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STEROIDS – CLINICAL ASPECT

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Contributors

Kumiko Toyoshima, Hajime Fukui, Erin King, Kwong-Kwok Wong, Hirofumi Shimomura, Tetsuo Nishikawa, Małgorzata Kotula-Balak, Barbara Bilińska, Anna Hejmej, Julio Calleja-Gonzalez, Marko Stojanovic, Sergej Ostojic, Rosário Monteiro, Cidália Pereira, Isabel Azevedo, Maria João Martins, Ettore Cicinelli, Ronald H. F. Hunter, Niels Einer-Jensen

Notice

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Meet the editor

Professor Hassan Abduljabbar obtained his Medical degree from King Abdulaziz University, Jeddah, Saudi Arabia on June 1980. He then pursued his postgraduate training in Obstetrics and Gynecology at the University of Western Ontario, Canada. On Nov 1986 he became a Fellow of the Royal College of Physicians Surgeons of Canada. He obtained his American board in OBGYN on 1988. Currently he is a Professor and the chairman of the Department of Obstetrics and Gynecology at King Abdulaziz University Hospital, Jeddah, Saudi Arabia. He also is the Medical Director of United Doctors Hospital, Jeddah, Saudi Arabia from 1998 till present. He is a member of American Fertility Socitey, American Socitey of Gynecological laparscopy and The Saudi Obstetrics and Gynecology Society. His main interests are infertility, Stem Cells, and physiological and biochemical changes in the human body during pregnancy. He has a number of publications in different journals in Obstetrics and Gynecology.

Contents

Preface

The history of steroids is richly immersed and runs long and deep. The modern history of steroids started in the early 20th century, but its use has been traced back to ancient Greece where athletes ingested animal testicles to boost their energy and performance. This book covers the modern understanding and clinical use of steroids.

We divided the book into two sections. The first section contains information about steroids in the human body. The second section will cover the relationship of steroids.

Topics covered in the first section include the concentration of the steroids in various parts of the body, effects of steroids on body composition of athletes, the metabolic syndromes, and the effect of steroids on the development of the male reproductive system. The second section contains various clinical scenarios that involve steroids.

We hope that this book will contribute further to the literature available about steroids and enables the reader to further understand this interesting and rapidly evolving science.

Hassan Abduljabbar
Professor of Obstetrics and Gynecology,
King Abdulaziz University,
Kingdom of Saudi Arabia

Part 1

Steroid in the Body

1

The Concentration of Steroid Hormones in Blood and Peritoneal Fluid Depends on the Site of Sampling

N. Einer-Jensen[1], R.H.F. Hunter[2] and E. Cicinelli[3]
[1]*Institute of Molecular Medicine, University of Southern Denmark, Odense M,*
[2]*Institute for Reproductive Medicine, Hannover Veterinary University, Hannover,*
[3]*Dept Obstet Gynecol, University Hospital, Bari,*
[1]*Denmark*
[2]*Germany*
[3]*Italy*

1. Introduction

The concentration of steroid hormones in arteries varies according to the site of blood sampling. The hormone concentration is higher in the ovarian and testicular arteries than in the aorta resulting in a high impact on the target organs, opening a route for local hormonal regulation between organs. Local application of drugs may induce a potential method for semi-specific treatments. Vaginal application of hormones will therefore induce relatively higher concentrations in the uterus and urinary bladder area than a peripheral application. Likewise, nasal application will induce a higher relative concentration in brain arterial blood than peripheral application. This is due to local counter-current transfer between venous and arterial blood, and between the lymphatic and arterial vessels. Similarly, the concentration of steroids in peritoneal fluid varies according to the site of sampling.

2. The third way of humoral communication: local counter-current transfer

Humoral communication between cells can be either through local diffusion in the interstitial fluid between neighbouring cells or through the vascular system. The present paper will discuss a third possibility that is part of the vascular distribution. The paper will concentrate on local communication between organs belonging to the reproductive system and steroid hormones, but will also touch other organs (the adrenal, the brain and the peritoneal cavity) where local transfer of steroids seem to be involved in the physiological regulation.

McCracken et al. (1972) initiated the hormonal transfer investigations in the female. The anatomical structure had, however, been known for hundreds of years (Blancardi 1687, published in 1739). Ginther (1967) described the functional importance of a close connection between the ovarian artery and veins. The work involving steroid transfer in males was started by Jacks and Setchell (1973) and Einer-Jensen (1974). The documentation comes from investigations in several animal species as well as in man (see

Einer-Jensen et al., 1989; see Krzymowski, 1990; see Einer-Jensen and Hunter, 2005). Despite the anatomical differences between the species (one ovulation versus 10-20 ovulations, large uterine body and small uterine horns versus small uterine body and large horns), the picture of transfer is similar.

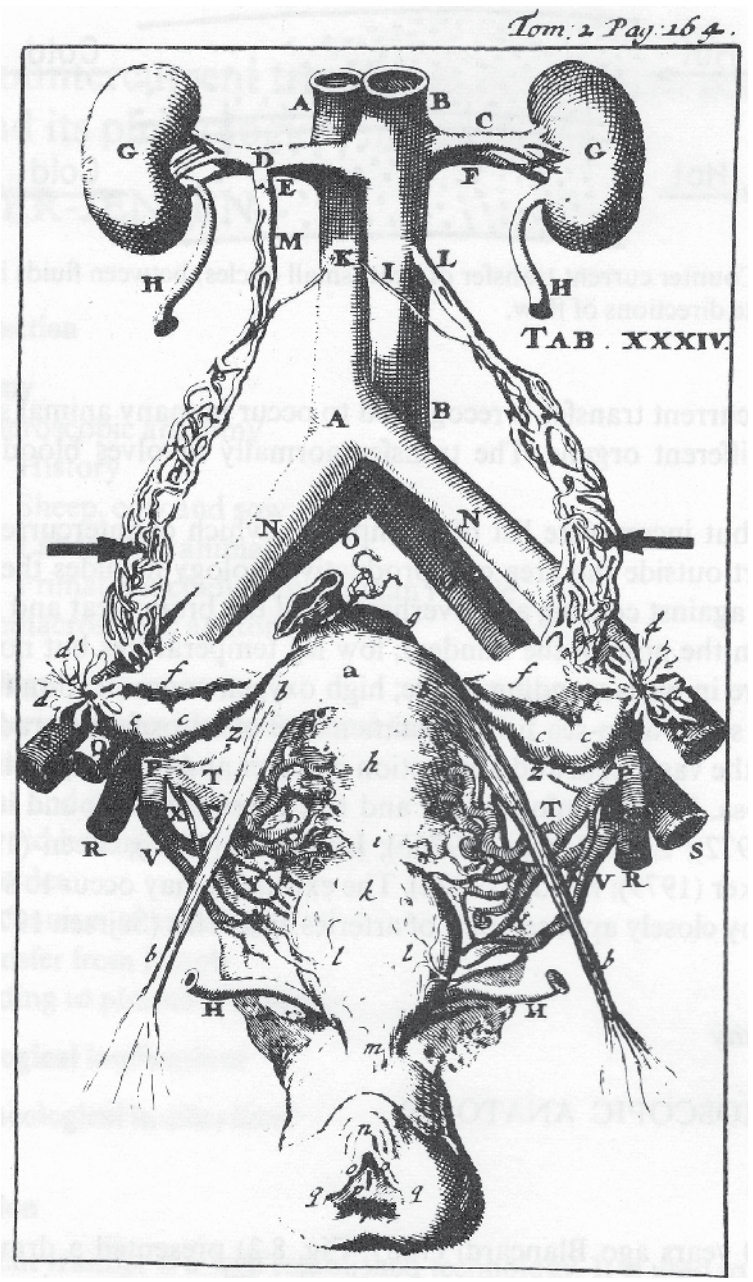

Fig. 1. The human genital organs and their vascular supply. The plexus formed by the ovarian artery and vein can be seen (arrows). (From Blancardi 1687).

Signal substances such as hormones produced in an organ will diffuse to the surrounding lymph and blood capillaries. Thus the signal substance will be present in the content of local lymph and blood vessels removing fluid and blood from the organ. The concentration will, of course, be high here compared to the peripheral fluids since no dilution has taken place (Einer-Jensen and Hunter, 2005).

In some hormone producing organs, the vessels removing the fluid from the organ are very intimately arranged with the artery supplying the organ. This is the case for steroid producing organs such as the gonads in both male and female. It is well known that the temperature in the extra-abdominal testis is a few degrees Centigrade lower than the general body temperature due to cooling through the scrotal wall and maintenance of the temperature gradient by counter-current transfer of heat energy. The transfer is expected to take place between the venous plexus (the Pampiniform plexus) and the convoluted testicular artery. The efficacy of the heat transfer is very high, close to 100% (Glad Sørensen et al., 1991), thus the cooling through the scrotal wall can be kept at a low level – which will diminish the waste of energy from the body. In most mammals, the testis is an organ positioned outside the abdomen, but even in animals with intra-abdominal testes the close apposition between the vessels is found, e.g. in small whales (Einer-Jensen, pers. com.). This strongly suggests that cooling is not the only reason for the vascular arrangement

The ovaries are always positioned in the abdomen and one would not expect temperature gradients within their tissues. The vessels to and from the ovary are closely apposed in a way similar to the male, indicating the potential for of a transfer system. However, the temperature of the pre-ovulatory follicles tends to be lower than deep body temperature (Hunter and Einer-Jensen, 2005; Hunter et al., 2006). Heat "consuming" proteins induce lower temperature and the temperature decrease is maintained by a very local heat-exchange mechanism in the vessels to and from the follicle.

3. Counter-current exchange

When blood or lymph flows through arteries, and veins and lymph vessels, and the vessels are in close contact, the flow can be described as counter-current flow.

Counter-current exchange along with Concurrent exchange comprise the mechanisms used to transfer some property of a fluid from one flowing current of fluid to another across a semipermeable membrane or thermally-conductive material between them. The property transferred could be heat, concentration of a chemical substance, or others. Counter-current exchange is a key concept in chemical engineering thermodynamics and manufacturing processes, for example in extracting sucrose from sugar beet roots. (Wikipedia, the figure below is also from Wikipedia).

The present authors expect the transfer to be passive; no active transfer mechanisms have been detected or proposed (to the best knowledge of the authors). The laws of physics, the respective rates of flow, the diffusion distance between the vessels, the chemical nature of the substances, especially the lipophility, will determine the rate of exchange. In general, a system will transfer heat at almost 100% (Glad Sørensen et al., 1991), whereas the exchange of tritiated water may be 20%, and the rate of steroid hormone transfer a few per cent.

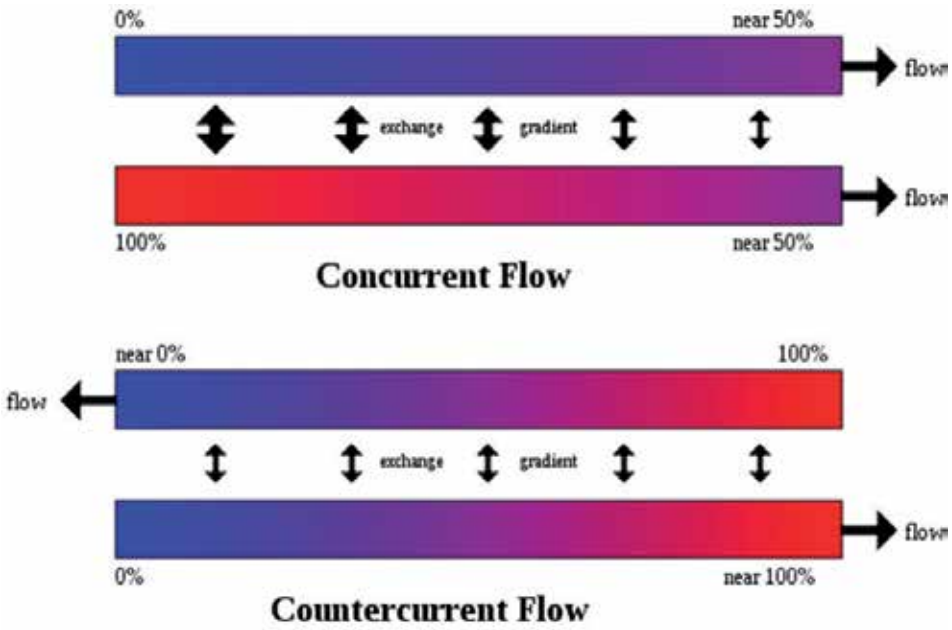

Fig. 2.

Many of the first experiments evaluating the transfer were performed with radioactive gases (^{133}Xenon and ^{85}Krypton). The efficacy of the transfer is similar to that of tritiated water. The advantage of using gas was the lack of recirculation since more than 95% of the gas is cleared during the first passage.

Even a limited transfer of steroids may have a marked physiological impact. Only non-protein bound steroids are biologically active. The hormone transferred will reach the arterial blood as free hormone and, because the binding takes some time (seconds), the steroid may reach the capillaries before it is bound to the plasma proteins (Einer-Jensen, 1984, 1989).

4. Steroid transferred from the gonads will reach the epididymis and the Fallopian tube

Like heat energy, substances may be transferred in areas with a close connection between lymph vessels, veins and arteries. The gonads are typical examples. The close connection between the winding testicular artery and the Pampiniform plexus is well known. It is not, however common knowledge that the blood supply to the first part of the epididymis also originates from the testicular artery. Thus, the epididymis is involved in the local transfer system and transfer of steroids will act to stimulate the epididymis more than indicated by the content of testosterone in peripheral blood. An intramuscular injection of testosterone may produce a high peripheral concentration of the hormone and produce a strong negative feedback on the pituitary gland. However, it will not produce the concentration difference between the blood in the testicular artery and any other arterial sample.

In the female, the arterial supply to the Fallopian tube and the proximal part of the uterus originates from the ovarian artery. Any hormone in or transferred to the ovarian artery will also reach the tube and part of uterus (Stefańczyk-Krzymowska et al., 1998; Einer-Jensen et al., 2002; Cicinelli et al., 2004a). The ovarian production of individual steroids is cyclic and the amount of hormone transferred will therefore fluctuate (Cicinelli et al., 2004c). The increased production of oestradiol shortly before ovulation and of progesterone in the days after ovulation may be especially important, since transfer to the blood supply of the Fallopian tube and proximal part of uterus will influence tissue function. The transfer has been documented in both experimental animals and in man. The border between the blood supply from the uterine and tubal arteries shifts during the ovulatory cycle in man, probably due to the local vasodilatory effect of oestrogens in the tubal artery (Cicinelli et al., 2004b; Cicinelli et al., 2005).
Cooling of the vagina induces a temperature fall in the vesica and corpus of uterus but not the tubal part, probably through a counter-current transfer mechanism (Einer-Jensen et al., 2001a). Application of steroids in the vagina will induce a semi-selective effect in the vesica and uterus (Cicinelli et al., 2001). Cycle dependent variations in transfer of ^{133}Xenon from vagina to uterus was found in rats (Zhao and Einer-Jensen, 1998).

5. Other organs may also have a local transfer mechanism

An important steroid producing gland, the adrenal, does not have the external artery-veins complex needed for a counter-current transfer. It is, however, known that glycogenic steroids potentiate the production of adrenalin. It is tempting to think that a local exchange mechanism exists within the adrenal gland. There is some anatomical evidence, but the hypothesis has not been documented sufficiently to exclude doubt (Einer-Jensen and Carter, 1995).
Local counter-current transfer between the brain blood vessels has been found in experimental animals (Krzymowski, 1992; Einer-Jensen and Larsen, 2000a and b; Einer-Jensen et al., 2001b; Einer-Jensen et al., 2002). The brain is (probably) not a steroid producing organ, but some neurons have steroid receptors. Nonetheless, the effect on the brain may be semi-selective when treated with nasal application of steroids as based on the following knowledge. When animals exercise intensively, the body temperature tends to increase. The brain is the first body organ to be damaged after a rather small increase of 3-5^{o} Centigrade. Nature has developed a brain cooling mechanism. Large airflows through the nose will cool the nasal mucous membrane and the capillary blood. The venous blood will leave the head either through a superficial or a deep vein before reaching the jugular vein, the route being decided by an autoregulated mechanism. The higher the temperature, the more blood will reach the deeper vein. This vein is at one point in close connection with the carotid. In some animals, an arterial plexus (Rete Mirabile) is formed by the carotid creating a very effective transfer system which decreases the temperature of the carotid blood and therefore of the brain. Transfer of steroid hormones has been found in experiments involving nasal application in isolated, perfused heads from pigs (Skipor et al., 2003). The transfer mechanism may also be present in man.

6. The importance of high progesterone concentrations in peritoneal fluid

The peritoneal cavity, its lining membrane and fluids are active participants in local regulation of the reproductive processes. In women, peritoneal fluid was collected during

abdominal surgery by means of cotton swaps in nine women all with an active corpus luteum. Several samples were collected during the same operation (over the active corpus luteum, over the opposite ovary, at the right left and right paracolic gutter and at the pouch of Douglas). Progesterone concentrations close to the corpus luteum were 4 times (range 1.4 – 9.2) higher than in the other peritoneal samples and, on average, 5 times higher than in the systemic blood (Cicinelli et al., 2009). Progesterone would be expected to enter the peritoneal cavity locally (close to the corpus luteum). The authors know of no similar investigations in farm or experimental animals. One may speculate on the physiological importance:

Nonetheless, female genital tissues and their mesenteries are bathed in fluid with an elevated concentration of steroid hormones and, in many species, peritoneal fluid also enters the Fallopian tube ostium around the time of ovulation. This is principally due to the ab-ovarian beat of the cilia on the inner surface of the fimbriated infundibulum. In addition, evidence from pigs indicates that vital dyes irrigated onto the mesometrium and mesosalpinx enter the lymphatic vessels bordering the genital tract of oestrous animals (Hunter, 2011). There is little doubt that steroid hormones would do likewise, eventually influencing the activity of the endosalpinx and its secretions at a time when gametes and/or embryos could be present in the lumen.

The contralateral ovary is bathed in the high progesterone-containing fluid, which may influence follicular development. Other steroids such as oestrogens may behave comparably. Although alternating ovulation between the two ovaries is far from obligatory, signal transfer via the peritoneal fluid may have an influence on such a phenomenon (Hunter et al., 2007).

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2

Steroid Prohormones: Effects on Body Composition in Athletes

Sergej M. Ostojic, Julio Calleja-Gonzalez and Marko Stojanovic
Center for Health, Exercise and Sport Sciences, Belgrade
Serbia

1. Introduction

Androgenic-anabolic steroid hormones (AAS) are synthetic derivatives of the male hormone testosterone and for many years have been popular among athletes both for performance enhancement, due to physiological and psychological effects, and for aesthetic reasons (Evans 2004; Hartgens & Kuipers 2004). The anabolic action of AAS is particularly interesting since its affects protein metabolism by stimulation of protein synthesis and inhibition of protein breakdown, which could induce muscle growth and enhance adaptation to resistance training (Yesalis & Bahrke 1995; Brown et al. 2006). Since the AAS use in sport is banned, different nutritional strategies have been developed in the past decades to circumvent this problem and administer other exogenous testosterone analogues (King et al. 1999).

In the past 2o years, different steroid prohormones or prosteroids (e.g. androstenedion, dehydroepiandrosterone, androstenediol, 19-nor androstenediol, 19-nor androstenedione, 1-testosterone) have been developed and aggressively marketed in athletic environment as legal nutritional supplements that are expected to convert to active anabolic steroid hormones in the body and enhance exercise performance (Brown et al. 1999; Brown et al. 2000; Earnest et al. 2000; Leder et al. 2000; Brown et al. 2001; Kanayama et al. 2001). The efficacy and safety of these prohormones are not well established but are highly promoted to have the same androgenic effects on building muscle mass and strength as AAS (Baulieu et al. 2000; Brown et al. 2006). A typical steroid prohormone is intended to be a precursor to both testosterone and estrogens, through different biochemical pathways (Figure 1), typically resulting in the action of dehydrogenases in skeletal muscle, adipose tissue, skin, prostate and adrenal gland (Griffin 2004).

Dehydroepiandrosterone (3β-hydrohy-5-androsten-17-one; DHEA) seems to be the master steroid prohormone due its precursor function and its conversion to other hormones (Brown et al. 2006). Although the mechanism of action of DHEA or other prosteroids is not completely understood, it could be hypothesized that DHEA could increase testosterone production (at least as an acute response) if supplemented in diet, and due to its anabolic action may affects nitrogen balance and protein synthesis (Morales et al. 1998).

Testosterone is synthesized through either the Δ-4 or Δ-5 pathway (Broeder 2003) with the effects of newly synthesized testosterone in humans occur by way of 2 main mechanisms: by activation of the androgen receptor (directly or as 5α-dihydrotestosterone), and by conversion to estradiol and activation of estrogen receptors (Wilson 1988). Free testosterone

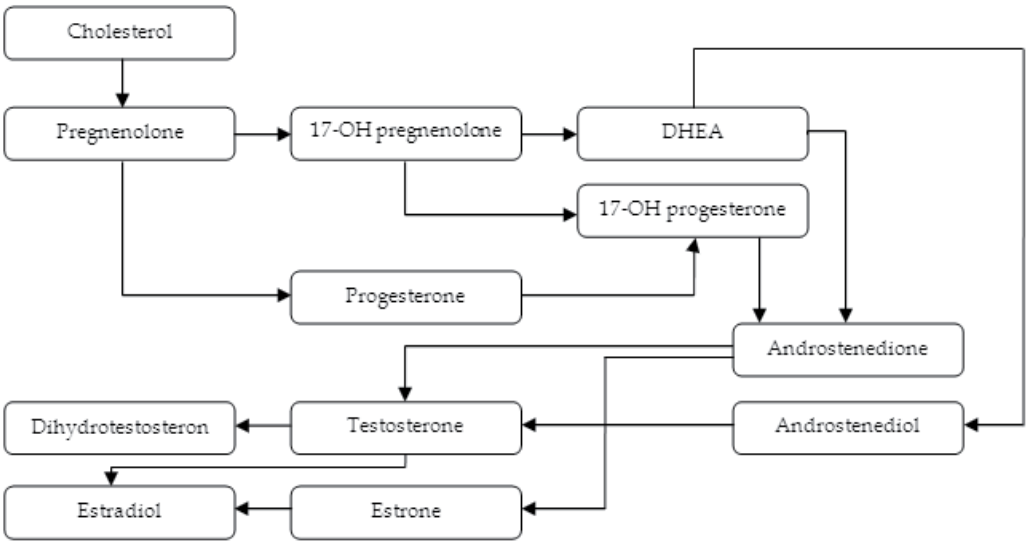

Fig. 1. Androgens biosynthesis from cholesterol to testosterone/dihydrotestosterone and estrogens (estrone and estradiol) via prohormone precursors (e.g. DHEA, androstenedione, androstenediol). *Abb.* DHEA – dehydroepiandrosterone.

is transported into the cytoplasm of target tissue cells, where it can bind to the androgen receptor, or can be reduced to 5α-dihydrotestosterone (DHT) by the cytoplasmic enzyme 5-alpha reductase (Hartgens & Kuipers 2004). DHT binds to the same androgen receptor even more strongly than testosterone, so that its androgenic potency is about 5 times that of testosterone (Breiner et al. 1986). The testosterone-receptor or DHT-receptor complex undergoes a structural change that allows it to move into the cell nucleus and bind directly to specific nucleotide sequences of the chromosomal desoxiribonucleic acid. The areas of binding are called hormone response elements, and influence transcriptional activity of certain genes, producing the anabolic effects (Saartok et al. 1984). Theoretically, the fewer interconversion steps a prohormone must complete in the syntheisis pathway to testosterone, the greater potential for enhancing active hormone production (Broeder 2003); for example, androstenedione converts to testosterone more rapidly than DHEA.

Existing data suggest that acute oral ingestion of DHEA, androstenediol or androstenedione modestly and transiently increase serum testosterone concentration; however, this is accompanied by greater increase in other steroids as well (i.e. estrogens, luteinizing hormone) (Ziegenfuss et al. 2002). Yet, it is questionable if this acute elevation in testosterone concentration induced by prosteroids necessarily result in enhanced transcriptional activity (Broeder 2003). Furthermore, research has shown that prosteroids may have significant biological activity by itself (Ostojic et al. 2009, Ostojic et al. 2010), affecting resting metabolic rate through futile cycling (Tagliaferro et al. 1986), increasing the flux of fatty acids through β-oxidation (Mohan et al. 1988), and alter the level of serotonin and dopamine (Cleary 1991; Ebeling et al. 1994; Kroboth 1999). Yet, the mechanism of action of prosteroids may differ between compounds because of variations in the steroid molecules (Hartgens & Kuipers 2004). These differences could be responsible for differences in the specificity of binding to receptor proteins or to interactions with various steroid-metabolizing enzymes (Wilson 1988; Creutzberg & Schols 1998) with future research needed to clarify the explaining physiological effects due to prosteroids use in humans.

2. History of steroid prohormones use in sport and exercise

Although the first documented reports of misuse of AAS by athletes stem from the 1950s (Yesalis 1999), the use of prosteroids in the athletic environment is rather new. Prosteroids were indirectly introduced with US Anabolic Steroid Control Act of 1990 (21 USCS Section 802), which defined anabolic steroids as *"…any drug or hormonal substance that promotes muscle growth in a manner physiologically similar to testosterone…"*, while steroid prohormones were not classified as anabolic steroids and could be purchased legally as dietary supplements (Broeder 2003; Brown et al. 2006). This document didn't appear to make a significant decrease in the use of anabolic steroids (Brainum 2008), but concern about the side effects linked to steroid use did lead to the development of another popular anabolic offering: prohormone nutritional supplements (Brown et al. 2003).

In December of 1996, androstenedione became available for over-the-counter sales in United States (Ziegenfuss et al. 2002), with subsequent availability of several other prohormones (e.g. androstenediol, DHEA, norandrostenedione). The non-critical promotion era of prosteroids in sport began with the disclosure of androstenedione use by Mark McGwire in 1998, who at the time was elite baseball player, which stimulated extensive media attention and dramatically increased the sales of prohormones among recreative and professional athletes (Brown et al. 2003, Brown et al. 2006).

In the past decades, prohormones have been highly marketed in the field of sport as lean body mass builders, fat reduction agents, and anticatabolic compounds. Recently, concerns over the safety of the prohormones use induced changes in US Anabolic Steroid Control Act in 2004 (21 USCS Section 802, amended), redefining anabolic steroids and classify prohormones as controlled substances. While the 2004 amendment specifically mentioned most of the current prohormones by name, that didn't stop some companies from marketing at least one anabolic steroid that the lawmakers had overlooked (Brainum 2008).

At the moment, it seems that legal status of prohormones is different throughout the world. In USA, Canada or Australia prosteroids are recognized as controlled substances and it is illegal to own or sell the product without prescription. On the other hand, no prescription is required for prosteroids purchase in several European countries, Russia or Japan, with products recognized as over-the-counter dietary supplements.

3. Epidemiology of steroids use among athletes: scope of the problem

Anabolic steroid usage has been recognized as a serious health and ethical problem among athletes for several decades (Foster & Housner 2004). Numerous examples of steroid usage rules violations have been highly publicized and have lead to the suspension and stripping of medals from international athletes, as well as many professional athletes (International Olympic Committee 1997; Wroble et al. 2002). Elite athletes are not the only population of individuals that use steroids. Recreational athletes also use steroids to enhance performance and to improve personal appearance (Wroble et al. 2002). Furthermore, evidence indicates that steroid usage often starts during high school (Yesalis et al, 1989; Kerr and Congeni 2007). Use of anabolic steroids is widespread in the athletic environment, particularly in power events and disciplines (e.g. football, track and field, body building, power lifting) (Foster & Housner 2004).

It seems that athletes have used AAS for more than 50 years, with first anecdotal evidence of use of animal testicular extracts even in 1890s (Yesalis 1999). Despite educational and

preventive measures, steroid use increases (Windsor & Dumitru 1988). The prevalence of AAS abuse has been reported in several populations. The highest estimates have come from male bodybuilders with even more than 50% regularly using steroids (Tricker et al. 1989; Lindstrom et al. 1990). Lower rates have been reported among intercollegiate athletes, ranging from 15% to 20% (Dezelsky et al. 1985). Rates of steroids use vary greatly across individual sports and are used in higher frequency and higher doses by strength athletes (Sturmi & Diorio 1998). By contrast, only 1% of their nonathletic university student counterparts reported steroids use (Dezelsky et al. 1985). Perhaps the most surprising and alarming finding is the rather high rate of steroids abuse among high school students (Mulcahey et al. 2010). The typical rate reported in male students is between 5% and 6%, but rates as high as 11% have been reported (Johnson et al 1989). The most recent estimate reported a range of 5% to 15% for steroids use among high school boys. In female high school students, steroids abuse rates tend to be lower, but quite worrisome at 1% to 3% (Harmer 2010). Wroble and co-workers (2002) indicated that less than one percent (0.7%) of youth sports participants reported current or previous usage of anabolic steroids; the rate of usage was higher in males than females. Three percent of athletes had been offered steroids at some time with 22% of them admitted to using steroids. Of the reported anabolic steroid users, 27% admitted they used anabolic steroids for athletic performance; 18% used to improve personal appearance; 18% used for bodybuilding; and 18% took due to peer pressure. Twelve percent of all athletes said that they personally know someone who was using or had used steroids.

Although several prohormones are considered as AAS, and are banned by many sports governing bodies, including the International Olympic Committee (IOC), they are semi-legal substances available from many retail outlets, including internet health food stores. The overall prevalence of prohormones use is not known, although several reports indicated that prohormones are among the most popular dietary supplements especially in adolescent athletes (Smurawa & Congeni 2007). Even though usage has decreased by over 50% since 1989, steroid use in sport is still a serious problem. Insufficient knowledge and inappropriate attitudes regarding the benefits and risks of using anabolic steroids is also a major concern (Schwingel et al. 2011).

4. Testosterone production and musculotrophic effects of prosteroids

The most prevalent reason for athletes initiating AAS or prosteroids use is to promote muscle mass and strength (Yen et al. 1995). From long list of previous studies (for review see Hartgens & Kuipers 2004) it could be concluded that steroids administration may increase muscle mass. Exogenous testosterone administration (> 125 mg/week), with and without strength training program, may lead to increments of muscle volume and/or muscle fibre size. Yet, lower doses of testosterone (e.g. 25, 50 or 100 mg/week) had no effect on muscle fibre cross-sectional area. It seems that musculotrophic effect of steroids is dose-dependent. Therefore, the effects of prosteroids on muscle size and/or strength is highly influenced by its potential to increase serum testosterone after administration.

Existing data on testosterone-boosting effects of prosteroids are equivocal; it seems that age and basal serum testosterone concentration may influence the response to prosteroids intake (Brown et al. 2000). Several studies reported that serum sex steroid levels in both mature and young men were not significantly affected by prohormone supplementation, with only a minimal amount converted to testosterone and more to estrogen (Vogiatzi et al. 1996;

Morales et al. 1998; Kroboth et al. 1999; Yamada et al. 2007). No changes in levels of testosterone and estradiol were observed for men after supplementation with 50 mg of DHEA for 3, 6 and 12 months (Von Muhlen et al. 2008). In 19 young men (23 ± 1 yr old) participating in an 8-week resistance training, ingestion of 150 mg/day of DHEA did not affect serum testosterone and estrogen concentrations (Bowers 1999). On the other hand, ingesting 100 mg of androstenedione t.i.d. for 28 days increases serum-free testosterone concentration by 40% (Brown et al. 2000; Brown et al. 2001), while 200 mg of androstenediione increases testosterone area under the curve by approximately 15% during the 90 min post-administration (Earnest et al. 2000). In recent study (Ostojic et al. 2010) intake of DHEA resulted in significant increase of total testosterone in treated subjects after 28-days of supplementation. Accordingly, Wolf et al. *(*1997) reported 1.3-fold increase in testosterone levels after supplementation with 50 mg oral DHEA for 2 weeks in 25 men. Furthermore, serum estradiol levels were significantly elevated, indicating that a significant portion of the ingested prosteroids underwent aromatization. It seems that both the magnitude of the dose administered and the route of administration affect the extent of change in concentrations of sex hormones (Ziegenfuss et al. 2002). Furthermore, several studies confirm the importance of extraadrenal and extragonadal 3ß-hydroxysterodi dehydrogenase activity in the synthesis of androgens and estrogens after prohormones administration (Nestler et al. 1991; Kroboth et al. 1999).

Not all subjects respond to prohormones in same fashion, suggesting that additional factors (i.e. age, gender, diet, type and intensity of exercise) influence these responses. For example, research demonstrates that prohormone supplementation may acutely increase testosterone levels in women, thus producing a virilizing effect (Bahrke & Yesalis 2004). Furthermore, exercise could result in increased DHEA and DHEA-S concentrations (Bernton et al. 1995) and these elevated levels in athletes could influence response to supplementation, which requires further investigation. The recent study (Ostojic et al. 2010) reported an increase of total testosterone and estradiol while free testosterone is normal. These data can be consistent with an increase of sex hormone binding globulin (SHBG) by prosteroids administration (Nestler et al. 1991). Total testosterone was increased to keep normal free testosterone or in alternative free testosterone was normal due to an increase activation of 5-alpha reductase. Measuring SHBG luteinizing and folicle-stimulating hormone in future studies should prove these hypotheses. However, it is important to point out that simply producing an acute elevation in a particular hormone concentration (i.e. testosterone) does not necessarily result in increases in muscle mass or lean body mass. Prosteroids do not appear to have functional benefits when taken in daily concentrations up to 300 mg per day in young, middle-aged or older men (Wallace et akl. 1999; Ballantyne et al. 2000; Broeder 2003). Although oral DHEA intake enhanced testosterone production for 30%, Ostojic and co-workers (2010) did not found changes in total muscle mass or regional muscularity.

It seems that effect of prosteroids on serum hormones was not mediated by an effect on body composition. Increasing DHEA or other prohormones levels may not provide the optimal anabolic environment desired in spite of elevated total testosterone level, due to several possible mechanisms (i.e. genetic polymorphism of the androgen receptor, potential hormonal interconversions at the paracrine level) (Nestler et al. 1991). Whether the increase of testosterone after intake of prohormones translates into a meaningful change in body composition or rates of muscle protein synthesis is debatable. Studies must be evaluated in terms of the relative potency of various testosterone enhancers with varying effects on

different tissues according to receptor-binding properties of the compound and its metabolites (Ebeling & Koivisto 1994). The relative potency of prosteroids seems to be small with inconsiderable advantageous anabolic properties. Several authors hypothesized that an important part of the musculotrophic effect of prosteroids may not be directly mediated through androgen receptors but instead involves interference with catabolic effects produced by glucocorticoid hormones binding to their specific receptors (Bernton et al. 1995; Morales et al. 1998). With an incomplete understanding of how prohormones exert their effects on skeletal muscle, further studies should analyze nitrogen balance indicators as noninvasive approximate index of muscle protein status.

5. Fat mass alteration and steroid prohormones intake

In the field of sports and exercise nutrition, prosteroids (DHEA in particular) are often promoted as fat-burning agent that could enhance body physique and estetize appearance (Kroboth et al. 1999). However, clear evidence supporting the use of prosteroids in athletic environment remains less clear. It is well known that age-related decreases in DHEA are associated with increases in obesity and a decline in fat free mass (Morales et al. 1998) yet the potential usefulness of DHEA as a slimming agent is mostly indicated by previous research in animals, particularly lower mammals (Cleary 1991). In the rat plasma concentration of DHEA ranges between 14 and 80 nM while in the plasma of humans DHEA-concentration ranges between 5 and 24 nM and DHEAS-concentration is up to 9 μM (Svec & Porter 1998). The anti-obesity effect of DHEA in animals could be due to several possible mechanisms (Cleary 1991; Ebeling & Koivisto 1994; Kroboth et al. 1999). However, studies that have investigated the effects of oral prosteroids supplementation on body composition in humans produced equivocal results, particularly in young men.

Nestler et al. (1988) reported that 28-day supplementation with DHEA (1600 mg/day) reduced body fat by 31% with no change in body mass in five normal men. Serum total testosterone, free testosterone, sex hormone-binding globulin, estradiol, and estrone levels did not change while serum DHEA-S and androstenedione rose 2.0- to 3.5-fold in DHEA group. Morales et al. (1998) founded that 100 mg of DHEA for 6 months induced decrease in body fat mass (6.1 ± 2.6%) in healthy non-obese men. On the other side, several investigators showed that body composition was not affected by prosteroids treatment in young and adult men, both obese and non-obese (Usiskin et al. 1990; Welle et al. 1990; Wallace et al. 1999). Vogiatzi et al. (1996) suggested that DHEA 40 mg administered sublingually twice daily for 8 weeks has no positive effects on body composition in obese young adults. In recent DAWN trial (Von Muhlen et al. 2008) no beneficial effects of 50 mg daily oral DHEA supplementation on body composition were found in 110 healthy mature men. In accordance with above research, the recent study (Ostojic et al. 2010) failed to show any beneficial effects of oral DHEA administration on body mass and body composition in non-obese young athletes. Authors did not found significant reduction in body fat of young soccer players after DHEA supplementation. Other indicators of body fatness (i.e. body mass index, waist-to-hip ratio) remained unchanged during the study in both DHEA and placebo group, indicating that treatment with DHEA does not result in significant changes to justify its use as an antiobesity or slimming agent. As in the case of cognition, negative results in healthy volunteers can be attributed either to a true lack of DHEA effect or to body composition too close to ideal at the study start to detect changes in the small numbers of subjects studied.

Although most studies found no beneficial effects of prosteroids supplementation on body composition in athletes, several investigators underlined possible beneficial effects of prosteroids supplementation for elderly. Hernandez-Morante et al. (2008) demonstrated for the first time in vitro that DHEA-S stimulates lipolysis in 85 obese patients, preferably in subcutaneous fat in women and in visceral fat in men. A study by Ho et al. (2008) suggested that low DHEA-S is associated with increased waist-to-hip ratio and reduced insulin sensitivity with aging while Hsu *et al.* (2008) had reported that body composition and insulin sensitivity can change with aging in early lifetime. Benefits of prosteroids supplementation in this regard for early middle-aged people requires more clinical investigation.

Although the body composition changes induced by steroids or prosteroids administration are rather small, after the drug withdrawal the alterations of body composition fade away in slow manner, but may be presented in part for period up to 3 months (Kuipers et al. 1991; Hartgens et al. 2001; Brown et al. 2006). Yet, the final net results of short-term steroids or prosteroids administration on body composition seems to be minute (Hartgens & Kuipers 2004). This is particularly true for all athletes who are not capable of maintaining the nutritional intake and training workload of the level required for significant body composition changes (Hartgens et al. 1996). It may be important that steroid cessation is followed by a period of hypogonadism, while testicular function gradually returns to normal, over a period of weeks or several months (Hartgens & Kuipers 2004). Although this has not been specifically studied, reduced circulating androgen during this period may help to accelerate the loss of any anabolic steroid-induced gains. How much of the gain can be sustained by physical training following drug cessation remains to be studied (Yesalis & Bahrke 2000).

6. Known and potential health risks of prosteroids administration

During the past 20 years, researches suggested that potential risk factors associated with prosteroids use were similar to those observed with anabolic steroids (Broeder 2002). The altered hormonal milieu caused by prohormone intake is similar to the hormonal milieu observed in men with gynecomastia, prostate cancer, testicular cancer and pancreatic cancer (Fyssas et al. 1997; Chang et al. 2005). Yet, no documented cases exist of these endocrine-related diseases caused by prohormone supplementation (Brown et al. 2006). Furthermore, it seems that athletes who regularly use prosteroids experienced several side effects (e.g. fatigue, headache, nasal congestion, acne, increased aggressiveness, increased blood pressure, masculinization in women, gynecomastia and testicular wasting in men) (Broeder et al. 2000). Although prohormones induces small decreases in high-density lipoprotein cholesterol (HDL-C), long-term implications of transient negative changes in blood lipids (e.g. 3-6 mg/dL reductions in HDL-C) have yet to be elucidated as the risk of sustaining a cardiac event (Ziegenfuss et al. 2002).

Regarding unfavourable body composition changes, it has been noted that prohormones could lead to changes in hydration of the fat free mass (via sodium and water retention), which could be interpreted as *hyperhydration* effect (Casaburi et al. 1996). Whether more prolonged (> 8-12 weeks) prohormones supplementation is safe or useful remains uncertain, but appears unlikely (Ziegenfuss et al. 2002). Although some health risks have been noted, thus far none of the prohormones tested appear to be overly toxic as no elevations in clinically relevant tissue enzymes (e.g. alanine aminotransferase, creatine kinase, aspartate

aminotransferase, gamma-glutamyltransferase, lactate dehydrogenase) have been observed (Brown et al. 2000; Ziegenfuss et al. 2002). However, due to the lack of efficacy of oral prosteroids supplementation in athletes, its theoretical risks seem to fat outweight any potential benefits on body composition and should be discouraged (Earnest 2001).

7. Summary

In the past 2o years, different steroid prohormones or prosteroids have been aggressively marketed in athletic environment as legal nutritional supplements that are expected to convert to active anabolic steroid hormones in the body and enhance exercise performance. Although the mechanism of action of prosteroids is not completely understood it has been promoted that prosteroids increases testosterone production if supplemented in diet and due to its anabolic action may affects nitrogen balance and protein synthesis. Although popular among athletes, studies have demonstrated repeatedly that acute and long-term administration of these oral testosterone precursors does not effectively increase serum testosterone levels and fails to produce any significant changes in lean body mass, muscle strength, or performance improvement compared with placebo. It seems that increasing prohormone levels in athletes may not provide the optimal anabolic environment desired in spite of elevated total testosterone level (at least acutely), due to several possible mechanisms (i.e. genetic polymorphism of the androgen receptor, potential hormonal interconversions at the paracrine level). The relative potency of prosteroids seems to be small with inconsiderable advantageous anabolic properties. Furthermore, recent studies indicates that treatment with prosteroids does not result in significant changes to justify its use as an antiobesity or slimming agent. Not all subjects respond to prohormones in same fashion, suggesting that additional factors (i.e. gender, diet, type and intensity of exercise) influence these responses. Although some health risks have been noted, thus far none of the prohormones tested appear to be overly toxic. Yet, due to the lack of efficacy of oral prosteroids supplementation in athletes, its theoretical risks seem to far outweight any potential benefits on body composition and should be discouraged. Although the understanding of testosterone precursors as performance-enhancing drugs continues to advance, there are likely to be more revelations as scientific investigations continue.

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3

11β-Hydroxysteroid Dehydrogenase Type 1 and the Metabolic Syndrome

Cidália D. Pereira, Maria J. Martins, Isabel Azevedo and Rosário Monteiro
Dept. of Biochemistry (U38/FCT), Faculty of Medicine, University of Porto, Portugal

1. Introduction

In the past few years, efforts are being made to unravel the mechanisms endowed with the metabolic disturbances associated with obesity that predispose to the metabolic syndrome (MetSyn). The special pathogenic role of liver and visceral adipose tissue (VAT) functions has been particularly intriguing, and many hypotheses have been advanced to explain this association. Tissue-specific actions of glucocorticoids (GCs) go far beyond the circulating levels of the hormones and can be controlled by local intracellular enzymes. In the past few years, evidence is being gathered not only on the relevance of such enzymes to GC physiological actions but also on their involvement in the pathophysiology of certain chronic disease states, in which circulating GC levels are not necessarily altered. These enzymes are 11β-hydroxysteroid dehydrogenases (11β-HSDs, EC 1.1.1.146) which interconvert inactive GCs, such as cortisone and dehydrocorticosterone, and the active hormones, cortisol and corticosterone.

2. Brief overview of the hypothalamus-pituitary-adrenal (HPA) axis

The regulation of tissue GC levels is critical for the maintenance of homeostasis, playing a central role in essential physiological processes, such as stress responses, energy metabolism, electrolyte levels, blood pressure, immunity, cell proliferation and differentiation and cognitive functions (Atanasov & Odermatt, 2007). Cortisol release by the adrenal gland is under the control of the HPA axis. Briefly, corticotrophin releasing hormone (CRH) is produced by parvicellular hypothalamic neurons and acts on anterior pituitary cells increasing the production and release of adenocorticotrophic hormone (ACTH) into the blood stream in a pulsatile fashion and with circadian rhythm: peak in the morning and valley later in the afternoon (Gathercole & Stewart, 2010; White B., 2008b). Cortisol is synthesized in the cells of the zona fasciculata of the adrenal cortex. Under the influence of ACTH, cholesterol esters, stored in the foamy cytoplasm of these cells, are unsterified by cholesterol ester hydrolase and converted to cortisol (Tomlinson et al., 2004; White B., 2008a). GCs are able to bind and activate GC receptors (GR) and mineralocorticoid receptors (MR), which are ligand-regulated nuclear receptors and members of the steroid hormone receptor family (Gathercole & Stewart, 2010). Cortisol and the principal GC in rodents, corticosterone, are active steroids whereas cortisone and 11-dehydrocorticosterone, the latter in rodents, are inactive (Tomlinson et al., 2004). Cortisol is metabolized in the liver

through conjugation with glucuronide and sulfate for posterior renal excretion (Tomlinson et al., 2004; White B., 2008a). Moreover, in the liver, 5α- and 5β-reductases inactivate cortisol and cortisone, in conjunction with 3α-HSD, to tetrahydrometabolites: 5α-tetrahydrocortisol (5α-THF), 5β-tetrahydrocortisol (5β-THF) and tetrahydrocortisone (THE) (Campino et al., 2010).

3. 11β-HSDs – enzymology, tissue expression and physiological role

3.1 11β-HSD type 2 (11β-HSD2)

Because cortisol and aldosterone have the same *in vitro* affinity for the MR (Gathercole & Stewart, 2010), 11β-HSD2, that catalyzes the inactivation of cortisol to inert cortisone, in humans, or of corticosterone to 11-dehydrocorticosterone, in rodents, avoids MR actions of GCs. 11β-HSD2 was the first isoform to be identified and is a NAD^+ dependent dehydrogenase. 11β-HSD2 is present in high amounts in the distal convoluted tubule of the kidney, colon, salivary and sweat glands as well as in other locations such as the human placenta and vascular wall to avoid deleterious actions of active GC overstimulation (Anagnostis et al., 2009; Andrews et al., 2003; Edwards et al., 1988; Ferrari, 2010; Funder et al., 1988; Gathercole & Stewart, 2010; Palermo et al., 2004). The importance of 11β-HSD2 activity is illustrated in the case of congenital deficiency of 11β-HSD2 in humans (Gathercole & Stewart, 2010; Stewart et al. 1996), transgenic deletion in mice (Kotelevtsev et al., 1999) or by its pharmacological inhibition which produces the apparent mineralocorticoid excess (AME) syndrome in which the lack of cortisol inactivation in the kidney allows its mineralocorticoid action, producing sodium retention, hypertension and hypokalemia, despite normal circulating levels of cortisol and an intact HPA axis (Anagnostis et al., 2009; Andrews et al., 2003; Edwards et al., 1988; Gathercole & Stewart, 2010; Monder et al., 1986; Mune et al., 1995; Palermo et al., 2004; Quinkler & Stewart, 2003; Stewart et al., 1996; Walker & Andrew, 2006). Thus AME has been considered 'Cushing's disease of the kidney' where there are normal circulating levels of cortisol but a tissue-specific excess at the site of MR action (Stewart, 2005).

3.2 11β-HSD type 1 (11β-HSD1)

Pre-receptor metabolism of GCs by 11β-HSD1 amplifies intracellular levels of GCs, through the reduction of inactive cortisone in humans (11-dehydrocorticosterone in rodents) back into active cortisol (corticosterone in rodents) (Anagnostis et al., 2009; Espindola-Antunes & Kater, 2007). 11β-HSD1 is mostly expressed in the liver, adipose tissue (AT), bone, lung and central nervous system. However, its expression can be present in other tissues including pancreas, kidney cortex, adrenal cortex, cardiac myocytes, bone, placenta, uterus, testis, oocytes and luteinized glanulosa cells of the ovary, eye, pituitary, fibroblasts and immune, skeletal and smooth muscle cells (Anagnostis et al., 2009; Bujalska et al., 1997; Cooper & Stewart, 2009; Espindola-Antunes & Kater, 2007; Stewart & Krozowski, 1999; Tomlinson et al., 2004; Whorwood et al. 2001). This enzyme is located in the endoplasmic reticulum, facing the lumen (Gathercole & Stewart, 2010), where there is a high concentration of NADPH owing to the activity of hexose-6-phosphate dehydrogenase (H6PDH), that regenerates NADPH from $NADP^+$ (Atanasov et al., 2008; Bujalska et al., 2005; Draper et al., 2003).

11β-HSD1 is bidirectional, able to act as both a reductase (activating GCs) and a dehydrogenase (inactivating GCs) (Cooper & Stewart, 2009; Tomlinson et al., 2004). However, its main function is as a reductase on intact cells such as hepatocytes (Jamieson et

al., 1995), myocytes (Whorwood et al., 2001) and adipocytes (Bujalska et al., 2002a; Bujalska et al., 2002b), supported by a higher affinity for cortisone than cortisol (Stewart et al., 1994). *In vitro*, when deprived of NADPH regeneration (Seckl & Walker, 2001; Walker & Andrew, 2006) or in certain physiological or developmental states, it may work as a dehydrogenase. For example, in human omental adipose stromal cells, 11β-HSD1 switches from a dehydrogenase to a reductase upon differentiation (Bujalska et al., 2002a; Bujalska et al., 2002b). In the H6PDH null mouse, hepatic or AT, 11β-HSD1 acts mainly as a dehydrogenase (Bujalska et al., 2008b; Lavery et al., 2006). Most studies on the regulation of 11β-HSD1 have been performed on rodent tissues showing that GCs, CCAAT/enhancer binding proteins, peroxisome proliferator-activated receptor (PPAR) agonists and some pro-inflammatory cytokines [tumor necrosis factor-α (TNF-α) and interleukin-1β] increase 11β-HSD1 expression. On the other hand, growth hormone (via insulin-like growth factor-1) and liver X receptor (LXR) agonists inhibit its expression. Some other factors that may influence 11β-HSD1 expression include sex steroids, insulin and thyroid hormone, but effects vary in different tissues and between species (Tomlinson et al., 2004).

Human 11β-HSD1 congenital deficiency has been described as the apparent cortisone reductase deficiency syndrome (Phillipov et al., 1996). The phenotype is related to the lack of regeneration of cortisol in peripheral tissues with compensatory activation of the HPA axis. This results in increased secretion of androgens by the adrenals, and affected females present hirsutism and oligomenorrhea. 11β-HSD1 congenital deficiency does not appear to protect against obesity. The syndrome does not seem to arise only from mutations of HSD11B1, but rather from the co-inheritance of deleterious mutations in both HSD11B1 and H6PDH (Draper et al., 2003), decreasing NADPH supply and switching 11β-HSD1 to the dehydrogenase activity (Lavery et al., 2006).

4. Chronic GC deficiency or excess

The involvement of GCs in human obesity, particularly visceral obesity, and its related metabolic complications, is becoming increasingly evident. As we will discuss further, this is evident not only in subjects with disturbances in the HPA axis, but also in conditions where tissue GCs are locally modified. To illustrate the first case, two conditions reflect the involvement of circulating cortisol on body weight regulation in opposite extremes: Addison's disease (hypocortisolism) and Cushing's syndrome (hypercortisolism) (Rutters et al., 2010). As to the second case, the clinical entity that aggregates visceral obesity along with several metabolic abnormalities in glucose and lipid metabolism as well as in blood pressure, and known as the MetSyn (Reaven, 2011), may constitute the best example. Chronically elevated GC levels cause obesity, type 2 diabetes mellitus (T2DM), heart disease, mood disorders and memory impairments (Wamil & Seckl, 2007). This is demonstrated in Cushing's syndrome, in which elevated GC levels are a result of increased pathological secretion from the adrenal cortex (endogenous) or from prolonged anti-inflammatory GC treatment (iatrogenic) (Newell-Price et al., 2006). A particular case of Cushing's syndrome is Cushing´s disease that consists of hypercortisolism driven by increased ACTH secretion from pituitary adenoma (Cushing, 1932; Stewart, 2005). Patients with Cushing's syndrome are hypertensive, have visceral obesity, insulin resistance (IR; 50% develop T2DM or impaired glucose tolerance) and may present hepatic steatosis (Stewart, 2005), muscle weakness, dyslipidemia, mood disturbances and infertility as well as features more specific to Cushing's syndrome (e.g. easy bruising, facial plethora and violaceous striae) (Carroll & Findling, 2010; Newell-Price et al., 2006).

The increase of AT in states of GC excess, such as Cushing's syndrome, may not seem straightforward. Indeed, one might predict from cortisol metabolic actions that it would increase the availability of energetic substrates, as is seen with its lipolysis-stimulating effects. However, in these settings, particularly if there is positive energy balance, the chronic increase in GCs is concomitant with the increase in insulin (Dallman et al., 2004). This favors fatty acid re-esterification over lipolysis, which, along with pro-adipogenic effects of insulin and GCs (Rosen & MacDougald, 2006), increases AT depots. This is seen particularly on VAT depots, rather than other AT locations, probably for two reasons: higher expression of GR (Bronnegard et al., 1990) and increased reactivation of circulating cortisone due to high 11β-HSD1 expression and/or activity (Alberti et al., 2007; Simonyte et al., 2009).
Diagnostic features of GC excess in Cushing's syndrome overlap many of the MetSyn components suggesting that GCs may contribute to the pathogenesis of both states (Anagnostis et al., 2009; M. Wang, 2011). It has been demonstrated that circulating cortisol concentrations are higher in patients with MetSyn compared with healthy subjects, both in basal conditions and during dynamic stimulation (Duclos et al., 2005; Misra et al., 2008; Phillips et al., 1998; Sen et al., 2008; Weigensberg et al., 2008). Furthermore, increased 11β-HSD1 activity in VAT may generate increased cortisol levels within AT and liver and thereby promote features of the MetSyn (Walker & Andrew, 2006). This effect has been termed 'Cushing's disease of the omentum' (Bujalska et al., 1997; Stewart, 2005).

5. MetSyn definition

In the past few decades, there has been a worldwide increase in the prevalence of obesity and associated metabolic disorders including glucose intolerance, IR, dyslipidemia and hypertension. In the clinical practice, the presence of these conditions defines the MetSyn, which comprises an increased risk of atherosclerotic cardiovascular events and T2DM (or is associated with T2DM) and, additionally, is characterized by a pro-inflammatory and a pro-thrombotic state and occurrence of non-alcoholic fatty liver disease (NAFLD) (Feldeisen & Tucker, 2007; Gathercole & Stewart, 2010; Johnson & Weinstock, 2006; Reaven, 2011).
Distinct organizations have established their own definitions of the MetSyn: the World Health Organization (WHO), in 1998, the Adult Treatment Panel III (ATP III) of the National Cholesterol Education Program (NCEP), in 2001 (updated in 2005), and the International Diabetes Federation (IDF), in 2005 (Johnson & Weinstock, 2006; Reaven, 2011). In the attempt to harmonize MetSyn definitions, ATP III and IDF, joined by several other prestigious organizations, reviewed the criteria. Meeting any three of the following criteria is sufficient for the diagnosis: elevated waist circumference (abdominal obesity), triglycerides, blood pressure and fasting glucose (glucose intolerance) and low HDL-cholesterol levels. The cut points for an elevated waist circumference are not the same for all population groups (with population-specific reference values) and drug treatment is sufficient to meet the criteria for the other four components. The latest WHO report states that the 'MetSyn should not be a clinical diagnosis', but rather viewed as 'a pre-morbid condition, and should thus exclude individuals with established T2DM or cardiovascular disease' (Reaven, 2011).

6. 11β-HSD1 and MetSyn components: evidence from human and animal studies

Evidence has been accumulated that strongly argues for an etiological role of 11β-HSD1 in obesity, T2DM and MetSyn (Cooper & Stewart, 2009; Gathercole & Stewart, 2010; London &

Castonguay, 2009; Masuzaki & Flier, 2003; Morton, 2010; Staab & Maser, 2010; Tomlinson & Stewart, 2007; van Raalte et al., 2009; Wamil & Seckl, 2007).

Initial studies in obese humans, that measured the ratio of cortisol to cortisone metabolites in urine as an indirect index of total body 11β-HSD activity, produced inconsistent results (such ratios, however, are inadequate as they may be influenced by other enzymes involved in cortisol metabolism). Recently, more trustworthy results from various more tissue-specific measures were obtained (Andrews et al., 2002; Andrews et al., 2003; Desbriere et al., 2006; Gathercole & Stewart, 2010; Karlsson et al., 2010; Morton, 2010; Paulmyer-Lacroix et al., 2002; Rask et al., 2001; Rask et al., 2002; Sandeep et al., 2005; Stewart & Tomlinson, 2009; R. Stimson et al., 2011; Tomlinson et al., 2008; Valsamakis et al., 2004; Wamil & Seckl, 2007). After studies in men and women, representing a wide range of body compositions and insulin sensitivities (but without T2DM), 11β-HSD1 activity is found selectively increased in the abdominal subcutaneous AT (SAT) in obese humans (Rask et al., 2001; Rask et al., 2002; Sandeep et al., 2005), to a similar degree as the increase in transgenic overexpressing mice (Andrews et al., 2003), but impaired in the liver (Rask et al., 2001; Rask et al., 2002; Stewart et al., 1999). This decrease in hepatic tissue may represent a compensatory mechanism to preserve insulin sensitivity and to decrease hepatic glucose output (Gathercole & Stewart, 2010; Morton, 2010; Valsamakis et al., 2004; Wamil & Seckl, 2007). Increased adipose 11β-HSD1 activity results from increased 11β-HSD1 mRNA expression (Desbriere et al., 2006; Paulmyer-Lacroix et al., 2002) in the abdominal SAT in adipocytes, and also in the VAT in both adipocytes and stroma (Paulmyer-Lacroix et al., 2002). Valsamakis et al. report a lack of inhibition of 11β-HSD1 activity with increasing body mass index (BMI) in diabetic patients *versus* non-diabetic BMI- and age-matched controls (where the inhibition is closely associated with VAT mass), and suggest that a reduction in 11β-HSD1 activity might act as an autocrine protective mechanism to prevent increasing adiposity and increased hepatic glucose output with advancing obesity. This adaptive mechanism of reduced cortisol regeneration does not occur in obesity-associated T2DM and might contribute to the underlying pathogenesis of the disease (Gathercole & Stewart, 2010; Morton, 2010; Valsamakis et al., 2004; Wamil & Seckl, 2007). In contrast, in lean patients with T2DM (controlled by diet alone) a relatively small decrease in hepatic 11β-HSD1 activity, and no change in gluteal SAT enzyme activity, has been reported, but only by one group (Andrews et al., 2002; Andrews et al., 2003). Abdominal SAT 11β-HSD1 expression is higher in obese women with impaired glucose tolerance than in obese women with normal glucose tolerance, despite AT (total and regional) being similar between the two groups, and positively correlated with glucose area under curve levels across an oral glucose tolerance testing (Tomlinson et al., 2008). Whole-body 11β-HSD1 activity is increased in obese men with T2DM, compared to healthy normal-weight control subjects, whereas liver 11β-HSD1 activity is sustained, unlike in euglycemic obesity (R. Stimson et al., 2011). The evidences presented raise the hypothesis that hepatic 11β-HSD1 inhibition in obese people who develop impaired glucose tolerance may protect from progression to T2DM (Gathercole & Stewart, 2010; Morton, 2010; Wamil & Seckl, 2007). Additionally, in line with this, myotubes established from obese T2DM subjects show an increased expression of 11β-HSD1 mRNA compared to healthy obese subjects (Abdallah et al., 2005). SAT 11β-HSD1 mRNA levels decrease during very low calorie diet (16 weeks) and anthropometric measurements and metabolic parameters are associated with 11β-HSD1 mRNA levels in obese subjects without the MetSyn (following the WHO definition). However, in obese subjects with the MetSyn these associations were lost or in the opposite direction. In another cohort, this difference is also observed in skeletal muscle (vastus lateralis) between subjects with T2DM or with normal glucose tolerance (Karlsson et al., 2010).

6.1. Findings of 11β-HSD1 biology from rodent models

6.1.1 The aP2-HSD11B1 transgenic rodent model

Transgenic mice with 2–3-fold overexpression of 11β-HSD1, comparable to that seen in obese humans, in white AT have been generated, exploiting the murine adipocyte fatty acid binding protein (aP2) promoter. These aP2-HSD11B1 transgenic mice have elevated corticosterone levels in the AT, but unaltered systemic plasma concentrations, and many features of the MetSyn: glucose intolerance and IR [exacerbated further by high-fat (HF) feeding], dyslipidemia, apparent leptin resistance, truncal obesity and hypertension associated with activation of the circulating renin-angiotensin system. 11β-HSD1 expression correlates strongly and positively with adipocyte size (London & Castonguay, 2009; Masuzaki & Flier, 2003; Masuzaki et al., 2001; Masuzaki et al., 2003; Morton, 2010; Staab & Maser, 2010; van Raalte et al., 2009; Wamil & Seckl, 2007). TNF-α and leptin are elevated whereas resistin and insulin-sensitizing adiponectin are reduced. aP2-HSD11B1 transgenic mice are hyperphagic and obese, predominantly in the VAT. Expression of the GR-α is higher in VAT compared to SAT, while the expression of the transgene HSD11B1 is similar in all AT depots. The greater effects in VAT may reflect the higher GR and/or higher lipoprotein lipase in mesenteric AT. aP2-HSD11B1 transgenic mice have elevated corticosterone and free fatty acids (FFA) levels in the hepatic portal vein that drains blood from VAT to the liver (Masuzaki et al., 2001; Masuzaki et al., 2003; Morton, 2010; Wamil & Seckl, 2007). The aP2-HSD11B1 model shows that altered AT metabolism of GCs (similar to human MetSyn levels) could be the primary driver of many features of this disease (Masuzaki & Flier, 2003; Masuzaki et al., 2001; Masuzaki et al., 2003; Morton, 2010).

6.1.2 The apolipoprotein E (apoE)-HSD11B1 transgenic rodent model

To examine the impact of elevated liver GCs, mice overexpressing 11β-HSD1 selectively in that tissue under the control of the human apoE promoter have been generated. Transgenic lines with 2- and 5-fold-elevated 11β-HSD1 activity exhibit unaltered systemic corticosterone, modest IR (but lacking glucose intolerance), unaltered AT mass (lacking obesity or central adiposity), hepatic fat accumulation (mainly as triglycerides) and dyslipidemia (elevated circulating FFA and HF diet-induced dyslipidemic cholesterol lipoprotein profile), with increased hepatic lipid synthesis/flux associated with elevated hepatic LXR-α and PPAR-α expression as well as impaired hepatic lipid clearance. Increased expression of GC-inducible cholesterol 7α-hydroxylase present in apoE-HSD11B1 transgenic livers may drive increased bile acid synthesis, contributing to stimulation of LXR-α-regulated pathways (and further potentiation of cholesterol 7α-hydroxylase expression) as well as PPAR-α. ApoE-HSD11B1 transgenic mice also have a marked, transgene-dose-associated hypertension, paralleled by incrementally increased liver angiotensinogen expression. Elevated 11β-HSD1 hepatic expression may relate to the pathogenesis of specific fatty liver, insulin-resistant and hypertensive syndromes without obesity in humans as may occur in, possibly, the metabolically obese normal-weight individual (Paterson et al., 2004).

6.1.3 The HSD11B1 knockout (KO) rodent model

HSD11B1 KO mice have been generated, which are viable and healthy but unable to convert inert 11-dehydrocorticosterone to corticosterone. Despite compensatory adrenal hyperplasia and increased adrenal secretion of corticosterone, on fasting, HSD11B1 KO mice have attenuated activation of the hepatic gluconeogenic enzymes, presumably, because of relative

intra-hepatic GC deficiency. The HSD11B1 KO mice resist hyperglycemia provoked by obesity or stress (Kotelevtsev et al., 1997). HSD11B1 KO mice, fed *ad lib*, have markedly lower plasma triglyceride levels, driven by increased hepatic expression of enzymes of fat catabolism and PPAR-α. HSD11B1 KO mice also have increased plasma HDL-cholesterol, with elevated liver mRNA and serum levels of apoAI. Conversely, hepatic Aα-fibrinogen expression is decreased. Upon fasting, the normal elevation of hepatic PPAR-α mRNA is lost in HSD11B1 KO mice, consistent with attenuated GC induction. Despite this, crucial oxidative responses to fasting are maintained. Refeeding (4 h and/or 24 h) shows more rapid and/or marked induction of genes encoding lipogenic enzymes/transcription factors and a more rapid and/or marked suppression of genes for fat catabolism in HSD11B1 KO mice, implying increased liver insulin sensitivity. PPAR-α is suppressed by 4 h of refeeding (similarly in wild type and HSD11B1 KO mice), but PPAR-α levels are higher after 24 h of refeeding in HSD11B1 KO mice when compared to wild type mice, reestablishing the *ad lib*-fed pattern. Concordant with this, 24 h refed HSD11B1 KO mice have higher plasma triglycerides than 24 h refed wild type mice and *ad lib*-fed HSD11B1 KO mice. 24 h Refed HSD11B1 KO mice have lower plasma glucose levels than 24 h refed wild type mice and *ad lib*-fed HSD11B1 KO mice. HSD11B1 KO mice also have improved glucose tolerance. 11β-HSD1 deficiency may produce an improved lipid profile, hepatic insulin sensitization and a potentially atheroprotective phenotype (Morton et al., 2001). HSD11B1 KO mice on the control diet express, compared to wild-type mice, lower leptin, resistin and TNF-α but higher PPAR-γ, adiponectin and uncoupling protein-2 (UCP-2) mRNA levels in epididymal AT, indicating insulin sensitization. On the control diet, in mesenteric VAT, PPAR-γ mRNA is elevated in HSD11B1 KO mice, though leptin, resistin, TNF-α, adiponectin and UCP-2 mRNA levels are unaltered, compared to wild-type mice. With HF feeding, the elevated PPAR-γ mRNA level in control-fed HSD11B1 KO mice is further increased selectively in VAT, what does not happen in the epididymal AT depot of HSD11B1 KO or wild-type mice. HSD11B1 KO mice also show a HF–mediated induction of UCP-2 selectively in VAT, which is greater than that observed in wild-type mice. Isolated adipocytes from HSD11B1 KO mice exhibit higher basal and insulin-stimulated glucose uptake. HSD11B1 KO mice also display reduced VAT accumulation upon HF feeding. HF-fed HSD11B1 KO mice rederived onto the C57BL/6J strain (obesity/T2DM/metabolic disease-susceptible) resist T2DM and weight gain despite consuming more calories. These data provided the first *in vivo* evidence that AT 11β-HSD1 deficiency beneficially alters AT distribution and function (Morton et al., 2004), complementing the just above-described effects of hepatic 11β-HSD1 deficiency or data presented further bellow regarding 11β-HSD1 pharmacological inhibition. Since PPAR-γ ligands cause insulin sensitization and AT redistribution to the periphery, a mechanism for the beneficial AT redistribution is suggested, on the assumption that increased circulating FFA during HF feeding act as endogenous ligands for PPAR-γ receptors. Further, UCP-2 levels are higher in HSD11B1 KO mice AT, consistent with GC and PPAR-γ regulation. This higher PPAR-γ-responsive UCP-2 expression in HSD11B1 KO mice AT may drive increased energy dissipation within the adipocytes (Morton, 2010). Interestingly, when mice are fed a HF diet they preferentially gain weight in peripheral AT rather than in VAT what can be explained by an increased expression of PPAR-γ and UCP-2 in VAT (Morton, 2010; van Raalte et al., 2009; Wamil & Seckl, 2007).

Mice overexpressing the cortisol inactivating enzyme specifically on the AT (aP2-HSD11B2 mice) are phenotypically similar to HSD11B1 KO mice, exception only for food intake, what

emphasizes the importance of AT as a target for enzyme inhibition (Wamil & Seckl, 2007). 11β-HSD1 gene deficiency is associated with a number of improvements of adipose and hepatic functions, what highlights the importance of adipose and hepatic 11β-HSD1 in the development of metabolic disease.

6.2 11β-HSD1 and T2DM/IR

A role for 11β-HSD1 in exacerbating IR and T2DM has been proposed. Animals with targeted deletion of HSD11B1 manifest increased hepatic and adipose insulin sensitivity (Kotelevtsev et al., 1997; Morton et al., 2001; Morton et al., 2004), and when backcrossed onto the C57BL/6J strain appear to resist the development of IR in response to HF feeding (Morton et al., 2004). Additionally, specific 11β-HSD1 inhibitors improve insulin sensitivity (glycemic control and/or glucose and/or insulin levels) in animal models (associated or not with HF feeding) of hyperglycemia, obesity (by damage of feeding center or diet-induced), T2DM (also ob/ob) and combined T2DM, dyslipidemia and atherosclerosis (Alberts et al., 2002; Alberts et al., 2003; Barf et al., 2002; Gathercole & Stewart, 2010; Hermanowski-Vosatka et al., 2005; Morgan et al., 2009; Park et al., 2011; X. Zhang et al., 2009b).

It is well known that excess GCs increase IR and can, in susceptible individuals, precipitate T2DM. In line with this, it has been suggested that the increased production of cortisol from VAT seen in obesity could drain through the portal circulation to the liver and pancreas contributing to IR (Cooper & Stewart, 2009; Masuzaki et al., 2001; Morton, 2010; R. Stimson et al., 2009; Walker & Andrew, 2006; Wamil & Seckl, 2007). This hypothesis was investigated *in vivo* in humans by Stimson et al. by quantifying, for the first time, selectively, the contributions of SAT, visceral tissues and liver to whole-body cortisol production by 11β-HSD1. Stimson et al. confirmed that splanchnic cortisol production is substantial, originating entirely from the 11β-HSD1 activity in the liver. However, although release of cortisol by 11β-HSD1 into the portal vein, which drains a number of visceral organs, is not detected, a significant cortisol release into veins draining exclusively SAT has been found. So, cortisol release from SAT into the systemic circulation is unlikely to have effects in other organs because the feedback control by the HPA axis will adjust adrenal cortisol secretion to maintain circulating cortisol concentrations. Therefore, the most likely impact of this source of cortisol will be intracrine or paracrine in the local AT environment (R. Stimson et al., 2009).

Skeletal muscle represents a key target tissue for insulin-stimulated glucose uptake, metabolism and utilization (Abdul-Ghani & DeFronzo, 2010; Benito, 2011; Van Cromphaut, 2009). There are just a few studies regarding 11β-HSD1 in skeletal muscle from T2DM, although with non-consensual results (Cooper & Stewart, 2009). Whorwood et al. found, with kinetic analysis, that 11β-HSD1, in intact cultured human skeletal myoblasts (from both lean-moderately overweight and obese adult men, few with T2DM but without therapy), acts exclusively as a reductase and is down-regulated by insulin, which may maintain insulin sensitivity in skeletal muscle tissue by diminishing GC antagonism of insulin action (Whorwood et al., 2001). Cortisone reduces glucose uptake in myotubes established from obese T2DM men (treated either by diet alone or in combination with sulfonylurea or metformin, withdrawn one week before performing the biopsy), what could be mediated by an increased mRNA 11β-HSD1 expression (previously mentioned) emphasizing that the local conversion of inactive to active GCs may be important in IR pathogenesis (Abdallah et al., 2005). Accordingly, Zhang et al., in an animal model of T2DM (Wistar rats with HF feeding, combined with multiple low dose streptozotocin injection), report increased 11β-

HSD1 mRNA and protein levels in skeletal muscle extracts of the diabetic animals *versus* the non-diabetic animals, what may be related to disturbances in insulin signaling pathway observed in the skeletal muscle (M. Zhang et al., 2009a). Jang et al. demonstrated that the activities of skeletal muscle 11β-HSD1 and 11β-HSD2 (in vastus lateralis biopsies) are altered in T2DM patients (treated by diet alone or oral hypoglycemic agents) *versus* healthy age- and sex-matched controls (altogether overweight and obese subjects): 11β-HSD1 activity is reduced and 11β-HSD2 activity is higher in T2DM subjects (negative correlation between both enzyme activities; with similar mRNA levels in T2DM and control subjects for both enzymes), and, more importantly, 11β-HSD1 reductase activity is significantly lower in T2DM subjects whereas 11β-HSD1 dehydrogenase activity is significantly higher in the T2DM group (with very low levels of 11β-HSD1 dehydrogenase activity in both groups). Together these results may indicate a reduced intracellular cortisol generation, potentially conferring metabolic protection (Jang et al., 2007). In what regards the AT, Balachandran et al. demonstrated that insulin stimulates adipocyte 11β-HSD1 activity and expression both *in vitro* (in 3T3-L1 adipocytes) and *in vivo* (Wistar rat white AT) (Balachandran et al., 2008).
Morgan et al. established a strong connection between a key player in insulin signaling, the insulin receptor substrate 1 (IRS1), and 11β-HSD1 in skeletal muscle: in KK/Ta Jcl mice (an hyperglycemic model) treated with A2, inducing selective 11β-HSD1 inhibition, skeletal muscle $pSer^{307}$IRS1 decreases, $pThr^{308}$Akt/PKB increases and lipogenic and lipolytic gene expression decreases (Morgan et al., 2009). 11β-HSD1 has also been proposed to have effects on insulin secretion itself. Davani et al. report 11β-HSD1 mRNA expression in human and ob/ob mice (non-insulin-dependent diabetes model) pancreatic β-cells, and also characterize the 11β-HSD1 activity in intact pancreatic rodent islets (where the reductive reaction prevails). In ob/ob mice islets, in the absence of carbenoxolone, 11-dehydrocorticosterone markedly inhibits insulin release, whereas a reversal of this effect is noted in the presence of carbenoxolone, indicating an important role of 11β-HSD1 in the regulation of insulin release (Davani et al., 2000). A more recent report describes a similar effect of dehydrocorticosterone on insulin release in human and murine pancreatic cells, but it appears that enzyme expression is absent in β-cells, with this effect being mediated indirectly through expression within α-cells. This α-cell expression additionally inhibits insulin-stimulated glucagon secretion (Cooper & Stewart, 2009; Swali et al., 2008).

6.3 11β-HSD1 and hypertension

GC hormones act on the cardiovascular system (Nussinovitch et al., 2010; Raff & Findling, 2003; Walker et al., 2000; Wallerath et al., 1999). Cortisol and 11β-HSDs have been implicated in hypertension (Anagnostis et al., 2009; Andrews et al., 2003; Campino et al., 2010; Cicala & Mantero, 2010; Edwards et al., 1988; Ferrari, 2010; Franks et al., 2004; Funder et al., 1988; Gathercole & Stewart, 2010; Y. Liu et al., 2008; Malavasi et al., 2010; Masuzaki et al., 2003; Millis, 2011; Monder et al., 1989; Morales et al., 2008; Mune et al., 1995; Palermo et al., 2004; Paterson et al., 2004; Quinkler & Stewart, 2003; Raff & Findling, 2003; S. Shah et al., 2011; Stewart et al., 1996; Walker & Andrew, 2006; Walker et al., 1993; Wallerath et al., 1999; White et al., 1997).
The fact that 11β-HSD2 is important in protecting MR in the distal nephron from stimulation by GCs revealed its role in the regulation of arterial blood pressure. Pharmacological inhibition or genetic deficiency of 11β-HSD2 leads to the development of hypertension (Anagnostis et al., 2009; Andrews et al., 2003; Edwards et al., 1988; Ferrari, 2010; Funder et al., 1988; Gathercole & Stewart, 2010; Palermo et al., 2004; Walker et al., 1993; White et al.,

1997). HSD11B2 can be epigenetically regulated, what is also involved in hypertension development (Millis, 2011). In the same line, defects and polymorphisms in HSD11B2 have also been shown to play a role in human hypertension and cardiovascular disease [e.g. essential hypertension (Soro et al., 1995; Walker et al., 1993) and 'salt-sensitive' hypertension (Lovati et al., 1999)] (Bailey et al., 2008; Cooper & Stewart, 2009; Henschkowski et al., 2008).
Campino et al. reported a high percentage of alterations in the cortisol metabolism at the pre-receptor level in hypertensive patients, previously misclassified as having essential hypertension, where 18% of the patients present reduced 11β-HSD2 activity or imbalance of 11β-HSD1 activity in comparison to 11β-HSD2 (Campino et al., 2010). As referred above, hypertension is induced in mice genetically modified to overexpress 11β-HSD1 either in the liver or AT (Masuzaki et al., 2003; Paterson et al., 2004). HSD11B1 polymorphisms have been described, affecting enzyme expression and activity *in vitro* and/or *in vivo*, and/or being associated with hypertension (Franks et al., 2004; Malavasi et al., 2010; Morales et al., 2008). Variants of HSD11B1 were associated with the risk of hypertension in Pima Indians (Franks et al., 2004). Liu et al. showed that suppression of 11β-HSD1 expression in the renal medulla attenuates salt-induced hypertension in Dahl salt-sensitive rats (Y. Liu et al., 2008). Taking into consideration that diet is one important factor on MetSyn development, it is interesting to mention that in Dahl salt-sensitive hypertensive rats, fed a high-salt diet for 4 weeks, peri-renal AT corticosterone concentration and 11β-HSD1 activity as well as GR, 11β-HSD1 and TNF-α expression increase when compared with Dahl salt-resistant rats fed the same diet (Usukura et al., 2009).

6.4 11β-HSD1 and NAFLD

NAFLD is being increasingly recognized as a common liver disorder that represents the hepatic manifestation of the MetSyn. NAFLD is more frequent among people with T2DM and obesity, and it is almost universal amongst T2DM patients who are morbidly obese (Bellentani et al., 2000; Fabbrini et al., 2010; Gupte et al., 2004; Konopelska et al., 2009; Ratziu et al., 2010; Wree et al., 2010). Non-alcoholic steatohepatitis (NASH) is the progressive form of liver injury that carries a risk of progressive fibrosis, cirrhosis and end-stage liver disease. There is strong evidence that IR and increased FFA are a major cause of NASH (Brunt, 2004; Konopelska et al., 2009; Ratziu et al., 2010; Scheen & Luyckx, 2002). Inflammation plays an important additional role with increased production of reactive oxygen species and pro-inflammatory cytokines. In addition, several studies support a link between VAT and NASH (Kern et al., 2003; Konopelska et al., 2009; McCullough & Falck-Ytter, 1999). Konopelska et al., for the first time in patients with elevated liver enzymes (that after liver biopsies had histological diagnosis of normality, steatosis, NASH and other forms of hepatitis or cirrhosis), found no association between increased liver fat accumulation or different stages of liver inflammation and hepatic 11β-HSD1 expression, suggesting that, probably, there is no major role of this enzyme in the inflammatory process from fatty liver to NASH in humans (Konopelska et al., 2009). In contrast, as mentioned before, transgenic mice with hepatic overexpression of 11β-HSD1 develop fatty liver and dyslipidemia (Paterson et al., 2004). 11β-HSD1 expression correlated positively with H6PDH expression in the liver and negatively with waist-to-hip ratio in women (this being in accordance to obesity results we have mentioned previously). No evaluation of 11β-HSD1 and H6PDH protein or activity levels was done (Konopelska et al., 2009).
Given all the above evidences, 11β-HSD1 has thus emerged as a major potential drug target for the treatment of obesity and its associated metabolic abnormalities.

7. 11β-HSD1 inhibition studies

Several and distinct selective 11β-HSD1 inhibitors are being produced, developed and tested *in vitro*, *ex vivo* and *in vivo*, in normal animals, rodent models of metabolic alterations or disease (hyperglycemia, dyslipidemia, atherosclerosis, IR, T2DM, obesity, diet-induced obesity and/or MetSyn) and some of them already in humans, healthy or not (Alberts et al., 2002; Alberts et al., 2003; Barf et al., 2002; Bhat et al., 2008; Bujalska et al., 2008a; Cho et al., 2009; Cooper & Stewart, 2009; Coppola et al., 2005; Courtney et al., 2008; Feig et al., 2011; Gathercole & Stewart, 2010; Ge et al., 2010; Hale et al., 2008; Hale & Wang, 2008; Hermanowski-Vosatka et al., 2005; Hollis & Huber, 2011; Hughes et al., 2008; Hult et al., 2006; Johansson et al., 2008; Julian et al., 2008; J. Liu et al., 2011; Morgan et al., 2009; Morgan & Tomlinson, 2010; Morton, 2010; Park et al., 2011; Rosenstock et al., 2010; S. Shah et al., 2011; U. Shah et al., 2010; Siu et al., 2009; Stewart & Tomlinson, 2009; Tiwari, 2010; Tu et al., 2008; van Raalte et al., 2009; Véniant et al., 2010; S. J. Wang et al., 2006; Webster et al., 2010; Yuan et al., 2007; X. Zhang et al., 2009b). Besides inhibition of 11β-HSD1 reductase activity, increase of 11β-HSD1 dehydrogenase (oxidase) activity, without inhibition of 11β-HSD2, may provide a better therapeutic strategy for T2DM, obesity and MetSyn (Ge et al., 2010). 11β-HSD1 is also inhibited by natural compounds, such as an active ingredient of various Chinese herbs (emodin), derivatives or analogues of the licorice root, coffee extract, flavanone (and the monohydroxylated flavonoid 2′-hydroxyflavanone), endogenous steroids and their metabolites and bile acids (Andrews et al., 2003; Atanasov et al., 2006; Chalbot & Morfin, 2006; Classen-Houben et al., 2009; Diederich et al., 2000; Feng et al., 2010; Gathercole & Stewart, 2010; Hollis & Huber, 2011; Latif et al., 2005; Livingstone & Walker, 2003; Maeda et al., 2010; Monder et al., 1989; Morris et al., 2004; Odermatt & Nashev, 2010; Sandeep et al., 2005; Schweizer et al., 2003; Su et al., 2007; Taylor et al., 2008; Tomlinson et al., 2007; van Raalte et al., 2009; Walker et al., 1995a; Wamil & Seckl, 2007). Glycyrrhetinic acid, the active pharmacological ingredient of the licorice root and some of its derivatives, as well as its steroidal synthetic analogue carbenoxolone (hemisuccinate derivative of glycyrrhetinic acid) are inhibitors of both 11β-HSD1 and 11β-HSD2 (the magnitude of the effect being dependent on *in vitro versus in vivo* environment, dose, administration mode, tissue and specie as well as compound structure) (Abdallah et al., 2005; Andrews et al., 2003; Classen-Houben et al., 2009; Gathercole & Stewart, 2010; Hollis & Huber, 2011; Jellinck et al., 1993; Livingstone & Walker, 2003; Monder et al., 1989; Sandeep et al., 2005; Su et al., 2007; Taylor et al., 2008; Tomlinson et al., 2007; van Raalte et al., 2009; Walker et al., 1995a; Wamil & Seckl, 2007). Both 7-oxygenated steroids and 7-ketocholesterol modulate 11-HSD1 activity (Balázs et al., 2009; Odermatt & Nashev, 2010; Wamil et al., 2008; Wamil & Seckl, 2007). From all the bile salts tested *in vitro* and found to inhibit 11β-HSD1, Diederich et al. reported that chenodesoxycholic acid does not affect *in vivo* the activity of 11β-HSD1 when given in therapeutic doses to healthy men (Diederich et al., 2011).

7.1 Human 11β-HSD1 inhibition studies

In a study with carbenoxolone it is observed, in healthy non-diabetic men, a small (although significant) increase in whole body insulin sensitivity (Hollis & Huber, 2011; Walker et al., 1995a). Walker et al. infered that carbenoxolone, by inhibiting hepatic 11β-HSD1 and reducing intra-hepatic cortisol concentration, increases hepatic insulin sensitivity and decreases hepatic glucose production (Walker et al., 1995b). Further developing their research on carbenoxolone 11β-HSD1 inhibition, Walker et al. report decreased glucagon-stimulated glucose production

and glycogenolysis in T2DM men (non-obese normotensive, treated with diet alone), but not in healthy subjects, and decreased total cholesterol in healthy subjects, but not in T2DM patients. Carbenoxolone has no effect on gluconeogenesis, peripheral glucose uptake or insulin-mediated reduction of plasma FFA (Andrews et al., 2003). So, as just described, carbenoxolone enhances hepatic insulin sensitivity in healthy men and in non-obese normotensive T2DM. However, Sandeep et al. describe later, in non-diabetic obese men, a highly effective inhibition of whole-body 11β-HSD turnover by carbenoxolone, but without inhibiting the conversion of cortisone to cortisol in SAT or modifying insulin sensitivity (Sandeep et al., 2005). Nevertheless, 11β-HSD1 inhibition in AT by carbenoxolone has been reported. After both a single dose and posterior 72 h of continuous treatment with carbenoxolone, in healthy male volunteers, Tomlinson et al. observe a decrease not only on serum cortisol generation, after oral administration of cortisone acetate (although only significantly for continuous treatment), but also on cortisol concentrations, after oral cortisone acetate, and glycerol concentrations, after oral prednisone, both within SAT interstitial fluid (in the latter location being indicative of inhibition of GC-mediated lipolysis) (Tomlinson et al., 2007). It is important to mention that 11β-HSD2 inhibition, with licorice or carbenoxolone, can lead to cortisol-dependent mineralocorticoid excess, with hypertension, sodium retention, hypokalemia and fluid retention (Andrews et al., 2003; Edwards et al., 1988; Ferrari, 2010; Gathercole & Stewart, 2010; Palermo et al., 2004; Stewart et al., 1990; Stewart et al., 1987). 11β-HSD2 is expressed principally in the distal nephron, where it inactivates cortisol to cortisone and thereby protects MR from cortisol (Andrews et al., 2003; Edwards et al., 1988; Ferrari, 2010; Funder et al., 1988; Palermo et al., 2004). PF-915275 is a potent and selective 11β-HSD1 inhibitor, without adverse side effects in a wide range of orally tested doses, that is selective for the human and primate enzymes (Bhat et al., 2008; Courtney et al., 2008). A modest pharmacodynamic effect of PF-915275 on 11β-HSD1 activity in the healthy human liver is reported, but experiments to assess its inhibitory effect in the AT have not been performed (Courtney et al., 2008; Hollis & Huber, 2011). So far, to our knowledge, there are no reports of PF-915275 activity in patients with T2DM or MetSyn (or any of the associated components). Bhat et al. showed, in normal cynomolgus monkeys, that PF-915275 dose-dependently inhibits 11β-HSD1-mediated conversion of prednisone to prednisolone and reduces insulin levels (Bhat et al., 2008). Hollis et al. reviewed the clinical results obtained with the selective 11β-HSD1 inhibitor INCB13739. In patients with T2DM inadequately controlled with metformin, INCB13739 treatment achieves significant reductions in hemoglobin A1c, fasting plasma glucose and HOMA-IR (homeostasis model assessment-IR), and improves hyperlipidemia and hypertriglyceridemia (when present). Adverse events (occurring in ≥ 3%: nasopharyngitis, headache, diarrhea, cough, nausea, arthralgia and upper respiratory tract infection) were similar across all treatment groups. Interestingly, those positive effects are observed primarily in subjects categorized as obese (BMI > 30 kg/m^2) and not in subjects categorized as overweight (BMI ≤ 30 kg/m^2), underscoring the likely importance of AT 11β-HSD1 activity to the cardiometabolic sequelae of obesity (Hollis & Huber, 2011; Rosenstock et al., 2010). Feig et al. showed that 11β-HSD1 selective inhibition with MK-0916 is generally well tolerated in patients with T2DM and MetSyn (NCEP ATP III-defined) (Feig et al., 2011; S. Shah et al., 2011). Although no significant improvement in fasting plasma glucose is observed with MK-0916 compared to placebo, modest improvements in hemoglobin A1c, body weight and blood pressure are observed (Feig et al., 2011). These patients were only mildly hypertensive, with 55% receiving ongoing anti-hypertensive therapy, and yet treatment with MK-0916 led to

reductions from baseline of 7.9 and 5.4 mmHg in systolic and diastolic blood pressure, respectively, relative to placebo (Feig et al., 2011; S. Shah et al., 2011). Further developing the research on 11β-HSD1 selective inhibition, Shah et al. reported that, in overweight-to-obese hypertensive patients, reduction in trough sitting diastolic blood pressure with MK-0736 is not statistically significant. Nonetheless, MK-0736 is well tolerated and appears to modestly improve other blood pressure endpoints as well as LDL-cholesterol and body weight. The 24 h ambulatory blood pressure measurements data (from the subset of patients who participated in ambulatory blood pressure measurements) suggest that MK-0736 has blood pressure-lowering efficacy over a 24 h period not adequately represented by measuring sitting diastolic blood pressure and sitting systolic blood pressure, notably a greater blood pressure-lowering effect during daytime than during night-time (S. Shah et al., 2011). 11β-HSD1 inhibitors may improve a number of metabolic disturbances, unlike current available anti-diabetic compounds, that occur in obesity, T2DM and/or MetSyn patients, as seen from genetically engineered animal studies (Kotelevtsev et al., 1997; Morton et al., 2001; Morton et al., 2004) as well as from animal (Alberts et al., 2002; Alberts et al., 2003; Barf et al., 2002; Cooper & Stewart, 2009; Feng et al., 2010; Gathercole & Stewart, 2010; Hermanowski-Vosatka et al., 2005; Johansson et al., 2008; J. Liu et al., 2011; Livingstone & Walker, 2003; Morgan et al., 2009; Park et al., 2011; Taylor et al., 2008; Véniant et al., 2010; S. J. Wang et al., 2006; X. Zhang et al., 2009b) and human 11β-HSD1 inhibition studies (Andrews et al., 2003; Courtney et al., 2008; Feig et al., 2011; Gathercole & Stewart, 2010; Hollis & Huber, 2011; Morton, 2010; Rosenstock et al., 2010; Sandeep et al., 2005; S. Shah et al., 2011; Tomlinson et al., 2007; van Raalte et al., 2009; Walker et al., 1995a; Wamil & Seckl, 2007). Taking this into account, pharmacological inhibition of 11β-HSD1 to lower intracellular cortisol concentrations in the liver and AT, without altering circulating cortisol concentrations or responses to stress, is an exciting potential therapy in those conditions and likely to be most effective in obese T2DM patients (Andrews et al., 2003; Courtney et al., 2008; Feig et al., 2011; Gathercole & Stewart, 2010; Hollis & Huber, 2011; Morton, 2010; Rosenstock et al., 2010; Sandeep et al., 2005; S. Shah et al., 2011; R. Stimson et al., 2011; Tomlinson et al., 2007; van Raalte et al., 2009; Walker et al., 1995a; Wamil & Seckl, 2007).

8. Conclusion

According to the rising prevalence of the MetSyn and the burden of its associated cardiometabolic complications, the study of the mechanisms of disease as well as of possible prophylactic and therapeutic approaches is becoming increasingly necessary. The recognition of the involvement of GCs and 11β-HSD1, as likely etiological factors, adds new avenues for MetSyn management. Lately, research focusing on 11β-HSD1 inhibition has shown promising results. The role of dietary patterns on MetSyn development and of dietary components on 11β-HSD1 modulation for the prevention and/or treatment of metabolic disorders is now starting to be unraveled and may be a worthwhile investigation.

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4

Antiandrogenic and Estrogenic Compounds: Effect on Development and Function of Male Reproductive System

Anna Hejmej, Małgorzata Kotula-Balak and Barbara Bilińska
Department of Endocrinology, Institute of Zoology, Jagiellonian University
Poland

1. Introduction

In the last 50 years the increase in the frequency of male reproductive abnormalities has been observed in human (Auger et al., 1995; Bergström et al., 1996; Carlsen et al., 1992; Skakkebaek et al., 2001; Thonneau et al., 2003). Epidemiological studies have shown increasing trends in the incidence of cryptorchidism (undescended testis) and hypospadias (abnormal location of the urethral opening) in several regions of Australia, Europe, and the United States (Acerini et al., 2009; Boisen et al., 2004; Källén et al., 1986; Nassar et al., 2007; Paulozzi, 1999; Toppari et al., 2001). Moreover, several reports indicated that semen quality have declined during last century (Auger et al., 1995; Carlsen et al., 1992; Swan et al., 2000; Sharpe & Irvine, 2004). Decreasing sperm concentration and percentage of motile spermatozoa, and increasing number of spermatozoa with morphological alterations were observed in European population between 1940 and 1990. For instance, it has been found that the prevalence of an abnormally low sperm count in young men reaches even 15–20% (Andersson et al., 2008; Jørgensen et al., 2006, 2011). In earlier study by Jørgensen et al. (2001) significant geographical variations in semen quality have been also described. Although, the reason for these regional differences is not fully elucidated, some data indicate that a correlation exists between impaired semen quality and exposure to pesticides used in agricultural areas (Swan et al., 2003). Interestingly, it has been noticed that in the industrial areas, where peoples are exposed to high levels of industrial chemicals, the birth sex ratio can be altered; in some region of Canada male birth sex ratio (i.e. number of male births per total number of births) have reached only 0.3 during the period 1990 – 2003 (Mackenzie et al., 2005).

In 2001 Skakkebaeck and co-workers have suggested that cryptorchidism, hypospadias, testicular cancer and oligozoospermia are interrelated disorders comprising a single syndrome, called the testicular dysgenesis syndrome (TDS) (Skakkebaeck et al., 2001; Skakkebaeck & Jørgensen, 2005). This idea arose from the observation that cryptorchidism and hypospadias are closely linked to testicular cancer, because in men with a history of one of these anomalies significantly increased risk of testicular cancer was described (Davenport et al., 1997; Dieckmann & Pichlmeier, 2004; Sharpe & Irvine, 2004). Moreover, oligozoospermia is frequently found in men, who develop testicular cancer (Møller & Skakkebaek, 1999). The disorders included in TDS are believed to result from disruption of

hormone synthesis or action during fetal development of reproductive system. Indeed, numerous experimental studies have demonstrated that prenatal exposure to some environmental chemicals may disrupt the endocrine system in males and thus interfere with hormone-dependent development (Delbès et al., 2006; Fisher, 2004a; Gray et al., 2006).

Male reproductive system anomalies have been also reported in wild living animals (Vos et al., 2000). In fish, sexual reversal, decreased sperm count and motility, and spermatogenesis impairment were noticed (Barnhoorn et al., 2004; Jobling et al., 2002; Vajda et al., 2008). Feminization and abnormal gonadal development were observed in reptiles and birds (De Solla et al., 1998, 2006; Fry, 1995; Guillette et al., 1994), whereas in mammals, such as panthers or polar bears cryptorchidism and reduced size of reproductive organs were found (Mansfield & Land, 2002; Sonne et al., 2006). An interesting example of the species in which environmental pollutants may be the cause of reproductive system abnormalities is Sitka Black-Tailed Deer. It was reported that in the population living in the Aliulik Peninsula of Kodiak Island extraordinary high percentage (75%) of the males exhibited cryptorchidism when compared with males living elsewhere on the Kodiak Archipelago, among which only 12% were cryptorchid (Bubenik et al., 2001; Veeramachaneni et al., 2006a). Additionally, abnormal antlers and testicular neoplasia were frequently observed in cryptorchid deer from Aliulik Peninsula. The authors hypothesized that it was likely that testis and antler dysgenesis resulted from exposure of pregnant female (or alternatively, historic exposure of founders) to some estrogenic endocrine disrupting agent(s) present in the environment (Veeramachaneni et al., 2006a).

Although the substances affecting endocrine system were studied from 1950', the term "endocrine disruptor" was introduced in 1991 at Wingspread Conference, organized to evaluate the adverse effects observed in wildlife in the Great Lakes region in North America (Colborn & Clement, 1992; Colborn et al., 1993). According the World Health Organization (2006) endocrine disruptor (ED) is "an exogenous chemical substance or mixture that alters the function(s) of the endocrine system and thereby causes adverse effects to an organism, its progeny, or a (sub)population". In 2009, The Endocrine Society presented the Scientific Statement in which endocrine disruptor was defined as "a compound, either natural or synthetic, which through environmental or inappropriate developmental exposure alters the hormonal and homeostatic systems that enable the organism to communicate with and respond to its environment" (Diamanti-Kandarakis et al., 2009).

2. Role of androgens and estrogens in male reproductive tract development and function

Androgens are steroid hormones that play a central role in the development and function of male reproductive system (Dohle et al., 2003). The principal androgens are testosterone and dihydrotestosterone (DHT). High amounts of testosterone are produced in the testes from early stages of fetal development until birth. During prenatal period testosterone is necessary for the differentiation of Wolffian duct into the epididymis, vas deferens and seminal vesicles. It is also involved in the process of testis descent. DHT, synthesized from testosterone by the action of 5α-reductase, mediates the masculinization of external genitalia and prostate. Studies by Welsh et al. (2008) revealed the existence of a fetal "masculinization programming window", a period within which androgens action is necessary to ensure correct later development of the male reproductive system. Blockade of androgen action

only during this critical period by using androgen receptor antagonists (e.g., flutamide) suppresses development of the male accessory glands and disrupts testis descent leading to cryptorchidism (Macleod et al., 2010; Welsh et al., 2008). In rat masculinization programming window occurs at 15.5–18.5 gestational days, whereas in human it spans from approximately 8 to 14 weeks of gestation (Welsh et al., 2008). In neonates testosterone level is high for a short time, then its production decreases and is maintained at low level until puberty, when rising androgen level mediate growth and function of accessory sex glands, initiation of spermatogenesis and development of secondary male sex characteristics. In mature males androgen action is essential for the maintenance of male phenotype and fertility (Dohle et al., 2003).

The discovery that aromatase (the enzyme converting androgens to estrogens) and estrogen receptors α and β (ERα and ER β) are expressed in male reproductive tract and studies on transgenic mouse models with inactivated estrogen receptor α (α ERKO) or aromatase genes (ArKO) led to the conclusion that not only androgens, but also estrogens are important for development and physiology of male reproductive system (Bilinska et al., 1997; Carreau et al., 2003; Levallet et al., 1998; Lubahn et al., 1993; Kuiper et al., 1996; Robertson et al., 1999). It was demonstrated that during fetal and neonatal life estrogens are involved in control of gametogenesis, promoting germ cell and seminiferous tubule development, and in the regulation of fetal Leydig cell steroidogenesis (Albrecht et al., 2009; Delbés et al., 2005; Vigueras-Villaseñor et al, 2006). Aromatase and ERs are transiently expressed in the hippocampus of newborn males, suggesting that estrogens are involved in brain masculinization (McEwen & Alves, 1999). In the reproductive system of adult males the role of ERs is associated with the maintenance of fluid reabsorption in the excurrent ducts of the testis (Hess, 2000; Hess et al., 1997). Data from studies on male mice with knockout of ERα suggested that long-term atrophy of the testes, observed in these animals, was caused by backpressure of the accumulating luminal fluids. Moreover, estrogens appear to have direct effects on the Leydig cell, controlling testosterone synthesis, and possibly on the seminiferous epithelium (Akingbemi et al., 2003; Hess, 2003). In male, estrogens play also a physiological role in non-reproductive tissues and organs such as bone and cardiovascular system (Oettel, 2002).

Although endogenous estrogens are necessary for normal male fertility, excessive production of these hormones or exposure to exogenous estrogens during fetal or neonatal life could produce adverse outcomes, affecting reproductive system development and adult reproductive functions. Destructive effects of estrogen overexposure on the development of post-meiotic germ cells and testicular atrophy was observed in rodents and humans (Gancarczyk et al., 2004; Toyama et al., 2001; Williams et al., 2001). Moreover, cryptorchidism, spermatogenic arrest, Leydig cell hyperplasia, and decreased serum follicle-stimulating hormone (FSH) and testosterone levels have been reported in the transgenic mouse model with aromatase overexpression (Fowler et al., 2000; Li et. al., 2001).

3. Antiandrogens

Antiandrogens are defined as chemicals that interfere with androgen action or production. The compounds shown to have antiandrogenic properties include pharmaceuticals (e.g., flutamide, ketoconazole) as well as environmental contaminants: pesticides (e.g., vinclozolin, linuron) and industrial chemicals (e.g., di(n-butyl) phthalate).

3.1 Flutamide as a model antiandrogen

Flutamide, a pharmaceutical used in therapy of androgen-dependent prostate cancer, and its active metabolite hydroxyflutamide, are non-steroidal synthetic androgen receptor (AR) antagonists, which display pure antiandrogenic activity, without exerting agonistic or any other hormonal activity (Neri, 1989; Singh et al., 2000). Flutamide is regarded as a model antiandrogen and in experimental studies it is often used as a positive control in screening assays used for the identification of endocrine disruptors (O'Connor et al., 1998).

In utero exposure to flutamide was shown to alter reproductive development and function in male rat offspring (Mikkila et al., 2006). Recently, it was reported that flutamide interferes with desert hedgehog (Dhh) signaling in the fetal testis, resulting in impaired fetal Leydig cell differentiation. Leydig cell dysfunction was reflected by suppressed levels of insulin-like factor 3 (Insl-3) and testosterone and reduced expression of steroidogenic enzymes, cytochrome P450scc and 3β-hydroxysteroid dehydrogenase (3β-HSD) (Brokken et al., 2009). Insufficient levels of testosterone and Insl-3 in the fetal testis could, in turn, prevent full masculinization. Decrease in gonad and accessory sex glands weight, cryptorchidism, testicular histological lesions and increased germ cell apoptosis have been reported in adult male rats exposed to flutamide during fetal period, indicating that flutamide exerts long-term antiandrogenic effects (Omezzine et al., 2003).

In our recent studies flutamide (50 mg/kg bw) was injected into pregnant gilts during gestational days 20–28 and 80–88, and into male piglets on postnatal days 2–10. We found no changes in testicular morphology of neonatal pigs *in utero* exposed to flutamide, whereas in prepubertal males some of the seminiferous tubules were altered, exhibiting reduced number of Sertoli cells and dilated lumina (Durlej et al., 2011; Kopera et al., 2010). Testes of adult pigs exposed to flutamide *in utero* exhibited moderate alterations of the spermatogenic process: seminiferous tubules showed degeneration of germ cells and their extensive sloughing into the lumen of the seminiferous tubules, however all generations of germ cells could be recognized in the seminiferous epithelium. Testes of neonatally exposed boars contained severely altered seminiferous tubules, exhibiting drastic increase in the number of apoptotic germ cells, hypospermatogenesis or spermatogenic arrest at the spermatocyte level. Alterations of normal histological structure were accompanied by decreased expression and/or disturbed localization of intercellular junction proteins, connexin 43, N-cadherin, β-catenin and ZO-1 in the seminiferous epithelium (Hejmej et al., 2011a; Kopera et al., 2011). Also interstitial tissue was adversely affected; Leydig cells displayed hyperplasia or hypertrophy, increased expression of aromatase and reduced expression of LH receptor. Dysfunction of Leydig cells led to disruption of androgen-estrogen balance (Kotula-Balak et al., submitted for publication). These data suggest that in pigs flutamide acting during fetal, and especially, neonatal period can reprogram the development of testicular cells, leading to morphological and functional alterations of the testis at adulthood.

Interestingly, flutamide exposure has also long-term effects on sperm morphology. Our data showed that in sperm derived from neonatally-treated boars either flattened head or abnormal sperm with altered shape of the acrosome and abnormal packaging of sperm chromatin were frequently observed. Prepuberal treatment with flutamide resulted in an increased number of sperm displaying abnormal midpiece or tail defects (Lydka et al., submitted for publication)

Several studies demonstrated the effects of short-term androgen blockage induced by the administration of flutamide to immature or mature males. In immature rats structure of interstitial tissue and seminiferous epithelium, and the expressions of steroidogenesis-related genes, *Cyp11a1* and *StAR*, were significantly affected by flutamide treatment (Vo et al., 2009). When administered to pubertal animals, flutamide accelerated testes maturation, causing degeneration and detachment of primary spermatocytes and round spermatids (Maschio et al., 2010). In adult males, germ-cell degeneration, alterations in ectoplasmic specialization between the Sertoli cell and spermatids, and premature detachment of spermatids, as well as increase in the relative volume of Leydig cells were observed (Anahara et al., 2008; Maschio et al., 2008). Moreover, our *in vitro* results showed that pig sperm incubated with hydroxyflutamide (50 and 100 µg/mL) displayed disorders in sperm phospholipid membrane, decreased oxidative capability of sperm mitochondria and decreased sperm membrane integrity (Lydka et al., submitted for publication)

3.2 Environmental antiandrogens

3.2.1 Pesticides: procymidone, vinclozolin, prochloraz, linuron and p,p'DDE

Procymidone is used as a fungicide for the control of plant diseases. High quantities of this compound were found in rice, tomatoes and grapes (Gebara et al., 2011; US Environmental Protection Agency annual report, 1994). When administered to pregnant rats, the male pups displayed a reduced anogenital distance, nipple retention, hypospadias, cleft phallus, and reduced sex accessory gland size (Gray et al., 1999; Ostby et al., 1999). Moreover, in prostate and seminal vesicles fibrosis, cellular infiltration and epithelial hyperplasia were observed (Ostby et al., 1999). Chronic treatment of male rats with procymidone inhibited the negative feedback exerted by androgens on the hypothalamus and/or the pituitary, causing enhanced luteinizing hormone (LH) secretion and Leydig cell steroidogenesis, and in consequence, increased serum testosterone level (Hosokawa et al., 1993; Svechnikov et al., 2005). Such a long-term hyperstimulation of Leydig cells induces Leydig cell tumors (Murakami et al., 1995).

Vinclozolin is a dicarboximide fungicide used in the control of *Botrytis cinerea*, *Sclerotinia sclerotiorum*, and *Moniliniam spp* on vegetables, fruits and ornamental plants. Vinclozolin and its two active metabolites, M1 and M2, compete for androgen binding to AR and inhibit AR transactivation and androgen-dependent gene expression (Wong et al., 1995). Administration of vinclozolin to pregnant rats resulted in abnormalities of androgen-regulated sexual differentiation in male offspring, including reduced anogenital distance, nipple retention, hypospadias, cryptorchidism, decreased sex accessory gland growth as well as in induction of prostate inflammation and reduced sperm production at adulthood (Cowin et al., 2010; Gray et al. 1994; 1999). Vinclozolin has also been implicated in epigenetic modifications of male reproductive tract via changes in DNA methyltransferase expression (Anway et al., 2008; Anway & Skinner, 2008). The most sensitive period of rat fetal development to the effects of vinclozolin was found to be gestational days 16-17, whereas less severe malformations were seen in males exposed during gestational days 14–15 and 18–19 (Wolf et al. 2000). Peripubertal exposure resulted in delayed pubertal maturation, decreased sex accessory gland and epididymal growth concomitantly with increased serum levels of LH and testosterone (Monosson et al., 1999).

Prochloraz is an imidazole fungicide widely used in gardening and agriculture which acts as both AR antagonist and inhibitor of fetal testosterone production. In addition to

antiandrogenic action, prochloraz antagonizes the estrogen receptor, agonizes the aryl hydrocarbon (Ah) receptor and suppresses aromatase activity (Andersen et al., 2002; Vinggaard et al., 2006). Gestational exposure significantly reduces testosterone production by inhibiting activity of cytochrome P450c17, decreases reproductive organ weights, increases nipple retention and induces malformations (e.g., hypospadias) in androgen-dependent tissues of male offspring (Blystone et al. 2007; Laier et al., 2006; Noriega et al., 2005; Vinggaard et al., 2005).

Linuron is a herbicide employed to control of weeds in crops and potatoes (Gray et al., 2006). It binds AR and inhibits dihydrotestosterone induced gene expression *in vitro* (Lambright et al., 2000). Fetal exposure to linuron resulted in epididymal and testicular abnormalities, reduced anogenital distance and nipple retention; however, in contrast to other AR antagonists, it does not induce hypospadias and cryptorchidism. Moreover, linuron was shown to decrease testosterone production by fetal Leydig cells (McIntyre et al., 2000, 2002a, 2002b; Wilson et al., 2009). Thus its mechanism of action resembles those of phthalates (Gray et al., 2006). Interestingly, when administered to sexually immature and mature rats, linuron decreased weights of accessory sex organs, increased serum estradiol and LH levels, and produced Leydig cell tumors (Cook et al., 1993).

p,p'-DDE (dichlorodiphenyldichloroethylene) is a metabolite of the persistent pesticide, DDT (dichlorodiphenyltrichloroethane). DTT is now banned in most countries, since in 1960' it was discovered that it has endocrine disrupting properties and causes birth defects in human and animals. However, it is still used in some regions to prevent malaria and other tropical diseases spread by insects (van den Berg et al., 2009). p,p'-DDE acts as AR antagonist both *in vivo* and *in vitro* (Kelce et al., 1995). Fetal treatment with this compound was shown to affect male development, leading to reduced anogenital distance, nipple retention and hypospadias (You et al., 1998). Recently, it was reported that p,p'-DDE induces testicular apoptosis in pubertal rats through the involvement of Fas/FasL, mitochondria and endoplasmic reticulum-mediated pathways (Shi et al., 2011).

3.2.2 Phthalates

The diesters of 1,2-benzenedicarboxylic acid, called phthalates, are widely used as plasticizers in the production of toys, medical devices, rainwear, food packaging, and certain cosmetics (Schettler, 2006). Di-n-butyl phthalate (DBP) and di(2-ethylhexyl) phthalate (DEHP) and their metabolites have been shown to cause antiandrogenic effects, however, without binding to AR (Frederiksen et al., 2007). Although, the exact mechanism of action is not yet fully elucidated, it was demonstrated that phthalates interfere with Leydig cell function, reducing the expression of most of genes involved in testosterone biosynthesis (Barlow et al., 2003). Fetal exposure to phthalates results in reduced anogenital distance, hypospadias, cryptorchidism, malformed epididymis, and nipple retention (Mylchreest et al., 1999, 2002; Mylchreest & Foster, 2000). At the histological level, multinucleated gonocytes, detachment of gonocytes from the seminiferous epithelium, Sertoli cell-only tubules and Leydig cell hyperplasia were found in the testes of males exposed to DEHP and DBP (Fisher et al., 2003; Mylchreest et al., 2002; Parks et al., 2000). Some of these alterations were permanent and affected testicular function in adulthood, resulting in low testosterone level and reduced sperm count. It is worth noting that histological changes induced in rat by *in utero* exposure to phthalates resemble those observed in patients with TDS (Fisher, 2004a).

4. Xenoestrogens

Compounds with estrogenic activity, called xenoestrogens, comprise a broad range of synthetic chemicals (e.g., diethylstilbestrol, bisphenol-A, octylphenol, nonylphenol), naturally occurring phytoestrogens (e.g., genistein, resveratrol) and heavy metals (e.g., cadmium, lead, and boron).

4.1 Diethylstilbestrol (DES)

DES is synthetic potent non-steroidal estrogen used as a supplement in cattle and poultry feed and as a pharmaceutical (Rubin, 2007). DES was given to pregnant women to prevent miscarriages or premature deliveries from about 1940 to 1970. It was restricted in 1971 because of increased risk of a rare reproductive tract cancer, vaginal clear cell adenocarcinoma, in daughters of women who had taken DES (Gill et al., 1979; Melnick et al., 1987). Further studies have reported multiple adverse effects in males and females as a result of prenatal DES exposure. In males decreased fertility and anatomical malformations of reproductive organs such as cryptorchidism, epididymal cysts and prostatic squamous metaplasia were observed (Driscoll & Taylor, 1980; Marselos & Tomatis, 1992; Mittendorf, 1995).

Nowadays, experimental animals exposed to DES during fetal and neonatal development are useful models for studying mechanisms of endocrine disruption caused by exogenous estrogenic compounds (Diamanti-Kandarakis et al., 2009). In male mice exposed to DES during gestation, cryptorchidism, hypospadias, as well as underdeveloped epididymis, vas deferens and seminal vesicles were observed (McLachlan et al., 2001). Similarly, neonatal treatment of male rats with DES induced a wide range of reproductive abnormalities, including delay of testicular descent, retardation of pubertal spermatogenesis, reduction in testis weight, infertility, and gross morphological alterations in the rete testis, efferent ducts, epididymis and accessory sex glands (Atanassova et al., 1999, 2000; Fisher et al., 1999; McKinnell et al, 2001; Williams et al., 2001). Testes of adult rats neonatally exposed to DES displayed suppression of Leydig cell development and steroidogenesis, reduced Sertoli cell proliferation and spermatogenic impairment. It was shown that DES has both direct and pituitary-mediated effects on the developing testis, leading to decreased expression of AR and reduced FSH level (Sharpe et al., 1998, 2003). Studies on transgenic mouse models with inactivated ERs suggest that DES elicits its toxic effects in the male reproductive tract through an ERα-mediated mechanism (Prins et al., 2001).

4.2 Environmental xenoestrogens

4.2.1 Industrial xenoestrogens: bisphenol A and alkylphenols

Bisphenol A (BPA) is one of the most important industrial chemicals, which worldwide production is over 500 000 tons per year. It is found mainly in plastic food containers, baby bottles, the resins lining food cans, dental sealants, cardboards, and as an additive in other plastics (Richter et al., 2007). BPA is structurally similar to DES and can act by binding to ERα and ERβ, and through other mechanisms, since some effects differ from those observed in response to activation of estrogen receptors. *In vivo* and *in vitro* experiments revealed that BPA mimics estrogen action, however it is also able to antagonize the activity of estradiol, acting as a selective estrogen receptor modulator (SERM) (Welshons et al., 2006). In high concentrations BPA can bind to AR and inhibit the androgen action (Lee et al., 2003). Although BPA is approximately 1000- to 2000-fold less potent than estradiol, exposure to

environmentally relevant doses impacts the reproductive system development and function in male rodents (Richter et al., 2007). It was demonstrated that rodents exposed to BPA during fetal and/or neonatal life had decreased weights of the epididymis and seminal vesicles, but increased weights of the prostate and preputial glands, decreased epithelial height in the efferent ducts and decreased levels of testicular testosterone (Akingbemi et al., 2004; Fisher et al., 1999; vom Saal et al., 1998). Alterations in ectoplasmic specialization between the Sertoli cell and spermatids, abnormalities in the acrosomal granule and nucleus of spermatids, reduced percentage of motile sperm, and increased incidence of sperm malformations were also observed (Aikawa et al., 2004; Toyama et al., 2004). Similar changes in the seminiferous epithelium and reduced fertility were found in adult males treated with BPA (Toyama et al., 2004). BPA was found to act directly on Leydig cell steroidogenesis, affecting the expression of cytochrome cytochrome P450 17α-hydroxylase/C_{17-20} lyase (P450c17) and aromatase enzymes and interfering with LH receptor-ligand binding (Akingbemi et al., 2004; Svechnikov et al., 2010).

Alkylphenols, such as 4-nonylphenol and 4-*tert*-octylphenol, are used to manufacture the alkylphenol polyethoxylates, non-ionic surfactants used as detergents, plasticizers, emulsifiers and modifiers in paints, pesticides, textiles, and personal care products. Alkylphenols present in the environment, mainly in wastewater and rivers, derive from the release of unreacted alkylphenols during manufacturing as well as from degradation of the alkylphenol polyethoxylates in the environment (Blake et al., 2004; Staples et al., 2001). Currently, alkylphenols have been found in human urine and breast milk (Ademollo et al., 2008; Calafat et al., 2008,). Octylphenol and nonylphenol has been reported to exhibit weak estrogenic activity as demonstrated by its ability to bind and activate the estrogen receptors (Kuiper et al., 1998; Lee, 1998; Safe et al., 2000). Although these chemicals are between 100 and 10000-fold less estrogenic than 17β-estradiol, the widespread use of these compounds causes that they largely contribute to the environmental estrogen pool (Blake & Bookfor, 1997).

Maternal exposure to octylphenol was shown to affect the expression of genes essential for reproductive system development, such as steroidogenic factor-1 (SF-1) and steroidogenic enzymes in rat testes (Majdic et al., 1996, 1997). In the lamb, it was demonstrated to inhibit the secretion of FSH in the fetus with a concomitant decrease in testis size and Sertoli cell number at birth (Sweeney et al., 2000). In adult males exposed *in utero* or neonatally to alkylphenols abnormalities in reproductive organs histology, reduced weight of the testis, epididymis and prostate, reduced testosterone level as well as increased number of abnormal sperm and decreased sperm production were observed (Aydoğan & Barlas, 2006; Jie et al., 2010; Lee, 1998; Yoshida et al., 2001). These alterations may result from both modulation of the hypothalamus-pituitary axis and direct estrogenic action in reproductive tissues (Yoshida et al., 2001). Importantly, all these effects were observed only when relatively high doses (400 mg/kg bw) of alkylphenols were used (Atanassova et al., 2000; Sharpe et al., 2003).

Administration of high doses of alkylphenols to adult males resulted in reduced size and function of the testis, epididymis and male accessory glands, decreased serum LH, FSH and testosterone concentrations, increased apoptosis of germ cells and reduced sperm count (Blake & Boockfor, 1997; Boockfor & Blake, 1997; Han et al., 2004; Gong & Han, 2006; Kim et al., 2007). However, reports on the effects of lower doses (<200 mg/kg bw) of octylphenol on male reproductive system are contradictory (Bian et al., 2006; Kim et al., 2007).

Recently, bank vole, a seasonally breeding rodent, was used to investigate the effects of 4-tert-octylphenol on testes and seminal vesicles, depending on the length of exposure and reproductive status of animals. Adult bank vole males kept under long or short photoperiod were orally administered octylphenol (200 mg/kg bw) for 30 or 60 days. We found that treatment for 30 days had no effect on the reproductive organs, whereas treatment for 60 days adversely influenced sperm morphology as well as weights and histological structure of the testes and seminal vesicles. In these tissues, expression of 3β-HSD and AR, and testosterone levels were decreased, concomitantly with increased expression of aromatase and ERα, and elevated estradiol levels, resulting in androgen-estrogen imbalance. These data indicate that long-term exposure to octylphenol is necessary to affect male reproductive organs histology and hormonal milieu. Furthermore, a subtle difference in the sensitivity to octylphenol between voles kept in different light conditions was noted (Hejmej et al., 2011b). In a further study negative effects of this compound on MA-10 Leydig cells *in vitro* have been reported. In cell cultures treated with different octylphenol concentrations, dose-related changes in the cytoarchitecture of MA-10 cells, including cytoplasm vacuolization and altered size and distribution of lipid droplets, were visible. Moreover, it was shown that high doses attenuate 3β-HSD and AR expression, concomitantly with the reduction of progesterone synthesis. Based on this results it was hypothesized that octylphenol besides binding to ERs may use other potential routes of action such as effects on the AR (Kotula-Balak et al., 2011).

4.2.2 Phytoestrogens

Phytoestrogens are plant compounds, structurally similar to 17β-estradiol and thus exhibiting estrogenic or antiestrogenic activity. There are four main classes of phytoestrogens: isoflavones (genistein, daidzein, biochanin A, naringenin), coumestans (coumestrol), lignans (matairesinol) and stilbene (resveratrol). Phytoestrogens are present in fruits, vegetables and leguminous plants, but the main source of these compounds in human diet are soy-based products, i. a. soy-based infant formula, that contain high concentration of genistein and daidzein (Reinli & Block, 1996; Setchell et al., 1997). It is believed that isoflavones exert beneficial effects in prevention of cancer, cardiovascular diseases and osteoporosis, however it was reported that they can adversely affect development and function of male and female reproductive function (Lee et al., 2004; Suthar et al., 2001). This may be of special concern in case of infants fed with soy formula milk. Although, phytoestrogens binding affinity to the estrogen receptors is 1000-10000-fold lower compared with the 17β-estradiol, in infants, which consume even 9 mg/kg/day of isoflavones, mainly genistein, blood concentrations of the isoflavones exceed 1000 times those of endogenous estradiol and are higher than the amount reported to produce hormonal effects in adult women (Henley & Korach et al., 2010; Schmitt et al., 2001; Setchell et al., 1997). Therefore in recent years multiple studies on animal models were undertaken to elucidate the mechanism of action and the consequences of exposure to genistein. In rodents dietary administration of genistein induced Leydig cell hyperplasia and decrease of testosterone level by down-regulation of the expression of steroidogenic enzymes (e.g., cytochrome P450scc) (Svechnikov et al., 2005). *In vivo* and *in vitro* data indicate that genistein is able to signal through both ERα and ERβ, depending on the specific tissue (Mueller et al., 2004).
In recent years resveratrol, a stilbene found in grapes and wine, has been widely used to prevent cardiovascular diseases, since it was shown to inhibit oxidation of LDL cholesterol,

platelets aggregation and synthesis of eikozanoids (Kris-Etherton et al., 2002). However, resveratrol appeared to have adverse effect on Leydig cell steroidogenesis through suppression of the expression of StAR and cytochrome P450c17 (Svechnikov et al., 2009).
Estrogenic activity is also attributed to several other compounds derived from plants, for example lavender oil and tea tree oil, frequently used in cosmetics, such as lotions, gels, and creams. It is supposed that exposure to these chemicals may induce prepubertal gynecomastia in humans. *In vitro* experiments revealed that apart from estrogenic activity both lavender and tea tree oil possess antiandrogenic properties (Henley et al., 2007; Henley & Korach et al., 2010).
Interestingly, based on the analysis of published data concerning correlations between exposure to different endocrine disruptors and decrease in sperm counts and increase in testicular cancer rate, Safe (2004) suggested that dietary phytoestrogens, rather than synthetic environmental endocrine disruptors may by involved in induction of reproductive tract disorders in human.

4.2.3 Methoxychlor

Methoxychlor was introduced in 1944 to substitute more persistent and more toxic insecticide, DDT. It is used on agricultural crops, livestock, animal feed, grain storage, home gardens, and on pets. Methoxychlor exhibits mixed estrogenic and antiandrogenic activity: the most active estrogenic metabolite is HPTE [2,2-bis-(*p*-hydroxyphenyl)-1, 1, 1-trichloroethane], whereas other metabolites have antiandrogenic activity (Cummings, 1997; Dehal & Kupfer, 1994; Kelce & Wilson, 1997). HPTE has differential effects on ERs, being an ERα agonist and ERβ antagonist (Gaido et al., 1999, 2000). In cultured Leydig cells from immature and adult rats, HPTE was shown to inhibit both basal and hCG-stimulated testosterone production, and these effects were reported to be mediated through the ER (Murono & Derk, 2005). Recently, a direct inhibitory activity of methoxychlor and HPTE on 3β-HSD and 17β-hydroxysteroid dehydrogenase (17β-HSD) was reported (Hu et al., 2011). Exposure to methoxychlor during gestation or neonatal period affected embryonic testis cellular composition, Sertoli and germ cell numbers, germ cell survival and epididymal sperm count, reducing spermatogenic potential of males (Chapin et al., 1997; Johnson et al., 2002; Suzuki et al., 2004). In adult rat testis methoxychlor induced apoptosis via mitochondria- and FasL-mediated pathways (Vaithinathan et al., 2010).

4.2.4 Heavy metals

Numerous heavy metals (e.g., cadmium, lead, arsenic, boron, mercury, antimony, aluminum, cobalt, chromium, lithium) have been demonstrated to adversely affect the reproductive function of human and experimental animals. For example, cadmium, used in battery electrode production, galvanizing, plastics, alloys and paint pigments, has potent estrogen- and androgen-like activities *in vivo* and *in vitro* (Sikka et al., 2008; Takiguchi & Yoshihara, 2006). In mice exposed to cadmium during late gestation and puberty markedly reduced weights of testes, epididymides, prostate and seminal vesicles, and decreased testosterone levels were observed. Moreover, testicular expression of StAR and steroidogenic enzymes, such as cytochrome P450scc, 17*a*-HSD and 17*β*-HSD, was down-regulated (Ji et al., 2010, 2011). In the seminiferous tubules, cadmium caused disruption of the blood-testis barrier and oxidative stress, leading to germ cell degeneration, seminiferous tubules vacuolization, and aberrant morphology and apoptosis of Sertoli cells (de Souza

Predes et al., 2010; Zhang et al., 2010). Epidemiological and animal studies have additionally demonstrated a carcinogenic effect of cadmium on the prostate (Nakamura et al., 2002).
Lead, another metal widespread in the environment, has adverse reproductive effect on the testes and the hypothalamic-pituitary axis. In animal studies, lead has been shown to reduce serum testosterone and FSH levels, disrupt spermatogenesis and induce oxidative cellular damage in epididymis (Foster at al., 1998; Marchlewicz et al., 2004; Sokol et al., 1985). Clinical studies have associated exposure to lead with reduced libido, reduced sperm motility and sperm count, chromosomal damage, infertility, and changes in serum testosterone (Braunstein et al., 1978; Winder, 1989).

5. Mechanisms of action

Endocrine disruptors affect cellular processes by different modes of action. They can act by mimicking the action of naturally produced hormones, blocking their receptors in target cells or altering the synthesis or metabolism of hormones and hormone receptors. It is important to note, that many endocrine disruptors have more than one mechanism of action (e.g., methoxychlor) (Gaido et al., 2000). Some can be metabolized to hormonally active compounds, exhibiting different properties (e.g., DDT and its metabolite DDE) (Kelce et al., 1995). Moreover, even compounds with the same supposed mechanism of action can induce different effects after exposure. It was also demonstrated that action of some xenoestrogens may be different in various tissues; thus they can act as SERMs (e.g., BPA, resveratrol, naringenin) (Gehm et al., 1997; Gould et al., 1998; Yoon et al., 2001).

5.1 Interaction with hormone receptors

Endocrine disruptors can bind to specific hormone receptors and act via agonistic or antagonistic mechanism. Numerous xenoestrogens (e.g., BPA, alkylphenols, genistein) activate estrogen receptors, interacting with their binding pockets (Lehraiki et al., 2011; Mueller, 2004; Singleton & Khan, 2003). It is possible due to structural similarities of these compounds to estradiol. The affinity of xenoestrogens to the estrogen receptor and/or their ability to initiate nuclear retention and transcriptional effects is usually lower than those of estradiol. It is worth noting, however, that weak activity via receptor-dependent pathway does not necessarily predict the potency of the chemical acting via another signaling pathway. Moreover, many xenoestrogenic compounds bioaccumulate in fat tissues, resulting in prolonged exposure (Watson et al., 2011). Several estrogenic chemicals, among others flavonoids and resveratrol, have been shown to interact not only with ERs, but also with aryl hydrocarbon receptor (AhR) (Revel et al., 2003; Van der Heiden, et al., 2009).
Antiandrogens, such as flutamide, vinclozolin, prochloraz and linuron, repress AR-mediated transcriptional activation, by competitive inhibition of endogenous androgens binding to their receptor (Gray et al., 1999; Lambright et al., 2000; Mohler et al., 2009; Noriega et al., 2005; Vinggaard et al., 2002). Binding of antiandrogen may result in a conformational change of ligand binding domain of AR appropriate for the interaction with co-repressors, instead of coactivators (Berrevoets et al., 2002; Hodgson et al., 2008).
Besides classical intracellular steroid hormone receptors, several membrane steroid receptors, capable to mediating non-genomic steroid actions, have been described (Thomas & Dong, 2006; Watson et al., 2007). BPA has been shown to bind to membrane-bound form of ERα (mER) and a transmembrane G protein-coupled receptor 30 (GPR30) (Watson et al., 2005). This GPCR-mediated non-genomic action included activation of cAMP-dependent

protein kinase and cGMP-dependent protein kinase pathways and a rapid phosphorylation of the transcription factor cAMP response-element-binding protein (CREB) (Bouskine et al., 2009). Recent results revealed the possibility that BPA may have adverse effects on spermatogenesis via activation of extracellular signal-related kinases 1 and 2 (ERK1/2) (Izumi et al., 2011). Also alkylphenols and phytoestrogens appear to activate non-genomic pathways, signaling via calcium influx and activation of mitogen-activated protein kinases (MAP kinases) (Bulayeva & Watson, 2004; Wozniak et al., 2005).

5.2 Alterations in synthesis, metabolism and transport of hormones or their receptors

It was reported that some endocrine disruptors can interfere with steroid synthesis or metabolism, acting via non-receptor mediated mechanisms (Fisher, 2004b). Phthalates induce antiandrogenic effects, however they do not interact with the AR (Lehraiki et al., 2009; Stroheker et al., 2005,). It was demonstrated that DBP and DEHP decrease fetal testosterone synthesis by reducing the expression of steroidogenic genes, such as *Cyp17*, *Cyp11a* and *StAR* (Barlow & Foster, 2003; Borch et al., 2006; Howdeshell et al., 2007; Parks et al., 2000). Phthalates were also shown to decrease the expression of Insl-3, a factor produced by fetal Leydig cells. Insl-3 is an important regulator of testicular descent and phthalate-induced reduction of Insl-3 is consistent with the high incidence of cryptorchidism (Gray et al., 2006; Laguë & Tremblay, 2008; Wilson et al., 2004). In contrast to phthalates, *in utero* exposure to prochloraz decreases testosterone production by direct inhibition of the activity of steroidogenic enzymes without affecting the mRNA expression of these enzymes (Blystone et al., 2007; Wilson et al., 2008).

As mentioned above, biosynthesis of estrogens is catalyzed by the enzyme aromatase. Various endocrine disruptors were reported to alter the expression or activity of aromatase, leading to testosterone-estradiol imbalance. Enhanced expression of aromatase was found in testes of males exposed to octylphenol and BPA (Hejmej et al., 2011b; Kim et al., 2010), whereas prochloraz reduced aromatase expression (Vinggaard et al., 2006). Estradiol level can also be influenced by inhibition of SULT 1A1 and 2E1 enzymes, which catalyze inactivation of estrogens by sulphation. It was shown that alkylphenols and phthalates, suppressing these enzymes, cause a rise in the levels of the free active endogenous estrogens (Waring & Harris, 2005).

Some endocrine disruptors may additionally influence the expression levels of hormone receptors, shifting the balance between concentrations of endogenous ligand and its receptor. For instance, it was reported that exposure to DES (McKinnell et al., 2001; Williams et al., 2001) and octylphenol (Hejmej et al., 2011b; Kotula-Balak et al., 2011) results in up-regulation of ERα and down-regulation of AR in male reproductive tissues.

In case of steroid hormones, the level of bioavailable hormone is determined not only by the level of synthesis and metabolism, but also by concentration of steroid hormone-binding globulin (SHBG), protein involved in transport of steroids in the blood. Studies revealed that endocrine disruptors may influence SHBG level, altering the level of free, bioavailable hormone (Bagchi et al., 2009; Sikka & Wang, 2008).

It should be mentioned, that xenoestrogens and antiandrogens affect reproductive functions not only acing directly on reproductive organs, but also disturbing hypothalamus-pituitary-testicular axis. For example, in adult male rats exposed to BPA during pre- and early postnatal periods, LH serum levels showed no changes, whereas FSH and testosterone levels decreased significantly (Cardoso et al., 2011). Secretion of FSH was also reduced following prenatal octylphenol and vinclozolin exposure (Sweeney et al., 2000; Veeramachaneni et al., 2006b).

5.3 Epigenetic mechanisms

Epigenetic modifications are regulators in numerous biological processes, including spermatogenesis. Key mechanism in establishing epigenetic change is DNA methylation, which usually suppresses expression of the gene. Several studies revealed that endocrine disrupting chemicals are implicated in epigenetic programming and DNA methylation (McLachlan, 2001; Skinner & Anway, 2005). Indeed, hypermethylation found in several genes in the sperm DNA (i. a. *Mest*, *Snrpn*, *Peg1* and *Peg3*) was accompanied by the reduction of semen quality (Stouder & Paoloni-Giacobino, 2010). These changes may be heritable, if they occur during certain stages of development (Crews & McLachlan, 2006). It was demonstrated that methoxychlor and vinclozolin when administered during prenatal period interfere with testis development and lead to increased spermatogenic cell apoptosis and decreased fertility in the adult males. These spermatogenic defects were also evident in subsequent generations (Chang et al., 2006; Skinner & Anway, 2005). Also maternal exposure to BPA resulted in postnatal changes in DNA methylation status and altered expression of specific genes in offspring (Bernal & Jirtle, 2010; Kundakovic & Champagne, 2011).

Taken together, estrogenic and antiandrogenic compounds act by multiple mechanisms of toxicity disrupting the interactions among the interconnected signaling pathways in reproductive tissues. Importantly, in the environment organisms are usually exposed to mixtures of multiple endocrine disruptors, which can produce cumulative effects, regardless of the mode of action of the individual mixture component (Gray et al., 2006).

6. Conclusion

Experimental studies clearly suggest that estrogenic and antiandrogenic compounds could cause alterations of sexual differentiation and impairment of male reproductive functions. Although the process of spermatogenesis is directly vulnerable to exposure to endocrine disrupting agents only in sexually mature males, above-mentioned data imply that exposure during the period of reproductive system development may have subsequent impact on the reproductive functions in adulthood. Fetal and neonatal exposures might result in the reprogramming of the developmental process of testicular cells, leading to their irreversible dysfunction. In contrast, adverse effects on the process of spermatogenesis in adulthood can be reversible (Sharpe, 2010; West et al., 2005). It is likely, therefore, that fetal and neonatal periods are of critical importance, when considering the role of hormonally active chemicals in male reproductive functions.

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Part 2

Steroid Clinical Correlation

5

Relationship Between Steroid Hormones and *Helicobacter pylori*

Hirofumi Shimomura
Jichi Medical University
Japan

1. Introduction

Helicobacter pylori is a Gram-negative microaerobic curved-rod possessing polar flagella as the motility organ. This bacterium colonizes human gastric epithelium and causes chronic gastritis and peptic ulcers (Graham, 1991; Warren & Marshall, 1983; Wyatt & Dixon, 1988). Via longer periods of colonization in the human stomach, it also contributes to the development of gastric cancer and marginal zone B-cell lymphoma (Forman, Eurogast Study Group, 1993; Wotherspoon et al., 1991). Approximately half of population in the world is infected with *Helicobacter pylori*, and the majority of infected persons develop atrophic gastritis with or without symptoms. Among *Helicobacter pylori*-infected individuals, about 10% persons develop gastric and duodenal ulcers, 1% to 3% persons develop gastric adenocarcinoma, and 0.1% or less person develops gastric mucosa-associated lymphoid tissue (MALT) lymphoma (Fukase et al., 2008; Peek & Blaser, 2002; Peek & Crabtree, 2006; Stolte et al., 2002; Uemura et al., 2001).

The bacterial species belonging to the genus *Helicobacter* have a unique feature of free-cholesterol (FC) assimilation into the membrane lipid compositions (Haque et al., 1995, 1996). *Helicobacter pylori* aggressively absorbs free-cholesterol supplemented to a medium, or extracts free-cholesterol from the lipid raft of epithelial cell membrane when the organism adhered onto the epithelial cell surface (Wunder et al., 2006). The free-cholesterol assimilated into the *Helicobacter pylori* membranes is glucosylated via the enzymatic action, and the organism utilizes as the membrane lipid components both free-cholesterol itself and the glucosylated cholesterols. Previous study by our group has identified the following three types of glucosyl cholesterols in the membrane lipid compositions of *Helicobacter pylori* (Hirai et al., 1995): cholesteryl-α-D-glucopyranoside (CGL), cholesteryl-6-*O*-tetradecanoyl-α-D-glucopyranoside (CAG), and cholesteryl-6-*O*-phosphatidyl-α-D-glucopyranoside (CPG). One of the enzymes involved in the biosynthesis of glucosyl cholesterols is HP0421 protein, a cholesterol α-glucosyltransferase encoded by HP0421 gene in *Helicobacter pylori* (Lebrun et al., 2006). The HP0421 protein adopts as the glucose source a uridine diphosphate-glucose (UDP-Glc) and catalyzes the dehydration condensation reaction between a 1α-hydroxyl (OH) group of D-glucose (Glc) molecule and a 3β-OH group of free-cholesterol (FC) molecule, and thereby CGL is synthesized. The other enzymes involved in the biosynthesis of CAG and CPG have still not been identified (Fig. 1).

Though it is almost special cases that bacterial species produce glucosyl sterols, plants and fungi universally produce various glucosyl sterols such as glucosyl sitosterol and glucosyl

ergosterol (Kim et al., 2002; Oku et al., 2003; Peng et al., 2002; Warnecke et al., 1994, 1997, 1999). As with *Helicobacter pylori*, the bacterial species that produce glucosyl cholesterol have been only reported in *Borrelia hermsi*, *Acholeplasma axanthum*, *Spiroplasma* spp., and *Mycoplasma gallinarum* (Livermore et al., 1978; Mayberry & Smith, 1983; Patel et al., 1978; Smith, 1971). Recent studies have shown that *Borrelia burgdorferi*, *Borrelia garinii*, and *Borrelia afzelii* possess the galactosyl cholesterol that binds to the cholesterol a D-galactose as the sugar molecule in place of a D-glucose (Ben-Menachem et al., 2003; Schröder et al., 2003; Stübs et al., 2009). Plants and fungi carry out the biosynthesis of sterols by themselves, and thereafter attach a D-glucose molecule to the sterols biosynthesized via the catalytic action of sterol β-glucosyltransferase. In contrast, bacterial species including *Helicobacter pylori* do not have the anabolic pathway for cholesterol. Therefore, *Helicobacter pylori* must absorb free-cholesterol from the outside environments to biosynthesize glucosyl cholesterols. In addition, there is the structural difference between glucosyl cholesterols of *Helicobacter pylori* and the other glucosyl sterols. The D-glucose molecule in glucosyl cholesterols of *Helicobacter pylori* is attached to the cholesterol molecule with α-configuration, whereas the D-glucose molecule of phytogenic and fungal glucosyl sterols is attached to the sterol molecule with β-configuration. This structural difference is resulting from the enzymatic action catalyzing the binding of D-glucose into the sterol. In sum, the HP0421 protein of *Helicobacter pylori* catalyzes the α-glucosidic linkage between the 1α-OH group of D-glucose molecule and the 3β-OH group of free-cholesterol molecule, whereas the sterol β-glucosyltransferase of plants and fungi catalyzes the β-glucosidic linkage between the 1β-OH group of D-glucose molecule and the 3β-OH group of free-sterol molecule.

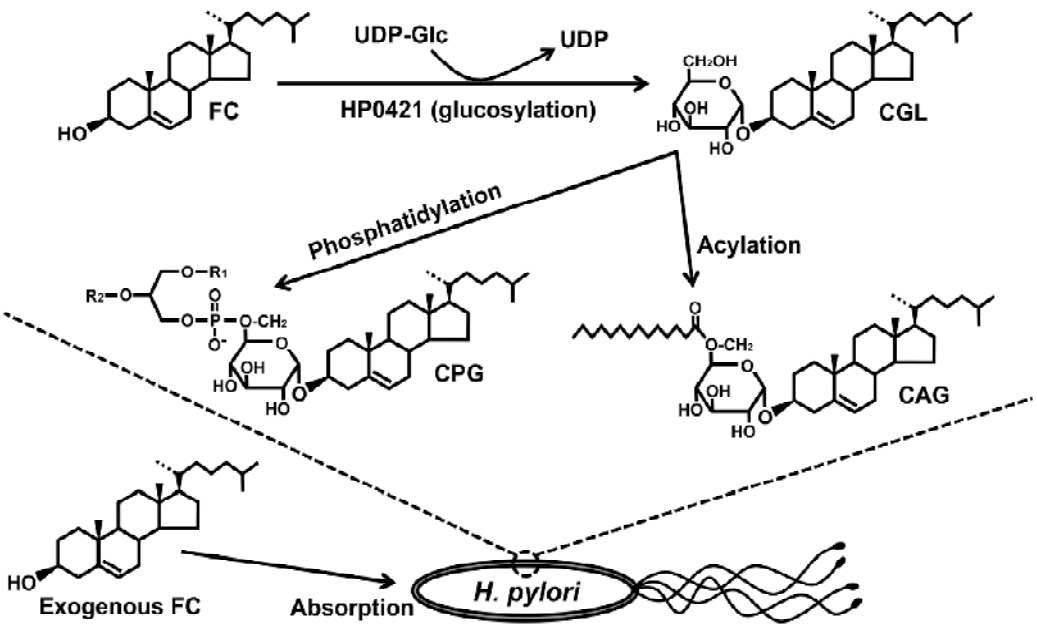

Fig. 1. The free-cholesterol (FC) assimilation of *Helicobacter pylori*

For many years, the biological significance of cholesterol glucosylation in *Helicobacter pylori* remained to be clarified. Recently, it has been, however, elucidated that *Helicobacter pylori*

induces the glucosylation of free-cholesterol absorbed into the membranes to evade the host immune systems (Wunder et al., 2006). The HP0421 gene-knockout *Helicobacter pylori* mutant, which lacks the capability to biosynthesize the glucosyl cholesterols and retains free-cholesterol without glucosylation, easily succumbs to the phagocytosis of macrophages, and strongly induces the activation of antigen-specific T cells, compared to the wild type *Helicobacter pylori*. In addition, when the HP0421 gene-knockout mutant is inoculated into the mouse via oral administration, the organism is promptly excluded from the murine gastric epithelium. The abnormal wild type *Helicobacter pylori*, which fails to induce the biosynthesis of enough amount glucosyl cholesterols by artificial insertion of excessive free-cholesterol into the membranes, also succumbs to the phagocytosis of macrophages and induces the activation of antigen-specific T cells, to similar to the cases of HP0421 gene-knockout mutant. In contrast, the normal wild type *Helicobacter pylori* and the HP0421 gene-reconstructed organism resist the phagocytosis of macrophages, control the induction of antigen-specific T cell activation, and colonize longer periods onto the gastric epithelium of mouse. These findings indicate that the glucosylation of free-cholesterol absorbed into the membranes of *Helicobacter pylori* plays an important role in survival and colonization of the organism in host.

However, it remains to be clarified about why *Helicobacter pylori* aggressively absorbs exogenous free-cholesterol into the membranes. If *Helicobacter pylori*, as with the general bacterial species, did not absorb free-cholesterol into the membranes, the organism will not need to glucosylate it. In addition, free-cholesterol is rather harmful for the survival of *Helicobacter pylori*, because the organism possessing free-cholesterol without glucosylation strongly activates the macrophages and the antigen-specific T cells, and is eradicated from the gastric epithelium by inducing the host immune responses. Moreover, whether the free-cholesterol is the only sterol absorbed into the membranes of *Helicobacter pylori* is also unsolved. Our group, thus, assumed that the free-cholesterol (or steroid) absorption in *Helicobacter pylori* has other some physiological role to maintain the viability of the organism.

To elucidate these unsolved points, our group has initiated the investigations as to the capability of *Helicobacter pylori* to use steroid hormones. Steroid hormones, such as sex hormones and corticoids, are typical sterol analogues that are derived from free-cholesterol in mammals. A number of investigations have demonstrated that the enzymes involved in the biosynthesis and the activation of sex hormones are also expressed in human stomach tissue (Javitt et al., 2001; Miki et al., 2002; Takeyama et al., 2000; Turgeon et al., 2001). In addition, the expression of sex hormone receptors has been found in gastric cancer (Kominea et al., 2004; Matsuyama et al., 2002; Takano et al., 2002). These studies indirectly indicate that sex hormones exist in the human stomach environment. We know that *Helicobacter pylori* colonizes the human stomach. In sum, there is possibility that *Helicobacter pylori* has contact with sex hormones in the human stomach. No earlier studies, however, have investigated the assimilation of sex hormones in *Helicobacter pylori*, and/or the influence of sex hormones on the viability of the organism.

Based on the findings from our current basal research, this chapter summarizes the capability of *Helicobacter pylori* to assimilate various sex hormones, and the bactericidal activity of certain sex hormones targeting selectively the *Helicobacter pylori*.

2. The 3β-OH steroids and *Helicobacter pylori*

Of the steroid hormones including steroid pre-hormone, pregnenolone (PN), dehydroepiandrosterone (DEA), and epiandrosterone (EA) possess a hydroxyl group (3β-

OH) with β-configuration at the carbon-3 position of steroid framework, as with free-cholesterol (FC). First of all, our group has, therefore, examined the capability of *Helicobacter pylori* to assimilate the 3β-OH steroid hormones. After *Helicobacter pylori* (10^5 CFU, colony-forming unit/ml) was cultured for 24 hours with pregnenolone (50 μM concentration), dehydroepiandrosterone (50 μM concentration), or epiandrosterone (50 μM concentration) in a serum-free medium (10 ml) with continuous shaking under microaerobic conditions (an atmosphere of 5% O_2, 10% CO_2, and 85% N_2 at 37˚C), the membrane lipids of the organisms were purified via the Folch method (Folch et al., 1957) and analyzed by thin-layer chromatography (TLC). This paragraph summarizes the assimilation of 3β-OH steroid hormones in *Helicobacter pylori*.

2.1 Pregnenolone and *Helicobacter pylori*

Though the structure at the carbon-17 position of pregnenolone framework differs from that at the same carbon position of free-cholesterol framework, the membranes of *Helicobacter pylori* aggressively absorbed the exogenous pregnenolone, and the organism induced the glucosylation of this 3β-OH steroid pre-hormone: the TLC analysis detected the three spots of glucosyl pregnenolones equivalent to the three spots of glucosyl cholesterols (CGL, CAG and CPG) in the membrane lipid compositions of *Helicobacter pylori* (Hosoda et al., 2009).

The recombinant HP0421 protein expressed in *Escherichia coli* has been shown to catalyze α-glucosylation of various phytogenic and fungal sterols (Lebrun et al., 2006). Moreover, *Helicobacter pylori* lacks the gene that encodes sterol β-glucosyltransferase in plants and fungi. Therefore, the glucosyl pregnenolones detected in the membrane lipid compositions of *Helicobacter pylori* are easily guessed to be all α-glucosyl pregnenolones. As with free-cholesterol (FC), the functional group to which a D-glucose molecule can be attached via the catalytic action of HP0421 protein is the only 3β-OH group in the pregnenolone (PN) framework (Fig. 2). The spot of glucosyl pregnenolone corresponding to the CGL spot detected in the TLC analysis must, in sum, be a 3β-(α-D-glucosyl)-pregnenolone. In addition, the spot of glucosyl pregnenolone corresponding to the CAG spot is guessed to be a 3β-(6-*O*-tetradecanoyl-α-D-glucosyl)-pregnenolone, and the spot of glucosyl pregnenolone corresponding to the CPG spot is guessed to be a 3β-(6-*O*-phosphatidyl-α-D-glucosyl)-pregnenolone.

2.2 Dehydroepiandrosterone and *Helicobacter pylori*

As with pregnenolone, the structure at the carbon-17 position of dehydroepiandrosterone framework also differs from that at the same carbon position of free-cholesterol framework. *Helicobacter pylori*, however, absorbed the exogenous dehydroepiandrosterone into the membranes and induced the glucosylation of this androgen. Though the TLC analysis detected the three spots of glucosyl dehydroepiandrosterones, the detection level of the glucosyl dehydroepiandrosterone corresponding to the CGL was remarkably lower than the detection levels of the other glucosyl dehydroepiandrosterones corresponding to the CAG and CPG.

Our previous study has demonstrated that the detection level of CGL, a basic structure of glucosyl cholesterols, in the membrane lipid compositions of *Helicobacter pylori* undergoing the long-term cultures reduces via the conversion to both CAG and CPG that are produced by modifying the CGL molecule with an acyl group and a phosphatidyl group, respectively, and thereby the detection levels of CAG and CPG increase in the membrane lipid

compositions of organism (Shimomura et al., 2004). Our findings as to the assimilation of this androgen, in sum, suggest that *Helicobacter pylori* promptly converts the 3β-(α-D-glucosyl)-dehydroepiandrosterone, which is a fundamental structure of the glucosyl dehydroepiandrosterones, to those modified by an acyl group or a phosphatidyl group. However, the transferases that attach an acyl group or a phosphatidyl group to the CGL molecule have still not been identified in *Helicobacter pylori*. Investigations into the CGL acyltransferase and CGL phosphatidyltransferase will be, therefore, required to elucidate the anabolic pathway in glucosyl cholesterols and glucosyl steroid hormones.

2.3 Epiandrosterone and *Helicobacter pylori*

The only structural difference between dehydroepiandrosterone and epiandrosterone is in the part of double bond between the carbon-5 position and the carbon-6 position in the steroid molecule: dehydroepiandrosterone has the double bond between those carbon positions, while epiandrosterone lacks its double bond. The TLC analysis detected the three spots of glucosyl epiandrosterones in the membrane lipid compositions into which *Helicobacter pylori* assimilated this androgen, but the detection level of the glucosyl epiandrosterone corresponding to the CGL was remarkably lower than the detection levels of the other glucosyl epiandrosterones corresponding to the CAG and CPG, as with the case of dehydroepiandrosterone. These results also suggest that *Helicobacter pylori* promptly converts the 3β-(α-D-glucosyl)-epiandrosterone to those modified by an acyl group or a phosphatidyl group.

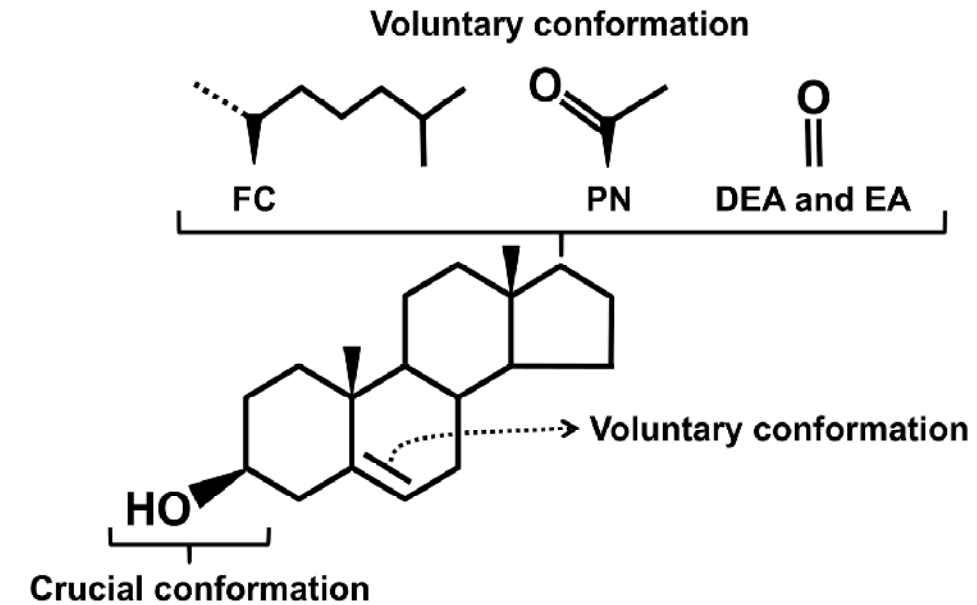

Fig. 2. The 3β-OH: a crucial conformation for steroid glucosylation by *Helicobacter pylori*

These findings from our recent study show that *Helicobacter pylori* glucosylates not only free-cholesterol, but also various 3β-OH steroid hormones. The only common structure among pregnenolone (PN), dehydroepiandrosterone (DEA), epiandrosterone (EA), and free-cholesterol (FC) is a 3β-OH group in the steroid framework (Fig. 2). This, thus, indicates that

the 3β-OH of steroids is a crucial conformation required for the steroid glucosylation by *Helicobacter pylori*. Further conformation analyses will be required to identify the chemical structures of glucosyl steroid hormones in more detail.
Our group has demonstrated the first report to describe the capability of *Helicobacter pylori* to glucosylate steroid hormones (Hosoda et al., 2009). In addition, no earlier studies have reported that the glucosyl sex hormones were detected in eukaryotes, prokaryotes, and/or environments. Further investigations will be necessary to analyze the hormonal actions or biological activities of these glucosyl sex hormones produced by *Helicobacter pylori*.

3. The 3-OH steroids and *Helicobacter pylori*

The three female hormones, namely, estrone (E1), estradiol (E2), and estriol (E3) possess a flat hydroxyl group (3-OH) at the carbon-3 position of the steroid framework. Our recent studies have revealed that these 3-OH steroid hormones have the different influences on the viability of *Helicobacter pylori*. This paragraph describes the relationship between the 3-OH steroid hormones and *Helicobacter pylori*.

3.1 Estrone and *Helicobacter pylori*

When *Helicobacter pylori* (10^5 CFU/ml) was cultured for 24 hours with estrone at the 50 μM concentration in a serum-free medium (10 ml) with continuous shaking under microaerobic conditions, the membranes of organism efficiently absorbed the estrone (Hosoda et al., 2009). These findings indicate that *Helicobacter pylori* aggressively uses as the membrane lipid components not only 3β-OH steroids (including free-cholesterol), but also 3-OH steroid estrone. However, the organism has failed to induce the glucosylation of estrone absorbed into the membranes. In combination with the results of glucosylation observed in the 3β-OH steroid hormones, they strongly indicate that *Helicobacter pylori* recognizes only the 3β-OH conformation of steroid molecule and glucosylates only the 3β-OH steroids with or without the other structural differences.

3.2 Estradiol and *Helicobacter pylori*

Our group has recently clarified the inhibitory effect of estradiol on the growth of *Helicobacter pylori*. When *Helicobacter pylori* (10^5 CFU/ml) was cultured for 24 hours with estradiol at the 50 and 100 μM concentrations in a serum-free medium (3 ml) with continuous shaking under microaerobic conditions, the estradiol at these concentrations made the division and proliferation of *Helicobacter pylori* stagnate: the levels of colony-forming unit (CFU), which indicates the number of living bacterial cells in the meaning of the wide sense, at that time maintained the baseline CFU level (10^5 CFU/ml) immediately after the culture initiation (Hosoda et al., 2011). Estradiol has been, in sum, found to exhibit the bacteriostatic action to *Helicobacter pylori*. This estrogen seems to act to *Helicobacter pylori* by attaching to the cell surface of the organism, since it is contained in the membrane lipids purified from the *Helicobacter pylori* incubated in the presence of estradiol. Incidentally, estrone has no influence on the growth of *Helicobacter pylori*, and the proliferation capability of the organism even in the presence of estrone at the 100 μM concentration is comparable to that of the organism in the absence of its estrogen.
Earlier investigations (including our own) have shown that *Helicobacter pylori* morphologically converts from a bacillary form to a coccoid form when the organism

exposed to various stresses such as excessive oxygen, alkaline pH, or long-term culture (Benaïssa et al., 1996; Catrenich & Makin, 1991; Donelli et al., 1998; Shimomura et al., 2004). For many years, there is a controversy as to whether the coccoid-converted *Helicobacter pylori* cells are maintaining a viable state. Cells that had changed to a coccoid form lack the ability to form colonies on an agar plate, which it makes very difficult to accurately determine the CFU in coccoid-converted *Helicobacter pylori*. Our group has, therefore, examined whether estradiol confers the bacteriostatic action to *Helicobacter pylori* by promoting the induction of coccoid-conversion in the bacterial cells. Though the cell morphologies of *Helicobacter pylori* were observed with a differential interference microscope after the organisms (10^5 CFU/ml) were incubated for 24 hours with estradiol at the 100 μM concentration in a serum-free medium (3 ml) with continuous shaking under microaerobic conditions, the coccoid *Helicobacter pylori* cells were unobserved. This indicates that estradiol inhibits the proliferation of *Helicobacter pylori* via a certain bacteriostatic mechanism but not the induction of coccoid-conversion against the bacterial cells.

Epidemiological studies and animal models have suggested that female hormones, particularly estrogens, play a protective role in gastric cancer (Campbell-Thompson et al., 1999; Freedman et al., 2007; Furukawa et al., 1982; Ketkar et al., 1978; Sipponen & Correa, 2002). *Helicobacter pylori* infection is also one of the risk factors in developing of gastric cancer in humans. Recent study by others has demonstrated that estradiol somehow protects against the development of *Helicobacter pylori*-induced gastric cancer in a mouse model (Ohtani et al., 2007). The bacteriostatic action of estradiol may play some role in mechanisms preventing the development of *Helicobacter pylori*-induced gastric cancer. Further investigations will be necessary to elucidate the relationship between estradiol and *Helicobacter pylori in vivo*.

3.3 Estriol and *Helicobacter pylori*

When *Helicobacter pylori* (10^5 CFU/ml) was cultured for 24 hours with estriol at the 50 and 100 μM concentrations in a serum-free medium (10 ml) with continuous shaking under microaerobic conditions, estriol had no influence on the growth of *Helicobacter pylori*, and the CFU levels of the organism cultured in the presence of estriol (both 50 μM and 100 μM) were comparable to the control CFU level (10^8 CFU/ml) of the organism cultured in the absence of estriol. In addition, this estrogen was undetectable in the membrane lipid compositions of the organism in the TLC analysis. *Helicobacter pylori* has, thus, failed to use as the membrane lipid component estriol.

4. Membrane distribution of steroids assimilated by *Helicobacter pylori*

In general, Gram-negative bacteria including *Helicobacter pylori* have two membranes that are composed of the phospholipid bilayer, namely an inner membrane and an outer membrane. The phospholipid components constituting both the inner and outer membranes are phosphatidylethanolamine, phosphatidylglycerol and cardiolipin. The outside lipid layer of the outer membrane also contains as the major glycolipid component a lipopolysaccharide (LPS) (Rietschel et al., 1994). LPS is composed of an *O*-polysaccharide chain, an outer core saccharide, an inner core saccharide, and a lipid A regions. The lipid A is composed of two phosphorylated glucosamine molecules linked by β (1→6)-glycosidic bond and plural fatty acid molecules (5 to 7 molecules) attached to the glucosamine molecules, and is buried in the outside lipid layer of the outer membrane. Meanwhile, the *O*-polysaccharide chain region has

direct contact with the outside of the bacterial cells and maintains the membrane permeability against exogenous lipophilic compounds. The outer core saccharide and the inner core saccharide regions are located between *O*-polysaccharide chain region and lipid A region.
In addition to LPS, *Helicobacter pylori* retains the glucosyl cholesterols (CGL, CAG and CPG) as another glycolipid components into the membranes when the organism assimilated exogenous free-cholesterol. Our previous study has demonstrated that the percentage of total glucosyl cholesterols is greater than 20% in the total lipids (except for LPS) composing the *Helicobacter pylori* membranes (Shimomura et al., 2004). Glycerophospholipids, such as phosphatidylethanolamine and phosphatidylglycerol, have been known in Gram-negative bacteria to be in both the inner membrane and the outer membrane. No earlier reports have, however, investigated the localization of glucosyl cholesterols (CGL, CAG and CPG) in *Helicobacter pylori* membranes.
To elucidate the membrane distribution of glucosyl cholesterols in *Helicobacter pylori*, we have divided the two membranes into the inner membrane fraction and the outer membrane fraction via sucrose-gradient centrifugation method, purified the lipids from each membrane fraction by the Folch method, and analyzed the lipid compositions obtained from these membrane fractions by thin-layer chromatography (TLC) (Shimomura et al., 2009). The TLC analysis detected the spot of free-cholesterol itself at a similar density in both the inner membrane fraction and the outer membrane fraction obtained from *Helicobacter pylori* ingested free-cholesterol from the medium. In contrast, the glucosyl cholesterols (CGL, CAG and CPG) produced via the absorption of free-cholesterol were detected at clearly higher levels in the outer membrane fraction than in the inner membrane fraction. In sum, the steroid itself was distributed into both the inner membrane and the outer membrane, whereas the glucosyl steroids were distributed into the outer membrane rather than into the inner membrane. We have also fractionated the inner membrane and the outer membrane of *Helicobacter pylori* that was cultured in the presence of estrone, and analyzed the lipids purified from each membrane fraction by TLC. As with the free-cholesterol itself, the spot of estrone absorbed by *Helicobacter pylori* was also detected at a similar density in both the outer and inner membrane fractions.
These findings indicate that *Helicobacter pylori* assimilates exogenous steroids into the outer and inner membranes and uses the glucosylated steroids as major lipid components constituting the outer membrane. This membrane distribution of steroids also suggests a possibility that *Helicobacter pylori* possesses a certain membrane transport system for the steroids: the steroids absorbed into *Helicobacter pylori* are shifted from the outer membrane to the inner membrane, and thereafter, the steroids glucosylated in the inner membrane are transported to the outer membrane. There is, however, no evidence that HP0421 protein catalyzing the α-glucosylation of steroids is distributed into the inner membrane of *Helicobacter pylori*. Therefore, it is important to ascertain the localization of HP0421 protein in the *Helicobacter pylori* membranes. In addition, further investigations will be necessary to clarify whether *Helicobacter pylori* possesses such a steroidal membrane transport system.

5. The physiological role of steroid assimilation in *Helicobacter pylori*

Though *Helicobacter pylori* aggressively assimilates various exogenous steroids into the membrane lipid compositions, the organism divides and proliferates even in the absence of steroids as well as the other bacterial species that have no ability to assimilate the exogenous steroids into the membranes. Our recent study has elucidated why *Helicobacter pylori*

physiologically requires steroids to survive (Shimomura et al., 2009). This paragraph describes an importance of steroid assimilation in maintaining the viability of *Helicobacter pylori*.
Phosphatidylcholine, the most prevalent phospholipid in mammals, is much higher in concentration than free-cholesterol in the blood plasma of humans; phosphatidylcholine exists at a concentration of approximately 144 mg/dl, whereas free-cholesterol exists at a concentration of approximately 60 mg/dl. The fundamental chemical structure of phosphatidylcholine is 1,2-diacyl-*sn*-glycero-3-phosphocholine (Fig. 3). In general, the carbon-1 (C1) position in the glycerol backbone of phosphatidylcholine carries a saturated fatty acid such as palmitic acid ($C_{16:0}$) or stearic acid ($C_{18:0}$), whereas the carbon-2 (C2) position in the glycerol backbone carries an unsaturated fatty acid such as oleic acid ($C_{18:1}$), linoleic acid ($C_{18:2}$), or arachidonic acid ($C_{20:4}$). Lyso-phosphatidylcholine (LPC) is a monoacyl-type phosphatidylcholine and generally lacks an unsaturated fatty acid at the carbon-2 (C2) position of the glycerol backbone. A number of investigations have demonstrated that unsaturated fatty acids and lyso-phosphatidylcholines have the potential to kill various microorganisms including *Helicobacter pylori* (Bruyn et al., 1996; Conley & Kabara, 1973; Constance et al., 1992; Kabara et al., 1972; Kanai & Kondo, 1979; Kanetsuna, 1985; Knapp & Melly, 1986; Kondo & Kanai, 1985; Nieman, 1954; Steel et al., 2002; Thompson et al., 1994). Thus, these individual lipophilic compounds constituting phosphatidylcholine act as antimicrobial agents against the various microorganisms. It remains unclear, however, whether the phosphatidylcholine itself also confers an antimicrobial action against the microorganisms.

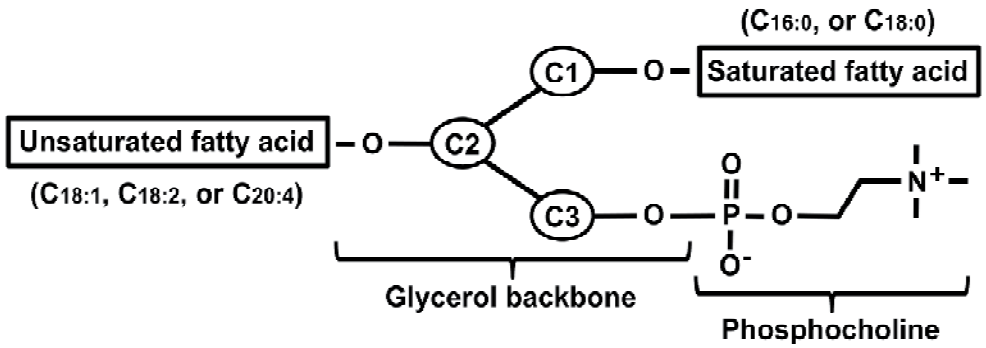

Fig. 3. The fundamental chemical structure of phosphatidylcholine

A previous study by others has shown that the concentration of phosphatidylcholine is 124.8 ± 62.6 μM in gastric juice from eight healthy volunteers (Berstad et al., 1992). We know that *Helicobacter pylori* colonizes the human gastric epithelium. In sum, this study indicates that *Helicobacter pylori* is surrounded by phosphatidylcholine *in vivo*. If phosphatidylcholine itself affects the survival of *Helicobacter pylori* that has failed to assimilate exogenous steroids such as free-cholesterol or steroid hormones, it may explain the importance of steroid assimilation in the organism. We have, therefore, investigated the antibacterial activity of phosphatidylcholine itself against *Helicobacter pylori* (Shimomura et al., 2009).

5.1 Antibacterial effects of phosphatidylcholine variants on the steroid-free *Helicobacter pylori*

When the steroid-free *Helicobacter pylori* (10^7 CFU/ml) that has no steroid in the membrane lipid compositions was incubated for 24 hours with various phosphatidylcholine variants

carrying different fatty acid molecules at the carbon-2 position of the glycerol backbone in a serum-free medium (3 ml) with continuous shaking under microaerobic conditions, the phosphatidylcholine variants attaching either a linoleic acid ($C_{18:2}$) molecule or an arachidonic acid ($C_{20:4}$) molecule to the carbon-2 (C2) position in the glycerol backbone conferred an antibacterial action fatal to the steroid-free *Helicobacter pylori*. In contrast, the phosphatidylcholine variants attaching either an oleic acid ($C_{18:1}$) molecule or a palmitic acid ($C_{16:0}$) molecule to the carbon-2 (C2) position in the glycerol backbone had no influence on the viability of *Helicobacter pylori*.

To ascertain the antibacterial potencies of phosphatidylcholine-themselves, we have also investigated the antibacterial activity of compositional constituents of the two phosphatidylcholines that exhibited the anti-*Helicobacter pylori* action, a linoleic acid ($C_{18:2}$), an arachidonic acid ($C_{20:4}$), and a LPC (1-palmitoyl-*sn*-glycero-3-phosphocholine). When the steroid-free *Helicobacter pylori* (10^7 CFU/ml) was incubated for 24 hours with linoleic acid (10 μg/ml), arachidonic acid (10 μg/ml), or LPC (10 μg/ml) in the serum-free medium (3 ml) containing a 0.2% dMβCD (2,6-di-*O*-methyl-β-cyclodextrin) with continuous shaking under microaerobic conditions, the two fatty acids and LPC had no influence on the viability of the steroid-free organism. The dMβCD has, thus, completely counteracted the antibacterial action of these lipophilic compounds against the steroid-free *Helicobacter pylori*. Incidentally, the linoleic acid (10 μg/ml), arachidonic acid (10 μg/ml), and LPC (10 μg/ml) in the absence of dMβCD confer the antibacterial action fatal to the steroid-free *Helicobacter pylori*. Intriguingly, the dMβCD had no influence on the anti-*Helicobacter pylori* action of the phosphatidylcholine variants carrying either a linoleic acid molecule or an arachidonic acid molecule at the carbon-2 position of the glycerol backbone, and these two phosphatidylcholine variants (concentrations ranging from 10 μg/ml to 100 μg/ml) conferred the bactericidal action to the steroid-free *Helicobacter pylori* even in the presence of the 0.2% dMβCD, as with had being done in the absence of dMβCD.

The dMβCD is a cyclic oligomer consisting of seven 2,6-di-*O*-methyl-α-D-glucose molecules linked by α (1→4)-glycosidic bonds and has the ability to solubilize lipophilic compounds through the formation of molecular inclusion complexes (Ohtani et al., 1989). To examine whether dMβCD inhibits the adsorption of unsaturated fatty acids, or phosphatidylcholines onto the steroid-free *Helicobacter pylori* cells, we carried out the following experiments. The heat-killed *Helicobacter pylori* cells that have no steroid in the membranes were incubated for 24 hours with a linoleic acid (100 μg/ml), an arachidonic acid (100 μg/ml), or phosphatidylcholine variants (100 μg/ml), to which either a linoleic acid molecule or an arachidonic acid molecule is attached as the acyl group, in the serum-free medium containing a 0.2% dMβCD with continuous shaking at 37°C, and thereafter the membrane lipids were purified from the heat-killed cells recovered via the Folch method and analyzed by TLC. The membrane lipids obtained from the heat-killed cells incubated in the absence of dMβCD contained tremendous amounts of linoleic acid and arachidonic acid, whereas the membrane lipids obtained from the heat-killed cells incubated in the presence of dMβCD (0.2%) contained negligible amounts of those fatty acids. Surprisingly, the phosphatidylcholines contained in the membrane lipids of the heat-killed cells incubated in the presence of dMβCD (0.2%) were larger amount than those contained in the membrane lipids of the heat-killed cells incubated in the absence of dMβCD. In sum, dMβCD has inhibited the binding of the unsaturated fatty acids to the membranes of *Helicobacter pylori* but promoted the binding of the phosphatidylcholines to the membranes of the organism.

These results indicate that dMβCD obstructs the hydrophobic interaction between the unsaturated fatty acids and the *Helicobacter pylori* membranes by solubilizing those fatty acids, and thereby protects the organism from the bactericidal action of the unsaturated fatty acids, and probably LPC (Fig. 4). It is unclear why dMβCD promotes the adsorption of the two phosphatidylcholines onto the *Helicobacter pylori* membranes. These findings, however, indicate that the anti-*Helicobacter pylori* action originates in the potencies of phosphatidylcholine-themselves and that the unsaturated fatty acids (linoleic acid and arachidonic acid), and the LPC (1-palmitoyl-*sn*-glycero-3-phosphocholine), which result from the hydrolysis of the phosphatidylcholines, do not contribute to this action. We have, thus, elucidated the bactericidal activity of phosphatidylcholine (PC) variants carrying either a linoleic acid molecule or an arachidonic acid molecule at the carbon-2 position of the glycerol backbone against the steroid-free *Helicobacter pylori*.

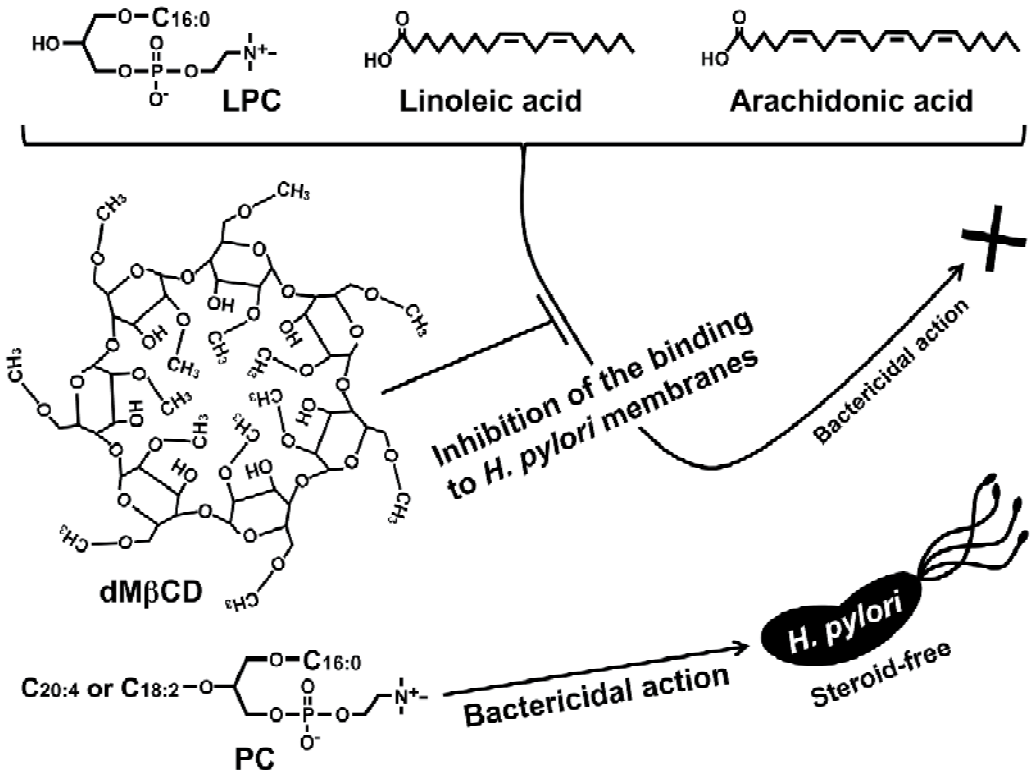

Fig. 4. The role of 2,6-di-*O*-methyl-β-cyclodextrin (dMβCD) on the anti-*Helicobacter pylori* action of lipophilic compounds

5.2 Bacteriolysis in the steroid-free *Helicobacter pylori* caused by the cell adsorption of phosphatidylcholines

To examine the antibacterial mechanism of phosphatidylcholine variants carrying either a linoleic acid molecule or an arachidonic acid molecule at the carbon-2 position of the glycerol backbone, we performed the following experiments. After the steroid-free *Helicobacter pylori* (10^8 CFU/ml) was incubated for 8 hours with each phosphatidylcholine

(100 μg/ml) in a serum-free medium (3 ml) with continuous shaking under microaerobic conditions, the supernatant recovered was subjected to the purification of proteins by an anion-exchange chromatography, and the proteins purified were analyzed by a sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). A number of protein bands with tremendous high densities were detected in the supernatant obtained from the steroid-free *Helicobacter pylori* incubated with the phosphatidylcholines, in the SDS-PAGE analysis. Among those protein bands detected, the band for flavodoxin (FldA) protein was also contained. As FldA is an electron acceptor of the oxidoreductase that catalyzes acetyl-CoA synthesis in *Helicobacter pylori* cell (Hughes et al., 1995), we can assume that FldA is the intracellular protein. These results, in sum, indicate that the phosphatidylcholine variants attaching either a linoleic acid or an arachidonic acid as the acyl group to the carbon-2 position in the glycerol backbone exert deleterious effect on the cell membranes of steroid-free *Helicobacter pylori* and induce the bacterial cell lysis, resulting in abundant leakage of intracellular proteins (especially FldA protein) to outside of the bacterial cells. Incidentally, the SDS-PAGE analysis detected only negligible amount of proteins in the supernatant obtained from the steroid-free *Helicobacter pylori* incubated without either the phosphatidylcholines.

5.3 Acquirement of phosphatidylcholine resistance in *Helicobacter pylori* conferred by assimilating steroid

We next investigated whether the *Helicobacter pylori* with the assimilated steroid succumbs to the bactericidal action of the phosphatidylcholines, as with the steroid-free organism. When the steroid-free *Helicobacter pylori* is cultured for 24 hours in the serum-free medium containing free-cholesterol (50 μM concentration) with continuous shaking under microaerobic conditions, the organism recovered retains both free-cholesterol itself and glucosyl cholesterols (CGL, CAG and CPG) in the membranes. We, therefore, examined the anti-*Helicobacter pylori* action of the two phosphatidylcholine variants possessing the antibacterial action fatal to the steroid-free *Helicobacter pylori* by using the organism retaining the assimilated free-cholesterol that was prepared via the above culture procedure. When *Helicobacter pylori* (10^7 CFU/ml) with the assimilated free-cholesterol was incubated at various time points in a serum-free medium (3 ml) with shaking under microaerobic conditions in the presence or absence of each phosphatidylcholine (100 μg/ml), which carries either a linoleic acid molecule or an arachidonic acid molecule at the carbon-2 position of the glycerol backbone, the *Helicobacter pylori* did not succumb to the antibacterial effects of the two phosphatidylcholine variants: the time-dependent growth-decline curve of *Helicobacter pylori* with each phosphatidylcholine roughly corresponded to the time-dependent growth-decline curve of the organism without either the two phosphatidylcholines, when the CFU values ($\log_{10}$ CFU/ml: vertical axis) and the incubation times (hour: horizontal axis) were plotted in a graph. *Helicobacter pylori* that had assimilated free-cholesterol (FC) has been, thus, found to resist the bactericidal action of phosphatidylcholine variants carrying either a linoleic acid molecule or an arachidonic acid molecule at the carbon-2 position of the glycerol backbone.

As described above, *Helicobacter pylori* retains free-cholesterol in the form of glucosyl cholesterols (CGL, CAG and CPG). This raises the question as to whether the glucosyl cholesterols are more important rather than the free-cholesterol itself in the expression of phosphatidylcholine resistance in *Helicobacter pylori*. To resolve this question, we examined

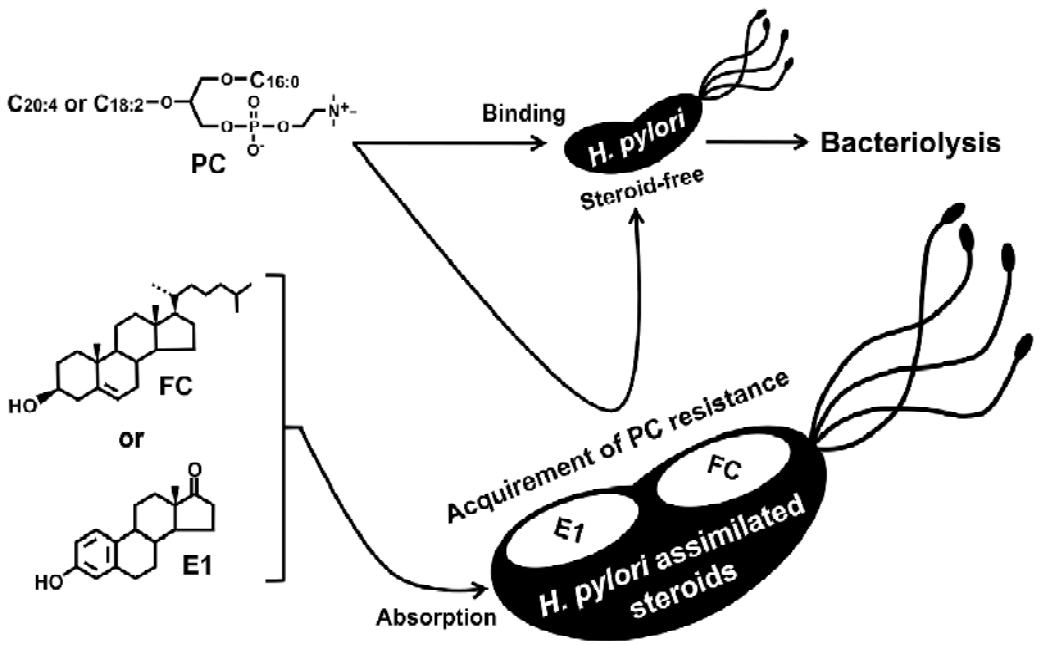

Fig. 5. The expression of phosphatidylcholine (PC) resistance in *Helicobacter pylori* with the assimilated steroids

the phosphatidylcholine resistance in *Helicobacter pylori* with another assimilated steroid. We have shown that *Helicobacter pylori* efficiently absorbs and retains the female hormone estrone into the membranes, but fails to glucosylate the estrogen (Hosoda et al., 2009). In addition, we have also found that other female hormone estriol is not absorbed into the membranes of *Helicobacter pylori*. Therefore, we decided to use estrone and estriol as steroid tools that are not glucosylated by *Helicobacter pylori*. After the steroid-free *Helicobacter pylori* was cultured for 24 hours with estrone (50 μM concentration) in a serum-free medium with continuous shaking under microaerobic conditions, the recovered organism (10^7 CFU/ml) that had assimilated estrone without glucosylation in the membranes was incubated for further 24 hours with each phosphatidylcholine (100 μg/ml), which carries either a linoleic acid molecule or an arachidonic acid molecule at the carbon-2 position of the glycerol backbone, in a serum-free medium (3 ml) with continuous shaking under the same conditions. As with the *Helicobacter pylori* that assimilated free-cholesterol into the membranes, the organism with the assimilated estrone also resisted the bactericidal action of the two phosphatidylcholine variants, and the CFU was maintained to a high level (> 10^6 CFU/ml) comparable to the control CFU (10^7 CFU/ml) of *Helicobacter pylori* incubated for 24 hours without either the phosphatidylcholines. *Helicobacter pylori* has, in sum, expressed phosphatidylcholine resistance even when assimilated estrone (E1) without glucosylating it. In addition, this finding indicates that the glucosylation of steroid is so far not important in conferring resistance to the bactericidal action of phosphatidylcholine upon *Helicobacter pylori*, although the glucosylation of steroid is essential for *Helicobacter pylori* to evade the host immune systems. In contrast, the *Helicobacter pylori* treated for 24 hours with estriol (50 μM concentration) succumbed to the bactericidal action of the two phosphatidylcholine variants as with the steroid-free organism, and the CFU level reduced from 10^7 CFU/ml to <

10^3 CFU/ml, when the estriol-treated organism was incubated for 24 hours in the serum-free medium containing the respective phosphatidylcholine variants (100 μg/ml) to which either a linoleic acid or an arachidonic acid is attached as the acyl group. These results, together with our findings on the free-cholesterol assimilation in *Helicobacter pylori*, indicate that bacteria of this species acquire a resistance against the bacteriolytic activity of phosphatidylcholine by assimilating the exogenous steroids into the membranes (Fig. 5).
Phosphatidylcholine is not a single molecule, but a family of variants with different fatty acid compositions attached to the glycerol backbone of the phosphatidylcholine (Fig. 3). The predominant phosphatidylcholine in human serum has been known to carry a palmitic acid ($C_{16:0}$) molecule and a linoleic acid ($C_{18:2}$) molecule, and recently, the predominant phosphatidylcholine in the human gastric mucus has also been shown to carry the same two fatty acids (Orihara et al., 2001). One of the two phosphatidylcholines investigated as to the anti-*Helicobacter pylori* effect by our group is exactly its variant carrying a palmitic acid molecule and a linoleic acid molecule: 2-linoleoyl-1-palmitoyl-*sn*-3-phosphocholine. In sum, the phosphatidylcholine attaching a palmitic acid and a linoleic acid to the carbon-1 position and to the carbon-2 position in the glycerol backbone is the most prevalent phosphatidylcholine in humans. *Helicobacter pylori* colonizes the human gastric epithelium and inhabits the human stomach for many years. On this basis, we can assume that *Helicobacter pylori* is constantly exposed to various phosphatidylcholine variants, particularly the phosphatidylcholine carrying a palmitic acid molecule and a linoleic acid molecule. Our recent study, in sum, indicates that the steroid assimilation in *Helicobacter pylori* plays an important role in reinforcing the membrane lipid barrier and conferring resistance to the bacteriolytic action of hydrophobic compounds such as phosphatidylcholine.

6. The 3=O steroids and *Helicobacter pylori*

Testosterone, androstenedione and progesterone possess an oxo (3=O) group at the carbon-3 position of the steroid framework. Our recent studies have revealed that *Helicobacter pylori* cannot use as the membrane lipid components these 3=O steroids and rather succumbs to the antibacterial action of certain 3=O steroids. This paragraph describes the 3=O steroids as bactericidal agents to *Helicobacter pylori*.

6.1 Testosterone and *Helicobacter pylori*

Like estriol, testosterone also was not utilized as the membrane lipid component of *Helicobacter pylori*: the TLC analysis did not detect testosterone in the membrane lipid compositions of *Helicobacter pylori* cultured for 24 hours with this androgen at the 50 μM concentration (Hosoda et al., 2009). Testosterone did not, therefore, contribute to the phosphatidylcholine resistance upon *Helicobacter pylori* (Shimomura et al., 2009). In addition, this 3=O steroid at the 50 μM concentration hardly affected the growth of *Helicobacter pylori*.

6.2 Androstenedione and *Helicobacter pylori*

When *Helicobacter pylori* (10^5 CFU/ml) was cultured for 24 hours in the serum-free medium (3 ml) containing androstenedione at concentrations ranging from 10 to 100 μM with continuous shaking under microaerobic conditions, this 3=O steroid exhibited inhibitory effect on the growth of *Helicobacter pylori* at concentrations grater than 50 μM. Androstenedione was, however, relatively low potency in inhibiting the growth of *Helicobacter pylori*. The decrease in CFU (10^4 CFU/ml) of *Helicobacter pylori* cultured with

androstenedione at the 100 μM concentration was slight compared to the baseline CFU (10^5 CFU/ml) immediately after the culture initiation (Hosoda et al., 2011).

6.3 Progesterone and *Helicobacter pylori*

Of the three 3=O steroid hormones (testosterone, androstenedione, and progesterone) investigated, the progesterone has demonstrated the most effective anti-*Helicobacter pylori* action. Progesterone efficiently inhibited the growth of *Helicobacter pylori* by a manner dependent on the greater doses added into the medium, and the CFU of the organism in the presence of progesterone at the 100 μM concentration was below the limits of detection (< 10 CFU/ml), when *Helicobacter pylori* (10^6 CFU/ml) was cultured for 24 hours in the serum-free medium (3 ml) containing progesterone at concentrations ranging from 10 to 100 μM with continuous shaking under microaerobic conditions (Hosoda et al., 2011).

6.4 Progesterone derivatives and *Helicobacter pylori*

We have discovered the effective anti-*Helicobacter pylori* action of progesterone. Progesterone has at least two derivatives, namely, 17α-hydroxyprogesterone and 17α-hydroxyprogesterone caproate. The derivatives, 17α-hydroxyprogesterone and 17α-hydroxyprogesterone caproate are modified by a hydroxyl group and an acyl group (caproic acid), respectively, at the carbon-17 position of the progesterone framework. 17α-hydroxylprogesterone is a natural progesterone derivative, while 17α-hydroxyprogesterone caproate is a synthetic progesterone derivative. Noting this, we have examined the anti-*Helicobacter pylori* action of these progesterone derivatives. When *Helicobacter pylori* (10^6 CFU/ml) was cultured for 24 hours in a serum-free medium (3 ml) containing 17α-hydroxyprogesterone with continuous shaking under microaerobic conditions, surprisingly, this natural progesterone derivative had no influence on the growth of *Helicobacter pylori*: even in the presence of 17α-hydroxyprogesterone at the 100 μM concentration, the CFU was comparable to the control CFU (10^8 CFU/ml) of *Helicobacter pylori* cultured for 24 hours without steroid. In contrast, 17α-hydroxyprogesterone caproate had a stronger anti-*Helicobacter pylori* action than progesterone, and the CFU was below the limits of detection (< 10 CFU/ml), when the organism (10^6 CFU/ml) was cultured for 24 hours with 17α-hydroxyprogesterone caproate at the 10 μM concentration in a serum-free medium with continuous shaking under microaerobic conditions. Incidentally, caproic acid ($C_{6:0}$), a constituent of 17α-hydroxyprogesterone caproate, did not affect the viability of *Helicobacter pylori* even when added into the bacterial cell suspension at a 100 μM concentration (Hosoda et al., 2011). These findings suggest that the acylation at the carbon-17 position in the progesterone framework plays an important role in reinforcing the anti-*Helicobacter pylori* action of progesterone.

6.5 Antibacterial effects of progesterone and its derivative on *Helicobacter pylori*

To ascertain the antibacterial potencies of progesterone and 17α-hydroxyprogesterone caproate, we investigated the time-dependent antibacterial effects of these two gestagens on *Helicobacter pylori* (Fig. 6A). When *Helicobacter pylori* (approximately 10^7 CFU/ml) was incubated with progesterone (100 μM) or 17α-hydroxyprogesterone caproate (100 μM) in a serum-free medium (3 ml) at various time points with shaking under microaerobic conditions, the CFUs of the organism incubated with progesterone (PS) moved along a

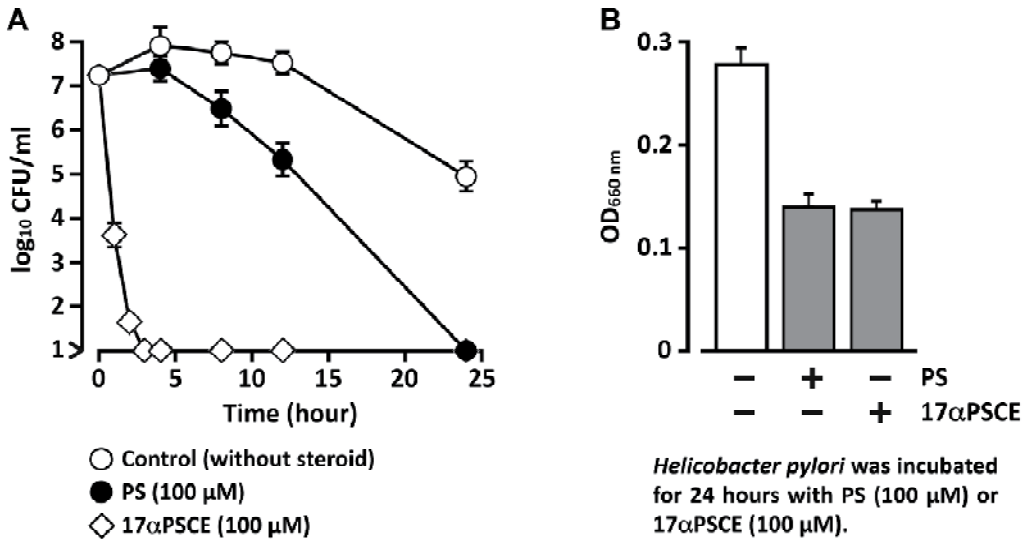

Fig. 6. Antibacterial effects of progesterone (PS) and 17α-hydroxyprogesterone caproate (17αPSCE) on *Helicobacter pylori*

gently-sloping curve, falling below the limits of detection (< 10 CFU/ml) by 24 hours after the start of incubation. In contrast, the CFUs of *Helicobacter pylori* incubated with 17α-hydroxyprogesterone caproate (17αPSCE) dropped off sharply, falling the limits of detection within 4 hours after the start of incubation. In sum, 17α-hydroxyprogesterone caproate (17αPSCE) has been found to be much more prompt in conferring the antibacterial action fatal to *Helicobacter pylori* than progesterone (PS).

6.6 Bacteriolysis in *Helicobacter pylori* caused by the cell surface binding of progesterone and its derivative

To clarify the antibacterial mechanism of progesterone and 17α-hydroxyprogesterone caproate against *Helicobacter pylori*, we measured an optical density ($OD_{660\ nm}$) in the bacterial cell suspensions after *Helicobacter pylori* (10^8 CFU/ml) was incubated for 24 hours with progesterone (100 μM) or 17α-hydroxyprogesterone caproate (100 μM) in a serum-free medium (3 ml) with continuous shaking under microaerobic conditions. The decline of $OD_{660\ nm}$ means that the bacterial cells in suspension had been lysed via certain physical or chemical actions. As it turned out, the $OD_{660\ nm}$ of the bacterial cell suspension incubated with progesterone or 17α-hydroxyprogesterone caproate declined to half value of that of the control cell suspension of *Helicobacter pylori* incubated for 24 hours in the absence of steroid (Fig. 6B).

To confirm the cell lysis of *Helicobacter pylori*, we examined the bacterial morphologies using a differential interference microscope (Fig. 7). When *Helicobacter pylori* (10^7 CFU/ml) was incubated for 24 hours in a serum-free medium in the presence or absence of the two 3=O steroids, the control cell suspension of *Helicobacter pylori* incubated without the steroids harbored the organisms in both mixed rod and coccoid forms. In contrast, the cell suspension of the *Helicobacter pylori* incubated with progesterone (100 μM) or 17α-hydroxyprogesterone caproate (100 μM) harbored hardly any organisms, although objects

such as cellular debris were observed. These results, together with the findings from the measurement of $OD_{660\ nm}$ in the bacterial cell suspension, suggest that *Helicobacter pylori* cells are lysed by a certain action of progesterone (PS) and 17α-hydroxyprogesterone caproate (17αPSCE).

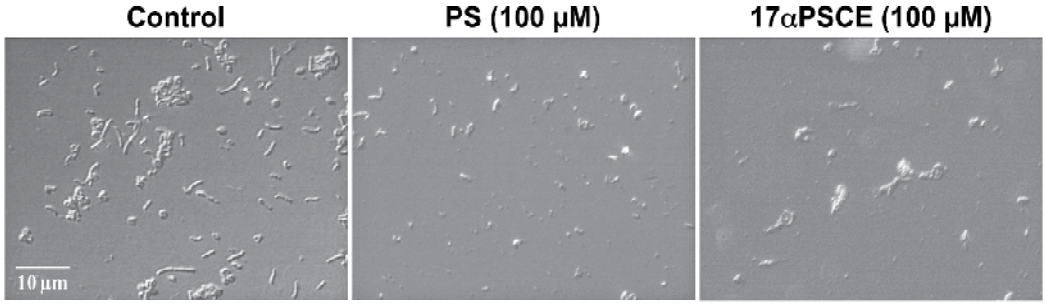

Fig. 7. Cell lysis on *Helicobacter pylori* induced by progesterone (PS) and 17α-hydroxyprogesterone caproate (17αPSCE)

Next, we carried out a series of experiments to examine whether progesterone and 17α-hydroxyprogesterone caproate induce the cell lysis of *Helicobacter pylori* via membrane injury. After *Helicobacter pylori* (10^9 CFU/ml) was incubated for 5 hours with progesterone (100 μM) or 17α-hydroxyprogesterone caproate (100 μM) using phosphate-buffered saline (PBS: 10 ml), in place of the serum-free medium, with continuous shaking under microaerobic conditions, the proteins in the bacterial cell supernatant were analyzed by SDS-PAGE. The protein bands detected in the cell supernatant of *Helicobacter pylori* incubated with progesterone or 17α-hydroxyprogesterone caproate were considerably denser than the protein bands detected in the control cell supernatant of *Helicobacter pylori* incubated for 5 hours without steroid. A band for flavodoxin (FldA), an intracellular protein, was also found among the other protein bands. Though progesterone conferred the remarkable antibacterial effect to *Helicobacter pylori* suspended into the PBS, the potency of progesterone to decrease the CFU of *Helicobacter pylori* was somewhat lower than that of 17α-hydroxyprogesterone caproate. In addition, the control CFU of *Helicobacter pylori* suspended into PBS without steroid was also decreased, but the decrease magnitude in the CFU was slight. The amount of FldA protein detected in the bacterial cell supernatant correlated closely with the decreases of CFU: the FldA protein band became more noticeable when the CFU decreased by a greater magnitude. In sum, a large amount of FldA protein has leaked from the *Helicobacter pylori* cells to outside, when the organism was exposed to progesterone and 17α-hydroxyprogesterone caproate. These results indicate that progesterone and 17α-hydroxyprogesterone caproate injure the membranes of *Helicobacter pylori* and thereby induce the cell lysis more promptly than autolysis (Hosoda et al., 2011).

6.7 Antibacterial effects of progesterone and its derivative on other Gram-positive and Gram-negative bacteria

To estimate the antibacterial effects of progesterone and 17α-hydroxyprogesterone caproate against other representative Gram-positive and Gram-negative bacteria, we have

determined the minimum inhibitory concentrations (MICs) of these 3=O steroids by the following method. Progesterone or 17α-hydroxyprogesterone caproate was serially diluted 2-fold with a dimethyl sulfoxide (DMSO) solution and added to agar plates of serum-free medium. Bacterial cell suspension (10 μl) adjusted to approximately 10^7 CFU/ml was dotted onto agar plates containing progesterone or 17α-hydroxyprogesterone caproate (from 1.6 μM to 100 μM) and cultured for 1 week under microaerobic conditions. The MICs (μM) of progesterone and 17α-hydroxyprogesterone caproate for the four *Helicobacter pylori* strains (NCTC 11638, ATCC 43504, the clinical isolates A-13 and A-19), *Escherichia coli* strain NIH JC-2, *Pseudomonas aeruginosa* strain ATCC 10145, *Staphylococcus aureus* strain FDA 209D, and *Staphylococcus epiderimidis* strain sp-al-1 were determined by confirming the growth of colonies from the organisms on the agar plates. As it turned out, the MICs of progesterone and 17α-hydroxyprogesterone caproate for the four *Helicobacter pylori* strains were 50 μM and 3.1 μM, respectively (Table 1). Intriguingly, progesterone and 17α-hydroxyprogesterone caproate had no influence on the growth of the other four bacterial species, namely, *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Staphylococcus epiderimidis*: all four species grew even in the presence of progesterone or 17α-hydroxyprogesterone caproate at 100 μM (the highest concentration examined). The antibacterial spectra of progesterone and 17α-hydroxyprogesterone caproate have, thus, been remarkably narrow. The four bacterial species, *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Staphylococcus epiderimidis* have no capability to incorporate exogenous steroids into the membranes. Given the unique feature of *Helicobacter pylori* as an aggressive assimilator of exogenous steroids, we can assume that progesterone and 17α-hydroxyprogesterone caproate attacked *Helicobacter pylori* without targeting the other four bacterial species.

Bacterial species	MIC (μM)	
	PS	17αPSCE
Helicobacter pylori	**50**	**3.1**
Escherichia coli	**> 100**	**> 100**
Pseudomonas aeruginosa	**> 100**	**> 100**
Staphylococcus aureus	**> 100**	**> 100**
Staphylococcus epiderimidis	**> 100**	**> 100**

Table 1. MICs of progesterone (PS) and 17α-hydroxyprogesterone caproate (17αPSCE) for various bacterial species

7. Investigation of the steroid-binding site on *Helicobacter pylori*

As described above, we have demonstrated the relationship between *Helicobacter pylori* and steroids. Certain steroids such as free-cholesterol and estrone have been found to be beneficial for the survival of *Helicobacter pylori*. Conversely, other steroids such as estradiol and progesterone have been found to impair the viability of *Helicobacter pylori*. From these findings, in sum, *Helicobacter pylori* seems to bind various steroids to the identical regions on the cell surface. In light of this, we hypothesized that progesterone and free-cholesterol act

to steroid-binding sites existing on the *Helicobacter pylori* cell surface. To verify this hypothesis, we carried out the following experiments (Hosoda et al., 2011). After a 24-hour preculture of *Helicobacter pylori* (10^6 CFU/ml) with progesterone (5 μM or 10 μM) in a serum-free medium (30 ml), the *Helicobacter pylori* cells (10^8 CFU/ml) recovered were incubated for 4 hours in a serum-free medium (30 ml) containing free-cholesterol fixed-beads (free-cholesterol concentration: 250 μM). Thereafter, the amount of free-cholesterol absorbed into the *Helicobacter pylori* cells was quantified via the ferric chloride-sulfuric acid reagent method. The amount of free-cholesterol per CFU obviously tended to reduce by preculturing *Helicobacter pylori* with progesterone. These results suggest that progesterone strongly binds to the *Helicobacter pylori* cell surface and thereby obstructs the free-cholesterol absorption of *Helicobacter pylori* by inhibiting the cell surface binding of free-cholesterol. Incidentally, progesterone had no influence on the viability of *Helicobacter pylori* at the 5 and 10 μM concentrations: the CFUs of the *Helicobacter pylori* cultured for 24 hours with progesterone were similar to the control CFU (10^8 CFU/ml) of the *Helicobacter pylori* cultured for 24 hours without progesterone.

Helicobacter pylori glucosylates the absorbed free-cholesterol and synthesizes glucosyl cholesterols (CGL, CAG and CPG). With this in mind, we decided to examine the influence of progesterone on the glucosylation of free-cholesterol. After a 24-hour preculture of *Helicobacter pylori* (10^6 CFU/ml) in the presence or absence of progesterone (10 μM) in a serum-free medium (30 ml), the *Helicobacter pylori* cells (10^8 CFU/ml) recovered were incubated for 4 hours with free-cholesterol fixed-beads (free-cholesterol concentration: 250 μM) in a serum-free medium (30 ml), and the membrane lipids were purified to analyze the glucosyl cholesterol levels in the membrane lipid compositions by TLC. The TLC analysis detected the glucosyl cholesterols (CGL, CAG and CPG) in the membrane lipids of *Helicobacter pylori* precultured with progesterone, although no free-cholesterol was found to have accumulated within the lipids. Meanwhile, the glucosyl cholesterol levels detected in the membrane lipids of *Helicobacter pylori* precultured with progesterone were similar to the glucosyl cholesterol levels detected in the membrane lipids of *Helicobacter pylori* precultured without progesterone. Progesterone has been found to exert no inhibitory effects on the enzymes involved in the glucosyl cholesterol synthesis.

Next, we examined whether free-cholesterol conversely inhibits the anti-*Helicobacter pylori* action of progesterone. When the *Helicobacter pylori* (10^6 CFU/ml) was cultured for 24 hours with free-cholesterol fixed-beads at various volumes (free-cholesterol concentration: 30 to 90 μM) in a serum-free medium (15 ml) containing progesterone (30 μM), the free-cholesterol did not inhibit the anti-*Helicobacter pylori* action of progesterone: the CFU increase was not observed in any concentrations of free-cholesterol, and the CFU levels hardly altered from the control CFU (10^6 CFU/ml) of *Helicobacter pylori* cultured for 24 hours with progesterone in the absence of free-cholesterol fixed-beads. These results, at least, indicate that free-cholesterol does not competitively inhibit the anti-*Helicobacter pylori* action of progesterone. This compelled us, in sum, to examine the inhibitory effect of a high concentration of free-cholesterol on the anti-*Helicobacter pylori* action of progesterone. When the *Helicobacter pylori* (10^6 CFU/ml) was cultured for 24 hours with progesterone at concentrations ranging from 10 to 30 μM in a serum-free medium (15 ml) containing free-cholesterol fixed-beads (free-cholesterol concentration: 500 μM) or simple-beads (the volumes similar to the free-cholesterol fixed-bead volumes), free-cholesterol at the highest concentration (500 μM) had a noticeable influence on the anti-*Helicobacter pylori* action of the

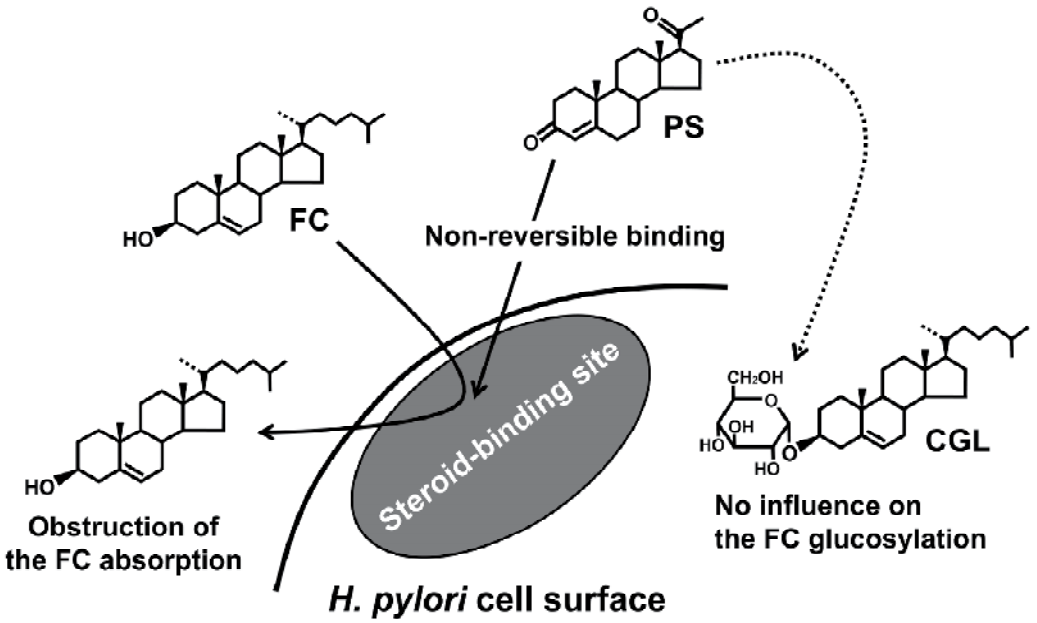

Fig. 8. The obstruction of free-cholesterol (FC) absorption in *Helicobacter pylori* by progesterone (PS)

progesterone: the growth-inhibitory curve of *Helicobacter pylori* cultured with progesterone in the presence of free-cholesterol fixed-beads shifted from the control growth-inhibitory curve of *Helicobacter pylori* cultured with progesterone in the presence of simple-beads to the right side, when the CFU values ($\log_{10}$ CFU/ml: vertical axis) and the progesterone concentrations (μM: horizontal axis) were plotted in a graph. These results indicate that free-cholesterol noncompetitively inhibits the anti-*Helicobacter pylori* action of progesterone. In combination with the results of the inhibitory effect of progesterone on the binding of free-cholesterol onto the *Helicobacter pylori* cells, they also strongly suggest that progesterone non-reversibly binds to the *Helicobacter pylori* cells and thereby induces the cell lysis, and/or inhibits the free-cholesterol absorption of the organism.

Our recent study has shown that progesterone inhibits the free-cholesterol absorption of *Helicobacter pylori*, and conversely, that a relatively high concentration of free-cholesterol inhibits the anti-*Helicobacter pylori* action of progesterone. Progesterone and free-cholesterol, in sum, seem to bind to identical sites on the *Helicobacter pylori* cell surfaces and thereby obstruct each other's effects (Fig. 8). This suggests that *Helicobacter pylori* may express a certain component, such as a steroid-binding protein, on the cell surface. Further investigations will be required to elucidate whether such a steroid-binding protein does indeed exist in *Helicobacter pylori*.

8. Conclusion

Our current basal research has revealed the following relationship between *Helicobacter pylori* and steroid hormones: pregnenolone (PN), dehydroepiandrosterone (DEA), epiandrosterone (EA), and estrone (E1) are absorbed into the membranes of *Helicobacter pylori* and play an important role to reinforcing the membrane lipid barrier, and thereby

Helicobacter pylori acquires the phosphatidylcholine resistance. Conversely, estradiol, androstenedione, and progesterone are harmful for the survival of *Helicobacter pylori*, and especially progesterone (PS) exhibit more effective antibacterial action to *Helicobacter pylori* than the other steroid hormones (Fig. 9). In addition, we have discovered that the acylation at the carbon-17 position of progesterone framework considerably augments the anti-*Helicobacter pylori* action of progesterone and that the hydroxylation at the same carbon position of progesterone framework conversely cancels out this action of progesterone. In sum, 17α-hydroxyprogesterone caproate (17αPSCE) exhibits much stronger anti-*Helicobacter pylori* action than progesterone, whereas 17α-hydroxyprogesterone has no anti-*Helicobacter pylori* action. These findings are expected to contribute to the development of a novel antibacterial steroidal medicine that targets *Helicobacter pylori* as an aggressive assimilator of exogenous steroids. Particularly, progesterone may be useful as a fundamental structure for designing new anti-*Helicobacter pylori* steroidal agents.

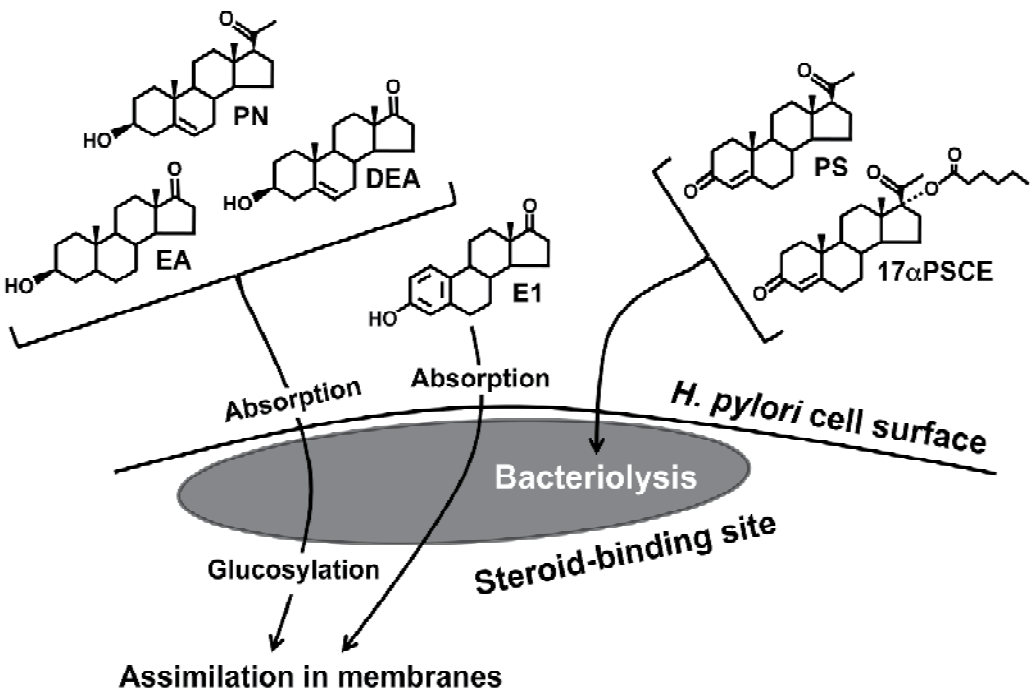

Fig. 9. The relationship between *Helicobacter pylori* and steroid hormones

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6

Steroid Hormones and Ovarian Cancer

Erin R. King and Kwong-Kwok Wong
The University of Texas MD Anderson Cancer Center, Houston, TX, United States of America

1. Introduction

Globally, ovarian cancer is the 6th most common malignancy in developed countries, responsible for 100,300 new cases and 64,500 deaths annually (Jemal et al., 2011). Approximately 90% of ovarian cancers arise within the ovarian surface epithelium (OSE) or the fallopian tube surface epithelium; the remainder of ovarian malignancies develops from other ovarian tissues (sex cord-stromal, germ cell, or mixed cell tumors). The overall prognosis for epithelial ovarian carcinoma (EOC) is poor: Diagnosis is typically late-stage due to the lack of effective screening methods and vague presenting symptoms, with 5-year survival at 40% for stage III and 20% for stage IV patients (Heintz et al., 2006). Despite excellent initial activity, the standard treatment consisting of cytoreductive surgery followed by platinum- and taxane-based chemotherapy often fails with a recurrence rate of over 80% in stage III and IV disease. Therefore, novel therapeutic approaches are needed to improve the outcomes in this population.

Research efforts have yielded insight into the etiology, signaling mechanisms, and progression of ovarian cancer, yet much remains poorly understood. Physiologically, steroid hormones are intimately involved in ovulation, reproduction, and function of normal OSE cells. There is growing evidence that estrogen, progesterone, and other hormones also play a role in the development and progression of ovarian cancer (Leung & Choi, 2007). "Incessant ovulation" with repetitive injury and repair of OSE and subsequent cumulative DNA damage is one of the hypothesized risk factors for ovarian cancer (Fathalla, 1971), yet this does not explain the occurrence of the majority of ovarian carcinomas well after the reproductive years (Berek & Hacker, 2010). Interestingly, although oral contraceptives (OCs), increasing parity, and prolonged breastfeeding all decrease cumulative risk (Gwinn et al., 1990; Risch et al., 1994), progestin-only contraceptives offer just as much protective benefit as estrogen-containing OCs without prohibiting ovulation (Risch, 1998). Another hypothesis for the development of ovarian cancer, the "gonadotropin hypothesis," stipulates that gonadotropins contribute to ovarian carcinogenesis through follicle stimulating hormone (FSH)- and luteinizing hormone (LH)-mediated excess stimulation of ovarian tissue. This hypothesis is consistent with the protective effect of OCs, and the observation that the majority of cases of epithelial ovarian cancer develop postmenopausally after a surge in gonadotropin levels. *In vitro,* gonadotropins such as FSH activate mitogenic pathways and stimulate ovarian epithelial cell proliferation (Choi et al., 2002). In addition to gonadotropins, excess androgens and estrogen have also been linked to the progression and possibly development of ovarian cancer. *In vivo* treatment of mice with

estrogen significantly increases tumor growth (Armaiz-Pena et al., 2009). These observations suggest that in addition to regulation of the menstrual cycle, certain steroid hormones may promote the progression—and possibly development—of ovarian cancer, while others offer a protective benefit. The objectives of this chapter are to summarize the signaling mechanisms involved in normal human OSE and its neoplastic counterparts, to highlight the effects of these steroid hormones on ovarian cancer cell growth, and to discuss the current clinical trials utilizing anti-hormonal approaches in ovarian cancer patients.

2. Steroid hormone signaling in normal ovarian surface epithelium and ovarian cancer

2.1 Steroid signaling in normal ovarian surface epithelium

Human ovarian surface epithelium is formed by a single layer of squamous to cuboidal cells covering the outermost layer of the ovary. While the exact function of OSE is unclear, with each ovulatory cycle, the OSE undergoes damage and repair. This damage incites an inflammatory response, the sequelae of which have been implicated in neoplastic transformation to tumor cells (Murdoch & Martinchick, 2004; Ness et al., 2000). Although steroid synthesis takes place elsewhere in the ovary, both normal and malignant OSE display estrogen, progesterone, and androgen receptors (Karlan et al., 1995; Lau et al., 1999; Li et al., 2003), and take part in steroid signaling. OSE also have FSH and LH receptors, though *in vitro* studies suggest these stimulate cellular growth and proliferation rather than steroidogenesis in OSE (Choi et al., 2004; Ji et al., 2004).

2.1.1 Estrogen and progesterone signaling in normal ovarian surface epithelium

Though the purpose of steroid signaling within the OSE remains uncertain, the effects of these steroids have been characterized *in vivo* and *in vitro*. The estrogen receptor (ER) and progesterone receptor (PR) are intracellular nuclear hormone receptors, which upon localization to the nucleus prompt transcriptional activity. Furthermore, the estrogen receptor participates in pathway crosstalk with known mitogenic pathways which have been associated with tumor progression and drug resistance, including transforming growth factor-Beta (TGF-β), human epidermal growth factor receptor 2 (HER-2/neu), and the insulin-like growth factor (IGF) receptors (Arpino et al., 2008; Band & Laiho, 2011; Fagan & Yee, 2008). In OSE, low doses of estrogen cause proliferation of ovarian surface epithelial cells *in vitro*, which involves the interleukin-6 (IL-6)/signal transducer and activator of transcription 3 (STAT-3) pathway (Syed et al., 2002). Others, however, have documented no effect at low doses of estrogen (Choi et al., 2001b; Karlan et al., 1995), or an inhibitory effect at high doses in OSE (Wright et al., 2005). *In vivo*, estrogen exposure causes rabbit OSE cell proliferation and an increase in the number of papillae but does not result in spontaneous development of tumors (Bai et al., 2000). In comparing normal OSE to ovarian carcinoma cell lines, Lau et al. (1999) found loss of ERα, PR, and androgen receptor (AR) mRNA in neoplastic cells compared to OSE, suggesting this loss may contribute to neoplastic transformation. Though the role of ERβ in Lau's work was uncertain, others assert that the ERβ receptor subtype promotes apoptosis in OSE (Bardin et al., 2004).

While estrogen has an unclear effect on cellular proliferation in OSE, progestins have a consistent effect of inhibiting cell growth and inflammation, and promoting apoptosis (Ivarsson et al., 2001; Karlan et al., 1995; Rae et al., 2004a). *In vivo*, macaques treated with progestin exhibited upregulation of apoptosis in OSE cells (Rodriguez et al., 1998). Though

the exact mechanism by which progesterone exhibits growth-inhibitory effects is not fully-understood, Syed and Ho (2003) demonstrated involvement of the caspase 8 Fas/FasL pathway in progesterone-mediated apoptosis in OSE.
Culturing OSE *in vitro* with the inflammatory mediator IL-1 results in upregulation of several inflammation-associated genes, specifically *HSD11B1*, which plays a role in conversion of cortisone to cortisol (Rae et al., 2004b). With well-documented anti-inflammatory effects, cortisol prohibits downstream inflammatory signaling and may therefore exhibit a protective effect on the OSE. Together with the anti-inflammatory and growth-inhibitory effects of progesterone, glucocorticoids and progestin may serve to protect the OSE from inflammatory damage resulting from ovulation (Rae & Hillier, 2005).

2.1.2 Gonadotropin signaling in normal ovarian surface epithelium

The gonadotropins FSH and LH are members of a glycoprotein hormone family which also include thyroid stimulating hormone (TSH) and human placental chorionic gonadotropin (hCG). Gonadotropin receptors belong to the G protein-coupled receptor family (GPCR), which harbor seven transmembrane domains and upon their activation convert guanosine diphosphate (GDP) to guanosine triphosphate (GTP). This subsequently results in downstream activation of the phosphatidylinositol-3-kinase (PI3K) pathway; a pathway whose involvement in oncogenesis has been well-characterized. The type I isoform of gonadotropin-releasing hormone (GnRH) and its receptor are found on OSE, and interestingly, GnRH analogs appear to inhibit growth of OSE and ovarian cancer cells *in vitro* (Kang et al., 2000). The GnRH type II isoform also has growth inhibitory effects *in vitro*. (Choi et al., 2001a). While the exact mechanism of growth inhibition is not fully-understood, it is discussed in more detail later in the chapter.
Synthesized in the anterior pituitary, the gonadotropins FSH and LH regulate the menstrual cycle: FSH stimulates follicular growth and the FSH receptor (FSHR) is expressed mainly in granulosa cells, while LH triggers ovulation and the LH receptor (LHR) is expressed mostly in theca but also by granulosa cells. FSHRs and LHRs are found in both OSE and ovarian cancer cells (Minegishi et al., 2000; Parrott et al., 2001). Leung and Choi (2007) characterized the signaling pathway of FSHR in preneoplastic immortalized OSE, and found that Extracellular-Related Signaling Kinase 1/2 (ERK 1/2), *c-myc*, and HER2/neu were upregulated, and cell growth was accelerated in response to FSHR overexpression. Epidermal growth factor receptor (EGFR) is also upregulated via the ERK 1/2 and PI3K/Akt pathways in immortalized OSE treated with gonadotropins (Choi et al., 2005). Others have similarly confirmed increased proliferation in OSE in response to FSH and LH administration (Choi et al., 2002; Syed et al., 2001). *In vitro,* treatment with FSH results in upregulation oncogenic genes and downregulation of tumor suppressor genes *RB1, BRCA1,* and *BS69* (Ji et al., 2004).

2.2 Steroid signaling in ovarian cancer

Steroid hormone signaling in ovarian cancer involves complex pathways which are not yet fully understood. Figure 1 depicts some of the established routes of signaling in EOC.

2.2.1 The role of estrogen in ovarian cancer

Although the influence of exogenous estrogen in OSE is ambiguous, it most certainly triggers proliferation and cellular growth in ovarian cancer cells (Choi et al., 2001b). This is

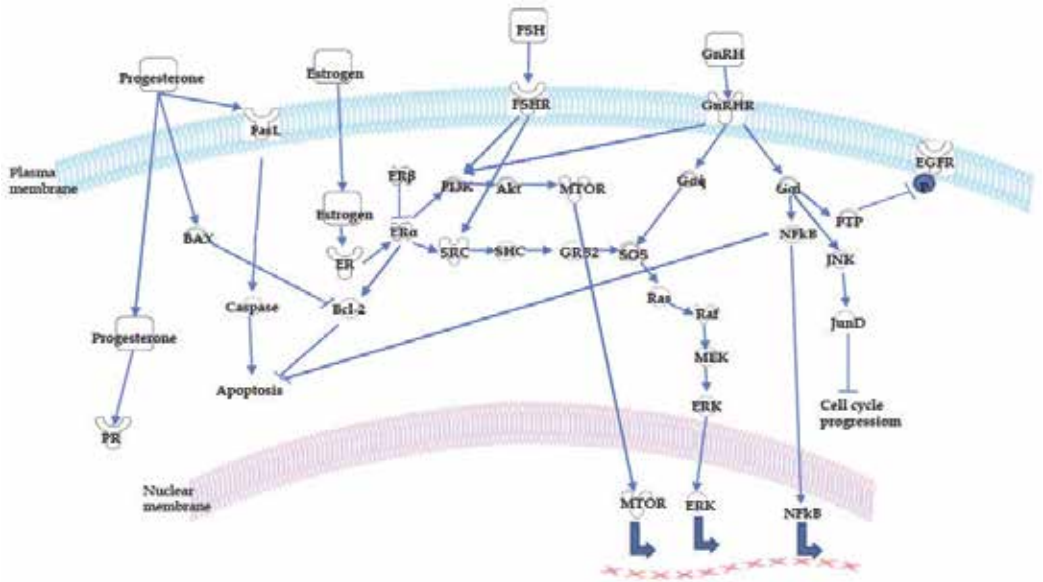

Fig. 1. Steroid hormone signaling in ovarian cancer.

accomplished in part through activation of the PI3K/Akt pathway and the transcription factor *c*-myc (Chien et al., 1994), and via estrogen receptor crosstalk with other pathways such as IGF-1, TGF-α, and EGFR (Simpson et al., 1998; Wimalasena et al., 1993). Estrogen also inhibits apoptosis via bcl-2, an anti-apoptotic protein (Choi et al., 2001b), and increases invasive capacity via upregulation of ezrin (Song et al., 2005), fibulin-1 (Galtier-Dereure et al., 1992), cathepsin D (Galtier-Dereure et al., 1992), and kallikreins (Yousef et al., 2003). In ovarian cancer cell lines treated with 17-B-estradiol, pro-angiogenic hypoxia-inducible factor-1 (HIF-1) expression was increased, the effect of which was abrogated by the Akt inhibitor snf and via Akt short-interfering RNA (siRNA) (Hua et al., 2009).

Both ER subtypes α and β are expressed in EOC. It has been suggested that ERα is mainly responsible for proliferation in the ovary, while ERβ modulates differentiation (Britt & Findlay, 2002). This is supported by the fact that ERβ is abundant in OSE and benign tumors, and ERα is mainly found in malignant ovarian tumors (Brandenberger et al., 1998; Hillier et al., 1998). An increased ERα: ERβ ratio has also been observed in ovarian cancer (Cunat et al., 2004), with an increase in ERα and inverse decrease in ERβ expression throughout tumor progression (Brandenberger et al., 1998). Overexpression of ERβ in an ovarian cancer cell line decreased proliferation by 50%.

In vivo studies support the role of 17-β-estradiol in accelerating tumor growth. Using a transgenic mouse model, Laviolette et al. (2010) showed that treatment of tumors with 17-β-estradiol resulted in the earlier onset of tumors, decreased survival time, and papillary histology, while treatment with progesterone resulted in no difference. Armaiz-Pena et al. (2009) similarly showed that treatment with 17-β-estradiol increased ovarian tumor growth, and inoculation with tumor cells during the proestrus when estrogen levels are high significantly increased tumor burden, compared to inoculation during the estrus phase. Furthermore, treatment with 17-β-estradiol resulted in increased vascular endothelial growth factor (VEGF), increased cell adhesion in ER positive cells, increased migratory potential, and mitogen-activated protein kinase (MAPK) upregulation.

Correspondingly, the estrogen antagonist tamoxifen abrogates the estrogen effect in epithelial ovarian cancer cell lines, and is surprisingly effective in both ER-positive and ER-negative and platinum-resistant ovarian carcinomas (Mabuchi et al., 2004; Markman et al., 1996). In ER-negative ovarian carcinomas, tamoxifen functions independently of estrogen via ERK, c-Jun N-terminal protein kinase (JNK), and p38 (Mabuchi et al., 2004).

2.2.2 The role of progesterone in ovarian cancer

Progesterone decreases cellular proliferation *in vitro* via multiple pathways. Blumenthal et al. (2003) demonstrated that progesterone activated the cyclin-dependent (CDK) pathway and promoted a more differentiated cell type. In the ovarian cancer cell line SKOV3, progesterone inhibited invasion and suppressed urokinase plasminogen activator (UPA), thereby decreasing the metastatic potential of cells (McDonnel & Murdoch, 2001). As in OSE cells, progesterone induces apoptosis via caspases and the FasL pathway (Syed & Ho, 2003), and enhances tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced cell death (Syed et al., 2007). Recent evidence supports that progesterone also causes apoptosis by upregulating the proapoptotic gene expression of p53 and BAX, and decreasing antiapoptotic gene expression of BCL-2 in ovarian cancer cells, thereby preventing oxidative damage (Nguyen & Syed, 2010).

In vivo, treatment of athymic mice with progesterone suppressed tumorigenesis after inoculation with the ovarian cancer cell line SKOV3 and increased survival (McDonnel et al., 2005). Furthermore, progesterone treatment in athymic nude mice inoculated with platinum-resistant SKOV3 enhanced platinum activity and sensitivity (Murdoch et al., 2008). However, the actions of progesterone in ovarian carcinomas is complex, and clinical trials utilizing progesterone have not proved as promising as *in vivo* and *in vitro* observations. In part, this may be due to the action of progesterone receptor membrane component 1 (PGRMC1), which recently *in vitro* was found to promote ovarian tumor cell proliferation, while its depletion slows cellular growth (Peluso et al., 2008). *In vivo*, PGRMC1-depleted mice had fewer and smaller tumors, again supporting the pro-oncogenic nature of PGRMC1 (Peluso et al., 2009).

As is the case with estrogen receptors, different progesterone receptor isoforms exist, including isoforms A and B. Akahira et al. examined the expression of PRA and PRB in the normal ovary, and in benign, borderline, and malignant ovarian tumors (2002). Using immunohistochemistry and RT-PCR in normal and malignant tissue and cell lines, they found that PRA receptor expression declined ($p<0.05$) during the transition from benign to borderline to malignant tissues, whereas there was no significant difference in PRB expression. They suggest that downregulation of PRA is associated with the development of ovarian cancer.

2.2.3 The role of gonadotropins in ovarian cancer

An alternative theory to the incessant ovulation hypothesis is that excessive gonadotropin stimulation leads to the development of ovarian cancer. Early animal models simulated hypogonadism, which prevented development of ovarian tumors in mice (Marchant, 1961). Oral contraceptives, which are proven to reduce the risk of EOC, also provide negative feedback on the gonadotropin axis and reduce circulating gonadotropins, which further supports this hypothesis. Additionally, the peak incidence of ovarian cancer occurs postmenopausally, with a temporal relationship to an increase in circulating gonadotropins (Choi et al., 2007).

Similar to estrogen, FSH promotes ovarian cancer cell growth *in vitro*. One way this occurs is through inhibition of apoptosis through activation of survivin via the PI3K/Akt pathway, and downregulation of the programmed cell death gene 6 (PCDG6) and death receptor 5 (DR5) (Huang et al., 2010). Promotion of cell growth through FSH also occurs through activation of the MAPK, PI3K/Akt pathways (Choi et al., 2002). Overexpression of the FSH receptor promotes well-established oncogenic signaling pathways including upregulation of EGFR (Choi et al., 2004), and angiogenesis promotion via VEGF upregulation (Schiffenbauer et al., 1997; Wang et al., 2002). In ovarian cancer cell lines, both FSH and LH exploit the PI3K/Akt pathway to upregulate cyxlooxygenase (COX)-1 and COX-2, resulting in increased cell motility and invasion (Lau et al., 2010). The FSH receptor (FSHR) is also prominent in ovarian carcinomas—Ji et al reported higher levels of the FSHR in cancerous ovarian tissue compared to normal controls (2004). The role of LH is controversial; in one study, LHR mRNA expression decreased in the transition from benign to malignant ovarian lesions (Lu et al., 2000). LH also appears to abrogate the proliferative effects of FSH (Zheng et al., 2000), but also has been reported to stimulate growth of OSE and contribute to tumor progression (Tashiro et al., 2003).

In vivo, treatment of rats with gonadotropins increased OSE proliferation (Stewart et al., 2004); similarly, mice treated with gonadotropins experienced increased ovarian cellular proliferation and decreased apoptosis (Burdette et al., 2006). Mice displaying hypergonadotropism from overexpression of FSH develop multicystic hemorrhagic ovaries, while overexpression of LH resulted in the formation of granulosa cell tumors (Kumar et al., 1999; Risma et al., 1995). However, it is important to note that these tumors formed in stromal or granulosa cells—not epithelial cells—and to date, no evidence exists establishing that gonadotropins can initiate malignant transformation of the ovarian epithelium.

2.2.4 The role of androgens in ovarian cancer

The role of androgens in promoting growth, tumorigenesis, and tumor progression in prostate cancer is well-established. In ovarian cancer cell lines, androgens 5α-dihydrotestosterone (DHT) and testosterone also cause cell proliferation which is mediated by upregulation of interleukin-6 (IL-6) (Syed et al., 2001; Syed et al., 2002). Levine and Boyd (2001) found that androgen receptor allele length influenced age at diagnosis with ovarian cancer; those patients with a shorter AR allele length were diagnosed 7 years earlier than those with normal allele length. *In vitro*, androgens decrease TGF-β receptors levels, which provides a mechanism for ovarian cancer cells to evade the growth inhibitory effects of TGF-β (Evangelou et al., 2000). Shi et al. (2011) examined the role of DHT, testosterone, and dehydroepiandrosterone (DHEA) in breast and ovarian cancer cell lines, and found that DHT but not the other androgens induced the degradation of the tumor suppressor p27 in both cell lines. Furthermore, in ovarian carcinoma cell lines, testosterone and androstenedione were found to increase cell viability, and induce telomerase activity which was blocked by PI3K inhibitors (Nourbakhsh et al., 2010). Sheach et al. (2009) found that the ovarian cancer cell lines OVCAR3 and OSEC2 expressed the androgen receptor, and treatment with androgen upregulated 121 genes, including G-protein-related genes Rab25 and Rab35. Level of gene expression correlated with tumor grade as well.

2.2.5 The role of GnRH in ovarian cancer

Two of twelve vertebrate GnRH isoforms exist in humans: GnRHI and GnRHII. GnRHI and its receptor are present in 80% of biopsies of ovarian carcinomas (Emons et al., 1989). While

GnRH functions in a paracrine manner systemically, there is evidence that GnRH functions in an autocrine manner in epithelial ovarian cancer, with GnRHI and GnRHII mRNA identified in both ovarian carcinoma and OSE (Choi et al., 2001a; Kang et al., 2000). Although the traditional gonadotropin GnRH signaling pathway involves $G_{\alpha q}$, protein kinase C, and MAP kinases, the autocrine mechanism of GnRH-related signaling in ovarian carcinomas differs (So et al., 2008). Interestingly, it appears that GnRH receptors in ovarian malignancy function via the pertussis toxin-sensitive protein $G_{\alpha i}$, and activate protein phosphatase (Imai et al., 2006). This alternate pathway is responsible for the antiproliferative effects of GnRH in ovarian carcinomas; however, the antiapoptotic function of GnRHI remains controversial. GnRHI and its agonists exert an inhibitory effect in ovarian cancer *in vitro* and *in vivo,* reducing cell growth and inducing cell cycle arrest in G_0/G_1 (Kim et al., 1999). However, GnRH agonists are in fact protective *against* apoptosis in ovarian cancer cell lines treated with doxorubicin (Sugiyama et al., 2005). Somewhat paradoxically, there is also emerging evidence that GnRH promotes tumor cell migration, metastasis, and invasion. *In vitro,* GnRHII enhances Akt pathway activation, and increased nuclear beta-catenin accumulation. This was reversed with siRNA targeting the GnRH receptor, and with treatment of cells with a PI3K/Akt inhibitor. GnRH treatment also increases type I matrix metalloproteinase (MT-1MMP) levels, thereby increasing cells' invasiveness and metastatic potential which increases cell migration and invasion during tumor progression through activation of Rho GTPases and accumulation of p120 catenin, with reversible effects upon inhibition of p120 (Cheung et al., 2010). *In vivo,* mice treated with a GnRH agonist displayed increased tumor weight (Romero et al., 2009). Thus, while GnRH and its agonists work via $G_{\alpha i}$ to decrease cellular growth and proliferation, it also enhances other pathways which promote cellular migration and invasion, essentially increasing metastatic potential.

3. Endogenous steroid hormones and ovarian cancer

3.1 Endogenous hormones

Population-based studies have supported the *in vitro* findings that progestins harbor protective effects against ovarian cancer. Conditions in which excess progesterone is present decrease cumulative risk of developing EOC, including multiple gestations, multiparity with an additional 10% decrease in risk with each birth, and breastfeeding (Lambe et al., 1999; Risch et al., 1994). While these findings support the incessant ovulation hypothesis by sparing the epithelium from monthly ovulations and repetitive damage, there is also evidence that progesterone independently increases the magnitude of benefit by promoting cellular apoptosis (Rodriguez et al., 1998).

3.2 Hormone receptors

Much effort has been directed at correlating hormone receptor status and outcomes in ovarian cancer. Geisler et al. (1996) examined estrogen and progesterone receptor status as prognostic indicators in patients with optimally cytoreduced stage IIIC serous carcinoma of ovary. Out of 96 patients, those with an estrogen receptor level <10 fmol/mg had better mean survival (41 vs 34 mos) than patients with higher levels of ER, whereas there was no correlation between PR status and survival. Others have found opposing results: One study examined ER mRNA expression in 35 stage III-IV ovarian carcinoma patients receiving neoadjuvant chemotherapy, and in multivariate analysis found that elevated baseline ER mRNA levels predicted prolonged progression-free survival ($p=0.041$) and overall survival

(p=0.01), independently of pathological grade and age (Zamagni et al., 2009). Another group examined hormone receptor status in older and younger patients with advanced papillary serous ovarian carcinoma (Liu et al., 2009). They reported a higher percentage of ER-positivity and PR-negativity in older patients, while both groups were largely Her2/neu receptor-negative. Although there was no significant association between receptor status and survival, for the younger cohort, those who expressed both ER and PR receptor types had better overall survival (OS) (p=0.056) at 51 months, compared to 35 months in those not expressing both receptors. In a large cohort of Danish patients with ovarian cancer, Hogdall et al (2007) examined the prognostic value of ER and PR status via immunohistochemistry and microarray analysis in 582 women with EOC and 191 patients with serous borderline ovarian tumors. They noted that ER positivity increased with stage (p=0.0003), PR positivity increased with increasing grade (p=0.0006), and tissue ER and/or PR expression greater than 10% pointed to a more favorable prognostic outcome.

Estrogen receptor subtypes have also received attention in predicting ovarian cancer outcome. Halon et al. (2011b) examined ERβ via immunohisto- and immunocytochemistry in 43 patients pre-chemotherapy and 30 patients post-chemotherapy with stage III ovarian cancer. They determined that patients with higher initial ERβ expression (>30% of cells) enjoyed longer OS and progression-free survival (PFS) (p=0.0016, p=0.032, respectively). Similarly, Halon et al. (2011a) looked at ERα expression in the same group of patients and found that loss of ERα expression predicted significantly shorter OS and PFS.

Another group (Yue et al., 2010) also examined steroid receptors as possible prognostic markers in EOC. They described the steroid and xenobiotic receptor (SXR, also known as the pregnane X receptor), which regulates gene transcription and triggers proliferation of ovarian cancer cells *in vitro* and *in vivo* and induces drug resistance (Gupta et al., 2008). In 141 cases of EOC, SXR immunostaining was correlated with older patient age, clear cell histology, higher grade, and ER- and PR-positive tissues. SXR expression correlated with a higher likelihood of recurrence, and worse disease-free and overall survival. However, ER and PR status were not associated with disease-free survival (DFS) or OS.

3.3 Circulating hormones

In general, there is no documented consistent association between circulating hormone levels and the risk of developing EOC. Studies have addressed serum FSH and LH, and have not found any relationship between serum levels and ovarian cancer incidence (Akhmedkhanov et al., 2001; Arslan et al., 2003). Although there is no known association between the development of EOC and LH levels, high LH levels have been observed in BRCA1 mutation carriers compared to controls without BRCA1 mutations, which may comprise part of the BRCA1 phenotype (Jernstrom et al., 2005). While serum circulating levels of gonadotropins are nonpredictive, high levels of gonadotropins in ovarian cysts and peritoneal fluid are associated with malignancy. In comparing patients with and without malignant ovarian neoplasms, there was no difference in serum levels of LH or FSH, but ovarian cyst fluid levels of LH and FSH were elevated in serous ovarian carcinomas compared to benign tumors (Kramer et al., 1998). Similarly, peritoneal fluid aspirates contained elevated levels of FSH and LH in ovarian carcinoma versus benign tumors (Chudecka-Glaz et al., 2004; Halperin et al., 2003). One recent study actually found that in 67 patients with ovarian cancer versus controls, increased circulating levels of FSH were correlated with a reduced prediagnostic risk of EOC, which argues against the excess gonadotropin hypothesis in ovarian carcinogenesis (McSorley et al., 2009).

In a large European study, 192 ovarian cancer cases and 346 matched controls were compared for serum levels of testosterone, androstenedione, dehydroepiandrostenedione, and sex hormone binding globulin (SHBG) (Rinaldi et al., 2007). Free testosterone levels were inversely related to risk of EOC (p=0.01) in postmenopausal women, whereas free testosterone was positively associated with EOC risk in women under age 55, though this was not statistically significant. Other circulating androgens were not associated with risk.
In a nested case-control study of 31 cases of ovarian cancer and 63 controls, FSH and LH were measured (Helzlsouer et al., 1995). Mean FSH was lower in the cases (p=0.04) compared to controls, and LH was also lower but the difference was not statistically significant. The risk of ovarian cancer increased with higher androstenedione levels (p=0.03) and higher DHEA levels (p=0.02). Lukanova et al. (2003) followed by evaluating pre-diagnostic levels of serum testosterone, DHEA-sulfate, estrone, and SHBG in a case-control study nested within three cohorts, including 132 patients with primary EOC matched with 2 controls per case. There was no apparent association between any of the five hormone levels and ovarian carcinoma risk. Increased levels of circulating androstenedione in premenopausal patients did increase overall risk, but the low number of subjects in that subgroup precluded a definitive association.
In nonepithelial ovarian cancer (NEOC), circulating levels of steroid hormones may provide information about risk of NEOC development (Chen et al., 2010). In a case-control study within the Finnish Maternity Cohort, serum specimens were obtained from women with a singleton pregnancy that preceded their diagnosis of NEOC: 41 women had sex cord stromal tumors (SCST), and 21 had germ cell tumors (GCT). Doubling of testosterone, androstenedione, and 17-hydroxyprogesterone were associated with a two-fold increased risk of SCST compared to matched controls, a trend which remained after exclusion of women with a 2-, 4-, or 6-year time lag between blood donation and SCST diagnosis.

4. Exogenous hormones and ovarian cancer

4.1 Infertility treatment and risk of ovarian cancer

The relationship between infertility and steroid hormones is a controversial one. Many initially speculated that exogenous treatment with FSH and LH in infertility patients would lead to subsequent epithelial proliferation and tumorigenesis. In a case-control study, Whittemore et al. (1992) concluded that infertility patients who used infertility medications were at an increased risk of EOC compared to infertile women not using infertility medications. Since that time, however, other studies have emerged refuting that theory, and in fact it seems that infertility itself rather than gonadotropin use is an inherent risk for ovarian cancer (Ness et al., 2000; Tworoger et al., 2007). Brinton et al. reported on the relationship between ovarian cancer and infertility in a retrospective cohort study of 12,193 subjects (2004a). There were 45 identified cases of ovarian carcinoma, and infertility patients had a significantly elevated risk when compared to the general population, with a higher rate for primary versus secondary infertility, and the highest rate for those patients with endometriosis (Relative Risk or Hazard Ratio: RR=2.72 for patients with primary infertility and endometriosis). Examining the same patient cohort, they also reported on the risk of ovarian cancer with ovulation-stimulating drugs (Brinton et al., 2004b). They concluded a "slight but insignificant elevation in risk associated with drug usage among certain subgroups," including those using clomiphene citrate, which warrants continued surveillance.

4.2 Hormone replacement therapy

The topic of hormone replacement therapy (HRT) use and ovarian cancer risk has sparked much debate and controversy. Several large cohort studies deserve mention.

In a large study as part of the Cancer Prevention Study II Nutrition Cohort, a group of 54,436 postmenopausal women were followed starting in 1992 (Hildebrand et al., 2010). Over 15 years of follow-up, 297 incident cases of EOC were identified. Relative to never users, estrogen-only HRT was associated with a twofold higher relative risk (RR=2.07) of ovarian cancer, each 5-year increment of use was associated with a 25% higher risk, and greater than or equal to 20 years with a threefold higher risk (RR=2.89). Neither current nor former combination estrogen and progesterone use was associated with an increased ovarian cancer risk.

The UK Million Women Study enrolled 948,576 women and followed them for an average of 5.3 years for incident ovarian cancer (Beral et al., 2007). They identified 2273 cases of ovarian cancer, with 1591 associated deaths, and reported that current users of HRT were significantly more likely to develop (RR=1.2, p=0.0002) and die (RR=1.23, p=0.0006) from EOC versus never users. As in other studies, risk increased with duration of use, but interestingly did not differ by type of HRT used, and past users were not at an increased risk.

In a large prospective study, Rodriguez et al. (2001) examined the effects of postmenopausal estrogen use and risk of ovarian cancer. Of 211,581 women prospectively enrolled in the American Cancer Society's Prevention Study II, 944 ovarian cancer deaths were recorded over 14 years. Ovarian cancer mortality in women using estrogen replacement therapy (ERT) was higher than never users (RR=1.51), and risk was slightly but not significantly increased in former ERT users (RR=1.16). They noted that duration of use was correlated to risk, baseline current use for ≥10 years resulted in an RR of 2.20, versus former use for ≥10 years which resulted in a decreased but still elevated relative risk of 1.59. Risk did, however, decrease with increasing time since last use. The increased risk of ovarian cancer mortality persisted for up to 29 years.

Another large prospective cohort involved 329 women who developed ovarian cancer from a pool of 44,241 women in the Breast Cancer Detection Demonstration Project (Lacey et al., 2002). After adjustment for age and OC use, ever use of ERT was the only form of HRT associated with ovarian cancer (RR=1.6), in a dose dependent manner with RR for 10-19 years at 1.8 and more than 20 years at 3.2 ($p<0.001$). The addition of progesterone in an estrogen-progesterone regimen after estrogen-only use was still associated with an elevated risk (RR=1.5), but was not significant for estrogen-progesterone use only (RR=1.1). Women who used estrogen-only therapy for greater than 10 years were at a significantly increased risk of ovarian cancer, whereas estrogen-progestin only users were not at an increased risk.

Data from the Women's Health Initiative also merits mention (Anderson et al., 2003). This was a randomized, double-blind, placebo-controlled trial with 16,608 postmenopausal women given a daily tablet of estrogen-progesterone versus placebo. They identified 32 cases of ovarian cancer, and the hazard ratio for HRT use was 1.58, suggesting that continuous combination therapy may increase EOC risk.

In the clear cell and endometrioid non-serous subtypes of epithelial ovarian cancer, endometriosis is a known precursor lesion to the development of these malignancies. Correspondingly, there have been reports that HRT use is more strongly associated with clear cell and endometrioid histologic subtypes than serous EOC (Riman, 2003; Risch, 2002).

In light of these large cohort studies and the lack of definitive molecular evidence that steroid hormones initiate carcinogenesis, it is most likely that exogenous hormones hasten and perhaps fuel pre-existing lesions. As Risch points out, given the long latency between HRT use and development of these neoplasms, it is unlikely that these agents cause malignant transformation (2002).

4.3 Oral contraceptives and BRCA carriers

Whereas hormone replacement therapy may increase risk of ovarian carcinoma, oral contraceptive use diminishes the risk of EOC. Use over five years decreases risk by up to 50% lasting 10 to 15 years (Gwinn, 1985; Gwinn et al., 1990). While the exact protective mechanism is unknown, it is hypothesized that the apoptotic effects of progesterone are responsible for this effect. In a case-control study of 546 women with ovarian cancer and 4228 controls, women who had used OCs had a 40% reduction in risk compared to those who had never used them, with a protective effect lasting for 15 years (The Centers for Disease Control, 1987). BRCA mutation carriers harbor an especially high lifetime risk of development of EOC, ranging from 40 to 45% in BRCA1 carriers and 15-20% amongst BRCA2 mutation carriers (Narod et al., 2002). Studies support the use of OCs in high-risk women. Narod et al. particularly addressed this subgroup, and conducted a cohort study on 207 women with hereditary ovarian cancer, with 161 of their sisters serving as controls (1998). Lifetime history of oral contraceptive use was obtained, and the investigators found a decreased odds ratio of 0.5 for ovarian cancer associated with oral contraceptive use, with adjustment for age and parity. As in prior studies, the risk decreased with increasing duration of use, with a 60% reduction in risk with use for 6 or more years. In specifically examining BRCA mutation status, OR for OC use and development of EOC for BRCA1 carriers was 0.5, and was 0.4 for BRCA2 mutation carriers. A subsequent study in 1311 matched pairs of women with BRCA1 and BRCA2 mutations, however, revealed a modest increase in breast cancers in BRCA1 mutation carriers (OR=1.2) compared to controls, whereas there was no increased risk for BRCA2 mutation carriers (OR=0.94) (Narod et al., 2002). While oral contraceptives undoubtedly provide benefit in ovarian cancer prevention and are recommended in young BRCA mutation carriers, there is a minimal increased risk of breast cancer which must be weighed against the benefits.

5. Clinical trials with hormonally-targeted therapeutics in ovarian cancer

Several clinical trials in ovarian cancer have examined the utility of hormonal therapy in ovarian carcinoma, including antiestrogens (tamoxifen, fulvestrant), aromatase inhibitors (letrozole), progestins and progesterone receptor antagonists (medroxyprogesterone acetate, megestrol acetate, mifepristone), GnRH agonists (leuprolide acetate, triptorelin, goserelin), and antiandrogens (flutamide). Virtually all the trials examine these agents in the recurrent setting or in combination with other therapies; rarely are they studied as a component of up-front therapy. Given the overall low response rate to chemotherapy in recurrent ovarian cancer, most hormonal agents have a modest effect, with tamoxifen used the most frequently. Hormone-directed therapy is particularly popular in the setting of "chemical recurrence," in other words a rising CA-125 without clinical or radiographic evidence of disease. In this situation, clinicians are reluctant to initiate cytotoxic chemotherapy as early initiation does not prolong OS (Rustin et al., 2010), while patients often experience anxiety waiting for full return of their disease. With much more acceptable side effects than cytotoxic chemotherapy,

hormonal therapeutics offer some activity and are generally well-tolerated. Not surprisingly, levels of endocrine receptor expression correlate positively with response; however, even patients without high receptor expression may enjoy clinical benefit.

5.1 Estrogen antagonists

5.1.1 Selective estrogen receptor modulators

Tamoxifen is a selective estrogen receptor modulator (SERM) with mixed estrogen agonist and antagonist activity. In the endometrium, it acts in an agonistic manner and has been associated with endometrial carcinoma (Bland et al., 2009); in the breast it is an antagonist. Use in ovarian carcinoma is generally restricted to the setting of recurrence or maintenance, with modest effects. Nonetheless, it is an attractive option for patients with biochemical recurrence and a rising CA-125 in the absence of obvious radiologic disease or symptoms. In this situation, early treatment offers no advantage and unnecessary toxicity, yet watchful waiting often causes patient anxiety. In this regard, tamoxifen may offer some activity while maintaining a favorable side effect profile.

Markman et al. explored this particular patient group in a retrospective review of patients with recurrent small-volume disease who received tamoxifen prior to initiation of cytotoxic chemotherapy (2004). Of 56 patients, the median duration of treatment was 3 months, but 42% of patients remained on tamoxifen for over 6 months, and 19% were still on tamoxifen at 9 months. They concluded that while tamoxifen is a reasonable treatment option, it is unknown whether the delay in chemotherapy resulted from the tamoxifen itself or the natural history of the patients' disease.

In a prospective trial, the Gynecologic Oncology Group (GOG) examined 105 patients with stage III or IV epithelial ovarian cancer whose disease recurred or persisted after surgery and were treated with tamoxifen 20 mg twice daily (Hatch et al., 1991). They reported an 18% response rate: 10% of patients had a complete response (CR) with a median duration of 7.4 months, while 8% experienced a partial response (PR), and 38% of patients had short-term disease stabilization. Median duration for PR or stable disease (SD) was 3 months. Of those with a complete response, 89% had elevated ER levels, versus 59% in SD or PR groups. Markman et al. (1996) followed with an ancillary report on the group, examining those patients with platinum-refractory disease. They reported an objective response rate of 13% in patients with platinum-resistant ovarian cancer, and a median duration of response of 4.4 months. The Mid-Atlantic Oncology Program (MAOP) conducted three separate phase II trials in patients with refractory ovarian carcinoma, and treated patients with either high-dose megestrol acetate, high-dose tamoxifen, or aminoglutethimide (Ahlgren et al., 1993). Of 30 patients who received high-dose megestrol acetate (800 mg/day for 30 days, then 400 mg/day thereafter), there were no identified responses. Among 29 patients treated with tamoxifen (80 mg /day for 30 days, then 40 mg/day thereafter), 17% responded, and two of those responses exceeded five years. Finally, aminoglutethimide was administered at a dose of 1g/day to 15 patients, and no responses were observed.

A more recent trial explored the role of tamoxifen versus thalidomide and its effects on VEGF expression in a randomized phase III trial in 138 women with stage III or IV EOC, primary peritoneal cancer, or fallopian tube carcinoma who were disease-free following first line chemotherapy and experienced a "biochemical" recurrence only as defined by rising CA-125 (Hurteau et al., 2010). Thalidomide was not superior to tamoxifen on interim analysis, with a similar risk of progression, higher toxicity, and an increased risk of death, and the trial was closed. VEGF expression was also not a prognostic factor in determining response.

Tamoxifen has also been explored in combination with cytotoxic agents. In a phase II trial, 50 patients with recurrent or progressive ovarian cancer after platinum-based chemotherapy received either 100 mg/m^2 cisplatin or 400 mg/m^2 carboplatin q3 weeks with tamoxifen 80 mg/day for 30 days followed by 40 mg/day thereafter (Benedetti Panici et al., 2001). Overall response rate was 50% with a 30% CR and 20% PR, with a higher response rate (64%) in the platinum-sensitive group compared to platinum-resistant cases (39%). Toxicity included nausea and vomiting, neuropathy, nephrotoxicity, and bone marrow suppression. While encouraging, it is difficult to interpret the effects of tamoxifen without a comparison group of platinum-alone, or platinum plus paclitaxel as is often used for recurrent platinum-sensitive disease.

In combination with the EGFR inhibitor gefitinib, tamoxifen was administered to 56 patients with platinum- and taxane-refractory EOC, peritoneal cancer, or fallopian tube cancer (Wagner et al., 2007). Sixteen patients had stable disease, although there were no tumor responses and in 10% the medications were discontinued secondary to adverse events, the most common of which were rash and diarrhea. Median time to progression was 58 days, with a median survival of 253 days. The investigators concluded that the drug combination was not efficacious.

5.1.2 Aromatase inhibitors

Letrozole is a non-steroidal aromatase inhibitor. It competitively and reversibly binds to aromatase and thereby prevents conversion of androgens to estrogen. In the setting of relapsed ovarian cancer, aromatase inhibitors can achieve a response in 35.7% of patients and stable disease in 20-42% of patients (Li et al., 2008). In a phase II setting, Smyth et al. (2007) investigated letrozole 2.5 mg orally daily in previously-treated patients with ER+ ovarian carcinoma. Of 42 patients, 17% had CA-125 response (defined as a decrease >50%), and 26% had not progressed (defined as doubling of CA-125). In terms of radiologic response, 9% of patients had a partial response, and 42% had stable disease at 12 weeks. Progression free survival of greater than 6 months was observed in 26% of patients. Response correlated to level of ER expression as defined by immunohistochemistry.

In another phase II trial examining patients with recurrent ovarian cancer, 50 patients received letrozole 2.5 mg daily (Bowman et al., 2002). Primary tumors were assessed for ER, PR, EGFR, erbB2, and HSP27 expression via immunohistochemistry. Though no PR or CR was observed, 10 patients experienced stable disease for at least 12 weeks. Those with stable disease exhibited significantly higher ER and PR levels, implying that endocrine receptor expression may help identify those patients most likely to benefit from treatment.

Walker et al (2007) aimed to explore estrogen-related gene expression and its predictive value in patient response to letrozole. Protein expression was measured via immunohistochemistry in tissue sections of tumors from patients treated with letrozole, and eight genes were significantly differentially expressed amongst patients who responded or had disease stabilization versus those who progressed. They concluded that these results might help identify those patients who would benefit most from endocrine therapy.

In 2010, Pan and Kao (2010) published a case report of two patients with endometrioid type histology. Both patients with advanced ER+ endometrioid ovarian carcinoma were treated with letrozole. The first patient had undergone optimal debulking, followed by completion of carboplatin and paclitaxel, had residual disease on second look surgery, and was subsequently disease-free for 30 months with letrozole treatment. The second patient was on her third recurrence and also experienced a 30 month remission with letrozole.

5.1.3 Estrogen receptor antagonists

Fulvestrant is a pure estrogen receptor antagonist without agonistic effects on other tissues. It competitively binds the ER, blocking estrogen binding and causing degradation and internalization of the estrogen receptor. In a phase II trial of fulvestrant in 26 women with ER positive recurrent ovarian or primary peritoneal carcinoma, patients received 500 mg IM on day 1, 250 mg IM on day 15, and 250 mg IM on day 29 and every 28 days thereafter (Argenta et al., 2009). The group had been heavily pretreated, with a median of 5 chemotherapy regimens prior to enrollment. Half of women experienced disease stabilization, though there was only one complete response and one partial response. Median time to progression was 62 days, and the regimen was well tolerated.

5.2 Progesterone

Given its promising apoptotic effects *in vitro* and *in vivo*, clinical trials with progestins have been disappointing. Several trials have used megestrol acetate or medroxyprogesterone acetate in the recurrent setting. Zheng et al. provide a nice summary of 13 trials, with 432 patients total (2007). Complete response was observed in 10 patients (2.3%), with a partial response in 4.9% of patients and stable disease in 47 or 10.9% of patients. They note that of the ten patients, 6 of them were reported in one study which also noted an overall 45% response rate (Geisler, 1985). A higher dose regimen does not appear to provide any additional benefit: In a phase II trial of 800 mg/day for 1 month followed by 400 mg/day thereafter, patients did not experience an overall increased benefit from higher doses, with an overall response rate of 10% (Veenhof et al., 1994) and 3 thromboembolic events.

Interestingly, combination therapy with medroxyprogesterone acetate and ethinyl estradiol yielded a partial response rate of 17% and stable disease in 24% of 25 patients with recurrent EOC, all patients had ER+ tumors (Fromm et al., 1991). Surprisingly, combination of progestins with tamoxifen does not appear to provide any clinical benefit (Jakobsen et al., 1987); nor does combination with chemotherapy. Based on *in vitro* evidence that megestrol acetate may reverse P-glycoprotein-mediated drug resistance, a phase I trial investigating the combination of megestrol acetate and paclitaxel was initiated (Markman et al., 2000). In 44 patients with paclitaxel-resistant EOC, four patients exhibited a response. However, 32% of patients experienced peripheral neuropathy, four patients developed venous blood clots, and one patient suffered from a stroke. Given the relatively low level of activity and significant toxicity, the authors did not recommend further study of the treatment regimen.

Mifepristone is a progesterone antagonist, more commonly known for its abortifacent properties. Rocereto et al. (2000) conducted a phase II study of mifepristone in the treatment of recurrent of persistent EOC, fallopian tube, or primary peritoneal cancer. Patients with persistent or recurrent disease less than 1 year after chemotherapy were eligible, and received mifepristone 200 mg daily for 28 day cycles. Of 44 patients enrolled, 34 were evaluable and 9 (26.5%) of patients experienced a response (9% CR, 17.5% PR). Duration of response was 1 to 3 months, except in one patient who continues to respond after three years. The major cited toxicity was rash.

5.3 GnRH agonists

GnRH agonists, principally leuprolide acetate, triptorelin, and goserelin, have been investigated in recurrent ovarian cancer. Emons & Schulz reported on 245 published phase II trials in patients with recurrent disease, mostly platinum-refractory, treated with GnRH

agonists: 9% had an objective remission and 26% experienced disease stabilization (2000). Compared to estrogen-directed endocrine therapy, responses are minimal and GnRH agonist therapy is generally not used in this setting anymore.

Initial studies with GnRH agonists were small, with 5 to 37 patients, and employed leuprolide acetate in the setting of recurrent EOC. Of 161 combined patients in 7 studies, there were 2 reported CRs, 15 PRs, and a disease stabilization rate of about 24% (So et al., 2008).

Triptorelin was the next generation GnRH agonist examined in EOC, and results were generally disappointing. In a large European Organisation for Research and Treatment of Cancer (EORTC) study, 74 patients with progressive ovarian cancer were treated with the LHRH agonist triptorelin via IM injections of 3.75 mg (on days 1, 8, and 28 followed by monthly injections thereafter) (Duffaud et al., 2001). There were no objective responses, and only 16% of patients experienced stable disease with a median PFS of 5 months for SD. Though the treatment was well-tolerated, the authors concluded that triptorelin exhibits only modest efficacy in this patient cohort. Emons et al. also conducted a large study, with the benefit of a prospective randomized double blind trial design (1996) in 135 patients with Stage III or IV EOC after cytoreduction. Patients received standard platinum-based chemotherapy and were randomized to placebo or triptorelin 3.75 mg IM. There was no difference in survival between the two groups.

Following triptorelin, cetrorelix—a GnRH antagonist—was found *in vitro* to have better activity in ovarian carcinoma, and was hypothesized to act directly on the GnRH receptors in the tumor as well as centrally (Yano et al., 1994). A phase II study utilized cetrorelix 10 mg subcutaneously daily in 17 patients with platinum-resistant recurrent EOC or mullerian carcinoma. Three patients had a PR of 9, 16, and 17 weeks, there was one grade 4 anaphylactic reaction, and two patients exhibited a 20% increase in cholesterol not requiring treatment. Stable disease was observed in 35% of patients for up to 62 months. They also examined LHRH receptor status, and two of the three responding patients were LHRH positive.

Goserelin, another GnRH agonist, was evaluated in combination with tamoxifen in a phase II trial for patients with recurrent advanced ovarian cancer (Hasan et al., 2005). Patients had received a median of 3 prior chemotherapy regimens, and 17 of 26 patients had platinum-resistant disease. Tamoxifen was prescribed at 20 mg orally daily, and goserelin was provided subcutaneously at 3.6 mg once monthly. The overall response rate was 50%, with one CR, 2 PRs, and 10 with SD. Median PFI was 4 months, while median OS was 13 months. The regimen was well-tolerated. Zidan et al. (2002) treated 15 patients with advanced recurrent disease with 3.6 mg of goserelin monthly; two of these patients had not received initial chemotherapy due to poor performance status. They reported one CR lasting 8 months, one PR lasting 14 months, and disease stabilization in 20% for a median of 7.5 months, and there was no significant toxicity. Most recently, a 2007 study considered the activity of goserelin 3.6 mg subcutaneously monthly and bicalutamide (an oral anti-androgen often used in prostate cancer) 50 mg orally daily in patients in their second or greater complete disease remission (Levine et al.). Of 35 patients, PFS for second disease remission was 11.4 months, and was 11.9 months for patients in their third or fourth disease remission. There was no association between androgen receptor expression and PFS. Toxicities included liver function abnormalities, fatigue, and hot flushes. They concluded that the combination did not prolong PFS in patients with second or greater disease remission.

Emons et al. recently reported on a novel compound, AEZS-108, which is composed of [D-Lys]LHRH linked to doxorubicin (2010). In a phase I dose escalation trial of 17 women with metastatic unresectable EOC, endometrial, or breast cancer and immunohistochemical LHRH positivity, a total of 6 patients exhibited responses, both in the highest dose group. Dose-limiting toxicities included leukopenia and neutropenia.

5.4 Antiandrogens in ovarian cancer

Few studies exist assessing the efficacy of antiandrogenic therapy in ovarian cancer. Flutamide, a nonsteroidal antiandrogen, has been studied in the phase II setting (Vassilomanolakis et al., 1997). In 24 patients with relapsed stage III or IV ovarian cancer, flutamide was given at a dose of 100 mg three times daily. There was one partial response lasting 3 months, and two patients with stable disease for 7 and 8 months. Reported toxicity was mild. Another trial utilized flutamide in 68 pretreated patients with EOC (median prior chemotherapy of two regimens), dosed at 750 mg/daily for at least 2 months (Tumolo et al., 1994). Of 68 patients, there was one complete and one partial response lasting 44 and 72 weeks, and 28% of patients experienced stable disease for a median of 24 weeks. Toxicities included nausea and vomiting in 34.5% of patients. The authors concluded that flutamide was ineffective in heavily pretreated patients, in light of the significant percentage of side effects.

5.5 Ongoing clinical trials with hormonally-targeted therapeutics in ovarian cancer

While there are many clinical trials open to ovarian carcinoma patients, there are not many currently investigating hormonally-directed agents. There is currently one phase II trial exploring tamoxifen in combination with the EGFR tyrosine kinase inhibitor ZD839 in patients with recurrent ovarian cancer refractory to platinum and taxane-based therapy (NCT00189358).

There are a few trials exploring the newer SERMs. One of the newer agents, arzoxifene, is a SERM with higher potency than raloxifene. A current study, NCT00003670, is examining arzoxifene in patients with metastatic refractory EOC or peritoneal cancer in the phase II setting, and aims to correlate response to serum estradiol, FSH, LH, and SHBG. Another SERM, toremifene citrate, has just finished assessment in the recurrent ovarian cancer population, but results have not yet been published (NCT00003865). Endoxifen, a tamoxifen-related compound with higher affinity for the estrogen receptor, is undergoing evaluation in the setting of hormone-receptor positive breast, solid, desmoid, or gynecologic tumors which have not responded to standard chemotherapy (NCT01273168).

Some studies with aromatase inhibitors have completed recruitment. One study with combination anastrozole and gefitinib, an EGFR inhibitor, has completed enrollment in a phase II trial for patients with relapsed ovarian cancer (NCT00181688). Exemestane has also been utilized in a phase II trial in patients with recurrent stage II to IV ovarian cancer (NCT00261027). The trial has completed enrollment, and initial results presented at the American Society of Clinical Oncology reported that in 24 patients, 36% experienced stable disease lasting a median duration of 23 weeks (Verma et al., 2006). One patient had stable disease lasting greater than 95 weeks.

Though there are no current trials investigating progesterone-based compounds and active disease, NCT00445887 is studying the use of oral levonorgestrel to prevent ovarian carcinoma in patients at high risk of developing EOC.

6. Granulosa cell tumors of the ovary

As aforementioned, while it is generally agreed upon that gonadotropins contribute to tumor progression in ovarian carcinoma, there is a lack of clear evidence that they can *initiate* carcinogenesis in EOC. Claims that gonadotropins may cause EOC are derived from *in vivo* studies, in which rodents treated with excess gonadotropins formed sex cord stromal tumors, specifically granulosa cell tumors. In α-inhibin deficient mice, which results in a lack of negative feedback on the gonadotropin axis and resultant excess gonadotropins, gonadal stromal tumors formed (Matzuk et al., 1992). However, the tumors failed to form in these α-inhibin deficient mice in the absence of gonadotropins (Kumar et al., 1996). Dorward et al. (2007) treated hypogonadotropic immunodeficient mice with grafted ovaries from prepubertal genetically susceptible mice with an LH analog or FSH, and found that LH-treated mice developed granulosa cell tumors, while FSH-treated mice did not.

The role of granulosa cells in the ovary is well-established: they convert androgens to estradiol via aromatase, and are stimulated to do so by FSH. They also produce progesterone during the later stages of the menstrual cycle. Similarly, steroid signaling in granulosa cells has been extensively studied *in vivo*. In granulosa cells, FSH activates several pathways, including ERK, MAPK, and PI3K (Hunzicker-Dunn & Maizels, 2006). Estrogen protects against FasL-mediated apoptosis in the G1 to S phase transition of the cell cycle (Quirk et al., 2006), and GnRH agonists stimulate apoptosis (Takekida et al., 2003). In malignant granulosa cell tumors (GCTs), ER2 is upregulated (Chu et al., 2000). Nearly all GCTs express progesterone receptors, while about 30% express estrogen receptors (Hardy et al., 2005).

Granulosa cell tumors comprise approximately 5% of all ovarian tumors (Schumer & Cannistra, 2003), and often present with symptoms associated with estrogen excess—such as vaginal bleeding or virilization in postmenopausal women, or precocious puberty in juveniles. Given their relative rarity, there is a paucity of clinical trials, but antihormonal therapy has had activity in recurrent disease. There have been reports of disease stabilization or response after administration of megestrol acetate, goserelin, leuprolide, and anastrozole (Fishman et al., 1996; Freeman & Modesitt, 2006; Malik & Slevin, 1991; Martikainen et al., 1989).

7. Conclusions

In summary, steroid signaling plays an important part in both normal ovarian surface epithelial cells and in malignant epithelial ovarian carcinoma. The pathways that promote growth and inhibit apoptosis in OSE are often exploited and upregulated in EOC, while protective components are downregulated, thereby allowing evasion of apoptosis as well as cellular migration and invasion. While estrogen and FSH promote cellular growth and tumor progression *in vitro* and *in vivo*, there is no definitive evidence that they can initiate ovarian carcinogenesis. Progesterone impressively enhances apoptosis, and is valuable in preventive efforts, yet treatment with progestins in ovarian cancer has not yielded equally impressive results in the clinical setting. The reasons for this remain unclear, and warrant further investigation into signaling pathways and receptor crosstalk mechanisms. Perhaps even more interesting, the GnRH signaling pathway appears to operate via dual and opposing mechanisms, inhibiting cell growth through one pathway yet increasing cellular migration and invasion through another. Efforts to exploit the growth inhibitory effects

while curbing the invasive component may generate more effective GnRH analogs. Despite our growing knowledge surrounding steroid hormone signaling in OSE and EOC, much still remains unknown.

While serum levels of steroid hormones are generally not useful as disease markers in determining disease risk or prognosis, ovarian tumors undoubtedly contain elevated levels of gonadotropins. Whether or not these elevated levels are a byproduct of aberrant signaling pathways or a contributor to carcinogenesis remains to be determined. Tumor expression of estrogen and progesterone receptors does appear to correlate to treatment response in patients, though results regarding estrogen receptor expression and prognosis are mixed. Certainly, the shift in ERα: ERβ expression and loss of PRB in tumor progression merits further investigation, and indeed has triggered exploration into targeted therapy. Inhibiting these receptor subtypes may result in more robust, specific responses in disease. While fulvestrant is an ERα antagonist, attention has now turned towards developing ERβ agonists. Benzopyran-derived selective estrogen receptor beta-agonist-1 (SERBA-1) is a selective ERβ receptor agonist which has been studied in mice and prostate hyperplasia, with promising effects (Norman et al., 2006). Monoaryl-substituted salicylaldoximes also show high ERβ affinity and are interesting new compounds (Bertini et al., 2011).

The role of exogenous hormones in ovarian cancer also remains unclear. As Risch suggests, at best HRT may accelerate the proliferation of pre-existing malignancy (Risch, 2002). Despite the completion of multiple large cohort studies, confounding and bias are likely responsible for ambiguous results. Still, the use of HRT is best avoided in women at high risk of developing EOC, such as BRCA mutation carriers, or women with a strong family history of ovarian cancer. Conversely, use of oral contraceptive prophylaxis is recommended for women with a high risk of EOC, to be weighed against a perhaps slightly elevated risk of breast cancer with extended use.

Hormonally-targeted therapeutics offer modest benefit for women with recurrent ovarian cancer, along with a much more tolerable side effect profile when compared to cytotoxic chemotherapy. In all fairness, clinical trials have not included hormone antagonists as first-line agents; they are most frequently studied in recurrent disease when even cytotoxic chemotherapy yields little benefit. It is possible that antihormonals may offer even more activity when used up front in combination with cytotoxic compounds, and this is an area warranting further investigation.

As is the case with emerging targeted therapies and resistance, inhibition of one pathway often results in upregulation of another, requiring combination with other therapeutics. Combination therapy of hormone antagonists with novel targeted agents offers exciting opportunities for overcoming resistance and improving patient outcomes. For example, the estrogen receptor pathway participates in crosstalk with multiple other mitogenic pathways, and concomitant inhibition of these pathways could produce a synergistic effect. Targeting other portions of the hormone receptors themselves has also evoked interest. Small molecule inhibitors target alternative binding sites on the estrogen and androgen receptor, which may result in improved selectivity or novel interactions (Shapiro et al., 2011). Likewise, while estrogen and progesterone receptor expression does correlate with response to antihormonal therapy, those patients without receptor expression also experience some benefit. Thus, better markers for response are needed. Post-translational modification of steroid receptors—particularly the estrogen receptor—include phosphorylation, ubiquitination, sumoylation, methylation, and palmitoylation, and affect receptor stability, localization, and perhaps drug resistance (Le Romancer et al., 2011).

Steroid hormones and their receptors participate in complex signaling which is not yet fully understood. Gaining insight into these interactions and downstream effectors is paramount to developing new targeted therapies and advancing the treatment of ovarian cancer.

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7

Salivary Cortisol Can Reflect Adiposity and Insulin Sensitivity in Type 2 Diabetes

Yoko Matsuzawa[1,2], Kenichi Sakurai[1], Jun Saito[2],
Masao Omura[2] and Tetsuo Nishikawa[2]
[1]*Clinical Cell Biology and Medicine, Graduate School of Medicine, Chiba University,*
[2]*Division of Endocrinology and Metabolism, Endocrinology and Diabetes Center,*
Department of Medicine, Yokohama Rosai Hospital,
Japan

1. Introduction

Glucocorticoids are well known to play an important role in the regulation of most essential physiological processes (Atanasov & Odermatt,2007). Patients with Cushing`s syndrome show central obesity with insulin resistance, caused by hypersecretion of cortisol (F) (Arnaldi et al.,2004). Obese patients with type 2 diabetes often have symptoms usually observed in patients with Cushing`s syndrome, and F levels might reflect the severity of complications and metabolic abnormalities in diabetes (Chiodini et al.,2007). High levels of F are associated with activation or dysregulation of the hypothalamic-pituitary-adrenal (HPA) axis and increased volume of the adrenal glands (Pasquali et al.,2006, Godoy-Matos et al.,2006).

Moreover, various metabolic abnormalities induced by enhanced glucocorticoid activity were found to be not only due to accelerated function of the HPA axis, but also by an impairment in 11β-hydroxysteroid dehydrogenase (11β-HSD) enzymes within the target cells (Godoy-Matos et at.,2006). 11β-HSD has two isoforms: 11β-HSD type 1 (11β-HSD1) mainly works as a reductase which converts inactive cortisone (E) to active cortisol (F) in F target tissues (Walker & Andrew,2006). 11β-HSD type 2 (11β-HSD2) is expressed in mineralocorticoid target tissues such as the distal nephron, colon, and salivary glands (Tannin et al.,1991, Draper & Stewart, 2005, Edwards et al., 1988), converting F to E to protect mineralocorticoid receptors from activation by F.

Animal models demonstrated that activation of 11β-HSD1 exhibited features of metabolic syndrome (Masuzaki et al.,2004, Morton et al.,2004). It was also reported that 11β-HSD1 is increased in subcutaneous adipose tissue in obese patients (Rask et al.,2002, Paulmyer-Lacroi et al.,2002), and higher 11β-HSD1 activity in adipose tissue is associated with features of metabolic syndrome in Caucasians and Pima Indians (Lindsay et al.,2003). Thus, it is suggested that F may play a crucial role in the regulation of adiposity in type 2 diabetes with obesity. On the other hand, the exact role of abnormal glucocorticoid metabolism in the pathogenesis of obesity has not fully been clarified yet. Salivary cortisol has been reported to be in closer agreement with the real adrenocortical function than serum cortisol concentration (Bolufer et al.,1989). Measurement of salivary cortisol was also reported to have several advantages, such as directly reflecting free cortisol level (Vining et al.,1983), and non-invasiveness for sampling (Chen et al.,1985). We, therefore, analyzed samples from

serum, saliva, and 24h-collected urine from obese type 2 diabetic patients as well as healthy subjects in order to evaluate clinical usefulness of salivary cortisol accurately measured by liquid mass spectroscopy in obese patients with type 2 diabetes.

2. Subjects and methods

2.1 Subjects

Eighteen Japanese men without underlying diseases cooperated as the healthy subject group. As the patient group, 23 Japanese male patients with type 2 diabetes, admitted to the Department of Endocrinology and Metabolism in Yokohama Rosai Hospital between March 2006 and March 2007, who met the following conditions were selected:1) waist circumference of 85 cm or greater, 2) stage 1 or 2 diabetic nephropathy, 3) not treated with oral biguanide or thiazolidine derivatives, and 4)understanding the objective of this study and giving written consent. This study was approved by the research ethics committee of Yokohama Rosai Hospital.

2.2 Measurement of serum and salivary steroids

Five ml of blood and 1 ml of saliva were collected before breakfast and supper on the same day under regular conditions of daily life from the controls and patients. Furthermore, 5ml of blood and 1ml of saliva 2 hours after breakfast, lunch and supper, and 20 ml of 24-h accumulated urine were collected on the same day during hospitalization on 7 days after admission from the patient group. The diet consisted of 25kcal/kg for ideal body weight distributed in three meals.

Blood samples were immediately centrifuged, and the sera were stored at -30°C until measurement. Saliva and accumulated urine samples were stored with no processing at -30°C. Cortisol and cortisone in the samples were measured by liquid chromatography-tandem mass spectrometry (LC-MS/MS) (Teikoku Hormone Mfg.-Asuka Pharmaceutical Co., Tokyo, Japan).

2.3 Glucose clamp

Euglycemic-hyperinsulinemic glucose clamping was performed in 14 of the patient group using an artificial pancreas (STG-22, Nikkiso, Tokyo, Japan), following the method described previously (Bergman et al.,1985, Nishikawa et al.,1996). Average age, HbA1c and BMI of these patients were not significantly different from those of the whole cases in

	Diabetic subjects	Healthy Volunteers	P value
n	23	18	
Age (yr)	54.7±13.6	48.3±14.3	0.32
BMI(kg/m^2)	28.2± 5.4	22.1±2.0	<.001
Waist circumference (cm)	96.6±12.4	80.8±5.6	<.001
Duration of diabetes (yr)	7.0±4.7	—	—
HbA1c(%)	10.6±1.9	—	—

(data expressed as mean ±SD)

Table 1. Anthropometric measures and background of the subjects

patient group. In patients under drug treatment for diabetes, administration of sulfonylurea and a long-acting insulin preparation was suspended from the previous evening to avoid their influence on the test.

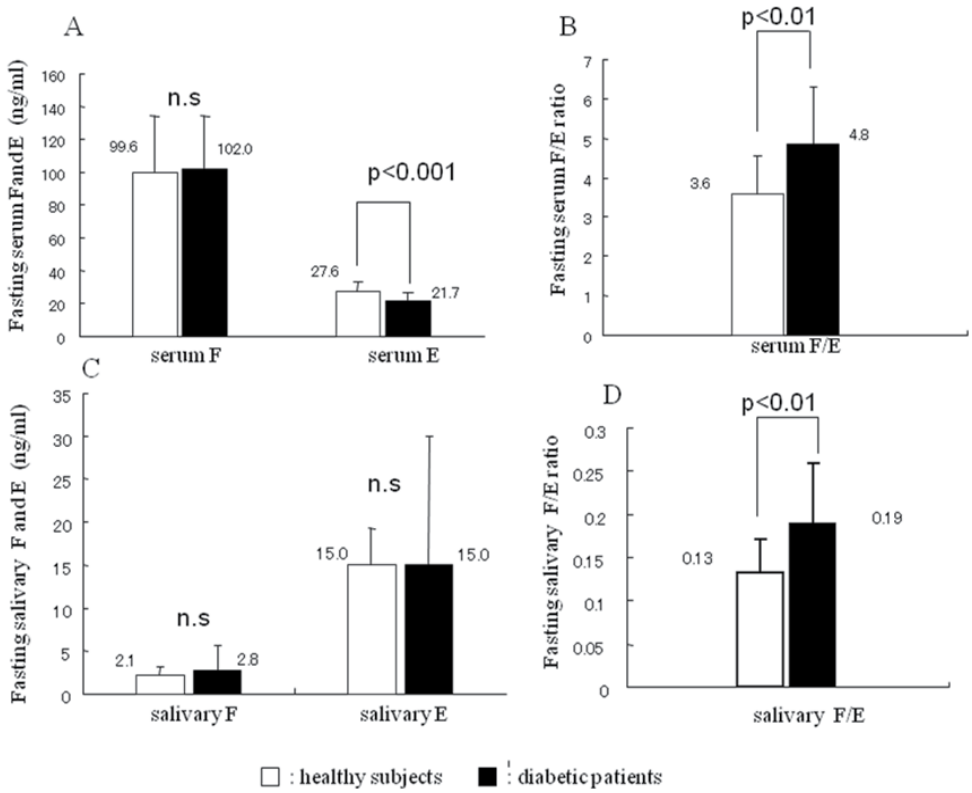
2.4 Statistical analysis

The subjects' backgrounds are presented as the means ± standard deviation, and the t-test was used to compare healthy subjects with patients. To analyze the correlation between 2 variables, Spearman's correlation coefficient was used, and a level of less than 5% was regarded as significant.

3. Results

3.1 Subject characteristics

The characteristics of the healthy and patient group, including age and BMI, are described in Table 1. The mean duration of illness was 7.0 ± 4.7 years in the patient group, and 14 (60.9%) and 8 (34.8%) patients were under treatment with insulin and oral drugs, respectively at the time of sample collection.



Serum levels of F and E were not different between healthy subjects and diabetic patients (A), and the ratio of F to E was significantly higher in the patient group (B). A similar result was observed for saliva (C, D). Data are expressed as the means±S.D. Statistical significance between healthy and diabetic subjects is described inside the figure.

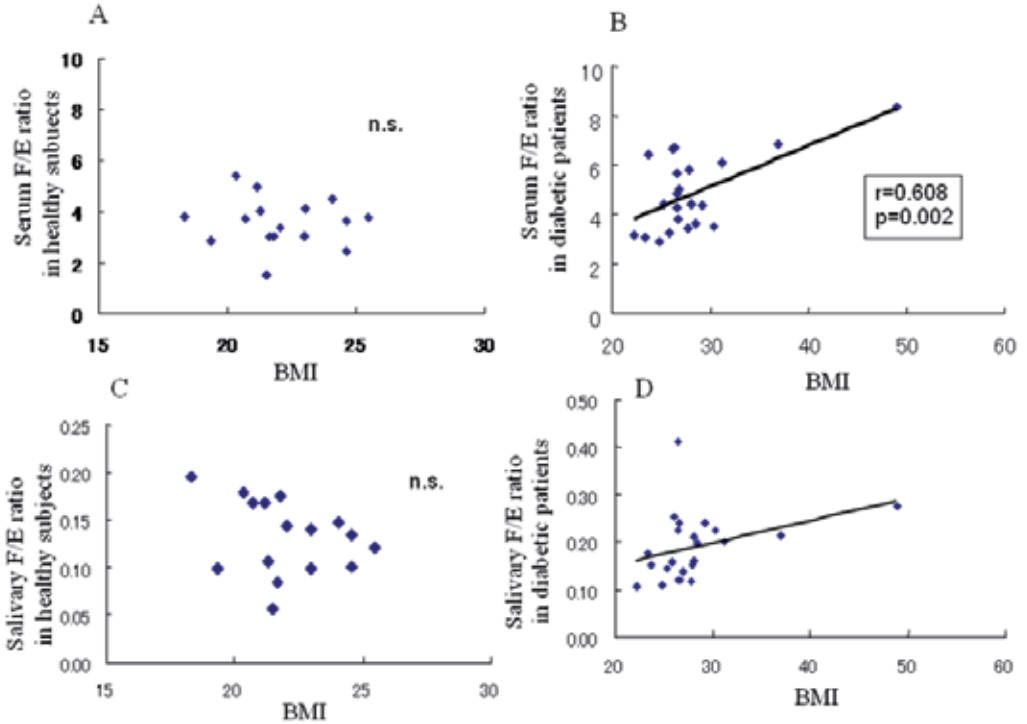
Fig. 1. Comparison of F, E, and F/E ratios between healthy subjects and diabetic patients in the fasting phase in the morning.

3.2 Relationship between F/E ratio and body weight

As shown in Fig. 1, no significant difference was noted in the blood cortisol (F) level before breakfast between the healthy and patient group, but the cortisone (E) level was lower in the patient group. Accordingly, the ratio of F to E (F/E) in blood was significantly higher in the patient group (3.6 vs. 4.8, respectively, $p<0.01$).

There was no significant difference in F or E level in saliva before breakfast between the healthy and patient group. Unlike the blood levels, E level was higher than F level in saliva. This is considered to be the influence of 11β-HSD2 expressed in the salivary gland (Tannin et al.,1991). However, the F/E ratio in saliva was significantly higher in the patient group, as in blood (0.13 vs. 0.19, respectively, $p<0.01$).

There was no correlation between the blood F/E ratio before breakfast and body mass index (BMI) in healthy subjects (Fig.2A), while there was a strong positive correlation between the blood F/E ratio and BMI in the patient group (Fig.2B). This correlation also remained significant after correction with age, HbA1c and blood glucose level before breakfast. Furthermore, a similar result was observed in salivary samples. The salivary F/E ratio tended to show a positive correlation with BMI in diabetic patients, but not in healthy subjects (Fig.2C, D).

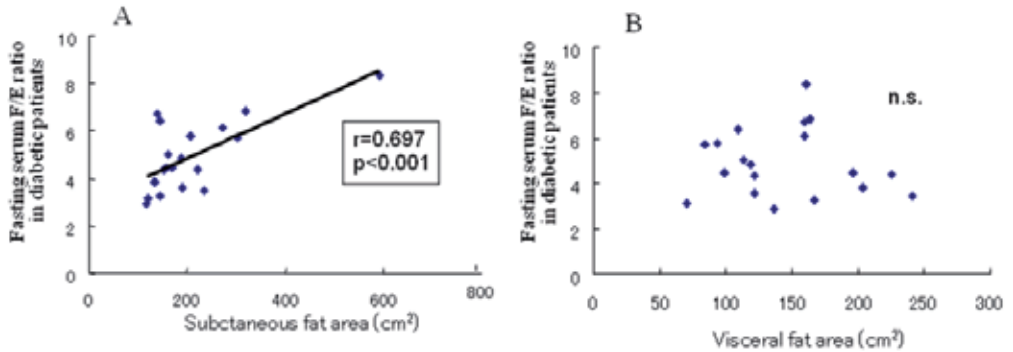


F/E ratio in blood was positively correlated with BMI only in diabetic patients (A, B). F/E ratio from salivary samples also showed a similar tendency, although the correlation was not statistically significant in diabetic patients (C, D).

Fig. 2. Correlation between fasting F/E ratio and BMI.

Fig. 3 shows the relationship between the fasting blood F/E ratio and fat volume assessed by abdominal CT in the patient group. The F/E ratio was positively correlated with the

subcutaneous fat area, but was not correlated with the area of visceral fat. Furthermore, the fasting blood and salivary F/E ratio was strongly correlated with serum leptin level ($r=0.652$, $p<0.01$ for blood, $r=0.469$, $p<0.05$ for saliva), but was not correlated with the plasma concentration of high molecular weight adiponectin or the severity of insulin resistance measured by glucose clamping (data not shown).

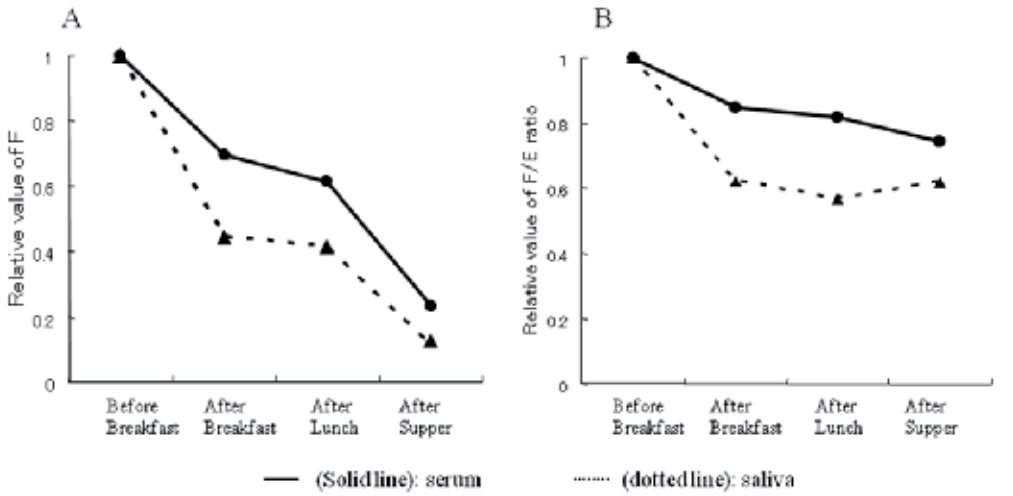


Fasting blood F/E ratio positively correlated with subcutaneous fat area (A), but no correlation was found between F/E ratio and visceral fat area (B).

Fig. 3. Relationship between fasting blood F/E ratio and body fat area assessed by CT scan.

3.3 Relationship between salivary cortisol and insulin sensitivity

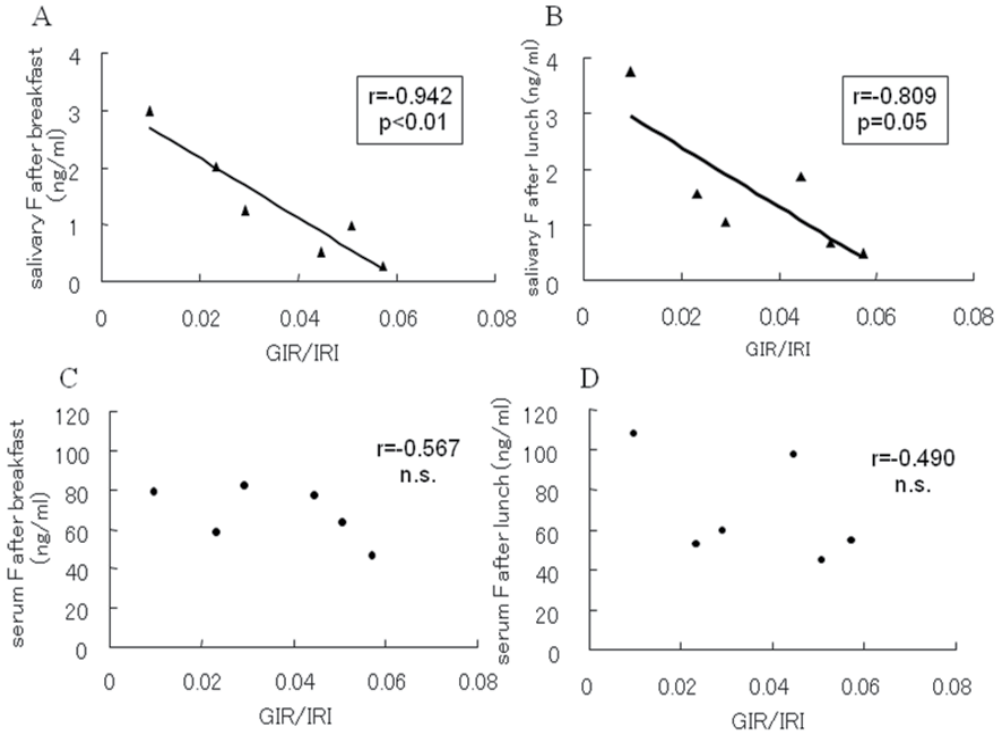
Blood and saliva were collected at 4-time points: before breakfast and 2 hours after each meal in the patient group. Fig. 4 shows the diurnal variation of F after assessing the level before breakfast as 1.0. The F level significantly decreased with time and the variation was larger in saliva than in blood. The within-day variation of the F/E ratio was smaller than that of the F level.



Solid line represents blood samples, and dotted line represents salivary samples. Both blood and saliva had a circadian rhythm in the patients, but salivary F fluctuated more dynamically than blood cortisol within a day.

Fig. 4. Circadian rhythm of F (A) and F/E ratio (B) in diabetic patients.

The salivary F level after breakfast was strongly correlated with the severity of insulin resistance measured by the glucose clamp (Fig. 5). This relationship with insulin resistance was not significantly noted in blood F.



Salivary F after breakfast showed the strongest negative correlation with GIR/IRI (A, B), while serum F(C, D) does not show significant correlation with insulin sensitivity.

Fig. 5. Relationship between insulin sensitivity (GIR/IRI) and F.

4. Discussion

The present study clearly demonstrated that obese patients with type 2 diabetes had a higher fasting F/E ratio in blood and saliva in the morning, comparing with healthy control subjects. Consistent with our results, an elevated blood F/E ratio in patients with type 2 diabetes has been reported (Valsamakis et al.,2004, Homma et al.,2001, Sinha & Caro 1998). Moreover, this is the first report demonstrating a significant increase in the F/E ratio in obese diabetic patients in saliva as well as in blood. Thus, it is suggested that overweight seems to induce much more F formation rather than E production via some mechanism(s) of changing steroidogenic enzymes, including 11β-HSD1 and 2.

Our data also showed a positive relationship between the fasting F/E ratio and BMI, between the fasting F/E ratio and total fat volume, and between the fasting F/E ratio and leptin, in diabetic obese patients. It is, therefore, suggested that the fasting F/E ratio may reflect the severity of adiposity accumulated to both of subcutaneous and visceral areas in obese diabetics, since circulating leptin levels are reported to be the best predictor of total body fat mass (Sinha & Caro,1998). Thus it can be postulated that increased fat mass may

lead to increased F production, at least partly mediated by 11β-HSD1 in adipose tissue, resulting in an elevated F/E ratio after overnight fasting. It was also reported that the expression of 11β-HSD1 is increased in adipose tissue in simple obesity (Rask et al.,2002, Paulmyer-Lacroi et al.,2002, Lindsay et al.,2003), suggesting that the ratio of F to E is a biomarker for assessing the adiposity in obese patients.

Oltmanns et al. (Oltmanns et al.,2006) recently reported that the level of salivary F of which sample was taken between breakfast and lunch was significantly related to metabolic findings in type 2 diabetes, such as fasting and postprandial blood glucose, urinary glucose, and glycosylated hemoglobin, although insulin sensitivity was not directly assessed in their study. Our data demonstrated that a circadian rhythm of F was apparently observed both in blood and saliva, and salivary F 2 hours after breakfast was shown to significantly correlate with insulin sensitivity assessed by euglycemic glucose clamping in obese patients with type 2 diabetes. Salivary F has been reported to have a circadian rhythm, highest in the morning, with lunch followed by a peak (Rosmond et al.,1998), suggesting that the salivary level of F usually decreases before lunch under normal conditions. Thus, it is suggested from our data that the level of salivary F before lunch, reflecting insulin sensitivity, may be up-regulated by food intake after breakfast despite of decreasing ACTH level by diurnal rhythm, since eating was reported to stimulate F secretion and women with abdominal obesity have also been reported to have a greater rise of F in response to food than those with peripheral obesity in simple obesity (Pasquali et al.,1998, Duclos et al.,2005, Korbonits et al.,1996). It had been recently reported that U-shaped associations were apparent between diurnal slope in salivary F and both BMI and waist circumference (Kumari et al.,2010), and also that 6 wk of supplementation with fish oil significantly increased lean mass and decreased fat mass, which were significantly correlated with a reduction in salivary F following fish oil treatment (Noreen et al.,2010).

Moreover, we should consider why salivary F could reflect insulin resistance in diabetic patients, while blood F could not. First, F measured in blood was the total of the free form and protein-bound form, while F in saliva reflected the biologically active blood unbound F level (Vining et al.,1983). The level of blood F can be altered by the concentration of blood F binding globulin (CBG), and CBG levels are shown to correlate negatively with BMI, waist-to-hip ratio, and HOMA (Fernandez-Real et al.,2002). Hence, salivary F is supposed to indicate the level of 'real' F activity in vivo, and thus correlates strongly with insulin resistance. Second, as shown in Fig. 4, salivary F fluctuates more dynamically than blood F within a day. None of our patients were diagnosed as overt or subclinical Cushing syndrome, and their levels of F were within the normal range. However, even a small increase in blood F within the normal range may contribute to abnormal glucose metabolism in metabolic syndrome and type 2 diabetes (Khani & Tayek,2001), and salivary F seems to be more sensitive than blood F to detect subtle changes in the metabolic state.

On the other hand, our results demonstrated that there is not significantly positive correlation between salivary F after breakfast and visceral fat mass. Visceral fat volume is known to be one of the most important factors to determine insulin resistance (Bergman et al.,2007), but our data suggest that F has a significant influence on insulin resistance, and intimately related to total fat volume. Furthermore, many factors including levels of glucose and insulin are supposed to modulate F metabolism after meals. Therefore, measuring salivary F after breakfast in obese diabetic patients may be a useful and noninvasive simple method to predict overall insulin sensitivity and severity of adiposity in such patients.

In conclusion, we tried to investigate the role of blood and salivary F and E in the regulatory mechanisms of obesity in obese men with type 2 diabetes. They had a higher fasting F/E ratio in both blood and saliva than that in healthy controls. Moreover, the fasting F/E ratio showed a significant correlation with total fat volume, suggesting that the fasting ratio of F to E in saliva and blood seems to directly reflect the adiposity in type 2 diabetic patients with obesity. Our data also demonstrated that salivary F after breakfast is suggested to be one of the most useful markers of insulin sensitivity in these patients. On the other hand, our study was conducted with a cross-sectional design, which does not allow us to assume any direct causality of F to insulin resistance. The role of F metabolism in adipogenesis and insulin resistance in type 2 diabetes will require further investigation with a prospective design.

5. Acknowledgment

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8

Music and Steroids – Music Facilitates Steroid–Induced Synaptic Plasticity

Hajime Fukui and Kumiko Toyoshima
Nara University of Education
Japan

1. Introduction

Music and medicine have always been closely related. This remains true even in hunter-gatherer cultures that are thought to reflect primitive human forms, as clarified by cultural anthropological and ethno-musicological studies (Lee & Daly, 2005; Merriam & Merriam, 1964). Interestingly, music has also been used for the treatment of neuropsychiatric disorders in hunter-gatherer cultures (Lee & Daly, 2005; Merriam & Merriam, 1964). However, in westernized societies, no established music therapy exists for neuropsychiatric disorders such as stress disorders, mood disorders (depression), and dementia. Experience has shown that music has certain therapeutic effects on neuropsychiatric disorders (both functional and organic disorders), and music therapy is currently being used in the United States and Europe in clinical and welfare settings. However, the mechanisms of action underlying music therapy remain unknown.

Various studies have examined the effects of listening to music on the brain (Bermudez & Zatorre, 2005; Nan et al., 2008). The study by Rauscher et al. on the "Mozart effect" is one of the most famous studies and has had both positive and negative impacts on music therapy (Rauscher et al., 1993). However, many subsequent studies have questioned the reliability of those results, and Chabris et al. published a study disproving the Mozart effect (Chabris, 1999). However, the fact that music affects the human body and mind was not disproved. In fact, more scientific studies on music have been conducted in recent years, mainly in the field of neuroscience, and the level of interest among researchers is increasing (Zatorre, 2003; Zatorre & McGill, 2005). Results of past studies have clarified that music influences and affects cerebral nerves in humans from fetuses to adults (Abbott, 2002).

The most significant finding has been that music enhances synaptic changes in the brain. In other words, studies comparing musicians and non-musicians and music learners and non-learners have clarified that music brings about cerebral plasticity. Music affects neuronal learning and readjustment (response of brain cells to sound and music stimuli, and changes in cell counts), and this effect lasts for a long period (Abbott, 2002). For example, even when neurodegenerative diseases such as Alzheimer's disease cause memory loss, patients can still remember music from the past, and listening to music can facilitate the recovery of other memories. This type of memory recovery is accompanied by the reconfiguration of existing neuron networks, which may allow access to long-term memory. However, most studies have been based on brain imaging modalities such as positron emission tomography (PET) or functional magnetic resonance imaging (fMRI). The effects of music at a cellular

level have not been clarified, and the mechanisms of action for the effects of music on the brain have not been elucidated.

The effects of steroids on changes in the brain have been documented in many animal species. For instance, vocal communication is a common characteristic among many vertebrates, and steroid hormones are implicated in the formation of neural mechanisms of vocal behavior in fish, amphibians, birds, and mammals (including primates) (Bass, 2008). The most fully known relationship between steroids and cerebral plasticity is vocal (singing) behavior in birds. The development of vocal behavior in singing birds involves complicated processes including neurons and muscles, and steroid hormones (testosterone and 17β-estradiol) are involved during many steps, such as neuron organization, neuron survival, and neural song-system formation (Fusani & Gahr, 2006; Nottebohm, 1981).

In humans, steroid hormones are associated with spatial perception and cognition. The relationship between testosterone and cognitive abilities is negative in men and positive in women (Gouchie & Kimura, 1991; Grimshaw et al., 1995a; Grimshaw et al., 1995b; Kimura & Hampson, 1994; O'Connor et al., 2001; Silverman et al., 1999). In women, the equilibrium of testosterone and 17β-estradiol associated with the menstrual cycle alters cognitive abilities (Silverman et al., 1999; Silverman & Phillips, 1993). Furthermore, in women, age-related decreases in 17β-estradiol are thought to be involved in cognitive dysfunction, memory disorder, learning disorder, depression, and other mood disorders. Numerous studies have also examined the relationship between 17β-estradiol and Alzheimer's disease accompanied by marked cognitive dysfunction (Gillies & McArthur, 2011; Wharton et al., 2001). The level of 17β-estradiol is lower for Alzheimer patients than for healthy individuals, and this decrease in estrogen level may lead to the progression of Alzheimer's disease and facilitate amyloid beta accumulation, one of the causes of memory disorders. Furthermore, testosterone administration to elderly men reportedly improves cognitive function (Gruenewald & Matsumoto, 2003).

The correlation between musical ability and spatial cognition is well recognized (Cupchik, 2001; Hassler, 1992; Hassler & Birbaumer, 1984). Many studies have investigated the relation between musical ability and spatial perception and cognition in humans. The assumption that some correlation exists between musical ability and steroid hormones also appears reasonable. In fact, Hassler discovered that the relationship between testosterone and musical ability (music composition) corresponded to that between testosterone and other forms of spatial perception and cognition (Hassler, 1991, 1992).

Furthermore, the relationship between music and steroid hormones is not confined to musical ability. Many studies in the field of behavioral endocrinology and neuroendocrinology have documented that musical stimulation (listening) affects various biochemical substances (Hassler et al., 1992; Kreutz et al., 2004; VanderArk & Ely, 1993).

2. Music and human physiology

The fact that music has an effect on the human body, particularly on stress and easing pain or anxiety, has been generally known since the Greeks (Aristoteles). Music influences the endocrine system to keep the body normal, as shown by many studies (e.g., Gardner et al., 1960; Logan & Roberts, 1984; Maslar, 1986; Standley, 1986; Hodges, 1996). Musical behavior is believed to invigorate several parts of the nervous system, as auditory information passes through the limbic and paralimbic systems including the thalamus, the hypothalamus, and amygdala, to the neocortex, and influences the pituitary gland; as a result, various

physiological effects are induced. Much research has been done regarding the physiologic effects of music, with results showing increases or decreases in respiration, heart rate, blood pressure, skin temperature, GSR (galvanic skin response), and electroencephalogram findings (Hodges, 1996). However, because of problems with experimental methods, results are inconsistent. Unfortunately, there is still no unified concept regarding the physiological effects of music, although the fact that music causes physiological effects in the human body is well accepted. Enormous advances have been made in recent years toward an understanding of the brain structures involved in music. Using the brain imaging techniques of PET, fMRI, and magnetoencephalography, the brain structures and activity related to music were clarified (Koelsch, 2010; Zatorre, 2003). Interestingly, these structures (limbic and paralimbic structures) are involved in the initiation, generation, detection, maintenance, regulation, and termination of emotions that have survival value for the individual and the species (Koelsch, 2010). Needless to say, emotions are deeply affected by steroids (Garcia-Segura, 2009).

3. Effects of steroids on auditory and musical behavior

Recently, endocrinologic research on human behavior has progressed. Evidence to date suggests that hormones not only have organizational effects but also affect cognition, perception, and other behaviors. Because the endocrine and nervous systems do not function in isolation but as an integrated whole, many aspects of neuronal functioning are affected by hormones (gonadal steroids). However, we still lack data regarding the effect of music on hormones. In addition, although knowledge regarding endocrinologic function and music has begun to accumulate, results are contradictory. Some studies indicate that music influences humans endocrinologically and other studies indicate that hormones influence musical behavior. Below we will review the substances that have been examined thus far and explore the physiological function of music.

Many reports support the correlation between hormones and hearing or vocal behaviors. The fact that testosterone influences growth of the larynx is well known. It is believed that testosterone also influences the auditory sense and the vocal organ (Kelley & Brenowitz, 1992; Marler et al., 1988; Silver, 1992). Reports also indicate that the utterance of song birds is influenced by testosterone (Nottebohm, 1972; Marler et al., 1988) and point to the existence of similarities between the vocal tract of song birds and humans (Bridgeman, 1988). The fact that the auditory sense of the human females undergoes cyclical changes affected hormones has been reported (Wynn, 1971). Moreover the female voice is also influenced by 17β-estradiol (Abitbol et al., 1989). The hypothesis that the perception of sound is influenced by hormones is based on the idea that hormones influence dorsal division and reticular formation in the auditory pathways.

Testosterone influences the development of the neural pathways of the brain and stimulates cerebral lateralization (Geschwind & Galaburda, 1985). As Lovejoy said, this provides males with right brain superiority, which results in making him proficient in spatial ability, such as securing food and adapting to the environment (Lovejoy, 1981). Other reports also show that spatial ability is influenced by sex hormones; for example, men with lower testosterone levels performed better than men with higher testosterone levels whereas women with higher testosterone levels performed better than women with lower testosterone levels on spacial ability tests (Gouchie & Kimura, 1991; Hampson & Kimura, 1992; Nyborg, 1983). Further a relation between spatial ability and musical ability has been reported (Hassler et

al., 1985), and listening to music has been shown to improve spatial ability (IQ) (Rauscher et al., 1993).

4. Musical behavior and steroids

4.1 Cortisol

Psychological and physiological stress affects testosterone and cortisol levels in both sexes. Generally, cortisol levels increase significantly in the presence of stress. It has been reported that music eases stress responses psychologically, physiologically, and endocrinologically. It is well known that listening to music reduces uneasiness (Gerdner & Swanson, 1993; Kaminski & Hall, 1996), depression, and fatigue (Field et al., 1997; Hanser & Thompson, 1994), changes mood (Cadigan et al., 2001; Gfeller & Lansing, 1991; McCraty et al., 1998; Sousou, 1997), and suppresses pain (Allen et al., 2001; Browning, 2000; Maslar, 1986). However, some reports that compare listening to music with other relaxation methods show no differences in alleviation of anxiety, depression, and fatigue (Field et al., 1997; Hanser & Thompson, 1994) or reduction of heart rate (Guzzetta, 1989; Scheufele, 2000). In addition, some authors have reported that there is differences in psychological and physiological responses among different genres of music (classical, hard rock, "favorite music," "relaxation music") (Allen & Blascovich, 1994) and others have reported no such differences (Burns, 1999).

Most of these studies have been on the stress-reducing effects of listening to music, and listening to music has been reported to cause a reduction in the cortisol levels. Cortisol is involved in many vital functions such as glucose metabolism and immune function, but in cases of chronic stress, it has been known to induce symptoms such as hypertension and impaired cognitive function (Lundberg, 2005). In addition, increasing cortisol levels with age may lead to a decline in memory or progression of Alzheimer's disease (Huang et al., 2009). Thus, the reduction of cortisol through the passive activity of listening to music may be useful for the treatment and prevention of diseases and disabilities.

Listening to music for short periods could lower cortisol regardless of the subject' s mental state (Field et al., 1998; Möckel et al., 1994), and music has been shown to significantly lower (Escher et al., 1993; Miluk Kolasa et al., 1994; Rider et al., 1985) or suppress cortisol levels (Schneider et al., 2001) even during surgery. Other papers reported that not only listening to music but also playing music (percussion instruments) lowered cortisol levels (Bittman et al., 2001; Burns et al., 2001). In addition, studies have shown that cortisol responses differed by music experience, such as (Vander Ark & Ely, 1992, 1993) and the subject's preference (Gerra et al., 1998). However, so far results are contradictory and there is no consensus regarding the relation between cortisol and music category or preference. However, judging from published research results, listening to one's favorite music decreases cortisol levels (Fukui, 1996).

4.2 Testosterone

Contrary to cortisol, several investigations have been conducted on the relationship between music and testosterone.

Testosterone has been shown to influence musical ability (Hassler, 1991), and its effects produce discrepancies between the sexes (Schumacher & Balthazart, 1986). Hassler hypothesized the existence of an optimal testosterone level in proportion to musical ability. Reports also discuss the existence of a correlation between hormone levels (testosterone)

and musical ability in puberty (Durden-Smith & Simone, 1983; Hassler, 1987; Hassler and Birbaumer 1987). Another report indicates that during puberty, children show poor results in music tests because of low testosterone levels at this stage (Serafine, 1988). Moreover, there is a report that composition has a seasonality that might be influenced by the circannual rhythm of testosterone (Fukui, 1995). In addition, most composers are male, and composers tends to show a low level of testosterone compared with control (Hassler et al., 1990). Further reports show that musicians have a tendency to demonstrate relatively low levels of sex-role stereotyping (Kemper, 1990), which again, may be related to testosterone levels. The point is that testosterone influences musical ability.

Regarding musical ability and testosterone, there is a high positive correlation between spatial cognitive ability and musical ability (talent) (Rauscher et al., 1993). A high correlation is also found between spatial cognitive ability and testosterone (Nyborg, 1983). Furthermore, these correlations differ between males and females. Hassler reported that male composers had relatively low testosterone levels, and that testosterone values increased as musical ability increased in female composers (Hassler, 1991).

On the other hand, only one report is available on sex-related differences in testosterone responses associated with music playing or listening. Fukui examined testosterone level changes between before and after listening to a wide variety of music, including favorite music, pop, jazz, and classical, in male and female students, and showed sex-related differences (Fukui, 2001). Specifically, testosterone values decreased in males and increased in females after listening to music, regardless of genre. Interestingly, the sex-related difference in testosterone levels while listening to music were the same as sex-related differences in stress responses**.** Grape et al. compared between patients with irritable bowel syndrome who took part in singing in a choir with those who took part in a group discussion and found that testosterone levels decreased in the singing group (Grape et al., 2010).

So far, there is no research investigating music and estrogen.

5. Hypothesis and the study

We propose that listening to music facilitates the regeneration and repair of cerebral nerves by adjusting the secretion of steroid hormones in both directions (increase and decrease), ultimately leading to cerebral plasticity. Music affects levels of cortisol, testosterone, and 17β-estradiol, and we believe that music also affects the receptor genes related to these substances and related proteins.

5.1 Methods

Subjects were recruited from healthy elderly women aged 60 or older who were participating in a 90 min. singing group (choir) as part of disease prevention and health-promoting activities hosted by the local government. The session was taught by a music therapist. Inclusion criteria were as follows: attended all four sessions (once a month); healthy; and not taking any medications such as steroids that could affect endocrinologic factors. A total of 50 volunteers (8 males and 42 females) were enrolled, however because of missing value, male's data was not possible to use. Finally, 42 female volunteers were enrolled. Mean age was 72.9 years (range: 64-83 years).

After obtaining informed consent, subjects participated in four choral sessions, once a month for 4 consecutive months. The sessions took place at a facility owned by the local

government. The flow of the experiment was as follows: first, health status and medication were checked. Then a saliva sample was collected before and after each session (about 90 min). Saliva samples were stored at -20°C in the freezer immediately after collection, and cortisol, testosterone, and 17ß-estradiol levels were measured by enzyme immunoassay. Intra- and inter-assay coefficients of variation ranged between 3.35–3.65% and 3.75–6.41% for cortisol, 2.5–6.7% and 7.9–8.6% for testosterone, and 6.3–8.1% and 6.0–8.9% for 17β-estradiol.

The musical preference of subjects was ascertained before the study, and several pieces of music were selected for the session. The contents of all four sessions were the same.

To assess depression and anxiety as psychological states, the Japanese version of the Profile of Mood States (POMS) as executed before and after the session (Yokoyama & Araki, 1994). The POMS is a highly reliable test that is often used in studies on mood states and the test we used were revised for use by the elderly in terms of terminology and style. In the present study, of the six subscales of POMS ("tension/anxiety" (TA), "depression/dejection" (DD), "anger/hostility," "vitality," "fatigue," and "confusion," the DD and TA subscales were used.

Cognitive tests were carried out at the same time as the POMS. Tests performed were as follows: 1) Digit Symbol-Coding (WAIS: Wechsler Adult Intelligence Scale III) memory task (Silverman and Eals' Object Location Memory Task), and 3) mental rotations test (Vandenberg and Kuse Mental Rotations test "3-dimensional").

5.2 Results

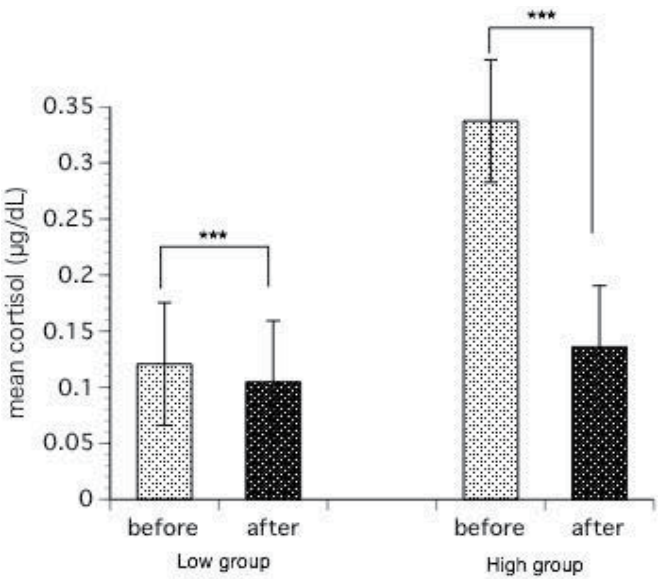
Cortisol, testosterone, and 17ß-estradiol levels for subjects were biphasic, and subjects were divided into two groups (high and low) with respect to pre-session median values. Mean hormone levels prior to the choral session were 0.2243 μg/dL for cortisol, 66.0579 pg/mL for testosterone, and 6.1332 pg/mL for 17β-estradiol.

Analysis of variance (ANOVA) was conducted on changes in cortisol, testosterone, and 17β-estradiol levels before and after each session, between the high and low groups, and between each session.

In terms of cortisol levels, the main effect of cortisol changes before and after the choral session was significant ($F(1,122)=28.16$, $p=0.0000$). In addition, the main effect between the high and low groups ($F(1,122)=35.05$, $p=0.0000$) was significant. Cortisol significantly decreased after each session in both the high and low groups (Fig. 1).

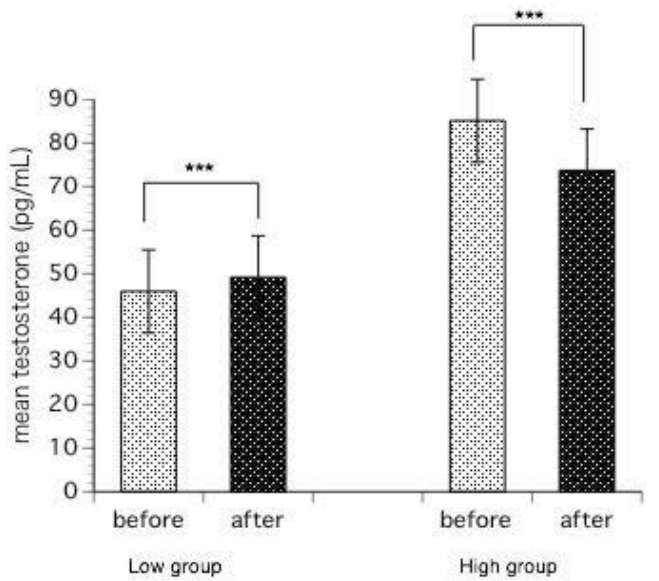
The main effect of testosterone changes before and after the choral session ($F(1,284)=4.26$, $p=0.0399$), the main effect between the high and low groups ($F(1,284)=289.99$, $p=0.0000$), and the interaction between testosterone changes and the high and low groups ($F(1,244)=15.04$, $p=0.0001$) were significant. In the high group, testosterone levels significantly decreased after the choral session; in contrast, in the low group, testosterone levels increased significantly after the session (Fig. 2).

The main effect of 17β-estradiol changes ($F(1,244)=23.23$, $p=0.0000$), the main effect between the high and low group ($F(1,244)=193.72$, $p=0.0000$), and the interaction between 17β-estradiol changes and the high and low groups ($F(1,244)=58.27$, $p=0.0000$) were significant. In the high group, 17β-estradiol levels significantly decreased after the choral session. Conversely, in the low group, 17β-estradiol levels significantly increased after the session (Fig. 3).



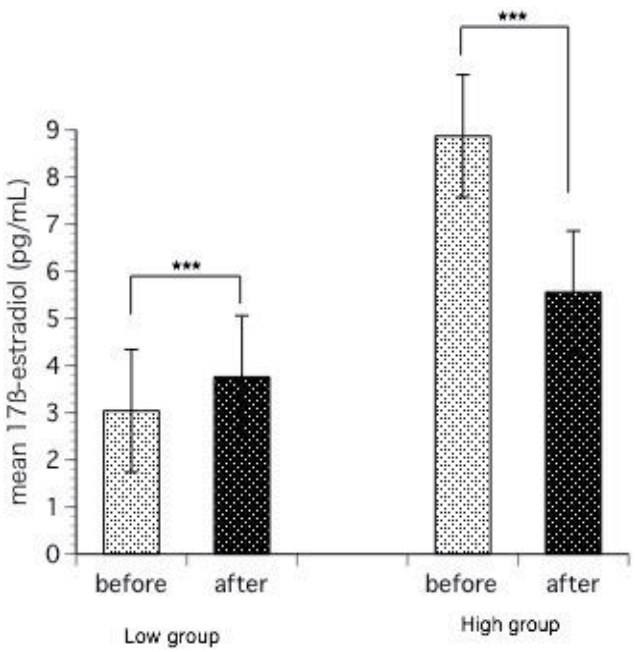
The main effect of cortisol changes before and after the choral session was significant (F (1,122)=28.16, p=0.0000). In addition, the main effect between the high and low groups (F (1,122)=35.05, p=0.0000) was significant. Cortisol decreased after each session in both the high and low groups.

Fig. 1. Cortisol levels of 42 female subjects



The main effect of testosterone changes before and after the choral session (F (1,284)=4.26, p=0.0399), the main effect between the high and low groups (F (1,284)=289.99, p=0.0000), and the interaction between testosterone changes and the high and low groups (F (1,244)=15.04, p=0.0001) were significant. In the high group, testosterone levels decreased after the choral session; in contrast, in the low group, testosterone levels increased after the session.

Fig. 2. Testosterone levels of 42 female subjects

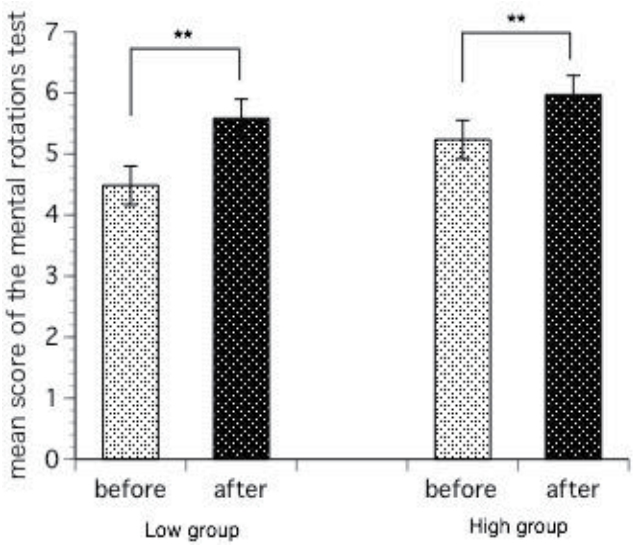


The main effect of 17β-estradiol changes (F (1,244)=23.23, p=0.0000), the main effect between the high and low group (F (1,244)=193.72, p=0.0000), and the interaction between 17β-estradiol changes and the high and low groups (F (1,244)=58.27, p=0.0000) were significant. In the high group, 17β-estradiol levels decreased after the choral session. Conversely, in the low group, 17β-estradiol levels increased after the session.

Fig. 3. 17ß-estradiol levels of 42 female subjects

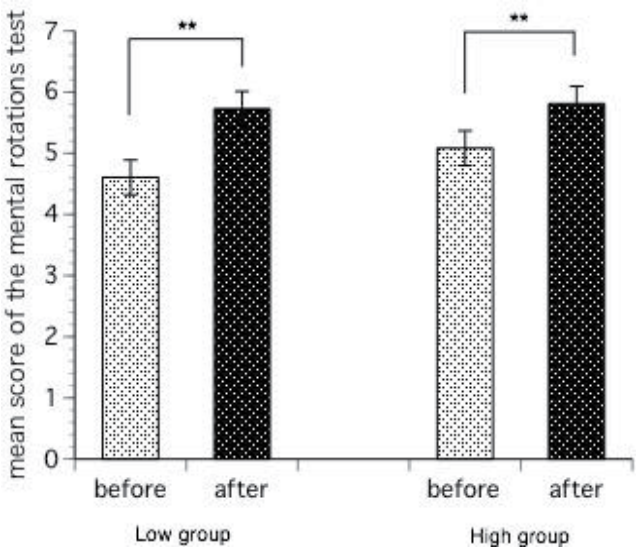
ANOVA was conducted on changes in cortisol, testosterone, and 17β-estradiol levels before and after each session, between the high and low groups, and between each session. No significant difference was found in any factor of the TA scores. The main effect of the DD scores was significant for cortisol (F (1,122)=4.02, p=0.0473), testosterone (F (1,117)=3.70, p=0.05), and 17β-estradiol (F (1,119)=4.25, p=0.0414). The scores of high and low groups significantly decreased after the session.

ANOVA was conducted on changes in cortisol, testosterone, and 17β-estradiol levels before and after each session, between the high and low groups, and between each session. No significant difference was found in any factor of the Digit Symbol-Coding. For the memory task, only the main effect of high and low groups for 17ß-estradiol was significant (F (1,120)=10.85, p=0.0013). The main effect of the mental rotations test was significant for cortisol (F (1,123)=9.16, p=0.0030), testosterone (F (1,116)=8.48, p=0.0043), and 17β-estradiol (F (1,120)=9.03, p=0.0032). The scores of high and low groups significantly increased after the session (Fig. 4, 5, 6).



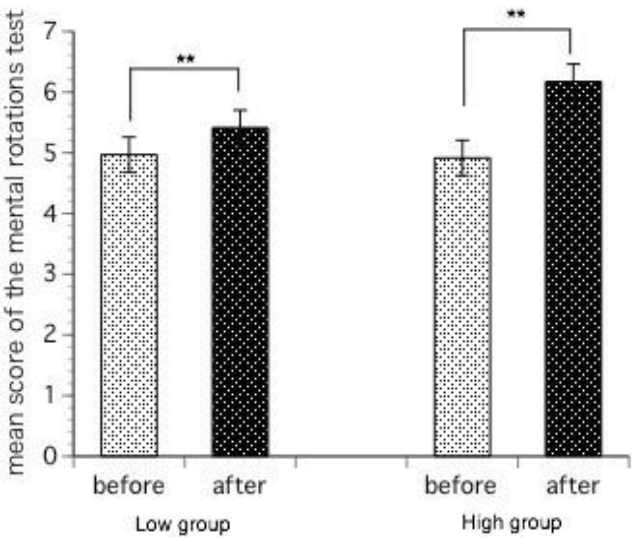
The main effect of the mental rotations test was significant for cortisol (F (1,123)=9.16, p=0.0030). The scores of high and low groups increased after the session.

Fig. 4. Mean score of the mental rotations test in cortisol levels



The main effect of the mental rotations test was significant for testosterone (F (1,116)=8.48, p=0.0043). The scores of high and low groups increased after the session.

Fig. 5. Mean score of the mental rotations test in testosterone levels



The main effect of the mental rotations test was significant for 17β-estradiol (F (1,120)=9.03, p=0.0032). The scores of high and low groups increased after the session.

Fig. 6. Mean score of the mental rotations test in 17ß-estradiol levels

6. Discussion

Coristol, testosterone, and 17β-estradiol were affected by musical behavior (chorus). Cortisol levels decreased after each choral session, whereas changes in testosterone and 17β-estradiol levels were dependent on the subject's baseline hormone level. After all sessions, testosterone and 17β-estradiol levels increased in the low groups and decreased in the high groups.

Anxiety and depression score (POMS) decreased after all sessions. The interesting finding was a result of the cognitive test (mental rotations test). The score increased regardless of the initial hormone level. Mental rotation involves spatial ability and is localized to the right hemisphere and is associated with intelligence (Hertzog & Rypma, 1991); Johnson, 1990; Jones & Anuza, 1982). It has been documented that music training improves verbal, mathematical, and visuo-spatial performance in children and adults (Brochard, 2004; Ho et al., 2003). However, no study has investigated music and mental rotation beside the "Mozart effects," especially in elderly people (Cacciafesta, et al., 2010). Our study is the first report to show that music improved mental rotation ability in elderly women.

Results of this study clarified that musical activities affect steroid secretion in elderly women and are likely to alleviate psychological states such as anxiety and tension. Furthermore, levels of steroids changed in both directions, increasing in subjects with low hormone levels and decreasing in subjects with high hormone levels. Thus, the hypothesis that listening to music affects the steroid hormone cascade and facilitates neurogenesis, regeneration, and repair of neuron appears highly plausible.

This study has several limitations, including the fact that only data obtained from a small number of elderly women were analyzed. However, the finding that musical behavior (chorus in this study) altered steroid levels agrees with results of previous studies that have documented strong correlations between steroids and spatial perception and cognition and the effects of listening to music on steroid secretion.

At this point, the effects of music on steroids are unclear, but music appears to be involved with steroid production via the pathway from the auditory system to the auditory area, particularly the neural pathway (emotion circuits) mediated by the cerebral limbic system (hypothalamic-pituitary-adrenal axis and amygdaloid complex). In recent years, the possible involvement of nerve damage in neuropsychiatric disorders has been suggested, and musical activities may enable the protection, repair, and even regeneration of human cerebral nerves.

Music is noninvasive, and its existence is universal and mundane. Thus, if music can be used in medical care, the application of such a safe and inexpensive therapeutic option is limitless.

7. Acknowledgment

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