Published in final edited form as:

J Nutr Biochem. 2011 March ; 22(3): 212–218. doi:10.1016/j.jnutbio.2010.01.004.

Effects of Apigenin on Steroidogenesis and Steroidogenic Acute Regulatory Gene Expression in Mouse Leydig Cells

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Abstract

Previous studies reported that the age-related decline in testosterone biosynthesis is associated with a decrease in the steroidogenic acute regulatory (StAR) protein which regulates the rate-limiting step of testosterone biosynthesis. To explore the possibility of delaying this decline using a dietary approach, we have examined the effect of a natural flavonoid, apigenin, on StAR gene expression in mouse Leydig cells. Incubation of these cells with the flavonoid enhanced cyclic AMP (cAMP)-induced steroidogenesis and StAR protein expression. The results from the analyses of StAR mRNA by reverse transcription-polymerase chain reaction and the luciferase assays of StAR promoter activity indicated that this flavonoid enhanced StAR gene expression at the level of transcription. Further studies showed that apigenin blocked the thromboxane A2 receptor and interrupted the signaling through the cyclooxygenase-2-thromboxane A synthase-thromboxane A2-receptor pathway, resulting in a reduction of DAX-1 (dosage sensitive sex reversal-adrenal hypoplasia congenita critical region on the X chromosome, gene-1) protein, a transcriptional repressor of StAR gene expression. When DAX-1 protein was reduced, the sensitivity of the Leydig cells was dramatically enhanced, with sub-threshold level of cAMP being able to induce maximal levels of StAR protein expression and steroid hormone production. The present study suggests a potential application of apigenin to improve StAR protein expression and steroidogenic sensitivity of aging Leydig cells.

Keywords

apigenin; flavonoid; steroidogenesis; testosterone; StAR

1. Introduction

During the course of male aging, circulating levels of testosterone decline [1,2], resulting in decreases in muscle function, bone density, sex function and other physiological functions [3-5]. It was also observed that serum testosterone concentrations were significantly lower in

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men with Alzheimer's disease in comparison to non-demented and age-matched men [6,7]. Supplementation with testosterone reduced β -amyloid peptide and hyperphosphorylation of τ protein, two bio-markers of the disease [8-10]. The studies suggested that low blood testosterone is a possible risk factor for development of Alzheimer's disease [11]. To improve the health of aging males, especially those suffering from age-associated hypogonadism, we have been attempting to determine if it is possible to delay the age-related decline in blood testosterone concentration.

Testosterone is mainly synthesized in testicular Leydig cells from substrate cholesterol and then released into the circulation [12]. It is known that the levels of blood testosterone are affected by multiple physiological and biochemical factors associated with aging [13]. It has also been shown that the primary site for the decline in blood testosterone appears to be at the level of testosterone biosynthesis in aging Leydig cells [14]. The rate-limiting step in testosterone biosynthesis is the transfer of the substrate cholesterol from the outer to the inner mitochondrial membrane to initiate the steroidogenic process [15]. Previous studies reported that a newly synthesized protein induced by trophic hormone, namely the steroidogenic acute regulatory (StAR) protein, plays a critical function at this step by facilitating the mitochondrial cholesterol transfer [16-18]. A large body of evidence demonstrated that the levels of StAR protein expression strongly affect testosterone production in Leydig cells [19]. However, StAR protein expression also decreases during the course of Leydig cell aging, and the cholesterol supply to the mitochondrial inner membrane is reduced in aged Leydig cells [20-22]. These studies implicated the involvement of an age-related decline in StAR gene expression in the decrease in testosterone production.

In addition, our studies have demonstrated that expression of cyclooxygenase-2 (COX2, an isoform of cyclooxygenase) increases during Leydig cell aging, a process that enhances the COX2-dependent inhibition of StAR gene expression. Consequently, the age-related increase in COX2 results in decreases in StAR gene expression and testosterone biosynthesis. When COX2 activity was inhibited, StAR protein expression and testosterone production were increased. Moreover, feeding aged rats with a selective COX2 inhibitor reversed the decreased StAR protein and blood testosterone concentration [23,24].

Further studies showed that the observed COX2-dependent inhibition of StAR gene expression involves the negative signaling through an autocrine loop consisting of COX2-thromboxane A synthase (TBXAS)-thromboxane A₂ (TBX A₂)-receptor, in which TBX A₂ generated by the co-action of COX2 and TBXAS is released from Leydig cells, and then binds to its receptors [25,26]. These studies further indicated that the TBX A₂-receptor complex regulate the expression or stability of DAX-1 (dosage sensitive sex reversal-adrenal hypoplasia congenita critical region on the X chromosome, gene-1) protein, a transcriptional repressor of StAR gene expression. Blocking the COX2-dependent signaling through this loop reduced DAX-1 protein and increased the sensitivity of Leydig cells to trophic hormone stimulation, resulting in dramatic increases in StAR gene expression and testosterone production in aged Leydig cells. These studies suggested that it is possible to delay the age-related decline in testosterone production by interrupting the signaling through this loop at any step, by either inhibiting the activity of COX2 or TBXAS, or by blocking the TBX A₂ receptor.

We have continued the studies in an attempt to identify natural compounds in food or food supplements that could enhance StAR gene expression in Leydig cells by intervention in the mechanism. After screening a group of compounds, the present study identified a natural flavonoid, apigenin that interrupted the COX2-dependent signaling by blocking the TBX A₂ receptor and increased StAR gene expression and steroidogenesis in mouse Leydig cells.

2. Materials and methods

2.1. Reagents

Apigenin, N⁶,2'-dibutyryladenosine 3',5'-cyclic monophosphate (dbcAMP), Human luteinizing hormone (LH), H89, GF-109203X (GFX) and Waymouth's MB/752 medium were purchased from Sigma (St. Louis, MO). Rabbit antiserum generated against StAR protein was a generous gift from Dr. D. B. Hales (University of Illinois, Chicago) [27]. The monoclonal antibody against DAX-1 protein was a generous gift from Dr. E. Lalli (Institut de Pharmacologie Moléculaire et Cellulaire, Valbonne, France). Donkey anti-rabbit IgG antibody conjugated with horseradish peroxidase was purchased from Biosource (Camarillo, CA). Horse serum was purchased from Invitrogen (Grand Island, NY). The Dual-Luciferase Reporter Assay System was purchased from Promega (Madison, WI). ³H-SQ29548 was purchased from Perkin Elmer (Boston, MA). Other common chemicals used in this study were obtained from either Sigma or Fisher Chemicals (Pittsburgh, PA).

2.2. MA-10 cell culture

The MA-10 mouse Leydig tumor cell line was a generous gift from Dr. Mario Ascoli (University of Iowa, Iowa City). The cells were cultured in 12-well culture plates in Waymouth's MB/752 medium containing 15% horse serum as previously described [28], in an incubator at 37°C and 5% CO₂. Before each experiment, the medium was replaced with serum-free Waymouth's medium.

2.3. Steroid hormone production

MA-10 cells were cultured for 30 min in serum-free Waymouth's medium with or without apigenin (as described in the Figure Legends), and then 0.1 mM dbcAMP or 10 ng/ml LH was added to the culture for 6 h. The medium was collected at the end of each experiment and stored at -20°C. The concentration of progesterone in the medium was determined by radioimmunoassay (RIA) [29].

2.4. Mouse Leydig cell isolation and cell culture

Four month-old C3H male mice (Taconic Farms) were euthanized in a chamber filled with carbon dioxide followed by cervical dislocation. Testes were collected from the mice. Leydig cells were isolated from the testes using density gradient centrifugation as previously described [30]. The cells were cultured overnight in 12-well plates with DMEM/F-12 medium containing 0.1% BSA, at 32°C and 5% CO₂. The cells were incubated with apigenin for 30 min in DMEM/F-12 medium without BSA, and then 0.01 mM dbcAMP was added to the culture for 6 hours. The culture medium and Leydig cells were collected and stored at -20°C. Testosterone concentrations in the medium were determined by RIA. All procedures were approved by the Texas Tech University Health Sciences Center Animal Care and Use Committee.

2.5. Western blot analysis

StAR protein and DAX-1 protein in Leydig cells were detected by Western blot analysis as described previously [31]. Western blot analysis experiments were performed at least three times and the results of one representative experiment are shown for each figure.

2.6. Transfection

MA-10 cells were cultured in 12-well plates (0.2×10^6 cells per well) overnight. The cells in each well were transfected with 0.5 µg DNA of the StAR promoter/luciferase plasmid PGL2/StAR expressing firefly luciferase driven by the -966 bp sequence of the StAR promoter [32]. Transfections also included 6.0 ng of the pRL-SV40 vector DNA (a plasmid

which constitutively expresses Renilla luciferase under the control of the SV40 promoter, Promega, Madison, WI). Transfections were performed using FuGENE HD Transfection Reagent (Roche, Indianapolis, IN) following the manufacturer's instructions. After 48 h in culture, the cells were utilized for further experiments.

2.7. Luciferase assays

Following experiments, the cells were washed with cold PBS and lysed with Passive Lysis Buffer (Promega, Madison, WI). The supernatants were utilized for luciferase assays using a Dual Luciferase Reporter Assay System following the manufacturer's instructions (Promega, Madison, WI). The Relative Light Units (RLU, determined by dividing the reading from the PGL2/StAR promoter by the reading from Renilla luciferase) were measured using a TD-20/20 luminometer (Turner Designers, Sunnyvale, CA).

2.8. Reverse transcription-polymerase chain reaction (RT-PCR)

In experiments designed to determine StAR mRNA expression, MA-10 cells were washed with cold PBS and used for total RNA purification using TRIzol reagent in accordance with the manufacturer's instructions (Invitrogen, Carlsbad, CA). The first-strand cDNA was synthesized from total RNA using the Reverse Transcription System (Promega, Madison, WI). Polymerase chain reaction (PCR) for StAR was performed as previously described [33]. β -actin was used as an internal marker.

2.9. Binding competition between apigenin and ^3H -SQ29548

To examine the binding competition between apigenin and ^3H -SQ29548 (a highly selective antagonist of the TBX A2 receptor), MA-10 cells in 6-well plates were rinsed twice with serum-free Waymouth's medium, and incubated with apigenin at increasing concentrations from 0 to 100 μM in 1 ml of the medium for 30 min, at 22°C, with shaking. Then, ^3H -SQ29548 was added to the final concentration of 1.2 nM in each well for 4 h with shaking. The cells were then rinsed three times with cold PBS and collected. The amount of ^3H -SQ29548 bound to the cells was determined using a scintillation counter. Non-specific binding of ^3H -SQ29548 was determined by co-incubation with 100 μM of unlabelled SQ29548.

2.10. Statistical analysis

Each experiment was repeated at least three times. Statistical analysis of the data was performed with ANOVA followed by Tukey's significant difference test using the GraphPad Prism 4 system (GraphPad Software, San Diego, CA). The data are shown as the mean $\pm$ standard error.

3. Results

3.1. Steroid hormone production

MA-10 mouse Leydig cells were incubated with increasing concentrations of apigenin for 30 min, followed by addition of 0.1 mM dbcAMP for 6 h to determine the effect of apigenin on steroidogenesis. Incubation of MA-10 cells with this flavonoid significantly increased progesterone production. Progesterone concentration in the culture medium was increased from 28.2 to 154.1 ng/ml as apigenin in the culture was increased from 0 to 10 μM (Fig. 1A). To determine if the observed increase in steroid hormone production was due to the increases in activities of steroidogenic enzymes, the water-soluble 22(R)hydroxylcholesterol was used as a substrate. It was shown that there was no significant difference in steroidogenesis among the treatments when 22(R)hydroxylcholesterol was added to the culture medium (Fig. 1A).

The steroidogenic affect of apigenin was verified by the experiments with LH-treated MA-10 cells. Co-action of 10 ng/ml LH and 10 μ M apigenin also induced significant increase in progesterone production (Fig. 1B). In the cells treated with 10 ng/ml LH, the apigenin-increased progesterone production was lower than that in the cells incubated with dbcAMP. Possibly, the level of endogenous cAMP induced by 10 ng/ml LH is lower than the levels of dbcAMP added to the culture. The effect of apigenin was further confirmed using Leydig cells isolated from mice. As shown in Fig. 1C, apigenin significantly increased testosterone production in mouse Leydig cells cultured in the medium containing 0.01 mM dbcAMP.

3.2. StAR protein expression

To further understand how apigenin enhanced steroidogenesis, Western blot analyses were performed to detect StAR protein expression in MA-10 cells treated with this flavonoid. Incubation of MA-10 cells with apigenin enhanced cAMP- or LH-induced StAR protein expression. Similar results were obtained in the experiments with Leydig cells isolated from mice. The increases in StAR protein expression in these experiments occurred concomitantly with the increases in steroid hormone production (Fig. 1).

3.3. StAR gene transcription

In order to study the mechanism responsible for the stimulatory effect of apigenin on StAR protein expression, luciferase assays of StAR promoter activity and RT-PCR analysis of StAR mRNA levels were performed on MA-10 Leydig cells treated with this compound. Incubation of MA-10 cells with increasing levels of apigenin induced concentration-dependent increases in StAR promoter activity. The promoter activities increased from 0.03 to 0.3 RLU when apigenin levels were increased from 0 to 10 μ M. Similar results were obtained in RT-PCR analyses of StAR mRNA, with StAR mRNA levels being increased in a concentration-dependent manner in the cells treated with apigenin (Fig. 2).

3.4. Synergistic interaction between apigenin and cyclic AMP

To determine the interaction between cAMP and apigenin, MA-10 cells were incubated with or without 10 μ M apigenin for 30 min, and then increasing concentrations of dbcAMP were added to the culture for a 6-h culture period. The results in Fig. 3 indicate that 0.05 or 0.1 mM dbcAMP alone did not induce significant increase in steroidogenesis, but in the presence of 10 μ M apigenin these low levels of dbcAMP dramatically increased StAR protein expression and steroid hormone production. Similarly, in the absence of cAMP, apigenin alone was unable to increase StAR protein expression and steroid hormone production, but in the presence of sub-threshold levels of dbcAMP (0.05 or 0.1 mM), apigenin dramatically increased StAR protein expression and steroidogenesis in MA-10 cells (Fig.3). In MA-10 cells treated with 0.1 mM dbcAMP, 10 μ M apigenin increased progesterone production 6.3 fold over that of the cells treated with 0.1 mM dbcAMP alone.

3.5. Binding competition between apigenin and 3 H-SQ29548

Binding assays were performed to examine the competition between apigenin and 3 H-SQ29548 for the TBX A2 receptors. As shown in Fig. 4A, apigenin blocked the binding of SQ29548 to the TBX A2 receptors in a dose dependent manner. Incubation with apigenin significantly reduced the 3 H-SQ29548 bound to the MA-10 cells. When the concentration of apigenin was increased to 50 μ M in the medium, the binding of SQ29548 to the TBX A2 receptors was reduced to 41.6%.

3.6. DAX-1 protein in apigenin-treated cells

To further understand how apigenin enhanced StAR gene transcription, the transcriptional repressor, DAX-1 protein, was analyzed by Western blot with MA-10 cells treated with apigenin and 0.1 mM dbcAMP. As shown in Fig. 4B, DAX-1 protein was present at high levels in MA-10 cells. While 10 μ M apigenin or 0.1 mM dbcAMP alone slightly reduced DAX-1 protein, co-action of the same levels of apigenin and dbcAMP dramatically reduced DAX-1 protein in MA-10 cells. When DAX-1 protein was reduced, StAR protein was markedly increased (Fig. 4C).

3.7. The effects of protein kinase A and protein kinase C on DAX-1 protein

The effects of protein kinase C (PKC) and protein kinase A (PKA) on DAX-1 protein were examined in MA-10 cells treated with a PKC inhibitor, GFX or a PKA inhibitor, H89. While incubation of MA-10 mouse Leydig cells with apigenin in the medium containing 0.1 mM dbcAMP reduced DAX-1 protein, inhibition of PKC activity with 20 μ M GFX or PKA activity with 25 μ M H89 reversed apigenin-reduced DAX-1 protein. When DAX-1 protein expression was reversed, the apigenin-increased StAR mRNA was also reversed (Fig. 5). In addition, StAR protein expression and steroid hormone production in MA-10 cell also changed concomitantly with the change of DAX-1 protein (Fig. 6).

4. Discussion

Apigenin is one of the natural flavonoids, a group of polyphenolic compounds widely distributed in food and food supplements, especially fruits and vegetables [34]. Flavonoids have been reported to have anti-oxidation [35,36], anti-cancer [37,38] and anti-inflammation activities [39,40].

The effect of apigenin on steroidogenesis was demonstrated in the present study by the dramatic increase in steroid hormone production in MA-10 mouse Leydig cells incubated with this compound. The results were corroborated by the experiments with Leydig cells isolated from mice. Also, the data illustrated in Fig. 1 indicated that majority of the increase in steroid hormone resulted from an increased supply of the substrate cholesterol to the mitochondrial inner membrane, not from increased activities of steroidogenic enzymes because that there was no significant difference in steroidogenesis among the treatments when a water soluble substrate, 22(R)hydroxylcholesterol, was added to the cultures. To verify these observations, StAR protein expression in MA-10 cells was measured. As expected, increasing levels of apigenin enhanced StAR protein expression in a concentration-dependent manner, suggesting that apigenin increase steroidogenesis in Leydig cells mainly by enhancing StAR protein expression.

The results from luciferase assays of StAR promoter activity further indicated that apigenin enhanced StAR gene expression at the level of transcription. This was confirmed by the results obtained from RT-PCR analysis of StAR mRNA levels in MA-10 cells treated with this compound, with StAR mRNA level being increased in a concentration-dependent manner when the level of apigenin in the culture medium was increased.

In addition, the results from the present study indicated that apigenin enhanced steroidogenesis by increasing the sensitivity of Leydig cells to cyclic AMP (cAMP) stimulation. Usually, sub-threshold levels of cAMP (0.05 and 0.1 mM), are unable to induce significant increases in steroid hormone and StAR protein expression in MA-10 cells [26]. However, in the presence of apigenin, the effectiveness of cAMP was dramatically enhanced, with the low levels of cAMP being able to induce significant increases in steroid hormone and StAR protein expression. Although, apigenin enhanced the effectiveness of cAMP stimulation, in the absence of cAMP apigenin alone was unable to induce

steroidogenesis and StAR gene expression. These results suggested that this flavonoid itself is unable to increase StAR protein, but rather synergistically interacts with cAMP and reduces the threshold required for StAR gene expression, resulting in an increase in the steroidogenic sensitivity of Leydig cells.

Surprisingly, these observations are quite similar to those reported in murine Leydig cells when the COX2-dependent signaling was interrupted by inhibiting the activity of either COX2 or TBXAS, or by blocking the TBX A2 receptor [23,25,26]. This led us to determine if apigenin acts on the COX2-dependent signaling system that inhibits StAR gene expression. The results from binding competition between apigenin and ³H-SQ29548 indicated that this flavonoid blocked the TBX A2 receptors on MA-10 mouse Leydig cells. These results corroborated previous studies describing the action of apigenin as an antagonist of the TBX A2 receptors in platelets [41].

We have reported similar steroidogenic effect of another natural flavonoid [42], chrysin that was demonstrated as a COX2 inhibitor [39,43]. The present and previous observations suggested that flavonoids may enhance StAR gene expression through different mechanisms, possibly due to the differences in the chemical structures of their side chains. Some of them, such as chrysin, may block COX2-dependent signaling by inhibiting expression or activity of COX2, while apigenin blocks the TBX A2 receptors.

It is known that blocking the TBX A2 receptors reduces DAX-1 protein in murine Leydig cells cultured with 0.1 mM cAMP [25]. DAX-1 protein is a transcriptional repressor that binds to a hairpin structure of StAR promoter and depresses StAR gene transcription [44]. It is possible that apigenin enhanced StAR gene transcription by reducing DAX-1 protein. This hypothesis was demonstrated to be correct, as the results from the present study indicated that apigenin markedly reduced DAX-1 protein in MA-10 mouse Leydig cells cultured in a medium containing 0.1 mM dbcAMP.

This observation suggested a mechanism for the apigenin-increased steroidogenic sensitivity of Leydig cells to cAMP stimulation, although other unknown mechanisms might also be involved. It is generally accepted that cAMP stimulation of Leydig cells activates PKA. Then, PKA phosphorylates the transcription factors that increase StAR promoter activity and StAR gene transcription. However, the effectiveness of cAMP stimulation is reduced by transcriptional repressors, such as DAX-1, that bind to the StAR promoter and depress StAR gene transcription. It is also known that DAX-1 protein is constitutively expressed in MA-10 mouse Leydig cells and generates a tonic inhibition of StAR gene expression [26,44]. Thus, reduction of DAX-1 protein by the interaction between cAMP and apigenin attenuated the tonic inhibition and improved the effectiveness of cAMP stimulation. Therefore, in the presence of apigenin sub-threshold levels of cAMP were able to induce significant increases in StAR gene expression and steroid hormone production in Leydig cells.

Although, the evidence available is not sufficient to completely elucidate the mechanism for the observed effect of apigenin on DAX-1 protein, the results from the present study indicated that both PKA and PKC activities are required for the effect, since inhibiting the activity of either PKA or PKC reversed the reduction of DAX-1 protein and reduced StAR protein in the cells incubated with apigenin and cAMP. Also, it can readily be seen that an interaction between apigenin and cAMP is required for the reduction in DAX-1 protein and the increase in StAR gene expression. In this interaction, apigenin appears to be unable to increase PKA activity as this flavonoid alone failed to increase steroidogenesis. Based on our PKA activity assays using ³²P incorporation into protein in previous studies [23,42], cAMP at the low concentration is able to induce a low level of PKA phosphorylation in MA-10 cells. While this low level of PKA activity alone is not sufficient to induce

significant increase in StAR gene expression, it was demonstrated to be critical and also sufficient for apigenin to reduce DAX-1 protein. Regarding PKC activity, it is possible that blocking the TBX A2 receptor by apigenin interrupted receptor-mediated signaling and subsequently affected PKC activity. Alternatively, the combined action of PKA and the downstream signaling of the TBX A2 receptor may be responsible for the regulation of PKC activity.

The present study in conjunction with our previous studies [42] suggest a possibility to delay the age-related decline in testosterone biosynthesis through the action of natural flavonoids. This possibility is supported by an earlier study that reported improved reproductive functions in 24 month-old male rats when the animals were orally administered natural flavonoids for 30 days [45]. In addition, the study with 30 to 58 year-old men reported significant increases in free testosterone and dihydrotestosterone in serum after a 4-week ingestion of a nutritional supplement containing chrysin [46]. However, consumption of propolis and honey, substances that are rich in chrysin failed to increase urinary testosterone in another study with 25 to 30-year old human males [47]. The differing results between these studies may result from the difference in bioavailability of the flavonoid in each specific diet [48,49]. Also, the difference in the ages of the males between these two studies might affect the response of Leydig cells to the flavonoid that acts by blocking COX2-dependent signaling, because COX2 levels are different between young and aging Leydig cells [24,25].

In summary, the present study demonstrates that apigenin is able to enhance StAR gene expression and steroid hormone production in mouse Leydig cells by blocking the TBX A2 receptor and reducing the transcriptional repressor DAX-1. It also suggests the potential for a dietary approach for the enhancement of steroidogenesis in aging Leydig cells. Further studies are needed to examine the efficacy of dietary apigenin on testosterone biosynthesis and its effects on the health of aging males.

Acknowledgments

The authors would like to acknowledge the support of NIH Grant AG025349 to X.J.W.

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Fig. 1.

Effects of apigenin on steroidogenesis and StAR protein expression in mouse Leydig cells. **A**, MA-10 cells were cultured with or without 25 μ M of 22(R)hydroxycholesterol (22R+ or 22R-), in the medium containing increasing concentrations of apigenin for 30 min. Then 0.1 mM dbcAMP was added to the culture for 6 h. The cells were collected and 20 μ g of cell lysate protein was used for Western blot analysis of StAR protein. Progesterone concentration in the medium was assessed by RIA. **, $p < 0.01$; ***, $p < 0.001$; $n=3$; compared with 0.1 mM dbcAMP alone. **B**, MA-10 cells were cultured with 10 μ M apigenin for 30 min and then 10 ng/ml LH was added for 6 h. StAR protein and progesterone production were determined as described above. ***, $p < 0.001$; $n=3$; compared with 10 ng/ml LH alone. **C**, Leydig cells were isolated from mice and incubated with 5 μ M apigenin for 30 min, followed by addition of 0.01 mM dbcAMP for 6 h. StAR protein and testosterone production were determined. **, $p < 0.01$; $n=3$; compared with 0.01 mM dbcAMP alone.

Fig. 2.

Effects of apigenin on StAR gene transcription in MA-10 mouse Leydig cells. MA-10 cells were cultured in serum-free Waymouth's medium with increasing concentrations of apigenin for 30 min and then 0.1 mM dbcAMP was added to the culture for 6 h. **A**, the cells were collected for total RNA isolation and StAR mRNA was analyzed by RT-PCR with β -actin as an internal marker. **B**, MA-10 cells were transfected with a StAR promoter/luciferase plasmid (PGL2/StAR) and a pRLSV40 vector, a plasmid that constitutively expresses Renilla luciferase. The cells were then treated with dbcAMP and apigenin as described above and the cell lysate was used for luciferase assays using a Dual-Luciferase Reporter Assay System. The Relative Light Unit (RLU) was determined by dividing the reading from the PGL2/StAR promoter by the reading from Renilla luciferase. ***, $p < 0.001$, $n=4$; compared with 0.1 mM dbcAMP alone.

Fig. 3.

Apigenin increased steroidogenic sensitivity of MA-10 mouse Leydig cells to cAMP stimulation. MA-10 cells were cultured for 30 min in serum-free Waymouth's medium with or without 10 μ M of apigenin, and then increasing concentrations of dbcAMP were added to the culture for 6 h. **A**, the cells were collected and 20 μ g of cell lysate protein was used for Western blot analysis of StAR protein. **B**, progesterone production in the culture medium was assessed by RIA. ***, $p < 0.001$, $n=4$, compared with the paired group stimulated with dbcAMP alone.

Fig. 4.

Apigenin blocked the thromboxane A2 receptor and reduced DAX-1 protein in MA-10 mouse Leydig cells. **A**, MA-10 cells were incubated with increasing levels of apigenin for 30 min. Then, $^3\text{H-SQ29548}$ was added to each well for 4 h. The cells were then rinsed and collected. The amount of $^3\text{H-SQ29548}$ bound to the cells was determined using a scintillation counter. Non-specific binding of $^3\text{H-SQ29548}$ was determined by co-incubation with 100 μM of unlabelled SQ29548. **, $p < 0.01$; ***, $p < 0.001$; $n=4$, compared with the total binding (100%). **B** and **C**, MA-10 cells were cultured in serum-free Waymouth's medium with 10 μM of apigenin for 30 min and then 0.1 mM dbcAMP was added to the culture for 6 h. The cells were collected and 20 μg of cell lysate protein was used for Western blot analyses of DAX-1 protein and StAR protein.

Fig. 5.

Effects of protein kinase A and protein kinase C on DAX-1 protein and StAR mRNA levels in the apigenin-treated MA-10 mouse Leydig cells. **A**, MA-10 cells were cultured in serum-free Waymouth's medium with 10 μ M apigenin, 20 μ M of PKC inhibitor GFX or 25 μ M of PKA inhibitor H89 for 30 min, and then 0.1 mM dbcAMP was added to the culture for 6 h. The cells were collected and 20 μ g of cell lysate protein was used for Western blot analysis of DAX-1 protein. **B**, the cells were treated as described above and collected for total RNA isolation. StAR mRNA was analyzed by RT-PCR with β -actin as an internal marker.

Fig. 6.

Effects of protein kinase A and protein kinase C on StAR protein expression and steroidogenesis in the apigenin-treated MA-10 mouse Leydig cells. **A**, MA-10 cells were cultured in serum-free Waymouth's medium with 10 μM apigenin, 20 μM of PKC inhibitor GFX or 25 μM of PKA inhibitor H89 for 30 min, and then 0.1 mM dbcAMP was added to the culture for 6 h. The cells were collected and 20 μg of cell lysate protein was used for Western blot analysis of StAR protein. **B**, progesterone production in the culture medium was assessed by RIA. ***, $p < 0.001$, $n=4$, compared with the group treated with apigenin and dbcAMP.