EFFECT OF ALCOHOL ON HORMONES IN WOMEN

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Were I to await perfection,
my book would never be finished.

Tai T'ung

CONTENTS

ABBREVIATIONS

ACTH	Adrenocorticotropic hormone
ADH	Alcohol dehydrogenase
ALDH	Aldehyde dehydrogenase
ANOVA	Analysis of variance
BMI	Body mass index
CBG	Corticoid-binding globulin
CNS	Central nervous system
CRF	Corticotropin-releasing factor
CYP2E1	Cytochrome P450-2E1
DHEA	Dehydroepiandrosterone
DHT	Dihydrotestosterone
FDA	Food and Drug Administration
FPM	First pass metabolism
FSH	Follicle-stimulating hormone
GGT	Gamma-glutamyltransferase
3α-HSD	3α-Hydroxysteroid dehydrogenase
11β-HSD	11β-Hydroxysteroid dehydrogenase
17β-HSD	17β-Hydroxysteroid dehydrogenase
20α-HSD	20α-Hydroxysteroid dehydrogenase
HPA-axis	Hypothalamic-pituitary-adrenal -axis
HPG-axis	Hypothalamic-pituitary-gonadal -axis
IRMA	Immunoradiometric assay
i.v.	Intravenous
LH	Luteinizing hormone
LHRH	Luteinizing hormone-releasing hormone
MEOS	Microsomal ethanol oxidizing system
4-MP	4-Methylpyrazole
NAD^+	Nicotinamide adenine dinucleotide
NADH	Nicotinamide adenine dinucleotide, reduced form
$NADP^+$	Nicotinamide adenine dinucleotide phosphate
NADPH	Nicotinamide adenine dinucleotide phosphate, reduced form
OC-	Premenopausal women not using oral contraceptives
OC+	Premenopausal women using oral contraceptives
p.o.	Per os
RIA	Radioimmunoassay
SD	Standard deviation
SEM	Standard error of mean
SHBG	Sex hormone-binding globulin
THF	Tetrahydrocortisol
aTHF	alloTetrahydrocortisol
THE	Tetrahydrocortisone
aTHE	alloTetrahydrocortisone

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications which are referred to in the text by their Roman numerals:

I Sarkola T, Mäkisalo H, Fukunaga T, Eriksson CJP. Acute effect of alcohol on estradiol, estrone, progesterone, prolactin, cortisol, and luteinizing hormone in premenopausal women. Alcohol Clin Exp Res 1999;23:976-982.

II Sarkola T, Fukunaga T, Mäkisalo H, Eriksson CJP. Acute effect of alcohol on androgens in premenopausal women. Alcohol Alcohol 2000;35:84-90.

III Sarkola T, Ahola L, von der Pahlen B, Eriksson CJP. Lack of effect of alcohol on ethinylestradiol in premenopausal women. Contraception 2001;63:19-23

IV Sarkola T, Adlercreutz H, Heinonen S, von der Pahlen B, Eriksson CJP. The role of the liver in the acute effect of alcohol on androgens in women. J Clin Endocrinol Metab (in press)

V Sarkola T, Adlercreutz H, Heinonen S, Eriksson CJP. Alcohol intake, androgens and glucocorticoid steroids in premenopausal women using oral contraceptives: an interventional study. J Ster Biochem Mol Biol (in press)

ABSTRACT

Long-term heavy alcohol drinking is associated with endocrinological abnormalities of which pseudo-Cushing´s syndrome, loss of sexual characteristics and function, and disturbances in bone metabolism are perhaps clinically the most prominent in women. In addition, alcohol intake has been associated with an increase in breast cancer. It seems reasonable to hypothesize that the mechanism of these effects would, at least in part, involve the effect of alcohol intake on the hormonal balance itself. The aim of the present work was to study the effects of alcohol on hormones in women and to elucidate mechanisms behind these effects.

The effect of alcohol intake on the hormonal balance was studied in healthy premenopausal women using oral contraceptives as well as in female non-users during different dose and time conditions. Plasma hormone levels were determined by radioimmunoassays. Urine steroid conjugates and plasma ethanol levels were determined by gas-liquid chromatography and headspace gas chromatography, respectively.

An acute increase in plasma testosterone was found after intake of alcohol. This increase was accompanied by decreases in plasma androstenedione and urine androsterone and etiocholanolone. The effect lasted throughout the elimination of ethanol and it was abolished during pretreatment with 4-methylpyrazole, an inhibitor of alcohol dehydrogenase. The magnitude of the testosterone elevation was not dependent on the alcohol dose (0.34-1.02 g/kg p.o.) and the acute effect was not modulated by a one week period of alcohol intake (0.8 g/kg/day p.o.), although an elevation in testosterone during non-intoxicated time points was observed in the end of the period. Acute increases in prolactin and estradiol, and an acute decrease in progesterone were found after intake of alcohol. No acute effects of alcohol on plasma cortisol, DHEA, dihydrotestosterone, and ethinylestradiol were observed. Minor effects were observed in certain urine androgen and glucocorticoid conjugate ratios during and after the drinking period.

In conclusion, the acute steroid effects are explained by a change in the metabolism of the steroids in the liver mediated by the alcohol induced shift in the redox state. The changes in the steroids observed during non-intoxicated time points may be explained by an effect of alcohol on the HPA-axis. The effect of alcohol on prolactin might involve the opioid peptides and dopamine in the hypothalamus-pituitary. The effects on the urine steroid conjugate ratios suggest an effect of alcohol drinking on the steroid metabolizing enzymes in the liver.

INTRODUCTION

Humans have been consuming alcohol for thousands of years. Nowadays alcohol is virtually drunk by all existing human populations. The patterns and amounts of drinking vary between different populations. Usually alcohol is consumed for pleasure, to live up the social situation. The J-shaped association between mortality and alcohol intake has been established by many prospective epidemiological studies (e.g. Doll et al 1994). Compared with abstinence, 1-2 standard drinks/day is associated with a minor reduction in mortality whereas an exponential increase in mortality is observed when alcohol consumption exceeds about 2 standard drinks/day. Long-term heavy alcohol drinking may lead to alcohol dependence and alcohol is by far the most widely abused drug in the world. In Finland it has been estimated to cause about 2500 deaths a year making it one of the major preventable environmental factors of mortality. The increase in alcohol consumption during the past decades and, as a consequence, the increase in social problems, morbidity and mortality related to alcohol makes it one of the most important issues in the health politics in this country (Huttunen 1998).

Long-term heavy alcohol drinking is associated with endocrinological abnormalities of which pseudo-Cushing´s syndrome (symptoms of truncal obesity, moon face, supra clavicular fat pads, buffalo hump, blue-red striae, easy bruisability, hypertension, and proximal myopathy), loss of sexual characteristics and function (symptoms of hirsutism and menstrual irregularities including anovulation, luteal-phase dysfunction, recurrent amenorrhea, and early menopause), and disturbances in bone metabolism (osteoporosis) are perhaps clinically the most prominent in women (Van Thiel and Lester 1979, Hugues et al 1980, Adler 1992, Veldman and Meinders 1996, Turner 2000). In addition, alcohol intake has been associated with an increase in breast cancer (Smith-Warner et al 1998, Longnecker 1993, Ginsburg 1999). The pathophysiological mechanisms of these clinical conditions are still unclear but it seems reasonable to hypothesize that they would, at least in part, involve the effect of alcohol intake on the hormonal balance itself.

Traditionally the acute effect of alcohol on steroids has been attributed to the effect of alcohol on the hypothalamic-pituitary-gonadal and -adrenal axes, the exact mechanisms of which are still unclear (see van Thiel and Gavaler 1990, Veldman and Meinders 1996 for reviews). The great majority of human studies published so far on the effect of alcohol on hormones have been performed on men. Thus, the aim of the present work was to study the effects of alcohol on hormones in premenopausal women and to elucidate mechanisms behind these effects.

REVIEW OF THE LITERATURE

2.1 ETHANOL PHARMACOKINETICS AND METABOLISM

2.1.1 Absorption, distribution and elimination of ethanol

After intake of alcohol per os ethanol is rapidly absorbed from the gastrointestinal tract by simple diffusion. Most of the ingested ethanol is absorbed in the duodenum and upper jejunum although some is metabolized and absorbed already in the ventricle. Factors that slow down gastric emptying (e.g. intake of food) delay the absorption phase (Roine et al 1991, Oneta et al 1998) which leads to lower maximal ethanol blood concentrations. Ethanol enters the portal vein and the liver before entering the general circulation. The magnitude of the first pass metabolism (FPM) occurring mainly in the ventricle and the liver and factors affecting FPM is a matter of debate. Studies on the rat show that elevated ethanol levels may be measured in the peripheral circulation and tissues including the brain already at 1 to 2 minutes from intake per os (Nurmi et al 1994).

Ethanol is distributed mainly in the body water due to the low solubility in lipids. As a consequence the distribution is largely governed by the water content of the various tissues (Wallgren and Barry 1970). The overall distribution volume is approximately 0.73 l/kg of body weight for men and 0.59 l/kg of body weight for women. The gender difference is due to the lower water content in women and as a consequence higher ethanol levels are found in women than in men after the intake of a standard amount of alcohol per body weight (Marshall et al 1983).

It is generally agreed that in mammals the major part, between 60% to 90% by most estimations, of the ethanol is eliminated in the liver (Lundsgaard 1938, Clark et al 1941, Larsen 1963, Utne and Winkler 1980). Almost all tissues are, however, capable of oxidizing some ethanol and important extra hepatic sites include the whole gastrointestinal tract, the airways and the lungs, and the kidneys (Pikkarainen et al 1980, Salaspuro 1996, Lieber 1997, Tillonen et al 1999). Ethanol is eliminated by oxidation to acetaldehyde which is further oxidized to acetate. A rough estimate is that the elimination of ethanol follows zero-order kinetics with a constant amount being eliminated during a constant period of time. At low blood ethanol levels (i.e. <2mM) when the capacity is non-saturated the elimination rate is nonlinear (Mumenthaler et al 2000). Among light to moderate drinkers the ethanol elimination rate is approximately 0.1 g/kg of body weight/hour. The elimination of acetaldehyde is far more effective than the production during ethanol oxidation and it has been

estimated that less than 1% of the acetaldehyde formed during ethanol intoxication in the liver enters the circulation (Nuutinen et al 1984, Eriksson and Fukunaga 1993). In the blood acetaldehyde is partly bound to proteins including hemoglobin and albumin (Donohue et al 1983, San George and Hoberman 1986, Niemelä et al 1987, Sillanaukee and Koivula 1990). The free blood acetaldehyde is eliminated within minutes of time (Stowell et al 1978) perhaps due to ALDH-activity contained in erythrocytes (Helander and Tottmar 1986). Consequently, venous blood free acetaldehyde levels are essentially non-detectable (i.e. < 1 μM) during alcohol intoxication (see Lindros 1989, Eriksson and Fukunaga 1993 for reviews). The acetate formed in the liver enters the blood and is rapidly converted to CO_2 and H_2O mainly in peripheral tissues (Lundqvist et al 1962, Skutches et al 1979). Minor amounts of ethanol are constantly produced endogenously, mainly by micro-organisms in the gastrointestinal tract, and effectively eliminated from the portal blood entering the liver (Krebs and Perkins 1970, Blomstrand 1971).

2.1.2 Ethanol oxidation

Three pathways have been described in the oxidation of ethanol to acetaldehyde: the alcohol dehydrogenase -pathway (ADH), the microsomal ethanol oxidizing system (MEOS) and the catalase -pathway.

2.1.2.1 Alcohol Dehydrogenase (ADH)

It is generally agreed that among healthy individuals the major part of ethanol is oxidized by the alcohol dehydrogenase enzyme (ADH). ADH is most abundant in liver tissue and is situated in the liver cell cytosol. The ADH isoenzymes are coded by seven genes (*ADH1A* to *ADH1C* and *ADH2* to *ADH5*) and they are traditionally grouped into five classes (class I to class V) based on similarities in function (Duester et al 1999). The class I isoenzymes are dimers of three different subunits ADH1A, ADH1B and ADH1C (formerly denoted α, β, γ; coded by the genes *ADH1A, ADH1B, ADH1C*) of which polymorphism is present for the latter two (ADH1B1, ADH1B2, ADH1B3 and ADH1C1, ADH1C2). The Km values for the class I isoenzymes are mainly in the micromolar level (range of 49 μM to 36 mM) and they are effectively inhibited by 4-methylpyrazole (Li and Theorell 1969, Ehrig et al 1990). The class II-V isoenzymes have Km values in the range of 34 mM to 1 M

and are, thus, of minor importance in the oxidation of ethanol (Ehrig et al 1990), except for the gastrointestinal tract (Lieber 1997). The inhibition of ethanol elimination by 4-methylpyrazole is competitive and the degree of the inhibition, thus, dependent on dose (Blomstrand and Theorell 1970). In vivo experiments with humans has demonstrated a 20 to 40% decrease in the ethanol elimination rate with a 4-methylpyrazole dose of 7 mg/kg intravenously (Salaspuro et al 1977, Salaspuro et 1978) or 10-20 mg/kg per os (Jacobsen et al 1996) with no adverse effects at this dose level (Salaspuro et al 1977, Salaspuro et al 1978, Jacobsen et al 1988). 4-methylpyrazole has recently been approved by the FDA and is currently in clinical use as an antidote for ethylene glycol poisoning (Brent et al 1999). Case reports indicate that it may successfully be used in the treatment of methanol poisoning (Burns et al 1997, Hantson et al 1999) and acetaldehyde-related flushing (Lindros et al 1981, Kupari et al 1983, Inoue et al 1984) as well.

2.1.2.1.1 The redox effect of ethanol

The oxidation of ethanol by ADH is coupled to the reduction of nicotinamide adenine dinucleotide (NAD^+) to NADH (Forsander et al 1958, Forsander 1970). The elevation in the ratio of NADH to NAD^+, the ethanol induced change in the redox state, is rather constant during different dose and time conditions, and leads to a number of acute changes in the liver metabolism including an increased lactate/pyruvate -ratio (hyperlactacidemia; Lundqvist et al 1962, Tygstrup et al 1965), an increased 3-hydroxybutyrate/acetoacetate -ratio (Lundqvist et al 1962, Tygstrup et al 1965), an increased 5-hydroxytryptophol/5-hydroxyindoleacetic acid -ratio (Davis et al 1967, Feldstein et al 1967), and an inhibition of the elimination of galactose (Salaspuro 1966). The monopolization of the liver metabolism during ethanol oxidation inhibits the citric acid cycle and as a consequence the production of carbon dioxide is markedly decreased (Forsander et al 1965). The major part of the liver energy metabolism is then accounted for by the regeneration of NAD^+ from NADH in the respiratory chain. The increase observed in the synthesis of triglycerides (triglyceridemia) after alcohol intake may be explained by a decrease in fatty acid oxidation as well as an increase in α-glycerophosphate also mediated by the alcohol-induced shift in the liver redox state (Nikkilä and Ojala 1963, Forsander 1970).

2.1.2.2 Microsomal ethanol oxidizing system (MEOS)

An increase in the ethanol elimination rate may be observed during long-term alcohol drinking. It is generally agreed that this increase is due to the increase in hepatic CYP2E1 which is regarded as the major component in the microsomal ethanol oxidizing system (Orme-Johnson and Ziegler 1965, Lieber and DeCarli 1968, Cederbaum et al 1979, Ingelman-Sundberg 1988, see Lieber 1999 for review). In the reaction the oxidation of ethanol is coupled to the reduction of oxygen (O_2) using nicotinamide adenine dinucleotide phosphate NADPH as a hydrogen donor. As a result water (H_2O) is produced.

2.1.2.3 Catalase

Minor amounts of ethanol may be oxidized by peroxisomal catalase. As its activity depends on the hydrogen peroxide (H_2O_2) production which is very low during normal circumstances in the liver (Boveris et al 1972), the role of catalase during ethanol oxidation is probably very small or negligible, perhaps with the exception of the brain (Cohen et al 1980, Aragon et al 1991). Some authors have nevertheless suggested that a significant proportion of the ethanol metabolism in the liver would be catalase-dependent (Thurman and Handler 1989).

2.1.3 Acetaldehyde oxidation

The acetaldehyde formed from ethanol is effectively oxidized further to acetate by aldehyde dehydrogenase (ALDH) within the liver. The oxidation of acetaldehyde is coupled to the reduction of NAD^+ to NADH adding to the redox change induced by ethanol oxidation in the hepatocyte. ALDH-activity is found both in the human hepatocyte mitochondria as well as the cytosol. The acetaldehyde formed from ethanol is primarily oxidized in the mitochondria (class 2 ALDH) where the isoenzyme has a micromolar Km for acetaldehyde (Greenfield and Pietruszko 1977, Cao et al 1988). The Km of the cytosolic enzyme (class 1 ALDH) is about 100 μM (Greenfield and Pietruszko 1977) and its role in the elimination of acetaldehyde in vivo is not clear. To date nine ALDH gene families (ALDH1 to ALDH9, including 16 genes) has been found of which only the first two families

seem to be important in acetaldehyde metabolism (Weiner 1996, Vasiliou and Pappa 2000). The gene coding the mitochondrial class 2 isoenzyme is polymorphic (ALDH2*1 and ALDH2*2) and the ALDH2*2 coding an inactive subunit is found exclusively in Oriental populations with 2% to 40% being heterozygote and 0% to 5% homozygote for the ALDH2*2 form (Goedde et al 1992). High acetaldehyde levels are found after alcohol intake among individuals carrying the ALDH2*2 allele generating discomfort including hypotension, tachycardia and facial flushing. As a consequence alcohol dependence is extremely rare among ALDH2*2 homozygotes whereas heterozygotes are able to drink some alcohol (Goedde and Agarwal 1987, Higuchi et al 1992). ALDH-inhibitors (e.g. disulfiram, calcium cyanamide and coprine) decrease voluntary alcohol intake both in rats (Sinclair et al 1980) and humans (Garbutt et al 1999) and as a consequence they (e.g. Antabus) are still recommended in the treatment of alcohol dependence.

2.2 EFFECT OF ALCOHOL ON HORMONES IN WOMEN

2.2.1 Effect of alcohol on sex steroids

2.2.1.1 Acute effect of alcohol on estrogens and progesterone

The fact that investigations on the hormonal effects of alcohol in women are complicated by the menstrual cycle and the use of hormonal preparations may explain why the acute effect of alcohol on steroids was not studied in women until the beginning of the 1980´s. No effects of alcohol were observed on sex steroids including estradiol and progesterone in these initial studies (McNamee et al 1979, Mendelson et al 1981, Välimäki et al 1983), although a tendency to elevated testosterone concentrations was displayed in one of them (Välimäki et al 1983). Later no effect of alcohol on progesterone could be found (Mendelson et al 1987, Teoh et al 1988, Mendelson et al 1989) but a significant alcohol-induced elevation in plasma estradiol was, however, reported in a number of papers originating from one group of researchers (Mendelson et al 1987, Mendelson et al 1988a, Teoh et al 1988, Mendelson et al 1989, Teoh et al 1990). Similarly, an acute elevation in estradiol was reported after alcohol intake among postmenopausal women using both transdermal as well as oral estrogen replacement therapy (Ginsburg et al 1995a, Ginsburg et al 1995b, Ginsburg et al 1996). Interestingly, the acute estradiol elevation was accompanied by a decrease in estrone levels (Ginsburg et al 1996).

2.2.1.2 Long-term effects of alcohol on estrogens and progesterone

The literature on alcohol consumption and estrogen levels (during non-intoxicated conditions) in women suggests a dose dependent effect and provides a possible link between alcohol consumption and a modest increase in the incidence of breast cancer (Longnecker 1993, Smith-Warner et al 1998, Ginsburg 1999, Colditz and Rosner 2000, Kuper et al 2000). Interestingly, the association between alcohol intake and the incidence of breast cancer has been reported to be particularly strong among postmenopausal women using estrogen replacement therapy (Colditz et al 1990, Gapstur et al 1992, Zumoff 1997). Taken together, the reports regarding light to moderate alcohol consumption and estrogens are, however, inconclusive. Among premenopausal women cross-sectional studies provide

evidence for both the lack of an association (Dorgan et al 1994) as well as for a positive association (Muti et al 1998) between moderate alcohol intake and estrogen levels. In the only interventional study available elevated estrogen levels were found in the morning after a 3-month-period of controlled intake of 2 to 3 standard drinks a day (Reichman et al 1993). For postmenopausal women not using estrogen replacement, a positive association between alcohol intake and estrogen levels has been observed in a minority of the cross-sectional studies published (see Purohit et al 1998 for review). Normal plasma estrogen and progesterone levels have been reported in non-cirrhotic female heavy alcohol drinkers with normal menstrual cycles (Mendelson and Mello 1988b, Välimäki et al 1990b, Välimäki et al 1995) although a tendency for lowered levels was observed in one of them (Välimäki et al 1990b). Decreased estrogen and progesterone levels are common in premenopausal amenorrheic heavy drinking women with or without alcoholic liver disease (Hugues et al 1980, Välimäki et al 1984a, Mendelson and Mello 1988b) as well as among pregnant women giving birth to infants with fetal alcohol syndrome (Halmesmäki et al 1987).

2.2.1.3 Acute effect of alcohol on androgens

Heavy alcohol intake leads to an acute decrease in plasma testosterone in men (Ylikahri et al 1974, Mendelson et al 1977, Välimäki et al 1990a). Recent reports demonstrate, however, that alcohol intake is associated with an acute elevation in plasma testosterone levels both in premenopausal women using oral contraceptives as well as among non-users. Acute elevations in plasma testosterone levels has been found among both premenopausal women in the midcycle phase of the menstrual cycle as well as among premenopausal women with regular use of oral contraceptives during the whole cycle (Eriksson et al 1994). The elevation was found to be pronounced among women using oral contraceptives. In line with this report is an observation of elevated plasma testosterone levels in four premenopausal women using oral contraceptives in the morning (14h) after the intake of a large dose of alcohol (2.0 g/kg) the previous evening (Karila et al 1996). Elevated testosterone levels has also recently been reported among drunken female adolescents entering an emergency department although the menstrual cycle phase or the use of hormonal preparations was not recorded in this study (Frias et al 2000).

2.2.1.4 Long-term effects of alcohol on androgens

In male alcoholics low androgen levels are frequently observed and this phenomenon has been attributed to the detrimental effect of long-term heavy alcohol intake on the hypothalamic-pituitary-gonadal axis (see Van Thiel and Lester 1979 for review). Several cross-sectional studies report, however, positive associations between androgen (testosterone, androstenedione, dehydroepiandrosterone) levels and alcohol intake among premenopausal women consuming moderate amounts of alcohol (Dorgan et al 1994, Cigolini et al 1996) as well as among premenopausal non-cirrhotic female alcohol abusers attending detoxification programs (Pettersson et al 1990, Välimäki et al 1990b, Välimäki et al 1995). No effect on plasma testosterone was, however, observed in the morning after a 3-month-period of controlled intake of two to three standard drinks a day among premenopausal women (Reichman et al 1993). Normal plasma testosterone levels has been reported among amenorrheic premenopausal women with alcoholic liver disease compared with postmenopausal controls (Välimäki et al 1984). Among pregnant women giving birth to infants with fetal alcohol syndrome elevated androstenedione and free testosterone but decreased dehydroepiandrosterone sulfate levels has been reported (Ylikorkala et al 1988).

2.2.1.5 Effect of alcohol on sex-hormone-binding globulin (SHBG)

In plasma sex steroids (i.e. estradiol and testosterone) are bound mainly to sex-hormone-binding globulin (SHBG) and to a minor extent to albumin with a small fraction (< 2%) remaining free and thus available for tissue uptake (Yen 1999). It is well known that the use of oral contraceptives increases SHBG-levels due to the estrogen effect of the pill (Kuhnz et al 1991, Wiegratz et al 1995). An acute effect of alcohol on the SHBG protein level in plasma seems, however, unlikely and there are no reports on this subject. Elevated SHBG levels during non-intoxicated conditions have been reported among male and female alcoholics with and without signs of liver cirrhosis (Lindholm et al 1978, Bahnsen et al 1981, Myking et al 1987, Becker et al 1991) with a concomitant decrease within days during abstinence (Iturriaga et al 1995, Iturriaga et al 1999). In contrast, decreased SHBG-levels have been reported among pregnant women giving birth to infants with fetal alcohol syndrome (Ylikorkala et al 1988).

2.2.1.6 Effect of alcohol on sex steroid synthesis in vitro

A number of studies performed primarily with rats indicate that alcohol inhibits steroid synthesis in the testis (Cicero and Bell 1980, Cobb et al 1980, Orpana et al 1990). Only a few reports have yet been published on the effect of alcohol on steroid synthesis in the ovaries. In these in vitro studies ethanol has been found to inhibit LH-stimulated secretion of estradiol and progesterone in human granulosa cells (Saxena et al 1990) possibly mediated by a decrease in LH-receptors (Wimalasena et al 1993). Adding to the complex picture, ethanol has also been reported to increase estradiol secretion during non-stimulated conditions in granulosa cells (Wimalasena et al 1993), as well as increase cAMP-stimulated estradiol and progesterone secretion in human trophoblast (Karl and Fisher 1993) and choriocarcinoma cells (Wimalasena 1994).

2.2.1.7 Effect of alcohol on sex steroid catabolism

The catabolism of sex steroids involve a series of reactions in the liver to transform them to water soluble conjugated forms that are consequently excreted in the urine (O´Malley and Strott 1999). The transformation includes the oxidation (inactivation) of the 17-hydroxyl group of testosterone and estradiol. This reaction, the oxidation of testosterone to androstenedione and the oxidation of estradiol to estrone, is catalysed by the 17β-hydroxysteroid dehydrogenase type 2 enzyme (17β-HSD2; Wu et al 1993, Andersson and Moghrabi, 1997) and in the reaction NAD^+ is reduced to NADH. The 17β-HSD2 enzyme has also been observed to possess 20α-hydroxysteroid dehydrogenase (20α-HSD) activity and catalyze the conversion of 20α-hydroxyprogesterone to progesterone (Wu et al 1993). The 17β-HSD2 enzyme is expressed in the microsomal fraction in many human tissues including liver, small intestine and colon, endometrium, placenta, kidney, breast, prostate (Casey et al 1994, Blomqvist 1995), and in the endometrium it has been found to be induced by synthetic progestins found in oral contraceptives (Tseng and Gurpide 1979). No data is available on the effect of progestins on 17β-HSD2 expression in the liver.

Androgens undergo ring A reduction which includes the reduction of the steroid A-ring by the NADPH-dependent 5α- or 5β-reductases and the reduction of the 3-keto group by the NADPH-dependent 3α-hydroxysteroid dehydrogenase (3α-HSD) enzyme (Dorfman and Ungar 1965, Penning et al 1996). As a result, androgens are transformed to androsterone and etiocholanolone (O´Malley and Strott 1999).

That alcohol oxidation is coupled to the reduction of conjugated 17-hydroxy- to 17-ketosteroids

in the human liver has been earlier confirmed using labelled ethanol (Andersson et al 1986a). An acute decrease during alcohol intake in androsterone and etiocholanolone, the main catabolic end products of androgens, has been reported in plasma (Cronholm and Sjövall 1970) and urine (Axelson et al 1981) among 3 to 6 men. An elevation during alcohol intake in the ratio of conjugated estradiol to estrone (sulphate and glucuronide conjugates) but not in the ratio of conjugated testosterone to androstenedione has also been demonstrated among four men (Andersson et al 1986b). Furthermore, among three pregnant women an acute alcohol-mediated increase in the ratio of 20α-hydroxy- and 20-ketosteroid monosulphates has been found (Cronholm et al 1969). Investigators have suggested that the shift in the ratio of NAD^+ to NADH in the liver might explain acute effects observed in unconjugated 17-hydroxy- and 17-ketosteroids (i.e. estradiol/estrone and testosterone/ androstenedione) as well although in these reports only an elevation in the corresponding 17-hydroxysteroid (i.e. estradiol and testosterone) has been demonstrated in women (Mendelson at el 1987, Ellingboe 1987) and in men (Phipps et al 1987).

Effects of alcohol on androgen conjugates has been reported among male alcoholics after withdrawal. An increase in the ratio of etiocholanolone to androsterone as well as an increase in the corresponding conjugated glucocorticoids (THF/aTHF) has been reported after withdrawal among alcoholic men (Cronholm et al 1994). This effect may be due to a decrease in 5α-reductase activity since a decrease in the liver 5α-reductase enzyme has been reported during long-term heavy alcohol intake in men (Gordon et al 1979).

2.2.2 Effect of alcohol on gonadotropins

No acute effects of alcohol have been demonstrated on plasma luteinizing hormone (LH) or follicle stimulating hormone (FSH) among premenopausal women (McNamee et al 1979, Mendelson et al 1981, Välimäki et al 1983, Mendelson et al 1987, Teoh et al 1988, Becker et al 1988, Mendelson et al 1989) or postmenopausal women (Mendelson et al 1985, Välimäki et al 1987).

Among non-cirrhotic female alcohol abusers with normal menstrual cycles LH and FSH levels are normal (Välimäki et al 1990b, Välimäki et al 1995). Low levels of LH and FSH in spite of low estrogen levels, but a normal response to LHRH, are found among female alcohol abusers with alcoholic liver disease indicating a disturbance at the hypothalamic level of the hypothalamic-pituitary-gonadal -axis (Hugues et al 1980, Välimäki et al 1984).

2.2.3 Effect of alcohol on prolactin

Among premenopausal women an elevation in plasma prolactin levels has been reported after acute intake of alcohol (Mendelson et al 1987, Teoh et al 1990) with a subsequent decline at two to four hours from the start of drinking (Välimäki et al 1983). Among postmenopausal women acute elevations in prolactin levels have been reported as well (Välimäki et al 1987, Ginsburg et al 1995). No effect of alcohol has, however, been observed during night-time (Ekman et al 1996).

The exact mechanisms of the effect of alcohol on plasma prolactin levels are unkown but they may involve the opioid peptides and dopamine which participate in the hypothalamic regulation of pituitary prolactin secretion (Tuomisto and Männistö 1985). The elevation in prolactin levels may, thus, reflect an activated opioid system as acute alcohol intake has been described to release β-endorphin peptides in the rat hypothalamus (Gianoulakis 1990) and elevate plasma β-endorphin in humans (Gianoulakis et al 1989). The subsequent decline in prolactin levels may, on the other hand, be the result of increased dopaminergic activity in the hypothalamus as has also been shown to occur in the rat (Ching and Lin 1994). Furthermore, the acute decreases in prolactin levels after intake of alcohol have been reported to be pronounced in males with a family history of alcohol dependence compared to controls suggesting a role of dopamine in the susceptibility to alcoholism (Schuckit et al 1983). A preliminary study on premenopausal women with and without a family history of alcoholism reported similar findings (Lex et al 1991).

Elevated plasma prolactin levels are commonly found in premenopausal female heavy drinkers (Mendelson and Mello 1988). Among premenopausal amenorrheic alcohol abusers with alcoholic liver disease prolactin levels have, however, been found to be normal compared with premenopausal controls but elevated compared with postmenopausal controls (Välimäki et al 1984). Among premenopausal non-cirrhotic alcohol abusers both elevated (Välimäki et al 1990b, Teoh et al 1992) and normal prolactin levels (Välimäki et al 1995) have been reported. Elevated prolactin levels have been reported among pregnant alcohol abusers as well (Halmesmäki et al 1987). The elevated prolactin levels observed among female heavy alcohol drinkers might be attributed to alcohol induced stress (see Delitala et al 1987 for review on stress and prolactin), the variability of which might explain the apparent discrepancies between the studies mentioned above.

2.2.4 Effect of alcohol on glucocorticoid steroids

2.2.4.1 Acute effect of alcohol on cortisol

Studies on healthy human volunteers including mainly men suggest that alcohol intake is associated with an activated hypothalamus-pituitary-adrenal axis mediated by an increase in the corticotropin-releasing factor (CRF) in the central nervous system (CNS) and subsequently elevated plasma cortisol levels (see Veldman and Meinders 1996 for review). The acute effects seem to occur rather late after the intake and/or mainly after higher doses (1.0-1.75 g/kg) in both women and men (Ylikahri et al 1978, Davis and Jeffcoat 1983, Välimäki et al 1984b, Gianoulakis et al 1989, Välimäki et al 1990a, Waltman et al 1993, Ekman et al 1994, Inder et al 1995) suggesting a link with hangover or withdrawal-induced stress. Although perfusion experiments with rat adrenals suggest a direct stimulating effect of alcohol on adrenal steroid synthesis (Cobb et al 1981) the overall evidence for a direct effect of alcohol on the adrenals in humans is insufficient (see Veldman and Meinders 1996 for review). The finding of a blunted plasma ACTH and cortisol in response to exogenous CRF, but no modulation of the cortisol response to exogenous ACTH, among healthy men after the intake of 0.75g/kg alcohol (Waltman et al 1993) adds to the complex picture of the effect of alcohol on the HPA-axis. Among male alcoholics a blunted ACTH-response to CRF has been reported during non-intoxicated conditions as well (Wand and Dobs 1991) suggesting the development of tolerance to the alcohol-induced HPA-activation during long-term heavy drinking.

Normal (Gianoulakis et al 1989, Gianoulakis et al 1996) and decreased cortisol as well as ACTH levels (Shuckit et al 1987, Schuckit et al 1988, Schuckit et al 1996) in response to alcohol intake has been reported among men with a family history of alcoholism compared with matched controls. Among women with a family history of alcoholism both elevated (Lex et al 1991) as well as normal levels (Gianoulakis et al 1989, Gianoulakis et al 1996) in response to alcohol intake has been reported. Differences have, however, been observed in the ACTH and cortisol response to the opioid receptor antagonist naloxone and adrenocorticotropin, but not to adrenal stimulation with the ACTH-analog cosyntropin, among men with a family history of alcoholism compared to matched controls suggesting a role of genetical factors and opioids in the CNS in the acute effect of alcohol intake on the HPA-axis (Waltman et al 1994, Wand et al 1999a, Wand et al 1999b).

2.2.4.2 Long-term effect of alcohol on cortisol

In interventional studies addressing long-term alcohol intake (0.4-0.8g/kg/day) both elevated and normal plasma cortisol levels have been observed among men and women (Prinz et al 1980, Bhathena et al 1995). Both elevated and normal cortisol levels in combination with or without altered circadian patterns are commonly found among alcoholic men during withdrawal and detoxification as well (Iranmanesh et al 1989, Wand et al 1991, Adinoff et al 1991). The prevalence rates for pseudo-Cushing´s syndrome among alcoholics range from less than 5% to 40% and there seems to be only a weak or no correlation between hormone levels and reported alcohol consumption (i.e., liver function tests) or between hormone levels and the clinical picture (see Veldman and Meinders 1996 for review). Most of the patients have normal plasma ACTH levels and a normal ACTH and cortisol response to the dexamethasone suppression test (Kirkman and Nelson 1988). In contrast to the classic Cushing´s syndrome, the signs and symptoms of pseudo-Cushing´s syndrome disappear within days to months of abstinence. In view of the literature pseudo-Cushing´s syndrome seems to be a CNS-mediated defect (Kirkman and Nelson 1988).

2.2.4.3 Effect of alcohol on cortisol-binding globulin (CBG)

The plasma cortisol is bound mainly to cortisol-binding globulin (CBG, transcortin) and to a minor extent to albumin with a small fraction (< 2%) remaining free and thus available for tissue uptake (Yen 1999). It is well known that the use of oral contraceptives increases cortisol and CBG levels due to the estrogen effect of the pill (Carr et al 1979, Kuhnz et al 1991, Wiegratz et al 1995). An acute effect of alcohol on the CBG protein level in plasma seems unlikely and there are no reports on this subject. There are also no reports on CBG levels among alcoholics with or without derangements in plasma cortisol. In one report alcohol was found to decrease cortisol binding (i.e., increase in free cortisol) to CBG, albumin, and lymphocyte glucocorticoid receptors in vitro (Hiramatsu and Nisula 1989).

2.2.4.4 Effect of alcohol on glucocorticoid steroid catabolism

The catabolism of glucocorticoids includes the transformation and conjugation of the steroid in order to be excreted in the urine (O´Malley and Strott 1999). An important step in the transformation is the oxidation (inactivation) of the 11-hydroxyl group of cortisol to cortisone in the human kidney and placenta (Albiston et al 1994, Krozowski et al 1999). The reaction is catalyzed by the NAD-dependent 11β-hydroxysteroid dehydrogenase type 2 isoenzyme (11β-HSD2). In the liver the $NADP^+$-dependent type 1 isoenzyme (11β-HSD1) is bidirectional but predominantly converts cortisone to cortisol (Tannin et al 1991, Krozowski et al 1999). Glucocorticoids (and androgens) undergo ring A reduction which includes the reduction of the A-ring by the NADPH-dependent 5α- or 5β-reductases and the reduction of the 3-keto group by the NADPH-dependent 3α-hydroxysteroid dehydrogenase (3α-HSD) enzyme (Dorfman and Ungar 1965, Penning et al 1996). As a results cortisol and cortisone are reduced to tetrahydro- and allotetrahydrocortisol (THF and aTHF) and respective cortisone metabolites (THE and aTHE) (O´Malley and Strott 1999). In addition, glucocorticoids undergo reduction at the 20-keto group by 20-hydroxysteroid dehydrogenase (20α-HSD and 20β-HSD) to form cortol and allocortol (cortolone and allocortol from cortisone)(Dorfman and Ungar 1965, McKerns 1969).

Although no acute changes in conjugated glucocortocoid metabolites has been observed during alcohol intake (Andersson et al 1986), abnormal conjugated urine glucocortocoid levels has been reported among male alcoholics during and after withdrawal. An increase in the ratio of THF/aTHF as well as an increase in the corresponding conjugated androgens (etiocholanolone/androsterone) has been reported after withdrawal among alcoholic men (Cronholm et al 1994). This effect may be due to a decrease in 5α-reductase activity since a decrease in the liver 5α-reductase enzyme has been reported during long-term heavy alcohol intake in men (Gordon et al 1979). An increase in the ratio of urine 20-hydroxy- to 20-ketosteroids has also been reported in male alcoholics during withdrawal (Cronholm et 1985) and suggests, on the other hand, an increased reduction of the 20-keto group of the conjugated glucocortocoids. Finally, an increase in the ratio of tetrahydrocortisol to tetrahydrocortisone (aTHF+THF/THE) has been reported in patients with alcoholic as well as non-alcoholic liver disease (Cronholm et al 1985, Cronholm et al 1994, Stewart et al 1993) but no effect was, however, observed among alcohol drinkers with no sign of liver injury (Mori et al 1991).

AIMS OF THE PRESENT STUDY

Alcohol-related endocrinological disorders are fairly common among alcoholic women but the mechanisms of the alcohol-induced changes in hormone levels are, however, unclear. The objective of this thesis was to study the effects of alcohol on hormones in premenopausal women and to elucidate mechanisms behind these effects. The aims were the following:

1. To study the acute effect of alcohol on steroids, LH and prolactin.
2. To study the mechanisms of the alcohol induced acute sex steroid changes in plasma and urine.
3. To study the kinetics of the alcohol induced androgen effects.
4. To investigate the acute androgen effects during moderate alcohol consumption.
5. To study the effect of alcohol on androgens and glucocorticoids in plasma and urine during moderate alcohol consumption at non-intoxicated conditions.
6. To study the effect of alcohol on ethinylestradiol found in oral contraceptives.

MATERIALS AND METHODS

4.1 Study subjects

In all the different studies healthy non-pregnant Caucasian female volunteers were recruited by advertising in a local newspaper and/or on a poster board at the dormitory of a local medical student association and/or by e-mail to different student organizations at the University of Helsinki. All studies were conducted in accordance with the guidelines proposed in The Declaration of Helsinki and approved by a local ethical committee. In addition, the use of 4-methylpyrazole for humans was approved by the Finnish National Agency for Medicines. A questionnaire for background data was sent to all subjects and participation was confirmed by obtaining signed informed consent together with the filled-in questionnaire. In addition, subjects were interviewed by phone in order to exclude subjects with alcohol abuse. All subjects included had a history of regular menses, and none used any medication other than oral contraceptives containing ethinylestradiol and a progestin (see original publications for doses). All subjects using oral contraceptives, but none of the subjects not using oral contraceptives, reported regular use for several months before entering the study. None of the study subjects had a record of hirsutism or any other disease.

4.2 Study procedures

The study design in all the substudies was a placebo-controlled interventional study with a cross-over design and the main outcome was the hormone level. The menstrual cycle phase of the participants was assessed by interview and hormone measurements as described in the original publications. In studies III-V the cycles of the participants with regular use of oral contraceptives were synchronized prior to the study and they were all provided with the same brand of an oral contraceptive containing 30 μg and 75 μg gestodene. Subjects received the different treatments (i.e. alcohol, 4-methylpyrazole and placebo) in random order and they were allocated in groups in order to keep the different treatments equally represented at each experimental session. All the treatments were given per os (see original publication for doses). 4-methylpyrazole was given double blind (see original publication for blinding procedures). Blood samples were taken from the cubital vein and spot urine samples collected before the treatment and at different time points during or after the different treatments.

4.3 Analytical procedures

Blood samples (10 ml) were collected into tubes containing 22.5 mg of sodium fluoride and 22.5 mg of potassium oxalate as anti-coagulants. Spot urine samples (40 ml) were collected in vials containing sodium azide (final concentration 1.0 g/l). Plasma and urine samples were stored at -70°C until determinations. All measurements were made in duplicate. Samples collected from one subject were measured in the same batch. No drift within batches was observed.

Ethanol levels were determined by headspace gas chromatography (Perkin Elmer, Sigma 2000). The intra-assay and inter-assay coefficients of variation were 4.0% and 5.1% at the level of 1.5 mmol/l (n=10), and the detection limit was about 0.02 mmol/l.

Testosterone levels (4-Androsten-17β-ol-3-one; within-assay variability 6.6% and between-assay variability 7.0% at the level of 0.96 nmol/l (n=10), detection limit 0.1 nmol/l), free testosterone levels (within-assay variability 4.3 % and between-assay variability 5.5 % at the level of 4.6 pmol/l (n=10), detection limit 0.5 pmol/l), androstenedione levels (4-Androsten-3,17-dione; within-assay variability 8.5% and between-assay variability 9.8% at the level of 5.3 nmol/l (n=10), detection limit 0.14 nmol/l), progesterone levels (4-Pregnen-3,20-dione; within-assay variability 7.9% at the level of 1.56 nmol/l and between-assay variability 8.1% at the level of 1.62 nmol/l (n=10)), cortisol levels (4-Pregnen-11β,17α, 21-triol-3,20-dione; within-assay variability 2.1% at the level of 146.0 nmol/l and between-assay variability 5.2% at the level of 31.2 nmol/l (n=10), detection limit about 7 nmol/l), Corticosteroid-Binding Globulin levels (CBG, within-assay variability 3.3% and between-assay variability 4.5% at the level of 107 μg/ml (n=15), detection limit about 0.3 μg/ml), estrone levels (1,3,5(10)-Estratrien-3-ol-17-one; within-assay variability 13.2% at the level of 32 pmol/l (n=14) and between-assay variability 6.8% at the level of 133 pmol/l (n=7)), luteinizing hormone levels (within-assay variability 2.9% at the level of 5.6 U/l and between-assay variability 11.0% at the level of 4.1 IU/l (n=10)), and prolactin levels (within-assay variability 9.5% at the level of 8 μg/l) were determined by standard radioimmunoassay (RIA) reagent sets (Orion Diagnostica, Finland, for testosterone, progesterone and cortisol; Diagnostic Products Corporation, Los Angeles, for free testosterone and androstenedione; BioSource Europe for CBG; Diagnostic System Laboratories (DSL-8700), USA, for estrone; Farmos Diagnostica, Finland, for LH; Kabi Pharmacia Diagnostics, Sweden, for prolactin).

Estradiol levels (1, 3, 5(10)-Estratrien-3,17β-diol; within-assay variability 9.7% at the level of 103 pmol/l and between-assay variability 5.1% at the level 394 pmol/l (n=10)) were determined for subjects not using oral contraceptives by a standard radioimmunoassay reagent set from Orion Diagnostica, Finland. In order to increase the sensitivity of the estradiol determinations for subjects

using oral contraceptives a standard radioimmunoassay reagent set from Diagnostic System Laboratories (DSL-39100), USA, was used (within-assay variability 14% at the level of 17 pmol/l and between-assay variability 22% at the level 30 pmol/l (n=5), detection limit 2.2 pmol/l).

Sex Hormone-Binding Globulin levels (SHBG, within-assay variability 5.5% and between-assay variability 6.9% at the level of 160 nmol/l (n=10), detection limit about 1.0 nmol/l) were determined using a standard non-competitive immunoradiometric assay (IRMA) reagent set (Orion Diagnostica, Finland).

Dehydroepiandrosterone levels (DHEA, 5-Androsten-3β-ol-17-one; within-assay variability 5.9% and between-assay variability 8.3% at the level of 7.6 nmol/l (n=38), detection limit 2 nmol/l) was determined by a method based on extraction into petroleum ether followed by a RIA assay using tritiated DHEA as the labelled antigen (Dehydroepiandrosterone,[1,2,6,7-^{3}H(N)]-, NET-814, 250 μCi/250μl ethanol, NEN^{R} Research Products) and Anti-DHEA (cat no. 07-129016 ICN) as the antibody. Sample and standard extracts were dissolved in buffer and incubated at +2 to +8°C overnight and dextran-coated charcoal (0.32% Norit A (active charcoal) and 0.032% Dextran T70 in 0.045 mol/l phosphate-buffered saline (PBS) with 0.1% gelatine, pH 7.0) was used to separate the bound and free steroids.

Dihydrotestosterone levels (5α-Androstan-17β-ol-3-one; within-assay variability 9.1% and between-assay variability 17.8% at the level of 2.5 nmol/l (n=10), detection limit 0.2 nmol/l) was determined as described (Apter et al 1976). Briefly, the method is based on extraction into ethylether-ethylacetate (7:3 v/v) twice followed by chromatographic separation on a Lipidex-5000 column (hydroxyalkoxypropyl Sephadex, petroleum-chloroform 98:2 as eluent) and a RIA assay using tritiated dihydrotestosterone (5α-dihydro(1,2,4,5,6,7-^{3}H)testosterone, Amersham TRK 443) as the labelled antigen and antisera raised in rabbits (for immunization procedures see Jänne et al 1976). Samples taken after start of drinking were pooled in equal volumes for dihydrotestosterone determinations.

Ethinylestradiol (17α-ethinyl-1,3,5-estratrien-3,17β-diol) was determined with an in-house radioimmunoassay method using antiserum from Biogenesis (UK, product 7010-7059), the standard from Steraloids, Inc. (UK, batch G745), and ethinylestradiol (17α-(6,7-^{3}H(N))) from NEN Life Science Products (NET-462) as the tracer. The tracer was purified prior to use on a 5 cm Sephadex LH20 column using methanol/toluene (15% v/v) as the eluent. Standards (112, 223, 447, 894, 1788, 3575 and 7150 pmol/l) were made in phosphate buffer (0.020 M, pH 7.4). Samples (250 μl) and standards (250 μl) were extracted with diethyl ether (2.7 ml). The organic phase was separated, evaporated to dryness with nitrogen, and redissolved in 250 μl of buffer. The recovery of the

extraction procedure was 93±9 % (n=7). One hundred microliters of tracer (concentration about 10000 cpm/100µl) and 100 µl of antiserum (4.5 ml distilled water added to lyophilised antiserum giving maximal binding of 40%) were added and incubated 30 min at 30 °C, and an additional 30 min at 4°C. Five hundred microliters of stirred charcoal (3.2 g charcoal (Norit A Neutral Pharmaceutical Grade, Drug & Chemical no. 64365-11-3) and 0.32 g dextran (Dextran T70, Pharmacia Fine Chemicals no. 17-0280-01) in 500 ml of buffer) were added and incubated 20 min at 4°C. The supernatant was separated by centrifugation at 2000g for 15 min, 3.6 ml of scintillation liquid (Opti Phase "HiSafe" III; Wallac OY, Turku, Finland) was added, and the radioactivity measured in a liquid scintillation counter. Ethinylestradiol was measured in plasma samples taken at time points 0,2,3,4 and 6 hours (time point 5 left out due to lack of antiserum). For accuracy, a control plasma sample was spiked to a final concentration of 1350 pmol/l (400 pg/ml). In the assay, a mean concentration of 1241 pmol/l (n=12) was obtained corresponding to a recovery (92%) comparable to the recovery of the extraction procedure. For specificity, no cross-reactivity was observed in samples from nine oral contraceptive users in the pill-free phase. According to information provided by the manufacturer the cross-reactivity of the antiserum with potential interfering steroids is negligible (all less than 1%). The intra-assay coefficient of variation was 13% (n=7) at the level of 326 pmol/l and the interassay coefficient of variation was 15% (n=6) at the level of 404 pmol/l. The detection limit of the assay (88 pmol/l) was determined by measuring 16 zero standards in a single run and the limit defined as the steroid level corresponding to the mean binding (B_0) - 3SD. The binding at the level of the detection limit (B/B_0) was 92% of the binding of the zero standards.

In order to check for possible changes of plasma androgen levels in vitro caused by alcohol as well as for possible interactions of alcohol with androgen assays, ethanol was added to fresh blood from 10 OC- and 6 OC+ 18 to 24 year old healthy female subjects to a final concentration of 10mM. No significant effects on androstenedione and total testosterone was observed. A negligible reduction of the dehydroepiandrosterone levels, confined to higher hormone levels, could be observed (16.8±2.9 nmol/l and 15.6±2.8 nmol/l, p=0.017, n=16), which was perhaps due to the procedure of extraction with petroleum ether.

Steroids in urine were determined by gas chromatography (GLC 8000 Top, CE Instruments, column SGE 25QC2/BP1 0.25, flame ionization detector, helium as the carrier gas) as earlier described (Hämäläinen et al 1990). The injector (splitless) and detector temperatures were 300 °C. The temperature program of the oven was the following: 160 °C/1 min, 160 to 205 °C (+79 °C/min), 205 to 230 °C (+1 °C/min), 230 to 300 °C (+5 °C/min) followed by 300 °C/15 min. The steroids were identified based on the retention time of standards and the specificity of the peaks in one sample from each subject was confirmed with gas chromatography-mass spectrometry (GLC 8000, Fisons, column

12QC2/BP1 0.25, detector: Fisons MD 1000). The neutral steroid profile obtained include the steroids displayed in Table 1 (original publication V). β-cortolone and β-cortol were contained in the same peak and the peak of 11β-hydroxyandrosterone was found to contain variable amounts of 17-hydroxypregnanolone. The accuracy of the method was checked by spiking urine samples with unconjugated standards (1.0, 5.0 and 15.0 μg/5 ml urine). The calculated recovery (hydrolysis excluded) was in the range of 80% to 129% for all steroids analyzed. The intra-assay coefficients of variation were in the range of 6.2% to 14.2% and those of inter-assay were in the range of 6.5% to 18.7% for all steroids at the levels found in the study samples.

The urine creatinine levels were determined with the VITROS 250 Chemistry System (Johnson & Johnson Clinical Diagnostics, USA). The intra-assay and inter-assay coefficients of variation were less than 5.0% at the level of 440 μmol/l (n=7) and the detection limit in the sample was 84 μmol/l.

4.4 Statistical methods

Results are reported as mean±SEM if not otherwise specified. The urine steroid to creatinine ratio was used to correct for dilution. Logarithmic transformations were occasionally used for urine steroid data to improve normality assumptions. Power calculations for matched-sample t´s were made as described (Howell 1992; see original publications for details). Statistical significance was tested using analyses of variance for repeated measures with drug (4-methylpyrazole/placebo), drink (alcohol/placebo), time and challenge (day 1/day 8) as within-group factors and oral contraceptive status as a between-group factor followed by paired t-test or Wilcoxon matched pairs test in case of non-normal distribution of data. F-values denote the drink per time interaction if not otherwise specified. In addition, Student´s t-test or the Mann-Whitney U-test, and Pearson´s or the Spearman´s rank-order correlations were used depending on distribution of data. In the correlation analyses the difference between the two r´s was tested by transforming r to r´ (Fisher´s transformation) as described (Howell 1992). Data was analysed using SPSS (version 10.0) and GraphPad Prism (version 2.0) statistical software.

RESULTS

5.1 Acute effect of alcohol on plasma androgen levels (II,IV,V)

An acute elevation in plasma total testosterone levels was observed in all the different studies among premenopausal women using oral contraceptives (OC+; F=40, p<0.001, figure 2 in II, and F=5.34, p=0.011, figure 6 in II; F=15.2, p=0.005, figure 1 in IV, and F=15.0, p<0.001, figure 4 in IV; F=15.2, p=0.005, figure 1 in V) as well as among non-users (OC-; F=7.9, p=0.008, figure 1 in II; F=4.6, p=0.002, figure 4 in IV) after intake of alcohol (**Figure 1**). An acute elevation relative to placebo was observed in 75% of the OC- subjects and in 95% of the OC+ subjects (II). The transient elevation closely followed the kinetics of plasma ethanol and returned to placebo levels when the ethanol had been eliminated (IV,V). The elevation in total testosterone was more pronounced among users of oral contraceptives compared with non-users (F=15.2, p<0.001, figure 1 and 2 in II; F=3.9, p=0.018, drink by time by oral contraceptive status interaction, figure 4 in IV).

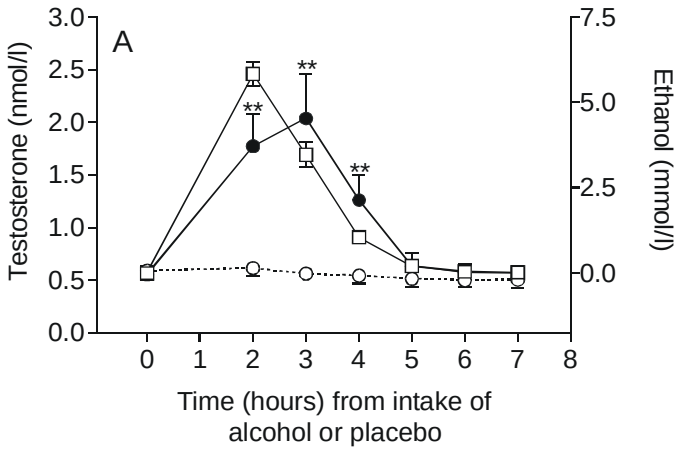

Figure 1. The acute effect of alcohol 0.4g/kg p.o.(closed circles) and placebo (open circles) on plasma testosterone levels in nine premenopausal women using oral contraceptives. Blood ethanol levels denoted with an open square. Mean±SEM. **p<0.01 compared with placebo. (Figure 1A in IV**,** reproduced with permission from The Endocrine Society)

The alcohol-induced elevation in total testosterone was reflected in the free testosterone fraction among both OC- (F=5.2, p=0.012 in II) and OC+ subjects (F=9.3, p=0.004 in II; F=5.2, p=0.013 in IV). The unbound fraction of testosterone expressed in percent as the ratio of free to total testosterone was seen to decline during alcohol compared with placebo sessions among both OC- (F=3.2, p=0.049, figure 3 in II) and OC+ subjects (F=4.3, p=0.018, figure 3 in II).

An acute decline in plasma androstenedione levels was observed among both OC- (F=8.3, p<0.001, figure 4 in II; F=3.5, p=0.01 in IV) and OC+ subjects (F=26.5, p<0.001, figure 4 in II; F=4.2, p=0.003, figure 1 in IV; F=10.0, p<0.001 in V). The transient decrease in androstenedione closely followed the kinetics of plasma ethanol and returned to placebo levels when the ethanol had been eliminated (figure 1 in IV, and V, **Figure 2**). A decline relative to placebo was observed in 69% of the OC- subjects and in 91% of the OC+ subjects (II).

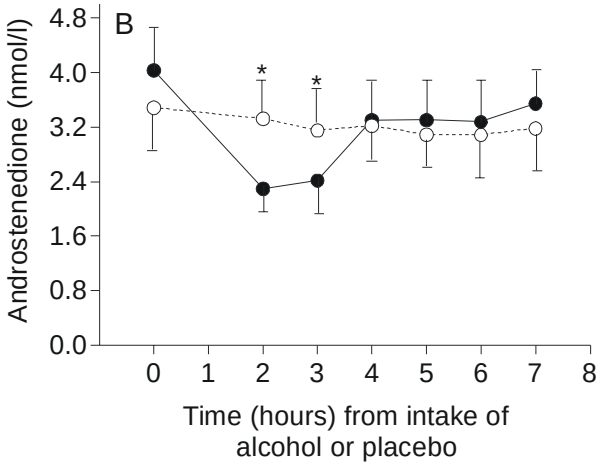

Figure 2. The acute effect of alcohol 0.4g/kg p.o.(closed circles) and placebo (open circles) on plasma androstenedione levels in nine premenopausal women using oral contraceptives. Mean±SEM. *p<0.05 compared with placebo. (Figure 1B in IV, reproduced with permission from The Endocrine Society)

The ratio of total testosterone to androstenedione was found to be elevated during alcohol relative to placebo sessions among both OC- subjects (F=30.1, p<0.001 in II; F=7.0, p<0.001 in IV) and OC+ subjects (F=45.3, p<0.001 in II; F=6.7, p<0.001 in IV; F=34.7, p<0.001, figure 1 in V). The transient increase in the ratio closely followed the kinetics of plasma ethanol and returned to placebo levels when the ethanol had been eliminated (IV,V).

The magnitude of the elevation in total testosterone levels was observed to be similar after the rapid intake of 0.34 g/kg, 0.68 g/kg, and 1.02 g/kg alcohol p.o. among OC+ subjects (F=1.2, p=0.3, dose by time interaction, figure 6 in II, **Figure 3**).

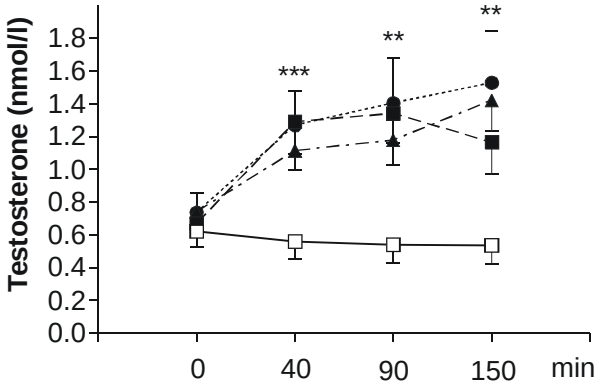

Figure 3. The acute effect of different alcohol doses (closed square: 0.34g/kg; closed pyramid: 0.68g/kg; closed circle: 1.02g/kg; all doses p.o.) and placebo (open square) on plasma testosterone levels in ten premenopausal women using oral contraceptives. Time from intake of alcohol or placebo. Mean±SEM. **p<0.01, ***p<0.001 compared with placebo (Figure 6 in II, reproduced with permission from Alcohol and Alcoholism)

After a seven day period of alcohol intake (0.8 g/kg/day p.o.), the transient acute effects in androstenedione and the ratio of testosterone to androstenedione were not statistically different than in the beginning of the period (F=2.6, p=0.15 for androstenedione in V; F=2.1, p=0.19 for the T/A -ratio, drink by time by experiment interaction, figure 1C and figure 1D in V), although a tendency for a shorter duration of the effect was observed for testosterone (F=3.6, p=0.087, drink by time by experiment interactions, figure 1A and figure 1B in V). After the seven day period an elevated baseline in testosterone was, however, observed with significantly elevated levels compared to placebo at non-intoxicated time points when ethanol had been eliminated (figure 1B in V). This difference in the baseline was, however, not observed in the ratio of testosterone to androstenedione (figure 1D in V and **Figure 4**).

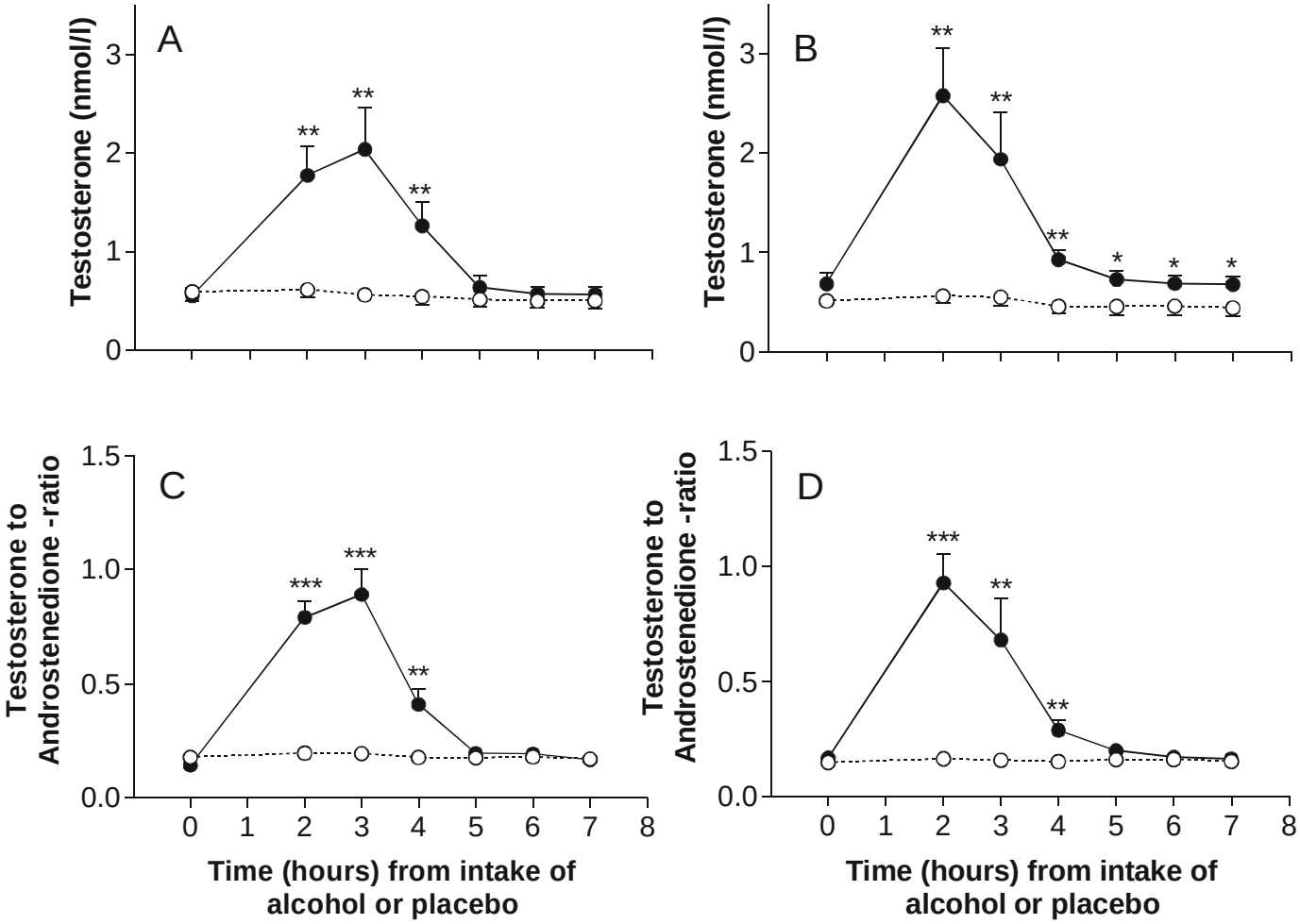

Figure 4. Plasma testosterone levels and the ratio of testosterone to androstenedione after acute intake of alcohol (0.4 g/kg p.o., filled circles) and placebo (open circles) before (A and C) and after (B and D) a seven day period of alcohol drinking (0.8 g/kg/day, mean±SEM, n=9). *p<0.05, **p<0.01, ***p<0.001 compared with the same time point. (Figure 1 in V, reproduced with permission from Elsevier Science)

The acute androgen effects in plasma were blocked during pretreatment with 4-methylpyrazole among both OC- and OC+ subjects (F=10.9, p<0.001, figure 4 in IV and **Figure 5** for testosterone; F=2.14, p=0.067 in IV for androstenedione; F=3.9, p=0.032 in IV for the testosterone to androstenedione -ratio; drug by drink by time interactions).

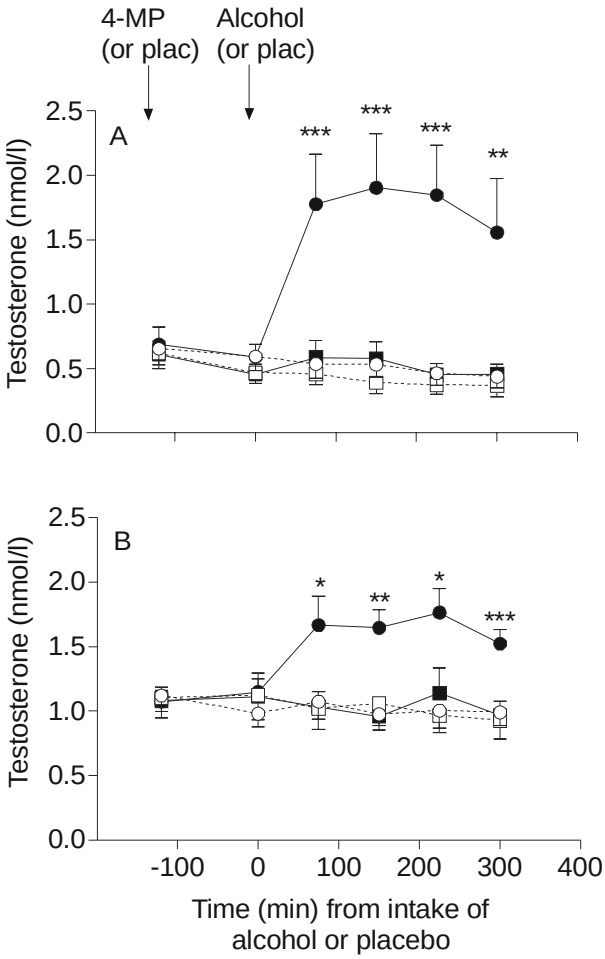

Figure 5. The acute effect of alcohol 0.5g/kg p.o. (closed symbols) and placebo (open symbols) during pretreatment with 4-methylpyrazole (squares) and placebo (circles) in 12 premenopausal women using oral contraceptives (A) and 10 premenopausal non-users (B). Mean±SEM. *p<0.05, **p<0.01, ***p<0.001 compared with the other treatments. (Figure 4 in IV, reproduced with permission from The Endocrine Society)

No acute effect of alcohol on plasma DHEA levels was observed among OC- (F=1.8, p=0.2, figure 5 in II) or OC+ (F=0.9, p=0.42, figure 5 in II) subjects compared to placebo although a decline was observed over time during both conditions (F=40.2, p<0.001, figure 5 in II).

No acute effect of alcohol in plasma dihydrotestosterone levels was observed among OC- (F=0.5, p=0.48, figure 5 in II) or OC+ (F=0.1, p=0.8, figure 5 in II) subjects compared with placebo although a decline was observed over time during both conditions (F=8.9, p=0.004, figure 5 in II).

In study II, the change in total testosterone levels (the average of changes seen at 45min and

90min) during placebo sessions was found to correlate with the change in androstenedione (r=0.49, p=0.003 for OC- and r=0.62, p=0.001 for OC+) and DHEA (r=0.31, p=0.07 for OC- and r=0.27, p=0.09 for OC+) levels. Among OC- subjects these correlations were similar during alcohol sessions (r=0.51, p=0.002 for androstenedione and r=0.29, p=0.08 for DHEA) whereas among OC+ subjects the correlations disappeared (r=-0.03, p=0.85 for androstenedione and r=-0.007, p=0.96 for DHEA). This reduction in the correlation coefficient among OC+ subjects was statistically significant for androstenedione (z=3.19, p=0.005) and a tendency was observed for DHEA (z=1.22, p=0.13).

5.2 Acute effect of alcohol on urine androgen and glucocorticoid conjugates (IV,V)

A decrease in urine androsterone (F=10.2, p=0.013 in IV) and etiocholanolone (F=13.7, p=0.006 in IV) levels was observed among OC+ subjects during alcohol intoxication (figure 2 in IV and **Figure 6**). After a seven day period of alcohol intake (0.8 g/kg/day p.o.), the acute effects on urine androsterone and etiocholanolone were not statistically different than in the beginning of the period (F=1.8, p=0.22 for androsterone; F=4.1, p=0.08 for etiocholanolone; drink by time by experiment interaction; unpublished observation). No acute effects in the other conjugated androgen, glucocorticoid or intermediate steroids, or in their ratios, which were included in the urine neutral steroid profile were observed during intoxication (IV,V).

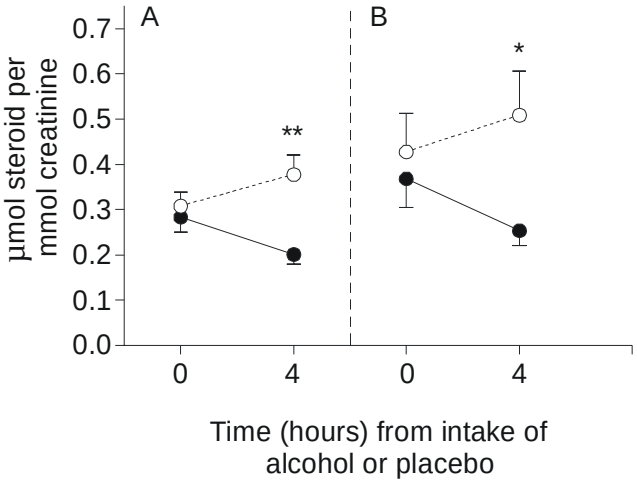

Figure 6. The acute effect of alcohol (filled circle) and placebo (circle) on urine androsterone (A) and etiocholanolone (B) levels in nine premenopausal women using oral contraceptives. Steroids expressed as the urine steroid to creatinine -ratio. Mean±SEM. * p<0.05 and ** p<0.01 compared with placebo. (Figure 4 in IV, reproduced with permission from The Endocrine Society)

5.3 Effect of long-term intake alcohol on plasma androgens and urine androgen conjugates (V)

In study V testosterone and androstenedione levels were investigated during a seven-day period of alcohol drinking (0.8g/kg/day p.o.). Plasma testosterone and androstenedione levels were elevated during the alcohol compared with the placebo drinking period in samples collected daily during non-intoxicated conditions at 4 p.m. ($F=5.8$, $p=0.04$ in V for testosterone; $F=7.1$, $p=0.03$ in V for androstenedione; drink effect; figure 2 in V and **Figure 7**). No effect was observed in the ratio of testosterone to androstenedione ($F=0.13$, $p=0.70$ in V; drink effect; mean ratio 0.16±0.04 at the beginning of the alcohol and placebo periods in V). However, in samples collected during non-intoxicated conditions at 8 a.m. at the beginning and the end of the drinking periods no significant differences in the plasma androgen levels were observed between alcohol and placebo (table 2 in V). A significant increase in plasma SHBG levels over time was observed ($F=51.9$, $p<0.001$; time effect) but the levels during alcohol and placebo drinking periods were not statistically different ($F=0.02$, $p=0.90$; drink effect; table 2 in V). No differences in samples collected daily in the morning (6.30-7.30 a.m.) during the alcohol and placebo periods were observed in urine androgen conjugates including androsterone, etiocholanolone and DHEA (see table 1 in study V for levels at beginning of the study period). The etiocholanolone to androsterone -ratio was not overall different between the alcohol and placebo periods ($F=0.7$, $p=0.4$ in V; drink effect), but a significant increase over time compared with the placebo ($F=2.4$, $p=0.025$ in V; drink per time interaction) with higher values during the post-alcohol period ($F=10.1$, $p=0.015$ in V; drink effect) was observed (figure 3 in V). No effects were observed in the intermediate urine metabolite conjugates including pregnanediol and pregnanetriol.

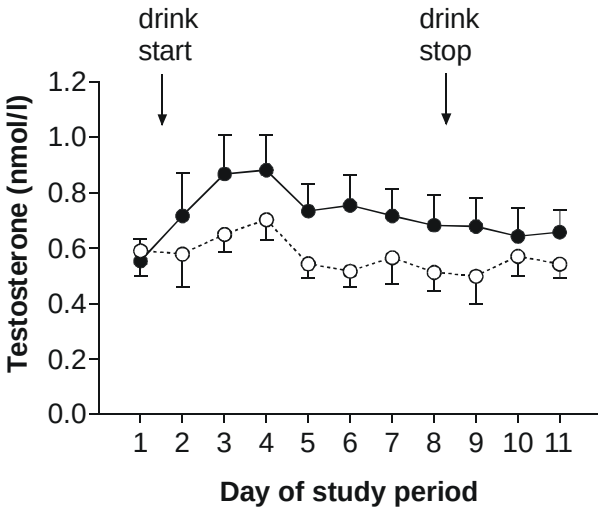

Figure 7. Plasma testosterone levels during alcohol (filled circles) and placebo (open circles) periods. Samples taken daily at 4 p.m.. Arrows denote the beginning and the end of the drinking period. Mean±SEM (n=9). (Figure 2 in V, reproduced with permission from Elsevier Science)

5.4 Acute effect of alcohol on plasma luteinizing hormone (II)

No significant effect of alcohol on LH levels was observed among OC- subjects (F=1.8, p=0.17, figure 5 in I). Among OC+ subjects a tendency for an overall effect was observed (F=2.4, p=0.11 in I) and in a subanalysis of menstrual cycle days 7 to 20 levels were significantly lower during alcohol compared with placebo at 120min (75±10% vs 112±10%, p=0.024, figure 5 in I).

5.5 Acute effect of alcohol on plasma estradiol, estrone, and ethinylestradiol (I,III)

A significant alcohol-mediated elevation in estradiol levels relative to placebo was observed among OC+ subjects (F=4.9, p=0.012, table 2 in I). No effect of alcohol was observed in estrone levels but the estradiol to estrone -ratio was significantly elevated relative to placebo (F=6.9, p=0.018 in I). No significant effect of alcohol on estradiol, estrone or the estradiol to estrone -ratio was observed among OC- subjects during midcycle (table 3 in I).

Ethinylestradiol levels were highly variable between OC+ subjects indicating considerable individual variation in its metabolism (III). No significant difference in the overall ethinylestradiol concentration was observed between the alcohol and placebo sessions (F=1.2, p=0.33 in III for drink effect at days 14 and 21 combined). An acute decline in the ethinylestradiol concentration over time was observed during both alcohol and placebo sessions (F=9.9, p=0.02 in III for time effect at days 14 and 21 combined). This decline was similar during both conditions (F=2.0, p=0.14 in III for drink by time interaction at days 14 and 21 combined). A tendency for higher levels at day 21 compared to day 14 of the menstrual cycle was observed (F=5.5, p=0.08 in III for challenge effect, figure 2 in III). At day 21 the ethinylestradiol levels were not statistically different between alcohol and placebo (F=3.6, p=0.12 in III for drug effect at day 21, figure 2 in III).

5.6 Acute effect of alcohol on plasma progesterone (I)

An alcohol-mediated decline in progesterone levels was observed among both OC+ and OC- subjects (I). A significant effect of alcohol on progesterone levels was observed in substudy A (F=3.4, p=0.04, table 2 in I) and tendencies in substudies B and C (F=1.79, p=0.17, table 2 in I and F=1.8, p=0.076 in I) among OC+ subjects. With relative values the effects were, however, significant in all

substudies with lower progesterone levels relative to placebo (F=4.3, p=0.049 for substudy A in I; F=4.1, p=0.050 for substudy B in I; F=6.7, p=0.002 for substudy C in I; table 2 and figure 3 in I). No effect of alcohol dose was observed (figure 3 in I). Among OC- subjects a decline in progesterone levels was observed in substudy A (F=10.1, p=0.004, table 3 in I) and a tendency for a decline in substudy B (F=1.9, p=0.16, table 3 in I). In the overall analysis with substudies A and B combined, the effect was significant (F=5.7, p=0.004 in I) among OC- subjects.

5.7 Acute effect of alcohol on plasma prolactin (I)

A transient increase in prolactin levels in the beginning of alcohol intoxication relative to placebo was observed (I). Among OC+ subjects a significant effect was observed in substudy A (F=8.1, p<0.001, figure 4 and table 2 in I). Among OC- subjects an overall significant effect was found with the two substudies combined (F=3.2, p=0.046; figure 4 and table 3 in I).

5.8 Acute effect of alcohol on plasma cortisol (I)

No significant differences in cortisol levels during alcohol and placebo sessions were observed for OC- and OC+ subjects in the separate substudies (tables 2 and 3, figure 3 in I) or with substudies A and B combined (F=1.5, p=0.23 in I for OC- subjects; F=0.2, p=0.82 in I for OC+ subjects).

5.9 Effect of long-term intake of alcohol on plasma and urine glucocorticoids (V)

Plasma cortisol levels were not statistically different during alcohol and placebo drinking periods in plasma samples collected daily during non-intoxicated conditions at 4 p.m. (F=2.2, p=0.18 in V for drink effect). Significantly higher levels where, however, observed during the three-day follow-up after alcohol compared with placebo (F=33.7, p<0.001 for drink effect, figure 2 in V and **Figure 8**). In plasma samples collected during non-intoxicated conditions at 8 a.m. at the beginning and the end of the drinking periods a tendency was observed for a drug by time interaction (F=4.3, p=0.07 in V) and a significant difference with higher levels during alcohol than placebo emerged at the end of the drinking period (table 2 in V). A significant increase in plasma CBG levels over time was observed

(F=19.8, p=0.002 in V for time effect) but the levels during alcohol and placebo periods were not statistically different (F=0.85, p=0.38 for drink per time interaction, table 2 in V). In urine samples collected daily in the morning (6.30-7.30 a.m.) no difference between alcohol and placebo was observed in the total urine conjugated glucocorticoids during the drinking period (F=1.6, p=0.3 in V for drink effect) or the three-day follow-up period (F=0.3, p=0.6 in V for drink effect). Minor non-significant tendencies were observed in the single glucocorticoid conjugates. Statistically significant differences emerged, however, when examining the ratios of the different conjugates. The tetrahydrocortisol/ allotetrahydrocortisol -ratio (F=9.6, p=0.021 in V for drink effect) as well as the 20-hydroxy/20-ketosteroid -ratio (F=10.7, p=0.014 in V for drink effect) were significantly higher during alcohol compared with the placebo drinking period (figures 3 and 4 in V). No consistent effect was observed in the ratio of (allotetrahydrocortisol + tetrahydrocortisol)/tetrahydrocortisone probably due to a difference in the beginning of the study periods (figure 4 in V).

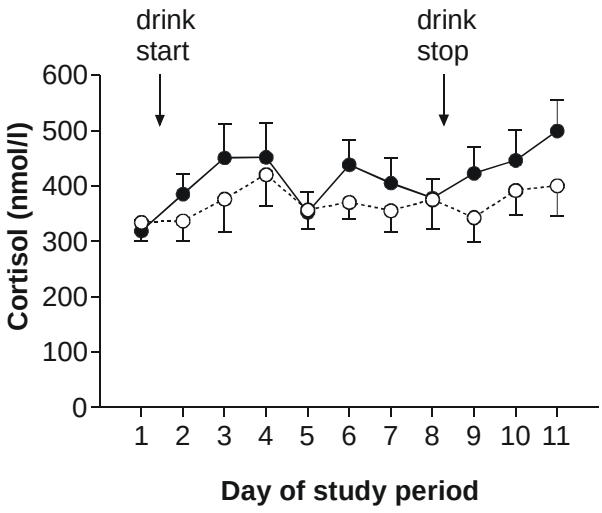

Figure 8. Plasma cortisol levels during alcohol (filled circles) and placebo (open circles) periods. Samples taken daily at 4 p.m.. Arrows denote the beginning and the end of the drinking period. Mean±SEM (n=9). (Figure 2 in V, reproduced with permission from Elsevier Science)

5.10 Effect of oral contraceptives on hormone levels (I,II,IV)

Plasma total and free testosterone levels were lower among OC+ subjects than OC- subjects in the midcycle phase (figures 1 and 2 in II; figure 4 in IV). Also the ratio of free to total testosterone was lower among OC+ subjects compared with OC- subjects (figure 3 in II). Plasma androstenedione, DHEA, dihydrotestosterone, estradiol, estrone, and progesterone levels were all lower among OC+ subjects than OC- subjects in the midcycle phase (figure 4 in II androstenedione, figure 5 in II for DHEA and dihydrotestosterone; table 1 in I for estradiol, estrone and progesterone). The ratio of testosterone to androstenedione was, however, similar among OC- and OC+ subjects (II,IV). LH levels were significantly lower among OC+ subjects than in OC- subjects (table 1 in II) and a decline was observed at the start of taking the oral contraceptive at day 8 of the menstrual cycle (figure 5 in II).

The baseline prolactin levels were correlated with the day of menstrual cycle among OC+ subjects ($r=0.43$, $p=0.02$; prolactin=4.9+0.12 x day, substudy A in II) and were significantly elevated relative to OC- subjects in the midcycle phase (table 1 in II). The baseline cortisol levels were significantly higher among OC+ than in OC- subjects in the midcycle phase (table 1 in II). The levels did not correlate with the day of menstrual cycle ($r=0.04$, $p=0.83$ for OC- and $r=0.17$, $p=0.36$ for OC+ in II).

5.11 Plasma ethanol levels after intake of alcohol: effect of long-term alcohol intake and pretreatment with 4-methylpyrazole (I-V)

In the different studies no ethanol (i.e. >0.04 mmol/l) was detected in plasma before intake of alcohol or placebo (I-V). An increase in the ethanol elimination rate of 24% (from 0.090±0.013 to 0.112±0.013 g/kg*h, $p=0.006$) was observed between the beginning (day 1) and the end (day 8) of the alcohol drinking period (0.8 g/kg/day) as calculated from the linear part of the elimination curve by extrapolation to zero ethanol (figure 1 in III, and V). No ethanol (i.e. > 0.04 mmol/l) was detected in the afternoon and morning blood samples collected during the alcohol drinking period (III,V).

During pretreatment with 4-methylpyrazole a decrease in the alcohol elimination rate was observed among both premenopausal women using oral contraceptives (33±13% decrease, from 0.087±0.008 to 0.056±0.008 g/kg*h, $p<0.001$) as well as among premenopausal non-users (31±8% decrease, from 0.077±0.013 to 0.053±0.008 g/kg*h, $p<0.001$) as determined by extrapolation of the pseudolinear part of the elimination curve to zero ethanol (figure 3 in IV).

DISCUSSION

The present work shows that alcohol intake is associated with changes in the hormonal balance in women. To the best of the authors knowledge, the original publications included in the thesis are the first reports on the effect of alcohol on the hormone balance in women using oral contraceptives. Furthermore, this is the first study including the use of 4-methylpyrazole in humans in vivo to elucidate the role of ethanol metabolism in the actions of alcohol on steroids.

6.1 Acute effect of alcohol on sex steroids

An acute elevation in plasma testosterone was observed after intake of alcohol among both premenopausal women using oral contraceptives as well as among non-users. The increase in the testosterone level was accompanied by a decrease in the androstenedione level in plasma as well as a decrease in the urine etiocholanolone and androsterone levels, the principal catabolic products of androgens. The effects lasted throughout the period of ethanol elimination, and were abolished during pretreatment with 4-methylpyrazole, an alcohol dehydrogenase (ADH) inhibitor. The testosterone effect in plasma was reflected in the free testosterone fraction as well. The magnitude of the alcohol induced elevation was similar after the intake of different amounts of alcohol and the data presented provides evidence for the lack of tolerance to the magnitude of the acute testosterone elevation in premenopausal women at a consumption level of five to six drinks a day.

The result suggests that the acute effect exerted by alcohol on testosterone is partly compensated by binding as the ratio of free to total testosterone was observed to decline after alcohol intake among both the oral contraceptive users as well as the non-users (i.e. the increase in free testosterone was smaller than what would have been expected from the increase in total testosterone). Testosterone is bound to SHBG and albumin in the ratio of 2:1 and the rate of the binding is rapid: the dissociation coefficient (t 1/2) for testosterone is only 20s for SHBG and 4s for albumin (Yen 1999). The levels of SHBG and albumin were not measured during acute alcohol intoxication, since acute changes in these proteins during alcohol intake seem unlikely during the present short time interval.

Testosterone levels can be affected either by changes in its synthesis in the adrenals or gonads, through changed peripheral conversion from androstenedione and DHEA(S) or through changed catabolism in the liver (Yen 1999). Since alcohol intake had no effect on DHEA, of which a major

part is of adrenal origin (Yen 1999), it seems that the testosterone elevation may not be caused by an acute increase in adrenal androgen synthesis. The findings that the testosterone effect was not alcohol dose dependent, that the effect follows the kinetics of plasma ethanol, and that it is blocked by 4-methylpyrazole implies that the mechanism of the testosterone elevation is coupled to the zero order mechanism of ethanol elimination and mediated by the change in the redox state. It is well known that this effect is rather constant during different dose and time conditions of ethanol oxidation (Forsander 1970). The facts that alcohol intake is associated with a decline in androstenedione levels, an elevation in testosterone levels, and an elevation in the testosterone to androstenedione -ratio suggest, on the other hand, a decrease in the testosterone to androstenedione conversion or an increase in the androstenedione to testosterone conversion. The positive correlation observed between the change in testosterone levels and the change in androstenedione levels over time after the intake of placebo can be explained by the similar circadian rhythm of these hormones in premenopausal women (Yen 1999). The fact that this positive correlation was significantly reduced during alcohol conditions among women using oral contraceptives, i.e. the group displaying the more pronounced testosterone effect, provides further evidence of a decreased testosterone to androstenedione or increased androstenedione to testosterone conversion superimposed on the circadian hormonal changes.

No acute effect of alcohol on dihydrotestosterone levels was observed during the present dose and time conditions. In view of the testosterone effect one could expect an elevation in dihydrotestosterone as well. The lack of an elevation in dihydrotestosterone is, however, not surprising in view of the fact that in women the main source of dihydrotestosterone in plasma is androstenedione with less than 20 per cent being derived from testosterone (Ito and Horton, 1971). The present dihydrotestosterone finding may thus be the net result of a decline and an elevation caused by androstenedione and testosterone, respectively.

That alcohol intake may be associated with an acute elevation in plasma estradiol levels in premenopausal women (Välimäki et al 1983, Mendelson et al 1987, Mendelson et al 1988, Teoh et al 1988, Mendelson et al 1989, Teoh et al 1990) as well as in postmenopausal women on estrogen replacement therapy (Ginsburg et al 1996), has earlier been demonstrated. Furthermore, estradiol has in both interventional and cross-sectional studies been positively associated with alcohol intake (Gavaler et al 1992, Reichman et al 1993, Muti et al 1998) providing a possible link between alcohol consumption and breast cancer (Smith-Warner et al 1998). In our study, the elevation in estradiol was only observed among women using oral contraceptives. No effect of alcohol was observed among premenopausal women not using oral contraceptives.

The present study provides evidence for the lack of an effect of alcohol on plasma ethinylestradiol in premenopausal women. No significant effects were observed after acute and long-term intakes of alcohol. A possible small effect can, however, not be excluded as this study was able to detect only major effects (e.g. a difference of 30% to 50% from mean) due to high interindividual variation and a fairly small sample size. Effects of lower magnitude may not be of clinical significance considering the variations in levels occurring during the day among oral contraceptive users.

The results from the present study indicate that alcohol intake is associated with an acute decline in progesterone levels among women using oral contraceptives as well as among non-users, and that the progesterone effect is not dose dependent. Earlier studies, with considerably lower statistical power, have demonstrated similar progesterone effects only during stimulatory conditions among premenopausal women not using oral contraceptives (Teoh et al 1990).

6.1.1 Coupling of alcohol and steroid metabolism

Ethanol oxidation has earlier been shown to be coupled to steroid reduction in the liver using labelled ethanol (Andersson et al 1986a). More specifically, ethanol oxidation was shown to cause an increased rate of the reduction catalysed by the liver NAD-dependent 17β-hydroxysteroid dehydrogenase type 2 enzyme (Wu et al 1993, Andersson and Moghrabi 1997) with a secondary change in the equilibrium between conjugated 17-hydroxy- and 17-ketosteroids. During normal conditions the 17-hydroxysteroid is oxidized to the 17-ketosteroid form and in the reaction NAD^+ is reduced to NADH. The competitive situation during alcohol intake seems, however, to be in favour of alcohol oxidation during which the elevated NADH level (and the decreased NAD^+) leads to an increased 17-ketosteroid to 17-hydroxysteroid -reaction. These findings on the conjugated steroids in men are similar to the present results on unconjugated testosterone/androstenedione and estradiol/estrone in premenopausal women. The finding that the effects in the present study were pronounced among women using oral contraceptives may be explained by the fact that the 17β-hydroxysteroid type 2 enzyme is induced by the synthetic progestins found in the contraceptive preparation (Tseng and Gurpide, 1979). Furthermore, this isoenzyme has also been found to catalyse the oxidation of 20α-dihydroprogesterone to progesterone (Wu et al 1993). Thus, the present results suggest that the sex steroid effects are the result of an alteration in the metabolism in the liver, i.e., a decreased overall oxidation of testosterone, estradiol (and 20α-dihydroprogesterone presumably) due to the increased reduction of androstenedione, estrone and progesterone, mediated by the alcohol-

induced elevation in the liver NADH to NAD^+ ratio (**Figure 9**). This mechanism provides also an explanation for the 3-fold acute transient elevation in estradiol caused by alcohol among postmenopausal women on estrogen replacement therapy (Ginsburg et al 1996). Interestingly, the 17β-hydroxysteroid dehydrogenase type 2 enzyme has also been found to be enriched in breast glandular epithelial cells (Blomquist 1995). An alcohol-induced redox change in the breast tissue leading to a local increase in estradiol could in addition to the overall estradiol elevation explain the link (Smith-Warner et al 1998) between alcohol consumption and the development of breast cancer.

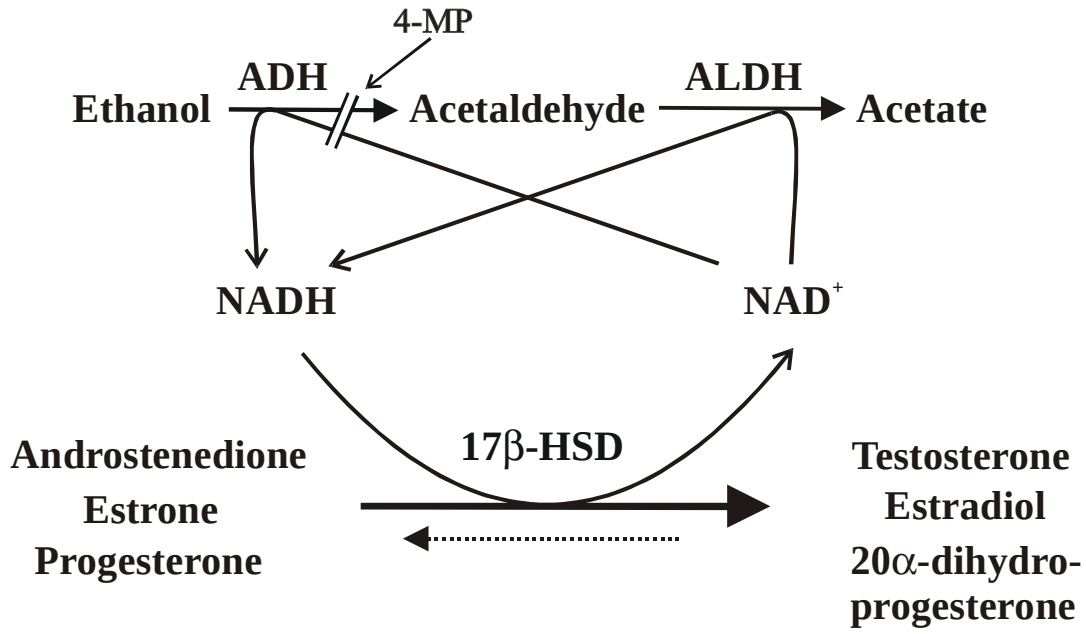

Figure 9. Action of ethanol on the steroid metabolism in the liver. ADH, Alcohol dehydrogenase; ALDH, Aldehyde dehydrogenase; 4-MP, 4-Methylpyrazole; NAD^+, oxidized nicotinamide adenine dinucleotide; NADH, reduced nicotinamide adenine dinucleotide; 17β-HSD2, 17β-Hydroxysteroid dehydrogenase type 2. The dashed arrow denotes a reduced reaction rate.

No statistically significant effect of tolerance to the alcohol-induced testosterone elevation was observed in the present study at a consumption level of five to six standard drinks a day. With a view to the suggested mechanism, metabolic tolerance with regards to the magnitude of the testosterone elevation might, however, be expected among alcoholics with inadequate nutrition and decreased alcohol dehydrogenase activity (Salaspuro et al 1978). Furthermore, among heavy drinkers with an increased CYP2E1-mediated alcohol elimination rate (Lieber et al 1999) metabolic tolerance would be expected to occur with a shortening of the duration of the testosterone elevation. An increased alcohol elimination rate combined with a tendency ($p=0.087$) for the shortening of the duration of the

acute testosterone effect was, in fact, observed in the present study.

That acute alcohol intake leads to decreased levels of testosterone in normal healthy men has been reported in a number of papers (Ylikahri et al 1974, Mendelson et al 1977, Välimäki et al. 1990) and these studies as well as studies carried out in the rat testis (Cicero et al 1980, Cobb et al 1980, Orpana et al 1990) provide evidence that this effect may be mediated by an inhibited testosterone synthesis. In women, in vitro studies indicate that alcohol may alter the steroid secretion of human granulosa cells as well (Saxena et al 1990). In our view, the acute elevation in the testosterone to androstenedione -ratio is not in contradiction with these earlier reports and would rather suggest an additional site in the acute actions of alcohol on steroids in humans. Thus, it is proposed that the effect on androgens in the venous blood is the net effect of an inhibited catabolism in the liver and an inhibited synthesis in the gonads, the result which will depend on different hormonal, dose and time conditions. In view of our results, the former effect is not dose dependent and seems to occur from the beginning of alcohol intoxication. The latter phenomenon may occur mainly after larger doses, e.g. 1.5 g/kg p.o. or more, and during the late descending phase of alcohol elimination or when the alcohol has been completely eliminated and the NAD^+ to NADH shift in the liver has recovered.

Ethinylestradiol is easily absorbed from the gastrointestinal tract, reaches peak levels within one to two hours from intake and has a elimination half-life of 13-27 hours (Brenner et al 1980, Goldzieher et al 1990). High inter- and intraindividual variance in the pharmacokinetics of the steroid is observed (Brenner et al 1980, Goldzieher et al 1990, Stanczyk et al 1990, Kuhnz et al 1991). The major pathway in the metabolism of ethinylestradiol involves 2-hydroxylation catalyzed by the CYP3A4 enzyme and 2-methylation catalyzed by the catechol o-methyltransferase enzyme (Guengerich 1990). Different metabolites are excreted in the urine mainly as glucuronide conjugates (Williams et al 1975). The metabolism of ethinylestradiol differs from that of 17β-estradiol in that the 17α-ethynyl group inhibits the oxidation of the 17-hydroxyl group of 17β-estradiol to estrone. Thus, it is suggested that the lack of an acute effect of alcohol on ethinylestradiol, comparable to that observed for estradiol in postmenopausal women on estrogen replacement therapy, may be due to the protection of the hydroxyl group at the 17-position.

6.2 Acute effect of alcohol on luteinizing hormone

No acute effect of alcohol on luteinizing hormone levels was observed among women not using oral contraceptives. However, among women using oral contraceptives an overall decreasing tendency was found and in a subanalysis of subjects in the midcycle phase a significant decline was

observed at 120min from intake. This finding is in contrast with earlier reports where no effects of alcohol have been observed in women or men (McNamee et al 1979, Mendelson et al 1981, Välimäki et al 1983, Mendelson et al 1987, Teoh et al 1988, Becker et al 1988, Mendelson et al 1989, Välimäki et al 1990a). The fact that none of the studies included women using oral contraceptives may well explain this discrepancy. The decline in LH levels might, at least in part, be mediated by a feedback response to the alcohol-induced increase in testosterone among these women.

6.3 Acute effect of alcohol on prolactin

A transient effect of alcohol on prolactin levels compared with the placebo was observed with a significant elevation at 60 min followed by a tendency for a decline at 120 min from the start of intake. The smaller difference in the levels in the second substudy indicates that the elevating effect of alcohol is short and peaks close to 60 min from the start of drinking. These findings are in line with the earlier studies where alcohol was found to elevate prolactin during human chorionic gonadotropin and naloxone-stimulation (Mendelson et al 1987, Teoh et al 1990) as well as among postmenopausal women (Ginsburg et al 1995). A decline at two to four hours from the start of drinking has also been described (Välimäki et al 1983). The exact mechanism of the prolactin effect cannot be provided by the present data but may involve the opioid peptides and dopamine which participate in the hypothalamic regulation of pituitary prolactin secretion (see Tuomisto and Männistö 1985 for review). The elevation in prolactin levels may, thus, reflect an activated opioid system as acute alcohol intake has been described to release β-endorphin peptides in the rat hypothalamus (Gianoulakis 1990) and elevate plasma β-endorphin in man (Gianoulakis et al 1989). The subsequent decline in prolactin levels may, on the other hand, be the result of an increased dopaminergic activity in the hypothalamus as has also been shown to occur in the rat (Ching and Lin 1994).

6.4 Acute effect of alcohol on plasma cortisol and urine glucocorticoids

No significant effects were observed in cortisol levels during the present dose and time conditions. The finding supports earlier conclusions that an acute effect of alcohol on cortisol levels can mainly be observed only late after the intake of higher doses (Ekman et al 1994) and may thus be due to withdrawal-induced stress (see review of literature).

6.5 Effect of long-term alcohol intake on plasma androgens

The elevated testosterone and androstenedione levels observed at non-intoxicated time points during the alcohol drinking period (0.8 g/kg/day) may not be explained by acute redox changes in the liver metabolism as no ethanol was present and no effect on the ratio of testosterone to androstenedione was observed. These findings are in line with earlier reported positive associations between reported alcohol consumption and plasma androgen levels among women consuming moderate amounts of alcohol (Dorgan et al 1994, Cigolini et al 1996) as well as among non-cirrhotic female alcohol abusers attending detoxification programs (Pettersson et al 1990, Välimäki et al 1990b, Välimäki et al 1995). In the present study elevated plasma testosterone and androstenedione levels were, however, observed only in the afternoon (4 p.m.) but not in the morning (8 a.m.) during the alcohol drinking period. No effects were observed in the conjugated androgen metabolites in the morning urine samples. This is in line with an earlier report where no effect on testosterone was observed in plasma collected in the morning after a 3-month-period of controlled intake of two to three standard drinks a day (Reichman et al 1993). Thus, the result suggests that alcohol intake may in addition to the transient acute redox-mediated effects alter circadian patterns of androgen levels in women perhaps by an effect on the hypothalamic-pituitary-adrenal axis. This is not surprising in view of the fact that in women, and in women using oral contraceptives with diminished ovarial function in particular, a substantial part (25-50%) of plasma androgens are derived from the adrenals (Yen 1999) the function of which has marked circadian variability (Lachelin et al 1979). Changes in the circadian patterns of plasma adrenal steroids have been reported earlier among male alcoholics (Iranmanesh et al 1989, Adinoff et al 1991).

No effect of alcohol was observed in plasma sex hormone-binding globulin in the present study. This may be due to the relatively low level of alcohol consumption (0.8 g/kg/day for seven days) during which there was no objective sign of liver injury as measured by serum γ-glutamyltransferase (see methods section in V). An elevation over time was, however, observed during the placebo and the alcohol drinking period which may be explained by the estrogen effect of the oral contraceptive (Jung-Hoffman et al 1988, Kuhnz et al 1991, Wiegratz et al 1995).

6.6 Effect of long-term alcohol intake on plasma cortisol

No effect of alcohol on plasma cortisol or urine total cortisol metabolites was observed during the alcohol drinking period (0.8 g/kg/day) in the present study. However, a tendency for an elevation in plasma levels over time during the alcohol period compared with the placebo was found reaching significance during the post-drinking period. Elevations in plasma cortisol levels have earlier been reported mainly following the acute intake of higher alcohol doses (1.0-1.5 g/kg; Välimäki et al 1990a, Ekman et al 1994). In interventional studies addressing long-term alcohol intake (0.4-0.8 g/kg/day) both elevated (Bhathena et al 1998) and normal (Prinz et al 1980) plasma cortisol levels have been observed. Among alcoholics elevated cortisol levels are frequently found during withdrawal and detoxification (Iranmanesh et al 1989, Wand et al 1991, Adinoff et al 1991). Thus, the elevation observed in the present study might be explained by an activation of the hypothalamo-pituitary-adrenal -axis due to withdrawal-induced stress although clinical symptoms where not recorded.

No effect of alcohol was observed in plasma cortisol-binding globulin. A similar elevation was observed during the placebo and alcohol drinking period which may be explained by the effect of the oral contraceptive.

6.7 Effect of long-term alcohol intake on urine androgen and glucocorticoid conjugates

The catabolism of steroids involve a series of reactions to transform them to water soluble conjugated forms that are consequently excreted mainly in the urine (O´Malley and Strott 1999). Changes during the alcohol drinking period were observed in the ratio of certain conjugated androgen and cortisol metabolites in the urine indicating changes in the metabolism of these steroids. This is perhaps not surprising in view of the observed increase in the alcohol elimination rate which may be explained by an induced CYP2E1-pathway (Lieber 1999). The increase in the ratio of etiocholanolone to androsterone as well as the increase in the ratio of tetrahydrocortisol to allotetrahydrocortisol (THF/aTHF) suggests a decrease or increase in the NADPH-dependent 5α-reductase or 5β-reductase, respectively. Of the two alternatives a decrease in the 5α-reductase activity seems more probable since a decrease in the liver 5α-reductase enzyme has been reported during long-term heavy alcohol intake (Gordon et al 1979). The present finding is also in line with increases in the same urine ratios in male alcoholics (Cronholm et al 1994). The increase in the ratio of 20-hydroxy- to 20-ketosteroids

observed in the present study and earlier among male alcoholics (Cronholm et al 1985) suggests, on the other hand, an increased reduction of the 20-keto group of the conjugated glucocortocoids. This reaction occurring in the liver and kidney is NADPH-dependent (McKerns 1969). No consistent effect in the ratio of tetrahydrocortisol to tetrahydrocortisone (aTHF+THF/THE) was observed in the present study. An increase in this steroid ratio has been reported in patients with alcoholic as well as non-alcoholic liver disease (Cronholm et al 1985, Stewart et al 1993, Cronholm et al 1994) but no effect was, however, observed among alcohol drinkers with no sign of liver injury (Mori et al 1991). The apparent discrepancy with the present results may then be explained by differences in the alcohol consumption level, the absence of liver injury, and/or gender factors.

6.8 Effect of oral contraceptives on the hormonal balance

It is well known that oral contraceptives alter the sex-hormone balance both by interfering with the release of gonadotropin-releasing hormone from the hypothalamus as well as by suppressing pituitary release of LH and FSH with diminished ovarial activity as a result (Mishell 1999). A decrease in LH after the initiation of the oral contraceptive approximately at day 8 of the cycle was observed in the present study. Among women using oral contraceptives the levels of estradiol and progesterone were similar or even lower than those observed in the follicular phase of a normal cycle. The reduced testosterone and androstenedione levels may be explained by an inhibition of the ovarian function as well (Jung-Hoffman et al 1988). The exact mechanism of the reduced basal DHEA level, which in major parts originates from the adrenals (Yen 1999), is unknown, but may be explained by the estrogen-induced elevation in plasma cortisol leading to decreased plasma ACTH (Carr et al 1979) and, as a consequence, a decreased DHEA synthesis. The fact that the use of oral contraceptives is associated with an estrogen-mediated increase in sex hormone-binding globulin (Jung-Hoffman et al 1988, Kuhnz et al 1991, Wiegratz et al 1995) may well explain the finding of a reduced free to total testosterone -ratio.

The elevation in cortisol binding globulin as well as the high plasma cortisol may be explained by the estrogen effect of the oral contraceptive as reported earlier (Doe et al 1964, Kuhnz et al 1991, Wiegratz et al 1995). The ratios of the urine androgen and glucocorticoid conjugates (i.e., etiocholanolone/androsterone, THF/aTHF, and aTHF+THF/THE) observed in the present study among premenopausal women using oral contraceptives are similar to those reported earlier for premenopausal non-users (Pfaffenberger and Horning 1977, Stewart et al 1993). Elevated prolactin levels among users of oral contraceptives have been reported earlier (Jung-Hoffmann et al 1988).

SUMMARY AND CONCLUSIONS

Alcohol intake is associated with hormonal changes in men and in women. In the present study the effect of alcohol on hormones was studied in premenopausal women during different conditions. The results imply that the liver, in addition to the hypothalamic-pituitary-gonadal and -adrenal axes, should be seen as a major site for the acute actions of alcohol on steroid levels in women. The key findings of this study were:

1. Acute alcohol intake was found to elevate plasma testosterone levels in premenopausal women. The magnitude of the elevation was not dose dependent (0.34-1.02 g/kg p.o.) and not modulated by a seven-day period of alochol intake of 0.8 g/kg/day per os. The elevation lasted throughout the period of ethanol elimination. The effect was pronounced among premenopausal women using oral contraceptives. The increase in testosterone was accompanied by a decrease in plasma androstenedione as well as urine androsterone and etiocholanolone levels. The acute androgen effects were abolished during pretreatment with 4-metylpyrazole, an inhibitor of alcohol dehydrogenase.

A small significant acute elevation in plasma estradiol as well as an elevation in the estradiol to estrone -ratio was observed among premenopausal women using oral contraceptives after intake of alcohol. No consistent effect on these was found among the non-users. No effect of alcohol was observed on plasma ethinylestradiol among premenopausal women with regular use of oral contraceptives.

An acute decline in plasma progesterone levels was found. The magnitude of the effect was not dependent on alcohol dose (0.34-1.02 g/kg p.o.)

The testosterone elevation seems to be the result of an inhibited catabolism in the liver: i.e., a decreased overall oxidation of testosterone due to a secondary shift in the equilibrium between androstenedione to testosterone. This is mediated by the alcohol-induced elevation in the ratio of NADH to NAD^+. The estrogen and progesterone effects may be explained by the same mechanism. The lack of an acute effect of alcohol on ethinylestradiol may be explained by the protection of the hydroxyl group at the 17-position.

2. A transient acute elevation in plasma prolactin was observed among premenopausal women using oral contraceptives as well as among non-users after intake of alcohol. The exact mechanism of the prolactin effect cannot be provided by the present data but may involve the opioid peptides and dopamine which participate in the hypothalamic regulation of pituitary prolactin secretion.

3. No acute effect of alcohol was observed on plasma cortisol levels in premenopausal women. The finding supports earlier conclusions in the literature that an acute effect of alcohol on cortisol levels can mainly be observed only late after the intake of higher doses and may thus be due to withdrawal-induced stress.

4. Elevated plasma testosterone and androstenedione levels was observed at non-intoxicated time points during an alcohol drinking period among premenopausal women using oral contraceptives. This effect was confined to the afternoon and not observed in samples collected in the morning suggesting an alteration in the CNS-mediated circadian rhythm of these plasma steroid levels. Elevated plasma cortisol levels was observed after the alcohol drinking period suggesting a withdrawal-induced change in the HPA-axis.

5. No acute or chronic effects in urine glucocorticoid conjugates was observed during alcohol drinking. However, significant changes were observed in certain glucocorticoid and androgen conjugate ratios suggesting a moderate effect of alcohol on liver steroid metabolizing enzymes.

Taken together, the results indicate that moderate alcohol drinking leads to altered glucocorticoid and androgen levels in premenopausal women using oral contraceptives as well as among non-users. The results show that the effects of alcohol on glucocorticoid and androgen steroids may not only be confined to heavy alcohol drinkers but may occur to a lesser degree among women at a moderate consumption level. The present results may be of relevance in the development of disturbances in the glucocorticoid as well as sex steroid balance leading to endocrinological disorders (pseudo-Cushing´s syndrome, loss of sexual characteristics and function, disturbances in bone metabolism, increase in breast cancer risk) among female heavy drinkers.

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