The Administration of Retinoic Acid Down-Regulates cAMP-Responsive Element Modulator (CREM) mRNA in Vitamin A-Deficient Testes

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Studies using genetics and vitamin A deficiency (VAD) have shown that vitamin A and retinoids play essential roles in spermatogenesis at the pre-meiotic stage. To understand the mechanisms of control in spermatogenesis by retinoic acid, we investigated whether retinoic acids regulate the expression of downstream transcription factors that are essential for spermatogenesis. In this study, we found that administration of all-trans retinoic acid (ATRA) or retinol to VAD rats down-regulates the testicular mRNA levels of the cAMP responsive element modulator (CREM), an essential transcription factor for spermatogenesis. Conversely, depletion of retinoids from the diet leads to an up-regulation of CREM expression in adult testes. In addition, RT-PCR analysis indicated that ATRA specifically represses the expression of the activator spliced variant of CREM (CREM/C28). These results suggest that retinoids function as a negative regulator of CREM expression in testes.

Key words: CREM; retinoic acid; spermatogenesis; testes; vitamin A deficiency

Spermatogenesis is a complex process of male germ cell development from spermatogonia to sperm that includes mitosis, meiosis, and differentiation. At each stage of spermatogenesis, stage-specific transcription factors are expressed that are thought to regulate the stage-specific expression of genes required for the determination of cell fate.1) Vitamin A has been implicated in a wide range of biological functions, such as reproduction, growth, differentiation, and vision.2–4) The all-trans- and 9-cis-isomers of retinoic acid (RA), the biologically active forms of vitamin A, are metabolized from retinol through dehydrogenization.5,6) These two RAs bind to specific nuclear receptors, RAR and RXR respectively. These complexes function as ligand-dependent transcription factors and regulate the transcription of target genes through binding to the retinoic acid responsive element (RARE).7–9)

Many reports have indicated that the retinoid signaling pathway is essential for spermatogenesis.10–13) Indeed, the depletion of retinol through a diet lacking it results in the arrest of spermatogenesis at the pre-meiotic stage.13–15) Conversely, the administration of retinol or ATRA to VAD rats quickly induces re-initiation of spermatogenesis.10,12,13,16–19) Additionally, both RAR and RXR have been shown to be expressed in male germ cells and testicular somatic cells.20–24) Mutant mice that show a disruption of the retinoic acid receptor (RARα, β, γ) gene have severe deficits in spermatogenesis.22,25) Similarly to the case of VAD, RARα knock-out mice also show an arrest in germ cell development at an early stage of spermatogenesis.25) These findings strongly suggest that the retinoid signaling pathway controls the progress of spermatogenesis by regulating target-gene expression through RAR and RXR.

In response to an increase in the intracellular concentration of cAMP and Ca2+, CREM regulates cAMP-responsive element (CRE)-mediated transcription through binding to CRE.26–28) Interestingly, the CREM gene generates both activators (CREMα, r1, r2) and repressors (CREMα, β, γ) by alternative splicing in both germ and somatic cells of the testes.29–33) More importantly, previous reports have found a developmental change in the expression pattern of CREM isoforms from repressor to activator during spermatogenesis.30,31) Repressor CREM isoforms are expressed at the pre-meiotic stage of spermatogenesis.30–32) In contrast, CREMα, an activator CREM isoform, is highly expressed in post-meiotic cells, activating the transcription of post-meiotic stage-specific genes, including RT7, protamine, and transition protein 1.30,34–36) In addition, mutant mice that carry a defective CREM gene show...
severe impairments in spermatogenesis, indicating that CREM is an essential transcription factor for spermatogenesis.

To examine the possibility that the retinoid signaling pathway regulates spermatogenesis by controlling the expression of downstream transcription factors, we examined testicular mRNA levels of CREM after the injection of retinoids into VAD rats. We found that the administration of retinoic acid into VAD rats down-regulates the expression of CREM mRNA in testes, suggesting that CREM is a candidate downstream transcription factor for retinoids in testes.

Methods

Vitamin A deficiency and administration of ATRA. Weaned male Wistar rats (3 weeks old) were depleted of retinoid by feeding them a retinol-deficient diet (VAD-diet), in which all other nutrients were at levels sufficient to support normal growth, for 38 d. The composition of the experimental diet was based on the AIN-76 diet. A control group was fed the same retinol-deficient diet, but were supplemented with 1.38 mg of dietary vitamin A/kg (supplied as retinyl acetate). Both the VAD and the retinoid-supplemented diet were freshly prepared weekly. The serum concentrations of total retinol and ATRA were determined by HPLC (Shimadzu, Japan) weekly. The serum concentrations of total retinol and when hepatic content was depleted to 2.5 mg/g of liver. An excess of either all-trans retinoic acid (ATRA; 500 μg/kg) or retinol (1 mg/kg) in 0.2 ml of soybean oil was given intragastrically to the VAD rats. Control rats were given 0.2 ml soybean oil alone. All animals were sacrificed at the indicated time for analyses. In another experiment (Fig. 5), adult male Wistar rats (3 months old) were fed either the retinol-deficient diet or the retinoid-supplemented one. All rats were treated in accordance with published NIH guidelines.

Northern blotting. Rat CREM1 cDNA and rat ATF-1 cDNA were obtained by reverse transcription (RT)-PCR using testis mRNA as a template. Because the C-terminal bZIP region in CREM1 has high sequence similarity with the corresponding region in CREB and ATF-1, we performed northern blotting analysis using the NcoI fragment encoding CREM1 (aa 3-152) or the BanHI–NcoI fragment encoding ATF-1 (aa 56-189) as a probe. This CREM probe hybridizes with all of the major CREM isoform mRNAs.

The rats were sacrificed by cervical dislocation. Total RNA was isolated using the acid guanidium thiocyanate/phenol/chloroform method. Poly(A)+ RNA was prepared using oligo(dT)-Latex (Oligotex-dT30; Roche, Mannheim, Germany). Poly(A)+ RNA (2 μg) was resolved by electrophoresis in 1.2% agarose gels containing 1.1 M formaldehyde and transferred onto nylon membranes (Gene Screen plus; NEM, Boston, MA). The blot was hybridized with DNA probes containing the CREM, ATF-1, or β-actin cDNA. These cDNAs were labeled with [35P]dCTP (Amersham, Piscataway, NJ) using random primers (Random primer DNA labeling kit; Takara, Japan). The membranes were prehybridized at 42 °C for 4 h in hybridization buffer containing 5 × SSPE (1 × SSPE = 0.15 M NaCl, 10 mM NaH2PO4, 1 mM EDTA, pH 7.0), 50% formamide, 0.2 mg of denatured salmon sperm DNA (ss DNA)/ml, and 1 × Denhardt’s solution. Following the pre-hybridization, the membranes were hybridized at 42 °C for 18 h in the above hybridization buffer containing 1 × 106 cpm/ml cDNA probe. The most stringent wash was performed at 65 °C in 1 × SSPE, 0.1% SDS, and 0.03% sodium pyrophosphate. The membranes were also rehybridized with a rat β-actin probe. The levels of CREM and ATF-1 mRNAs were normalized according to the β-actin signal. The intensity of the signal for each mRNA was measured using a Fuji Bio-image analyzer (BAS2000, Fuji-Film, Japan). The relative abundance of transcripts is indicated as the mean ± SD. for four animals (Figs. 2, 3 and 5).

Semi-quantitative RT-PCR. Testis poly(A)+ RNA (1 μg) was reverse-transcribed using oligo dT primers. PCR reactions were performed as previously described. The PCR products for specific rat CREM isoforms (CREMRα, 961 bp; CREMRβ, 773 bp; CREMRγ, 507 bp; CREMβ, 319 bp; and CREMY, 284 bp) were amplified using two primers (forward primer, TTTCCAGCGTCGACATTCTTTA, corresponding to exon 1; reverse primer, TTTTAGAGGAAAAATCAGA, corresponding to exon 5; reverse primer, GTTCTAGCTGACATTCTTTA, corresponding to exon 9b). PCR reactions were carried out for 17, 20, 23, 26, 29, and 32 cycles (one cycle was incubation at 94 °C for 1 min, at 56 °C for 1 min, and at 72 °C for 1 min). The PCR products were analyzed by southern blotting with the specific DNA probes for CREM or β-actin. The intensity of the signal was measured using a Fuji Bio-image analyzer. From comparison of the levels of PCR products after various cycles of amplification, the quantitative range of the PCR cycle number showing a linear amplification was determined (23–29 cycles for CREM mRNA and 20–26 cycles for β-actin mRNA). Finally, PCR reactions to examine CREM and β-actin mRNA levels were performed for 26 and 23 cycles respectively. The levels of CREM mRNA were normalized according to the signal for rat β-actin mRNA (forward primer, TCCG-TAAGACCTCCTATGCC, nucleotides 610-629; reverse primer, ACACAGAGTCATTGCCTCA, nucleotides 899-908 of the β-actin mRNA).
Results

Serum ATRA concentration in VAD rats

We generated VAD-rats using the standard protocol, as described in “Materials and Methods”. Three weeks old weaned male rats were depleted of retinoid by feeding them for 38 d with a VAD diet. Figure 1 shows the comparison of the serum ATRA concentration between VAD- and control rats (8 weeks old), and the time–course effects of ATRA administration to VAD rats on serum RA concentrations. ATRA was not detected in the serum of VAD rats following the depletion of retinol by feeding a VAD-diet for 38 d. In response to the administration of ATRA (500 mg/kg) or retinol (1 mg/kg), serum ATRA concentrations in VAD rats increased and reached a maximum at 4 h.

Expression levels of transcription factors in the testes of VAD rats

To examine whether retinoids regulate the expression of downstream transcription factors that control spermatogenesis, we examined the testicular mRNA levels of CREM and ATF-1 using northern blotting after the administration of ATRA (500 μg/kg) into VAD-rats for 4 h. As Fig. 2 indicates, testicular CREM mRNA levels in VAD rats decreased significantly after ATRA administration. In contrast, the mRNA levels of ATF-1, a CREB/CREM/ATF family member, were not influenced by ATRA administration. These results indicate that the administration of ATRA into VAD rats decreased CREM mRNA levels in the testes.

Time course analysis of CREM mRNA levels in VAD-testes after administration of retinoids

To examine further the expression regulation of CREM mRNA by ATRA, we monitored the expression levels of CREM mRNA in VAD-testes after administration of retinol or ATRA. Consistent with the results shown in Fig. 2, CREM mRNA levels were significantly decreased after administration of ATRA, and reached a minimum at 4–8 h. In addition, CREM mRNA levels also gradually decreased and reached a minimum at 16 h following retinol administration. This difference in the time required to reach a minimum CREM mRNA level between ATRA and retinol administration is thought to be due to enzymatic conversion from retinol to RA. Nevertheless, these results strongly indicate that CREM mRNA levels are down-regulated in VAD-testes by the retinoid signaling pathway, especially ATRA.

In the experiments shown in Figs. 1–3, prepubertal rats (3 weeks old) were fed a VAD-diet. This treatment is known to result in the arrest of spermatogenesis at the pre-meiotic stage. Therefore, VAD-testes are thought to be immature and lack post-meiotic germ cells, which express high levels of the activator isoform CREM. As above Fig. 3 shows that the expression level of CREM mRNA in VAD testes was much lower than that in normal adult testes (8 weeks old), which

![Fig. 1. Serum ATRA Concentration of VAD Rats after Oral Administration of ATRA or Retinol.](image1)

The serum concentrations of ATRA were measured (n = 4 animals/group) at 0, 2, 4, and 8 h after administration of retinol or ATRA to VAD rats. Control rats (C) were fed the same VAD diet supplemented with 1.38 mg of dietary vitamin A/kg, and did not receive administration of retinoids. ND, not detected.

![Fig. 2. Effects of Administration of ATRA on CREM and ATF-1 mRNA Levels in VAD Testes.](image2)

The left panels show representative Northern blotting autoradiograms of CREM, ATF-1, and β-actin mRNAs in VAD testes at 4 h after oral administration of ATRA or vehicle to VAD rats. In the right panels, CREM or ATF-1 mRNA levels are expressed as a ratio of vehicle group values, and indicate the means ±/− SD obtained from 4 animals per group. The level of CREM and ATF-1 mRNA was normalized according to the β-actin signal. *p < 0.05 compared to control group.

![Fig. 3. Time–Course Effects of Administration of ATRA or Retinol on CREM mRNA Levels in VAD Testes.](image3)

CREM mRNA levels are expressed as the ratio of values obtained from VAD testes immediately after the administration of ATRA (0h), and indicate the means ±/− SD obtained from 4 animals per group. The left panel also shows the level of testicular CREM mRNA in the control group (C). The level of CREM mRNA was normalized according to the β-actin signal. *p < 0.05 compared to 0h group.
contain all types of male germ cells. From previous findings, this significant difference in CREM mRNA levels between VAD-testes and normal testes might be caused by a lack of post-meiotic germ cells in VAD testes, rather than by expression regulation of the CREM gene by retinoids.

Expression analysis of CREM isoform mRNAs in VAD testes after administration of retinoids

Since the CREM gene encodes multiple CREM isoforms, including both activators and repressors, we examined which mRNA level of CREM isoforms was regulated in VAD testes by ATRA. To compare the expression ratio of multiple CREM isoforms in VAD testes before and after administration of ATRA, we performed a semi-quantitative RT-PCR using two primers that enable discrimination of various isoforms, including both activators and repressors, we examined which mRNA level of CREM isoforms was regulated in VAD testes by ATRA. To compare the expression ratio of multiple CREM isoforms in VAD testes before and after administration of ATRA, we performed a semi-quantitative RT-PCR using two primers that enable discrimination of various isoforms, including both activators and repressors, we examined which mRNA level of CREM isoforms was regulated in VAD testes by ATRA. To compare the expression ratio of multiple CREM isoforms in VAD testes before and after administration of ATRA, we performed a semi-quantitative RT-PCR using two primers that enable discrimination of various isoforms, including both activators and repressors. Figure 4 shows the expression levels of CREM mRNA were much higher than that of the other isoforms in VAD testes, including CREMα,β,γ. Moreover, ATRA-administration down-regulates the expression levels of CREM mRNA in VAD-testes. But the expression levels of other CREM isoforms were not altered, even after ATRA administration. Thus our results indicate that CREM mRNA levels are specifically decreased by ATRA-administration in VAD-testes.

Time course effect of retinol depletion on CREM mRNA levels in adult testes

Inducing severe VAD by feeding adult rats a VAD-diet is difficult, and hence we used juvenile rats (3 weeks old) in our previous experiment (Figs. 1–4). But we can examine the effects of retinol depletion from the diet on CREM mRNA levels in adult testes (3 month-old rats), which contain all types of germ cells. We examined the expression levels of testicular CREM mRNA in adult rats that were fed the retinol deficient diet. Figure 5 shows that CREM mRNA levels gradually increased after depletion of retinol from the diet, suggesting that depletion of retinol leads to up-regulation of testicular CREM mRNA level in adult testes. These results strongly support our previous observation that the retinoids down-regulate the expression level of CREM mRNA in VAD testes (Figs. 2–4). Interestingly, CREM mRNA levels were decreased at 48 d after feeding a retinol deficient diet (data not shown). As discussed above, this decrease in CREM mRNA expression is thought to be due to the disappearance of post-meiotic cells, but not to the direct effects of retinol depletion.

Discussion

In this study, we found that the expression level of CREM mRNA decreases in VAD testes after administration of ATRA. More importantly, RT-PCR analysis indicated that the expression of CREMα mRNA is specifically decreased in VAD testes by ATRA-administration. Furthermore, the depletion of retinol from the diet increased the CREM mRNA levels in adult testes. These results indicate that the retinoid signaling pathway down-regulates CREMα mRNA levels in testes, suggesting that CREM might be a downstream transcription factor of the retinoid signaling pathway in testes.

VAD causes inhibition of spermatogenesis, but the administration of retinoid to VAD rats quickly re-initiates spermatogenesis. These previous findings suggest that the decrease in expression levels of CREM mRNA after administration of ATRA coincides with re-initiation of spermatogenesis. Hence regulation of expression of CREM mRNA by retinoids might be a key process to control or re-initiate spermatogenesis.

VAD testes contain pre-meiotic germ cells and somatic testicular cells but not post-meiotic cells because VAD leads to the arrest of spermatogenesis at the pre-meiotic stage. Previous studies have found a developmental switch of expression of CREM
isoforms from repressor to activator during spermatogenesis.\(^{30,31}\) Moreover, repressor CREM isoforms (CREM\(\alpha,\beta,\gamma\)) are dominantly expressed in pre-meiotic male germ cells and testicular somatic cells, whereas the activator isoform, CREMr, is expressed at high levels only at the post meiotic stage.\(^{30,31}\) Judging by these previous observations, the down-regulation of CREMr mRNAs in VAD testes by ATRA might occur in pre-meiotic cells and/or testicular somatic cells, but not in post-meiotic cells. Therefore, ATRA might regulate CREMr mRNA expression to recover from abnormal to normal levels of expression in VAD testes because CREMr is not a major isoform in pre-meiotic and testicular somatic cells. Furthermore, previous findings that activator CREM isoforms activate the transcription of post-meiotic stage-specific genes\(^{30,34-36}\) raise the possibility that abnormal expression of CREMr in VAD testes indicates inhibitory effects on spermatogenesis and/or testes development. The repression of CREMr expression by retinoids might be necessary to maintain a constant expression level in normal testes, leading to normal spermatogenesis and/or testes development.

As described above, CREM isoforms are expressed in both germ and somatic cells of testes.\(^{30,31}\) In addition, previous studies have found that RARs and RXRs are also expressed in both types of cells.\(^{20-24}\) To clarify further the significance of expression regulation of CREM by retinoids, identification of cells in which retinoids regulate CREM gene expression using in situ hybridization or immunocytochemistry is required.

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