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Permalink

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Journal

Biology of Reproduction, 90(2)

ISSN

0006-3363

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Publication Date

2014-02-01

DOI

10.1095/biolreprod.113.114843

Peer reviewed

Comparison of Effects of Different Statins on Growth and Steroidogenesis of Rat Ovarian Theca-Interstitial Cells[1]

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ABSTRACT

Statins are competitive inhibitors of 3-hydroxy-3-methyl-glutaryl-CoA reductase, the rate-limiting enzyme of the cellular production of cholesterol and other products of the mevalonate pathway. Statins exert hepatic and extrahepatic effects, modulating the function of various tissues and organs, including ovaries. Previously, we have demonstrated that simvastatin inhibited cellular proliferation and reduced androgen production by ovarian theca-interstitial cells. The above actions are of translational relevance to the most common endocrine disorder among women in reproductive age: polycystic ovary syndrome. However, different statins may have distinctly different profiles of effects on cholesterol and androgens. The present study was designed to compare the effects of several statins on growth and steroidogenesis of rat theca-interstitial cells. The cells were incubated in the absence (control) or in the presence of simvastatin, lovastatin, atorvastatin, or pravastatin. Assessment of effects of statins on cell growth was carried out by evaluation of DNA synthesis and by estimation of the number of viable cells. Effects on steroidogenesis were evaluated by quantification of steroid production and expression of mRNA for the key enzyme regulating androgen production: *Cyp17a1*. Among tested statins, simvastatin exerted the greatest inhibitory effects on all tested parameters. The rank order of the effects of the tested statins is as follows: simvastatin > lovastatin > atorvastatin ≥ pravastatin. While the lipophilicity is likely to play a major role in determining the ability of statins to act on nonhepatic cells, other factors unique to individual cell types are also likely to be relevant.

Cyp17a1, ovarian theca-interstitial cells, proliferation, statins, steroidogenesis

INTRODUCTION

Statins are competitive inhibitors of 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase (HMGCR), the key enzyme regulating the mevalonate pathway. Inhibition of the mevalonate pathway leads to decreased cellular production of cholesterol and several other biologically important molecules, including substrates of isoprenylation: farnesyl pyrophosphate and geranylgeranyl pyrophosphate [1, 2]. The best-known property of statins pertains to the reduction of circulating cholesterol level: this effect is due to the inhibition of hepatic HMGCR, resulting in a compensatory increase in the expression of hepatic low-density lipoprotein (LDL) receptor and consequent uptake of LDL cholesterol [3]. However, statins exert also extrahepatic effects, modulating the function of various tissues and organs, including ovaries.

Previously, we have demonstrated that one of the statins, simvastatin, inhibits cellular proliferation and reduces androgen production by ovarian theca-interstitial cells [4, 5]. We also found that in these cells, simvastatin inhibits activity of HMGCR and reduces cholesterol synthesis in a concentration-dependent fashion [6]. The effect of simvastatin on the reduction of growth of theca-interstitial cells is not reversed in the presence of the cell- and mitochondrion-permeable forms of cholesterol (22-hydroxycholesterol and 25-hydroxycholesterol), indicating that the inhibition of proliferation of theca-interstitial cells is not due to reduced availability of cholesterol [4, 7]. However, the effects of simvastatin on cellular proliferation are partly abrogated in the presence of geranylgeranyl pyrophosphate and farnesyl pyrophosphate, indicating that simvastatin acts by inhibiting isoprenylation [4]. Mechanisms of action of simvastatin on steroidogenesis are more complex. A simvastatin-induced decrease of androgen production is likely to be due to a combination of two separate effects: reduction of the number of theca-interstitial cells and inhibition of *Cyp17a1* mRNA expression that is independent of the number of cells [5]. Inhibition of androgen production is reversed by 22-hydroxycholesterol as well as by geranylgeranyl pyrophosphate and farnesyl pyrophosphate, indicating that this action of simvastatin is the result of the reduced availability of cholesterol and substrates of isoprenylation [5].

The above actions of simvastatin on theca-interstitial cells are of translational relevance to the most common endocrine disorder among women in reproductive age: polycystic ovary syndrome (PCOS). This syndrome is characterized by enlargement of the ovarian theca-interstitial compartment, excessive androgen production, and ovulatory dysfunction [8, 9]. In clinical trials, we have demonstrated that administration of simvastatin to women with PCOS results in significant reduction of ovarian size, decrease of androgen levels, and restoration of ovulatory function [10–13]. Simvastatin also improved the profile of cardiovascular risk factors by reducing total cholesterol, LDL cholesterol, and hs-C-reactive protein level [13].

Recently, Kaya et al. [14, 15] compared effects of two statins—atorvastatin (20 mg daily) and simvastatin (20 mg daily)—on several endocrine and metabolic aspects of PCOS.

Interestingly simvastatin was more effective than atorvastatin in reducing the total testosterone level (by 30% vs. 18%). In contrast, simvastatin was less effective than atorvastatin in decreasing the LDL cholesterol level (by 6% vs. 18%). These observations indicate that different statins may have a distinctly different profile of effects on cholesterol and androgens. In view of these considerations, the present study was designed to compare the effects of several statins on growth and steroidogenesis of rat theca-interstitial cells.

MATERIALS AND METHODS

Animals

Female Sprague-Dawley rats were purchased at the age of 22 days from Charles River Laboratories (Wilmington, MA). The animals were housed in an air-conditioned environment and a 12L:12D cycle and received standard rat chow and water ad libitum. Starting at the age of 27 days, the rats received three daily injections of 17β-estradiol (1 mg in 0.3 ml of sesame oil s.c.) in order to promote ovarian development and growth of antral follicles. Twenty-four hours after the last injection, the animals were anesthetized using ketamine and xylazine (i.p.) and euthanized by intracardiac perfusion using 0.9% saline. All treatments and procedures were carried out in accordance with accepted standards of human animal care as described in the National Institutes of Health Guide for the Care and Use of Laboratory Animals and a protocol approved by the Institutional Animal Care and Use Committee at the University of California, Davis.

Cell Culture and Reagents

Ovarian theca-interstitial cells were isolated as described previously [16, 17]. Briefly, the ovaries were dissected from surrounding tissues under a dissecting microscope. Follicles were punctured, granulosa cells were released and washed out, and remaining ovarian tissues were minced and digested in collagenase and DNA-se for 60 min. Theca-interstitial cells were then finally purified using discontinuous Percoll gradient centrifugation. The cells were counted, and the viability assessed by the trypan blue exclusion test was routinely above 90%. In experiments evaluating cell proliferation and the number of viable cells, incubations were carried out for 48 h in 96-well plates at a density of 35 000 cells per well. In experiments evaluating steroidogenesis, theca-interstitial cells were incubated for 48 h in 24-well plates at a density of 400 000 cells/well. The cultures were carried out at 37°C in an atmosphere of 5% CO_2 in humidified air in serum-free McCoy 5A culture medium supplemented with 1% antibiotic/antimycotic mix, 0.1% bovine serum albumin, and 2 mM L-glutamine. The cells were incubated in the absence (control) or in the presence of simvastatin, lovastatin, atorvastatin, or pravastatin, each at a concentration of 1–10 μM. To optimize production of steroids, cultures were carried out in the presence of ovine luteinizing hormone (LH; 5 ng/ml). All the above chemicals were purchased from Sigma Chemical Co. (St. Louis, MO) except for LH, which was obtained from the National Hormone and Pituitary Program at the Harbor-UCLA Medical Center (Torrance, CA).

Cell Proliferation Assays

DNA synthesis was determined through a thymidine incorporation assay. Radiolabeled [³H] thymidine (1 μCi/well) was added to the cells 24 h before the culture was stopped. Subsequently, the cells were harvested with a multiwell cell harvester (PHD Harvester; Cambridge Technology, Inc., Watertown, MA), and radioactivity was measured in a liquid scintillation counter, Wallac 1409 (PerkinElmer, Shelton, CT).

Cell Viability Assays

The total number of viable cells was estimated using a CellTiter-Blue Cell Viability Assay (Promega, Madison, WI) 2 h before the end of the culture period. Fluorescence was determined with the use of a microplate reader (Fluostar Omega, BMG, Durham, NC). To validate the assay, a standard curve with a known number of cells was generated, and a linear correlation was verified ($r^2 = 0.99$, $P < 0.001$).

Total RNA Isolation and Quantitative Real-Time PCR

Isolation of total RNA was achieved using the MagMAX-96 Total RNA Isolation Kit (Applied Biosystems, Foster City, CA) and the KingFisher robot (Thermo Scientific, Vantaa, Finland). Reverse transcription of total RNA to cDNA was carried out using the High Capacity cDNA Reverse Transcription Kit for RT-PCR (Applied Biosystems). PCR were set up in 28-μl volumes, consisting of 5 μl cDNA, 4.5 μl forward and 4.5 μl reverse 900 nM primers, and 14 μl of 2× SYBR Green PCR Master Mix (Applied Biosystems).

Quantitative real-time PCR reactions were performed in triplicate using the ABI 7300 Real-Time PCR System (Applied Biosystems). Separate cDNA dilutions were included in each PCR run to generate standard curves. Data were analyzed using SDS 1.4 software (Applied Biosystems). The relative amount of target mRNA was expressed as a ratio normalized to hypoxanthine phosphoribosyltransferase (HPRT). The primer sequences were as follows: rat *Cyp17a1* forward (5′-ACT GAG GGT ATC GTG GAT GC-3′) and reverse (5′-CCG TCA GGC TGG AGA TAG AC-3′) and rat *Hprt* forward (5′-TTG TTG GAT ATG CCC TTG ACT-3′) and reverse (5′-CCG CTG TCT TTT AGG CTT TG-3′).

Quantification of Steroids

Preparation of samples and details of the analytical procedures have been described previously in greater detail in our previous publication [5]. Briefly, aliquots of each sample (300 μl) were placed in a 2.0-ml autosampler vial and spiked with 150 μl of internal standard solution, that is, androsteneione-d7 and testosterone-d3. Detection and quantitation of all analytes was accomplished using selective reaction monitoring. Androstenedione, androsterone, progesterone, and the deuterated derivative of androsteneione-d7 were obtained from Steraloids (Newport, RI), whereas testosterone-d3 was obtained from Cerillient (Round Rock, TX). Acetonitrile and methanol were HPLC grade and obtained from Burdick and Jackson (Muskesgon, MI). Acetone, isopropanol, and ammonium hydroxide were Optima grade and obtained from Fisher (St. Louis, MO). Formic acid was ACS grade and obtained from EMD (Gibbstown, NJ). Steroid levels were determined using novel turbulent flow chromatography (TFC) HPLC-MS/MS method facilitating the simultaneous detection of androstenedione, androsterone, and progesterone. The concentration of each of these steroids in each sample (e.g., calibrators, quality control, and unknowns) was determined by an internal standard method using the peak area ratio and linear regression analysis. The responses for androstenedione, androsterone, and progesterone were linear and gave correlation coefficients (R^2) of 0.99 or better. To account for statin-induced effects on the cell number, steroid production was expressed as percentage of control per unit of protein content in each individual culture.

Statistical Analysis

Statistical analysis of the data was carried out using JMP 10.0 software (SAS, Cary, NC). Data are presented as the mean ± SEM. Comparison of means was performed by analysis of variance followed by post hoc pairwise testing using the Tukey HSD test. When appropriate, data were logarithmically transformed. A value of $P < 0.05$ was considered statistically significant.

RESULTS

Effects of Statins on Growth of Cells

Assessment of effects of several statins on growth of theca-interstitial cells was carried out by evaluation of proliferative activity of cells and by estimation of the number of viable cells. Proliferation was quantified by determination of DNA synthesis using radiolabeled thymidine incorporation assay. As presented in Figure 1, all tested statins induced a concentration-dependent inhibition of DNA synthesis. The greatest effects were observed in response to simvastatin (1–10 μM), which induced inhibition by 40–77% below control levels ($P < 0.001$ for all tested concentrations). Lovastatin had no significant effect at 1 μM, whereas at 3 and 10 μM, it reduced DNA synthesis by 29% and 61%, respectively (both at $P < 0.001$). Atorvastatin had no effect at 1 μM, while it inhibited thymidine incorporation at 3 and 10 μM, respectively, by 22% and 52% (both at $P < 0.01$). Finally, pravastatin had no effect at 1 μM, but at concentrations of 3 and 10 μM, it decreased DNA synthesis by 26% and 38% (both at $P < 0.001$). Effects of simvastatin were significantly greater than the effects of all other statins at all tested concentrations ($P < 0.05$).
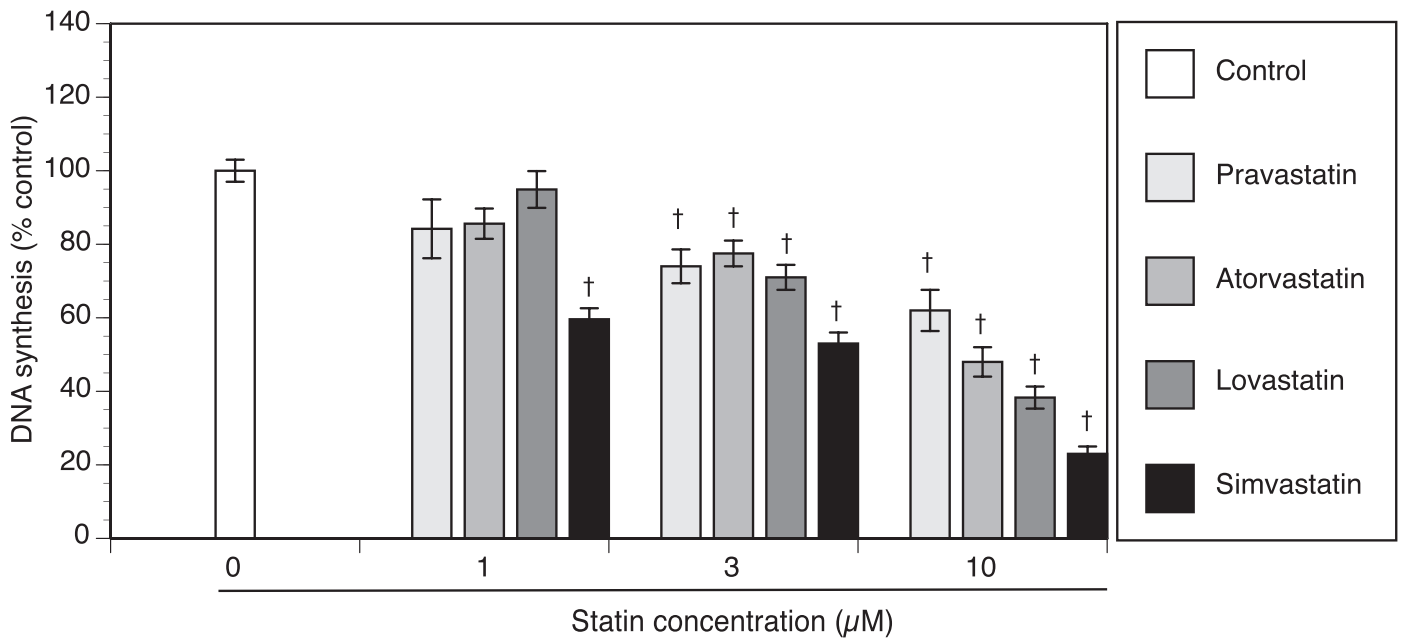
FIG. 1.   Effects of statins on DNA synthesis. The cells (35 000 cells/200 µl) were cultured in 96-well plates in chemically defined media supplemented with ovine LH (5 ng/ml) for 48 h in the absence (control) or in the presence of simvastatin, lovastatin, atorvastatin, or pravastatin at concentrations of 1, 3, and 10 µM. DNA synthesis was evaluated by radiolabeled thymidine incorporation assay. The number of viable cells was determined by MTS assay. Each bar represents the mean ± SEM; means significantly different from control values are denoted by * ($P < 0.05$) and † ($P < 0.01$).

The effects of statins on the number of viable cells are presented in Figure 2. At all tested concentrations, simvastatin was the most effective statin, reducing the number of viable cells in a concentration-dependent fashion by 13–52% ($P < 0.01$). Lovastatin also decreased the number of viable cells at all concentrations by 11–36% ($P < 0.05$). Atorvastatin and pravastatin were less effective and induced significant reduction of viable cell number only at 10 µM: atorvastatin by 21% ($P < 0.001$) and pravastatin by 10% ($P < 0.05$).

Effects of Statins on Steroidogenesis

Comparison of the effects of individual statins on steroidogenesis was carried out at a concentration of 1 µM. Summary of three separate experiments is presented in Figures 3 and 4. At this concentration, statins had minimal effect on the number of viable cells (Fig. 2). Figure 3 presents the concentrations of steroids detected in culture media and the amount of protein from the attached cells. To account for variability of steroid production in individual experiments and
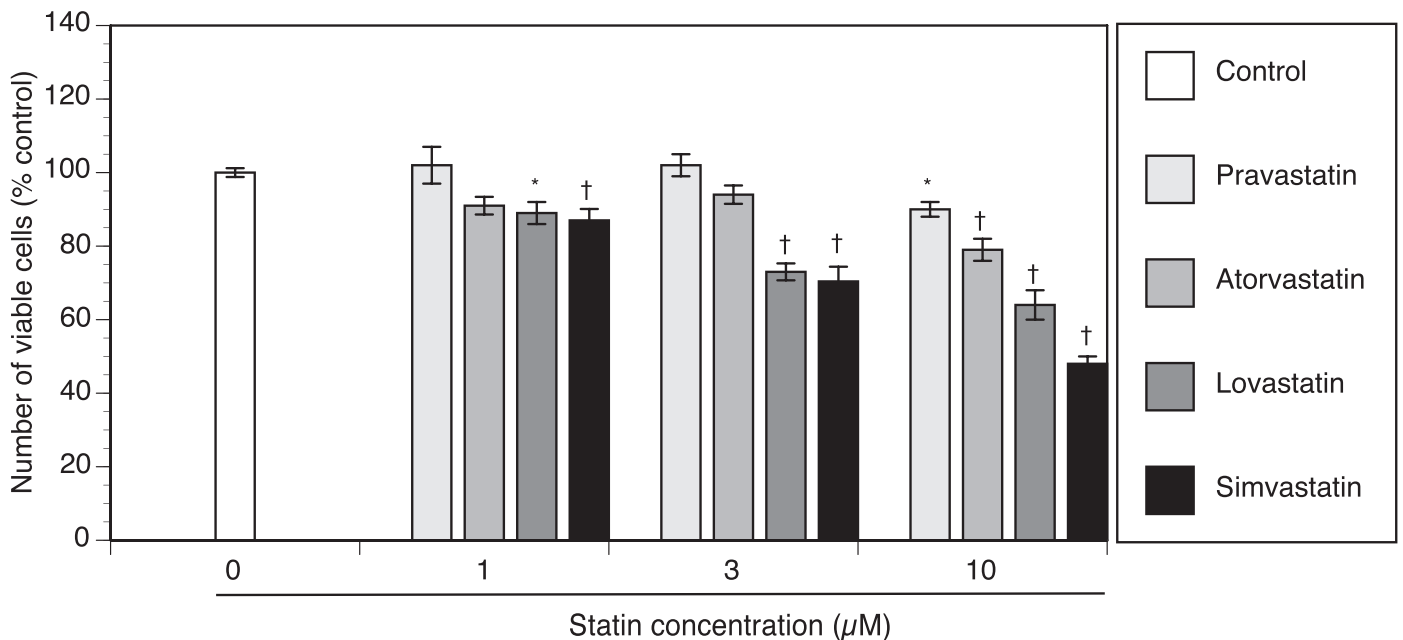

FIG. 2.   Effects of statins on the number of viable theca-interstitial cells. The cells were cultured as described in Figure 1 legend. The number of viable cells was determined by MTS assay. Each bar represents the mean ± SEM; means significantly different from control values are denoted by * ($P < 0.05$) and † ($P < 0.01$).
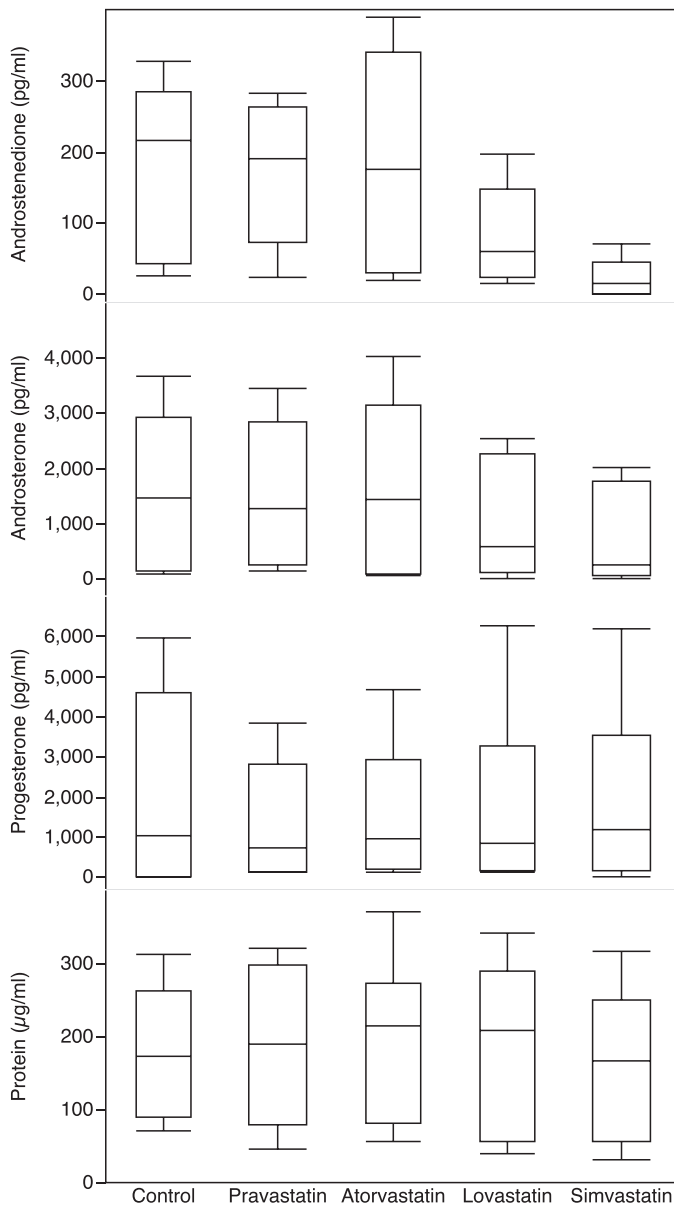
FIG. 3. Levels of androstenedione, androsterone, and progesterone in spent media and quantification of protein from attached cells. The graph presents box plots summarizing observations of three independent experiments. The cells (400 000 cells/ml) were cultured in 24-well plates in chemically defined media supplemented with ovine LH (5 ng/ml) for 48 h in the absence (control) or in the presence of simvastatin, lovastatin, atorvastatin, or pravastatin at concentrations of 1 μM.

to account for the effect of statins on the amount of theca-interstitial cells, levels of steroids were subsequently expressed per milligram of protein from the attached cells and normalized, that is, expressed as percentage of control cultures (Fig. 4). Simvastatin and lovastatin significantly inhibited concentrations of androstenedione (Fig. 4A), respectively, by 54% and 44% (both at $P < 0.05$). Similarly, simvastatin and lovastatin reduced concentrations of androsterone (Fig. 4B), respectively, by 52% and 40% (both at $P < 0.05$). In contrast, atorvastatin and pravastatin had no significant effect on androgen concentrations. Progesterone concentrations (Fig. 4C) were not affected by any of the statins.

Figure 5 illustrates relative expression of mRNA for the key enzyme regulating androgen production: *Cyp17a1*. Consistent
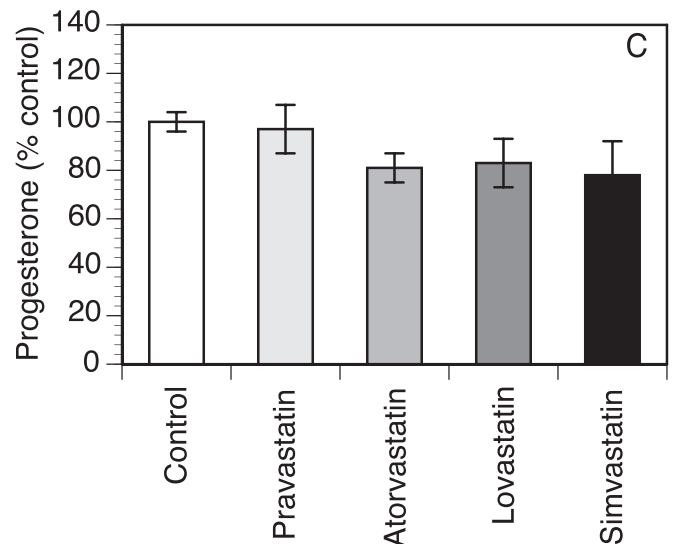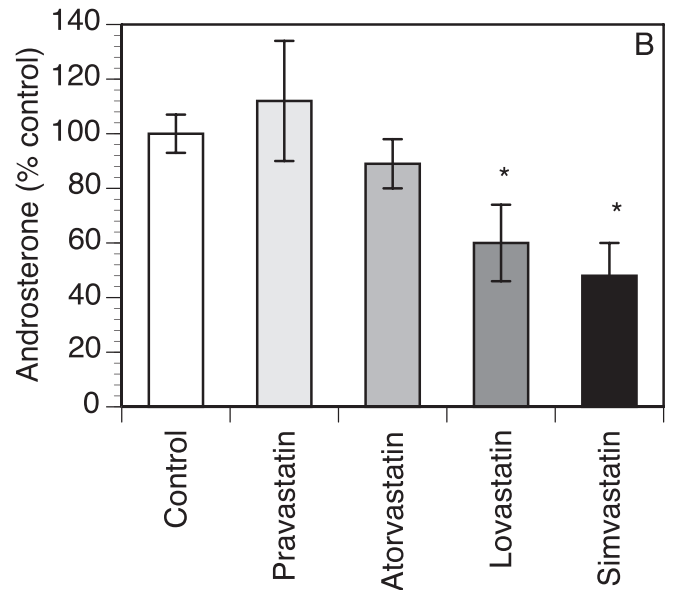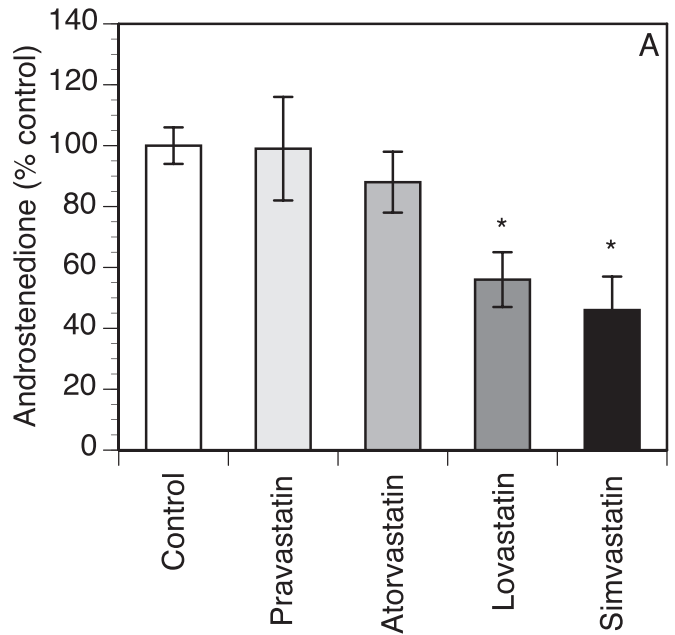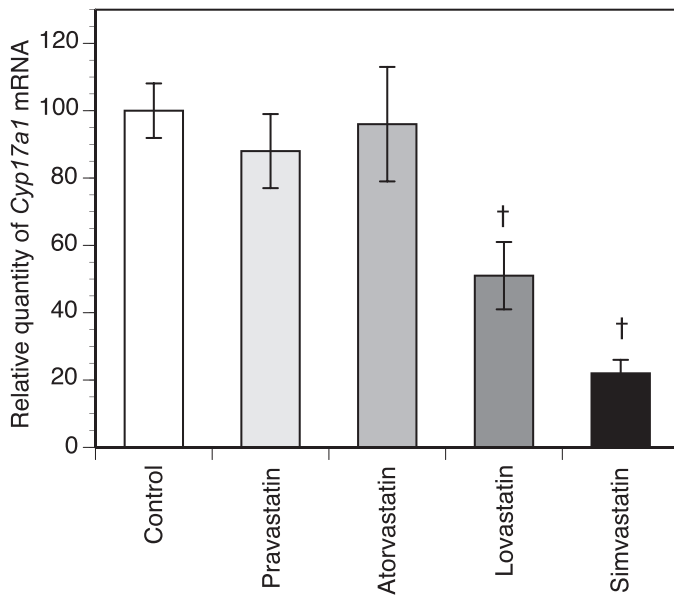
FIG. 5. Relative expression of mRNA for *Cyp17a1* in response to simvastatin, lovastatin, atorvastatin, and pravastatin. Experiments were performed as described in Figure 4. Housekeeping gene was *HPRT*. Each bar represents the mean ± SEM; means significantly different from control values are denoted by † ($P < 0.01$).

with other effects of statins, simvastatin induced the greatest inhibition of *Cyp17a1* expression by 78% ($P < 0.01$), whereas lovastatin reduced *Cyp17a1* expression by 49% ($P < 0.01$). Atorvastatin and pravastatin had no significant effect.

DISCUSSION

This study demonstrates that statins vary greatly with regard to their effects on growth and steroidogenesis of rat ovarian theca-interstitial cells. It is apparent that among tested statins, simvastatin exerts the greatest inhibitory effects on DNA synthesis, the number of viable cells, production of androgens, and relative expression of *Cyp17a1* mRNA. The rank order of the effects of the tested statins on cell growth and androgen production is as follows: simvastatin > lovastatin > atorvastatin ≥ pravastatin. This rank order is distinctly different from that of the effects of these statins on lipid profile in animal models and in clinical trials. For example, in a mouse model of hyperlipidemia, the plasma cholesterol- and triglyceride-lowering effects of these statins were in the following order: pravastatin > atorvastatin > simvastatin > lovastatin [18]. Again, a different order of potency of the effects of these statins was noted in a large clinical trial evaluating reduction of LDL cholesterol: atorvastatin > simvastatin > pravastatin ≥ lovastatin [19]. Notably, in a cell-free system using purified human HMG-CoA catalytic domain, the IC50s of simvastatin and atorvastatin were comparable at 9 and 10 nM [20]. The above observations demonstrate that relative effects of individual statins differ depending on the biological model and are not related solely to the actual potency of inhibition of HMGCR.

It is very likely that the key factor contributing to the presently observed differences in the effects of individual statins on theca-interstitial cells is the difference in the ability of statins to enter the cell due to the molecules' lipophilicity versus hydrophilicity. Lipophylic statins may traverse cell membranes via passive diffusion, whereas hydrophilic statins may be relatively inaccessible to most cells with the notable exception of hepatocytes, which possess specific organic anion transporters [21, 22]. Simvastatin, lovastatin, and pravastatin are structurally similar; however, simvastatin and lovastatin are lipophilic, whereas pravastatin is hydrophilic due to the presence of a polar hydroxyl group [23, 24]. Atorvastatin is structurally different from all other presently tested statins, and while it is considered lipophilic, it is less lipid soluble than simvastatin: a direct comparison of relative lipophilicity of several statins has been studied under physiological pH conditions demonstrating the following rank order: simvastatin > lovastatin ≈ atorvastatin > > pravastatin [25].

The above order is nearly identical to the order of potency of statins observed in the present study with the exception of significantly greater effects of lovastatin than atorvastatin on both the growth and the steroidogenesis of theca-interstitial cells. Comparable differences of effectiveness of different statins on cell proliferation and apoptosis were observed in pulmonary artery smooth muscle cells whereby the rank order of potency was simvastatin > atorvastatin > pravastatin [26]. While the lipophilicity is likely to play a major role in the determining the ability of statins to act on nonhepatic cells, other factors unique to individual cell types are also likely to be highly relevant. For example, in a study on proliferation of human saphenous vein smooth muscle cells, inhibitory effects of atorvastatin were significantly greater than the effects of either simvastatin or lovastatin [27]. We speculate that factors other than lipophilicity that may play a role in modulating actions of statins in different tissues may involve differences in intracellular metabolism and stability individual statins as well as variability in direct, not HMGCR-mediated antioxidant activity of statins [28]. Indeed, our previous studies on theca-interstitial cells have demonstrated that antioxidants such as vitamin E succinate and ebselen exert effects comparable to statins by inhibiting proliferation of cells, whereas moderate oxidative stress stimulates proliferation [29].

A noted limitation of this study pertains to the interpretation of the effects of statins on androgen production. Since statins also reduce proliferation of theca-interstitial cells, decreased accumulation of androgens in spent media is at least in part due to the lower number of cells. This issue was addressed as follows. First, the concentration of statins used to evaluate effects on steroidogenesis (1 µM) had a relatively small impact on the number of viable cells whereby the most potent statin, simvastatin, reduced the number of viable cells by 13% (Fig. 2) and reduced the concentration of androstenedione and androsterone, respectively, by 88% and 55% (Fig. 3). Second, as presented in Figure 4, relative steroid levels were expressed per amount of protein from viable-attached cells. Third, the expression of the mRNA of the key enzyme regulating

FIG. 4. Effects of several statins on the level of androstenedione (**A**), androsterone (**B**), and progesterone (**C**). The cells (400 000 cells/ml) were cultured in 24-well plates in chemically defined media supplemented with LH (5 ng/ml) for 48 h in the absence (control) or in the presence of simvastatin, lovastatin, atorvastatin, or pravastatin at concentrations of 1 µM. Quantification of steroids in spent media was performed by mass spectrometry, as described in *Materials and Methods*. To account for the effect of statins on the number of cells and for the variability of steroid production in individual experiments, the concentrations of steroids are expressed per milligram of protein content of attached cells and normalized to control cultures, that is, expressed as percentage of control. Each bar represents the mean ± SEM; means significantly different from control values are denoted by * ($P < 0.05$).

androgen production (*Cyp17a1*) was greatly reduced when compared to the expression of mRNA of a reference gene (*hprt*). Another relevant observation pertains to the levels of steroids detected in this study when compared to other studies evaluating theca explants or purified theca-interstitial cells. While the levels of progesterone in the present study were comparable to previous reports, levels of androstenedione and androsterone were typically lower [30–33]. This difference is likely related to the use of precise HPLC-MS/MS in this study (vs. radioimmunoassay in previous reports) and variability of culture conditions, especially differences in concentrations of LH.

In summary, present findings demonstrate that theca-interstitial cells have a unique set of responses to individual statins with regard to modulation of growth and steroidogenesis; these responses may explain differences in observed effects of different statins on cholesterol versus in androgen levels [15].

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