Involvement of the Thromboxane A2 Receptor in the Regulation of Steroidogenic Acute Regulatory Gene Expression in Murine Leydig Cells

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Recent studies suggested an involvement of thromboxane A2 in cyclooxygenase-2-dependent inhibition of steroidogenic acute regulatory (StAR) gene expression. The present study further investigated the role of thromboxane A2 receptor in StAR gene expression and steroidogenesis in testicular Leydig cells. The thromboxane A2 receptor was detected in several Leydig cell lines. Blocking thromboxane A2 binding to the receptor using specific antagonist SQ29548 or BM567 resulted in dose-dependent increases in StAR protein and steroid production in MA-10 mouse Leydig cells. The results were confirmed with Leydig cells isolated from rats. StAR promoter activity and StAR mRNA level in the cells were also increased after the treatments, suggesting an involvement of the thromboxane A2 receptor in StAR gene transcription. Furthermore study indicated that blocking the thromboxane A2 receptor reduced dosage sensitive sex reversal-adrenal hypoplasia congenita critical region on the X chromosome, gene 1 protein, a transcriptional repressor of StAR gene expression. Specific binding of the antagonists to the receptors on cellular membrane was demonstrated by binding assays using 3H-SQ29548 and binding competition between 3H-SQ29548 and BM567. Whereas SQ29548 enhanced cAMP-induced StAR gene expression, in the absence of cAMP, it was unable to increase StAR protein and steroidogenesis. However, when the receptor was blocked by the antagonist, subthreshold levels of cAMP were able to induce maximal levels of StAR protein expression, suggesting that blocking the thromboxane A2 receptor increase sensitivity of MA-10 cells to cAMP stimulation. Taken together, the results from the present and previous studies suggest an autocrine loop, involving cyclooxygenase-2, thromboxane A synthase, and thromboxane A2 and its receptor, in cyclooxygenase-2-dependent inhibition of StAR gene expression. (Endocrinology 150: 3267–3273, 2009)

Steroidogenic acute regulatory Protein (StAR) is critical in cholesterol transfer to the inner mitochondrial membrane to initiate steroidogenesis (1–3). In testicular Leydig cells, it is known that LH stimulation induces cAMP formation that activates protein kinase A (PKA), followed by phosphorylation of transcription factors regulating StAR gene transcription (4, 5). LH also induces arachidonic acid (AA) release (6–9). AA metabolites transduce signals to the nucleus to regulate StAR gene expression (10, 11). Both cAMP-PKA-phosphorylation and AA-mediated signaling pathways are required with neither pathway alone being sufficient for StAR gene expression and steroid production (12).

In the AA-mediated signaling pathway, AA is mainly metabolized by three enzymes, the lipoxigenase, epoxygenase, and cyclooxygenase enzymes, respectively (13). Whereas the AA metabolites produced by lipoxigenase and epoxygenase transduce positive signals to the nucleus to enhance StAR gene expression (14, 15), cyclooxygenase-2 (COX2; an isoform of cyclooxygenase) generates negative signals that inhibit StAR gene expression (16). Further studies demonstrated that COX2 increased progressively in Leydig cell aging and the increased COX2 inhibited StAR gene expression and testosterone biosynthesis (17, 18). It is well known that the decline in testosterone biosynthesis is

Abbreviations: AA, Arachidonic acid; COX2, cyclooxygenase-2; DAX-1, dosage sensitive sex reversal-adrenal hypoplasia congenita critical region on the X chromosome, gene 1; dbcAMP, N6,2-dibutyryladenosine cAMP; IOD, integrated OD; PKA, protein kinase A; StAR, steroidogenic acute regulatory protein; TBX, thromboxane; TBXAS, TBX A synthase.
associated with decreased bone density, muscle function, sexual function, and other physiological functions (19). Also, low level of blood testosterone is a possible risk factor for the development of Alzheimer’s disease (20). However, the mechanism responsible for the COX2-dependent inhibition of StAR gene expression has not been completely elucidated.

Recently, we described the inhibitory effect of an enzyme downstream of COX2, thromboxane A synthase (TBXAS), on StAR gene expression. The study suggested an inhibitory effect of thromboxane (TXB) A2, an AA metabolite generated by COX2 and TBXAS, on steroidogenesis (21). We continued the studies by investigating the role of the TBX A2 receptor in StAR gene expression in murine Leydig cells.

Materials and Methods

Reagents

N₂,2-dibutylryladenosine cAMP (dbcAMP) was purchased from Sigma (St. Louis, MO). Furegrelate, SQ29548, BM567, antibody against TBX A2 receptor, and TBX A2 receptor blocking peptide were purchased from Cayman (Ann Arbor, MI). Rabbit antisemur generated against StAR protein was a generous gift from Dr. D. B. Hales (University of Illinois, Chicago, IL) (22). The monoclonal antibody against dosage sensitive sex reversal-adenral hypoplasia congenita critical region on the X chromosome, gene 1 (DAX-1) protein was a generous gift from Dr. P. Sassone-Corsi (Universite´ Louis Pasteur, Strasbourg, France). The dual-luciferase reporter assay system was purchased from Promega (Madison, WI). ³H-SQ29548 was purchased from PerkinElmer (Boston, MA). Other common chemicals used in this study were obtained from either Sigma or Fisher Chemicals (Pittsburgh, PA).

Cell culture

The MA-10 mouse Leydig tumor cells (a cell line from Dr. Mario Ascoli, University of Iowa, Iowa City, IA) were cultured in Waymouth’s MB/752 medium containing 15% horse serum as previously described (23) in an incubator at 37 C and 5% CO₂. The culture medium was replaced with serum-free Waymouth’s medium before experiments. Also, Mouse Leydig cell lines, MLTC-1 and TM3 (American Type Culture Collection, Manassas, VA), were cultured in 12-well plates and collected for Western blot analysis of the TBX A2 receptor.

Steroid production

MA-10 cells were incubated for 30 min in serum-free Waymouth’s medium in the presence or absence of SQ29548, BM567, or furegrelate (as described in the figure legends) followed by addition of 0.1 mM dbcAMP to the cultures for 6 h. Progesterone concentrations in the medium were determined by RIA (24).

Rat Leydig cell isolation and cell culture

Brown Norway rats at ages of 3 and 20 months (National Institute of Aging, Bethesda, MD) were killed in a chamber filled with carbon dioxide followed by cervical dislocation. Leydig cells were isolated from the testes using density gradient centrifugation as previously described (25). The cells were cultured in six-well plates with DMEM/F-12 medium containing 0.1% BSA, at 32 C and 5% CO₂. After 2 h of culture, the cells were incubated with SQ29548 for 30 min in DMEM/F-12 medium without BSA and then 0.1 mM dbcAMP was added to the culture for 4 h. 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.

Transfection

MA-10 cells were cultured in 12-well plates (0.2 × 10⁶ cells/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 (26). Transfections also included 6.0 ng of the pRL-SV40 vector DNA (a plasmid that constitutively expresses Renilla luciferase; Promega). Transfections were performed using FuGENE6 transfection reagent (Roche, Indianapolis, IN) following the manufacturer’s instructions. After 48 h in culture, the cells were used for further experiments.

Luciferase assays

After experiments, cells were washed with cold PBS and lysed with passive lysis buffer (Promega). The supernatants were used for luciferase assays using a dual-luciferase reporter assay system following the manufacturer’s instructions (Promega). The relative light unit (determined by dividing the reading from the PGL2/StAR promoter by the reading from Renilla luciferase) was measured using a TD-20/20 luminometer (Turner Designs, Sunnyvale, CA).

RT-PCR

Total RNA from Leydig cells was extracted using TRZol reagent (Invitrogen, Carlsbad, CA) in accordance with the manufacturer’s instructions. mRNA was then transcribed to first-strand cDNA using the reverse transcription system (Promega). PCR for StAR was performed as previously described (27), using β-actin as an internal marker. The StAR- and β-actin-specific bands were quantitated as integrated OD (IOD) and expressed as average of StAR to β-actin IOD ratio for each treatment.

Detection of TBX A2 receptor on cellular membrane

The cell surface proteins of intact MA-10 cells were labeled with biotin, and then the biotinylated proteins were isolated with avidinagarose beads using the Pierce cell surface protein isolation kit following the manufacturer’s instruction (Pierce, Rockford, IL). The isolated cell surface proteins were used for Western blot analysis of TBX A2 receptor protein.

Western blot analysis

TBX A2 receptor protein, StAR protein, and DAX-1 protein were detected by Western blot analysis as described previously (28). In the Western blot analysis of TBX A2 receptor, TBX A2 receptor blocking peptide was used in the negative control following the manufacturer’s instruction (Cayman). The specific bands of TBX A2 receptor, StAR, and DAX-1 were quantitated as IOD and expressed as average of StAR to β-actin IOD ratio for each treatment. Western blot analysis experiments were performed at least three times, and the results of one representative experiment are shown for each figure.

Binding of the antagonists to the TBX A2 receptor

To determine the binding of SQ29548 to the TBX A2 receptor, MA-10 cells cultured in six-well plates were incubated with ³H-SQ29548 at increasing concentrations from 0 to 18.4 nM in 1ml of Waymouth’s medium for 4 h, at 22 C, 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. Nonspecific binding at each concentration of ³H-SQ29548 was determined by coincubation with 100 µM of unlabeled SQ29548. To examine the competition between SQ29548 and BM567 for the receptor, MA-10 cells in six-well plates were incubated with BM567 at increasing concentrations from 0 to 20 µM in 1 ml of the medium for 30 min, at 22 C, with shaking. Then ³H-SQ29548 was added to 4.6 nM in each well for 4 h with shaking. Finally, the cells were rinsed and collected for counting.

Statistical analysis

Each experiment was repeated at least three times with the treatments in triplicate. Statistical analysis of the data were performed with ANOVA
followed by Tukey’s significant difference test using the Prism 4 system (GraphPad Software, San Diego, CA). The data are shown as the mean ± se.

Results

Steroid hormone production and StAR protein expression

To study the role of the TBX A2 receptor in the COX2-dependent negative signaling in steroidogenesis, MA-10 cells were incubated with increasing concentrations of the receptor antagonist, SQ29548 and 0.1 mM dbcAMP for 6 h. Blocking the TBX A2 receptor with SQ29548 induced a concentration-dependent increase in steroid production. As SQ29548 concentrations in the cultures were increased from 0 to 25 μM, progesterone production by the cells increased from 35 to 208 pg/μg cellular protein (Fig. 1). Similar results were obtained when another receptor antagonist, BM567, was used (supplemental Fig. 1, published as supplemental data on The Endocrine Society’s Journals Online web site at http://endo.endojournals.org). To further determine whether the antagonist enhanced activities of steroidogenic enzymes, a water-soluble substrate, 22(R)hydroxycholesterol, was added to the cultures. In the presence of 22(R)hydroxycholesterol, there were no significant difference in steroid production among the treatments (Fig. 1), suggesting that the enhanced steroidogenesis was due to an increased supply of the substrate cholesterol. This was verified by Western blot analysis of StAR protein in the cells, with StAR protein expression being markedly enhanced, paralleling the increase in steroid production when the TBX A2 receptor was blocked using either antagonist (Fig. 1 and supplemental Fig. 1).

Because the COX2-dependent signaling is involved in the aged-related decline in testosterone biosynthesis (18), aged Leydig cells were used to examine whether blocking the TBX A2 receptor is able to reverse the decreased testosterone production. Interestingly, incubation of aged rat Leydig cells with 25 μM SQ29548 significantly enhanced StAR protein expression. Testosterone production by the aged cells also increased significantly from 86 to 146 pg/μg cellular protein (Fig. 2A). In comparison with young cells (supplemental Fig. 2), the levels of StAR protein and testosterone production were lower in aged cells. However, addition of 25 μM SQ29548 to the cultures with 0.1 mM dbcAMP increased StAR protein by 46% in the aged cells but only 23% in the young cells. Similar results were obtained in testosterone production. Possibly the difference between aged and young Leydig cells in response to SQ29548 may reflect the age-related difference in COX2-dependent negative signaling (18).

StAR gene transcription

RT-PCR analysis of StAR mRNA levels and luciferase assays of StAR promoter activity were performed to determine how the receptor antagonists enhanced StAR protein expression. As shown in Fig. 2B, incubation of aged Leydig cells with SQ29548 significantly increase StAR mRNA level. The results were confirmed by RT-PCR analyses of StAR mRNA in MA-10 cells (Fig. 3), which indicated a concentration-dependent increase in StAR mRNA. The increase in StAR mRNA occurred concomitantly with the significant increase in StAR promoter activity. When the
concentration of SQ29548 was increased to 25 μM, StAR promoter activity was increased to 1.7-fold of that in the cells treated with 0.1 mM dbcAMP alone. The observations were verified with BM567, with cAMP-induced StAR promoter activity and StAR mRNA level being significantly increased when MA-10 cells were treated with this receptor antagonist (supplemental Fig. 3).

Detection of the TBX A2 receptor

To detect the TBX A2 receptor in Leydig cells, Western blot analyses were performed with the mouse Leydig cell lines, MA-10, MLTC-1, and TM3. The results indicated that the TBX A2 receptor is expressed in the Leydig cells of these three cell lines (Fig. 4A). Furthermore, to detect the receptor on the cellular membrane, MA-10 cell surface proteins were isolated for Western blot analysis of TBX A2 receptor protein. As clearly shown in Fig. 4B, TBX A2 receptor proteins were detected in the cell surface proteins. In these Western blot analyses, the TBX A2 receptor-specific bands were verified using an antibody-specific blocking peptide that blocks TBX A2 receptor-antibody complex formation. In the presence of the blocking peptide, the receptor-specific bands were not detected.

Specific binding of the antagonists to the TBX A2 receptor

MA-10 cells were incubated with increasing concentrations of 
^3H-SQ29548 to examine the binding of the antagonist to the cells. The results in Fig. 4C show that binding of 
^3H-SQ29548 to MA-10 cells increased as its concentration in the medium was increased from 0 to 18.4 nM. Specific binding of SQ29548 to the TBX A2 receptors on the cellular membrane was demonstrated by binding competition between 
^3H-SQ29548 and BM567 for the receptors. When the concentration of BM567 in the medium was increased from 0 to 10 μM, 
^3H-SQ29548 bound to the cells was reduced to 3% (Fig. 4D). In this competition, IC_{50} of BM567 in inhibition of 
^3H-SQ29548-binding to MA-10 cells is 150 nM.

Synergistic interaction between the cAMP and the TBX A2 receptor antagonist

To determine the interaction between cAMP and the TBX A2 receptor antagonist, MA-10 cells were incubated with or without 25 μM SQ29548 for 30 min, followed by addition of increasing concentrations of dbcAMP to the cultures for 6 h. In the absence of SQ29548, 0.1 mM dbcAMP did not induce significant increase in steroidogenesis. However, addition of SQ29548 significantly
increased steroid hormone production by 10-fold over that of the cells treated with 0.1 mM dbcAMP alone. Also, StAR protein expression was markedly increased by addition of the antagonist to the cells treated with 0.05, 0.1, or 0.25 mM dbcAMP (Fig. 5). Whereas SQ29548 enhanced dbcAMP-induced steroidogenesis, in the absence of dbcAMP, this receptor antagonist alone did not increase StAR protein expression and steroid hormone production (Fig. 5).

**DAX-1 protein**

To understand how the receptor antagonist enhanced StAR gene transcription, DAX-1 protein in aged rat Leydig cells and MA-10 cells were detected by Western blot analysis. The results in Fig. 6 show that blocking TBX A2 receptor reduced the levels of DAX-1 protein. Similar results were obtained when TBXAS activity was inhibited. Furthermore, the reduction of DAX-1 protein was associated with marked increase in StAR protein expression (Fig. 6).

**Discussion**

After the previous study that suggested an inhibitory effect of TBX A2 on steroidogenesis (21), the present study further indicates an involvement of the TBX A2 receptor in the COX2-dependent negative signaling that depresses StAR gene expression in Leydig cells.

The TBX A2 receptor was detected in several mouse Leydig cell lines and on the cellular membrane of MA-10 cells. The role of the TBX A2 receptor in StAR gene expression was supported by the experiments using a highly selective antagonist, SQ29548 (29), to block the TBX A2 receptor, in which the increasing levels of SQ29548 induced a concentration-dependent increase in StAR protein expression in MA-10 cells cultured in a medium containing 0.1 mM dbcAMP. In concomitant with the increase in StAR protein, steroid hormone production by the cells was significantly enhanced. The observations were corroborated by use of another receptor antagonist, BM567 (30). As expected, BM567 dramatically increased StAR protein expression and steroid hormone production similar to those observed in the experiments using SQ29548. In addition, the specific bindings of the both antagonists to the receptors on the MA-10 cell membrane were demonstrated by the binding assays with $^3$H-SQ29548 and binding competition between $^3$H-SQ29548 and BM567. These results indicate that blocking the TBX A2 binding to its receptor enhanced StAR protein expression and steroid hormone production in MA-10 Leydig cells.

Furthermore, the studies measuring StAR promoter activities indicate that blocking the TBX A2 receptor using either of the antagonists significantly enhanced StAR gene transcription. This observation was strengthened by RT-PCR analysis of StAR mRNA levels, which showed a significant increase in StAR mRNA when the receptor was blocked. How the receptor antagonists acted on the StAR promoter and enhanced StAR gene transcription is not clear. However, it is clear that blocking TBX A2-binding to its receptor or reduction of the receptor ligand, TBX A2, by inhibiting the activity of either COX2 (16) or TBXAS (21) is able to significantly increase StAR promoter ac-
tivity and StAR mRNA levels. These observations suggest that StAR promoter activity is regulated by the signaling through AA, COX2, TBXAS, TBX A2, and the TBX A2-receptor. Moreover, we previously reported that inhibition of TBXAS activity enhanced StAR gene transcription by reduction of the transcriptional repressor, DAX-1 (21). Therefore, we reasoned that blocking TBX A2 binding to its receptor might reduce DAX-1 protein by interrupting this signaling pathway. This hypothesis was demonstrated to be correct. The results from the present study showed that DAX-1 protein was markedly reduced when MA-10 cells or aged Leydig cells were incubated with the receptor antagonist SQ29548, an observation similar to that seen in the cells treated with the TBXAS inhibitor. These results suggest that the AA-COX2-TBXAS-TBX A2-receptor signaling pathway is involved in the observed change of DAX-1 protein. The manner in which this signaling pathway affected DAX-1 protein is unknown. It is possible that the TBX A2-receptor complex triggered downstream signaling that regulated the expression or stability of DAX-1 protein by unknown mechanisms.

The role of the AA-COX2-TBXAS-TBX A2-receptor signaling pathway in StAR gene expression was further supported by the dramatic increase in the steroidogenic sensitivity of MA-10 cells to cAMP stimulation in the cultures treated with the receptor antagonist, SQ29548. Usually subthreshold levels of cAMP are unable to induce significant increases in steroid hormone and StAR protein expression in MA-10 cells. However, the effectiveness of cAMP-stimulation was dramatically enhanced when the TBX A2 receptor was blocked. In the presence of SQ29548, a low level of cAMP is able to induce steroid production and StAR protein expression to maximal levels. On the other hand, whereas the receptor antagonist effectively enhanced steroidogenesis, in the absence of cAMP, it did not increase steroid hormone and StAR protein expression in MA-10 cells. These results indicate that the receptor antagonist is unable to induce StAR protein expression by itself but, rather, increased the sensitivity of MA-10 cells to cAMP stimulation. Importantly, these observations are consistent with those obtained in previous studies, in which inhibition of COX2 activity or TBXAS activity dramatically increased steroidogenic sensitivity of MA-10 cells to cAMP stimulation (16, 21). These studies indicate that COX2, TBXAS, TBX A2, and the TBX A2 receptor transduce the same signal, but at a different step, in the AA-COX2-TBXAS-TBX A2-receptor signaling pathway and control the threshold of cAMP-stimulated StAR protein expression and steroidogenesis in Leydig cells.

Although the mechanism responsible for this interaction between cAMP and the receptor antagonist has not been completely elucidated, it is known that cAMP-PKA-phosphorylation signaling is critical for the activities of the transcription factors, such as cAMP response element-binding protein, GATA-4, and steroidogenic factor 1, which bind to StAR promoter and increase promoter activity (31). This signaling pathway also regulates the interaction and cooperation among the transcription factors, including CCAAT/enhancer-binding protein-β, activator protein-1, steroidogenic factor 1, and sterol regulatory element-binding protein, resulting in increase in StAR gene transcription. However, the effectiveness of cAMP-PKA-phosphorylation is reduced by negative signals, such as the signal transduced through COX2-TBXAS-TBX A2-receptor pathway that regulates the level of DAX-1 protein, as mentioned above. It is known that DAX-1 protein is constitutively expressed in MA-10 cells and generates a tonic inhibition on StAR gene transcription (21, 32). The reduction of DAX-1-generated tonic inhibition by interrupting this negative signaling using the receptor antagonist increases the effectiveness of cAMP-PKA-phosphorylation signaling. Thus, subthreshold level of cAMP is able to significantly stimulate StAR gene expression in the cells treated with the receptor antagonist.

It was previously reported that the age-related increase in COX2 enhanced the negative signaling that depressed StAR gene expression (18). The above observations indicated that TBX A2 receptor is involved in this negative signaling, which regulates DAX-1 protein level and depressed StAR protein expression in aged Leydig cells. The results from the study further indicated that it is possible to delay the decline in testosterone biosynthesis in aging Leydig cells by blocking their TBX A2 receptors. Because the age-related decline in testosterone is associated with decreases in many physiological functions, the present study suggests a potential for developing new drugs to improve the health of aging males suffering from hypogonadism.

Collectively, the results from the present and previous studies suggest that the COX2-dependent inhibition of StAR gene expression involve the negative signaling through the autocrine loop of COX2-TBXAS-TBX A2-receptor, in which TBX A2 generated by the coaction of COX2 and TBXAS is released from Leydig cells and then binds to its receptors. It is possible that the TBX A2-receptor complex induces downstream signaling that in turn regulates the expression or stability of DAX-1 protein. Blocking the signaling through this loop reduced DAX-1 protein and increased the sensitivity of Leydig cells to cAMP stimulation, resulting in dramatic increases in cAMP-stimulated StAR gene expression and steroid hormone production.

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