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Combined oral contraceptive synergistically activates mineralocorticoid receptor through histone code modifications

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ABSTRACT

Clinical studies have shown that the use of combined oral contraceptive in pre-menopausal women is associated with fluid retention. However, the molecular mechanism is still elusive. We hypothesized that combined oral contraceptive (COC) ethinyl estradiol (EE) and norgestrel (N) synergistically activates mineralocorticoid receptor (MR) through histone code modifications. Twelve-week-old female Sprague-Dawley rats were treated with olive oil (control), a combination of 0.1 μg EE and 1.0 μg N (low COC) or 1.0 μg EE and 10.0 μg N (high COC) as well as 0.1 or 1.0 μg EE and 1.0 or 10.0 μg N daily for 6 weeks. Expression of MR target genes in kidney cortex was determined by quantitative real-time polymerase chain reaction. MR was quantified by western blot. Recruitment of MR and RNA polymerase II (Pol II) on promoters of target genes as well as histone code modifications was analyzed by chromatin immunoprecipitation assay. Treatment with COC increased renal cortical expression of MR target genes such as *serum and glucocorticoid-regulated kinase 1* (*Sgk-1*), *glucocorticoid-induced leucine zipper* (*Gilz*), *epithelial Na^+channel* (*Enac*) and *Na^+–K^+–ATPase subunit α1* (*Atp1a1*). Although COC increased neither serum aldosterone nor MR expression in kidney cortex, it increased recruitment of MR and Pol II in parallel with increased H3Ac and H3K4me3 on the promoter regions of MR target genes. However, treatment with EE or N alone did not affect renal cortical expression of *Sgk-1*, *Gilz*, *Enac* or *Atp1a1*. These results indicate that COC synergistically activates MR through histone code modifications.

1. Introduction

The regulation of body fluid volume is critically important in maintaining life. Fluid retention is the main clinical feature in several pathological conditions, including a number of renal and cardiovascular disorders (Cotter et al., 2008; Kalantar-Zadeh et al., 2009). Combined oral contraceptive is used by more than 100 million women worldwide (Mosher et al., 2004) and has been implicated to cause fluid retention (Borges et al., 2006; Stachenfeld et al., 1999).

Oral contraceptives are a reliable form of contraception. Two forms are widely available: the combined oral contraceptive pill, containing essentially estrogen and progestin (progesterone), and the progestin-only pill. Combination pill not only prevents ovulation by inhibiting gonadotropin secretion via effect on both pituitary and hypothalamic centers, but inhibits endometrial implantation via alterations in tubal peristalsis, endometrial receptivity, and cervical mucus secretions. The two most common estrogens that are used in combined oral contraceptive pills are the synthetic products ethinyl estradiol and mestranol (Larsson et al., 1992; Thorogood and Villard-Mackintosh, 1993). The progesterone component is usually supplied by norethindrone, norgestrel, norethindrone acetate, norgestimate, desogestrel, or gestodene (Larsson et al., 1992; Maitra et al., 2004).

Fluid retention is usually caused by compromised regulatory mechanisms for sodium transport or excessive dietary sodium intake. The mineralocorticoid receptor (MR) is a nuclear receptor that is critical for controlling sodium and potassium transport in epithelial cells, most notably in the kidney and colon (Pearce et al., 2003). The MR is capable of binding multiple classes of steroids

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with high affinity, including the mineralocorticoids, aldosterone and deoxycorticosterone; the glucocorticoids, cortisol (in humans) or corticosterone (in rodents); and progesterone (Sutanto and de Kloet, 1991). While aldosterone is considered the primary physiological MR ligand in humans (Bledsoe et al., 2005), in some tissues, cortisol may be the primary ligand for MR, whereas endogenous progesterone behaves as a predominant antagonist (Fagart et al., 1998). The activation of MR plays an important role in the altered volume regulation or body fluid retention (Francis et al., 2001). MR becomes activated upon binding of its ligand aldosterone, resulting in its dissociation from the chaperone proteins, homodimerization, and translocation to the nucleus where it directs the transcription of specific target genes, including *serum and glucocorticoid-regulated kinase 1 (Sgk-1), glucocorticoid-induced leucine zipper (Gilz), Epithelial Na^+channel (Enac)* and *Na^+–K^+–ATPase subunit α1 (Atp1a1)* (Hellal-Levy et al., 2000; Viengchareun et al., 2007).

Recent studies have shown that treatment with oral contraceptive is associated with epigenetic regulation. Oral contraceptives modify DNA methylation and monocyte-derived macrophage function (Campesi et al., 2012). Studies have also demonstrated that histone code modifications (posttranslational modifications of histones via acetylation, methylation, phosphorylation and ubiquitinylation) play critical roles in regulating the transcriptional activity of MR (Kang et al., 2015; Lee et al., 2013). Despite the wide use of combined oral contraceptive (including estrogen and progestin) in pre-menopausal women, the molecular mechanism by which it induces fluid retention is still elusive. Therefore, we hypothesized that combined oral contraceptive ethinyl estradiol (EE) and norgestrel (N) synergistically activates MR through histone code modifications.

2. Materials and methods

2.1. Animals

The investigation was conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and was approved by the institutional review board of Kyungpook National University School of Medicine and effort was made to minimize both the number of animals used and their suffering. Twelve-week-old female Sprague-Dawley rats were treated with olive oil (control), a combination of 0.1 μg ethinyl estradiol, EE and 1.0 μg norgestrel, N (low COC) or 1.0 μg EE and 10.0 μg N (high COC) as well as 0.1 or 1.0 μg EE and 1.0 or 10.0 μg N daily for 6 weeks. Treatment with COC or E or N was given in olive oil and was administered orally once daily as previously reported (Olatunji and Soladoye, 2008a, 2008b, 2010). The low COC formulation used in this study is comparable to the clinical dose in women (Supplemental Table S1). At the end of treatment, the rats (4 in each group) were anesthetized with pentobarbital sodium. Kidneys were removed and the cortex was cut out. The tissues were frozen in liquid nitrogen and stored at −80 °C until further study.

2.2. Quantitative real-time PCR (qRT-PCR)

Tissues (about 100 mg) were homogenized in liquid nitrogen with a glass homogenizer. RNA was extracted by using Trizol Reagent (Invitrogen, Carlsbad, CA) according to manufacturer's recommendations from homogenized tissues. Total RNA (2 μg) was reverse-transcribed into cDNA by using RevertAidTM first strand cDNA synthesis kit (Fermentas, EU) in 20 μl reaction volume according to manufacturer's instructions. Quantitative real-time-PCR (qRT-PCR) was performed using the ABI Prism 7500 sequence detection system (Applied Biosystems; Foster City, CA). Ten micro liter of SYBR Green PCR master mix (TaKaRa, Japan), 4 μl of cDNA, and 200 nmol/l primer set were used for amplification in 20 μl reaction volume. All samples were amplified in triplicates in a 96-well plate and the cycling conditions were as follows: 2 min at 50 °C, 10 min at 95 °C, and 40 cycles at 95 °C for 15 s followed by 1 min at 60 °C. The relative mRNA expression level was determined by calculating the values of Δcycle threshold (ΔCt) by normalizing the average Ct value compared with its endogenous control (*Gapdh*) and then calculating $2^{-\Delta\Delta Ct}$ values. All primer sets used in qRT-PCR are shown in Supplemental Table S2.

2.3. Chromatin immunoprecipitation (ChIP) assay

ChIP analysis was performed according to the manufacturer's instructions with minor modification using EZ ChIP kit (Upstate Biotechnology, Lake Placid, NY). In brief, tissues were fixed with 1% formaldehyde, and washed with with ice-cold phosphate-buffered saline (PBS). After homogenization, tissues were incubated with sodium dodecyl sulfate (SDS) lysis solution for 10 min on ice. The lysate was sonicated with 15 cycles at 100 amplitude for 10 s followed by cooling on ice for 50 s. The lysate was incubated with protein G agarose beads for 2 h and, after removal of beads, further incubated with antibodies at 4 °C overnight. After addition of protein G beads, the mixture was incubated with rotation for 1 h. The beads were washed serially with a low-salt solution, high-salt solution, LiCl solution, and TE solution twice. The antibody–chromatin complexes were eluted from the beads with a solution containing 1% of SDS and 0.1 mol/L of $NaHCO_3$. To reverse the crosslinking between DNA and chromatin, elutes were incubated at 65 °C for 5 h after addition of NaCl to a final concentration of 0.2 mol/L. The proteins were eliminated by digestion with proteinase K at 45 °C for 2 h, and the DNA was purified with a spin column. Antibodies to MR and trimethyl-H3-K4 were obtained from Abcam (Cambridge, UK). Antibodies to acetyl-histone H3 and trimetyl-H3-K9 were obtained from Upstate Biotechnology. Specific promoter DNA was quantified by real-time PCR. All samples were amplified in triplicate in a 96-well plate and the cycling conditions were as follows: 2 min at 50 °C, 10 min at 95 °C, and 40 cycles at 95 °C for 15 s, followed by 1 min at 60 °C. The primer set used in the ChIP assay is shown in Supplemental Table S2.

2.4. Immunoprecipitation and western blot

The frozen tissues were homogenized in lysis buffer (20 mmol/l Tris, 150 mmol/l NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, and 1x proteinase inhibitor cocktail). Homogenized tissues were precleared with protein G agarose at 4 °C for 2 h. The supernatant were incubated with 1 μg of MR antibody (Cell Signaling Technology, Beverly, MA) at 4 °C for overnight. The immunocomplexes were washed three times with lysis buffer, and subjected to western blotting analysis. For western blotting analysis, protein-matched samples (Bradford assay) were electrophoresed (SDS-PAGE), and then transferred to nitrocellulose (NC) membranes. The NC membranes were blocked with 5% skim milk in TBS (25 mmol/l Tris base and 150 mmol/l NaCl) for 2 h at room temperature, and then incubated with 0.2 μg/ml of MR antibody at 4 °C overnight. Secondary antibody (1:2000 diluted) was incubated at room temperature for 1 h, and then washed three times, 10 min each in TBST. The target proteins were detected with ECL plus detection reagents (Amersham, Pittsburgh, PA). The expression levels were quantified by an optical densitometry, Image J software.