to day 1 cultures, respectively (Fig. 7). When day 4 cultures were compared to days 8 and 10, there was a significant increase in GnRHI and GnRHII mRNA levels with increasing time in culture. However, there was no significant difference between either day 8 and day 10 cultures or day 4 and day 1 cultures for GnRHI and GnRHII mRNA levels.

6.3 Dose and time dependent effects of E2 on GnRHI and GnRHII mRNA levels in cultured hGLCs

In the dose dependent studies, after 24h, GnRHI mRNA levels from all treatment groups decreased significantly compared to the control. However, there was no significant difference between different treatment groups (Fig. 8). One nM of E2 decreased GnRHI mRNA levels by 55% ($P < 0.05$) relative to the control. Ten nM and 100nM doses of E2 resulted in a decrease of 62% ($P < 0.05$) and 71% ($P < 0.05$) in GnRHI mRNA levels compared to the control (Fig. 8). In contrast, as a result of a 24h dose dependent treatment with E2, GnRHII mRNA levels of different treatment groups increased significantly compared to the control (Fig. 9). A 294% ($P < 0.05$), 382% ($P < 0.05$) and 156% ($P < 0.05$) increase in GnRHII mRNA level was observed with 1nM, 10nM and 100nM of E2 respectively. The mRNA level of the 100nM treatment group was significantly lower than the other two treatment groups (Fig. 9).

In the time dependent studies, 6h and 12h treatment with 1nM E2 had no significant effect on GnRHI mRNA levels. GnRHI mRNA levels decreased significantly compared to the 0h treatment group, only 24 and 48h after treatment (Fig. 10). Twenty four and 48h treatment with 1nM E2 resulted in 48% ($p < 0.05$) and 77% ($p < 0.05$) decrease in GnRHI mRNA level compared to the 0h treatment group (Fig. 10). GnRHI mRNA level of the 48h treatment group was not significantly different compared to the 24h treatment group (Fig. 10). In contrast, GnRHII mRNA level increased significantly for every treatment group, compared to the 0h treatment group (Fig. 11). Treatment with 1nM of E2 for 6h, 12h, 24h and 48h, resulted in 105% ($p < 0.05$), 204% ($p < 0.05$), 280% ($p < 0.05$) and 150% ($p < 0.05$) increase in GnRHII mRNA level compared to the 0h treatment

group, respectively (Fig. 11). In addition, the GnRHII mRNA levels of the 12h and 24h treatment groups were significantly higher compared to the 6h treatment group. The GnRHII mRNA level of the 48h treatment group was significantly lower compared to the 24h treatment group but still significantly higher than the 0h treatment group (Fig. 11). For each time point a control was included to take into account the changes during time in culture (data not shown).

6.4 Dose and time dependent effects of RU486 on GnRHI and GnRHII mRNA levels in cultured hGLCs

In the dose dependent studies, after 24h, there was no significant difference between the vehicle treated group and the RU486 treated groups in terms of GnRHI mRNA levels (Fig. 12). In contrast, GnRHII mRNA level showed a dose dependent increase with an increasing concentration of RU486 (Fig. 13). A 75% ($p < 0.05$), 220% ($p < 0.05$), 189% ($p < 0.05$) and 330% ($p < 0.05$) increase in GnRHII mRNA level was observed at doses of 0.01, 1, 100 and 10000nM of RU486, respectively (Fig. 13). The 1 and 100nM treatment groups had a significantly higher GnRHII mRNA level compared to the 0.01 nM treatment group as well as compared to the control group. The level of GnRHII mRNA in the 10000nM treatment group was also significantly higher than all other treatment groups (Fig. 13).

In the time course studies, there was no significant difference between different RU486 treated groups and the 0h treatment group in terms of GnRHI mRNA level (Fig. 14). However, all RU486 treated groups had a significantly higher level of GnRHII mRNA compared to the 0h treatment group (Fig. 15). A 243% ($p < 0.05$), 191% ($p <$

0.05), 362% ($p < 0.05$) and 414% ($p < 0.05$) increase was observed in GnRHII mRNA level after 6, 12, 24, 48h treatment with 10000nM of RU486, respectively (Fig. 15). The 24 and 48h treatment groups had a significantly higher level of GnRHII mRNA compared to the 6 and 12h treatment groups as well as compared to the 0h treatment group (Fig. 15). For each time point a control was included to take into account the changes during time in culture (data not shown).

6.5 Effects of co-treatment with E2 and tamoxifen on GnRHI and GnRHII mRNA levels in cultured hGLCs

Treatment with 100nM of E2 significantly decreased GnRHI mRNA level compared to the no treatment group (Fig. 16). When the cells were co-treated with 10nM of tamoxifen and 100nM of E2, the mean GnRHI mRNA level increased but no significant change was observed compared to the E2 only treatment group. However, equimolar treatment with E2 and tamoxifen reversed the down-regulating effect of E2 (Fig. 16). Therefore, no significant difference was observed between the equimolar treatment group and the no treatment group but there was a significant difference between the E2 only treatment group and the equimolar treatment group (Fig. 16). Treatment with 100nM of E2 significantly increased GnRHII mRNA level compared to the no treatment group (Fig. 17). When the cells were co-treated with 10nM of tamoxifen and 100nM of E2, there was no significant difference between this treatment group and the E2 only treatment group; however, the mean mRNA level of GnRHII was lower compared to E2 only treatment group. The GnRHII mRNA level of this co-treatment group was significantly higher compared to the no treatment group (Fig. 17). The GnRHII mRNA level of the

equimolar co-treatment group (100nM of tamoxifen plus 100nM of E2) was not significantly different from the no treatment group. However, the mRNA level of the equimolar co-treatment group, was significantly lower from the other co-treatment group and the E2 only treatment group (Fig. 17).

6.6 Changes in the incidence of apoptosis with time in cultured hGLCs

Human GLCs were cultured for 1, 4, 8 and 10 days. All time points were standardized to the level of apoptosis at day 1. Maximum levels of DNA fragmentation (51%, $p<0.05$) were observed at day 8 (Fig. 18). The levels remained elevated until the termination of these studies at day 10. DNA fragmentation levels at days 8 and 10 were not significantly different from each other but were significantly higher than day 4. The level of apoptosis at day 4 was not significant compared to day1 (Fig. 18).

6.7 Time and dose dependent effects of GnRHI on apoptosis in cultured hGLCs

In the time dependent studies, 12h treatment with 10^3nM of GnRHI significantly increased DNA fragmentation (205%; $p < 0.05$) compared to the 0h treatment group (Fig. 19). The levels of DNA fragmentation remained elevated for the 24 and 48h treatment groups. There was no significant difference between the 6h and the 0h treatment group (Fig. 19).

In the dose dependent studies, 12h treatment only with 10^3nm of GnRHI significantly increased DNA fragmentation compared to the no treatment group (Fig. 20). Equimolar co-treatment with GnRHI and antide (10^3nM) completely blocked the effect of

GnRHI on apoptosis. Antide treatment alone did not have any effect on apoptosis (Fig. 20).

6.8 Time and dose dependent effects of GnRHII on apoptosis in cultured hGLCs

In the time course studies, 6h treatment with 10^3nM of GnRHII significantly increased DNA fragmentation (316%; $p < 0.05$) compared to the 0h treatment group (Fig. 21). The levels of DNA fragmentation remained elevated for the 12, 24 and 48h treatment groups (Fig. 21).

In the dose dependent studies, 12h treatment only with 10^3nm of GnRHII significantly increased DNA fragmentation compared to the no treatment group (Fig. 22). Equimolar co-treatment with 10^3nm of antide completely blocked the effect of GnRHII on apoptosis. Antide treatment alone did not have any effect on apoptosis (Fig. 22).

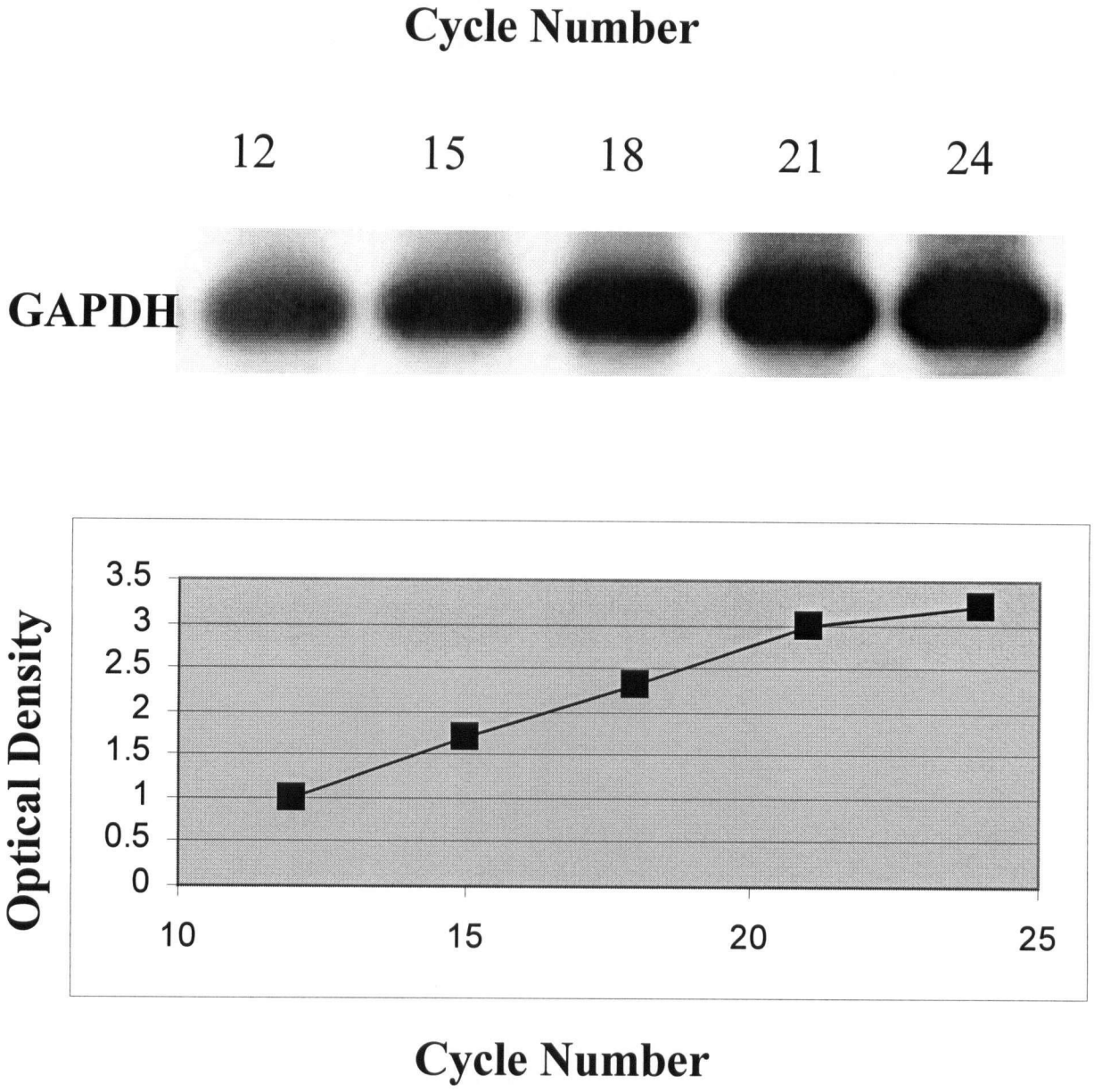

Fig3. Validation of semi-quantitative RT-PCR for GAPDH from hGLCs. Total RNA was extracted from the cells and 1μg of the RNA was subjected to reverse transcription. As a result of doing PCR a 372-bp fragment was obtained by agarose gel electrophoresis. In order to determine the linear phase of PCR amplification, GAPDH was amplified from the cDNA under increasing cycle numbers. A linear relationship between the cycle number and optical density was found between cycles 12 to 21. 18 cycles was chosen for quantitation of GAPDH mRNA from hGLCs.

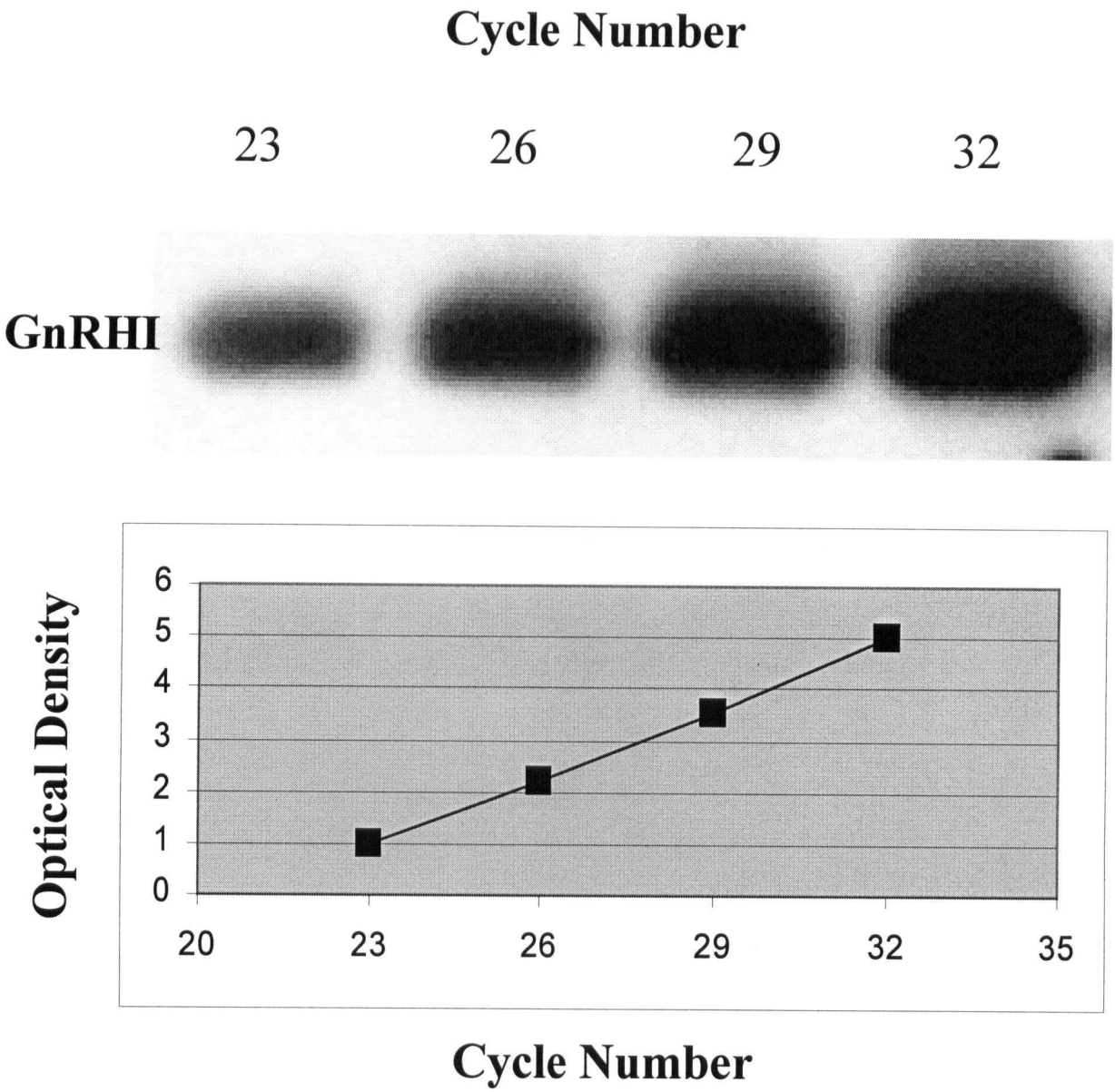

Fig4. Validation of semi-quantitative RT-PCR for GnRHI from hGLCs. Total RNA was extracted from the cells and 1μg of the RNA was subjected to reverse transcription. As a result of doing PCR a 380-bp fragment was obtained by agarose gel electrophoresis. In order to determine the linear phase of PCR amplification, GnRHI was amplified from the cDNA under increasing cycle numbers. A linear relationship between the cycle number and optical density was found between cycles 23 to 32. 26 cycles was chosen for quantitation of GnRHI mRNA from hGLCs.

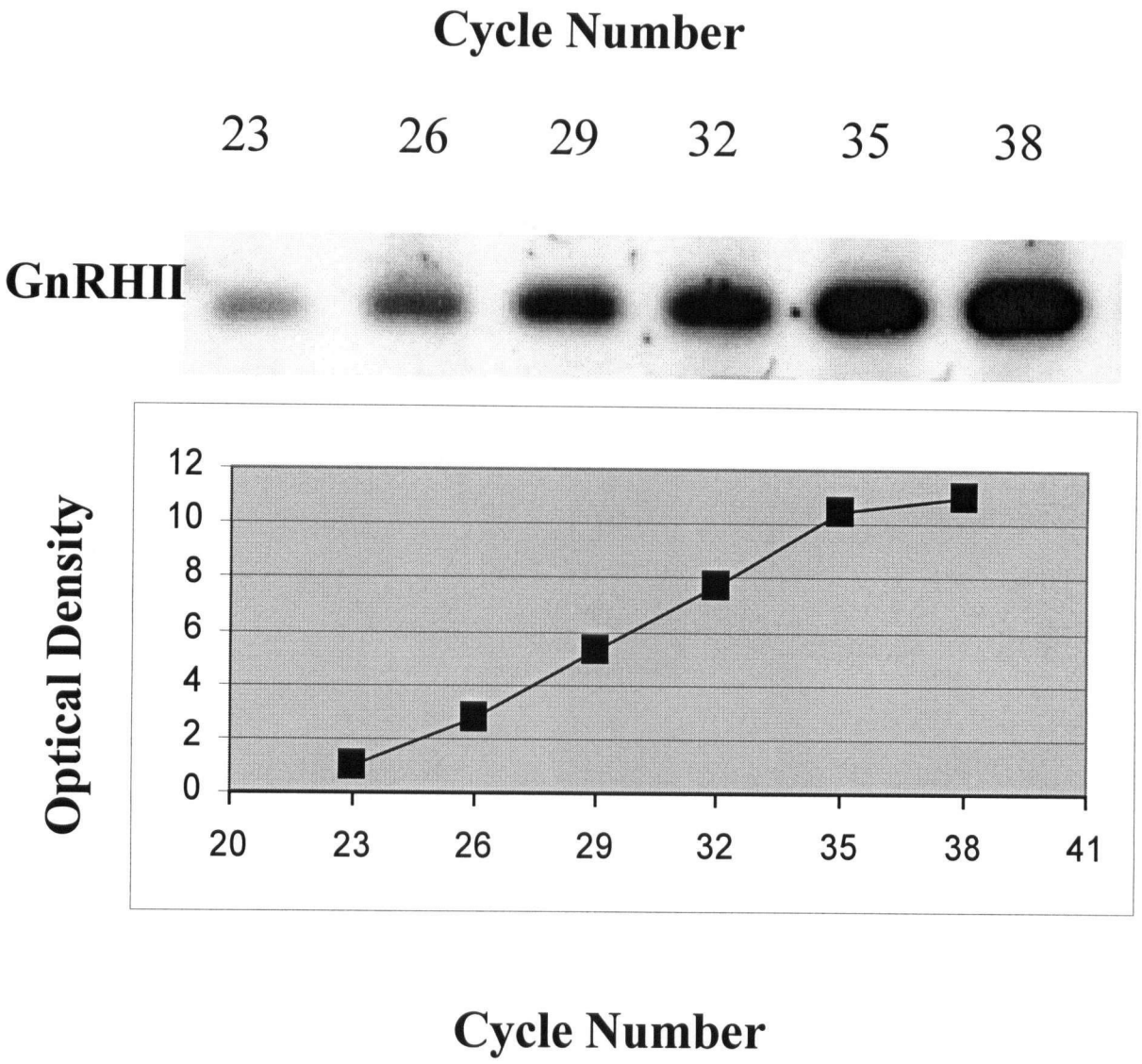

Fig5. Validation of semi-quantitative RT-PCR for GnRHII from hGLCs. Total RNA was extracted from the cells and 1μg of the RNA was subjected to reverse transcription. As a result of doing PCR a 327-bp fragment was obtained by agarose gel electrophoresis. In order to determine the linear phase of PCR amplification, GnRHII was amplified from the cDNA under increasing cycle numbers. A linear relationship between the cycle number and optical density was found between cycles 23 to 35. 26 cycles was chosen for quantitation of GnRHII mRNA from hGLCs.

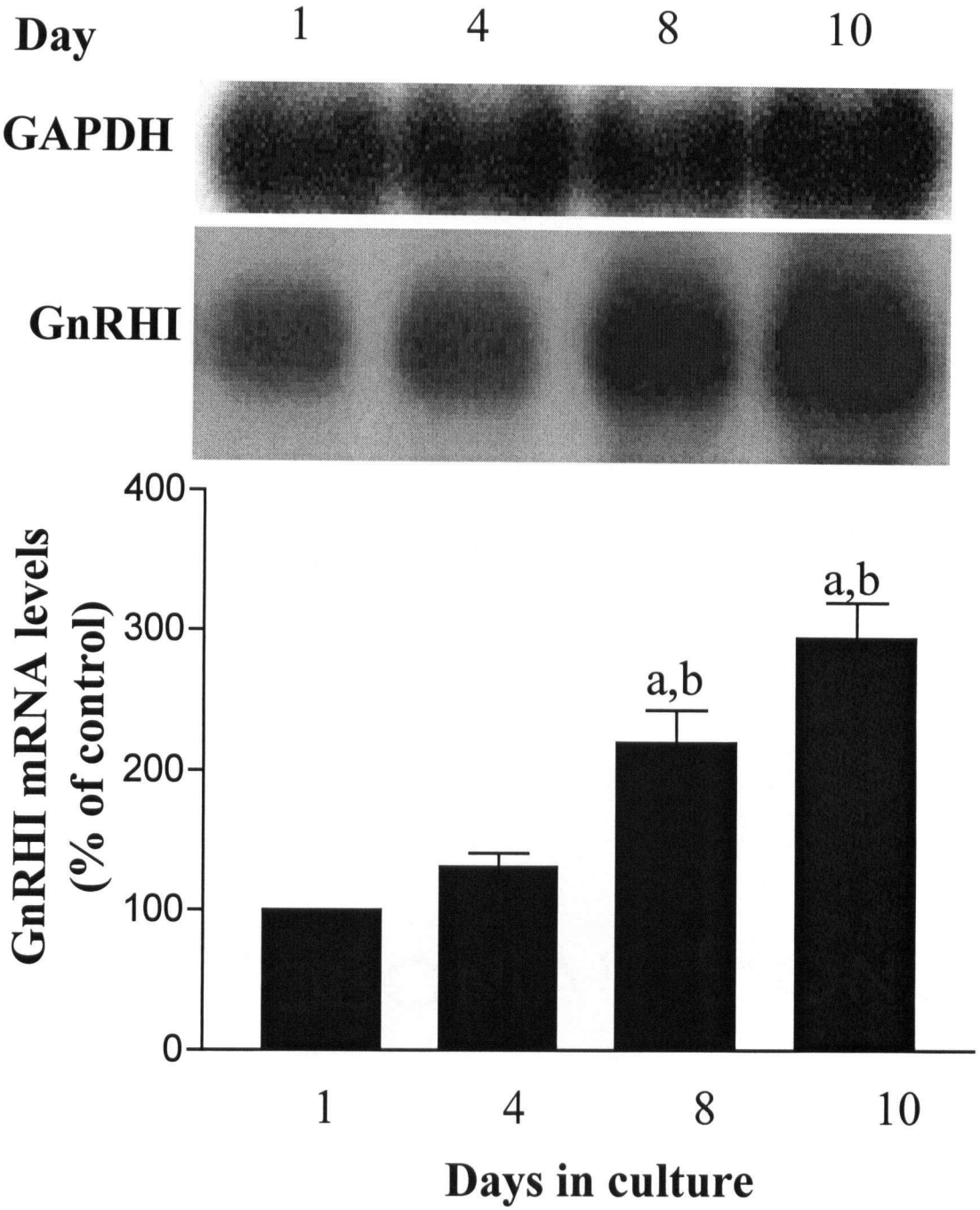

Fig6. Changes in GnRHI mRNA expression with time in culture. Total RNA was extracted from hGLCs (n=3) on days 1,4, 8, 10 in culture. One microgram of total RNA was reverse transcribed and RT-PCR was performed. The GnRHI mRNA levels were estimated by semi-quantitative RT-PCR and were normalized against the GAPDH mRNA. Data were expressed as percent change relative to control and represent the mean +/-SD of three different patients. a, $p<0.05$, significantly different from the control day 1 cultures. b,$p<0.05$, significantly different from the 4 day cultures.

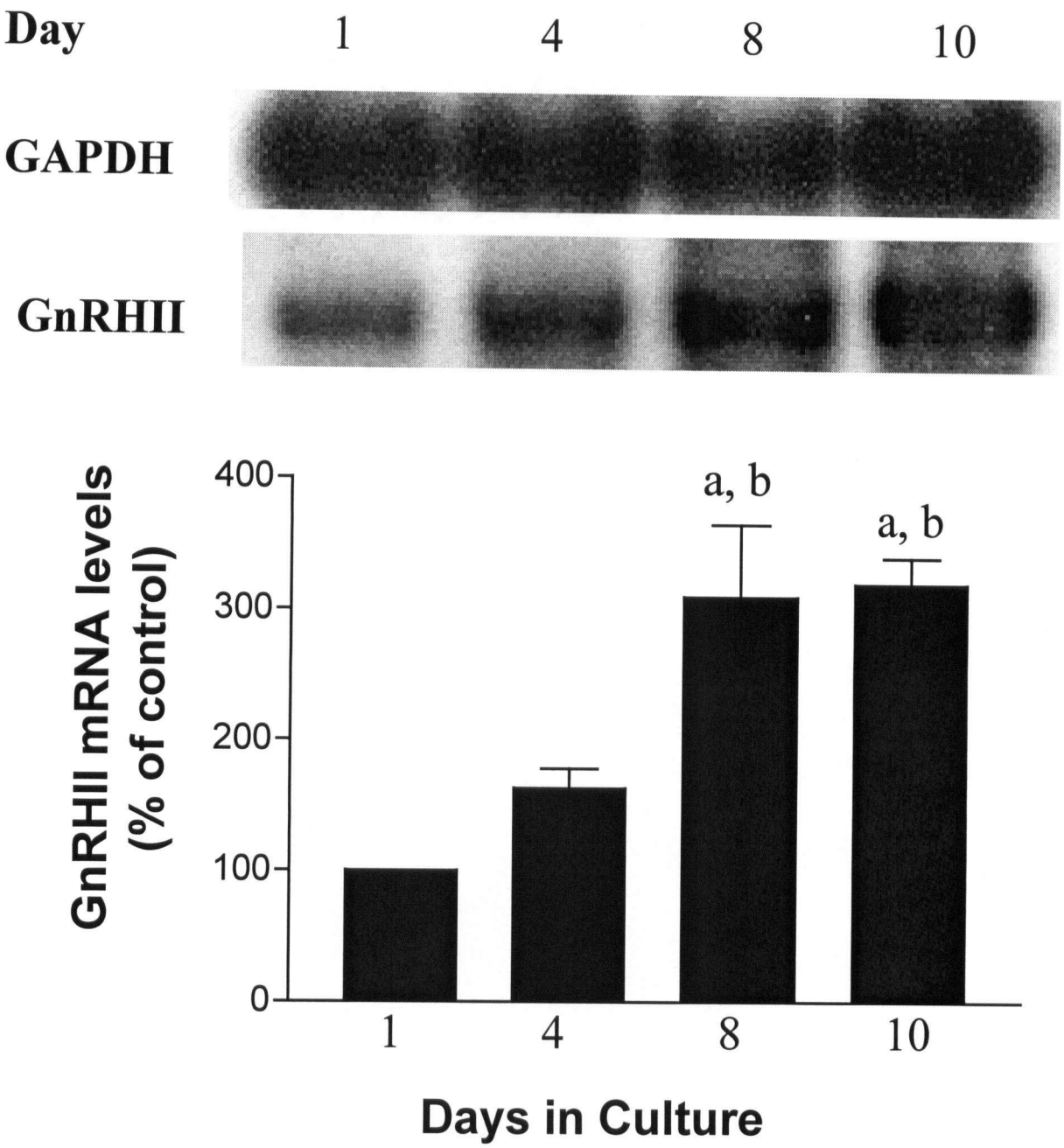

Fig7. Changes in GnRHII mRNA expression with time in Culture. Total RNA was extracted from hGLCs (n=3) on days 1, 4, 8, 10 in culture. One microgram of total RNA was reverse transcribed and PCR was performed. The GnRHII mRNA levels were estimated by semi-quantitative RT-PCR and were normalized against the GAPDH mRNA. Data were expressed as percent change relative to control and represent the mean +/-SD of three different patients. a, $p<0.05$, significantly different from the control day 1 cultures.

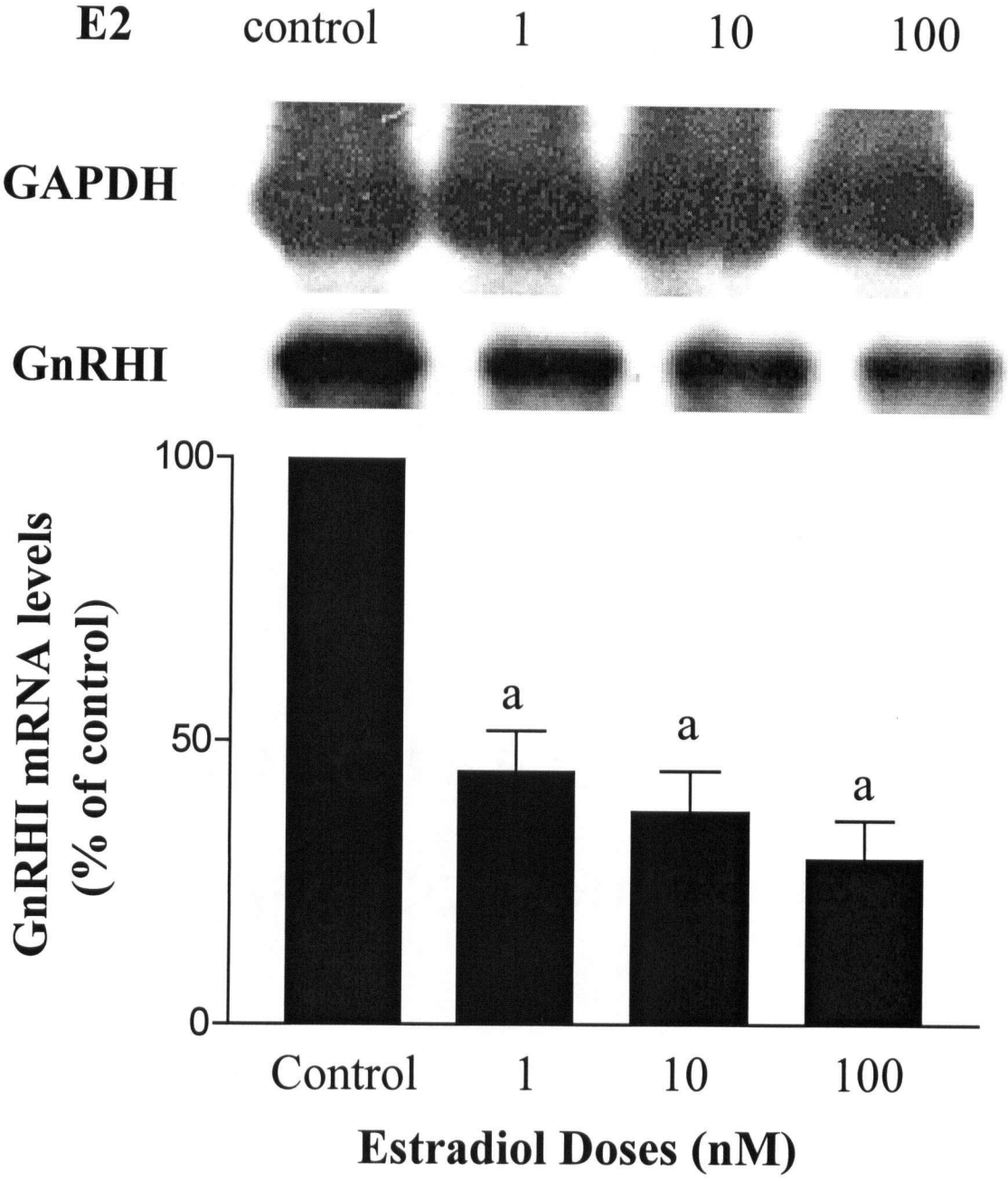

Fig8. The effects of varying concentrations of 17ß-estradiol on GnRHI mRNA levels in cultured hGLCs. On day 5 of the culture cells were treated for 24 hours with different doses of estrsadiol (0-100nM). GnRH mRNA levels were estimated by semiquantitative RT-PCR and were normalized against GAPDH mRNA. Data were expressed as percent change relative to control and represent the mean +/- SD of three different experiments from three different patients. a, $p<0.05$, significantly different from control.

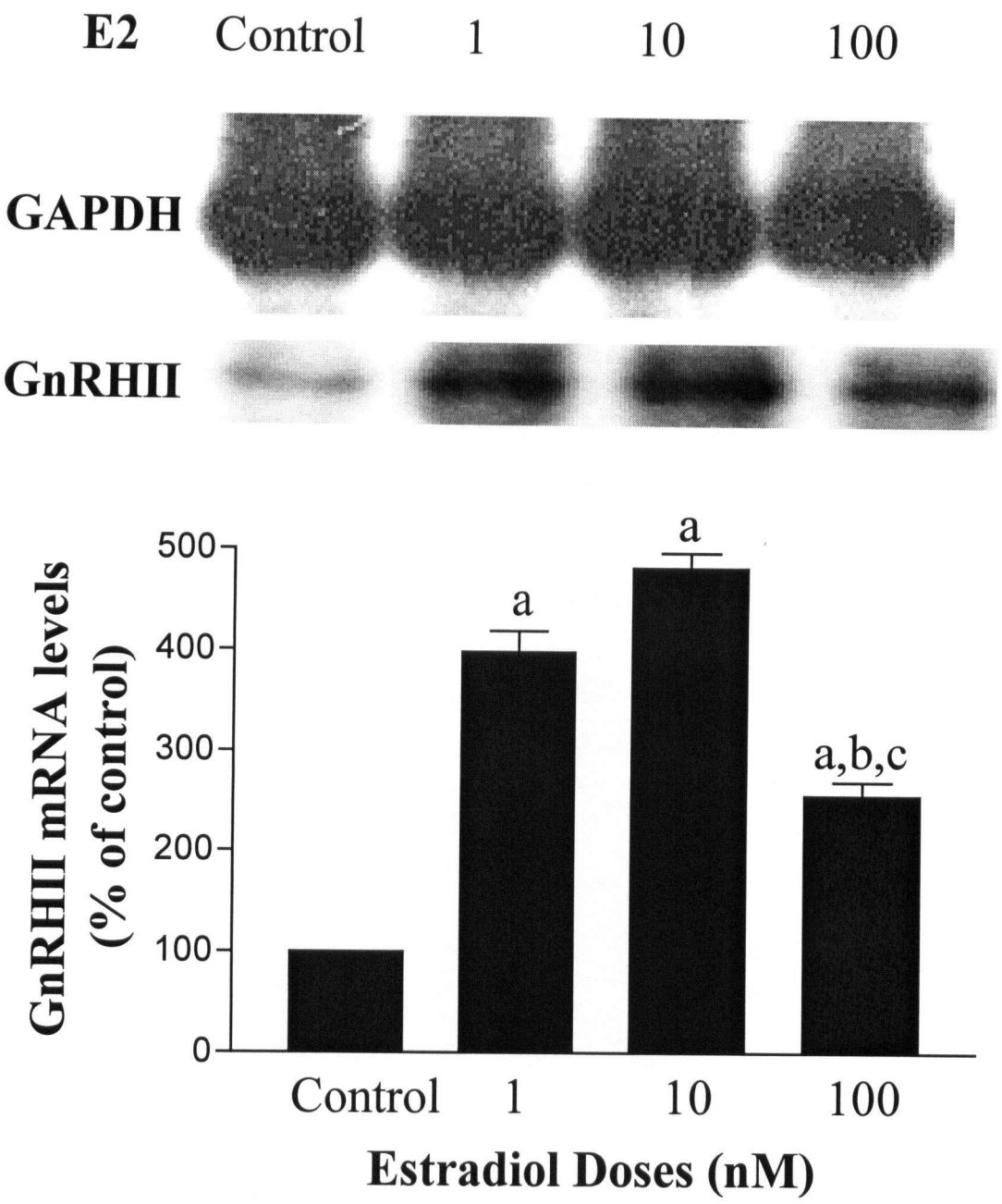

Fig9. The effects of varying concentrations of 17ßestradiol on GnRHII mRNA levels in cultured hGLCs. On day 5 of the culture cells were treated for 24 hours with different doses of estrsadiol (0-100nM). GnRHII mRNA levels were estimated by semi-quantitative RT-PCR and were normalized against GAPDH mRNA. Data were expressed as percent change relative to control and represent the mean +/- SD of three different experiments from three different patients. a, $p<0.05$, significantly different from control. b, $p<0.05$, significantly different from 1 nM treatment group. C, $p<0.05$, significantly different from 10 nM treatment group.

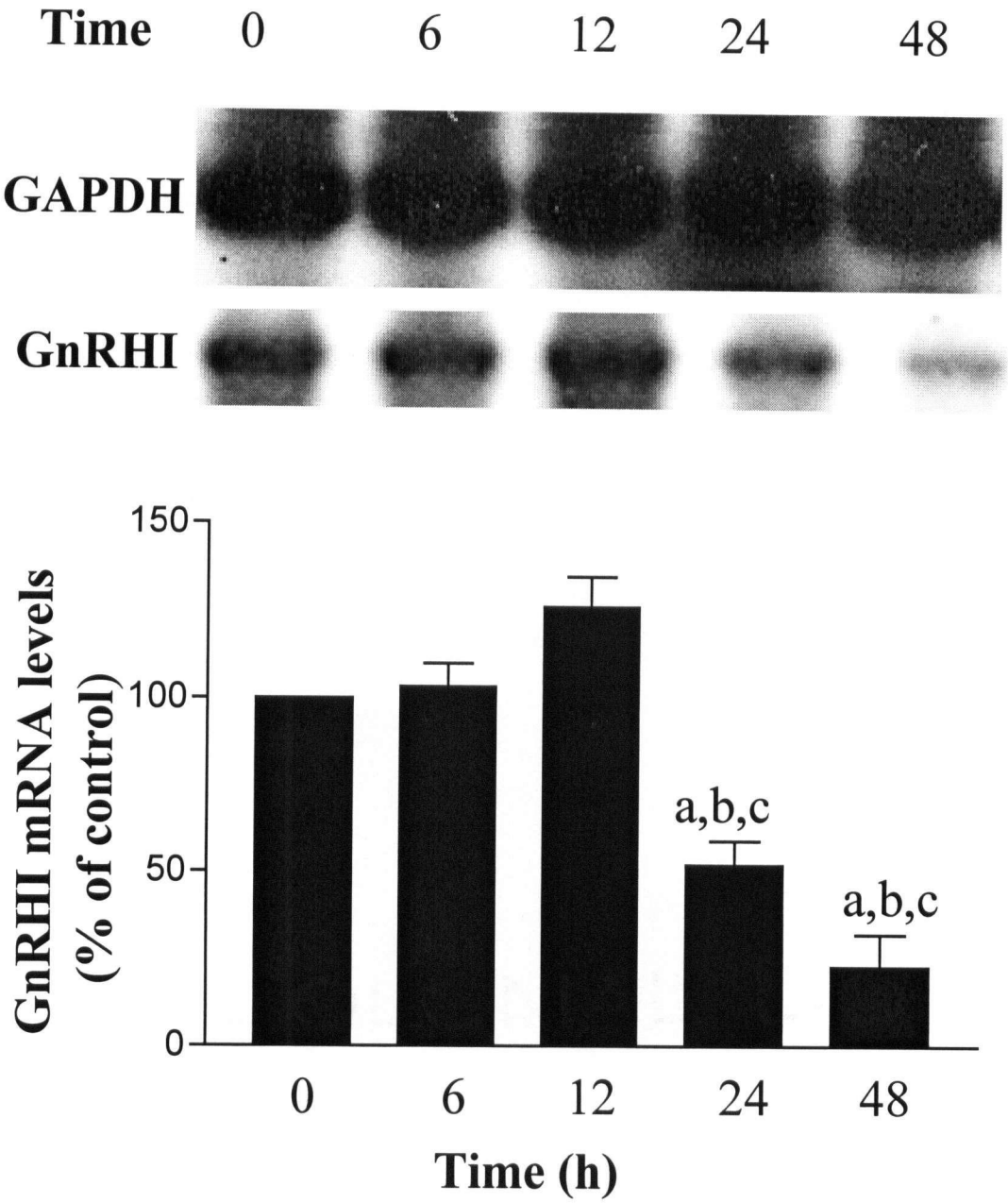

Fig10. Time dependent effects of 17ßestradiol on GnRHI mRNA levels in cultured hGLCs. On day 5 of the culture cells were treated with 1nM of estrsadiol for 0, 6, 12, 24, 48 hours. GnRHI mRNA levels were estimated by semi-quantitative RT-PCR and were normalized against GAPDH mRNA. Data were expressed as percent change relative to control and represent the mean +/- SD of three different experiments from three different patients. a, $p<0.05$, significantly different from 0 hour treatment group. b, $p<0.05$, significantly different from 6 hour treatment group. C, $p<0.05$, significantly different from 12 hour treatment group.

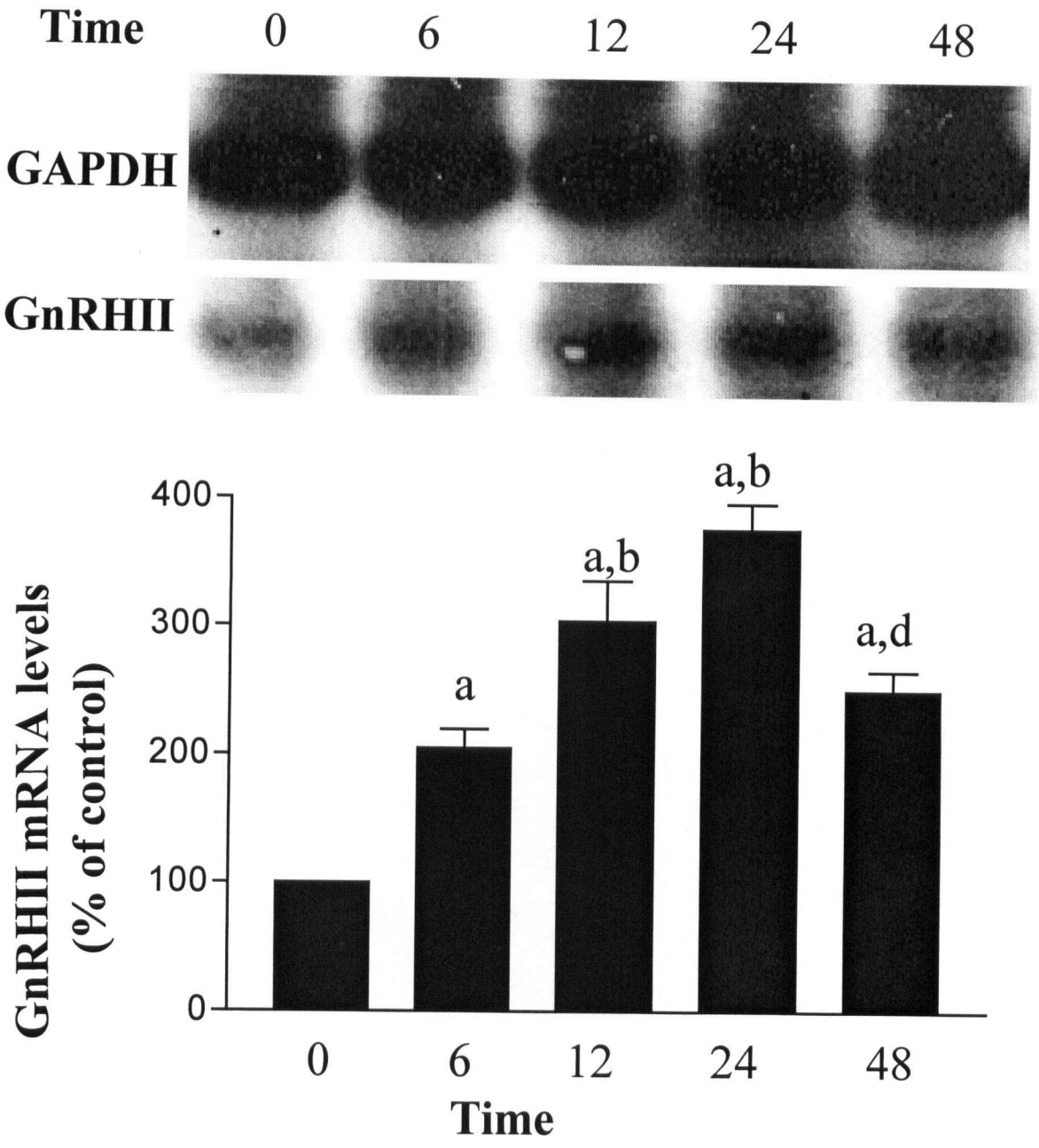

Fig11. Time dependent effects of 17ßestradiol on GnRHII mRNA levels in cultured hGLCs. On day 5 of the culture cells were treated with 1nM of estrsadiol for 0, 6, 12, 24, 48 hours. GnRHII mRNA levels were estimated by semi-quantitative RT-PCR and were normalized against GAPDH mRNA. Data were expressed as percent change relative to control and represent the mean +/- SD of three different experiments from three different patients. a, $p<0.05$, significantly different from 0 hour treatment group. b, $p<0.05$, significantly different from 6 hour treatment group. C, $p<0.05$, significantly different from 12 hour treatment group. d, $p<0.05$, significantly different from 24 hour treatment group.

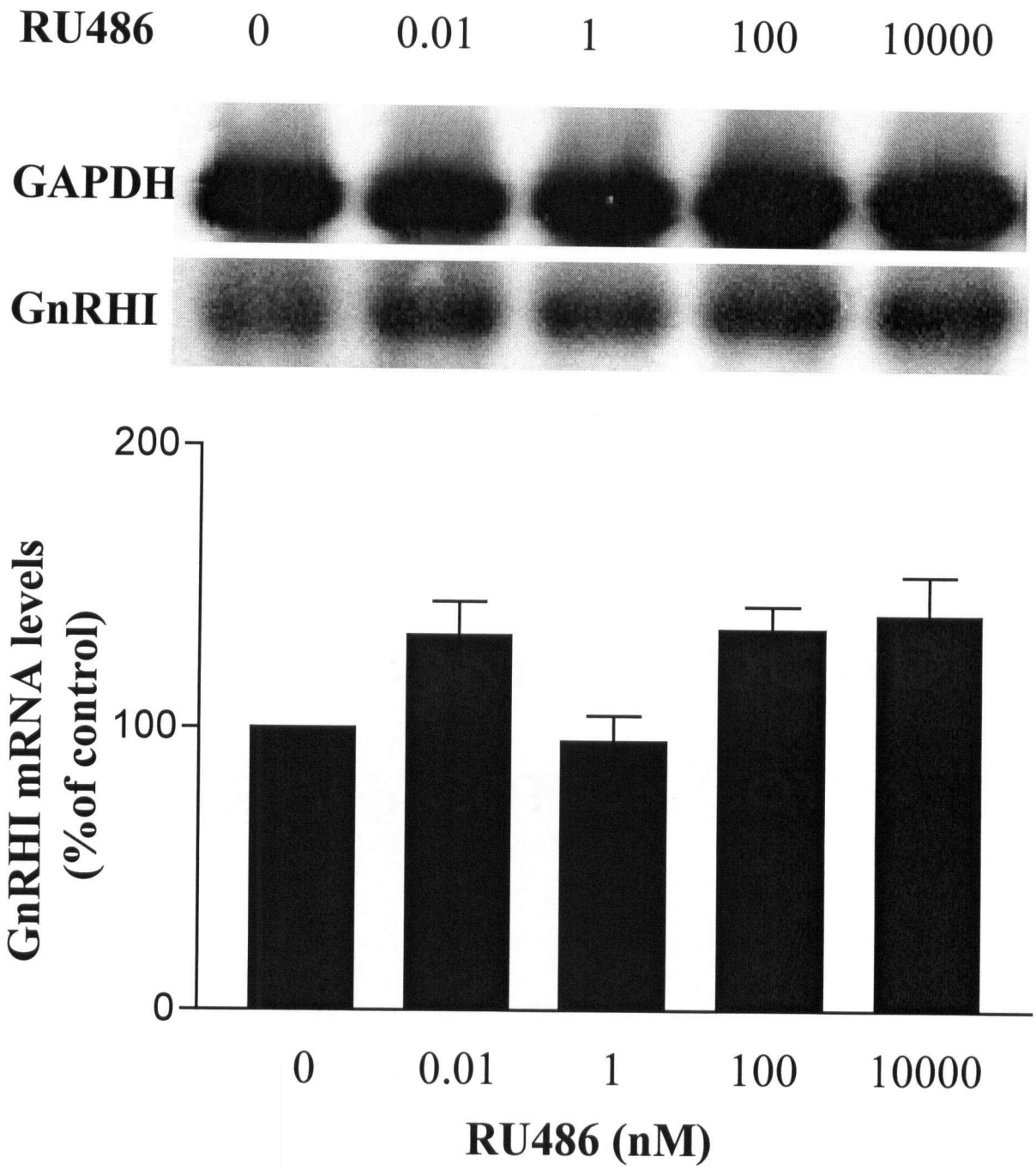

Fig12. The effects of varying concentrations of RU486 on GnRHI mRNA levels in cultured hGLCs. On day 5 of the culture cells were treated for 24 hours with different doses of RU486 (0-10000nM). GnRHI mRNA levels were estimated by semi-quantitative RT-PCR and were normalized against GAPDH mRNA. Data were expressed as percent change relative to control and represent the mean +/- SD of three different experiments from three different patients.

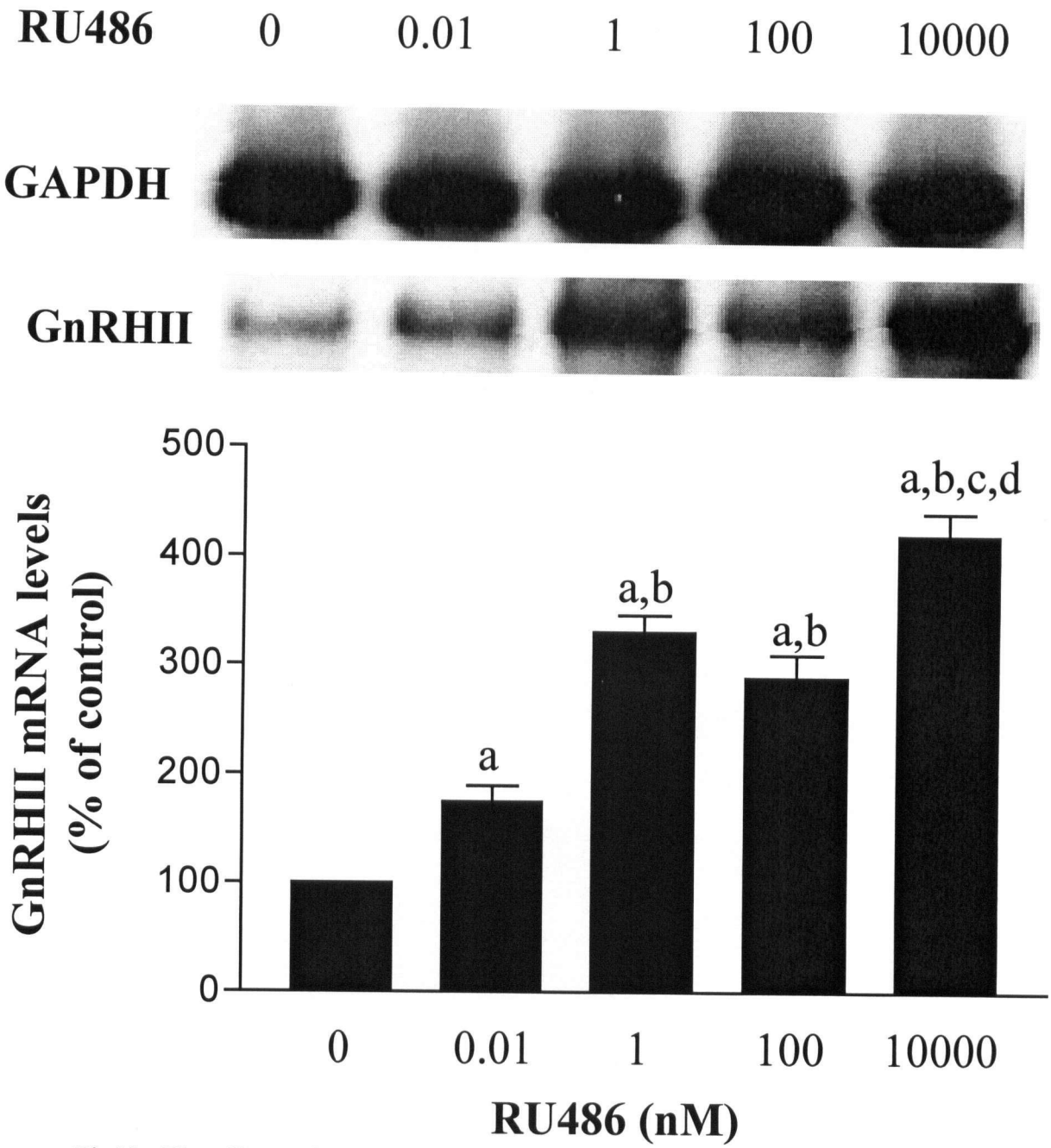

Fig13. The effects of varying concentrations of RU486 on GnRHII mRNA levels in cultured hGLCs. On day 5 of the culture cells were treated for 24 hours with different doses of RU486 (0-10000nM). GnRHII mRNA levels were estimated by semi-quantitative RT-PCR and were normalized against GAPDH mRNA. Data were expressed as percent change relative to control and represent the mean +/- SD of three different experiments from three different patients. a, $p<0.05$, significantly different from control. b, $p<0.05$, significantly different from 0.01 nM treatment group. C, $p<0.05$, significantly different from 1 nM treatment group. d, $p<0.05$, significantly different from 100 nM treatment group.

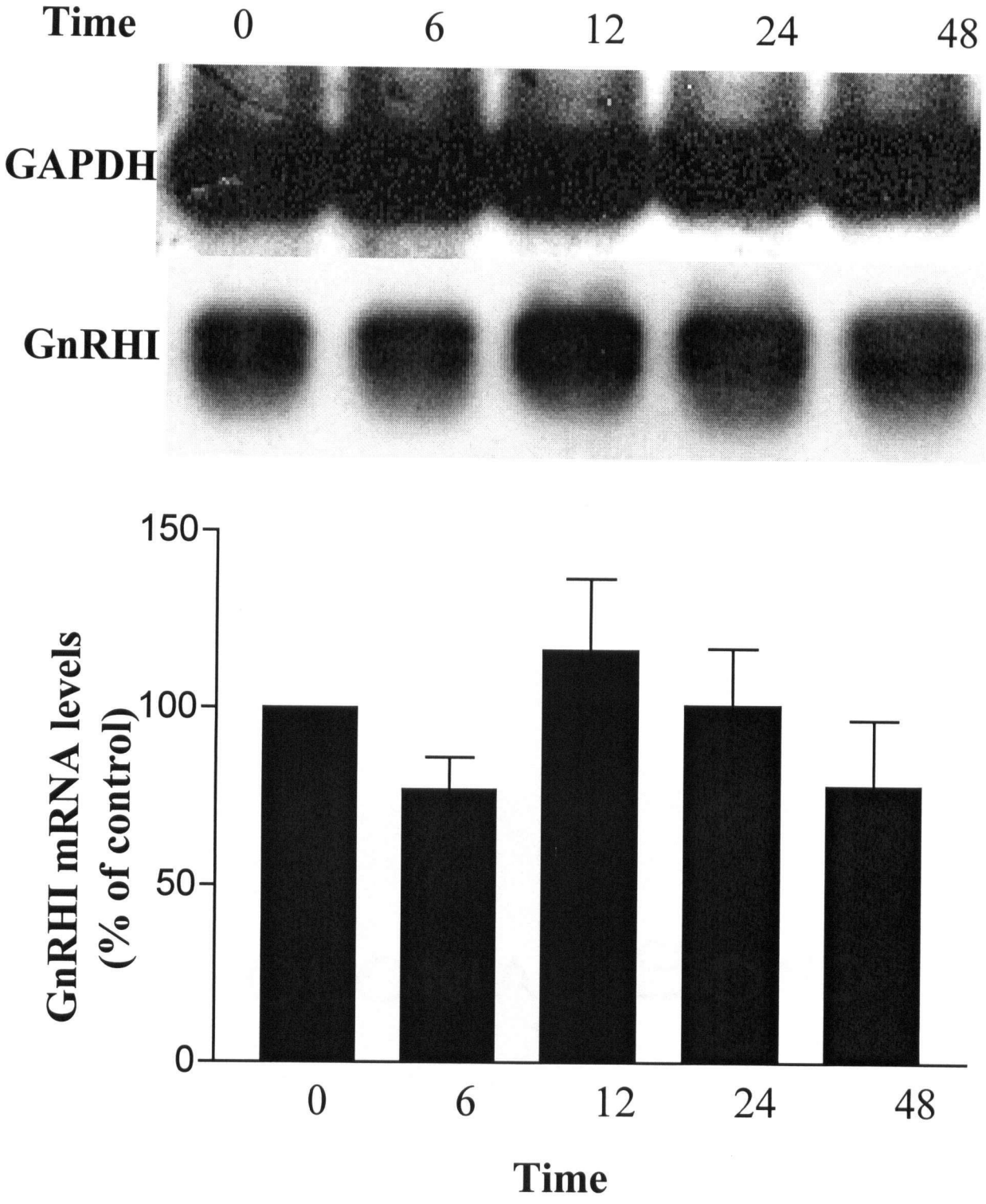

Fig14. Time dependent effects of RU486 on GnRHI mRNA levels in cultured hGLCs. On day 5 of the culture cells were treated with 10μM of RU486 for 0, 6, 12, 24, 48 hours. GnRHI mRNA levels were estimated by semi-quantitative RT-PCR and were normalized against GAPDH mRNA. Data were expressed as percent change relative to control and represent the mean +/- SD of three different experiments from three different patients.

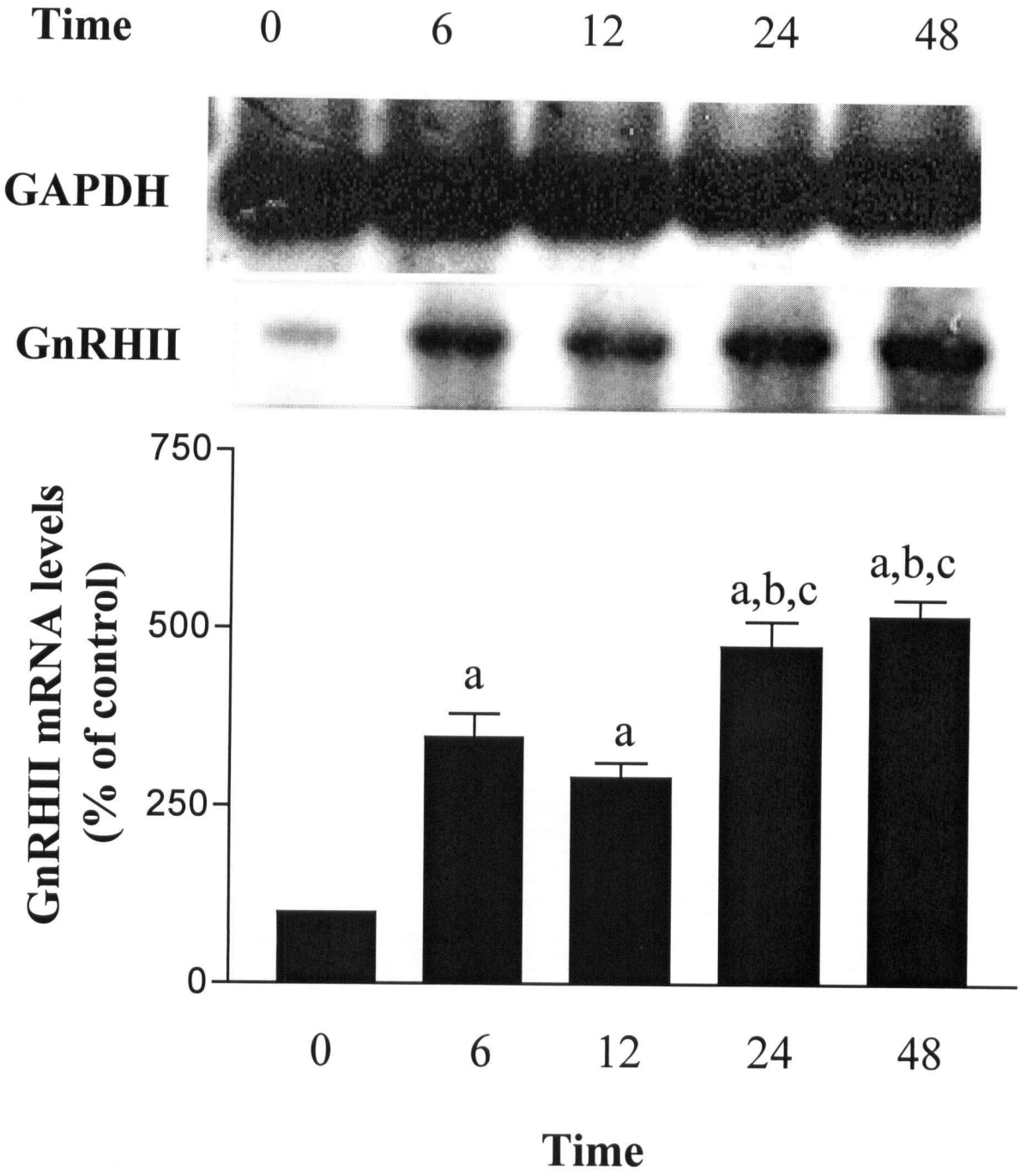

Fig15. Time dependent effects of RU486 on GnRHII mRNA levels in cultured hGLCs. On day 5 of the culture cells were treated with 10μM of RU486 for 0, 6, 12, 24, 48 hours. GnRHII mRNA levels were estimated by semi-quantitative RT-PCR and were normalized against GAPDH mRNA. Data were expressed as percent change relative to control and represent the mean +/- SD of three different experiments from three different patients. a, $p<0.05$, significantly different from 0 hour treatment group. b, $p<0.05$, significantly different from 6 hour treatment group. C, $p<0.05$, significantly different from 12 hour treatment group.

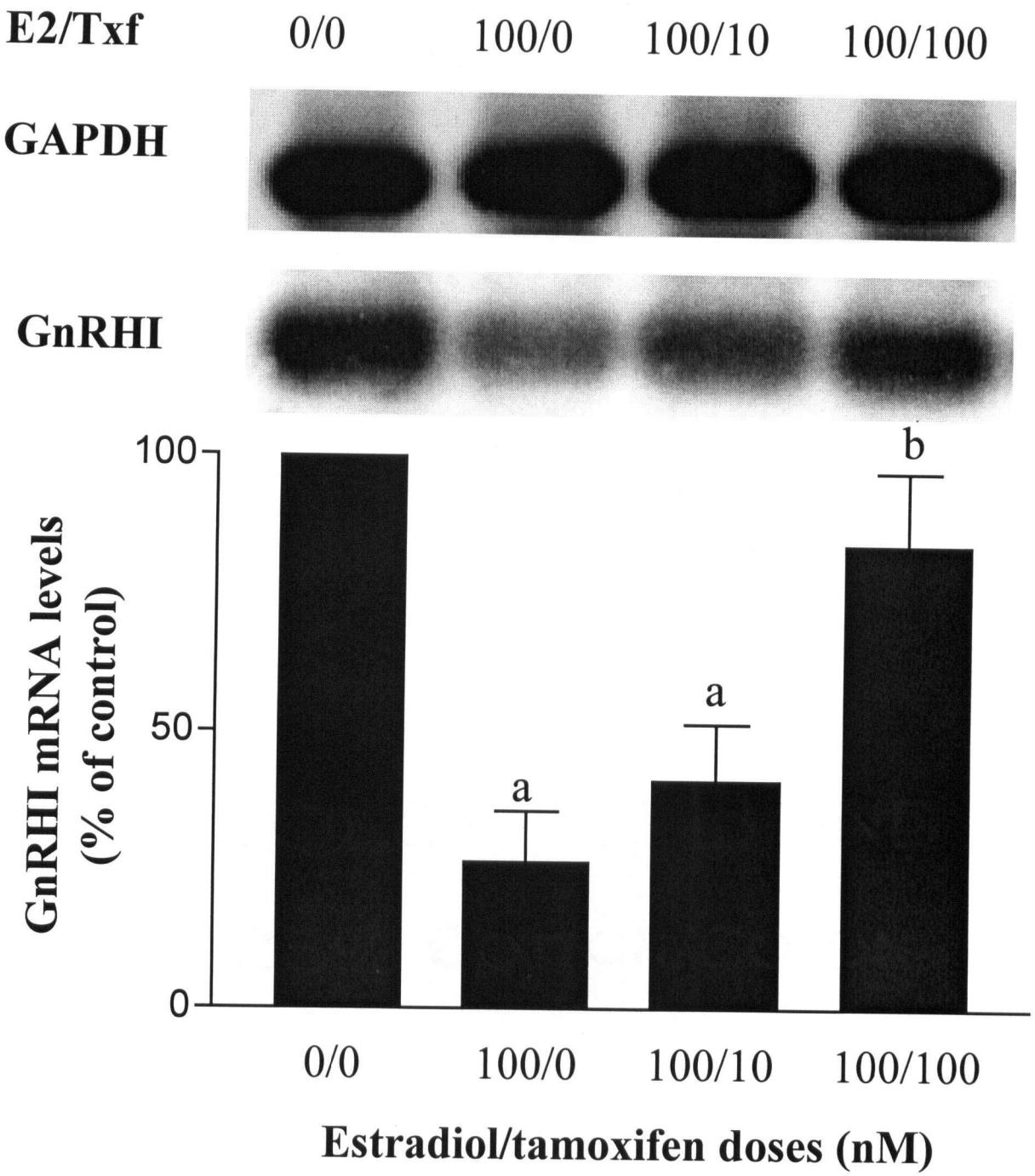

Fig16. The effect of 17ß-estradiol and tamoxifen co-treatment on GnRHI mRNA levels in cultured hGLCs. Cells were treated on day 5 with a 100 nM dose of estradiol in combination with varying doses of tamoxifen (0-100nM) for 24 hours. GnRHI mRNA levels were estimated by semi-quantitative RT-PCR and were normalized against GAPDH mRNA. Data were expressed as percent change relative to control and represent the mean +/- SD of three different experiments from three different patients. a, $p<0.05$, significantly different from the control group. b, $p<0.05$, significantly different from the E2 treatment group.

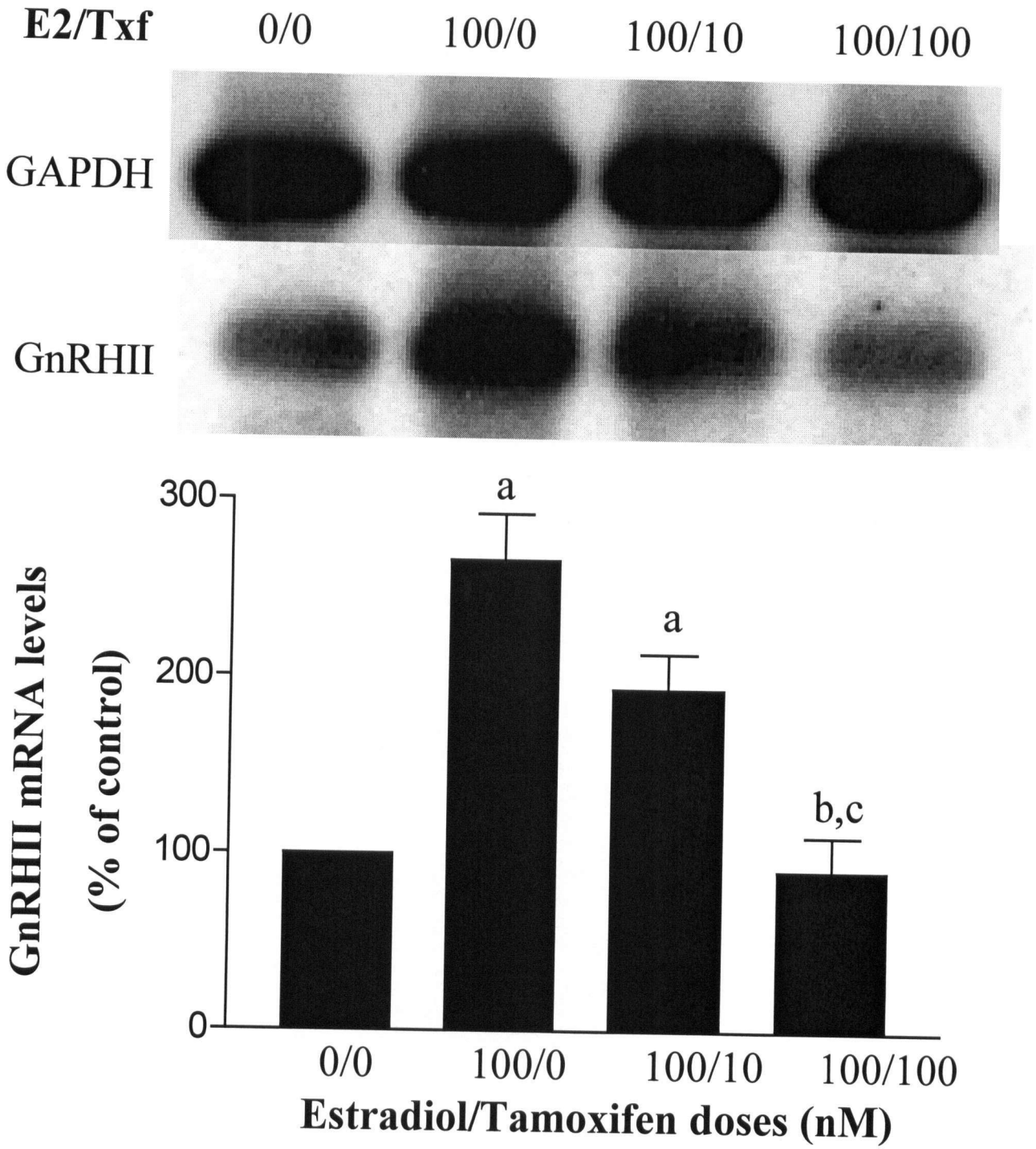

Fig17. The effect of 17ß-estradiol and tamoxifen co-treatment on GnRHII mRNA levels in cultured hGLCs. Cells were treated on day 5 with a 100 nM dose of estradiol in combination with varying doses of tamoxifen (0-100nM) for 24 hours. GnRHII mRNA levels were estimated by semi-quantitative RT-PCR and were normalized against GAPDH mRNA. Data were expressed as percent change relative to control and represent the mean +/- SD of three different experiments from three different patients. a, $p<0.05$, significantly different from the control group. b, $p<0.05$, significantly different from the E2 treatment group. c, $p<0.05$, significantly different from the 100/10 nM treatment group

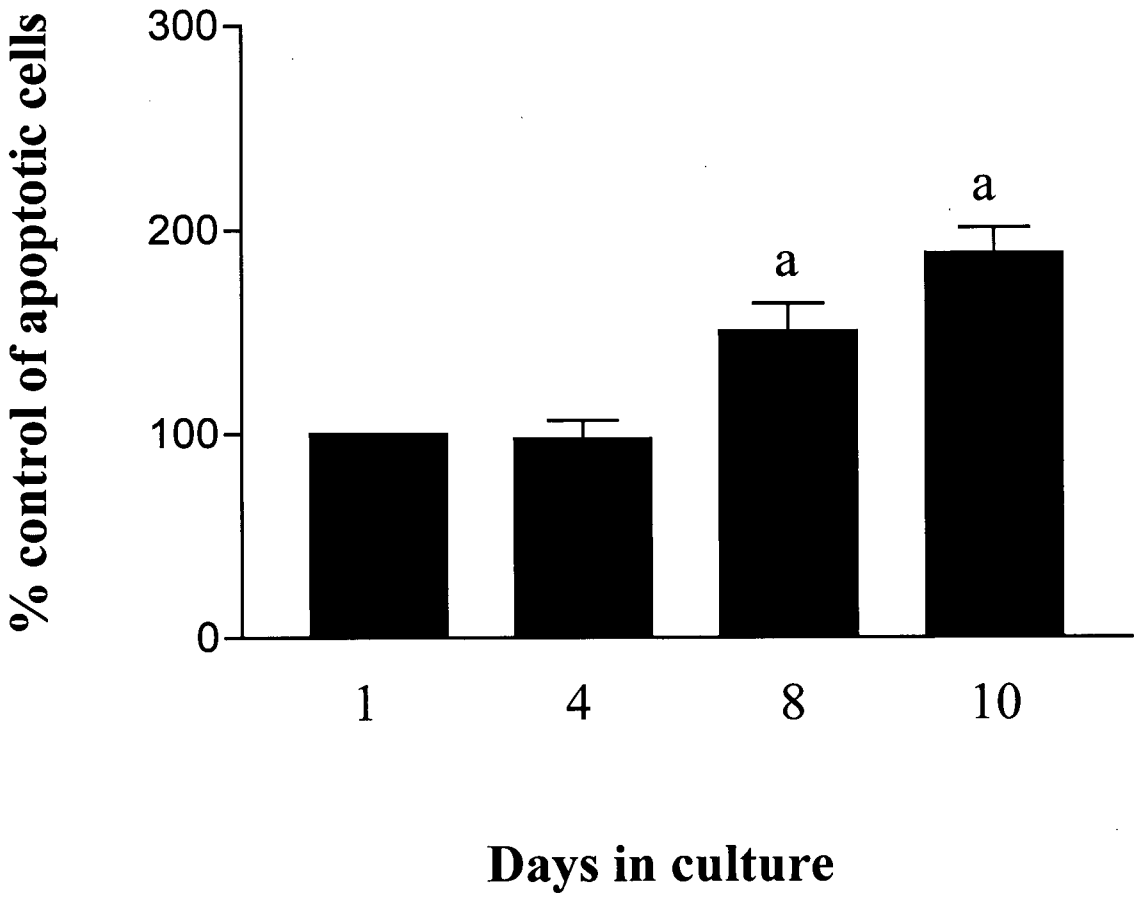

Fig 18. Changes in the incidence of apoptosis with time in cultured hGLCs. Cells were cultured for 1, 4, 8, 10 days and DNA fragmentation was measured by cell death detection ELISA. Data were expressed as percent change relative to control and represent the mean+/-SD of three experiments from three different patients. a, $p<0.05$, significantly different from the control group.

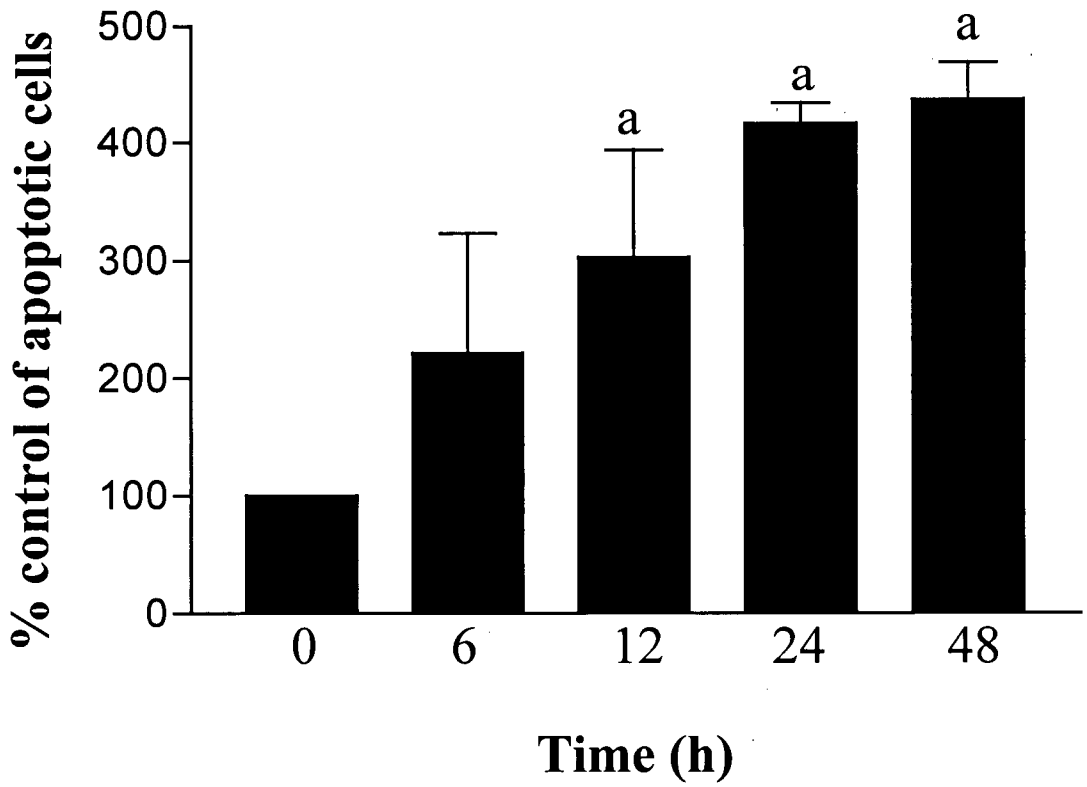

Fig 19. Time dependent effects of GnRHI on apoptosis in cultured hGLCs. To examine the role of GnRHI in apoptosis, DNA fragmentation was measured by cell death detection ELISA. To quantify the level of apoptosis, on day 5, cells were treated with 10^3 nM of GnRHI for 0,6,12,24,48 hours. Data were expressed as percent change relative to control and represent the mean+/-SD of three experiments from three different patients. a, $p<0.05$, significantly different from the 0 hour group.

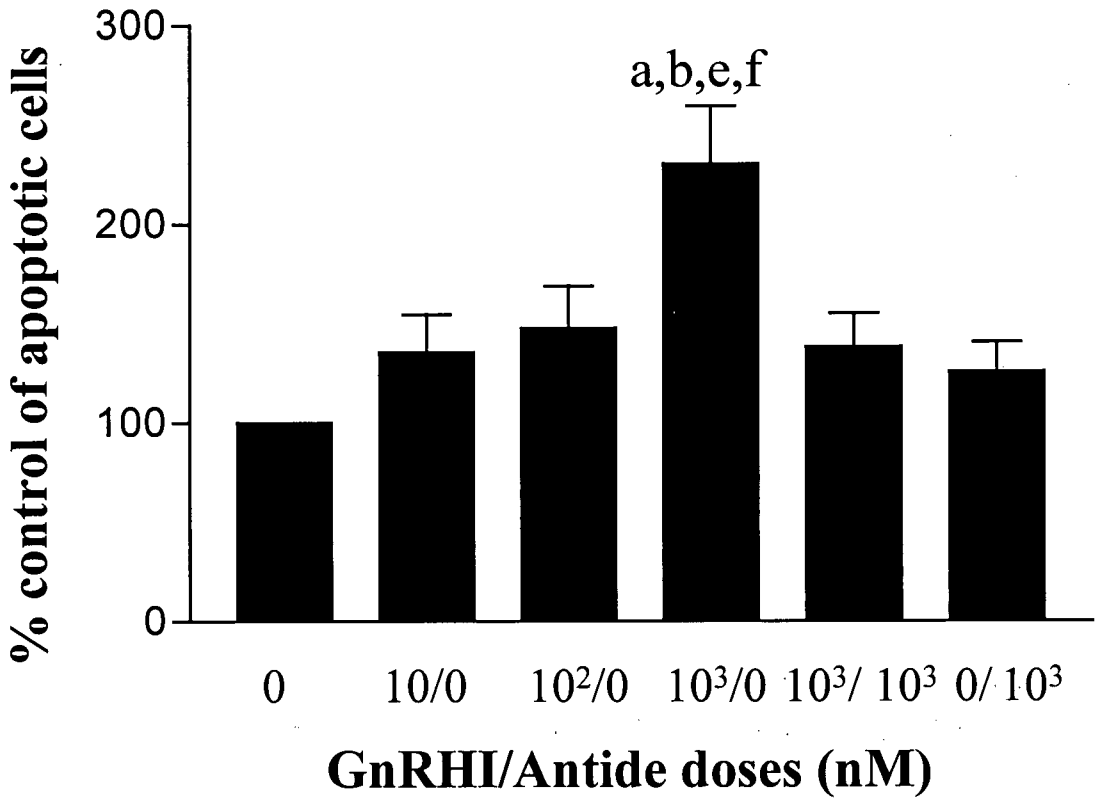

Fig 20. Dose dependent effects of GnRHI on apoptosis in cultured hGLCs. To examine the role of GnRHI in apoptosis, DNA fragmentation was measured by cell death detection ELISA. To quantify the level of apoptosis, on day 5, cells were treated for 12 hours with different doses of GnRHI and also in combination with antide. Data were expressed as percent change relative to control and represent the mean+/-SD of three experiments from three different patients. a, $p<0.05$, significantly different from the control group. b, $p<0.05$, significantly different from 10 nM treatment group. e, $p<0.05$, significantly different from the $10^3/10^3$ nM treatment group. f, $p<0.05$, significantly different from the $0/10^3$ nM treatment group

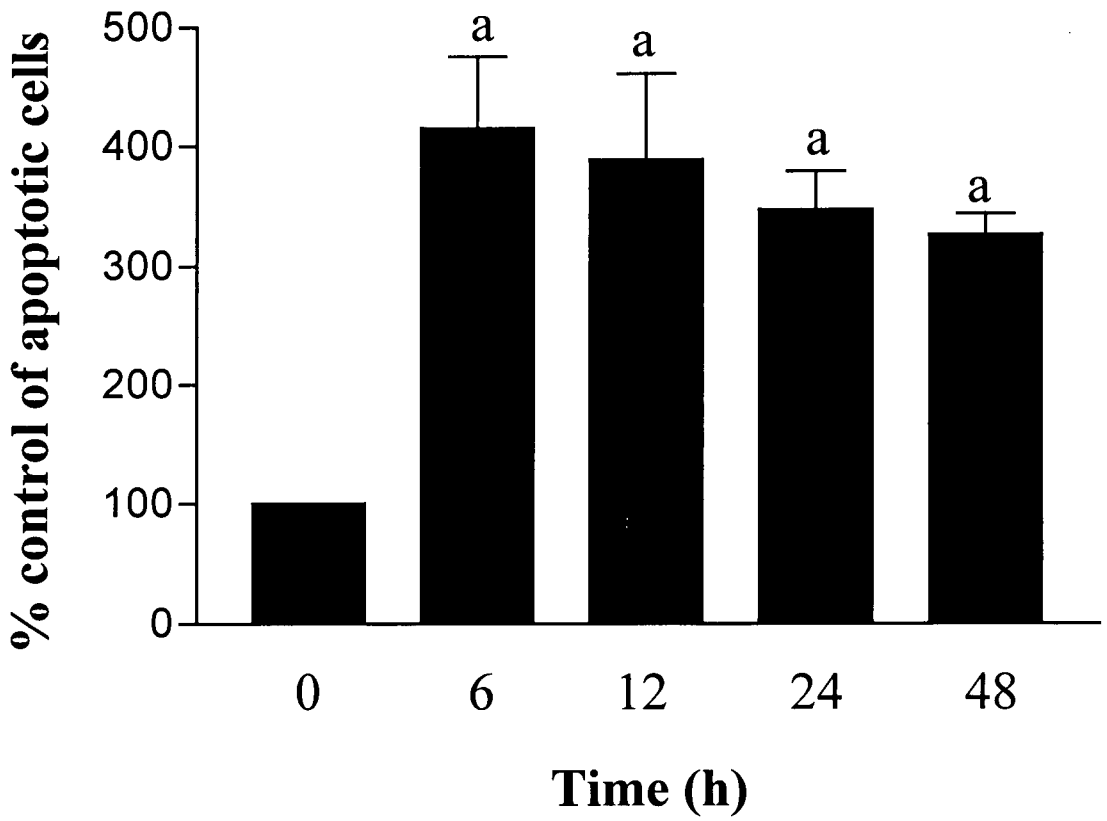

Fig 21. Time dependent effects of GnRHII on apoptosis in cultured hGLCs. To examine the role of GnRHII in apoptosis, DNA fragmentation was measured by cell death detection ELISA. To quantify the level of apoptosis, on day 5, cells were treated with 10^3 nM of GnRHII for 0,6,12,24,48 hours. Data were expressed as percent change relative to control and represent the mean+/-SD of three experiments from three different patients. a, $p<0.05$, significantly different from the 0 hour group.

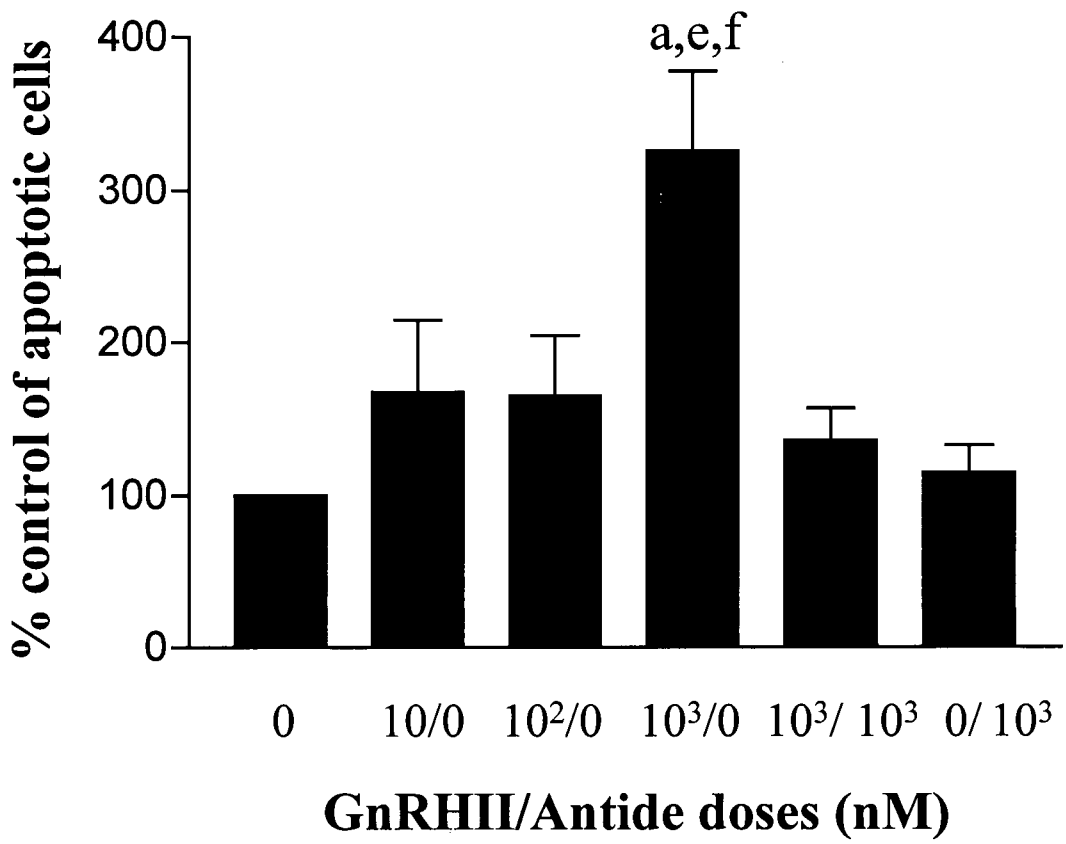

Fig 22. Dose dependent effects of GnRHII on apoptosis in cultured hGLCs. To examine the role of GnRHII in apoptosis, DNA fragmentation was measured by cell death detection ELISA. To quantify the level of apoptosis, on day 5, cells were treated for 12 hours with different doses of GnRHII and also in combination with antide. Data were expressed as percent change relative to control and represent the mean+/-SD of three experiments from three different patients. a, $p<0.05$, significantly different from the control group.e, $p<0.05$, significantly different from the $10^3/10^3$ nM treatment group. f, $p<0.05$, significantly different from the $0/10^3$ nM treatment group.

7. DISCUSSION

In the present study, in order to investigate the function and regulation of GnRHI and GnRHII in the ovary, primary cultures of hGLCs were used as our model system. Other studies showed that hGLCs in culture express the components of the GnRH system and are a good model for studying the regulation and the autocrine/paracrine function of GnRH in the ovary (Peng et al., 1994; Tureck et al., 1982). Primary cultures of GCs, unlike ovarian cell lines, retain hormonal responsiveness resembling the in vivo situations. (Hsueh et al., 1984). Isolation of hGLCs from the follicular aspirate is a very simple procedure and does not require the use of proteolytic enzymes or other agents that may cause some physiological changes in the cells (Hsueh et al., 1984). The hGLCs have all the enzymes for de novo synthesis of P4 (Hsueh et al., 1984); however these cells do not have the enzyme P450c17 that is necessary for making androgens (Sasano, 1994). Since there is no androgen substrate present in our culture system, no endogenous production of E2 will affect our exogenous E2 treatment. This idea is further supported by the observation that the cultured rat GCs were able to produce E2 only after treatment with androstenedione (Welsh et al., 1984). One of the problems with our culture system is that the cells come from women undergoing IVF procedures. Therefore, the locus and the reason for infertility are not known. Consequently, sometimes the results obtained may not reflect the real physiological responses expected from normal cells. Nevertheless, many investigators accepted this culture system as an appropriate model system among other models available (Bradley et al., 1995, Zhao et al., 2000, Billig et al., 1994).

There are a number of studies available regarding the regulation of GnRH gene expression by E2. It was shown that in the GT1-7 GnRH neurons, 1nM of E2 down regulated GnRH mRNA levels to approximately 55% of basal level over a 48h time course. This effect appeared to happen via an ER mediated mechanism because ICI 182,780, a complete ER antagonist, blocked the repression of GnRH mRNA levels by E2. The same study indicated that GT1-7 cells expressed both ER α and β (Roy et al., 1999). According to Kang et al. (2001a), treatment with E2 induced a significant down-regulation of GnRH mRNA in the ovarian cancer cell lines but not in human ovarian surface epithelial cells. This study demonstrated the expression of both ERs in these two cell lines. However, the expression levels were much higher in ovarian cancer cell lines than in the other cell line. The same study showed that tamoxifen alone had no effect on GnRH mRNA levels; however, E2 and tamoxifen co-treatment reversed the effect of E2, suggesting that E2 action was mediated via ERs. Wierman et al. (1992) showed that E2 could negatively regulate the rat GnRH promoter activity in placental tumor cells. Similarly Dong et al. (1996) demonstrated that E2 negatively regulated the GnRH promoter activity in a dose dependent manner in human placental cells. These results, particularly the ones from experiments on extrapituitary tissues, support our findings demonstrating the down regulation of GnRHI mRNA by E2 in hGLCs.

There are also some *in vivo* studies at the hypothalamic-pituitary level trying to find out the relationship between E2 and GnRH gene expression. For example, some studies showed that in the rat there is a direct relationship between the E2 levels in the plasma and GnRH gene expression in the hypothalamus (Roberts et al., 1989). On the other hand, others demonstrated that during the rat estrus cycle there is a reverse

relationship between the E2 levels and GnRH gene expression in the hypothalamus (Zoeller and Young, 1988). The conflicting results may be due to the time rats were killed, the location difference of the GnRH neurons within the medial septal-preoptic hypothalamic regions, and also the differences in the sensitivities of the techniques used.

There is only one study available regarding the regulatory effects of E2 on GnRHI but not GnRHII, in hGLCs in vitro. In this study Nathawani et al. (2000) demonstrated that E2 decreased GnRHI mRNA levels in a time and dose dependent manner, supporting the results obtained in our experiments. Additionally, differential regulation of GnRHI and GnRHII by E2 was demonstrated in human neuronal cell lines (Chen et al., 2002). This study demonstrated that E2 increased endogenous GnRHII mRNA levels and decreased endogenous GnRHI mRNA levels. These findings very well agree with the results obtained in the present study, suggesting that regulation of GnRHI and GnRHII by E2 in humans may be similar in the brain and in the ovary.

The biological effects of E2 are mediated through several different pathways (Nilsson et al., 2001). Through the classical mechanism of E2 action, E2 diffuses through the plasma membrane and forms complexes with specific cytosolic or nuclear receptors. The E2-ER complexes then bind to estrogen response elements (EREs) in target promoters, leading to an up or down-regulation of gene transcription and subsequent tissue responses (Carson-Jurica et al., 1990). The effects of E2 can also be mediated through ERE-independent genomic actions of ER. Through this mechanism, agonist-bound ER can lead to gene regulation in the absence of direct DNA binding. E2-ER complexes alter transcription of genes containing alternative response elements such as AP-1 through association with other DNA-bound transcription factors such as Fos and

Jun, which tether the activated ER to DNA, resulting in an up-regulation of gene expression (Paech et al., 1997; Webb et al., 1995, 1999). Another potentially important pathway of E2 action is constituted by the very rapid, so called, non-genomic effects of E2. E2 activates a putative membrane-associated binding site, linked to intracellular signal transduction pathways that generate rapid tissue responses (Monje and Boland, 1999; Pappas et al., 1995). For example, in rat pituitary cells, a rise in the intracellular calcium level happened within minutes after E2 treatment. This E2-triggered calcium surge was not inhibited by pre-treating the cells with E2 antagonists. The striking rapidity of this response suggested a non-genomic action triggered by a signal-generating receptor on the cell surface, which was different from the conventional slowly acting, gene-stimulating nuclear ER (Watson et al. 1999). In the present study, It would be more appropriate if a pure E2 antagonist, such as ICI 182,780, were used instead of tamoxifen. However, as mentioned in the introduction, tamoxifen seems to act as an E2 antagonist in the ovary. Therefore, the present study showed that tamoxifen inhibited the effects of E2 on the regulation of GnRHI and GnRHII in hGLCs. This suggests that the E2-induced regulation of GnRHI and GnRHII in hGLCs is a receptor mediated event, indicating genomic effects of E2. This idea is further supported by the presence of both types of nuclear ERs in hGLCs (Misao et al., 1999). Similarly, studies on mice indicated that also the hypothalamic GnRH producing neurons posses nuclear ERs (Martini et al., 1997). Furthermore, the discovery of one ERE on the human GnRHI gene in the hypothalamus (Radovic et al., 1991) suggested that, like in the ovary, this gene in the brain may also be regulated via genomic action of E2.

GnRH regulation by P4 has also been studied at the hypothalamic-pituitary level. Studies done on the rat showed that P4 decreased the expression of hypothalamic GnRH mRNA levels (Toranzo et al., 1989). In the ewe, at the hypothalamic-pituitary level, P4 removal accelerated the GnRH pulse frequency and P4 administration slowed down the GnRH pulse frequency. These effects were shown to be mediated by P4 itself and not by its hydroxylated metabolites. The same experiments showed that P4 exerted its effects by interacting with PRs (Chabbert-Buffet et al., 2000). The present study examined the role of P4 in regulating ovarian GnRHI and GnRHII. Preliminary studies from our lab, measuring changes in GnRH as a result of treatment with P4, resulted in inconclusive and contradictory data. Since hGLCs have all the enzymes for de novo synthesis of P4 (Hseuh et al., 1984), the contradictory data may have been a consequence of variations in endogenous production of P4 from different patients. As a result, we used RU486 to indirectly examine the effects of P4 on the expression of GnRHI and GnRHII mRNA in hGLCs. Our findings indicated that RU486 had no significant effects on the mRNA expression of GnRHI. In contrast, there was an increase in GnRHII mRNA levels in response to RU486, suggesting that P4 decreased GnRHII mRNA in cultured hGLCs. The exact regulatory mechanism of the P4-induced regulation of GnRHII in hGLCs is not known. However, in the rat GT1-7 neuronal cells, P4 was shown to inhibit GnRHI transcription via direct PR binding on non-consensus DNA sequences in the proximal rat GnRH promoter (Kepa et al., 1995). The differences between the P4-induced regulation of GnRHI in hGLCs and in animals at the hypothalamic-pituitary level, may be attributed to tissue specific regulatory mechanisms or species differences. Different regulations of a gene, by one hormone, in different tissues of different species have been observed

before. For example, P4 was shown to increase GnRH receptor mRNA in cultured hGLCs (Nathwani et al., 2000); however, no change was observed in GnRH receptor mRNA in the rat hippocampus in response to P4 (Badr et al., 1988).

The present study demonstrated that GnRHI and GnRHII were differentially regulated by E2 and P4. Differential regulation of two forms of GnRH has been observed before. In hGLCs, FSH and LH were shown to down-regulate GnRHI mRNA and up-regulate GnRHII mRNA (Kang et al., 2001). In the brain of female silver eel, E2 up-regulated GnRHI and testosterone down-regulated GnRHII (Montero et al., 1995). In the gold fish the ratio between sGnRH (salmon GnRH) and GnRHII changed with sexual maturation. A smaller increase in the level of GnRHII than in sGnRH was observed in the pituitary (Rosenblum et al., 1993). In the chicken, castration led to a change in the level of only cGnRHI (chicken GnRHI) but not GnRHII (Sharp et al., 1990). Taken together, the differential regulation of two forms of GnRH by E2 and P4 in the present study, suggests that GnRHI and GnRHII may be temporally regulated by E2 and P4 during different phases of menstrual cycle.

There is now evidence that two or three forms of GnRH in one species may have similar physiological roles. For example, although the two forms of GnRH present in the goldfish are different in terms of potency, they both stimulate the release of growth hormone and gonadotropins (King et al., 1995; Gazourian et. al., 1997; Sharp et al., 1990). Like GnRHI, administration of GnRHII to the rhesus monkey resulted in an up-regulation of plasma LH level (Lescheid et al., 1997). Both kinds of GnRH present in the lamprey are capable of stimulating ovarian steroidogenesis (Gazourian et. al., 1997). It was also shown by Kang et al. (2001c) that both GnRHI and GnRHII down-regulated P4

secretion in hGLCs, suggesting that the role of GnRHII might be similar to GnRHI in terms of ovarian steroidogenesis. Taken together these observations support the results from the present study that indicate a similarity between GnRHI and GnRHII function in terms of the ability to induce apoptosis. Teleologically speaking, the advantages of a dual hormone system over a single hormone system seem obvious. Should one system be deficient or fail, the second system would provide a backup. Conversely, when both systems are working, amplification and fine tuning of the signal is improved. However, it should be kept in mind that GnRHI and GnRHII might also have some functions that are totally different from each other.

Hypothalamic GnRH mRNA levels have been demonstrated to increase steadily during postnatal development and puberty. This increase in GnRH mRNA levels is thought to be important for regulating the onset of puberty as premature administration of GnRH causes precocious puberty in immature animals, (Wildt et al., 1980). In the present study, the increase in the mRNA levels of the two GnRH forms in hGLCs with time in culture, suggests that these hormones may have an autocrine/paracrine role such as regulating luteolysis during the luteal phase of menstrual cycle. Although the mechanism of this increase in hGLCs is not known, in the mouse differential mechanisms such as gene transcription and mRNA stability are thought play a role in determining GnRH mRNA levels during different stages of development (Gore et al 1999).

In the present study, GnRHI and GnRHII were shown to be capable of promoting apoptosis in a time and dose dependent manner in hGLCs *in vitro*. There are a number of reports that support the ability of GnRHI and GnRHII to induce apoptosis in general and in hGLCs in particular. For example, In hypophysectomized E2-treated rats, treatment

with a GnRH agonist induced ovarian apoptotic DNA fragmentation with or without co-treatment with FSH. In situ hybridization indicated that apoptotic cell death was confined to the GCs (Billig et al., 1994). The same group indicated that GnRH agonist treatment increased DNA fragmentation in isolated GCs as well (Billig et al., 1994). One group also showed that GnRH induced apoptosis in hGLCs in vitro (Zhao et al., 2000). Induction of apoptosis in rat and porcine cultured GCs as a result of GnRH treatment was similarly shown by Yano et al. (1997) and Zhao et al. (2000). In goldfish, GnRHII (10^-7 M) induced apoptosis in follicle-enclosed oocytes (Andreu-Vieyra and Habibi, 2000). GnRH has also been indicated to induce apoptosis in eutopic endometrial cells from patients with endometriosis (Imai et al., 2000). It is known that in a number of cell types including hGLCs, Fas, a cell surface receptor protein, triggers apoptosis when cross linked with its ligand called Fas ligand (FasL) (Quirk et al., 1995; Roughton et al., 1999). Additionally, GnRH was shown to induce apoptosis in GnRH receptor bearing tumors including ovarian carcinoma cells, by increasing FasL expression (Imai et al., 1998a, b). Therefore, one of the ways by which GnRH promotes apoptosis in hGLCs, may be increasing the expression of FasL in these cells.

Some of the suggested mechanisms for luteolysis explain it by apoptosis. For example, Prostaglandin $F_{2\alpha}$ is the factor from the uterus in most non-primate species that initiates luteolysis (Hansel et al., 1973; McCracken et al., 1970). In women (Patwardhan et al., 1980), cows (Pate et al., 1998), rodents (Olofsson et al., 1992), ewes (Tsai et al., 1997), and sows (Guthrie et al., 1978) $PGF_{2\alpha}$ can be synthesized by corpora lutea and may act via a paracrine and/or autocrine mechanism to induce luteolysis (Auletta and Flint, 1988). Prostaglandin $F_{2\alpha}$ has been demonstrated to promote apoptosis in cells comprising

the corpus luteum (Niswender et al., 2000). Evidence for this apoptotic action is the appearance of oligonucleosomes in response to $PGF_{2\alpha}$ in luteal cells of women, rats, sheep, cattle, and pigs (Niswender et al., 2000). Another evidence for $PGF_{2\alpha}$ inducing apoptosis, is the elevated level of bax mRNA compared to unchanged level of Bcl-2 mRNA during luteolysis in cattle (Rueda et al., 1997). In the ewe, $PGF_{2\alpha}$ was shown to cause degeneration of luteal endothelial cells via the process of apoptosis (Niswender et al., 2000). Degeneration of luteal endothelial cells leads to reduction in capillary density and consequently, reduction in blood flow to the luteal parenchyma (Azmi et al., 1984). In the rat luteal cells, one action of $PGF_{2\alpha}$ seems to be increasing generation of reactive oxygen species (Sawda and Carlson, 1991), an event that has been linked to both a loss of P4 biosynthesis and the induction of apoptosis (Buttke and Sandstorm, 1994; Tilly and Tilly, 1995)

Down-regulation of N-cadherin (a calcium-dependent cell adhesion molecule) in GCs, is another suggested mechanism that explains luteolysis by apoptosis (Makrigiannakis et al., 1999). N-cadherin is expressed by human GCs and mediates cell-cell adhesion between GCs. N-cadherin is highly expressed by GCs in follicles of the resting pool, growing antral follicles and healthy corpora lutea. However, the expression is very low in GLCs of the late luteal phase and in GCs of atretic follicles (Makrigiannakis et al., 1999). Indeed, cell-cell adhesion provided by N-cadherin in GCs has been shown to be an important factor for preventing apoptosis. Consequently, one of the contributing factors to the process of luteolysis might be the down-regulation of N-cadherin in hGLCs (Peluso et al., 1996). At least part of this down regulation is mediated via the enzymatic cleavage of the extracellular domain of N-cadherin (Peluso et al.,

1996). Further, in support of the notion that luteolysis might happen via apoptosis, caspase-3 (a cell death protease) has been shown to be expressed in hGLCs (Kugu et al., 1998) and caspase-3 is much more abundant in luteal cells as opposed to follicular GCs in the adult human ovary (Krajewska et al., 1997).

GnRH receptor has been demonstrated to be expressed in hGLCs (Peng et al., 1994). The present study showed an increase in GnRHI and GnRHII mRNA levels with time in cultured hGLCs. Additionally, GnRHI and GnRHII were demonstrated to be capable of promoting apoptosis in hGLCs. As argued before, some of the suggested mechanisms for luteolysis explain it by apoptosis. Taken together these observations suggest that the increase of GnRHI and GnRHII during the luteal phase of menstrual cycle and subsequently, the promotion of apoptosis in hGLCs, may be one of the contributing factors to the degradation of the corpus luteum during the process of luteolysis. Previous reports have also implicated GnRH as a luteolytic factor. For example, hCG decreased the expression of GnRH receptor mRNA in the human and rat ovary (Peng et al., 1994; Olofsson et al., 1995). During the early stages of pregnancy, hCG is an important rescue factor for the maintenance of the corpus luteum. Thus, the down regulation of GnRH receptor by hCG might be involved in the maintenance of the corpus luteum during pregnancy. Furthermore, GnRHI and $PGF_{2\alpha}$ have been suggested to have a very similar or complementary role in the ovary. Indeed, GnRHI was shown to enhance the luteolytic effects of $PGF_{2\alpha}$ in hGLCs (Vaananen et al., 1997).

During the follicular phase of a normal menstrual cycle, there is a rise in the level of E2 and immediately prior to ovulation E2 reaches its highest level. According to the results obtained from our experiments, the rise in the E2 level may increase the GnRHII

level and decrease the GnRHI level. P4 level in the follicular phase is very low compared to E2 level. Consequently, the effects of E2 will probably prevail over that of P4 in terms of regulating the two GnRH forms. This means that, before ovulation, there will be an overall increase in the GnRHII level and a decrease in the GnRHI level. Since the highest concentration of GnRH receptor has been demonstrated in GCs of atretic follicles (Whitelaw et al., 1995), this increase of GnRHII in the follicular phase, may be a contributing factor to the selection of the dominant follicle by promoting apoptosis. Our results regarding the regulation of expression of GnRHI and GnRHII by gonadal steroids was demonstrated in hGLCs. However, we postulate above that the effect of sex steroids on luteinized and non-luteinized GCs, regarding the regulation of GnRHI and GnRHII, is the same. This needs further investigation. Nevertheless, the role of GnRH in follicular atresia via inducing apoptosis has been noticed by others (Cogo et al., 1995; Whitelaw et al., 1995).

During the luteal phase, P4 and E2 begin increasing and reach their maximum levels at about day 21-24. According to our data, The increase in E2 level leads to a decrease in the GnRHI level, and the increase in P4 has no effect on the GnRHI level. Therefore, the GnRHI level goes down in response to E2, and remains reduced. The increase of the P4 level during the luteal phase will result in a decrease in the GnRHII level. The P4 level is much higher than the E2 level at this phase of the menstrual cycle. As a result, the effect of P4 on GnRHII level probably covers the effect of E2. Therefore, we anticipate an overall decrease in the level of both GnRHI and GnRHII in the luteal phase until about day 21-24. According to our data, both GnRH forms can promote apoptosis. Since the level of both forms is low at this stage, corpus luteum is protected

from undergoing apoptosis by GnRHI and GnRHII. However, if pregnancy does not occur, both E2 and P4 decline. As a result of a decline in E2 we anticipate an increase in GnRHI and a decrease in GnRHII level. A decrease in P4 should result in no change in GnRHI. Therefore, GnRHI increases by decline in E2 and remains high. Decrease in P4 causes an increase in GnRHII level. Since the decrease in P4 level is more dramatic than the decrease in E2 level, the effects caused by the decrease in P4 should prevail over the effects caused by the decrease in E2. As a result, we anticipate an overall increase in the level of both GnRH forms. This increase might facilitate the process of luteolysis by promoting apoptosis.

The above model only explains the consequences of the relationship between gonadal steroids and the two GnRH forms, based on the results obtained from our *in vitro* experiments. However, we should keep in mind that the *in vivo* situation is always much more complicated and there are many other factors that may play different roles.

8. SUMMARY

GnRHI and GnRHII mRNA levels increased with time in culture in hGLCs. Time and dose dependent treatment of hGLCs with E2 resulted in a decrease in mRNA expression of GnRHI and an increase in mRNA expression of GnRHII. This effect was blocked by tamoxifen, meaning that E2 works via its nuclear receptors. On the other hand, time and dose dependent treatment with RU486 didn't make any changes in the GnRHI mRNA level but resulted in an increase in the GnRHII mRNA level in the hGLCs. We demonstrated that in hGLCs with time in culture, the percentage of cells undergoing apoptosis increased significantly in days 8 and 10 compared to days 1 and 4. We also showed that GnRHI and GnRHII can promote apoptosis in hGLCs.

In conclusion, since GnRHI and GnRHII are capable of promoting apoptosis in hGLCs and these GnRH forms are dynamically regulated by E2 and P4, the balance between E2 and P4 may play a role in regulating the fate of the corpus luteum.

8. FUTURE STUDIES

$PGF_{2\alpha}$ has been shown to be involved in luteolysis, at least in part, by inducing apoptosis in the corpus luteum. Down regulation of N-cadherin has also been demonstrated to play a possible role in luteolysis by inducing apoptosis in GLCs. Our data suggest that GnRHI and GnRHII might also be involved in luteolysis via promoting apoptosis in hGLCs. The interactions between GnRHI and $PGF_{2\alpha}$ in the ovary have been investigated before. However, Further studies examining the regulation of gene expression of N-cadherin by GnRHI and GnRHII, and also the interactions of $PGF_{2\alpha}$ and

GnRHII in comparison with GnRHI, might lead to a better understanding of the process of luteolysis.

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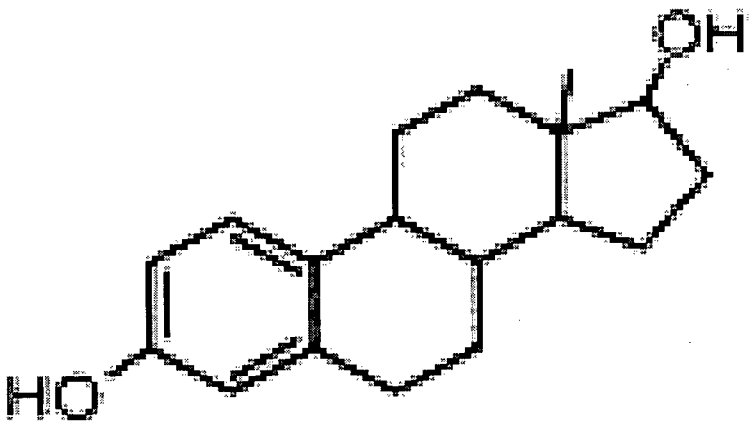
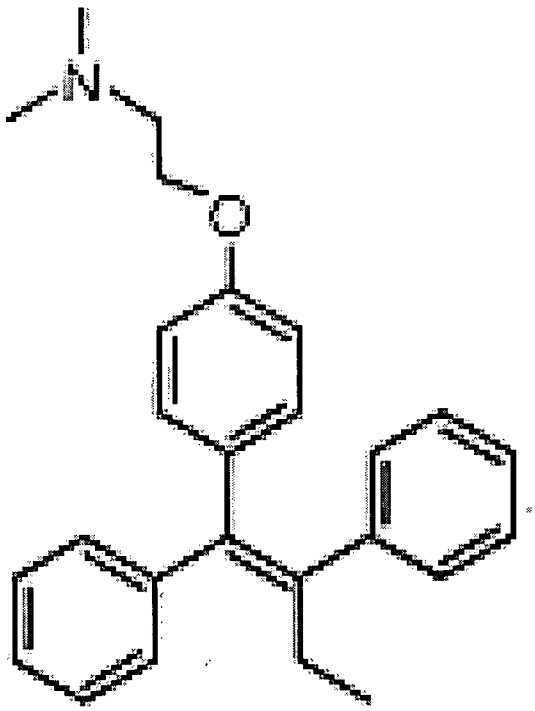
Appendix

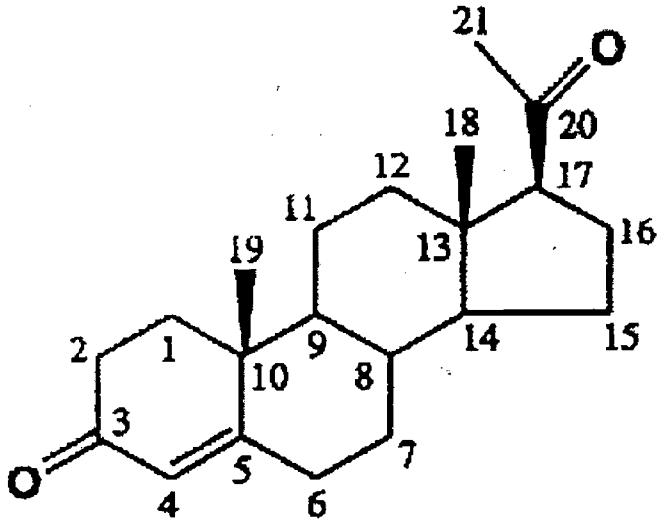
CHEMICAL STRUCTURES



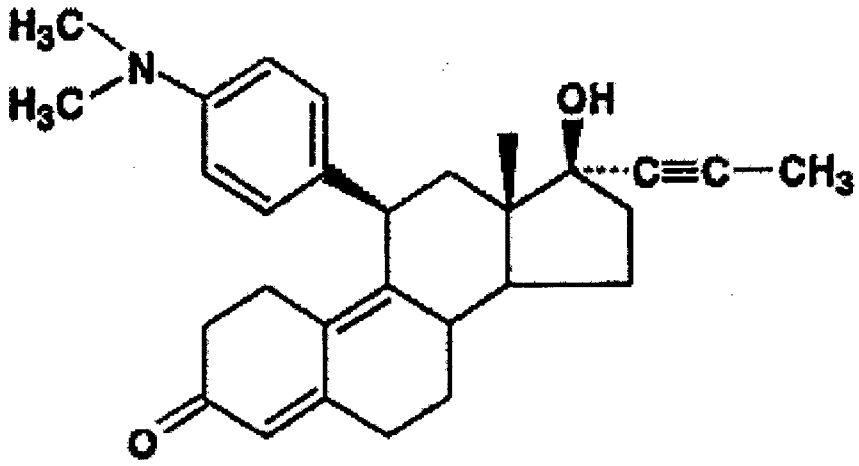
17 β-Estradiol



Tamoxifen



Progesterone



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