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Progestin isoforms provide different levels of protein S expression in HepG2 cells

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Highlights

- Progestin differentially upregulates PS expression depending on its isoform.
- The mechanism of progestin PS upregulation is mediated by transcription elongation.
- The difference in progestin may contribute to a variation in VTE risk between COCs.

Keywords

protein S, progestin, transcription elongation, combined oral contraceptive, venous thromboembolism

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Abstract

Introduction: Use of combined oral contraceptives (COCs) results in acquired protein S (PS) deficiency, a well-established risk factor for venous thromboembolism (VTE). The risk of VTE due to COCs containing newer-generation progestins is double compared with COCs containing older-generation progestins, although there is little difference in estrogen contents between the generations. In contrast, progestin-only contraceptives do not confer an increased risk of VTE. In this study, we aimed to investigate how different isoforms of progestin in COCs affect the risk of VTE by measuring PS expression.

Materials and Methods: The effect of progestin, levonorgestrel (LNG) or drospirenone (DRSP), on PS mRNA expression in HepG2 cells was measured using reverse transcription-quantitative PCR; PS level was determined using Western blot analysis. *PROS1* promoter activity, PS mRNA stability, and *de novo* synthesis of PS mRNA were examined in HepG2 cells after treatment with progestin.

Results and Conclusions: In the presence of progestins, PS mRNA and protein expressions were significantly upregulated in HepG2 cells due to the augmentation of *de novo* PS mRNA expression modulated by RNA polymerase II (Pol II), thereby facilitating PS transcription elongation. Moreover, the transcription elongation inhibitor blocked progestin-mediated *de novo* PS mRNA expression. Conversely, progestin did not affect *PROS1* promoter activity and PS mRNA stability. Pol II elongation efficiency in the newer-generation progestin (DRSP) treatment was not as strong compared with older-generation progestin (LNG), suggesting the difference in VTE risk between COC generations.

Introduction

Use of combined oral contraceptives (COCs) with progestin and the artificial estrogen, ethinylestradiol (EE), is associated with a three- to six-fold greater risk of venous thromboembolism (VTE)[1]. This increased risk is attributed to the serum levels of estrogen and the isoform of progestin found in the COCs[2,3]. In 2012, Lidegaard et al. summarized third- and fourth-generation combined contraceptives, containing newer-generation progestins, desogestrel (DSG), gestodene, drospirenone (DRSP), or cyproterone acetate. According to them, the aforementioned contraceptives doubled the risk of VTE compared with first- and second-generation contraceptive pills, that is, older-generation progestins containing norethisterone (NET), levonorgestrel (LNG), or norgestimate, representing a six-fold increase in risk compared with non-users[4]. In contrast, progestin-only contraceptives (POCs), which seem to have a similar contraceptive effect as COCs[5], did not alter the risk of VTE[5-8].

Reduced levels of anticoagulant factors, including antithrombin, protein C, and protein S (PS), contribute to increased risk of VTE. PS, a vitamin K-dependent plasma glycoprotein, is mostly synthesized in hepatocytes[9] and functions as a non-enzymatic cofactor for activated protein C (APC), increasing the inactivation efficiency of APC for coagulant factors Va and VIIIa[10-12]. Furthermore, PS inhibits the generation of thrombin in the absence of APC[13]. PS is also a cofactor for the tissue factor pathway inhibitor (TFPI)[14,15], where it enhances the ability of TFPI to inactivate factor Xa[16,17]. In human plasma, approximately 40% of PS circulates in a free form, whereas the remaining 60% is in a complex with the

C4b-binding protein (C4BP)[18-20]. Initially, it had been suggested that the C4BP–PS complex was an inactive form, and that the free form of PS exhibited anticoagulant activity. However, a subsequent study proposed that the complex works as a cofactor for APC[21]. Low levels of plasma PS are associated with an increased risk of VTE, particularly, deep venous thrombosis. Hereditary PS deficiency is an autosomal dominant disease, which is a well-established risk factor for VTE[22]. PS deficiency can occur due to various reasons such as use of anticoagulant, liver disease, pregnancy, and use of COCs[23-26].

In our previous study, we demonstrated that *PROS1* expression is downregulated by 17 beta-estradiol (E2)[27], which increases during the late stages of pregnancy. Similarly, we suspected that the mechanism responsible for the repression of PS is estrogen-dependent *PROS1* downregulation although this effect alone is not sufficient to account for the differences observed in VTE risk according to the progestin isoform in COCs. In this study, we aimed to investigate the effect of progestin isoforms on the expression of PS in HepG2 cells.

Materials and Methods

Materials

Actinomycin D, 5,6-dichlorobenzimidazole-1-β-D-ribofuranoside (DRB), 19-Norethindrone (norethisterone: NET), DRSP, DSG, and D(-)-norgestrel (levonorgestrel: LNG) were purchased from Sigma-Aldrich (Saint Louis, MO, USA). Dulbecco's modified Eagle's Medium (DMEM) with high glucose, L-glutamine, and phenol red; phenol red-free DMEM with high glucose, L-glutamine, and HEPES; and penicillin–streptomycin–amphotericin B suspension (×100) were purchased from Wako Pure Chemical (Osaka, Japan). Fetal bovine serum (FBS) was purchased from JRH Biosciences (Lenexa, KS, USA). Polyclonal rabbit anti-human PS IgG was a kind gift from Dr. Hiroko Tsuda (Nakamura Gakuen University, Fukuoka, Japan). *PROS1* promoter–reporter firefly luciferase plasmids (pPROS1/-1826 and pPROS1/-338) were cloned previously[27]. All vectors were purified from cultures using a Wizard® *Plus* SV Minipreps DNA Purification System (Promega, NSW, Australia) and the sequences were confirmed using an ABI PRISM 310 DNA sequencer (Applied Biosystems, Foster City, CA, USA).

Cell culture and treatment

HepG2, a human hepatoma cell line, was obtained from the American Type Culture Collection (Manassas, VA, USA) and routinely cultured in phenol red DMEM with high glucose and L-glutamine, supplemented with 6% v/v FBS and incubated at 37°C. For progestin assays, cells were cultured in phenol red-free DMEM with high glucose and L-glutamine supplemented with 6% v/v charcoal stripped FBS for 2 days. The following day, the cells were cultured in phenol red-free DMEM supplemented with 1% v/v charcoal stripped-FBS and treated with dimethyl sulfoxide (DMSO), 50 nM NET, 20 nM LNG、5 nM DSG or 100 nM DRSP for 48 h. We used different concentrations of each progestin according to the Cmax value data in a single clinical use (Suppl. Table).

Total RNA isolation and reverse transcription-quantitative PCR (RT-qPCR)

Total RNA was extracted from HepG2 cells using the RNeasy Mini Kit (QIAGEN, Hilden, Germany) and first-strand cDNA was prepared with 1 μg of total RNA using PrimeScript™ RT Master Mix (TaKaRa Bio,

Shiga, Japan). RT-qPCR was performed to determine mRNA levels of PS and Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) with SYBR® Premix ExTaq II (TaKaRa Bio) and the Thermal Cycler Dice Real Time System *II* (TaKaRa Bio). The level of mRNA expression in all experiments was calculated as relative values of the respective mRNAs normalized to GAPDH mRNA, a housekeeping gene. The following specific primers were used: PS, 5′-TGCTGGCGTGTCTCCTCCTA-3′ and 5′-CAGTTCTTCGATGCATTCTCTTTC-3′; GAPDH, 5′-CAGGAGCGAGATCCCTCCAA-3′ and 5′-CCCCCTGCAAATGAGCCC-3′.

Western blot analysis

PS was analyzed in cell extracts and HepG2-conditioned media by Western blotting as described previously[27]. Cells were lysed in SDS sample buffer (50 mM Tris-HCl, pH 6.8, 2% SDS, 850 mM 2-mercaptoethanol, 5% glycerol, and 0.001% bromophenol blue), and the protein concentration was determined using a Bio-Rad *DC*™ Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA, USA). Proteins were resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and electroblotted onto polyvinylidene fluoride membranes. After blocking, the membrane was incubated overnight with a primary antibody against human PS or β-actin (Cytoskeleton Inc., Denver, CO, USA) at 4 °C. Immunoreactivity was probed using a horseradish peroxidase-conjugated secondary antibody (Cell Signaling Technology, Danvers, MA), and then signal was visualized with an Immobilon Western chemiluminescent substrate (Millipore, Billerica, MA, USA).

Dual-luciferase reporter assay

Luciferase levels were measured according to the manufacturer's protocol (Promega). Cells were transiently transfected with 300 ng firefly luciferase reporter plasmid, and 40 ng renilla luciferase reporter plasmid (pGL4.74 vector) was used as an internal control to determine transfection efficiency. We confirmed positive expression of progesterone receptor isoforms A and B (PR-A and PR-B) in the HepG2 cells (Suppl. Fig.). Following the 24-h transfection period, the culture medium was replaced with fresh phenol red-free DMEM supplemented with 1% v/v charcoal-stripped FBS containing 20 nM LNG, 100 nM DRSP, or dimethyl sulfoxide (DMSO) alone as a vehicle control.

PS mRNA stability

HepG2 cells were pre-treated with 20 nM LNG, 100 nM DRSP, or DMSO for 24 h before the addition of actinomycin D (1 μg/mL) to the culture medium to block mRNA synthesis. RNA was isolated at different time points. Subsequently, PS and GAPDH mRNA levels were quantified by RT-qPCR.

Nuclear run-on assay

The nuclear run-on assay was performed as reported previously, with minor modifications[28]. This assay used a fluorescence-based approach to assess cellular gene expression and transcription rates. HepG2 cells were cultured in 100-mm dishes with or without progestin for 24 h. The cells were washed twice with chilled phosphate buffered-saline (PBS) and harvested in 1 mL of lysis buffer containing nonidet P-40 (NP-40) to collect intact nuclei. After washing with lysis buffer without NP-40, *in vitro* RNA synthesis was performed with a biotin RNA labeling mix (Roche, Mannheim, Germany) for 30 min at 29 °C. Total RNA

was then isolated using the RNeasy Mini Kit. The biotin-labeled RNA pull-down assay was performed using Dynabeads® M-280 Streptavidin (Invitrogen, Carlsbad, CA, USA). The RNA-bound beads were suspended in 16 μL of RNase-free water, and the bound RNA was used as a template for reverse transcription and subjected to RT-qPCR as described above.

Statistical analysis

Quantitative data are presented as means ± standard error (SE) of at least three independent experiments. Comparisons between two groups were made using Mann–Whitney U-test, whereas comparisons between multiple groups were made using Tukey one-way analysis of variance (ANOVA) or non-parametric ANOVA Wilcoxon (Kruskal–Wallis) test.

Results

The effect of progestin isoform on PS mRNA expression in HepG2 cells

First, we investigated the expression of PS in HepG2 cells incubated with progestins. The concentration of first- (NET), second- (LNG), third- (DSG) or fourth-generation progestin (DRSP) used was 50 nM, 20 nM, 5 nM or 100 nM, respectively, which is similar to the concentration of each COC found in serum. To assess whether the production of PS following LNG or DRSP treatment can be attributed to a transcriptional elevation, we studied PS mRNA expression in HepG2 cells by RT-qPCR. Stimulation of HepG2 cells using NET or LNG for 48 h resulted in a 2.1- or 2.3-fold increase in PS mRNA expression compared with vehicle control, respectively, whereas the effect of DSG (1.2-fold) or DRSP (1.4-fold) on PS mRNA expression was low (Fig. 1). Taken together, these results indicate that progestin treatment can upregulate PS mRNA expression although this activity varied according to the progestin isoform. Since very similar results were observed in the experiments using older- (NET or LNG) and newer-generation progestins (DSG or DRSP), respectively, we used LNG as an older- and DRSP as a newer-generation progestin in a further investigation.

PS expression is induced upon progestin treatment in HepG2 cells

Western blot analysis showed that the PS secreted in the conditioned media increased following 20 nM LNG or 100 nM DRSP treatment for 48 h (Fig. 2A). In our study, HepG2 cells secreted a small amount of doublet PS. Fair et al. reported that PS from HepG2 cells showed a smaller MW (75-77kDa) under reduced condition than the published measurements of 84kDa by Dahlback[9]. In addition, Dahlback reported that plasma PS migrated as a doublet of MW approx. 85kDa and 75kDa on reduction and thrombin converted the 85kDa chain of PS into a 75kDa[20]. The reason why the PS band patterns are different between in plasma and in HepG2 cell supernatants is unclear, but it might be possible that larger PS molecule from HepG2 cells would be converted into smaller PS by an unknown thrombin-like protease. Notably, a significant increase in the secretion of PS was observed in HepG2 cells with LNG treatment compared with vehicle treatment. Furthermore, we evaluated whether there was an elevation in the intracellular PS production due to progestin treatment. A low molecular weight PS compared with normal pooled plasma PS was detected in cell extracts. These differences in the molecular weight of PS are ascribed to the post-transcriptional modification of carbohydrate chains of PS[29]. Our results showed that in the presence of progestin, intracellular production of PS increased in both LNG and DRSP treatment; however, the effect of DRSP was smaller than LNG

treatment (Fig. 2B). These results indicate that intracellular production of PS production can be increased in HepG2 cells by treatment with progestin.

Characterization of the PROS1 promoter

It has been reported that *PROS1* promoter activity is upregulated by progestin in MCF-7 cells[30]. Ligand-activated progesterone receptor binds to a progesterone response element (PRE) located within the region spanning nucleotides −397 to −417 of the *PROS1* promoter[30]. This *cis*-regulatory element identified in the *PROS1* promoter led us to hypothesize that the production of PS increased upon treatment of HepG2 cells with progestin that is mediated by the elevation of promoter activity. To do this, we performed luciferase assay using *PROS1* promoter constructs: pPROS1/−1826 and −338. Our results showed that the progestin-dependent effect was not observed under our experimental conditions in the pPROS1/−1826 constructs, which contained at least one PRE (Fig. 3A). Taken together, these results suggest that the *cis*-regulatory element does not involve in a progestin-dependent PS transcriptional elevation.

Effect of progestin on PS mRNA stability

To explore whether increased PS transcripts in progestin-stimulated HepG2 cells resulted from resistance to mRNA degradation, we examined PS mRNA stability. To do this, HepG2 cells were stimulated with progestin for 24 h and subsequently actinomycin D was added. PS mRNA was decreased in a time-dependent manner, and the residual level was not found to be significantly different with or without progestin (Fig. 3B). These results indicated that PS transcripts were not be regulated at the post-transcriptional level following progestin treatment.

Progestin enhances PS expression through transcription elongation

Based on our observations, the induction of PS upon treatment of HepG2 cells with progestin was not mediated by *PROS1* promoter activity or enhancement of mRNA stability. Therefore, our next hypothesis was that progestin-mediated PS expression is due to activation of transcription elongation. To prove this, we performed *de novo* evaluation of PS mRNA transcripts using the nuclear run-on assay following progestin treatment. This assay measures transcription initiation rates of *PROS1*. The level of newly synthesized PS transcript was significantly increased with LNG treatment, whereas DRSP treatment brought about only minor changes in the PS transcript levels (Fig. 4A). Next, we assessed whether transcription elongation at late stage of transcription was promoted upon the addition of progestin in DRB-treated HepG2 cells (Fig. 4B). The phosphorylation of the C-terminal repeat domain (CTD) Serine2 (Ser2) residues of the largest RNA polymerase (Pol II) subunit is a good marker of an active elongation phase[31,32], with positive transcription elongation factor-b (P-TEFb) playing a pivotal role in Ser2 phosphorylation. DRB treatment inhibits P-TEFb kinase activity[33], which suppresses transcription elongation by Pol II. DRB abolished increased PS mRNA expression in HepG2 cells treated with progestin, suggesting that Pol II elongation activity is involved in progestin-dependent PS transcriptional elevation. These results indicate that transcription elongation mechanisms might contribute to the progestin-dependent PS upregulation.

Discussion

In this study, we showed that in HepG2 cells, PS mRNA and its secreted protein levels were upregulated by progestin treatment; these effects varied in an isoform-dependent manner. Moreover, our study suggested for the first time that the mechanism responsible for progestin-dependent PS upregulation is by the facilitation of transcription elongation. The newer-generation progestin, DRSP, exhibited this effect, but to a lesser degree than older-generation progestin, LNG. These results support the data of statistical surveys suggesting that users of newer-generation COCs had a higher risk of VTE than users of older-generation COCs[4]. Furthermore, we found that at lower doses of LNG treatment, PS expression remarkably increased compared with DRSP treatment, suggesting that the second-generation progestin induces PS more efficiently than the analogue DRSP at the transcriptional level in HepG2 cells.

Previously, we reported that *PROS1* gene expression was downregulated by E2 via estrogen receptor alpha (ERα)–Sp1 interaction. The ERα–Sp1 complex recruits RIP140; RIP140 is associated directly with the HDAC3 complex containing the nuclear receptor co-repressor 2/silencing mediator for retinoid or thyroid-hormone receptors co-repressors, which induce the deacetylation of histones in the *PROS1* promoter[27]. EE is commonly used as a medical property in COCs. However, *PROS1* promoter activity was also significantly reduced following stimulation with EE in HepG2 cells, which were transiently co-transfected with the ERα expression vector and the *PROS1* promoter–reporter gene constructs (unpublished observation). Despite the reduction of estrogen levels after the use of hormonal contraceptive pills, women who use newer-generation progestins have a greater risk of VTE than women who use older progestins[4]. Conversely, POCs do not increase the risk of VTE[5-8]; however, they have higher total PS levels in blood[34]. Thus, we hypothesized that progestin is involved in the upregulation of PS expression and PS elevation is critically dependent on progestin isoform.

The progesterone receptor (PR) is a member of the steroid/nuclear receptor superfamily of transcription factors and can bind to some progestins. The ligand-bound receptor translocates to the nucleus and binds to PREs in DNA leading to cellular responses. In 2007, Hughes et al. showed that progestin isoforms led to the upregulation of *PROS1* gene in MCF-7 cells[30]. MCF-7 is a triple-positive breast cancer cell line defined by the status of ERα, PR, and human epidermal growth factor receptor 2[35]. In our study, progestin did not effectively stimulate *PROS1* gene expression in HepG2 cells, indicating a different mechanism of PS regulation between hepatoma cells and breast cancer cells.

The 3'-untranslated region (UTR) of mRNA moderates its stability and is regulated by interactions with various factors, such as RNA-binding proteins and microRNAs (miRNAs)[36,37]. In a recent study, miR-494 negatively regulated PS mRNA at a *PROS1* 3′-UTR sequence containing three putative miR-494 binding sites in the human hepatocarcinoma cell line, HuH-7[38]. In our study, however, actinomycin D did not alter the kinetics of PS mRNA in HepG2 cells treated with or without progestin, suggesting that progestin's response dose not involve mRNA stability mediated by RNA-binding proteins or miRNAs in HepG2 cells.

Next, we examined the elongation stage of PS transcription. Transcription is classified into four stages: initiation, promoter clearance, elongation, and termination. There is an increased interest in the mechanism of the elongation phase, as numerous elongation factors have now been discoverd[39]. Previous studies reported that steroid receptor binding to their target genes triggers the recruitment of P-TEFb favoring Pol II recruitment[32,40], and the P-TEFb/Pol II complex maintains the elongation state. The

phosphorylation of Ser2 of the Pol II CTD is required to release paused Pol II into productive elongation[41]. CTD Ser2 is phosphorylated by CDK9 of P-TEFb. Based on our results, LNG treatment strongly increased nascent PS mRNA synthesis in HepG2 cells, which was prevented by DRB. In contrast, DRSP-mediated Pol II transcription elongation efficiency was much lower. This implies that progestin-dependent PS upregulation is mainly induced through the facilitation of transcription elongation, which is involved in P-TEFb complex formation.

In our study, PS expression was upregulated following stimulation with progestin, although the effect varied depending on the isoform of progestin. In contrast, we previously reported that estrogen treatment yielded a decrease in PS expression[27]. Therefore, these results led us to hypothesize of a new mechanism that the estrogen-dependent PS reduction will be overcome by progestin in COCs. Based on comparisons made between two different generations of progestin, users of fourth-generation COCs with DRSP might not sufficiently prevail compared with the users of second-generation pills. This mechanism, therefore, might be responsible for the observed differences in VTE risk between the COC generations and thus, these findings should contribute to the development of next-generation COCs with a low VTE risk.

The primary pathogenesis of VTE is classified by Virchow's triad: (1) endothelial injury, (2) stasis or turbulence of blood flow, and (3) blood hypercoagulability[42]. We have considered that the differences in VTE risk between COC generations cannot be solely explained on the isoform of progestin, because we have only approached VTE risk associated with COCs from aspect of pro-coagulant activity, especially due to PS alteration. Further studies are needed to understand the pathogenesis of COC-associated VTE.

In conclusion, our observations suggest that progestin increased PS expression by facilitating transcription elongation in HepG2 cells, and that this activity was more proficient with second-generation progestin treatments. Therefore, the type of progestin could be a crucial factor in selecting the appropriate COC generation in clinical practice.

Author contributions

T.Kozuka performed the majority of experiments, analyzed data, and drafted the manuscript. S.T., N.K., Y.N., R.H., A.M., Y.T., M.M., N.M., and A.T. interpreted the data and contributed to analytical methodology. T.Kojima designed the project, analyzed the data, and wrote the manuscript. All authors discussed the results and contributed to manuscript preparation.

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Conflict of Interest Statement

The authors have declared that no conflict of interest exists.

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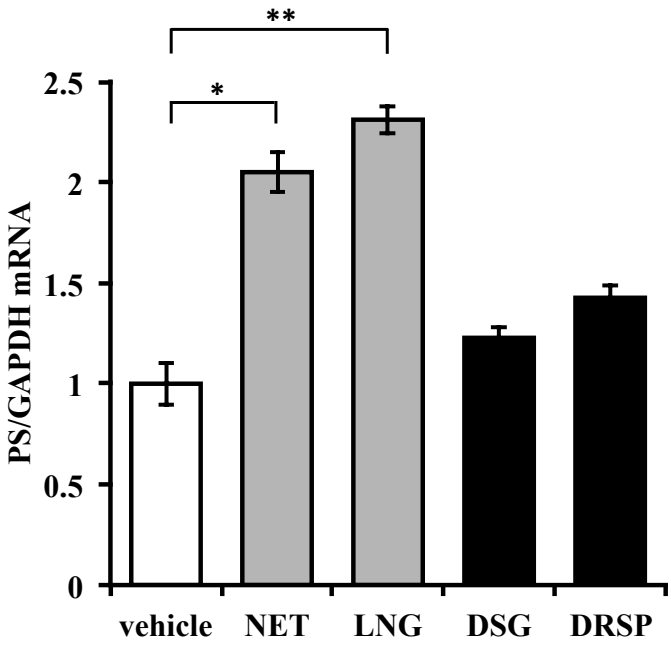


vehicle	NET	LNG	DSG	DRSP
1.00 ± 0.10	2.05 ± 0.10	2.31 ± 0.07	1.23 ± 0.05	1.43 ± 0.06

Fig. 1. Progestin upregulated PS mRNA expression in HepG2 cells.

HepG2 cells were treated with vehicle (DMSO), 50 nM NET, 20 nM LNG, 5 nM DSG or 100 nM DRSP. PS mRNA levels were measured after 48 h incubation using RT-qPCR. The expression levels of PS mRNA in progestin-treated cells were shown as relative values compared with vehicle treated cells (n = 4 per group). Data were normalized using GAPDH mRNA expression as an internal control.

The data are presented as mean ± standard error (SE). *, $p < 0.05$; **, $p < 0.01$.

DMSO = dimethyl sulfoxide, NET = norethisterone, LNG = levonorgestrel, DSG = desogestrel, DRSP = drospirenone, PS = protein S, GAPDH = glyceraldehyde-3-phosphate dehydrogenase.

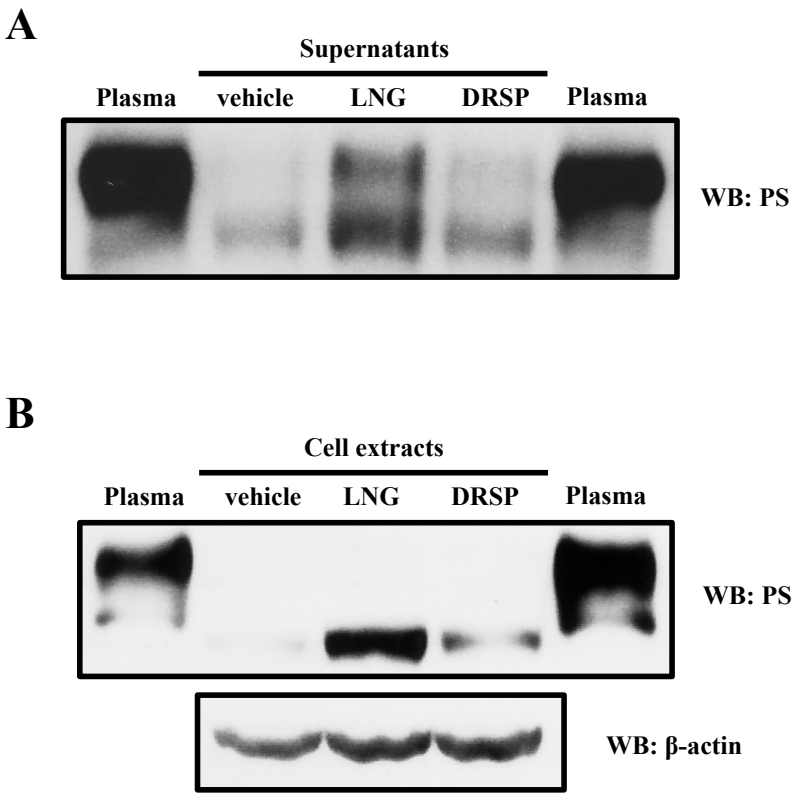
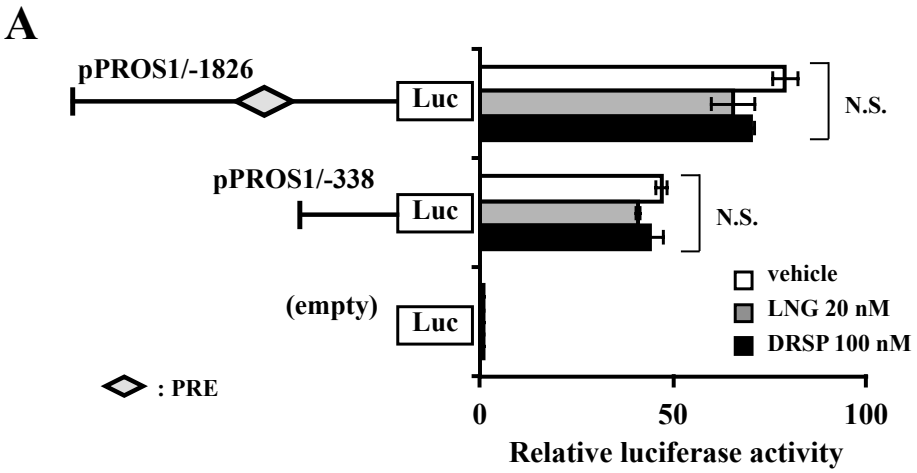


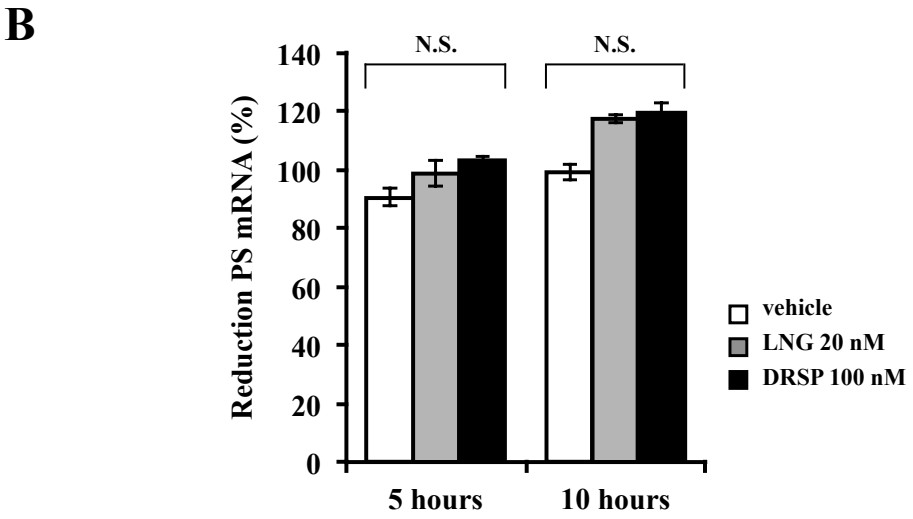
Fig. 2. Intracellular and secreted levels of PS after induction by progestin in HepG2 cells.

HepG2 cells were incubated with vehicle (DMSO), 20 nM LNG, or 100 nM DRSP for 48 h. (A) Secreted PS levels in conditioned media were analyzed by Western blot using rabbit anti-human PS IgG. Plasma PS (normal pooled plasma) was used as positive control. We applied 7 μL of 1/250 diluted normal pooled plasma and 20 μL of each progestin-treated cell culture media. (B) Intracellular PS in HepG2 cell extracts and normal pooled plasma were analyzed by Western blot using rabbit anti-human PS IgG. Western blot analysis was performed using anti-β-actin antibody as a loading control. We applied 8 μL of 1/250 diluted normal pooled plasma and 8 μL of each cell lysate sample containing 1.5 mg/mL protein.

DMSO = dimethyl sulfoxide, LNG = levonorgestrel, DRSP = drospirenone, PS = protein S.



	pPROS1/ -1826pGL3	pPROS1/ -338pGL3	pGL3-basic
vehicle	79.1 ± 3.2	47.1 ± 1.4	1.00 ± 0.03
LNG	65.4 ± 5.7	41.0 ± 0.6	0.84 ± 0.07
DRSP	70.3 ± 0.8	44.2 ± 3.3	0.87 ± 0.02

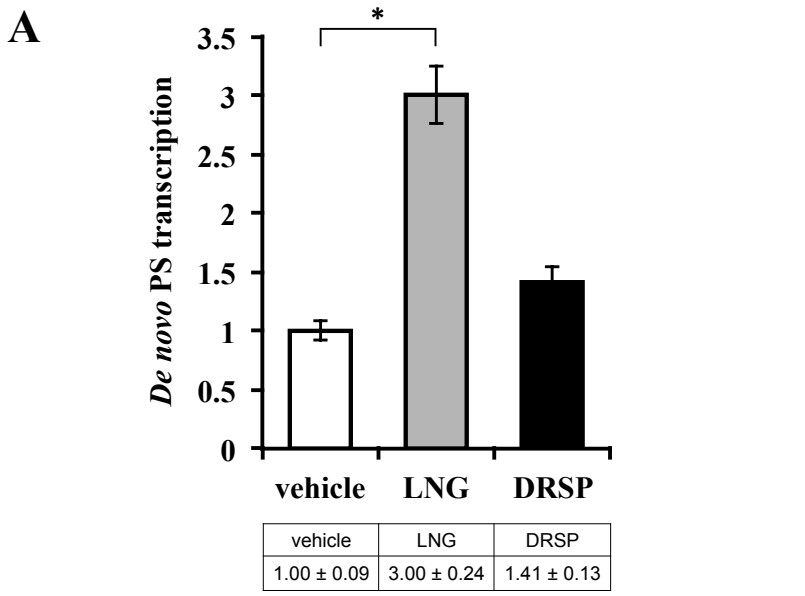


	5 hours	10 hours
vehicle	90.6 ± 2.9	99.3 ± 2.8
LNG-20nM	98.7 ± 4.3	117.4 ± 1.3
DRSP-100nM	103.1 ± 1.2	119.4 ± 3.5

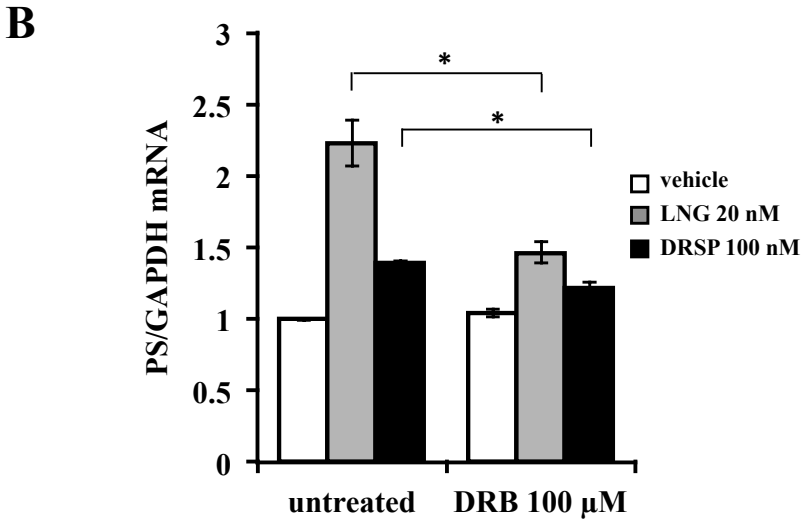
Fig. 3. Effects of progestin on *PROS1* promoter activity and PS mRNA stability.

(A) HepG2 cells transfected with pPROS1/−1826, pPROS1/−338, or pGL3-basic were cultured with vehicle (DMSO), 20 nM LNG, or 100 nM DRSP for 24 h. *PROS1* promoter activity was measured by dual-luciferase reporter assay systems. Results are expressed as normalized (Firefly/Renilla) relative luciferase activity (n = 4). (B) After pre-treatment with the respective progestin for 24 h, HepG2 cells were cultured for a further 5 or 10 h in the presence of actinomycin D (1 μg/mL) (n = 3). The data are presented as mean ± standard error (SE). N.S. indicates a non-significant difference.

DMSO = dimethyl sulfoxide, LNG = levonorgestrel, DRSP = drospirenone, PS = protein S.



vehicle	LNG	DRSP
1.00 ± 0.09	3.00 ± 0.24	1.41 ± 0.13



	untreated	DRB 100 µM
vehicle	1.00 ± 0.01	1.04 ± 0.02
LNG-20nM	2.23 ± 0.16	1.46 ± 0.08
DRSP-100nM	1.39 ± 0.01	1.21 ± 0.05

Fig. 4. *De novo* synthesis of PS mRNA after progestin treatment in HepG2 cells.

(A) Newly synthesized PS mRNA in HepG2 cells treated with vehicle (DMSO), 20 nM LNG or 100 nM DRSP were quantified. Thus, relative levels of PS mRNA normalized to GAPDH mRNA were determined *in vitro* by RT-qPCR (n = 3). (B) DRB blocks progestin-mediated upregulation of PS transcription elongation in HepG2 cells. HepG2 cells were treated with or without progestin for 4 h prior to the addition of DRB (100 µM) and cultured for a further 24 h. Total RNA was isolated, and PS mRNA levels were measured using RT-qPCR (n = 3). The data are presented as mean ± standard error (SE). *$p < 0.05$.

DMSO = dimethyl sulfoxide, LNG = levonorgestrel, DRSP = drospirenone, PS = protein S, GAPDH = glyceraldehyde-3-phosphate dehydrogenase, RT-qPCR = reverse transcription-quantitative PCR.

Supplementary Data.

Suppl. Table.

Cmax values of each progestin in a single clinical use of COCs.

	MW	Product name	Progestin dose (mg)	*Cmax (ng/mL)	*Cmax (nM)	Experimental conc. (nM)
norethisteron (NET)	298.42	LUNABELL®	1	12.4	41.6	50
levonorgestrel (LNG)	312.45	Triquilar®	0.05	6.2	19.8	20
desogestrel (DSG)	310.47	Marvelon®	0.15	1.05	3.4	5
drospirenone (DRSP)	366.49	YAZ®	3	37.4	102.0	100

*, in a single use of COCs

Data are derived from the Japanese interview form of each drug provided by the pharmaceutical companies.

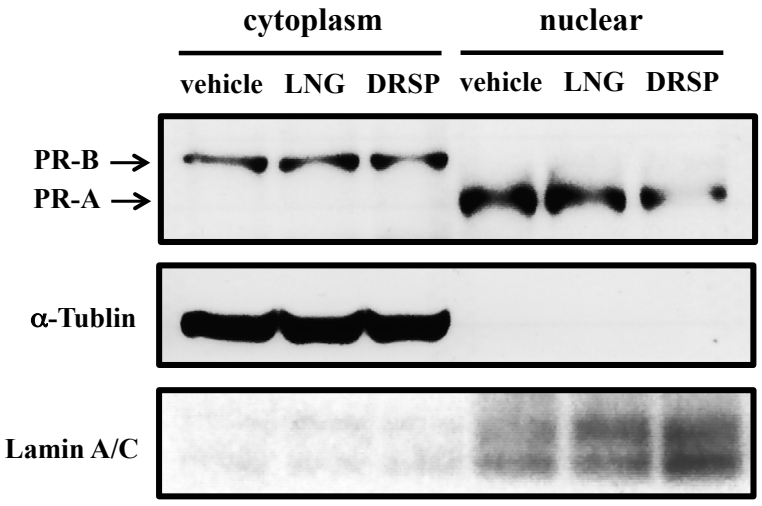
Suppl. Fig.

Methods

Cytoplasmic and nuclear proteins were prepared as nuclear run-on assay. Briefly, HepG2 cells were treated with vehicle (DMSO), 20 nM LNG, or 100 nM DRSP for 48 h. The cells were washed twice with chilled phosphate buffered-saline (PBS) and harvested in 1 mL of lysis buffer containing nonidet P-40 (NP-40) to collect intact nuclei. The supernatant was used to prepare cytoplasm proteins, and the pellets were used to prepare nuclear proteins. Cytoplasmic and nuclear proteins were run on a 10% SDS-PAGE, and Western blot analysis was performed using a primary antibody against progesterone receptor (C-20), α-Tublin (AA13) or Lamin A/C (H-110) (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) as described in PS Western analysis.

Results

We detected progesterone receptors (PR-A and PR-B) in HepG2 cells. With or without treatments of LNG or DRSP, PR-A was predominantly detected in the nucleus, whereas PR-B was in the cytoplasm.



Suppl. Fig. Expression of progesteron reseprors (PR-A and PR-B) in HepG2 cells.

Western blot analysis was performed using cytoplasmic and nuclear proteins isolated from HepG2 cells treated with vehicle (DMSO), 20 nM LNG, or 100 nM DRSP for 48 h. Lamin A/C and α-tubulin were used as loading controls for the nucleus and cytoplasm, respectively. DMSO = dimethyl sulfoxide, LNG = levonorgestrel, DRSP = drospirenone, PR-A = progesteron receptor-A, PR-B = progesteron receptor-B.