PERGAMON

Journal of Steroid Biochemistry & Molecular Biology 85 (2003) 159–166

The Journal of Steroid Biochemistry & Molecular Biology

www.elsevier.com/locate/jsbmb

Involvement of 5-lipoxygenase metabolites of arachidonic acid in cyclic AMP-stimulated steroidogenesis and steroidogenic acute regulatory protein gene expression☆

Xing Jia Wang*, Matthew T. Dyson, Youngah Jo, Darrell W. Eubank, Douglas M. Stocco

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

Abstract

To understand the mechanism for the role of arachidonic acid (AA) in steroidogenic acute regulatory (StAR) gene transcription, sections of the −1/−966 StAR promoter were deleted to produce constructs of −1/−426, −1/−211, −1/−151, and −1/−110 and inserted into the PGL3 vector to drive luciferase expression. Results indicated that −1/−151 StAR promoter contains the elements that are most responsive to AA. Electrophoretic mobility shift assays using nuclear extracts from AA-treated MA-10 Leydig tumor cells showed that AA enhanced specific binding of the nuclear extract to a 30 bp (−67/−96) sequence of the StAR promoter. Also, HPLC was used to identify AA metabolites involved in StAR gene transcription. It was found that 1 mM N6,2-O-dibutyryladenosine 3:5-cyclic monophosphate (dbcAMP) significantly increased the 5-lipoxygenase metabolites, 5-hydroperoxyeicosatetraenoic acid (5-HPETE) and 5-hydroxyeicosatetraenoic acid (5-HETE). Moreover, in the presence of 0.2 mM dbcAMP addition of 20 μM 5-HPETE or 5-HETE significantly enhanced StAR protein expression and progesterone production ($P < 0.05$). Similar results were obtained for StAR gene transcription with StAR mRNA levels and StAR promoter activities being significantly increased ($P < 0.05$) when 5-HPETE was added to MA-10 cell cultures. In summary, the present studies demonstrated that cyclic AMP (cAMP) stimulated the production of the AA metabolites, 5-HPETE and 5-HETE, and showed that these metabolites enhanced StAR gene expression and steroid hormone production. The results further suggested that the AA-responsive element resides in the −67/−96 region of the StAR promoter.

Keywords: Arachidonic acid; 5-HPETE; 5-HETE; 5-Lipoxygenase; StAR; Steroidogenesis

1. Introduction

It is well known that trophic hormones, such as luteinizing hormone (LH) and adrenocorticotrophic hormone (ACTH) induce second messenger cyclic AMP (cAMP) formation that in turn activates protein kinase A (PKA) and transduces the signal from the trophic hormone–receptor complex to the nucleus to regulate steroidogenesis. However, trophic hormones also stimulate the release of arachidonic acid (AA) and inhibition of this release from phospholipids inhibits steroid hormone biosynthesis [1,2]. Previous studies indicated that trophic hormone-induced AA release in rat Leydig cells was dependent on hormone-receptor interaction and was mediated through a G protein [3,4]. Recently, an ACTH-induced 43 kDa protein, termed the arachidonic acid-related thioesterase involved in steroidogenesis (ARTISt), was identified and cloned from rat adrenal cells. ARTISt was reported to have a role in steroidogenesis by regulating AA release from arachidonyl-CoA [5]. During the last two decades, the regulatory effects of AA and its metabolites in steroid hormone biosynthesis have been demonstrated in various steroidogenic cells from different species [6–9]. However, the mechanism for the role of AA remains unknown.

Several earlier studies reported that while inhibition of AA release with phospholipase A_2 (PLA_2) inhibitors inhibited steroidogenesis, these inhibitors failed to inhibit the conversion of 22-R(OH)cholesterol to steroids [10,11], suggesting that AA and its metabolites act on the rate-limiting step of steroid biosynthesis, cholesterol transfer from the outer to inner mitochondrial membrane. Steroidogenic acute regulatory (StAR) protein has been demonstrated to have a critical role in this cholesterol transfer [12–15]. It was therefore reasoned that StAR protein might be involved in the role of AA in the regulation of steroidogenesis. This hypothesis was demonstrated to be correct. Inhibition of

☆ Presented at the 11th International Congress on Hormonal Steroids and Hormones and Cancer, ICHS & ICHC, Fukuoka, Japan, 21–25 October 2002.

* Corresponding author. Tel.: +1-806-743-2505; fax: +1-806-743-2990.
E-mail address: xingjia.wang@ttmc.ttuhsc.edu (X.J. Wang).

0960-0760/$ – see front matter © 2003 Elsevier Science Ltd. All rights reserved.
doi:10.1016/S0960-0760(03)00189-4

AA release inhibited StAR gene expression and concomitant steroid production. Importantly, the inhibitory effects were reversed by addition of exogenous AA to the cell culture [16,17]. Previous studies have further indicated that the AA signaling pathway is different from the reported cAMP-PKA-phosphorylation pathway. These two signaling pathways are both required to co-regulate StAR gene expression and steroid production with neither one alone being sufficient for trophic hormone-stimulated steroidogenesis [17,18]. Moreover, the synergistic interaction between AA and cAMP increases the sensitivity of steroidogenesis to trophic hormone stimulation [19]. In the present study, in order to further understand the nature of the AA signaling pathway, we investigated StAR promoter DNA sequences and found a region in the promoter that was responsive to treatment with AA. Also, cAMP-induced AA metabolites were separated and identified using HPLC analysis. The results indicated that 5-lipoxygenase metabolites of AA are involved in trophic hormone-induced signaling and are stimulatory in StAR gene expression and steroid hormone biosynthesis.

2. Materials and methods

2.1. Materials

N6,2-O-dibutyryladenosine 3:5-cyclic monophosphate (dbcAMP) and arachidonic acid were purchased from Sigma (St. Louis, MO). 5-hydroperoxyeicosatetraenoic acid (5-HPETE) and 5-hydroxyeicosatetraenoic acid (5-HETE) were purchased from Cayman (Ann Arbor, MI). Waymouth's MB/752 medium, horse serum, trypsin-EDTA, antibiotics, and PBS were purchased from Gibco-BRL Life Technologies (Gaithersburg, MD). Rabbit antisera generated against StAR protein was a generous gift from Dr. W.L. Miller (Department of Pediatrics, University of California, San Francisco, CA). Donkey anti-rabbit IgG antibody conjugated with horseradish peroxidase was purchased from Amersham (Arlington Heights, IL). North2South Biotin Random Primer DNA Labeling kits and Chemiluminescent Nucleic Acid Hybridization and Detection kit were obtained from Pierce (Rockford, IL). The Dual-Luciferase Reporter Assay System was purchased from Promega (Madison, WI). Other common chemicals used in this study were obtained from either Sigma (St. Louis, MO) or Fisher Chemicals (Pittsburgh, PA).

2.2. Cell culture

MA-10 mouse Leydig tumor cells were a generous gift from Dr. Mario Ascoli (Department of Pharmacology, College of Medicine, University of Iowa, Iowa City, IA) and were cultured in 6-well culture plates in Waymouth's MB/752 medium containing 15% horse serum as previously described [20]. The cells were cultured in an incubator at 37 °C and 5% CO_2. Before each experiment, the medium was replaced with serum-free Waymouth's medium.

2.3. Steroid production

MA-10 cells were cultured for 30 min in serum-free Waymouth's medium containing AA, 5-HPETE or 5-HETE (as described in the legends of the figures) and then stimulated with 0.2 mM dbcAMP for 6 h. The medium was collected at the end of each experiment and stored at −80 °C. The cells were washed twice with cold PBS and stored at −80 °C. Progesterone concentrations in the medium were determined by RIA [21].

2.4. Plasmid construction of StAR promoter deletions

The plasmid PGL2/–966StAR [22] was used as a template for PCR to delete sections of the −966 StAR promoter to produce promoter constructs of −1/−426, −1/−211, −1/−151 and −1/−110 using the 5′-primers 5′-TAGCTCGAGATCTACTTGCCTCTGCCAGGGAGAG-3′, 5′-TAGCTCGAGGCCCCATCTCCGTGACCCCTGCTT-3′, 5′-TAGCTCGAGTCTGCTCCCTCCCACCTTGGCCA-GC-3′, 5′-TAGCTCGAGCAATCATTCCATCCTTGACCC-3′, respectively. This resulted in the introduction of a *Xho* I restriction site and a common 3′-primer 5′-CTAAAGCTTG-GCGCAGATCAAGTGCGCTGCCT-3′ was used to introduce a *Hin*d III site. The PCR fragments were digested with restriction enzymes *Xho* I/*Hin*d III and inserted into the PGL3 vector (Promega, Madison, WI) cut with the same enzymes to construct the plasmids PGL3/–426StAR, PGL3/–211StAR, PGL3/–151StAR and PGL3/–110StAR, respectively. The construction of the various PGL3/StAR promoter deletions were confirmed by DNA sequencing and activity assays.

2.5. Transfections

MA-10 cells (0.5×10^6 per well) were cultured in 6-well plates overnight. The cells in each well were transfected with 1.0 μg DNA of the PGL3/StAR expressing firefly luciferase driven by the StAR promoter deletion constructs described earlier. Transfections also included 12.5 ng of the pRL-SV40 vector DNA (a plasmid which constitutively expresses Renilla luciferase and acts as a control reporter under the regulation of the SV40 promoter, Promega, Madison, WI). Transfections were performed using FuGENE6 transfection reagent (Roche, Indianapolis, IN) following the manufacturer's instructions. After 48 h in culture the cells were utilized for experiments.

2.6. Luciferase assay

Following experiments, the cells were washed three times with ice-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,

expressed as the reading from the PGL3/StAR promoter divided by the reading from Renilla luciferase) were measured using a TD-20/20 luminometer (Turner Designers, Sunnyvale, CA).

2.7. Electrophoretic mobility shift assay

The oligonucleotides for sense and antisense corresponding to StAR promoter DNA sequences at −1/−48, −38/−76, −67/−96, −66/110, −106/141 and −135/−165 were synthesized, heated at 75 °C for 5 min and cooled at room temperature for 2 h to anneal in a buffer containing 100 mM NaCl, 1 mM EDTA, and 10 mM Tris–HCl, pH 7.5. The 5′ end GG overhangs in annealed DNA were labeled with α^{32}P-dCTP (Dupont NEN, Boston, MA) by fill-in reaction using Klenow (Promega, Madison, WI). The nuclear extracts were prepared from MA-10 cells treated with 0.25 mM db-cAMP and/or 150 mM AA as described [23]. Electrophoretic mobility shift assays (EMSA) were performed with the labeled DNA following the protocol described previously [23].

2.8. HPLC analysis of arachidonic acid metabolites

The methods previously described were modified for extraction and HPLC separation of AA metabolites [24,25]. MA-10 cells in 100 mm-culture plates were incubated in 5 ml Waymouth's medium containing 1.5% horse serum and 4 μCi/ml ^{3}H-AA for 4 h. The labeled cells were washed twice with 5 ml Waymouth's containing 0.1% fatty acid-free BSA. The cells were then incubated in 5 ml of Waymouth's medium and stimulated with 1 mM dbcAMP for 6 h. The stimulation was terminated by their transfer to −80 °C and the addition of 1.0 ml cold methanol containing 5 μl of 37% HCl, 15 μg of cold AA and a mixture of AA metabolites (3 μg each of: 5,6-EET; 8,9-EET; 11,12-EET; 14,15-EET; 5-HETE; 5-HpETE). The cells were collected and centrifuged at 8000 × *g* for 20 min. The resulting supernatants were loaded onto 3 ml/500 mg Discovery DSC-18 SPE tubes (Supelco, Bellefonte, PA) preconditioned with washes of 3 ml methanol followed by 3 ml water. The tubes were then eluted with 3 ml methanol, followed by 3 ml acetonitrile. The eluates from the tubes were dried under nitrogen and resuspended in 350 μl of 60% acetronitrile. The concentrated extracts were immediately analyzed by RP-HPLC with a Gradient Component System with two 515 HPLC pumps (Waters, Milford, MA) on a Discovery C18 column (4.6 mm × 25 cm, 5 μm particle size, Supelco) using a stepped gradient of solvent A, containing water/acetonitrile/methanol/phosphoric acid (75/12.5/12.5/0.01), and solvent B, containing water/acetonitrile/methanol/phosphoric acid (5/63/32/0.01). The gradient steps used are as follows: 0 min, 100% A, 1.0 ml/min; 0–10 min, 44%A + 56%B, 0.98 ml/min; 10–89 min, 40%A + 60%B, 0.96 ml/min; 98–140 min, 100%B, 0.96 ml/min. The sample was analyzed spectrophotometrically at 206 nm and 235 nm using a Waters 2487 Dual λ Absorbance Detector (Waters, Milford, MA). The column eluate was collected directly into scintillation vials and the radioactivities of the fractions were measured using a Beckman LS 6500 scintillation counter.

2.9. Northern blot analysis

In experiments designed to determine StAR expression at the mRNA level, cells were washed three times with cold PBS and used for total RNA purification using TRIzol reagent following the manufacturer's instructions (GibcoBRL, Grand Island, NY). The RNA was separated by electrophoresis in an agarose/formaldehyde gel (1%/6%) and blotted onto a Hybond-N$^+$ membrane (Amersham, Alington Heights, IL). StAR mRNA on the membrane was probed with biotin-labeled mouse StAR cDNA and detected using the North2South Chemiluminescent Nucleic Acid Hybridization and Detection kit following the manufacturer's instructions (Pierce, Rockford, IL). The membrane was stripped with a buffer containing 15 mM NaCl, 15 mM sodium citrate and 1% SDS, pH 7.0 for 30 min at 55 °C. 18S rRNA on the membrane was probed to adjust for the RNA loading in each lane.

2.10. Western blot analysis

StAR protein in MA-10 cells was detected by Western blot analysis as described previously [26]. Western blot analyses experiments were performed at least three times and the results of one representative experiment are shown.

2.11. Statistical analysis

Each experiment was repeated at least three times. Statistical analysis of the data was performed with ANOVA using the Stat View SE system (Abacus Concepts, Berkeley, CA). The data are shown as mean ± standard error.

3. Results

3.1. Arachidonic acid-enhanced activities of StAR promoter deletions

The constructed plasmids, PGL3/–426StAR, PGL3/–211StAR, PGL3/–151StAR, PGL3/–110StAR and PGL3/Basic, were transfected into MA-10 Mouse Leydig cells to test their response to AA. In the present of 0.2 mM db-cAMP 150 μM AA significantly enhanced the activities of PGL3/–426StAR ($P < 0.05$), PGL3/–211StAR ($P < 0.01$) and PGL3/–151StAR ($P < 0.01$). The activities of PGL3/–426StAR ($P < 0.05$), PGL3/–211StAR ($P < 0.01$) and PGL3/–151StAR were increased by 1.4-, 2.2- and 2.7-folds, respectively, over the paired group treated with dbcAMP alone (Fig. 1). However, removal of 41 bp of the

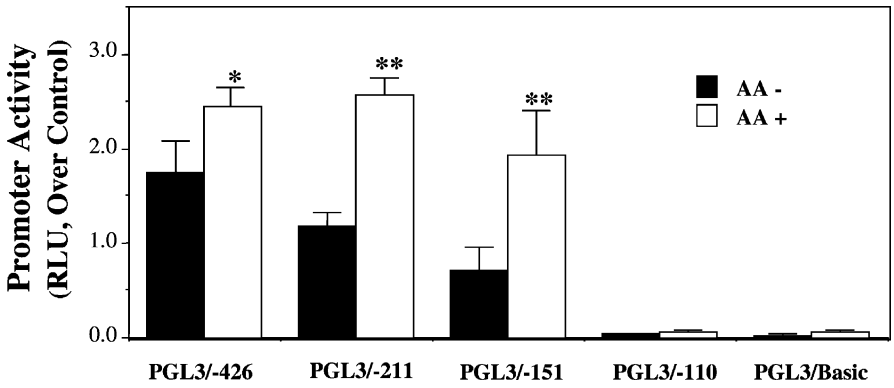

Fig. 1. Arachidonic acid-enhanced activities of StAR promoter deletions. The StAR promoter sequence −1/−966 was used as the template for PCR to delete the StAR promoter to produce promoter constructs of −1/−426, −1/−211, −1/−151 and −1/−110. The deletions were inserted into the PGL3 vector expressing the firefly luciferase reporter gene (Promega, Madison, WI) to construct plasmids PGL3/–426StAR, PGL3/–211StAR, PGL3/–151StAR and PGL3/–110StAR, respectively. The constructed PGL3/StAR plasmids and pRL-SV40 vector DNA, a plasmid which constitutively expresses Renilla luciferase, were transfected into MA-10 cells using FuGENE6 transfection reagent (Roche, Indianapolis, IN). After 48 h in culture the cells were treated for 6 h with 0.2 mM dbcAMP with or without 150 μM AA. The cell lysate was used for the luciferase assay using a Dual Luciferase Reporter Assay System (Promega, Madison, WI). StAR promoter activities were expressed as relative light units (RLU) over control (MA-10 cells transfected with the same DNA, without dbcAMP or AA). *: significantly different from stimulation with dbcAMP alone ($P < 0.05$). **: highly significantly different from stimulation with dbcAMP alone ($P < 0.01$).

StAR promoter DNA from positions −110 to −151 dramatically reduced the activity of PGL3/–151StAR to a level as low as that of PGL3/Basic.

3.2. Arachidonic acid-enhanced binding of nuclear extracts to the −67/−96 segment of the StAR promoter

EMSA was performed to determine if AA increases binding of nuclear proteins to StAR promoter sequences within −1/−151. As shown in the Fig. 2, 150 μM AA or 0.25 mM dbcAMP alone slightly increased binding of nuclear extract to the oligonucleotide corresponding to StAR promoter sequences from −67 to −96. Co-incubation of AA with dbcAMP remarkably increased the binding to this region. The AA-enhanced binding was eliminated by addition of 100-fold of the cold oligonucleotide corresponding to the −67/−96 sequence of the StAR promoter.

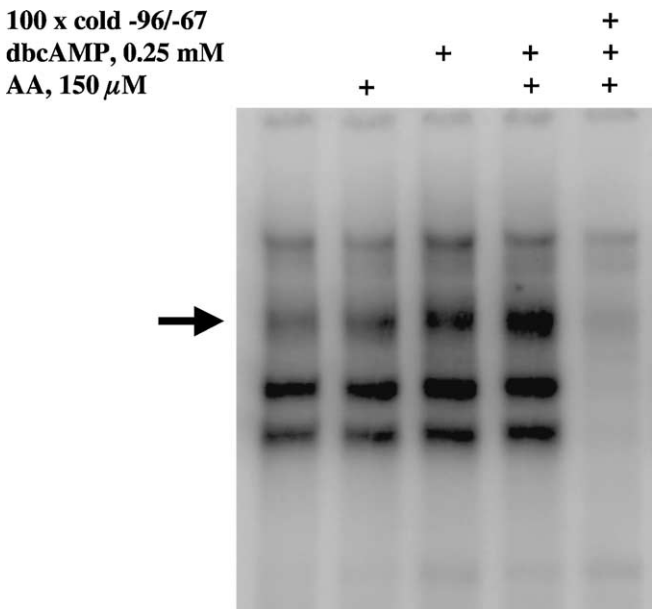

Fig. 2. Arachidonic acid-enhanced binding of MA-10 nuclear extract to the −67/−96 StAR promoter sequence. MA-10 cells were cultured in Waymouth's MB/752 medium with or without 150 μM AA for 30 min and then stimulated with 0.25 mM dbcAMP for 6 h. The cells were collected for the preparation of nuclear extracts. Five microgram of nuclear extract proteins were incubated with ^{32}P-labeled oligonucleotides representing the StAR promoter −67/−96 sequence. Unlabeled identical oligonucleotides in 100-fold molar excess were used as binding competitors. The resulting samples were used for electrophoresis in a 4% non-denaturing polyacrylamide gel. The gel was dried and the DNA–protein complexes were visualized by autoradiography.

3.3. dbcAMP-induction of 5-lipoxygenase AA metabolites

HPLC was used to separate cAMP-induced AA metabolites in MA-10 Leydig cells. Stimulation with 1 mM dbcAMP increased the production of the 5-lipoxygenase metabolites, 5-HPETE and 5-HETE which is demonstrated by an increase in these compounds as illustrated in Fig. 3A. The averages of the total radioactivities for the samples in the areas under the peaks from individual experiments indicated that 1 mM dbcAMP significantly increased 5-HETE and 5-HPETE by 2.02- and 2.87-fold over controls respectively (Fig. 3B).

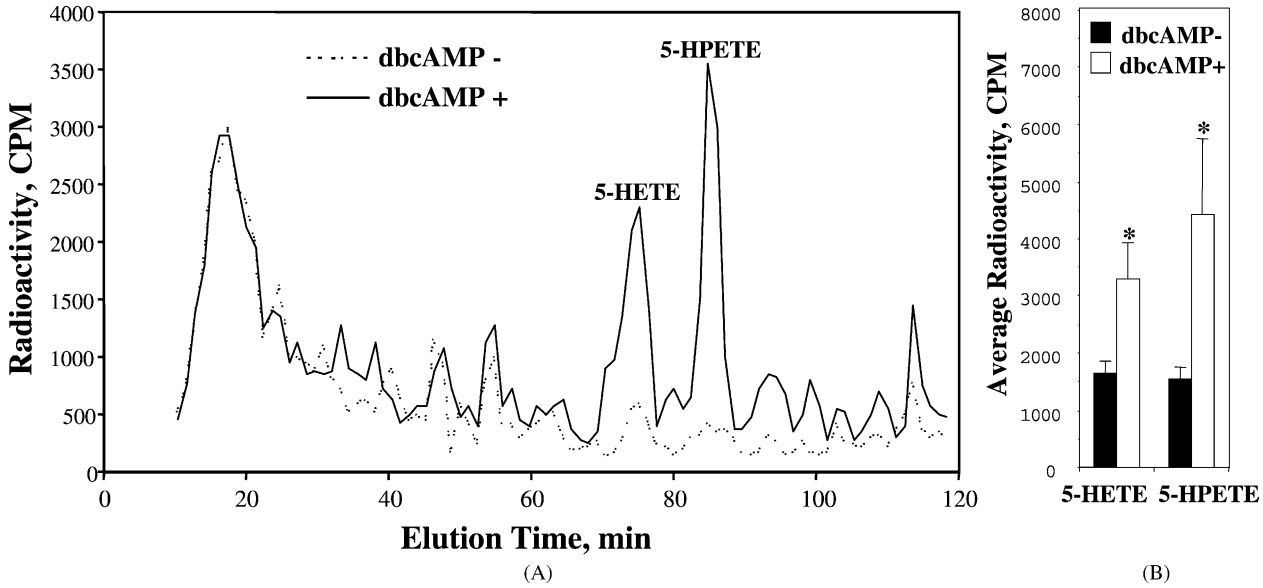

Fig. 3. HPLC analysis of dbcAMP-induced 5-HPETE and 5-HETE in MA-10 mouse Leydig cells. MA-10 cells pre-loaded with ^{3}H-AA were stimulated with 1 mM dbcAMP for 6 h. Stimulation was terminated by addition of 1 ml methanol containing a mixture of cold AA metabolites and freezing at −80 °C. AA metabolites were then extracted with a Discovery DSC-18 SPE cartridge (Supelco, Bellefonte, PA). The extracted samples were dried under nitrogen and resuspended in 350 μl of 60% acetronitrile. The AA metabolites in the resuspended solution were separated by HPLC using a Gradient Component System with two 515 HPLC pumps (Waters, Milford, MA) on a Discovery C18 column (4.6 mm × 25 cm, 5 μm particle size, Supelco) and a stepped gradient. The separated peaks were analyzed spectrophotometrically at 206 nm and 235 nm using a Waters 2487 Dual λ Absorbance Detector (Waters, Milford, MA). The column eluate was collected directly into scintillation vials and the radioactivities of the fractions were measured using a Beckman LS 6500 scintillation counter. (A): ^{3}H-5-HPETE and ^{3}H-5-HETE peaks separated by HPLC analysis; (B): the averages of the total radioactivities for the samples in the peak areas of 5-HPETE and 5-HETE from five individual experiments; *: significantly different from the paired group without dbcAMP ($P < 0.05$).

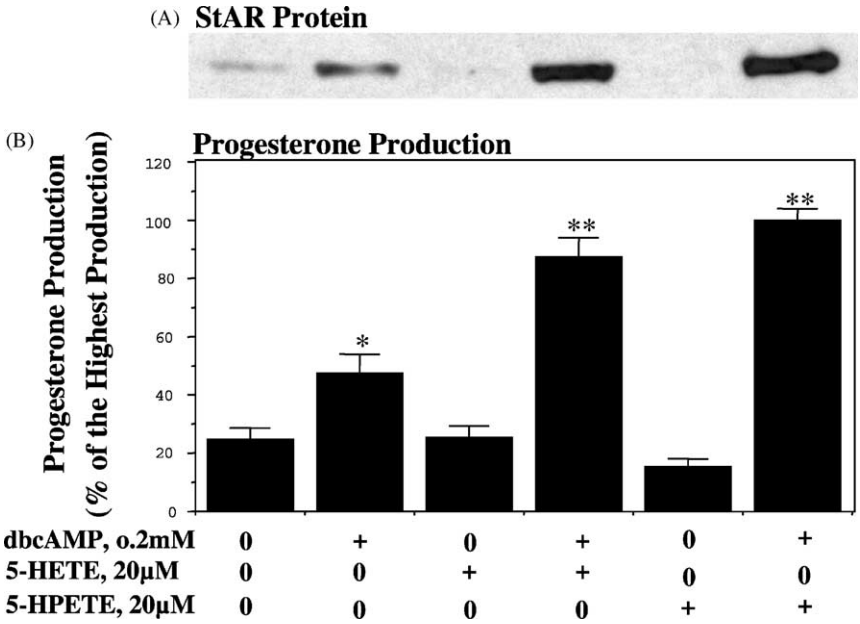

Fig. 4. Effects of 5-HPETE and 5-HETE on cAMP-stimulated StAR protein expression and steroid hormone production in MA-10 mouse Leydig cells. MA-10 cells were cultured for 30 min in serum-free Waymouth's MB/752 medium with or without 20 μM of 5-HPETE or 5-HETE and then stimulated for 6 h with 0.2 mM dbcAMP as indicated in the figure. (A): cells were collected and 25 μg of cell lysate protein was used to analyze StAR protein by Western blot; (B): progesterone production in the medium was analyzed by RIA and expressed as a percentage of the highest production; *: significantly different from controls ($P < 0.05$); **: highly significantly different from controls ($P < 0.01$).

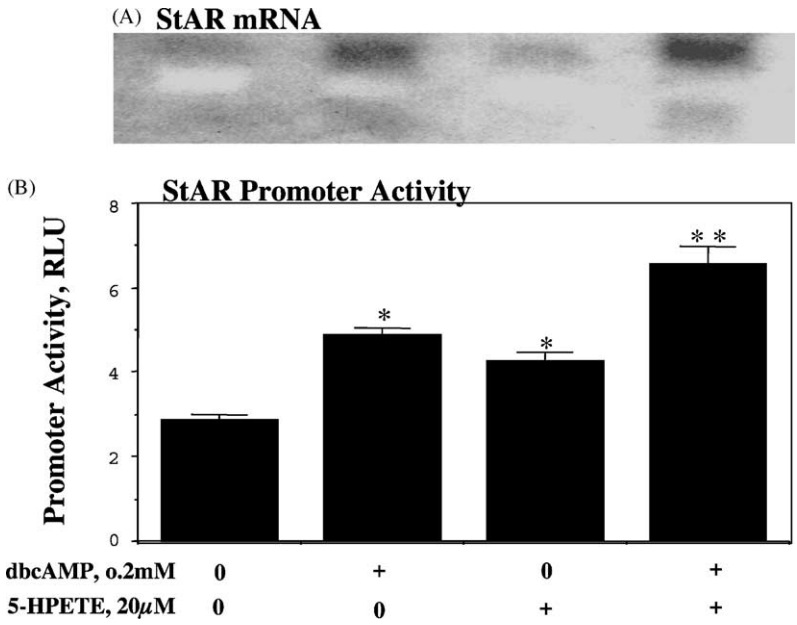

Fig. 5. Effects of 5-HPETE and 5-HETE on cAMP-stimulated StAR mRNA levels and StAR promoter activity in MA-10 cells. (A): MA-10 cells were cultured for 30 min in serum-free Waymouth's MB/752 medium with or without 20 μM of the 5-HPETE or 5-HETE and then stimulated for 6 h with 0.2 mM dbcAMP as indicated in the figure. Cells were collected for purification of total RNA. StAR mRNA expression was analyzed by Northern blot; (B): MA-10 cells were transfected with a PGL3/–151StAR plasmid and pRL-SV40 vector DNA, a plasmid that constitutively expresses Renilla luciferase. After 48 h in culture, the cells were treated as described earlier. The cell lysate was used for the luciferase assay using a Dual Luciferase Reporter Assay System as described in the Section 2. StAR promoter activities were expressed as relative light units (RLU); *: significantly different from controls ($P < 0.05$); **: highly significantly different from controls ($P < 0.01$).

3.4. 5-HPETE and 5-HETE-enhancement of StAR protein expression and steroid production

StAR protein levels in MA-10 cells cultured in medium containing 5-HETE or 5-HPETE were analyzed by Western blot analyses to determine if 5-HETE and 5-HPETE were involved in cAMP-induced StAR protein expression. While 0.2 mM dbcAMP slightly increased StAR protein, addition of 20 μM of 5-HETE or 5-HPETE dramatically enhanced StAR protein expression to much higher levels (Fig. 4A). With the increases in StAR protein, concomitant increases of steroid production in 5-HETE or 5-HPETE-treated cells were detected, with progesterone production being increased by 1.84- and 2.11-fold, respectively, over that in the cells treated with dbcAMP alone (Fig. 4B). In the absence of dbcAMP, 5-HETE or 5-HPETE increased neither StAR protein nor steroidogenesis. There was no significant difference in steroid production among the groups receiving no dbcAMP.

3.5. 5-HPETE-enhanced StAR gene transcription

Northern blot analysis was performed to determine the levels of StAR mRNA in 5-HPETE-treated MA-10 cells. As shown in Fig. 5A, while 0.2 mM dbcAMP slightly increased StAR mRNA, these transcripts were increased to higher levels by co-incubation of the cells with 20 μM 5-HPETE. In the absence of dbcAMP, 5-HPETE did not increase the levels of StAR mRNA. Similar results were obtained in promoter activity assays with activity of the PGL3/–151StAR promoter being significantly increased ($P < 0.01$) by the addition of 20 μM 5-HPETE into MA-10 cell culture containing 0.2 mM dbcAMP (Fig. 5B).

4. Discussion

Signal transduction through cAMP-PKA-phosphorylation is well known and is important for StAR gene expression and steroid hormone biosynthesis [27]. However, a high level of PKA activity in the absence of AA release is not sufficient to accomplish the physiological function of trophic hormone stimulation of steroidogenic cells, namely steroid biosynthesis [17]. Studies in recent years have demonstrated that a separate signaling pathway mediated by AA and its metabolites is also critical for trophic hormone-stimulated StAR gene expression and steroidogenesis [18]. However, the manner in which AA regulates StAR gene expression remains unknown.

The previous studies have demonstrated that AA regulates StAR gene expression at the level of transcription. In the presence of 0.2 mM cAMP analog, AA significantly increased the activity of the −1 to −966 bp segment of the wild type StAR promoter suggesting that this region contains

the element(s) responsive to AA [19]. In the present study, results from promoter activity assays further indicated that StAR promoter DNA sequences from −1 to −151 contained the most important elements responsible for the regulatory effect of AA on StAR gene transcription since the −1/−151 StAR promoter construct resulted in maximal activity in response to AA. Expression of the deletion construct, −1 to −110, reduced the promoter activity to basal levels, similar to results reported previously [22], and illustrated the importance of this region. Also, EMSA results with oligonucleotides corresponding to these 151 bp of StAR promoter sequences showed that AA synergistically increased the specific binding of nuclear extracts to the StAR promoter DNA sequences located between −67 and −96, suggesting the location of AA-responsive elements in this region. This region of the StAR promoter was reported to contain the binding sites for the transcription factors AP-1, C/EBPβ, SF-1 and also for transcription factors from the CREB family [28–30]. It is clear that this segment of the promoter is important for StAR gene transcription since mutations within the region significantly reduced dbcAMP-stimulated StAR promoter activity in MA-10 cells transfected with PGL3/–151StAR [30]. Whether the AA-enhanced binding of nuclear protein to this region is related to any of these transcription factors and which AA metabolites are involved in StAR gene transcription are currently unknown.

To further understand the mechanism for the role of AA in this process, we attempted to identify AA metabolites involved in trophic hormone-stimulated StAR gene transcription. As clearly shown by the presence of two peaks separated and identified following HPLC analysis, cAMP analog significantly increased 5-HPETE, and 5-HETE, two AA metabolites produced through the 5-lipoxygenase pathway. It was previously reported that trophic hormones induced AA release through G proteins [4] and also through cAMP-dependent mechanisms [19]. It is therefore possible that the cAMP-induced 5-HPETE and 5-HETE might be involved in trophic hormone-induced signal transduction in the steroidogenic pathway. This hypothesis is supported by the results from RIA of progesterone production and Western blot analysis of StAR protein expression in MA-10 mouse Leydig cells since progesterone production and StAR protein expression were increased significantly when MA-10 cells were incubated with exogenous 5-HPETE or 5-HETE. This observation corroborates similar results obtained in an earlier study with rat adrenal cells [31]. In that study, the authors reported that the ACTH-stimulated 5-HPETE production and inhibition of AA metabolism through the 5-lipoxygenase pathway inhibited ACTH-stimulated steroid biosynthesis. Moreover, the present study further demonstrated that 5-HPETE enhanced StAR gene expression at the level of transcription with StAR mRNA levels and StAR promoter activity being increased as 5-HPETE was added to the cell cultures. It will be most interesting to determine the manner in which this 5-lipoxygenase metabolite of AA interacts with the 30 bp sequence (−67/−96) of the StAR promoter DNA and how it enhances StAR gene transcription.

Previously, we have described a synergistic interaction between AA and cAMP which significantly enhanced StAR gene transcription and steroid hormone production in MA-10 mouse Leydig cells [19]. Taking into consideration the results in the present and previous studies, it can readily be seen that: (1) AA-enhanced binding of nuclear extract to the StAR promoter; (2) AA or 5-HPETE-increased StAR promoter activity; (3) StAR mRNA expression; (4) StAR protein expression; and (5) steroid hormone production are dependent on a scenario in which both AA and cAMP are required for maximal response. The co-regulation by these two signaling pathways remarkably increased the sensitivity of steroidogenesis to trophic hormone stimulation. These studies provide important information for understanding the roles of these two pathways. It is possible that maximal binding of as yet unidentified nuclear protein(s) to this 30 bp region (−67/−96) of the StAR promoter sequences requires both PKA-phosphorylation activity and the action of AA metabolite(s). AA or its metabolites might be involved in the induction or activation of transcription factor(s) or co-factor(s) that binds to this region of the StAR promoter and enhances StAR gene transcription.

In summary, the present study identified two cAMP-induced AA metabolites, 5-HPETE and 5-HETE, and demonstrated their stimulatory effects on StAR gene expression and steroid hormone production. Our results also demonstrated that AA enhanced the binding of nuclear protein(s) to the −67/−96 region of the StAR promoter DNA suggesting that the AA-responsive element is located within this DNA sequence.

Acknowledgements

The authors would like to acknowledge the support of NIH grant HD39308 to X.J.W. and the support of NIH grant HD17481 and funds from the Robert A. Welch Foundation to D.M.S.

References

[1] B.A. Cooke, G. Dirami, L. Chaudry, M.S.K. Choi, D.R.E. Abayasekara, L. Phipp, Release of arachidonic acid and the effects of corticosteroids on steroidogenesis in rat testis Leydig cells, J. Steroid Biochem. Mol. Biol. 40 (1991) 465–471.

[2] D.R.E. Abayasekara, A.M. Band, B.A. Cooke, Evidence for the involvement of phospholipase A_2 in the regulation of luteinizing hormone-stimulated steroidogenesis in rat testis Leydig cells, Mol. Cell. Endocrinol. 70 (1990) 147–153.

[3] P.F. Moraga, M.N. Llanos, A.M. Ronco, Arachidonic acid release from rat Leydig cells depends on the presence of lutenizing hormone/human chorionic gonadotrophin receptors, J. Endocrinol. 154 (1997) 201–209.

[4] A. M Ronco, P.F. Moraga, M.N. Llanos, Arachidonic acid release from rat Leydig cells: the involvement of G protein, phospholipase

A2 and regulation of cAMP production, J. Endocrinol. 172 (2002) 95–104.
[5] P. Maloberti, R.C. Lozano, P.G. Mele, F. Cano, C. Colonna, C.F. Mendez, C. Paz, E.J. Podesta, Concerted regulation of free arachidonic acid and hormone-induced steroid synthesis by acyl-CoA thioesterases and acyl-CoA synthetases in adrenal cells, Eur. J. Biochem. 269 (2002) 559–607.
[6] F. Romanelli, M. Valenca, D. Conte, A. Isidori, A. Negro-Vilar, Arachidonic acid and its metabolites effects on testosterone production by rat Leydig cells, J. Endocrinol. Invest. 18 (1995) 186–193.
[7] T. Yamazaki, K. Higuchi, S. Kominami, S. Takemori, 15-lipoxygenase metabolite(s) of arachidonic acid mediates adrenocorticotropin action in bovine adrenal steroidogenesis, Endocrinology 137 (1996) 2670–2675.
[8] J. Wang, P.C. Leung, Arachidonic acid as a stimulatory mediator of luteinizing hormone-releasing hormone action in the rat ovary, Endocrinology 124 (1989) 1973–1979.
[9] F. Mercure, G. Van der Kraak, Mechanism of action of free arachidonic acid on ovarian steroid production in the goldfish, Gen. Comp. Endocrinol. 102 (1996) 130–140.
[10] M.P. Lopez-Ruiz, M.S.K. Choi, M.P. Rose, A.P. West, B.A. Cooke, Direct effect of arachidonic acid on protein kinase C and LH-stimulated steroidogenesis in rat Leydig cells evidence for tonic inhibitory control of steroidogenesis by protein kinase C, Endocrinology 130 (1992) 1122–1130.
[11] P.G. Mele, L.A. Dada, C. Paz, I. Neuman, C.B. Cymeryng, C.F. Mendez, C.V. Finkielstein, F. Cornejo Maciel, E.J. Podesta, Involvement of arachidonic acid and lipoxygenase pathway in mediating luteinizing hormone-induced testosterone synthesis in rat Leydig cells, Endocrinol. Res. 23 (1997) 15–26.
[12] B.J. Clark, J. Wells, S.R. King, D.M. Stocco, The purification, cloning, and expression of a novel luteinizing hormone-induced mitochondrial protein in MA-10 mouse Leydig tumor cells. Characterization of the steroidogenic acute regulatory protein (StAR), J. Biol. Chem. 269 (1994) 28314–28322.
[13] D. Lin, T. Sugawara, F. Strauss III, B.J. Clark, D.M. Stocco, P. Saenger, A. Rogol, W.L. Miller, Role of steroidogenic acute regulatory protein in adrenal and gonadal steroidogenesis, Science 267 (1995) 1828–1831.
[14] D.M. Stocco, The steroidogenic acute regulatory (StAR) protein two years later. An update, Endocrine 6 (1997) 99–109.
[15] X.J. Wang, Z.M. Liu, S. Eimer, R. Timberg, A.M. Weiss, J. Orly, D.M. Stocco, Effect of truncated forms of the steroidogenic acute regulatory protein on intramitochodrial cholesterol transfer, Endocrinology 139 (1998) 3903–3912.
[16] X.J. Wang, L.P. Walsh, D.M. Stocco, The role of arachidonic acid on LH-stimulated steroidogenesis and steroidogenic acute regulatory protein accumulation in MA-10 mouse Leydig tumor cells, Endocrine 10 (1999) 7–12.
[17] X.J. Wang, P.L. Walsh, A.J. Reinhart, D.M. Stocco, The role of arachidonic acid in steroidogenesis and steroidogenic acute regulatory (StAR) gene and protein expression, J. Biol. Chem. 275 (2000) 20204–20209.
[18] X.J. Wang, D.M. Stocco, Cyclic AMP and arachidonic acid: a tale of two pathways, Mol. Cell. Endocrinol. 158 (1999) 7–12.
[19] X.J. Wang, M.T. Dyson, C. Mondillo, Z. Patrignani, O. Pignataro, D.M. Stocco, Interaction between arachidonic acid and cAMP signaling pathways enhances steroidogenesis and StAR gene expression in MA-10 Leydig tumor cells, Mol. Cell. Endocrinol. 188 (2002) 55–63.
[20] M. Ascoli, Characterization of several clonal lines of cultured Leydig tumor cells: gonadotropin receptors and steroidogenic responses, Endocrinology 108 (1981) 88–95.
[21] J.A. Resko, N.R. Norma, G.D. Niswender, H.G. Spies, The relationship between progestins and gonadotrophins during the late luteal phase of the menstrual cycle in Rhesus monkeys, Endocrinology 94 (1974) 128–135.
[22] K.M. Caron, Y. Ikeda, S.C. Soo, D.M. Stocco, K.L. Parker, B.J. Clark, Characterization of the promoter region of the mouse gene encoding the steroidogenic acute regulatory protein, Mol. Endocrinol. 11 (1997) 138–147.
[23] A.J. Reinhart, S.C. Williams, B.J. Clark, D.M. Stocco, SF-1 (steroidogenic factor 1) and C/EBPβ (CCAAT/enhancer binding protein-β) cooperate to regulate the murine StAR (steroidogenic acute regulatory) promoter, Mol. Endocrinol. 13 (1999) 729–741.
[24] W. Powell, Rapid extraction of arachidonic acid metabolites from biological samples using octadecylsilyl silica, Methods Enzymol. 86 (1982) 467–477.
[25] P. Borgeat, S. Picard, P. Vallerand, S. Bourgoin, A. Odeimat, P. Sirois, P.E. Poubele, Automated on-line extraction and profiling of lipoxygenase products of arachidonic acid by high-performance liquid chromatography, Methods Enzymol. 187 (1990) 98–116.
[26] D.H. Townson, X.J. Wang, P.L. Keyes, J.L. Kostyo, D.M. Stocco, Expression of the steroidogenic acute regulatory protein in the corpus luteum of the rabbit: dependence upon the luteotropic hormone, estradiol-17 beta, Biol. Reprod. 55 (1996) 868–874.
[27] A.J. Reinhart, S.C. Williams, D.M. Stocco, Transcriptional regulation of the StAR gene, Mol. Cell. Endocrinol. 151 (1999) 161–169.
[28] E. Silverman, S. Eimerl, J. Orly, CCAAT enhancer-binding protein beta and GATA-4 binding regions within the promoter of the steroidogenic acute regulatory protein (StAR) gene are required for transcription in rat ovarian cells, J. Biol Chem. 18 (1999) 17987–17996.
[29] C.R. Wooton-Kee, B.J. Clark, Steroidogenic factor-1 influences protein-deoxyribonucleic acid interactions within the cyclic adenosine 3,5-monophosphate-responsive regions of the murine steroidogenic acute regulatory protein gene, Endocrinology 141 (2000) 1345–1355.
[30] P.R. Manna, M.T. Dyson, D.W. Eubank, B.J. Clark, E. Lalli, P. Sassone-Corsi, A.J. Zeleznik, D.M. Stocco, Regulation of steroidogenesis and the steroidogenic acute regulatory protein by a member of the cAMP response-element binding protein family, Mol. Endocrinol. 16 (2002) 184–199.
[31] A. Hirai, K. Tahara, Y. Tamura, H. Saito, T. Terano, S. Yoshida, Involvement of 5-lipoxygenase metabolites in ACTH-stimulated corticosteroidogenesis in rat adrenal glands, Prostaglandins 30 (1985) 749–767.