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Glucocorticoids, master modulators of the thymic catecholaminergic system?

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Abstract

There is evidence that the major mediators of stress, i.e., catecholamines and glucocorticoids, play an important role in modulating thymopoiesis and consequently immune responses. Furthermore, there are data suggesting that glucocorticoids influence catecholamine action. Therefore, to assess the putative relevance of glucocorticoid-catecholamine interplay in the modulation of thymopoiesis we analyzed thymocyte differentiation/maturation in non-adrenalectomized and adrenalectomized rats subjected to treatment with propranolol ($0.4 \text{ mg} \cdot 100 \text{ g body weight}^{-1} \cdot \text{day}^{-1}$) for 4 days. The effects of β -adrenoceptor blockade on thymopoiesis in non-adrenalectomized rats differed not only quantitatively but also qualitatively from those in adrenalectomized rats. In adrenalectomized rats, besides a more efficient thymopoiesis [judged by a more pronounced increase in the relative proportion of the most mature single-positive $\text{TCR}\alpha\beta^{\text{high}}$ thymocytes as revealed by two-way ANOVA; for $\text{CD4}^+\text{CD8}^-$ $F(1,20) = 10.92$, $P < 0.01$; for $\text{CD4}^+\text{CD8}^+$ $F(1,20) = 7.47$, $P < 0.05$], a skewed thymocyte maturation towards the $\text{CD4}^+\text{CD8}^+$ phenotype, and consequently a diminished $\text{CD4}^+\text{CD8}^-/\text{CD4}^+\text{CD8}^+$ mature $\text{TCR}\alpha\beta^{\text{high}}$ thymocyte ratio (3.41 ± 0.21 in non-adrenalectomized rats vs 2.90 ± 0.31 in adrenalectomized rats, $P < 0.05$) were found. Therefore, we assumed that catecholaminergic modulation of thymopoiesis exhibits a substantial degree of glucocorticoid-dependent plasticity. Given that glucocorticoids, apart from catecholamine synthesis, influence adrenoceptor expression, we also hypothesized that the lack of adrenal glucocorticoids affected not only β -adrenoceptor- but also α -adrenoceptor-mediated modulation of thymopoiesis.

Key words: Catecholamines; Glucocorticoids; β -adrenoceptors; Propranolol; Thymocyte apoptosis; Thymocyte proliferation; Thymocyte differentiation

Introduction

It is well known that stress causes profound involution of the thymus gland (1). Increased secretion of catecholamines (CAs) and glucocorticoids (GCs), the end-products of the sympatho-adrenal and hypothalamo-pituitary-adrenal (HPA) axes, respectively, is the major trademark of the prototypical stress response (2,3).

Ample evidence from denervation and pharmacological studies (4-6) has implicated CAs in the fine-tuning of thymopoiesis (reviewed in Ref. 7). There is strong evidence that CAs exert thymus-dependent immunomodulatory effects via β -adrenoceptors (β -ARs) (reviewed in Refs. 7,8). However, there are data indicating that some of the effects of CAs on the thymus may be mediated via α -ARs (5,9).

It has been demonstrated that both thymic lymphoid and non-lymphoid cells express glucocorticoid receptors

(GRs) (10,11). Moreover, GCs have been implicated in the modulation of thymocyte development (12).

The sympatho-adrenal and HPA axes often act in concert, with the signaling pathways of CAs and GCs being interwoven (2). Furthermore, GCs stimulate: i) CA biosynthesis and release (13), ii) β -AR gene expression and the receptor downstream intracellular signaling (14), and iii) α_1 - and α_2 -AR gene expression (15,16). On the other hand, noradrenaline affects GR expression in a receptor-dependent manner (17).

On the basis of the above considerations, we hypothesized that CA modulation of thymopoiesis depends at least in part on circulating GC levels. To test this hypothesis we analyzed thymocyte differentiation/maturation in the absence and in the presence of the main source of

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endogenous GCs following a 4-day long β -AR blockade with propranolol.

Material and Methods

Animals

Adult male Dark Agouti rats bred at the Immunology Research Centre "Branislav Janković" in Belgrade were used. Animals were housed under standard laboratory conditions with free access to food pellets and tap water.

Chemicals and immunoconjugates

All monoclonal antibodies (mAbs): fluorescein-isothiocyanate (FITC)-conjugated anti-CD4 (clone W3/25), phycoerythrin (PE)-conjugated anti-CD8 (clone MRC OX-8) and peridinin chlorophyll protein (PerCP)-conjugated anti-TCR $\alpha\beta$ (clone R73) were purchased from Serotec (UK). The appropriate IgG isotype-matched controls were obtained from BD Biosciences Pharmingen, USA.

Experimental protocol

Circulating glucocorticoids were removed by bilateral adrenalectomy. Non-adrenalectomized (non-Adx) and adrenalectomized (Adx) rats were treated with propranolol hydrochloride (0.4 mg·100 g body weight⁻¹·day⁻¹, sc; Sigma Aldrich GmbH, Germany) or saline for 4 consecutive days.

Rats were bilaterally Adx *via* a single dorsal incision under sodium phenobarbitone anesthesia (Nembutal, 40 mg/kg, *ip*; Serva, Germany) and subjected to propranolol or saline treatment from day 4 post-adrenalectomy. All Adx animals were given 0.9% saline to drink. To avoid changes in corticosterone levels due to diurnal variation the rats were sacrificed between 9:00 and 10:00 am. All experimental procedures followed the principles of the European Community's Council Directive of November 24, 1986 (86/609/EEC) and were approved by the Immunology Research Center "Branislav Janković" Institutional Committee for Animal Care and Use.

Thymic cell suspensions

The thymi were removed, dissected free of parathyroid lymph nodes and adherent membranous tissue and weighed. Cell suspensions were prepared by grinding the thymic tissue between the frosted ends of microscope slides and passing the ensuing suspensions through a fine nylon mesh. The single-cell suspensions were washed in ice-cold PBS containing 2% fetal calf serum (Gibco, USA) and 0.09% sodium azide (Sigma Aldrich; PS medium) and counted using an improved Neubauer hemocytometer.

Flow cytometry analysis

Analysis of CD4/CD8/TCR $\alpha\beta$ expression. Aliquots of 1×10^6 thymocytes were incubated simultaneously with

FITC-conjugated anti-CD4, PE-conjugated anti-CD8 and PerCP-conjugated anti-TCR $\alpha\beta$ mAbs for 30 min on ice in the dark. Thymocytes were then washed with PS medium, fixed in 0.5 mL 1% paraformaldehyde (Sigma Aldrich) in PBS and kept at 4°C until analysis. All samples were analyzed with a FACScan flow cytometer (Becton Dickinson Immunocytometry Systems, USA). Non-specific IgG isotype-matched controls were used for each fluorochrome type to define background staining, while dead cells and debris were excluded from analysis by selective anterior and right-angle scatter gating. Twenty thousand cells per sample were analyzed using the CellQuest Software (Becton Dickinson).

Cell cycle analysis. Briefly, 10^6 - 10^7 thymocytes in 0.4 mL PBS were fixed by dropwise addition of 0.4 mL ice-cold absolute ethanol for 30 min, on ice. The cells were then centrifuged at 700 *g* for 5 min and 0.5 mL 1 mg/mL RNase I-A (Sigma Aldrich) in PBS was added to the pellet. The samples were incubated at 37°C for 20 min. After incubation, 0.5 mL 40 μ g/mL propidium iodide (Sigma Aldrich) in PBS was added, and the samples were incubated for the next 10 min at room temperature in the dark. Finally, the samples were passed through a fine nylon mesh and analyzed with a FACScan flow cytometer.

Analysis of apoptotic cells. The cell susceptibility to apoptotic signals was analyzed in freshly isolated thymocytes stained with merocyanine (MC) 540 (Sigma Aldrich) as described previously (6). Briefly, a 1 mg/mL stock solution of MC540 in twice-distilled water was filtered through a 0.22- μ m filter and 5 μ L MC540 solution was added to 10^6 - 10^7 thymocytes before FACS analysis. Samples were analyzed with a FACScan flow cytometer.

Statistical analysis

Data are reported as means \pm SEM. Statistical significance of the differences in propranolol-induced thymic effects between non-Adx and Adx rats was determined by two-way ANOVA followed by the Bonferroni test for *post hoc* comparisons using the GraphPad Prism 5.0 software (GraphPad Software, Inc., USA). Differences were considered to be significant when $P < 0.05$.

Results

Differential effect of propranolol on thymus weight and thymocyte yield in non-Adx and Adx rats

A 4-day propranolol treatment produced differential effects on thymus weight [adrenalectomy x propranolol interaction, $F(1,20) = 4.281$, $P < 0.05$] and thymocyte yield [adrenalectomy x propranolol interaction, $F(1,20) = 4.279$, $P < 0.05$] in non-Adx and Adx rats. Blockade of β -ARs affected neither thymus weight nor thymocyte yield in non-Adx rats, while it ($P < 0.05$) reduced the values of these parameters in Adx rats compared to the corresponding saline-injected controls (Table 1).

Differential effect of propranolol on thymocyte proliferative index in non-Adx and Adx rats

There was a significant [adrenalectomy x propranolol interaction, $F(1,20) = 4.479$, $P < 0.05$] difference in the effects of propranolol on the percentage of proliferating thymocytes (cells in the S/G2M phases of the cell cycle) in non-Adx and Adx rats. Namely, propranolol ($P < 0.05$) affected thymocyte proliferation in non-Adx rats, causing an increase in the percentage of proliferating cells, while it had no effect in Adx rats (Table 1).

Propranolol affected thymocyte apoptosis in Adx and non-Adx rats

Two-way ANOVA showed a significant effect of propranolol on thymocyte apoptosis, but no significant propranolol x adrenalectomy interaction [$F(1,20) = 3.486$, $P > 0.05$]. Namely, irrespective of the presence of adrenal glands, propranolol ($P < 0.01$) increased the percentage of apoptotic thymocytes (Table 1).

Differential effect of propranolol on the distribution of thymocyte subsets as determined by CD4/CD8/TCR $\alpha\beta$ expression in Adx and non-Adx rats

As previously shown (7), twelve thymocyte subsets at distinct developmental stages were distinguished by plotting CD4 vs CD8 within each thymocyte population as determined by TCR $\alpha\beta$ surface density (TCR $\alpha\beta^{\text{high}}$, TCR $\alpha\beta^{\text{low}}$ and TCR $\alpha\beta^-$).

TCR $\alpha\beta^-$ thymocytes. Regarding the relative proportions of all TCR $\alpha\beta^-$ thymocyte subsets, two-way ANOVA revealed a significant propranolol x adrenalectomy interaction [for CD4 $^-$ CD8 $^-$, $F(1,20) = 6.67$, $P < 0.05$; for CD4 $^+$ CD8 $^+$, $F(1,20) = 6.42$, $P < 0.05$; for CD4 $^+$ CD8 $^-$, $F(1,20) = 35.73$, $P < 0.01$; for CD4 $^-$ CD8 $^+$, $F(1,20) = 47.01$, $P < 0.01$]. Propranolol increased the percentage of CD4 $^-$ CD8 $^-$ double-negative (DN) cells in both non-Adx and Adx rats ($P < 0.01$), but this effect was more pronounced in Adx rats (Figure 1Aa,Ba). Following

propranolol treatment, the relative proportions of CD4 $^+$ CD8 $^+$ double-positive (DP) and CD4 $^+$ CD8 $^-$ single-positive (SP) cells were decreased ($P < 0.01$ and $P < 0.05$, respectively), but to a greater extent in non-Adx rats (Figure 1Aa,Ba). Propranolol had opposing effect on the relative proportion of CD4 $^-$ CD8 $^+$ SP cells in non-Adx and Adx rats. Compared with the corresponding saline-treated controls, the percentage of these cells was decreased ($P < 0.01$) and increased ($P < 0.01$) in non-Adx and Adx rats, respectively (Figure 1Aa,Ba).

TCR $\alpha\beta^{\text{low}}$ thymocytes. Compared with non-Adx rats, in Adx rats propranolol differently [$F(1,20) = 66.97$, $P > 0.01$] affected only the DP subset of TCR $\alpha\beta^{\text{low}}$ cells. Namely, although propranolol ($P < 0.01$) increased the percentage of DP cells in both non-Adx and Adx rats compared with their respective saline-injected controls, this effect was more pronounced in non-Adx rats (Figure 1Ab,Bb). Propranolol had no effect on the relative numbers of cells belonging to any of the other three subsets of TCR $\alpha\beta^{\text{low}}$ thymocytes in either non-Adx or Adx animals (Figure 1Ab,Bb).

TCR $\alpha\beta^{\text{high}}$ thymocytes. Regarding the relative proportions of all thymocyte subsets, two-way ANOVA revealed a significant propranolol x adrenalectomy interaction [for DN, $F(1,20) = 4.63$, $P < 0.05$; for DP, $F(1,20) = 30.76$, $P < 0.01$; for CD4 $^+$ CD8 $^-$, $F(1,20) = 10.92$, $P < 0.01$; for CD4 $^-$ CD8 $^+$, $F(1,20) = 7.47$, $P < 0.05$]. Propranolol ($P < 0.01$) reduced the percentage of DP thymocytes in non-Adx rats, but was ineffective in this respect in Adx rats (Figure 1Ac,Bc). On the other hand, although propranolol increased the relative proportions of CD4 $^+$ CD8 $^-$ ($P < 0.01$) and CD4 $^-$ CD8 $^+$ ($P < 0.05$) SP cells in both non-Adx and Adx rats, this effect was more pronounced in Adx rats (Figure 1Ac, Bc). Furthermore, a reduced ($P < 0.05$) CD4 $^+$ /CD8 $^-$ cell ratio in propranolol-treated Adx rats (2.90 ± 0.31) compared with propranolol-treated non-Adx rats (3.41 ± 0.21) suggested a particularly pronounced effect of propranolol on the size of the CD4 $^-$ CD8 $^+$ SP cell subset in Adx rats. In addi-

Table 1. Differential effect of 4-day propranolol treatment on thymus weight, thymocyte yield and proliferative index in adrenalectomized (Adx) and non-Adx adult rats compared to the corresponding saline-treated controls.

	Non-Adx rats		Adx rats	
	Saline	Propranolol	Saline	Propranolol
Thymus weight (g)	0.18 \pm 0.006	0.17 \pm 0.016	0.31 \pm 0.016	0.25 \pm 0.006*
Thymocyte yield ($\times 10^7$)	19.89 \pm 1.03	17.76 \pm 0.27	62.29 \pm 2.01	57.66 \pm 0.95*
Proliferative index (%)	9.32 \pm 0.38	13.48 \pm 0.47*	16.34 \pm 0.21	19.33 \pm 1.87
Apoptosis (%)	8.75 \pm 0.51	12.36 \pm 0.34*	3.27 \pm 0.35	4.98 \pm 0.35*

Data are reported as means \pm SEM (N = 6). Proliferative index = percent of cells in the S/G2M phases of the cell cycle. Apoptosis was measured with merocyanine 540. * $P < 0.05$ compared to the corresponding saline-treated controls (two-way ANOVA followed by the Bonferroni test for *post hoc* comparisons).

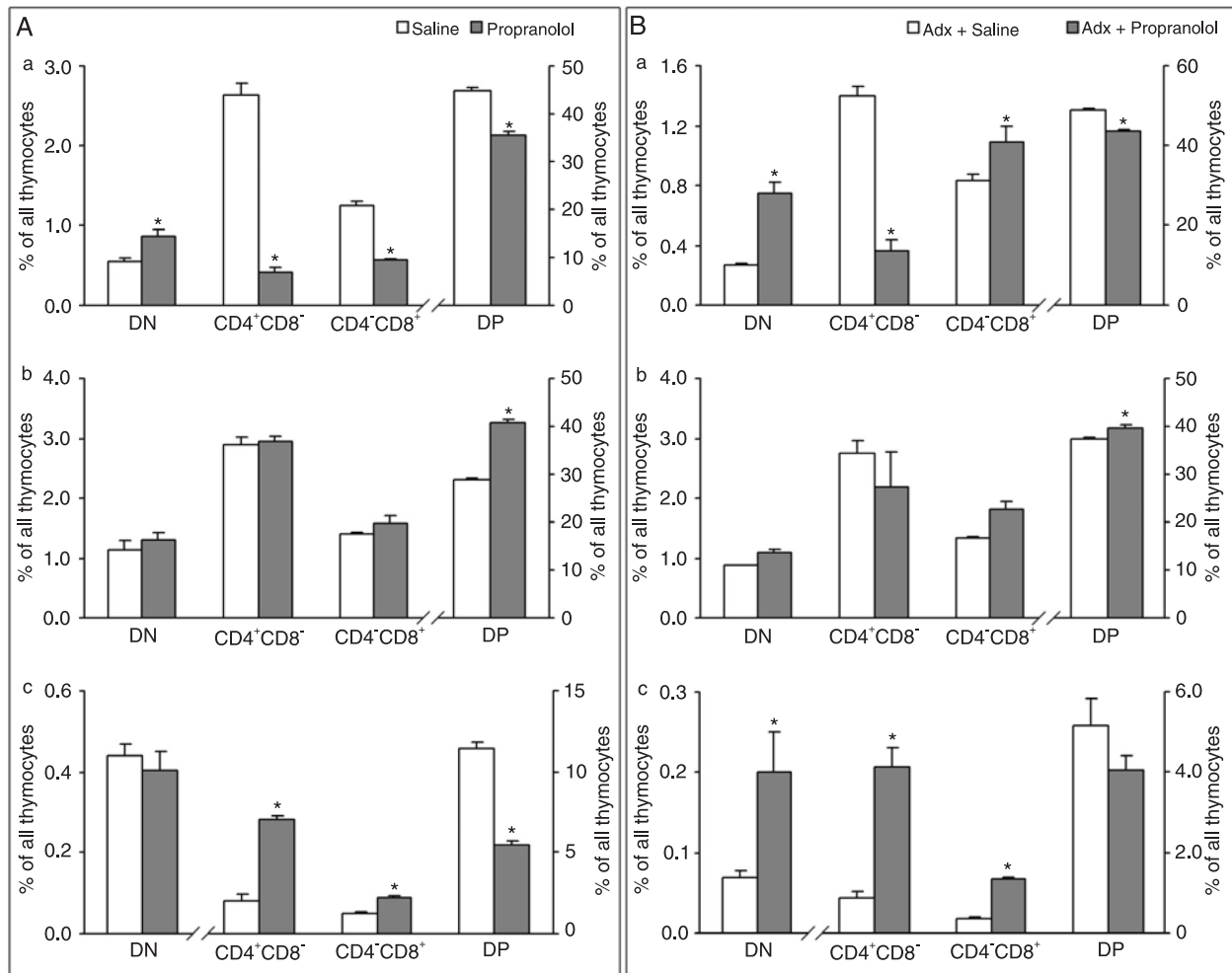


Figure 1. Differential effect of 4-day propranolol treatment on thymocyte subset distribution in non-adrenalectomized (non-Adx; panel A) and Adx adult male Dark Agouti rats (panel B). The histograms indicate the relative numbers of thymocyte subsets determined by CD4/CD8 expression with (a) undetectable (b) low and (c) high levels of TCR $\alpha\beta$ in non-Adx and Adx rats treated with propranolol or saline. Data are reported as means \pm SEM (N = 6). DN = CD4⁻CD8⁻, double-negative; DP = CD4⁺CD8⁺, double-positive. *P < 0.05 compared to the corresponding saline-treated control (two-way ANOVA followed by the Bonferroni test for *post hoc* comparisons).

tion, in Adx rats, propranolol increased the percentage of DN cells compared to saline-injected rats, while in non-Adx rats the percentage of these cells was unaltered following propranolol treatment (Figure 1Ac,Bc).

Discussion

The results of the present study showed that a 4-day propranolol treatment has a differential effect on thymus weight and thymocyte yield in non-Adx and Adx rats. Propranolol (most likely due to a differential effect on thymocyte proliferation) reduced both thymic weight and cellularity in Adx, with no similar effect in non-Adx rats. Since the adrenaline level in adult rat thymus has been found to be below detection level (18), it may be assumed that the thy-

mic changes in Adx rats mainly reflected a lack of adrenal GCs. This view is further supported by data showing that: i) noradrenergic nervous fibers represent the main source of CAs in the thymus (7,8), and ii) surgical stress does not affect any of the thymopoiesis parameters analyzed (12). The ineffectiveness of propranolol in enhancing thymocyte proliferation in the absence of adrenal GCs may be explained by the fact that thymocyte proliferation was measured in an unfractionated thymocyte suspension. Namely, in Adx rats, an impaired α_2 -AR-mediated stimulatory effect of CA on proliferation of an α_2 -AR expressing thymocyte subset [due to reduced α_2 -AR expression (16)], could mask the enhancing effects of the β -AR blockade on the proliferation of the thymocyte subset expressing β -ARs.

The analysis of thymocyte subset composition showed

that propranolol treatment led to an overrepresentation of the least mature DN TCR $\alpha\beta^-$ thymocytes in non-Adx rats, followed by the underrepresentation of their DP TCR $\alpha\beta^-$ progeny. Furthermore, in these rats, propranolol treatment increased the relative proportion of DP TCR $\alpha\beta^{\text{low}}$ thymocytes (the cells entering positive selection) and the most mature SP TCR $\alpha\beta^{\text{high}}$ thymocytes, while it reduced that of intermediate DP TCR $\alpha\beta^{\text{high}}$ cells (the cells that had just undergone positive selection). Taken together, these findings suggest a decelerated transition from the DN TCR $\alpha\beta^-$ to the DP TCR $\alpha\beta^-$ developmental stage, followed by increased positive/reduced negative selection and accelerated post-selection differentiation/maturation. In view of the fact that: i) propranolol abrogates dose-dependent noradrenaline-induced destabilization of Thy-1 mRNA in T-cell lines (19) and increases Thy-1 surface expression on thymocytes (7), and ii) Thy-1 $^{-/-}$ mice exhibit reduced positive/exaggerated negative selection and consequently a reduced proportion of mature SP cells (20), the alterations in thymocyte development in propranolol-treated non-Adx rats may be related at least in part to increased Thy-1 surface expression (data not shown).

In view of the fact that GCs increase CA synthesis and β -AR signaling (14,15), a more efficient β -AR blockade, and therefore more pronounced changes in thymopoiesis may be expected in thymi of Adx rats. Indeed, compared with non-Adx rats, in Adx rats propranolol caused a more pronounced increase in the proportion of the most immature DN TCR $\alpha\beta^-$ and the most mature (in particular CD4 $^+$ CD8 $^+$) SP TCR $\alpha\beta^{\text{high}}$ thymocytes. In addition, adrenalectomy partly (DP TCR $\alpha\beta^-$ and DP TCR $\alpha\beta^{\text{low}}$ cells) or entirely (DP TCR $\alpha\beta^{\text{high}}$ cells) abrogated the effects of β -AR blockade on the relative proportion of DP thymocytes. Generally, a more pronounced increase in the proportion of the least mature DN TCR $\alpha\beta^-$ cells than in the proportion of the most

mature SP TCR $\alpha\beta^{\text{high}}$ cells following propranolol treatment in Adx compared with non-Adx rats may suggest a more pronounced increase in positive selection/decrease in negative selection. Furthermore, it may be speculated that the alteration in thymocyte selection (most likely reflecting a greater increase in Thy-1 thymocyte surface density due to a more efficient β -AR blockade) in Adx rats not only compensated decelerated transition from the early DN TCR $\alpha\beta^-$ to the DP TCR $\alpha\beta^-$ developmental step but provided a substantially more pronounced increase in the relative proportion of the most mature SP thymocytes in these rats compared with non-Adx rats.

Moreover, given that: i) GCs enhance CA and α_1 -AR expression (14,16), and ii) the blockade of α_1 -ARs prevents the transition from the DN to DP thymocyte developmental steps and favors post-selection maturation towards mature CD4 $^+$ CD8 $^-$ SP cells (6), it is reasonable to assume that a more pronounced increase in the proportion of DN TCR $\alpha\beta^-$ cells and CD4 $^+$ CD8 $^+$ SP TCR $\alpha\beta^{\text{high}}$ cells following propranolol treatment in Adx rats compared with non-Adx rats reflected a substantially reduced α_1 -AR-mediated CA action.

In conclusion, the present study pointed to GC-dependent plasticity of CA-mediated fine-tuning of thymopoiesis, and indirectly suggested that GCs influence T-cell development, not only by a direct action on thymocytes and thymic non-lymphoid cells (11,12), but also indirectly, affecting CA availability and/or AR sensitivity to CA signaling.

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