Effects of genistein, resveratrol, and quercetin on steroidogenesis and proliferation of MA-10 mouse Leydig tumor cells

Yu-Chyu Chen[1,2], Madan L Nagpal[1,2], Douglas M Stocco[3] and Tu Lin[1,2]

[1]Medical and Research Services, WJB Dorn Veterans Medical Center, Columbia, South Carolina 29201, USA

[2]Department of Medicine, Medical Library Building, Suite 316, School of Medicine, University of South Carolina School of Medicine, Columbia, South Carolina 29208, USA

[3]Department of Cell Biology and Biochemistry, Texas Tech University Health Sciences Center, Lubbock, Texas 79430, USA

(Requests for offprints should be addressed to T Lin at Department of Medicine, University of South Carolina; Email: lin@med.sc.edu)

Abstract

This study was performed to compare the effects of three well-known phytoestrogens such as genistein, resveratrol, and quercetin on steroidogenesis in MA-10 mouse tumor Leydig cells. Addition of genistein or resveratrol to MA-10 cells resulted in decreases in the cAMP-stimulated progesterone secretion, but quercetin had an opposite response. Steroidogenic acute regulatory (StAR) mRNA expression and StAR promoter activity in transiently transfected MA-10 cells were significantly reduced by genistein or resveratrol, but increased by quercetin. Genistein was found to inhibit MA-10 cell proliferation, while resveratrol and quercetin had no effect. Quercetin-induced increase in cAMP-stimulated progesterone secretion was reversed by ICI 182,780, an estrogen receptor (ER) antagonist. However, ICI 182,780 had no effect on cAMP plus quercetin-stimulated StAR promoter activity. To examine whether non-ER factors are associated with quercetin-stimulated progesterone production, we treated MA-10 cells with EGTA to deprive them of extracellular Ca^{2+}. We found that EGTA inhibited quercetin- plus cAMP-stimulated progesterone secretion and StAR promoter activity. Blocking of Ca^{2+} influx through L- or T-type voltage-gated Ca^{2+} channels with verapamil or mibefradil respectively, attenuated quercetin-stimulated progesterone secretion, while they had no effect on quercetin- plus cAMP-stimulated StAR promoter activity. Blocking of intracellular Ca^{2+} efflux by sodium orthovanadate, a Ca^{2+}-pump inhibitor, blocked quercetin- plus cAMP-stimulated progesterone secretion and StAR promoter activity in MA-10 cells. Finally, EGTA or vanadate reduced quercetin and cAMP-increased in StAR mRNA expression in MA-10 cells, while ICI 182,780 had no effect. Taken together, these results indicate that phytoestrogens have differential effects on steroidogenesis in MA-10 cells.

Journal of Endocrinology (2007) **192,** 527–537

Introduction

Converging lines of evidence suggested that estrogen acts as an important paracrine factor to modulate testicular functions (O'Donnell *et al.* 2001). Estrogen receptor (ER)α is present in the Leydig cells and ERβ is detected exclusively in the Sertoli cells (Saunders *et al.* 1998, Pelletier *et al.* 2000). Estradiol treatment suppressed progesterone synthesis (Freeman 1985), but stimulated proliferation of mouse Leydig tumor cells (Sato *et al.* 1987, DuMond *et al.* 2001). Furthermore, mice with a genetic disruption of ERα (α estrogen receptor knockout (αERKO)) appeared infertile, had lower sperm counts and elevated serum testosterone levels (Eddy *et al.* 1996), and were not responsive to the estrogen benzoate-mediated decreases in both serum luteinizing hormone (LH) and androgen levels (Akingbemi *et al.* 2003). In addition, cytochrome P450 17α-hydroxylase (C17α), 17β-hydroxysteroid dehydrogenase (17β-HSD), and StAR mRNAs were higher in αERKO mice than in wild-type mice (Akingbemi *et al.* 2003). This finding suggested that estrogen and ER-mediated signals regulate Leydig cell steroidogenesis.

Phytoestrogens such as genistein, resveratrol, and quercetin are widely distributed in human and animal diet and have chemopreventive properties against estrogen-responsive diseases, including inhibition of tumor cell growth (Setchell & Cassidy 1999, Miodini *et al.* 1999, Mitchell *et al.* 1999, Xing *et al.* 2001), lowering serum cholesterol, and prevention of bone loss in rodents (Mizutani *et al.* 2000, Nakajima *et al.* 2001, Wattel *et al.* 2003). Genistein is the most potent estrogenic compound in soy and soy products. Resveratrol is a potent antioxidant in grapes and red wines. Quercetin is found in apples, onions, and vegetables. Importantly, the exposure of young animals to phytoestrogens has potential impact on male and female gonadal functions. For example, a dietary (Weber *et al.* 2001, Wisniewski *et al.* 2003, Svechnikov *et al.* 2005) or s.c. (Ohno *et al.* 2003) administration of male rats with genistein resulted in lower serum testosterone levels and demasculinization. Exposure of resveratrol to female rats led to

DOI: 10.1677/JOE-06-0087
0022–0795/07/0192–527
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the development of ovarian hypertrophy and to shorter estrous cycle (Henry & Witt 2002). All of these phenomena are believed to be due to the perturbation on gonadal steroidogenesis by the phytoestrogens. In fact, steroidogenic enzymes such as cytochrome P450 side-chain cleavage complex (P450scc) and 17β-HSD in the gonad and adrenal (Le Bail 2002, Ohno *et al.* 2002, Supornsilchai *et al.* 2005, Svechnikov *et al.* 2005) were altered by phytoestrogen treatments. As yet, reports for phytoestrogen-mediated effects on steroidogenic acute regulatory (StAR) protein expression are limited. Therefore, we focused on phytoestrogen-mediated effects on *StAR* gene expression and steroidogenesis.

In the gonad, the initial step of steroid synthesis depends on the delivery of cholesterol from cytosol into the mitochondria by the StAR protein (Clark *et al.* 1994, 1997), which is the rate-limiting step in steroidogenesis. The expression of StAR is especially abundant in testis, ovary, and adrenal glands (Sugawara *et al.* 1995, Clark *et al.* 1997), and its importance in steroidogenesis was clearly shown by the virtual absence of steroid synthesis in StAR knockout mice (Caron *et al.* 1997*b*) and in patients with congenital lipoid adrenal hyperplasia caused by mutations in the *StAR* gene (Lin *et al.* 1995, Bose *et al.* 1996). The expression of the *StAR* gene is regulated by numerous factors (Manna & Stocco 2005) and have been shown to be suppressed by 17β-estradiol in MA-10 cells (Houk *et al.* 2004). Apart from their estrogenic effects, genistein (Sargeant *et al.* 1993) and quercetin (Lloyd *et al.* 1995) are potent tyrosine kinase inhibitors and have effects in the alteration of cytoplasm Ca^{2+} level. Resveratrol has been characterized as a nonspecific cyclooxygenase (COX) inhibitor (Murias *et al.* 2004). The aim of the present study was to compare the effects of quercetin, resveratrol, and genistein on *StAR* gene expression and steroid hormone synthesis in MA-10 cells, in which progesterone is the main steroid product due to the lack of P450c17 expression.

Materials and Methods

Materials and reagents

ICI 182,780 was purchased from Tocris (Ellisville, MO, USA). EGTA, mibefradil, verapamil, BK4866, sodium orthovanadate, and 8-bromo-adenosine-cAMP (8-Br-cAMP) were obtained from Sigma. DNase I, Murine Moloney Leukemia Virus (M-MLV) reverse transcriptase, Dual Luciferase Assay System, pRL-SV40 *Renilla luciferase* vector, and CellTiter 96 Aqueous Non-Radioactive Cell Proliferation Assay kits were purchased from Promega. Progesterone Enzyme Immunoassay kits were obtained from Cayman (Ann Arbor, MI, USA). Bicinchoninic Acid Protein Assay kits were purchased from Pierce Biochemicals (Rockford, IL, USA). The Geneport transfection reagent was obtained from Gene Therapy System, Inc. (San Diego, CA, USA). Trizol reagent, horse serum, and Waymouth's MB572/1 culture medium were obtained from Invitrogen Life Technologies, Inc.

Cell line and culture condition

The MA-10 mouse Leydig tumor cell line (Ascoli 1981) was a generous gift from Dr Mario Ascoli (University of Iowa, Iowa City, IA). MA-10 cells were grown in complete Waymouth's MB572/1 medium containing 2·2 g sodium bicarbonate, 100 U/ml penicillin/streptomycin, and 15% horse serum in a 37 °C and 5% CO_2 incubator.

Progesterone assay

MA-10 cells (5×10^4/ml) were seeded into each well of 96-well plates. On the next day, the cells were incubated with 1, 10, 25, or 50 μM quercetin, resveratrol, or genistein. After 24 h, the cells were washed with Ca^{2+}-free phosphate buffer saline twice and replaced with assay medium (Waymouth MB572/1 containing 0·1% BSA (pH 7·4)) containing 0·2 mM 8-Br-cAMP for 4 h. The media were collected for the measurement of progesterone. Steroids were extracted by mixing 1 vol medium with 10 vol diethyl ether in 12×75 mm glass tubes. The steroid extracts were evaporated in a 34 °C water bath in a ventilated chemical hood. The amount of progesterone in the extracts was measured according to the protocol accompanying the Progesterone Enzyme Immunoassay Kit. Ten microliters of radio immunoprecipitation assay (RIPA) lysis buffer (50 mM Tris–HCl (pH 7·5), 150 mM NaCl, 1 mM EGTA, 1% NP-40, 0·25% sodium deoxycholate, 2 μg/ml aprotinin, 1 μg/ml leupeptin, and 100 μg/ml phenyl methyl sulfonyl fluoride (PMSF)) were added to each well containing cell monolayer and the protein concentration was determined using a Bicinchoninic Acid Protein Assay Kit.

StAR promoter–luciferase assay

MA-10 cells (1×10^5/ml) were seeded into each well of six-well culture plates for 2 days until they were 50–60% confluent. The cells in each well were transfected with 2·0 μg mouse StAR promoter (P-966)–luciferase reporter gene (Caron *et al.* 1997*a*), 0·2 μg pRL-SV40 *Renilla* luciferase vector, and 15 μl GenePORTER transfection reagent in serum-free Waymouth's MB752/1 medium for 5 h. Cells were replaced with complete Waymouth's MB752/1 medium and incubated at 37 °C, 5% CO_2. On the next day, the cells were incubated with fresh Waymouth's MB752/1 medium containing 0·5, 1, 10, 25, or 50 μM quercetin, resveratrol, or genistein for another 24 h. The cells were then treated with or without 0·2 mM 8-Br-cAMP for 6 h and the lysate was harvested using Passive Lysis Buffer (Promega). The luciferase activity of the cell lysate was determined according to the manufacturer's protocol of Dual-Luciferase Reporter Assay System. The light intensity of each sample was measured in an EG&G Berthold LB 9507 Luminometer (Berthold, Bad Wildband, German). The StAR P-996 luciferase activity was normalized to that of the *Renilla* luciferase vector in order to compensate for the variation in transfection efficiency. Each treatment group consisted of triplicate cultures, and each experiment was repeated at least thrice.

Cell proliferation assay

MA-10 cells ($2{\cdot}5\times10^4$/ml) were seeded into each well of 96-well plates. After 24 h, the cells were treated with 1, 10, 25, or 50 μM quercetin, resveratrol, or genistein. Cell growth was measured from days 0 to 5 with the MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) assay according to the manufacturer's protocol. Absorbance was recorded at 490 nm using a Bio-Rad Benchmark microplate reader (Bio-Rad). Each treatment contained four replicates, and this experiment was performed at least thrice.

Real-time reverse transcription-PCR

MA-10 cells were treated with 50 μM quercetin, resveratrol, or genistein and 0·2 mM 8-Br-cAMP for 24 h. Total RNA was prepared using Trizol reagent according to the manufacturer's protocol. RNA samples were incubated with RQ1 DNase at 37 °C for 30 min and then inactivated at 65 °C for 10 min. First strand cDNA was generated using 5 μg DNase-treated RNAs and M-MLV reverse transcriptase at 42 °C for 1 h. The first strand cDNA was used for real-time PCR amplification reactions that were carried out using the MyIQ system (Bio-Rad). Reactions were initiated with hot start at 94 °C for 5 min, followed by 48 cycles at 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 1 min with an extension of 5 min. The *StAR* gene was amplified using the primer pairs: forward: 5′-TTCA-AGCTGTGTGCTGG GAGCTCCTA-3′ and reverse: 5′-TTAACACTGGGCC TCAGAGGCAGGGCTGG-3′ which gave an amplification product of 247 bp. Primer S14 ribosomal protein was amplified using the forward: 5′-TTTGGTGTCTGCCACATCTTTG-3′ and reverse: 5′-ATGGGGGTGACATCCTCAAT-3′ sequences which yielded an amplification product of 323 bp (Mamchaoui *et al.* 2002). Reactions were performed in triplicate, and the average threshold cycle (C_T) was used in subsequent calculations to determine the StAR mRNA levels using the $^{\Delta\Delta}C_T$ method of MyIQ system (Bio-Rad). The mean relative values ± S.E.M. from three independent experiments were determined.

Statistical analysis

All data were analyzed by one-way ANOVA followed by Fisher's least significant difference test using the StatView 4·5 program (Abacus Concepts, Inc., Berkley, CA, USA) fitted for the Macintosh computer. The results were expressed as the mean ± S.E.M., and $P\leq0{\cdot}05$ was considered statistically significant.

Results

Effects of phytoestrogens on progesterone productions in MA-10 cells

In the present study, progesterone secretion of MA-10 cells in response to cAMP stimulation was decreased by genistein or resveratrol at concentrations higher than 25 μM (Fig. 1). On the other hand, quercetin increased progesterone synthesis (Fig. 1). These results suggested that there are differential effects of phytoestrogens on MA-10 cell steroidogenesis.

Effects of phytoestrogens on StAR mRNA levels and StAR promoter activity in transiently transfected MA-10 cells

We next evaluated the effects of genistein, resveratrol, and quercetin on StAR promoter activities in transiently transfected MA-10 cells. StAR promoter activity was inhibited by treatment with either genistein or resveratrol at concentrations higher than 10 or 25 μM respectively (Fig. 2), when compared with cells treated with cAMP only. On the other hand, StAR promoter activity was enhanced by quercetin (Fig. 2). Cyclic AMP-stimulated StAR mRNA levels in MA-10 cells were decreased by treatment with 50 μM genistein or resveratrol, but were increased by quercetin (Fig. 3). These results indicated that genistein and resveratrol have inhibitory effects, while quercetin has a stimulatory effect on MA-10 cell steroidogenesis.

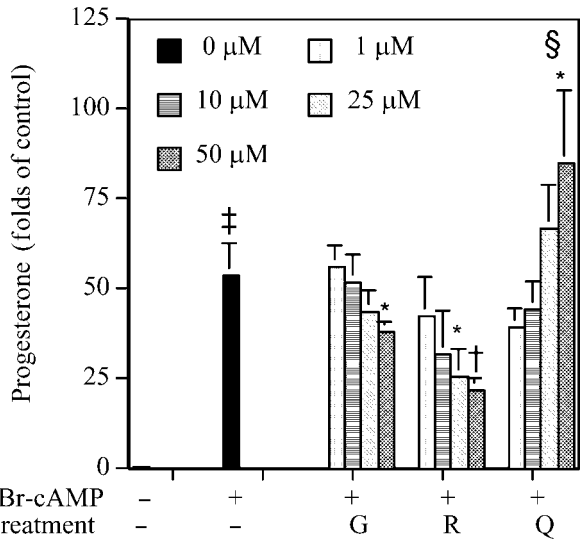

Figure 1 Effects of phytoestrogens on progesterone synthesis. MA-10 cells were incubated with indicated amounts of genistein (G), resveratrol (R), or quercetin (Q) for 24 h. The cells were then replaced with assay medium containing 0·2 mM 8-Br-cAMP. After 4 h, the medium was collected and subjected to the extraction with diethyl ether, and the cell lysate was prepared by adding RIPA solution to the monolayer in each well. The progesterone levels in the ether extracts were measured using progesterone EIA kit as described under 'Materials and Methods'. The progesterone concentration in each well was normalized to the amount of total cellular protein. Each group contained triplicate samples. This experiment was repeated at least thrice. * Or † denotes significant increase or decrease when compared with the cells treated with cAMP only ($P<0{\cdot}05$ or 0·01 respectively). ‡ Denotes significant increase when compared with the cells without cAMP treatment ($P<0{\cdot}001$). § Denotes significant difference when compared with the cells treated with cAMP plus genistein or resveratrol ($P<0{\cdot}01$).

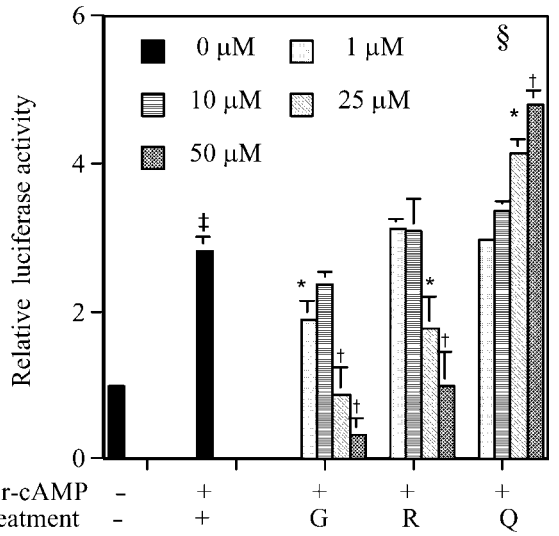

Figure 2 Effects of phytoestrogens on StAR transcription. MA-10 cells were transiently transfected with a StAR P-966 luciferase reporter construct and pRL-*Renilla* luciferase plasmid. After 16 h, the transfected cells were incubated with indicated amounts of genistein (G), resveratrol (R), and quercetin (Q) for another 24 h. The cells were then treated with 0·2 mM 8-Br-cAMP for 6 h. Both StAR-reporter luciferase and *Renilla* luciferase activities were measured in the cell lysates as described under 'Materials and Methods'. The activity of StAR P-966 luciferase was normalized to that of Renilla luciferase. For each experiment, the data were expressed as the -fold increase or decrease in treated cells relative to untreated cells that was set to a value of 1·0. The graph displays the mean values ± S.E.M. for normalized data from three independent transfection experiments for StAR luc/*Renilla* luc. Each treatment contained triplicate samples. * Or † denotes a significant increase or decrease when compared with the cells treated with cAMP only ($P<0·05$ or $P<0·1$ respectively). ‡ Denotes a significant increase compared with the cells without cAMP treatment ($P<0·001$). § Denotes a significant difference when compared with the cells treated with cAMP plus genistein or resveratrol ($P<0·01$).

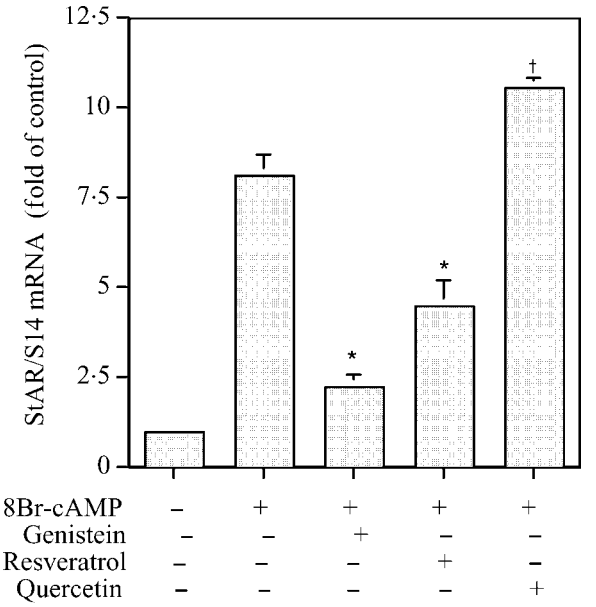

Figure 3 Effects of phytoestrogens on StAR mRNA expression. MA-10 cells were treated with 0·2 mM 8-Br-cAMP and 50 μM genistein, resveratrol, or quercetin. After 24 h, cell monolayer was used for the preparation of total cellular RNA. The first strand of cDNA was synthesized from DNase-treated RNA samples by M-MLV reverse transcriptase. The first strand of cDNA was used as the template for real-time RT-PCR analysis as described under 'Materials and Methods'. S14 rRNA was used as an internal standard to normalize StAR mRNA expression. The mRNA levels were expressed relative to control using $^{\Delta\Delta}C_T$ method and represented as means ± S.E.M. This experiment was repeated thrice using three independent sets of RNA samples. * Denotes a significant decrease when compared with the cells treated with cAMP ($P<0·01$). † Denotes a significant increase when compared with the cells treated with cAMP ($P<0·05$).

Effects of phytoestrogens on the proliferation of MA-10 cells

It has not been reported whether genistein, resveratrol, or quercetin affects MA-10 cell proliferation. In the present study, genistein inhibited MA-10 cell proliferation in a dose-dependent manner (Fig. 4A), while resveratrol or quercetin had no effect. The proliferation rate between days 1 and 5 in cells treated with genistein exhibited a gradual decline when compared with those treated with 0·1% dimethyl sulfoxide (DMSO). However, this phenomenon was not observed following treatment with either resveratrol or quercetin (Fig. 4B). During the first 48 h, incubation with 50 μM genistein did not show any obvious inhibition on MA-10 cell growth and its cytotoxicity became discernable after 72 h in the cultures.

The role of estrogen receptor in quercetin-stimulated steroidogenesis

To investigate the role of ER in quercetin-stimulated steroid hormone synthesis, ICI 182,780, a potent ER antagonist (Wakeling *et al.* 1991), was used to block endogenous ER activity in MA-10 cells. ICI 182,780 had no effect on cAMP-induced progesterone secretion, but reduced quercetin-plus cAMP-stimulated progesterone secretion by 31% (Fig. 5A). The effect of ICI 182,780 on quercetin-stimulated StAR reporter activity was next investigated. ICI 182,780 did not alter StAR promoter activity when cells were treated with cAMP alone or with cAMP plus quercetin (Fig. 5B). If activated ER has an inhibitory effect on MA-10 cell steroidogenesis, ER antagonists should increase MA-10 cell steroidogenesis. This result in the present study indicated that the effects of quercetin are not mediated by ER and are at a step beyond StAR.

The role of extracellular Ca^{2+} in quercetin-stimulated steroidogenesis

Steroid hormone synthesis in the gonads and adrenal glands is dependent on the trophic hormones and modulation by Ca^{2+} and various cytokines (Huhtaniemi & Toppari 1995, Rossier 1997). Whether extracellular Ca^{2+} plays a role in

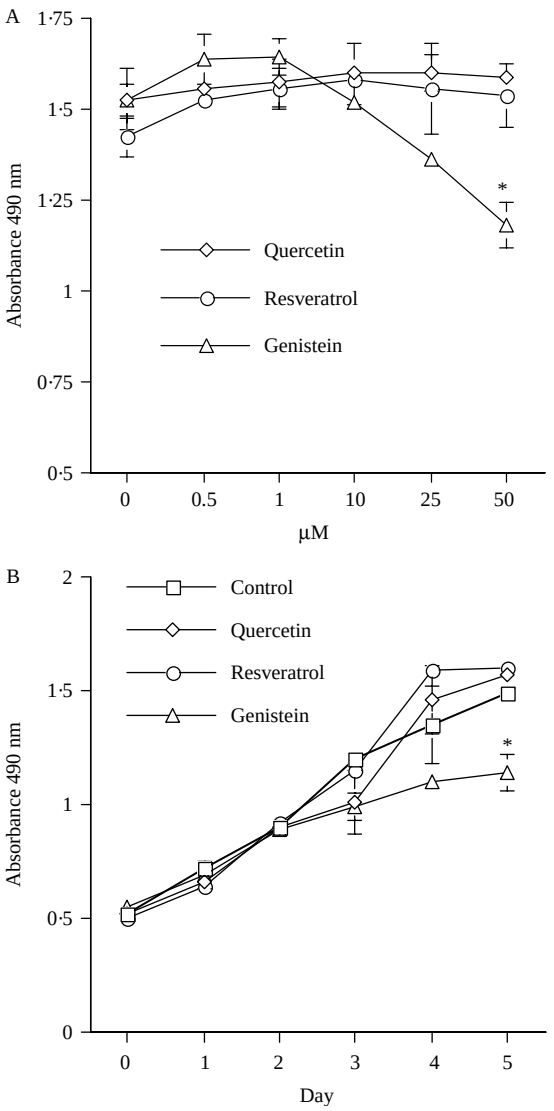

Figure 4 Effects of phytoestrogens on MA-10 cell proliferation. MA-10 cells (5×10^3 cells/well) were seeded onto the 96-well plates and allowed to grow for 5 days in the presence of (A) indicated amounts of genistein, resveratrol, or quercetin. Cell proliferation was measured using MTS assay as described under 'Materials and Methods'. * Denotes a significant difference when compared with the cells treated with 50 μM resveratrol or quercetin ($P<0·05$). Data show the mean ± S.E.M. of the values combined from three independent experiments. Four samples were used for each group. (B) The cells were incubated with 0·1% DMSO (control) or 50 μM of each phytoestrogens. Cell growth was measured from days 0 to 5. * Denotes a significant decrease when compared with the control cells ($P<0·05$). Data show the mean values ± S.E.M. combined from three independent experiments. Four samples were used for each group.

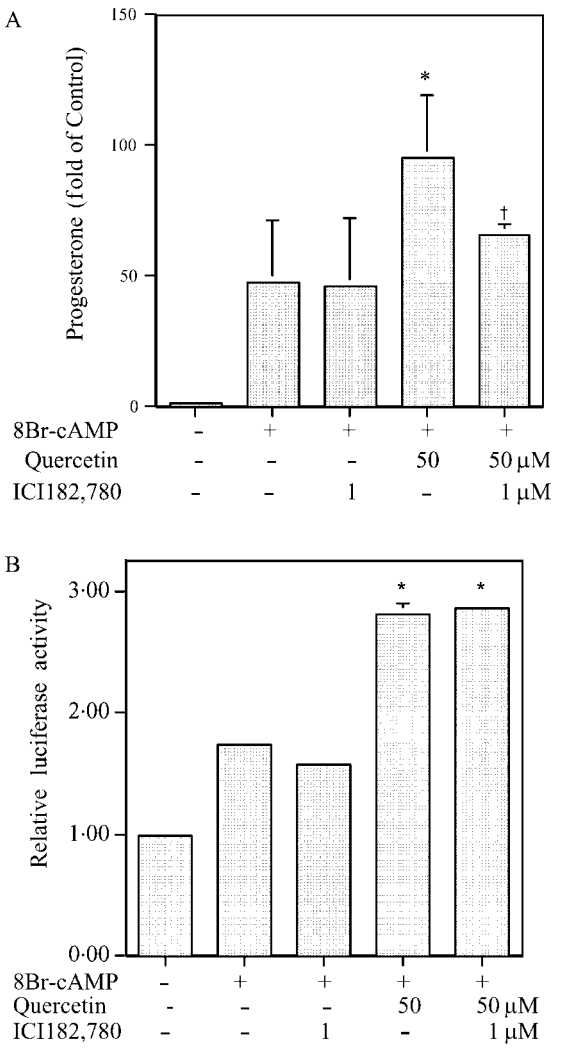

Figure 5 Effects of ICI 182,780 on quercetin-stimulated steroidogenesis and StAR transcription. MA-10 cells were treated with 0·1% DMSO (control) or 50 μM quercetin. After 24 h, cells were replaced with an assay medium containing 1 μM ICI 182,780 and 0·2 mM 8-Br-cAMP for another 4 h. The medium in each well was collected and progesterone level was measured as described in Fig. 1. (A) ICI 182,780 decreased cAMP-and quercetin-stimulated progesterone secretion. * Denotes a significant increase when compared with the cells treated with cAMP ($P<0·05$). † Denotes a significant decrease when compared with the cells treated with cAMP and quercetin ($P<0·05$). (B) MA-10 cells were subjected to transient transfection as mentioned in Fig. 2, except treatment with 1 μM ICI 182,780 and 0·2 mM 8-Br-cAMP for another 6 h. The relative luciferase activities were measured. Data show the mean values ± S.E.M. combined from three independent sets of RNA samples. Each treatment contained four replicates. * Denotes a significant increase when compared with the cells treated with cAMP only ($P<0·01$).

quercetin-stimulated steroid hormone synthesis remains to be determined. In the presence of cAMP, EGTA decreased progesterone production and attenuated quercetin-mediated increase in progesterone secretion by 28% (Fig. 6A). EGTA also reduced cAMP- and cAMP- plus quercetin-stimulated StAR promoter activities (Fig. 6B). These results indicated that extracellular Ca^{2+} is required for quercetin-stimulated steroidogenesis in MA-10 cells. Voltage-gated Ca^{2+} channels and calcium-release-activated Ca^{2+} (CRAC) channels provide the major routes for Ca^{2+} to enter the cells in response to a

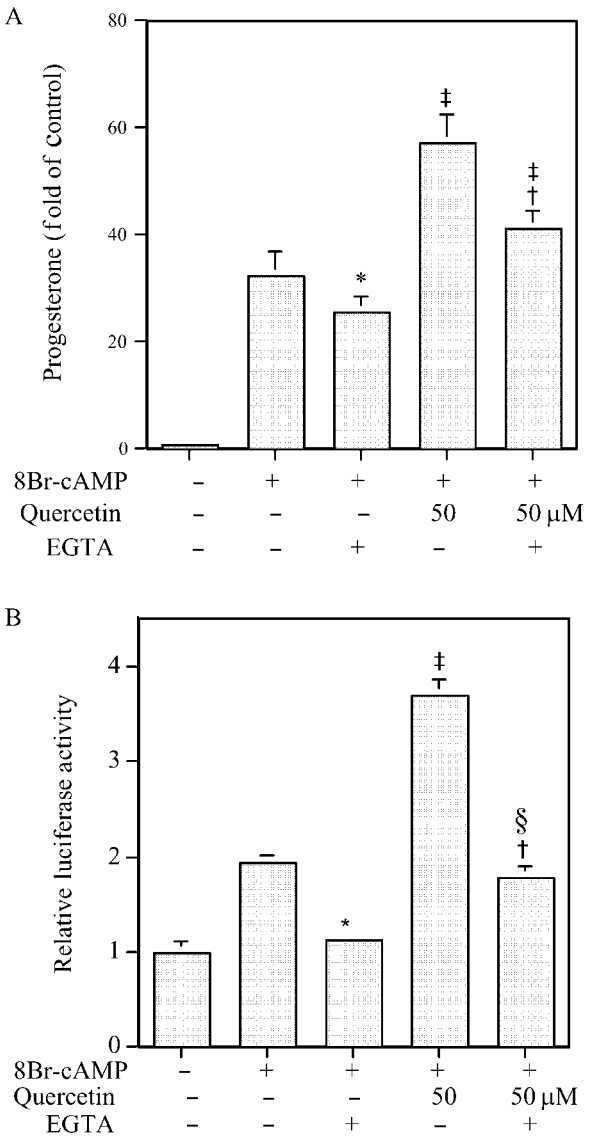

Figure 6 Effects of deprivation of extracellular calcium by EGTA on quercetin-stimulated progesterone secretion and StAR transcription. MA-10 cells were treated with 0·1% DMSO or 50 μM quercetin for 24 h. The cells were replaced with an assay medium containing 4 mM EGTA and 0·2 mM 8-Br-cAMP for 4 h. The medium in each well was collected and progesterone levels were measured as described in Fig. 1. (A) EGTA reduced cAMP- and quercetin-stimulated progesterone secretion. (B) MA-10 cells were subjected to transient transfection as mentioned in Fig. 2, except treatment with 4 mM EGTA and 0·2 mM 8-Br-cAMP for 6 h. The relative luciferase activities were measured as described in Fig. 2. * Denotes a significant decrease when compared with the cells treated with cAMP only ($P<0·05$). † Denotes a significant decrease when compared with the cells treated with cAMP and quercetin ($P<0·01$). ‡ Denotes a significant increase when compared with the cells treated with cAMP only ($P<0·01$). § Denotes a significant increase when compared with the cells treated with cAMP and EGTA ($P<0·05$). Data show the mean values±S.E.M.1 combined from three independent experiments. Four samples were used for each group.

variety of stimulations (Rossier 1997). We sought to determine if blockade of Ca^{2+} channels affects quercetin-mediated responses in MA-10 cells. We utilized mibefradil (Massie 1998) and verapamil (Atlas & Adler 1981) to block T- and L-type voltage-gated Ca^{2+} channel. Treatment of MA-10 cells with 10 μM of either mibefradil or verapamil had no effect on cAMP-stimulated progesterone synthesis, but significantly reduced quercetin-plus cAMP-stimulated progesterone secretion (Fig. 7A). However, neither mibefradil nor verapamil had an inhibitory effect on quercetin-plus cAMP-stimulated StAR promoter activity (Fig. 7B). Together, these results supported the view that extracellular Ca^{2+} influx is required for quercetin-plus cAMP-stimulated steroidogenesis in MA-10 cells and that Ca^{2+} acts at a step beyond StAR.

Effects of vanadate on quercetin-stimulated steroidogenesis

Calcium leakage through the plasma membrane (PM) Ca^{2+} pump or uptake by intracellular Ca^{2+} stores is essential for maintaining the homeostatic environment of the cytosol after elevation of cytosolic Ca^{2+} levels (Strehler & Treiman 2004). Vanadate and quercetin have been reported to inhibit the parathyroid hormone-stimulated PM Ca^{2+} pump activity in osteoblasts (Lloyd *et al.* 1995). We investigated the effect of sodium orthovanadate on MA-10 cell steroidogenesis. Treatment with 10 μM vanadate inhibited progesterone secretion by 28% and quercetin-stimulated progesterone secretion by 36% (Fig. 8A). In the parallel experiments, vanadate had no effect on cAMP-stimulated StAR promoter, but significantly inhibited quercetin-plus cAMP-stimulated StAR promoter (Fig. 8B). These results indicate that Ca^{2+} pump activity is required for quercetin-stimulated progesterone synthesis.

Effects of ICI 182,780, EGTA, and vanadate on quercetin-increased StAR mRNA expression in MA-10 cells

We next examined the effect of ICI 182,780, EGTA, or vanadate on quercetin-induced StAR mRNA expression in the MA-10 cells. We observed that vanadate alone significantly reduced cAMP-stimulated StAR mRNA expression, while EGTA or ICI 182,780 had no effect. Vanadate and EGTA reduced quercetin-plus cAMP-increased StAR mRNA level by 50 or 36% respectively, while ICI 182,780 had no effect (Fig. 9).

Discussion

In the present study, we found that the phytoestrogens, quercetin, resveratrol, and genistein have differential effects on steroid hormone synthesis in MA-10 cells. Resveratrol and genistein inhibited progesterone secretion through down-regulation of *StAR* gene expression at the transcriptional and mRNA levels. MA-10 cell proliferation was decreased by treatment with genistein, but was unaffected by resveratrol or

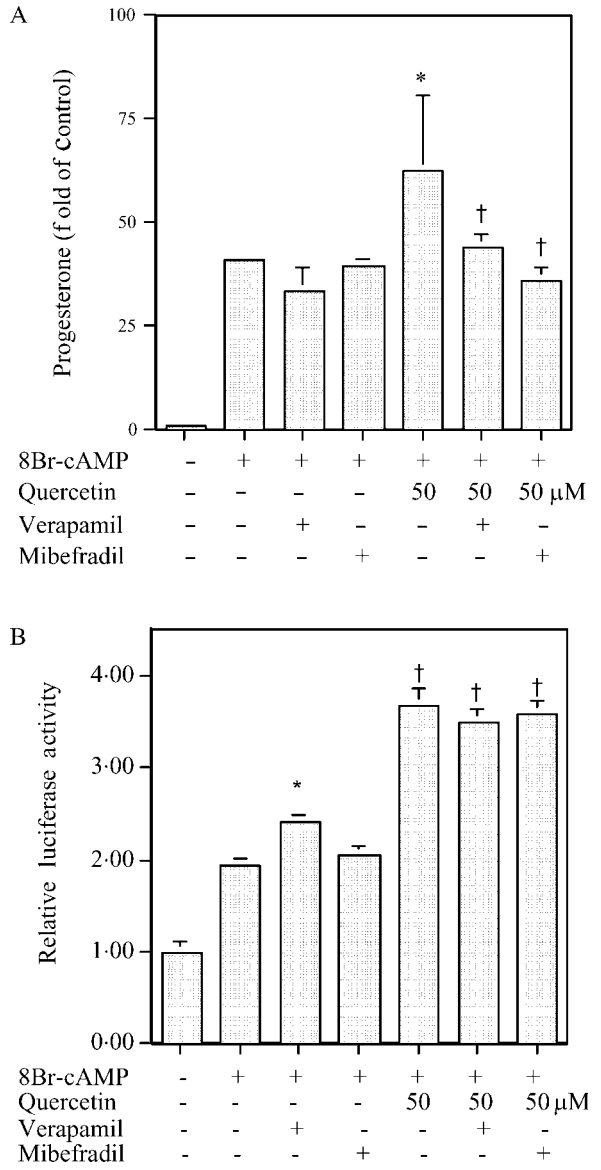

Figure 7 Effects of blockade of T- or L-type voltage-gated Ca^{2+} channel on quercetin-stimulated steroidogenesis and StAR transcription. MA-10 cells were treated with either 0·1% DMSO or 50 μM quercetin for 24 h. Cells were incubated with an assay medium containing 10 μM mibefradil or verapamil and 0·2 mM 8-Br-cAMP for another 4 h. The medium in each well was collected and progesterone levels were measured as described in Fig. 1. (A) Mibefradil or verapamil reduced cAMP-and quercetin-stimulated progesterone synthesis. * Denotes a significant increase when compared with the cells treated with cAMP only ($P<0·05$). † Denotes a significant decrease when compared with the cells treated with cAMP and quercetin ($P<0·05$). (B) MA-10 cells were subjected to transient transfection as mentioned in Fig. 2, except treatment with 10 μM mibefradil or verapamil for 6 h. * Or † denotes a significant increase when compared with the cells treated with cAMP only ($P<0·05$ or $P<0·01$ respectively). Four samples were used for each treatment. Data show the mean values ± S.E.M. combined from three independent experiments.

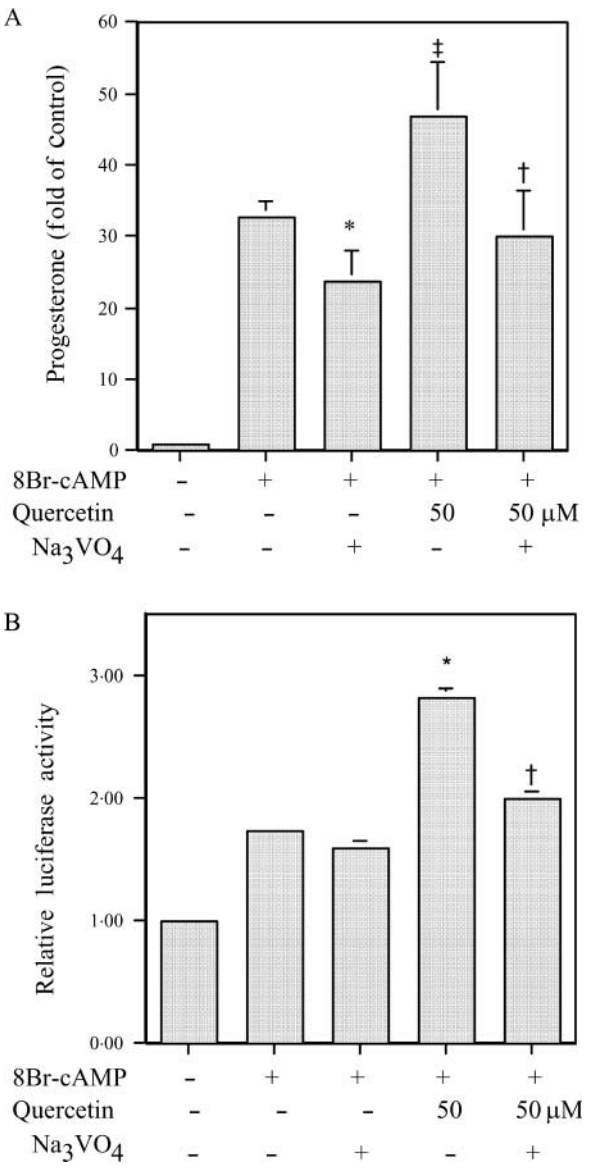

Figure 8 Effects of vanadate on quercetin-stimulated progesterone synthesis and StAR promoter activities in MA-10 cells. MA-10 cells were treated with either 0·1% DMSO or 50 μM quercetin for 24 h. Cells were incubated with an assay medium containing 10 μM sodium orthovanadate (Na_3VO_4) and 0·2 mM 8-Br-cAMP for another 4 h. The medium in each well was collected and progesterone levels were measured as described in Fig. 1. (A) Vanadate reduced cAMP- and quercetin-stimulated progesterone synthesis. * Denotes a significant decrease when compared with the cells treated with cAMP only ($P<0·05$). † Denotes a significant decrease when compared with the cells treated with cAMP and quercetin ($P<0·05$). ‡ Denotes a significant increase when compared with the cells treated with cAMP only ($P<0·05$). (B) MA-10 cells were subjected to transient transfection as mentioned in Fig. 2, except treatment with 10 μM Na_3VO_4 for 6 h. * Denotes a significant increase when compared with the cells treated with cAMP only ($P<0·01$). † Denotes a significant decrease when compared with the cells treated with cAMP and quercetin ($P<0·01$). Four samples were used for each treatment. Data show the mean values ± S.E.M. combined from three independent experiments.

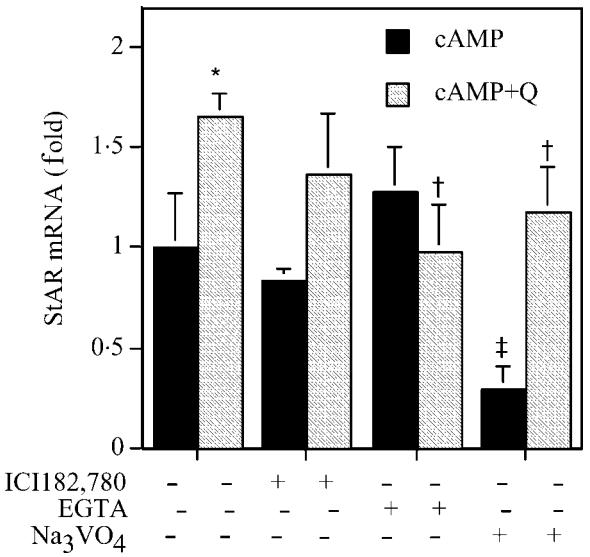

Figure 9 Effects of ICI 182,780, EGTA, and vanadate on quercetin-plus (cAMP+Q)-stimulated StAR mRNA expression. Cells were treated with 0·2 mM 8-Br-cAMP and 50 μM quercetin for 24 h, followed by treatment with 1 mM ICI 182,780, 4 mM EGTA, or 10 μM Na_3VO_4 for 4 h. The cell monolayer was used for the preparation of total cellular RNA. The first strand of cDNA was synthesized and subjected to real-time PCR analysis as mentioned in Fig. 3. The mRNA levels were expressed relative to cAMP treatment alone using $^{\Delta\Delta}C_T$ method and represent the mean ± S.E.M.. This experiment was repeated thrice using three independent sets of RNA samples. * Denotes a significant increase when compared with the cells treated with cAMP ($P<0·01$). † Denotes a significant decrease when compared with the cells treated with cAMP and quercetin ($P<0·05$). ‡ Denotes a significant decrease when compared with the cells treated with cAMP ($P<0·01$).

quercetin. On the contrary, quercetin stimulated steroid hormone synthesis by up-regulation of StAR promoter activity and mRNA expression. ICI 182,780 decreased quercetin-stimulated progesterone secretion, but had no effect on quercetin-induced StAR promoter activity and StAR mRNA expression in MA-10 cells, suggesting that its effect is at a step beyond StAR. The role of cytosolic Ca^{2+} in the quercetin-stimulated steroidogenesis was next investigated. We found that Ca^{2+} is involved in quercetin-stimulated steroid hormone synthesis. We observed that the quercetin-induced increase in progesterone secretion is reduced by (1) deprivation of extracellular Ca^{2+} by EGTA treatment and (2) blocking extracellular Ca^{2+} influx with either mibefradil or verapamil in MA-10 cells. Quercetin-plus cAMP increased StAR promoter activity and StAR mRNA expression were reduced by EGTA, while mibefradil or verapamil had no effect. Blocking intracellular Ca^{2+} efflux by vanadate suppressed the quercetin-induced progesterone secretion, StAR promoter activity, and StAR mRNA expression, suggesting that protein phosphatases may be involved in quercetin-mediated response in MA-10 cells. These findings suggest that phytoestrogens have differential effects on steroid hormone synthesis in MA-10 cells.

Feeding rats with a phytoestrogen- or genistein-rich diet resulted in lower serum testosterone levels, but had no obvious effect on testicular StAR protein levels when compared with those fed with a regular diet (Weber *et al.* 2001, Svechnikov *et al.* 2005). Serum genistein levels were approximately 1·5 μM in rats (Weber *et al.* 2001) and 10 μM in men (Busby *et al.* 2002) after consumption with a phytoestrogen-rich diet. However, it was not clear whether such concentrations of genistein are sufficient to suppress *StAR* gene expression. In the present study, we treated MA-10 cells with 0·5–50 μM genistein, resveratrol, and quercetin. We found that genistein and resveratrol in concentrations higher than 25 μM decreased cAMP-stimulated progesterone secretion through down-regulation of StAR expression, while quercetin had an opposite effect. Our results are comparable with the previous report that genistein had an inhibitory effect on steroid hormone synthesis in adrenal glomerulosa (Bodart *et al.* 1995, Mesiano *et al.* 1999, Aptel *et al.* 1999, Ohno *et al.* 2002), Leydig (Svechnikov *et al.* 2005), and ovarian cells (Lacey *et al.* 2005). Moreover, genistein, resveratrol, and quercetin have been shown to suppress the proliferation of several tumor cells (Miodini *et al.* 1999, Mitchell *et al.* 1999, Setchell & Cassidy 1999, Xing *et al.* 2001). Since our assays were performed by the incubation with genistein for less than 30 h and 50 μM genistein had no obvious inhibitory effect on MA-10 cell proliferation during the first 48 h in the cultures (Fig. 4B), therefore it is unlikely that genistein-mediated decreases on steroidogenesis and *StAR* gene expression were due to its cytotoxicity. On the other hand, we cannot rule out the possibility that genistein-mediated cytotoxicity has an impact on steroidogenesis in the animals receiving a long-term exposure to the higher doses of genistein. Feeding rats with resveratrol has been shown to decrease serum corticosterone levels (Supornsilchai *et al.* 2005) and our results showed that resveratrol decreased progesterone production in MA-10 cells. Resveratrol-mediated decrease in *StAR* gene expression is probably due to its estrogenic effect rather than its inhibitory effect on COX activities (Murias *et al.* 2004), because StAR expression in the MA-10 cells was increased by COX-2 inhibitors (Wang *et al.* 2003). Together, we conclude that genistein and resveratrol play a negative role in steroidogenesis. In contrast, we report for the first time that quercetin has a stimulatory effect on steroid production in MA-10 cells through up-regulation of StAR expression. In fact, quercetin alone was sufficient to induce a 2·5-fold increase in basal progesterone secretion in MA-10 cells (data not shown). Previously, quercetin was reported to have no suppressive effect on dibutyryl cAMP-stimulated cortisol secretion in H295R cells (Ohno *et al.* 2002), supporting our present finding.

Genistein, resveratrol, and quercetin are classified as phytoestrogens because of their capability to bind to both ERα and ERβ and to activate the transcription of several estrogen-responsive genes *in vitro* (Kuiper *et al.* 1998, Bowers *et al.* 2000). The hierarchy of the binding affinity for both ER subtypes is genistein > quercetin ≥ resveratrol when compared with 17β-estradiol. The differences in the binding

affinity for ERα and ERβ among these three phytoestrogens are unlikely to explain the discrepancy in quercetin-induced progesterone production in MA-10 cells. In the present study, ICI 182,780 did not affect cAMP-induced progesterone production but inhibited cAMP-plus quercetin-induced progesterone production. Our result is in agreement with the previous report that ICI 182,780 did not increase LH-stimulated testosterone production in isolated primary rat Leydig cells (Akingbemi *et al.* 2003). ICI 182,780 suppressed cAMP- plus quercetin-increased progesterone production without affecting StAR promoter activity and StAR mRNA expression, suggesting that the effect of ICI 182,780 is at a step beyond StAR.

Many factors such as trophic hormones, cytokines, Ca^{2+}, kinases, and phosphatases have been reported to affect *StAR* gene expression (Manna & Stocco 2005). Previously, genistein was reported to decrease AngII-stimulated aldosterone synthesis by diminishing Ca^{2+} influx in adrenal glomerulosa cells (Aptel *et al.* 1999). In the present study, EGTA significantly decreased quercetin-plus cAMP-stimulated StAR transcriptional activity and StAR mRNA expression that attenuated progesterone production in MA-10 cells, suggesting that Ca^{2+} signal is involved in quercetin-stimulated steroidogenesis. Verapamil significantly reduced human chorionic gonadotropin (hCG)-plus potassium (K^+)-stimulated progesterone synthesis and StAR mRNA levels in mLTC-1 mouse Leydig tumor cells (Manna *et al.* 1999). In the presence of quercetin plus cAMP stimulation, verapamil as well as mibefradil significantly reduced progesterone secretion, but fail to decrease StAR promoter activity and StAR mRNA expression in MA-10 cells. These results indicate that the major effect of Ca^{2+} influx through voltage-gated Ca^{2+} channels may contribute to quercetin-stimulated increases in steroidogenesis and its effect is at a step beyond StAR.

In the present report, vanadate suppressed cAMP- and cAMP-plus quercetin-stimulated progesterone secretion in MA-10 cells, implicating that cellular signals associated with vanadate may play a role in regulating quercetin-stimulated steroidogenesis. Vanadate did not alter StAR promoter activity, but reduced StAR mRNA expression when MA-10 cells were treated with cAMP, suggesting that there is a post-transcriptional regulation on *StAR* gene expression by vanadate. On the other hand, vanadate inhibited StAR promoter activity and StAR mRNA expression when the cells were treated with cAMP plus quercetin, suggesting that vanadate-sensitive cellular factors are responsible for quercetin effects on steroidogenesis. Vanadate is a well-known inhibitor for phosphoprotein phosphatase (PP) and alkaline phosphatase (ALP) (Gordon 1991) and is able to enhance the sensitivity of mouse Leydig cell proliferation in response to 17β-estradiol stimulation (Sato *et al.* 1987). Recently, PP was reported to play a role in regulating cAMP-mediated increase in StAR protein expression and steroidogenesis in mouse Y1 adrenocortical cells (Jones *et al.* 2000), while PPs are also involved in the elevation of mitochondria Ca^{2+} level that may be associated with K^+-stimulated aldosterone synthesis in H295R cells (Lalevee *et al.* 2003).

In conclusion, phytoestrogens have differential effects on MA-10 cell steroidogenesis. Genistein and resveratrol have a negative role, while quercetin has a stimulatory effect on steroidogenesis. The effects of quercetin are complex and may depend on the non-ER-mediated events and Ca^{2+} in MA-10 cells.

Acknowledgements

This work was supported by the Veterans Affairs Medical Research Fund (to T L), a South Carolina Cancer Research Award (to Y C C), and by NIH Grant HD17481 and funds from the Robert A Welch Foundation Grant B1-0028 (to D M S). The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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Received in final form 26 November 2006
Accepted 19 December 2006
Made available online as an Accepted Preprint
3 January 2007