FULL PAPER *Anatomy*

Decrease of Pituitary Adenylate Cyclase Activating Polypeptide and Its Type I Receptor mRNAs in Rat Testes by Ethanol Exposure

Phil-Ok KOH[1)]* and Chung-Kil WON[1)]

[1)]*Department of Anatomy, College of Veterinary Medicine, Institute of Agriculture and Life Sciences, Gyeongsang National University, 900 Gajwa-dong, Jinju 660–701, South Korea*

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ABSTRACT. The present study was designed to evaluate the effect of ethanol on pituitary adenylate cyclase activating polypeptide (PACAP) and its typ I (PAC_1) receptor expression in adult rat testes. Ethanol (3 g/kg i.p., 15% v/v in saline) was administrated to adult male rats for 10 days. Using northern blot analysis, the present study showed the reduction of PACAP mRNA levels in rat testes by ethanol administration. Also, ethanol decreased the expression level of PAC_1 receptor in testes. In particular, *in situ* hybridization clearly showed the decrease of PAC_1 receptor mRNA expression in Leydig cells, which produce testosterone. Furthermore, the serum level of testosterone was significantly decreased in the ethanol-treated group. In conclusion, our findings suggest that the decrease of PACAP and PAC_1 receptor expression in rat testes by ethanol exposure may partly contribute to the suppression of male reproductive activity.
KEY WORDS: ethanol, PACAP, testes.

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It is known that ethanol suppresses reproductive function and sexual behavior in laboratory animals and humans [1, 7]. In particular, ethanol decreases male reproductive ability through the reduction of testosterone production by inhibition of luteinizing hormone (LH) release from pituitary gland [23, 25]. In males, LH stimulates testosterone production through cAMP activation in testicular Leydig cells. Besides LH, growth hormone is reported to activate steroidogenic related enzymes and stimulate steroidogenesis in testicular Leydig cells [13]. Pituitary adenylate cyclase activating polypeptide (PACAP), like LH and growth hormone, is known to be associated with steroidogenesis in various cells, including ovarian granulosa cells, corpus luteal cells, and testicular Leydig cells [3, 17, 22].

PACAP was originally isolated from ovine hypothalamus and was known to stimulate the production of cAMP in anterior pituitary cells [18]. It has considerable homology with secretin, glucagon, vasoactive intestinal peptide (VIP), and growth hormone releasing hormone, and exists in two active forms, PACAP 38 and PACAP 27, which share the same N-terminal 27 amino acids [18, 19]. PACAP binds to three types of receptors: PAC_1, VIP1, and VIP2. Among these receptors, PAC_1 has a high affinity for PACAP 38 and PACAP 27, very low affinity with VIP [12]. But, VIP1 and VIP2 receptors have an approximately equal affinity for PACAP 38, PACAP 27, and VIP [24]. PACAP and its receptors have been found in various tissues, including brain, adrenal gland, testis, and ovary [4, 10, 15]. The highest level of PACAP was found in adult testes, and the amount of PACAP in two testes exceeds that of the whole brain [4]. Also, it is known that PACAP stimulates testosterone secretion through the PAC_1 receptor in isolated adult rat Leydig cells [21, 22]. Thus, we propose that ethanol suppresses male reproductive ability through the down-regulation of PACAP and PAC1 receptor in rat testes. To date, however, the effect of ethanol on PACAP and PAC_1 receptor expression in rat testes has not been elucidated. Therefore, the present study was performed to provide this information.

* CORRESPONDENCE TO: KOH, P.O., Department of Anatomy, College of Veterinary Medicine, Gyeongsang National University, Jinju 660–701, South Korea.

MATERIALS AND METHODS

Animals and tissue preparation: Male Sprague-Dawley rats (250–300 g, *n*=20) were purchased from Samtako Co. (Animal Breeding Center, Korea) and were randomly divided into two groups, saline-treated group and ethanol-treated group (*n*=10 per group). Animals were maintained under controlled temperature (25°C) and lighting (14/10 light/dark cycle), and were allowed to have free access to food and water. Ethanol (3 g/kg, 15% v/v in saline) was administrated daily (08:00–09:00 hr) intraperitoneally for 10 days, and the same volume of saline was administrated for the controls. The dose of ethanol was determined by review of the effect of i.p. injected ethanol on increment of blood alcohol levels in rats [20]. On the day after the last injection, rats (*n*=5 per group) were decapitated (09:00 hr), and trunk blood was collected to determine serum testosterone concentrations. Testes were removed quickly and frozen in liquid nitrogen for northern blot analysis and RT-PCR. For *in situ* hybridization studies, animals (*n*=5 per group) were perfused with 4% paraformaldehyde in 0.1 M phosphate buffered saline (PBS) through the left cardiac ventricle. Sections in 15 μm thickness were prepared on the probe-on plus-charged slides (Fisher Scientific, Pittsburgh, PA, U.S.A.), and stored at –70°C until use.

Northern blot analysis: Total RNA from tissues was extracted by the acid guanidium thiocyanate phenol chloro-

form method [5]. Thirty micrograms of total RNA was denatured, electrophoresed in a 1.2% agarose denaturing gel, and transferred to nylon membranes (Nytran, pore size: 0.45 μm; Schleicher & Schuell, Keene, NH, U.S.A.). The membrane was prehybridized with gentle shaking at 42°C for 2 hr in a buffer containing 50% deionized formamide, 5 × Denhardt's solution, 5 × sodium chloride/sodium phosphate/EDTA buffer (SSPE), and 0.1% SDS. The ^{32}P-UTP labeled PACAP probe (rat PACAP$^{971–1171}$) was prepared using *in vitro* transcription kit (Promega, Madison, WI, U.S.A.) [15]. The labeled probe was separated by a Sephadex G-50 nick column (Pharmacia Biotech, Uppsala, Sweden), added to the hybridization buffer, and hybridized at 60°C for 24 hr. The membrane was washed and exposed with X-ray films at –70°C for 2 days. The intensity analysis was carried out using SigmaGel 1.0 (Jandel Scientific, San Rafael, CA, U.S.A.).

Reverse-transcription PCR analysis: Complementary DNAs were prepared from 500 *n*g of total RNA using oligo(dT) primers and Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI, U.S.A.). The reaction components were incubated at 22°C for 10 min and at 37°C for 70 min, heated to 95°C for 5 min, and flash cooled to 4°C. The primers used for the amplication of the PAC_1 receptor were an upstream primer (5'-GCAGGATGCTGGGATATGAATG-3') and a downstream primer (5'-AGAGTAATGGTGGATAGTTCTG-3'). The RT product samples were subjected to 30 cycles of amplification in a Perkin-Elmer PCR Thermal Cycler (Perkin Elmer, Boston, MA, U.S.A.) with denaturation at 94°C for 30 sec, primer annealing at 63°C for 30 sec, and primer extension at 72°C for 15 min. PCR products were electrophoresed in a 1.5% agarose gel and were stained with ethidium bromide and photographed. β-actin was used as an internal control for procedual variation. For quantification, the intensity of PCR bands was measured densitometrically and analyzed using SigmaGel 1.0 (Jandel Scientific, San Rafael, CA, U.S.A.) and SigmaPlot 4.0 (SPSS Inc., Chicago, IL, U.S.A.).

In situ hybridization histochemistry: All solutions were made with sterile water, and glassware was autoclaved to prevent contamination by RNase. *In situ* hybridization histochemistry was carried out as described by Angerer *et al.* [2]. Tissue sections were treated with Proteinase K and an acetylation solution. Subsequently, tissue sections were covered with prehybridization buffer containing 50% formamide and incubated at 37°C for 1 hr. Hybridization with the ^{35}S-labeled PAC_1 probe (5 × 10^5 cpm/slide) was performed at 60°C for 24 hr. 35S-UTP labeled PAC_1 probe (rat PAC$^{1172–422}$) was prepared using *in vitro* transcription kit (Promega, Madison, WI, U.S.A.) [16]. The labeled probe was separated by a Sephadex G-50 nick column (Pharmacia Biotech, Uppsala, Sweden) and eluted with SET buffer containing 0.1% SDS, 1 mM EDTA, 10 mM Tris, and 10 mM DTT. Tissue slides were posthybridized in a posthybridization buffer. Tissue slides were treated with RNase A (20 μg/m*l*), washed, and dehydrated in an ascending alcohol series. The slides were dipped into NTB2 emulsion (Eastman Kodak Co., Rochester, NY, U.S.A.), exposed at 4°C for 2 weeks, developed in Kodak D19 developer, and counterstained with hematoxylin. The slides were observed under a dark and a bright field microscope, and then photographed.

Radioimmunoassay: Testosterone levels were assayed according to those described by Fortune [8]. [2, 4, 6, 7–^{3}H] testosterone (98 Ci/mmol) was obtained from Amersham Biosciences (Buckinghamshire, U.K.). The antiserum of the testosterone was evaluated and cross-reacted 14.0% with 5α- dihydrotestosterone, 6.0% with 5α-androstenediol, 0.8% with androstenedione, and less than 0.01% with other steroids. Intra- and inter-assay coefficients of variation were 7.4 and 9.4%, respectively.

Data analysis: All data are expressed as mean ± S.E.M. The results in each group were compared by one-way analysis of variance (ANOVA) followed by Student's *t* test. The difference for comparison was considered significant at * $P<0.05$.

RESULTS

Northern blot analysis of PACAP mRNA in testis: Ethanol administration significantly decreased the level of PACAP mRNA in rat testes (Fig. 1). PACAP transcripts appeared to have a size of 1.2 kb in rat testes. Ethanol exposure reduced the level of PACAP by 47.7%, compared with saline-treated group. The levels of PACAP mRNA were 3.52 ± 0.29 and 2.25 ± 0.15 in saline-treated and ethanol-treated animals, respectively.

Inhibitory effects of ethanol treatment on PAC_1 receptor mRNA expressions in Leydig cells: RT-PCR analysis showed the decrease of PAC_1 receptor expression by ethanol treatment (Fig. 2). The expression level of PAC_1 receptor was decreased by 42.3% in testes of the ethanol-treated group, compared to that of the control group. *In situ* hybridization histochemistry revealed the distribution of PAC_1 receptor mRNA in Leydig cells (Fig. 3A and 3B). Also, we observed that the positive cells decreased in Leydig cells of ethanol-treated animals (Fig. 3C and 3D). There were no detectable signals in the negative control with a sense probe (Fig. 3E).

Inhibitory effect of ethanol exposure on serum testosterone levels: We evaluated serum testosterone levels to confirm the effect of ethanol on male reproductive hormone release. The serum levels of testosterone were markedly decreased by ethanol treatment (Fig. 4). The mean levels were 154.2 ± 4.3 and 90.3 ± 5.8 in saline-treated and ethanol-treated animals, respectively.

DISCUSSION

PACAP stimulates cAMP production and steroidogenesis through the PAC_1 receptor, which is coupled to adenylate cyclase and phospholipase C pathways in various steroidogenic cells [12, 17, 24]. In particular, Rossato *et al.* [22] clearly demonstrated that PACAP stimulates testoster-

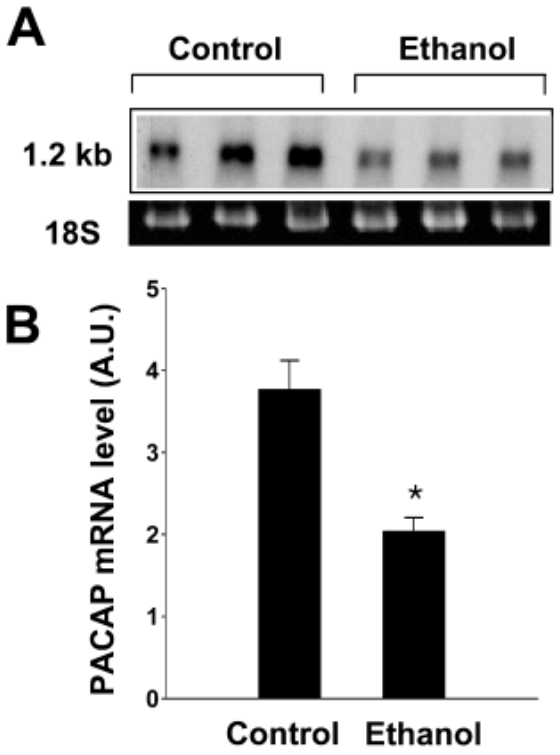

Fig. 1. Northern blot analysis of PACAP mRNA in the rat testis of saline- (Control) and ethanol-treated animals (Ethanol). A: Hybridization was performed with a ^{32}P-labeled PACAP probe. The lower panel shows the 18S ribosomal RNA on an agarose gel stained with ethidium bromide. Each lane represents an individual experimental animal. B: Densitometric analysis of PACAP mRNA levels is represented as an arbitrary unit (A.U.) that was normalized by 18S RNA. Represented data: mean ± S.E.M. (n=5). * P<0.05 (vs. control)

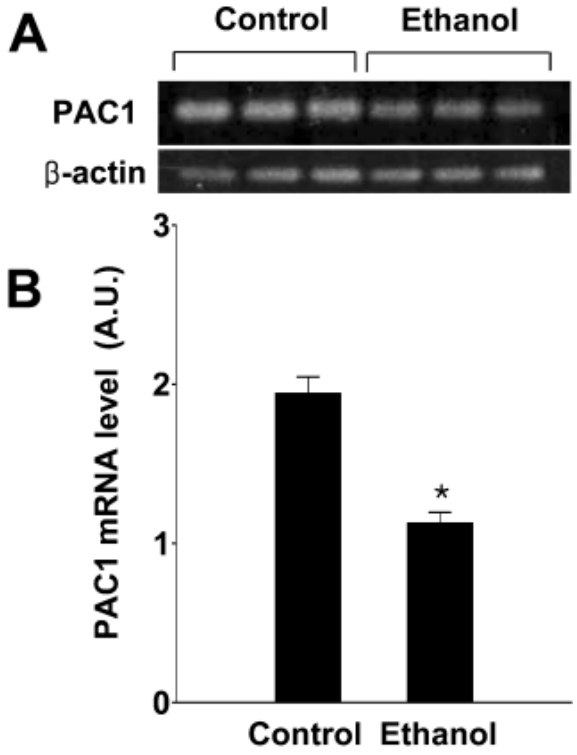

Fig. 2. RT-PCR analysis of PAC_1 receptor in the rat testis of saline- (Control) and ethanol-treated animals (Ethanol). A: The expression of PAC_1 receptor is significantly decreased in testes of the ethanol-treated group, compared to those of the saline-treated group. Each lane represents an individual experimental animal. B: Densitometric analysis of PAC_1 receptor mRNA levels is represented as an arbitrary unit (A.U.) that was normalized to β-actin. Represented data: mean ± S.E.M. (n=5). * P<0.05 (vs. control).

one secretion in isolated adult Leydig cells through the PAC_1 receptor. In this study, northern blot analysis showed a single band of PACAP transcript in rat testis. A 1.2 kb PACAP transcript has been previously detected in rat testis [11, 15]. Our result coincided well with previous studies. Also, this study showed that ethanol treatment significantly decreased the levels of PACAP mRNA in rat testes. Moreover, RT-PCR analysis and *in situ* hybridization showed that ethanol exposure significantly decreased the levels of PAC_1 receptor mRNA in testes. Until now, it was not reported the localization of PAC_1 receptor in Leydig cells by *in situ* hybridization technique. However, previous studies clearly demonstrated the existence of PAC_1 receptor in Leydig cells [21, 22]. In this study, we confirmed the presences of PAC_1 receptor in Leydig cells by *in situ* hybridization. Leydig cells are well-known as the primary cells for the production of testosterone. In our study, ethanol administration markedly decreased the expression levels of PACAP and PAC_1 receptor mRNAs in adult rat testes. Also, we confirmed that ethanol exposure significantly reduced serum testosterone levels in adult male rats. Previous studies demonstrated that ethanol suppresses male reproductive activity through the inhibition of testosterone release by reduction of LH secretion from the pituitary gland [23, 25]. Also, it has been shown that the reduction of LH levels by ethanol decreases testosterone release through the inhibition of steroidogenic acute regulatory protein expression in testes [14]. This study demonstrated that PACAP, such as LH, mediated down-regulation of testosterone by ethanol exposure. Thus, we suggest that LH receptor and PAC_1 receptor pathways mediate down-regulation of testosterone production in rat testis by ethanol.

Chronic ethanol exposure for 30 days has been shown to cause testicular atrophy and accessory reproductive organ dysfunction in adult male rats [6]. However, in this study, the testicular weight was 1.25 ± 0.15 g and 1.18 ± 0.17 g, in saline- and ethanol-treated rats. Our study did not demonstrate the significant change of testicular weight and the severe morphological defect in ethanol-treated rats. We consider that ethanol treatment for 10 days is insufficient for the testicular atrophy and the morphological defect. However, our previous study demonstrated that the degenerating

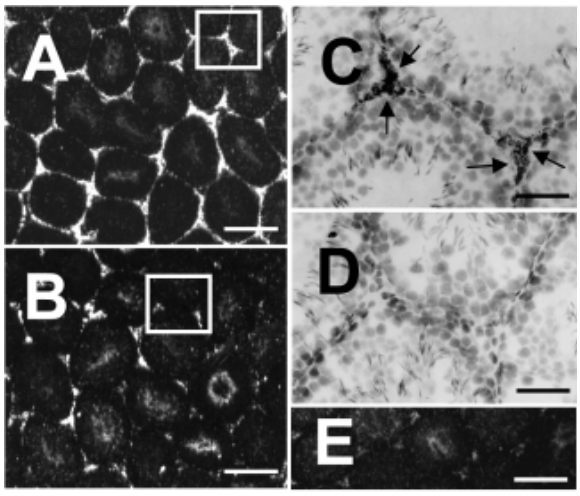

Fig. 3. Localization of PAC_1 receptor mRNA in Leydig cells of the rat testis by *in situ* hybridization. Dark-field (A, B, and E) and bright-field (C and D) photomicrographs show positive signals of PAC_1 receptor mRNA in Leydig cells of saline (A and C) and ethanol-treated groups (B and D). Bright field photomicrographs appear as the magnification of square areas in dark field. Arrows indicate positive signals. No positive signals were detected in the negative controls with a sense probe (E). Scale bars: A , B, and E,100 μm; C and D, 25 μm.

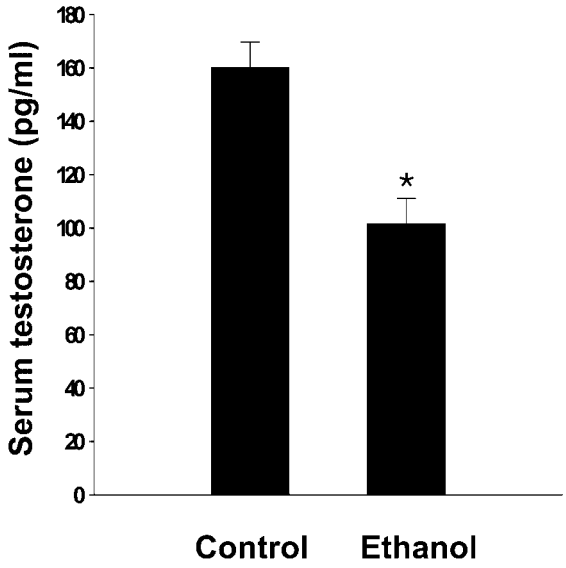

Fig. 4. Serum testosterone level in the rat testis of saline-(Control) and ethanol-treated animals (Ethanol). Trunk blood was collected for testosterone determination by radio-immunoassay. Each bar represents the mean ± S.E.M (n=5) serum testosterone level. * P<0.05 (vs. control).

germ cells increased in ethanol-treated animals, compared to that of saline-treated animals (unpublished data). Increased degenerating germ cells indicate the morphological defect including spermatogenesis impairment.

This study clearly showed that ethanol administration decreased the levels of PACAP and PAC_1 receptor in adult rat testes. Also, we confirmed the reduction of the testosterone release by ethanol exposure. However, this study did not directly show that how the decrease of PACAP and PAC_1 receptor expression contributes to the reduction of testosterone release in testicular Leydig cells. Nevertheless, previous studies provide strong supporting evidence that PACAP stimulates testosterone production through PAC_1 receptor in testicular Leydig cells [21, 22]. Therefore, we suggest that ethanol administration significantly decreases the levels of PACAP and PAC_1 receptor in testes, and the low expression levels of these genes contributes to the reduction of testosterone release.

In conclusion, our findings suggest that ethanol suppresses male reproductive activity, at least in part, through the decrease of PACAP and PAC_1 receptor expression in adult rat testes.

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