

# Cortisol, estradiol-17 $\beta$ , and progesterone secretion within the first hour after awakening in women with regular menstrual cycles

Ryun S Ahn, Jee H Choi<sup>1</sup>, Bum C Choi<sup>2</sup>, Jung H Kim<sup>3</sup>, Sung H Lee<sup>4</sup> and Simon S Sung<sup>5</sup>

Graduate School of Integrative Medicine, CHA Medical University, Yuksam-dong 605, Kangnamgu, Seoul 135-907, Republic of Korea

<sup>1</sup>Hormone Research Center, Chonnam National University, Gwangju 500-757, Republic of Korea

<sup>2</sup>Department of Obstetrics and Gynecology, CL Women's Hospital, Gwangju 502-210, Republic of Korea

<sup>3</sup>Department of Family Medicine, Chung-Ang University Medical Center, Seoul 156-755, Republic of Korea

<sup>4</sup>Department of Life sciences, Sangmyung University, Seoul 110-743, Republic of Korea

<sup>5</sup>Research Institute of Integrative Medicine, Honam Medical Center, Gwangju 506-813, Republic of Korea

(Correspondence should be addressed to R S Ahn; Email: ryunsup@yahoo.co.kr)

## Abstract

Cortisol concentration in both serum and saliva sharply increases and reaches a peak within the first hour after waking in the morning. This phenomenon is known as the cortisol awakening response (CAR) and is used as an index of hypothalamus–pituitary–adrenal (HPA) axis function. We examined whether ovarian steroid concentrations increased after awakening as with the CAR in the HPA axis. To do this, cortisol, estradiol-17 $\beta$  (E<sub>2</sub>), and progesterone (P<sub>4</sub>) concentrations were determined in saliva samples collected immediately upon awakening and 30 and 60 min after awakening in women with regular menstrual cycles and postmenopausal women. We found that both E<sub>2</sub> and P<sub>4</sub> concentrations increased during the post-awakening period in women with regular menstrual cycles, but these phenomena were not seen in any postmenopausal women. The area under the E<sub>2</sub> and P<sub>4</sub>

curve from the time interval immediately after awakening to 60 min after awakening (i.e. E<sub>2</sub>auc and P<sub>4</sub>auc) in women with regular menstrual cycles were greater than those in the postmenopausal women. E<sub>2</sub> and P<sub>4</sub> secretory activity during the post-awakening period was influenced by the phase of the menstrual cycle. E<sub>2</sub>auc in the peri-ovulatory phase and P<sub>4</sub>auc in the early to mid-luteal phase were greater than in the menstrual phase. Meanwhile, cortisol secretory activity during the post-awakening period was not influenced by menstrual status or the phase of menstrual cycle. These findings indicate that, as with the CAR in the HPA axis function, ovarian steroidogenic activity increased after awakening and is closely associated with menstrual status and phase of menstrual cycle.

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## Introduction

The measurement of steroid concentrations in saliva has become more common over the past several years. The collection of saliva is easier than a venipuncture and can readily be repeated at frequent intervals. These advantages provide for a better assessment of the diurnal rhythm-mediated secretion of cortisol and sex steroids.

The diurnal pattern of cortisol secretory activity is well characterized. Cortisol reaches a zenith around the time of awakening in the morning and a nadir after the onset of sleep. The most distinctive feature of the cortisol secretory rhythm is a sharp increase in cortisol levels in serum and in saliva within the first 30–45 min after awakening from nocturnal sleep (Pruessner *et al.* 1997, Wilhelm *et al.* 2007). This increase in the cortisol level after awakening is termed the cortisol awakening response (CAR; Federenko *et al.* 2004). An ACTH peak occurs prior to the CAR (Wilhelm *et al.* 2007) and the occurrence of the CAR is dependent on the waking

timing (Federenko *et al.* 2004, Dettenborn *et al.* 2007). Therefore, researchers believe that the CAR is a neuroendocrine activation of the hypothalamus–pituitary–adrenal (HPA) axis in response to awakening and the suprachiasmatic nucleus (SCN), which is the master pacemaker controlling circadian rhythmicity, is involved in the occurrence of the CAR (Fries *et al.* 2009, Clow *et al.* 2010). The CAR is used as a reliable index of the HPA axis function in various clinical research areas, and an altered CAR is known to be associated with health issues, such as fatigue, depression, and anxiety, and the prognosis of patients with a severe disease (Sephton *et al.* 2000, Chida & Steptoe 2009).

The timing of the preovulatory LH surge in rodents and humans has provided evidence that the SCN is also involved in regulating the function of the hypothalamus–pituitary–ovary (HPO) axis. The preovulatory LH surge occurs around the time of lights-off in nocturnal laboratory rodents (Legan & Karsch 1975, Seegal & Goldman 1975) and around lights-on in diurnal murid rodent (McElhinny *et al.* 1999). Although

a higher estradiol-17 $\beta$  (E<sub>2</sub>) concentration is an absolute prerequisite for generating the LH surge, the occurrence of a preovulatory LH surge is gated by a circadian oscillator in the SCN. Studies exploring the effect of the SCN in animals have shown that bilateral lesions of the SCN result in abolition of the preovulatory LH surge, induction of persistent estrus, and polyfollicular ovaries in gonadally intact female rats (Brown-Grant & Raisman 1977, Gray *et al.* 1978) and the elimination of daily afternoon surges of LH in estrogen-treated ovariectomized rats (Kawakami *et al.* 1980). In women, two studies have reported a possible role for the SCN in determining the preovulatory LH surge. One study reported that the preovulatory LH surge began at 0800 h in most women (80% of cases) who had normal menstrual cycles (Kerdelhue *et al.* 2002). Another study examining the timing of the LH surge found that the surges occurred between 0400 and 0800 h in 48% of cases and between 0000 and 0400 h in 37% of cases (Cahill *et al.* 1998).

Meanwhile, it is known that hormones produced in the HPA axis exert inhibitory effects on hormone secretion at multiple levels in the HPO axis under stress conditions (Chrousos *et al.* 1998, Kalantaridou *et al.* 2004). However, the relationship between these two endocrine systems is by no means unidirectional. Some studies have shown the temporal relationship between the HPA and HPO axes. For example, corticosterone and ACTH reach peak levels at the end of the light phase in female rat (Atkinson & Waddell 1997), and the preovulatory LH surge also occurs at the end of the light phase in the proestrus female rat and mice (Naftolin *et al.* 1972, Bronson & Vom Saal 1979). In humans, Kerdelhue *et al.* (2002) have reported that plasma cortisol reaches a peak when the preovulatory LH surge begins, e.g. at 0400 h when the LH surge begins at 0400 or 0800 h when it begins at 0800 h.

There is diurnal variation in the pattern of pulsatile LH secretion and ovarian E<sub>2</sub> and progesterone (P<sub>4</sub>) secretion in women. Studies on the LH profiles obtained by repeated blood sampling from adult women of a reproductive age have shown that the interpulse interval and pulse amplitude of LH are greater during nocturnal sleep than in day time and that LH concentrations increase sharply after awakening from nocturnal sleep (Kapen *et al.* 1981, Pincus *et al.* 1998, Hall *et al.* 2005). Although the acrophase (i.e. time of maximal concentration) of the E<sub>2</sub> diurnal rhythm is modulated by menstrual phases (Bao *et al.* 2003), studies on sex steroid secretion in normal cyclic women have suggested that E<sub>2</sub> and P<sub>4</sub> secretion exhibit a diurnal variation (Delfs *et al.* 1994, Bao *et al.* 2004).

The SCN receives direct light information via the optic tract (retinohypothalamic tract), translates this information into a daily pattern of activity, and transmits information about the time of day to hypophysiotropic corticotropin-releasing hormone (CRH)-synthesizing neurons in the paraventricular nucleus (PVN; Buijs *et al.* 2003) and to GnRH-synthesizing neurons in the preoptic area (POA; de la Iglesia *et al.* 1995). Transmission of the time-of-day signal from the SCN to CRH- and GnRH-synthesizing neurons is thought to be involved in the entrainment of the circadian

rhythm governing the activities of the HPA and HPO axes respectively (Kalsbeek & Buijs 2002).

As for the HPA axis function, the endocrine rhythm of the HPO axis is also regulated by the SCN. It could thus be speculated that increases in the E<sub>2</sub> and P<sub>4</sub> concentrations occur after an awakening from nocturnal sleep, similar to the CAR in the HPA axis function, if each component of the HPO axis is functioning normally. The menstrual cycle is regulated by the coordinated action between the ovaries and the hypothalamic–pituitary axis; the ovaries secrete E<sub>2</sub> and P<sub>4</sub> in response to gonadotropins and send feedback messages to the hypothalamic–pituitary axis (Buffet *et al.* 1998). Therefore, we hypothesized that any increases in E<sub>2</sub> and P<sub>4</sub> concentrations after the awakening period would occur in women with regular menstrual cycles. To test this hypothesis, this study examined whether the E<sub>2</sub> and P<sub>4</sub> concentrations increased within the first hour after awakening in women with a regular menstrual cycle. In addition, we examined whether the post-awakening increases in sex steroid concentrations differed throughout the menstrual cycle and whether these patterns differed between women with a normal menstrual cycle and those who have undergone menopause.

## Materials and Methods

### Subjects

National health insurance companies within Korea support periodical physical examinations for individuals who have not experienced hospitalization within 2 years after the previous health examination. Among the subjects receiving this periodical physical examination, healthy women aged 20–60 years were recruited between April 2009 and January 2011 from the Honam Medical Center (HMC). Information regarding medical history and menstrual cycle regularity, length, and history was obtained by face-to-face interview, and weight and height were measured at presentation. Exclusion and inclusion criteria were principally adopted from previous studies (Hall *et al.* 2000, Bao *et al.* 2003, 2004). Volunteers were excluded from the study if they met any one of the following criteria: 1) women who previously had a regular menstrual cycle but had experienced an irregular menstrual cycle two or more times in the 6 months preceding study entry and were aged 20–50 years (i.e. women with irregular menstrual cycles or perimenopausal women); 2) women undergoing hormone replacement therapy, taking oral contraceptives, tamoxifen, or other estrogen-, P<sub>4</sub>-, or glucocorticoid-containing drugs within the last 6 months; 3) women who were pregnant, lactating, showed evidence of infertility, or presence of sexually transmitted diseases or had been diagnosed with ovarian dysfunction; 4) night-shift workers, women diagnosed with chronic illness or abnormal body mass index (BMI  $\leq$  18 or  $\geq$  25); or 5) women taking antidepressant drugs or smokers. Subjects who last used oral contraceptives or steroid-containing drugs more than 6 months prior to this study were eligible.

Population-based studies have reported that menopause typically occurs in the late 40s (mean age, 48.5–49.4 years) in Korean women (Park *et al.* 2002, Ku *et al.* 2004). Women were classified into two groups based on their current menstrual status and age as follows: 1) women with a natural and regular menstrual cycle, with periods of 27–30 days for the 3 months preceding study entry, and age 20–40 years (women with regular cycles); and 2) women with no menses for more than 12 consecutive months preceding study entry, without other medical reasons for menses to stop, and age 50–60 years (postmenopausal women).

Because the subjects in the regular cyclic group were not synchronized in the phases of their menstrual cycles, they were divided into subgroups based on their menstrual phase on the day of saliva sample collection, as described previously (Bao *et al.* 2003): phase 1 subgroup, 2–3 days after the start of the menstrual cycle (menstrual phase;  $n=5$ ); phase 2 subgroup, 12–14 days (late follicular, peri-ovulatory phase;  $n=16$ ); phase 3 subgroup, 18–23 days (early to mid-luteal phase;  $n=14$ ); phase 4 subgroup, 25–28 days (late luteal phase;  $n=13$ ).

#### Saliva collection

One hundred and twenty-four women with regular menstrual cycles and 91 postmenopausal women were initially recruited in this study. Participants were asked to collect their saliva samples immediately upon awakening and 30 and 60 min after awakening for a single day, with a minimum volume of 1.5 ml saliva at each time point. They were asked not to alter their routine sleep–wake cycle. A saliva-collecting pack containing three collecting tubes, a zipper bag, and instructions was distributed or delivered to each subject.

Participants were instructed to collect saliva samples at the designated times (<5 min after awakening,  $30 \pm 5$  and  $60 \pm 5$  min after awakening) on workdays (Kunz-Ebrecht *et al.* 2004, Kim *et al.* 2010) and to note each collection time on the marking area of the collecting tube (Simport, Inc., Beloeil, QC, Canada). Saliva was collected without external stimulation but with muscle movement and expectoration into a collecting tube. All participants were asked not to drink alcohol on the previous night. During the sample collection period, patients were asked to refrain from eating food, drinking fluids, and brushing their teeth and to rinse their mouths with water before each sample collection. Steroid concentrations in saliva are stable after storage of saliva at  $-20^\circ\text{C}$  or  $-80^\circ\text{C}$  for up to 1 year, and freezing and thawing of samples up to four times before analysis does not affect measured concentrations (Groschl *et al.* 2001). Therefore, participants were asked to keep saliva samples in their own domestic freezers before sample submission.

Samples contaminated with blood, as determined by visual inspection, were excluded from the study. Samples collected outside of the designated times and those lacking a time or date on the marking area of the tube were also excluded, but this sample exclusion procedure was not informed to

participants in order to increase the veracity of subject's self-reports. After inspection procedures, saliva samples collected from 184 subjects (women with regular cycles:  $n=98$ ; postmenopausal women:  $n=86$ ) and accepted for the cortisol assay procedures.

The typical CAR was defined as an increase in cortisol levels at least  $2.5\text{ nmol/l}$  above the individual's baseline in compliant subjects (Wust *et al.* 2000, Kunz-Ebrecht *et al.* 2004). It is known that collecting the first sample with a delay of more than 10 min after awakening (i.e. non-compliant subject) in healthy subjects produces an absence of cortisol increase after awakening period (Kudielka *et al.* 2003). The typical CAR was not found and cortisol concentrations immediately upon awakening and 30 min after awakening in some subjects ( $n=28$ ) were  $16.4 \pm 3.8$ ,  $15.0 \pm 3.2$ , and  $10.2 \pm 2.8\text{ nmol/l}$  respectively. We considered these as non-compliant subjects. They were asked again to collect another batch of saliva samples and not to delay in taking the first saliva sample after awakening. Cortisol concentrations immediately upon awakening and 30 and 60 min after awakening were  $9.8 \pm 4.6$ ,  $18.3 \pm 7.5$ , and  $13.9 \pm 7.6\text{ nmol/l}$  ( $n=19$ ) respectively in the second batch of saliva samples. Other subjects ( $n=9$ ) did not participate in collection of the second batch of saliva samples. They were considered non-responders or non-compliant subjects, and their samples were excluded from this study.

Ultimately, the data obtained from saliva samples collected from 93 women with regular cycles (mean age,  $32.1 \pm 5.5$  years; BMI,  $21.5 \pm 2.1$ ) and 82 postmenopausal women (mean age,  $55.6 \pm 3.6$  years; BMI,  $22.2 \pm 1.8$ ) were included in this study. Women with regular cycles woke up  $0646 \pm 33$  min (range 0525–0737) and postmenopausal women woke up  $0701 \pm 29$  min (range 0615–0750).

The collected samples were stored at  $-70^\circ\text{C}$  after removal of debris. To precipitate mucins, the samples were thawed and centrifuged ( $10\,000\text{ g}$ , 15 min,  $4^\circ\text{C}$ ; Gozansky *et al.* 2005). The supernatant was collected and stored at  $-70^\circ\text{C}$  until the assay was performed. All participants gave their consent, and they were given information regarding their hormonal concentrations. This study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Hospital's Institutional Review Board (HMC). All saliva samples were analyzed at the Hormone Research Center at Chonnam National University.

#### Salivary steroid measurements

Steroid concentrations in saliva were determined using RIA as described previously (Ahn *et al.* 2007). Iodine-125-labeled cortisol (cortisol-3-(*O*-carboxymethyl oximino)-2- $^{125}\text{I}$ -iodohistamine) and estradiol ( $16\alpha$ -( $^{125}\text{I}$ )-iodo-3,estradiol) were obtained from PerkinElmer Life and Analytical Sciences (Waltham, MA, USA). Iodine-125-labeled progesterone (progesterone-11 $\alpha$ -hemisuccinyl-(2- $^{125}\text{I}$ )-iodotyrosine methyl ester) was obtained from Institute of Isotopes Company (Budapest, Hungary).

The reference standards for cortisol, E<sub>2</sub>, and P<sub>4</sub> were obtained from Sigma–Aldrich. A standard stock solution (10 nmol/l) of cortisol, E<sub>2</sub>, or P<sub>4</sub> was prepared in ethanol, and working standards were made by diluting the stock solution using 0.1% gelatin containing 50 mM PBS (pH=7.2).

Cortisol and E<sub>2</sub> antisera were purchased from AbD Serotec (Oxford, UK). Cortisol antiserum cross-reacts with aldosterone, 11-deoxycorticosterone, 11-deoxycortisol, 21-deoxycortisol, corticosterone, and other steroids, with cross-reactions of 0.001, 4.1, 5.7, 0.5, 1.2, and <0.01% respectively. E<sub>2</sub> antiserum cross-reacts with estrone, estriol, dihydrotestosterone, and other steroids, with cross-reactions of 0.4, 0.1, 0.05, and <0.01% respectively. P<sub>4</sub> antiserum was purchased from Abcam. P<sub>4</sub> antiserum cross-reacts with 17 $\alpha$ -hydroxyprogesterone, E<sub>2</sub>, corticosterone, 11 $\alpha$ -hydroxyprogesterone, and other steroids, with cross-reactions of 2.7, <0.1, <0.1, 15.9, and <0.01% respectively.

Exogenously added 5.5 and 22.0 nmol/l cortisol in charcoal-stripped saliva was determined to be  $5.4 \pm 0.7$  nmol/l ( $n=20$ ) and  $22.9 \pm 1.9$  nmol/l ( $n=20$ ) respectively. The inter-assay coefficients of variation (CV) as assessed from quality controls with mean cortisol concentrations of 3.6 and 10.9 nmol/l were 7.4 and 8.5% respectively ( $n=26$ ). The analytical sensitivity for cortisol was 0.4 nmol/l. Exogenously added 10.0 and 50.0 pmol/l E<sub>2</sub> in charcoal-stripped saliva was determined to be  $11.3 \pm 1.5$  pmol/l ( $n=20$ ) and  $52.4 \pm 4.5$  pmol/l ( $n=20$ ) respectively. The inter-assay CV as assessed from quality controls with mean E<sub>2</sub> concentrations of 18.4 and 183.6 pmol/l were 11.5 and 13.2% respectively ( $n=26$ ). The analytical sensitivity for E<sub>2</sub> was 3.7 pmol/l. Exogenously added 50.0 and 250.0 pmol/l P<sub>4</sub> in charcoal-stripped saliva was determined to be  $51.1 \pm 4.7$  pmol/l ( $n=20$ ) and  $258.1 \pm 8.3$  pmol/l ( $n=20$ ) respectively. The inter-assay CV as assessed from quality controls with mean P<sub>4</sub> concentrations of 159.0 and 795.0 pmol/l were 12.5 and 10.1% respectively ( $n=26$ ). The analytical sensitivity for P<sub>4</sub> was 31.8 pmol/l.

#### Data analysis

Two types of auxiliary index (i.e. mean increase in steroid concentrations and integrated steroid concentrations) were adapted to this study to analyze steroid secretory activity during the post-awakening period. After determination of cortisol, E<sub>2</sub>, and P<sub>4</sub> concentrations, the mean increases in steroid concentrations after awakening ( $MnInc$ ;  $AS_{30\ min} + AS_{60\ min} / 2 - AS_{0\ min}$ , where AS is awakening steroid concentrations) and the relative mean increases in steroid concentrations after awakening ( $MnInc$  %;  $AS_{30\ min} + AS_{60\ min} / 2 / AS_{0\ min} \times 100$ ) were calculated for each subject after modification of the previously proposed formula (Wust *et al.* 2000). Total cortisol, E<sub>2</sub>, or P<sub>4</sub> secretion during the post-awakening period was calculated as area under the curve (auc) with respect to ground from the time interval immediately after awakening to 60 min after awakening by

using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA, USA).

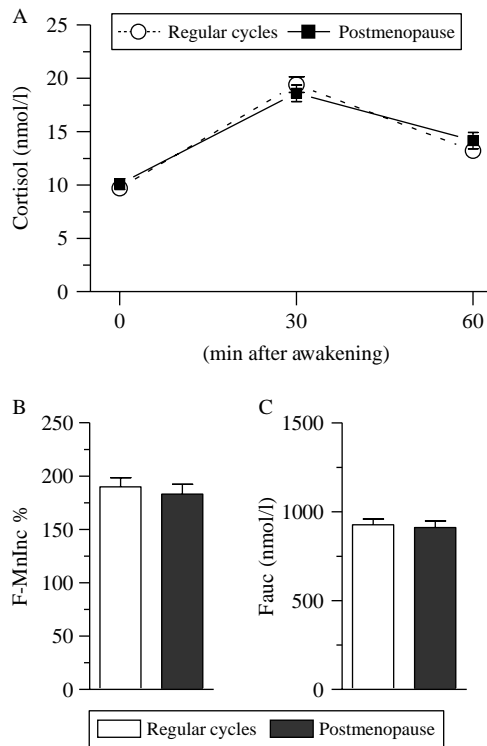
The Shapiro–Wilk normality test revealed that the variables, i.e. steroid concentrations at each examined time point, and auxiliary indices were not normally distributed. Differences in steroid concentrations among examined time points were analyzed using the Kruskal–Wallis test. Differences in auxiliary index ( $MnInc$  % and AUC) throughout four phases of the menstrual cycles were analyzed using the Kruskal–Wallis test, and when the one-way ANOVA test indicated a significant difference ( $P < 0.05$ ), Dunn's multiple comparisons test was performed to locate specific group differences. Between-group differences in the observed steroid concentrations at each time point and each auxiliary index ( $MnInc$  % or AUC) were analyzed using the Mann–Whitney test. The hormone values were square root transformed to normalize the sample distributions for a one-way repeated measure ANOVA. Statistical calculations were performed using SAS version 9.1 (SAS Institute, Inc., Cary, NC, USA), and a  $P$  value  $< 0.05$  was considered significant.

## Results

### Cortisol secretory activity within the first hour after awakening

Cortisol profiles within the first 1 h after awakening in women with regular cycles and postmenopausal women are presented in Fig. 1A, and indices for cortisol secretory activity during post-awakening period (F- $MnInc$  % and  $Fauc$ ) in each examined group are presented in Fig. 1B. Cortisol levels increased after awakening and reached a peak level 30 min after awakening in both groups of women (Fig. 1A). A one-way repeated measures ANOVA (group (regular cycles, postmenopause)  $\times$  time (three different time points)) with cortisol concentration as the dependent variable revealed that there was no significant group effect ( $F_{1, 519} = 0.11$ ,  $P = 0.74$ ), which reflected similar cortisol levels in examined groups. In addition, there was no significant interaction effect ( $F_{2, 519} = 0.99$ ,  $P = 0.37$ ), which indicated same patterns of cortisol secretion in both groups. We also observed an effect of time ( $F_{2, 519} = 101.9$ ,  $P < 0.001$ ), which reflected the changes in cortisol levels after awakening (Fig. 1A). The Mann–Whitney test revealed that there was no significant difference between two groups in cortisol concentration immediately upon awakening ( $P = 0.48$ ), 30 min ( $P = 0.22$ ), and 60 min ( $P = 0.71$ ; Fig. 1A).

No difference was found in the relative mean increases in cortisol concentrations after awakening (F- $MnInc$  %) and the integrated cortisol concentration ranging from the time immediately upon awakening to 60 min after awakening (i.e. area under the cortisol curve with respect to baseline,  $Fauc$ ) between both groups ( $P > 0.05$  for all analyses by the Mann–Whitney test; Fig. 1B and C).



**Figure 1** Changes in cortisol concentration within the first hour after awakening in women with regular menstrual cycles and postmenopausal women. Cortisol concentration at each examined time point is depicted in (A). The relative mean increase in cortisol concentration 30 and 60 min after awakening over the individual baseline (F-MnInc %) and the integrated cortisol concentration ranging from the time immediately upon awakening to 60 min after awakening (i.e. area under the cortisol curve with respect to baseline, Fauc) in each examined group are depicted in (B and C) respectively. Each data point in (A) and each bar in (B and C) represents mean  $\pm$  S.E.M.

#### *E<sub>2</sub> secretory activity within the first hour after awakening*

E<sub>2</sub> concentration was determined in the same saliva samples that were used for determining cortisol concentration. E<sub>2</sub> profiles within the first 1 h after awakening in women with regular cycles and postmenopausal women are presented in Fig. 2A. The E<sub>2</sub> profiles in women with regular cycles and postmenopausal women were analyzed using one-way repeated measures ANOVA (group (regular cycles, postmenopause)  $\times$  time (three different time points)) with E<sub>2</sub> concentration as the dependent variable. We found that there was a significant group effect ( $F_{1, 519} = 63.1$ ,  $P < 0.001$ ), with higher E<sub>2</sub> levels in the women with regular cycle than in postmenopausal women. In addition, there was a significant interaction effect ( $F_{2, 519} = 12.1$ ,  $P < 0.001$ ), which indicated differential patterns of E<sub>2</sub> secretion between both groups. We also observed an effect of time ( $F_{2, 519} = 101.9$ ,  $P < 0.001$ ), which reflected the changes in E<sub>2</sub> levels post-awakening period (Fig. 2A).

In women with regular cycles, E<sub>2</sub> concentrations 30 and 60 min after awakening were significantly higher than that immediately upon awakening ( $P < 0.001$  for all analyses by the Mann–Whitney test; Fig. 2A). However, E<sub>2</sub> concentrations at all the time point examined were similar to each other in postmenopausal women ( $P = 0.08$  by the Kruskal–Wallis test; Fig. 2A). E<sub>2</sub> concentration immediately upon awakening was comparable in both groups ( $P = 0.86$  by the Mann–Whitney test), but E<sub>2</sub> concentrations 30 and 60 min after awakening in women with regular cycle were significantly higher than those in the postmenopausal women ( $P < 0.001$  for all analyses by the Mann–Whitney test; Fig. 2A).

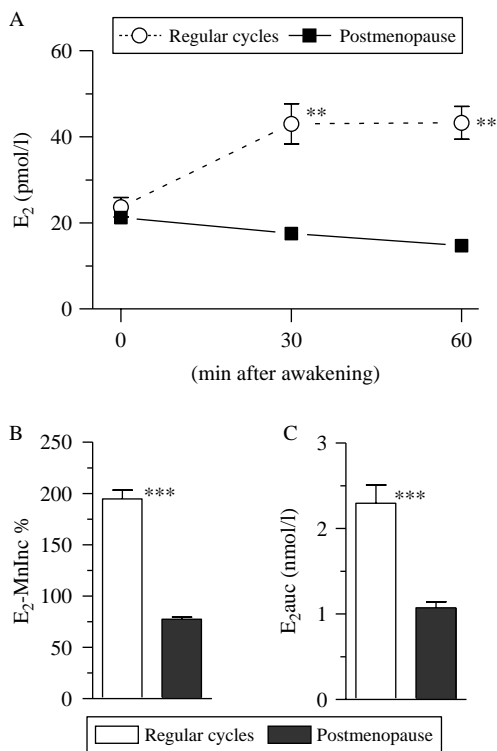
All women with regular menstrual cycles showed a positive E<sub>2</sub>-MnInc (range 0.4–93.0 pmol/l), but none of the postmenopausal women showed a positive E<sub>2</sub>-MnInc. The Mann–Whitney test revealed that the E<sub>2</sub>-MnInc % and E<sub>2</sub>auc in women with regular cycles were significantly higher than those in the postmenopausal women ( $P < 0.01$  for all analyses; Fig. 2B and C).

#### *P<sub>4</sub> secretory activity within the first hour after awakening*

P<sub>4</sub> concentration was also determined in the same saliva samples that were used for determining cortisol and E<sub>2</sub> concentrations. The P<sub>4</sub> profiles within the first 1 h after awakening in women with regular cycles and postmenopausal women are presented in Fig. 3A. The P<sub>4</sub> profiles in women with regular cycles and postmenopausal women were analyzed using one-way repeated measures ANOVA (group (regular cycles, postmenopause)  $\times$  time (three different time points)) with P<sub>4</sub> concentration as the dependent variable. We found that there was a significant group effect ( $F_{1, 519} = 50.6$ ,  $P < 0.0001$ ), with higher P<sub>4</sub> levels in the women with regular cycles than in the postmenopausal women. In addition, there was a significant interaction effect ( $F_{2, 519} = 6.04$ ,  $P < 0.01$ ), which indicated differential patterns of P<sub>4</sub> secretion between both groups. We also observed an effect of time ( $F_{2, 519} = 101.9$ ,  $P < 0.001$ ), which reflected the changes in P<sub>4</sub> levels post-awakening period (Fig. 2A).

In women with regular cycles, P<sub>4</sub> concentrations 30 and 60 min after awakening were significantly higher than concentrations immediately upon awakening ( $P < 0.001$  for all analyses by the Mann–Whitney test). However, the Kruskal–Wallis test revealed that P<sub>4</sub> concentrations at all the time points examined were comparable in women in menopause ( $P = 0.32$ ; Fig. 3A). P<sub>4</sub> concentration immediately upon awakening was compared between both groups ( $P > 0.05$  by the Mann–Whitney test), but the concentrations 30 and 60 min after awakening in women with regular cycles were significantly higher than those in the postmenopausal women ( $P < 0.001$  for all analyses by the Mann–Whitney test; Fig. 3A).

All women with regular menstrual cycles showed a positive P<sub>4</sub>-MnInc (range 6.24–1148.6 pmol/l), but 52% ( $n = 39$ ) postmenopausal women showed a positive P<sub>4</sub>-MnInc



**Figure 2** Difference in estradiol-17 $\beta$  (E<sub>2</sub>) concentrations within the first hour after awakening in women with regular menstrual cycles and postmenopausal women. E<sub>2</sub> concentration at each examined time point is depicted in (A). The relative mean increase in E<sub>2</sub> concentration 30 and 60 min after awakening over the individual baseline (E<sub>2</sub>-MnInc %) and integrated E<sub>2</sub> concentration ranging from the time immediately upon awakening to 60 min after awakening (i.e. area under the E<sub>2</sub> curve with respect to baseline, E<sub>2</sub>auc) are depicted in (B and C) respectively. Each data point in (A) and each bar in (B and C) represents mean  $\pm$  S.E.M. Asterisks denote the level of significance between both groups: \*\* $P < 0.01$ ; \*\*\* $P < 0.001$  (by the Mann-Whitney test).

(range 3.1–242.1 pmol/l). The Mann-Whitney test revealed that P<sub>4</sub>-MnInc % and P<sub>4</sub>auc in women with regular cycles were significantly higher than those in the postmenopausal women ( $P < 0.001$ ; Fig. 3B and C).

#### Variation of cortisol, E<sub>2</sub>, and P<sub>4</sub> secretory activities within the first hour after awakening throughout four phases of the menstrual cycle

The variation of cortisol, E<sub>2</sub>, and P<sub>4</sub> secretory activities within the first hour after awakening according to the phase of the menstrual cycle in women with regular cycles were analyzed using auxiliary indices for steroid secretory activity (MnInc % and AUC; Fig. 4).

The F-MnInc % was similar at each phase of the menstrual cycle (range 161.6–208.5%), as was the Fauc at each phase (range 831.4–988.7 nmol/l;  $P > 0.05$  for all analyses by the Kruskal-Wallis test; Fig. 4A).

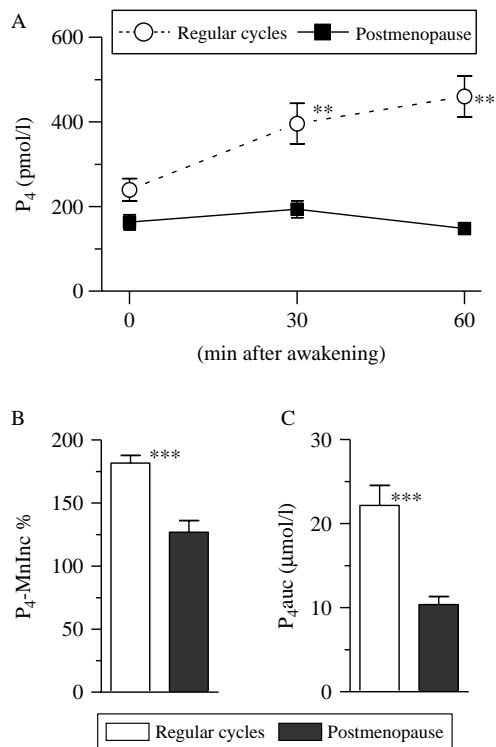
The E<sub>2</sub>-MnInc % in phase 2 of the menstrual cycle (peri-ovulatory phase) was slightly higher than in other phases, but this difference was not significant ( $P > 0.05$  by the Kruskal-Wallis test; Fig. 4B). Changes in the E<sub>2</sub>auc throughout the phases of the menstrual cycle were observed. Dunn's *post-hoc* tests revealed that the E<sub>2</sub>auc in phase 2 was significantly greater than that in phases 1 (menstrual phase) and 4 (late luteal phase;  $P < 0.05$ ), but significant differences in the E<sub>2</sub>auc were not found between other phases (Fig. 4B).

The P<sub>4</sub>-MnInc % was similar in each phase of the menstrual cycle ( $P > 0.05$  for all analyses by the Kruskal-Wallis test; Fig. 4C). Dunn's *post-hoc* test revealed that the P<sub>4</sub>auc in phase 3 (early to mid-luteal phase) was significantly greater than that in phases 1 and 2 ( $P < 0.05$  for all analyses), but significant differences in the P<sub>4</sub>auc were not found between other phases (Fig. 4C).

## Discussion

This study examined ovarian sex steroid and adrenocortical cortisol secretory activities within the first hour after awakening in women with regular menstrual cycles and postmenopausal women. We found that both the E<sub>2</sub> and P<sub>4</sub> concentrations were simultaneously increased after awakening in women with regular menstrual cycles. However, E<sub>2</sub> and P<sub>4</sub> concentrations did not increase in any of the postmenopausal women. The E<sub>2</sub>auc and P<sub>4</sub>auc in women with regular menstrual cycles were greater than in postmenopausal women. The sex steroid secretory activity after awakening was different throughout the menstrual phases; the E<sub>2</sub>auc at phase 2 (perioovulatory phase) and the P<sub>4</sub>auc at phase 3 (early to mid-luteal phase) of the menstrual cycle were significantly greater than in the menstruation phase. However, the effect of the menstrual status and menstrual phase on the cortisol secretory activity in the awakening period was negligible. The Fauc and F-MnInc % were comparable between both groups and throughout the phases of menstrual cycle.

The E<sub>2</sub> or P<sub>4</sub> concentration in saliva can be used as an index for ovarian function. Previous studies on salivary E<sub>2</sub> and P<sub>4</sub> concentrations have mainly focused on their fluctuations during the menstrual cycle using single or multiple time point samples (Choe *et al.* 1983, Bao *et al.* 2003, 2004, Celec *et al.* 2009). In this study, we determined the E<sub>2</sub> and P<sub>4</sub> concentrations in the post-awakening period to examine whether these sex steroid concentrations increased in response to awakening. This assessment was based on a method for determining the CAR (Pruessner *et al.* 1997), and adrenocortical and ovarian steroid secretory activities were examined together by the determination of cortisol, E<sub>2</sub>, and P<sub>4</sub> concentrations from the same saliva sample. This study found a simultaneous increase in cortisol, E<sub>2</sub>, and P<sub>4</sub> concentrations after women with regular menstrual cycles. This result implies that both the HPA axis and the HPO axis functions are activated together in response to awakening in women with regular menstrual cycles. Some studies have



**Figure 3** Difference in progesterone ( $P_4$ ) concentration within the first hour after awakening in women with regular menstrual cycles and postmenopausal women.  $P_4$  concentration at each examined time point is depicted in (A). The relative mean increase in  $P_4$  concentration 30 and 60 min after awakening over the individual baseline ( $P_4$ -MnInc %) and integrated  $P_4$  concentration ranging from the time immediately upon awakening to 60 min after awakening (i.e. area under the  $P_4$  curve with respect to baseline,  $P_4$ auc) are depicted in (A and B) respectively. Each data point in (A) and each bar in (B and C) represents mean  $\pm$  S.E.M. Asterisks denote the level of significance between both groups: \*\* $P < 0.01$ ; \*\*\* $P < 0.001$  (by the Mann-Whitney test).

reported similar observations in rats and humans with respect to the simultaneous occurrence of a preovulatory LH surge and the acrophase of the circadian corticosterone or cortisol rhythm (Raps *et al.* 1971, Chiappa & Fink 1977, Kerdelhue *et al.* 2002). Because the hormonal secretory rhythms in the HPA and HPO axes are coordinated by a circadian system, the hypothalamic-SCN system (Kriegsfeld & Silver 2006), a simultaneous increase in cortisol,  $E_2$ , and  $P_4$  concentrations after awakening is considered a result of an SCN-mediated co-activation of the HPA and HPO axes functions in women with regular menstrual cycles.

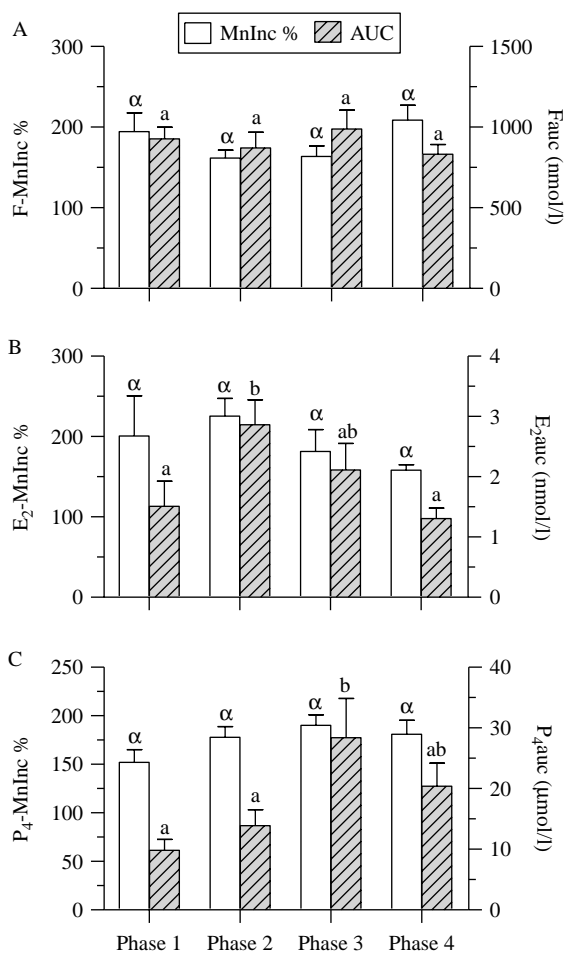
The increases in both  $E_2$  and  $P_4$  in women with regular menstrual cycles were absent in postmenopausal women. Because a dramatic loss of ovarian function starts during the perimenopausal period, one can postulate that the phenomenon in menopausal women could result from the lowered ovarian functionality. Indeed, postmenopausal women's ovaries are characterized by a high rate of ovarian follicle atresia, a depletion of non-growing follicles and a deficiency

in early-growing follicles (Gosden 1987, Gougeon *et al.* 1994, Faddy 2000, Hansen *et al.* 2008). Therefore, it is reasonable to propose that a deficiency of functionally active ovarian follicles (i.e. gonadotropin-responsive follicles) in postmenopausal women was a primary cause of the absence of an increase in either  $E_2$  or  $P_4$  concentrations after awakening.

There were differences in the  $E_2$  and  $P_4$  secretory activities throughout the phases of the menstrual cycle. The  $E_2$ - and  $P_4$ -MnInc % exhibited a rhythmic pattern throughout the menstrual cycle, but phase-specific increases in these indices were not found. The  $E_2$ auc and  $P_4$ auc also exhibited a rhythmic pattern throughout the menstrual cycle. The  $E_2$ auc at the periovulatory phase and the  $P_4$ auc at the early to mid-luteal phase were higher than in the menstrual phase. Throughout the menstrual cycle, the  $E_2$  and  $P_4$  secretory activities after awakening were similar to the previously published  $E_2$  and  $P_4$  secretory profiles determined in daily-collected saliva samples (Gann *et al.* 2001, Gandara *et al.* 2007). This result implies that the  $E_2$  and  $P_4$  secretory activities after awakening are influenced by the phase of the menstrual cycle.

In contrast to ovarian steroids, the F-MnInc % and Fauc did not significantly change with the menstrual status or phase. This result coincides well with previous studies on the changes in the HPA axis function throughout menstrual phases, which have shown that the CAR is not reduced or heightened by the phase of the menstrual cycle (Kudielka & Kirschbaum 2003) and that the secretion of ACTH and cortisol in response to stress is also not influenced by the phase of the menstrual cycle (Kirschbaum *et al.* 1999). Thus, we considered that the effect of ovarian steroids on the CAR may be negligible.

The CAR is initiated only after morning awakening from nocturnal sleep, not after awakening in the night or after a nap in the early night (Federenko *et al.* 2004, Dettenborn *et al.* 2007). This time dependency suggests the involvement of the SCN in mounting the CAR. Although the regulatory mechanism of the CAR is not fully understood, the transmission pathway of the circadian signal from the SCN to the adrenal glands in animals provides a clue in understanding the CAR in humans. In rodents, it is well documented that the SCN communicates with neuroendocrine neurons via neurotransmitters, such as arginine vasopressin (AVP) and vasoactive intestinal polypeptide (VIP; Buijs *et al.* 2003). The rhythmicity of AVP release from SCN terminals exerts a controlling effect on the rhythmic secretion of CRH and ACTH (Buijs *et al.* 2003, Perreau-Lenz *et al.* 2004, Kalsbeek *et al.* 2006). At the same time, the SCN transmits its circadian information to the adrenal gland via the intermediolateral column of the spinal cord and sympathetic outflow, which modulates adrenal sensitivity to ACTH (Buijs *et al.* 1999, Ishida *et al.* 2005, Ulrich-Lai *et al.* 2006). Because SCN-related neural pathways are similarly organized in rodents and humans (Buijs *et al.* 2003), it has been suggested that the SCN-mediated activation of the neuroendocrine pathway, which stimulates



**Figure 4** Changes in cortisol, estradiol-17 $\beta$ , and progesterone ( $P_4$ ) secretory activity throughout phases of the menstrual cycles in women with regular menstrual cycles. Cortisol (F), estradiol-17 $\beta$  ( $E_2$ ), and  $P_4$  secretory activity in each phase is presented in panels A–C respectively. The relative mean increases in steroid concentrations 30 and 60 min after awakening over the individual baseline (MnInc %) and the integrated steroid concentrations ranging from the time immediately upon awakening to 60 min after awakening (i.e. area under the curve (AUC) with respect to baseline) are depicted as mean  $\pm$  S.E.M. Bars with different Greek or Latin letters in each panel are significantly different from each other ( $P < 0.05$ ) by the Kruskal–Wallis test with Dunn's *post-hoc* test.

ACTH secretion, and the sympathetic nervous system, which modulates adrenal sensitivity for ACTH, are involved in the initiation of the CAR (Fries *et al.* 2009, Clow *et al.* 2010).

The SCN transmits information about the time of the day, which is involved in the regulation of estrus, via direct synaptic contacts with GnRH and estrogen receptor- $\alpha$  (ER $\alpha$ )-containing neurons (de la Iglesia *et al.* 1995, Watson *et al.* 1995). It is known that the majority of GnRH neurons and ER- $\alpha$ -containing neurons contacted by SCN efferents are located in the POA in rats and hamsters (de la Iglesia *et al.* 1995, Watson *et al.* 1995, van der Beek *et al.* 1997) and that the SCN coordinates the timing of the GnRH/LH secretion

via a rhythmic release of VIP and AVP (van der Beek *et al.* 1997, Kalsbeek *et al.* 2002).

Although no direct neuronal connection between the SCN neurons and the ovaries has been reported, the connections between the SCN and the preautonomic neurons of the PVN, which are involved in the autonomic innervation of the ovary, indicate that the SCN also uses the autonomic nervous system to control the circadian activity of the HPO axis (Gerendai *et al.* 1998, 2002). On the basis of these similarities between SCN-related neural and neuroendocrine pathways in the HPA and HPO axes, it can be said that the increases in  $E_2$  and  $P_4$  levels after awakening are a phenomenon analogous to the CAR in the HPA axis.

The menstrual cycle is strictly regulated by a coordinated action between the ovaries and the hypothalamus–pituitary axis. Gonadotropins act on the ovaries to stimulate follicular development and steroid secretion, but the secretion of hormones from the hypothalamus–pituitary axis is modulated by ovarian steroids (Buffet *et al.* 1998). The ovarian hormones  $E_2$  and  $P_4$  are known to be potent feedback modulators of GnRH and gonadotropin secretion (Yamaji *et al.* 1972). Because  $E_2$  exerts feedback action on the pituitary to alter the number of GnRH receptors and ERs (Shupnik *et al.* 1989, Kaiser *et al.* 1993) and because  $E_2$  also directly influences neurotransmitters in the brain that controls the pattern of the pulsatile secretion of GnRH (Smith & Jenness 2001), it seems likely that increased  $E_2$  and  $P_4$  levels after awakening provide feedback to the hypothalamus–pituitary axis to ensure the coordination of hormone secretion by the HPO axis and the ovarian cycle.

It is known that the CAR is influenced by various factors, such as stress-related factors (job stress and life stress), sleep-related factors (sleep duration and sleep quality), and awakening factors (forced awakening, light intensity during awakening, and wake-up time; Fries *et al.* 2009). Most of these factors have been shown to influence the CAR, which may be blunted or enhanced, but the CAR does not occur after taking a nap in the early evening (awakening: 1845–2030 h) or after nightly awakenings (Federenko *et al.* 2004, Dettenborn *et al.* 2007). It is known that bright light has a greater impact on the circadian rhythm of cortisol and the body temperature than other periodic behavioral stimuli, such as the sleep–wake schedule and social contact (Czeisler *et al.* 1986, Duffy *et al.* 1996). Similarly, early morning bright light exposure (0500–0800 h) was shown to result in an immediate inhibition of melatonin secretion and a robust increase in cortisol secretion in subjects who were kept in a state of continuous wakefulness for 36 h in dim light conditions, but bright afternoon light exposure (1300–1600 h) did not have any effect on hormone secretion (Leproult *et al.* 2001). The results of these studies suggest that the sensitivity of the SCN to bright light is time gated, but information regarding the minimum sleep duration required and time range of the responsiveness of the SCN to bright light is limited. If we accept the similarity in the transmission of time-of-day information from the SCN to the HPA axis and



to the HPO axis, it could be speculated that an increase in the E<sub>2</sub> and P<sub>4</sub> levels after awakening would also be influenced by various factors, such as sleep- and awakening-related factors.

Taken together, hormone secretion is regulated not only by feedback loops (i.e. concentration signals) but also by the SCN (i.e. temporal signals; Kriegsfeld & Silver 2006). The CAR is considered to be an SCN-mediated activation of the HPA axis function (Fries *et al.* 2009, Clow *et al.* 2010). We found that, as with the CAR in the HPA axis, waking up may be a potential stimulator of the HPO axis and may result in an increase in sex steroid levels within the first hour after awakening. However, unlike the situation for the HPA axis, the increases in the sex steroid levels after awakening were dependent on the woman's menstrual status, as such increases were not observed in any of the postmenopausal women. These results suggest that the measurement of sex steroid levels during the post-awakening period could provide information regarding the HPO axis response to awakening. The question whether altered (heightened or reduced) increases or the absence of increases in E<sub>2</sub> and/or P<sub>4</sub> after the awakening period is associated with menstrual disruption remains to be elucidated.

### Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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### Author contribution statement

R S A developed the idea for the paper. J H K and B C C participated in the study design and execution. J H K participated in the collection of saliva samples and interview of subjects. J H C determined steroid concentrations in saliva. S H L undertook the statistical analyses. R S A, B C C, and S S S participated in the analysis and interpretation of data and wrote the manuscript. All authors have approved the final version of the manuscript.

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