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Role of cAMP and Neutrophil Cyclooxygenase in Gonadotropin-Dependent Regulation of T Lymphocyte Proliferation

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Abstract—The effect of the main pregnancy hormone, chorionic gonadotropin (CG), on proliferation of peripheral blood mononuclear cells (PBMC) was studied in the presence of autologous neutrophils; also, hormone-dependent regulation of the cAMP levels in T lymphocytes and neutrophils was evaluated. PBMC proliferation in response to a mitogen is suppressed by physiological doses of CG (10, 50, and 100 IU/ml). Autologous neutrophils enhance the suppression induced by the low dose of CG (10 IU/ml), but when cyclooxygenase was inhibited this effect was not observed; this suggests that the anti-proliferative effects of the low dose of CG can be mediated by the products generated by neutrophil cyclooxygenase. The effect of CG was associated with increased cAMP levels in T lymphocytes and neutrophils. Comparison of functional and cAMP-related effects of CG in both cell populations indicates that cAMP is involved in the anti-proliferative effects of CG.

Key words: chorionic gonadotropin, T lymphocytes, neutrophils, proliferation, cAMP, cyclooxygenase

Immunomodulatory effects of reproductive hormones are important for reorganization of the immune system during gestation. Chorionic gonadotropin (CG) is the main peptide hormone secreted by the placenta during pregnancy. CG stimulates steroidogenesis by the fetal placenta and provides for growth and differentiation of the trophoblast [1, 2]. Moreover, CG significantly modulates the immune system, suppressing T lymphocyte proliferation is response to mitogens and alloantigens [3, 4]; this results in the decreased cell-mediated immunity observed during gestation [5]. Proliferation of T lymphocytes is regulated by neutrophils [6]; certain suppressive effects of CG on cultured splenocytes are detected only in the presence of macrophages and depend on the cyclooxygenase activity in these cells [7]; hence, the role of neutrophils should be considered in studies of the effect of CG on peripheral blood T lymphocyte proliferation.

The goal of the present work was to investigate spontaneous and mitogen-induced proliferation of peripheral blood mononuclear cells (PBMC) with autologous neutrophils in the presence of CG and a cyclooxygenase inhibitor, voltaren, and to analyze certain immunomodulatory mechanisms of CG effects on T lymphocytes and neutrophils.

MATERIALS AND METHODS

PBMC and neutrophils were isolated from peripheral blood of non-pregnant females obtained during the follicular phase of the menstrual cycle by centrifugation in a double density gradient of Ficoll-Paque (Pharmacia, Sweden; Spofa, Czech Republic). The densities of the upper and lower gradients were 1.077 and 1.112 g/cm^3, respectively [8]. PBMC were collected from the upper interface and neutrophils were collected from the lower interface. According to histological evaluation, neutrophil purity was 96% and viability of both populations was 97% determined by the trypan blue exclusion test (0.1% solution).

Chorionic gonadotropin (Profasi, I. F. Serono S.p.A., Italy) was used at 10, 50, or 100 IU/ml; these doses correspond to the CG levels in the peripheral blood of women during various trimesters of gestation [9]. Voltaren (Pliva, Croatia) was added at 0.015 mg/ml; this is the mean therapeutic concentration that efficiently inhibits cyclooxygenase [10].

Abbreviations: CG) chorionic gonadotropin; ICER) inducible cAMP early repressor; IL-2) interleukin-2; PGE_2) prostaglandin E_2; PKA) cAMP-dependent protein kinase; PKC) protein kinase C; PLCγ) phospholipase Cγ; AET) aminoethylisothiouronium bromide; MAPK) mitogen-activated protein kinase; PBMC) peripheral blood mononuclear cells.

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T lymphocytes were isolated from PBMC by rosette formation with sheep erythrocytes [11]. Washed erythrocytes ($2 \cdot 10^9$ cells/ml) were incubated for 15 min at 37°C with the rosette stabilizer aminoethylisothiouronium bromide (AET; Sigma, USA) at the final concentration 140 mM (pH 9); then the cells were washed and resuspended in the 199 medium. PBMC ($3 \cdot 10^6$ cells/ml) were mixed with an equal volume of 1% suspension of erythrocytes treated with AET and centrifuged at 200*g* for 7 min. The pellet was resuspended, and Ficoll-Paque (density 1.077 g/cm^3) was layered below the pellet; the tube was centrifuged at 200*g* for 30 min. Rosettes were collected on the bottom of the tube, and the erythrocytes were lysed by hemolytic shock. T lymphocyte purity was evaluated by immunofluorescent staining with monoclonal anti-CD3 antibody labeled with fluorescein isothiocyanate (Sorbent, Russia); it was 89% with viability 97% according to the 0.1% trypan blue exclusion test.

PBMC ($2 \cdot 10^6$ cells/ml) and autologous neutrophils ($6 \cdot 10^6$ cells/ml) were incubated for 1 h at a ratio corresponding to that detected in the peripheral blood; incubation was performed in 0.2 ml in the presence of CG and voltaren; then, phytohemagglutinin (Sigma) was added (10 μg/ml) and cells were cultured at 37°C in atmosphere of 5% CO_2 for 48 h. Proliferation was assayed as [^{3}H]thymidine incorporation (1 MBq added 18 h before incubation was terminated). The cells were harvested by a Titertek (Finland) harvester on glass fiber filters, which were then counted in 10 ml of ZhS-103, a toluene-based scintillator, using a Beta-2 counter (Russia).

Intracellular cAMP concentration was determined by the radioimmune method using KS-ATsF-N-3 kits (Izotop, Russia). CG was added to the culture of fractionated T lymphocytes or neutrophils ($1 \cdot 10^6$ cells/ml) and samples were incubated for 10, 30, and 60 min. Then, the phosphodiesterase inhibitor theophylline was added (3 mM final concentration) and the cells were pelleted by centrifugation (3000 rpm, 15 min). cAMP was isolated as described previously [12]. Radioactivity was determined with a Beta-2 counter (Russia) in the dioxane scintillator ZhS-8.

Results were statistically evaluated by Student's *t*-test.

RESULTS AND DISCUSSION

The data of Fig. 1A indicate that CG did not influence spontaneous proliferation of PBMC in the absence and in the presence of neutrophils. Addition of voltaren to the culture also had no significant effect (Fig. 1A). When the cells were stimulated with phytohemagglutinin, CG at three concentrations suppressed PBMC proliferation; the effect was dose-dependent and maximal in the case of the highest dose of CG (Fig. 1B). Neutrophils increased the mitogen-induced proliferation of PBMC and significantly enhanced the suppressive effect of a low dose of CG (10 IU/ml); however, in the presence of voltaren the effect was not detected (Fig. 1B).

Phytohemagglutinin is predominantly a T cell mitogen, and T lymphocytes are the major fraction of PBMC; hence, CG-dependent suppression can be associated with the effect of the hormone on T cells.

Thus, antiproliferative effects of CG can be due to direct CG action on T lymphocytes or at low CG concentration, they can be mediated by the products of neutrophil cyclooxygenase. Probably, prostaglandin E_2 (PGE_2) can be a mediator in this case because neutrophils produce PGE_2 [13], which has direct antiproliferative activity towards T cells [14].

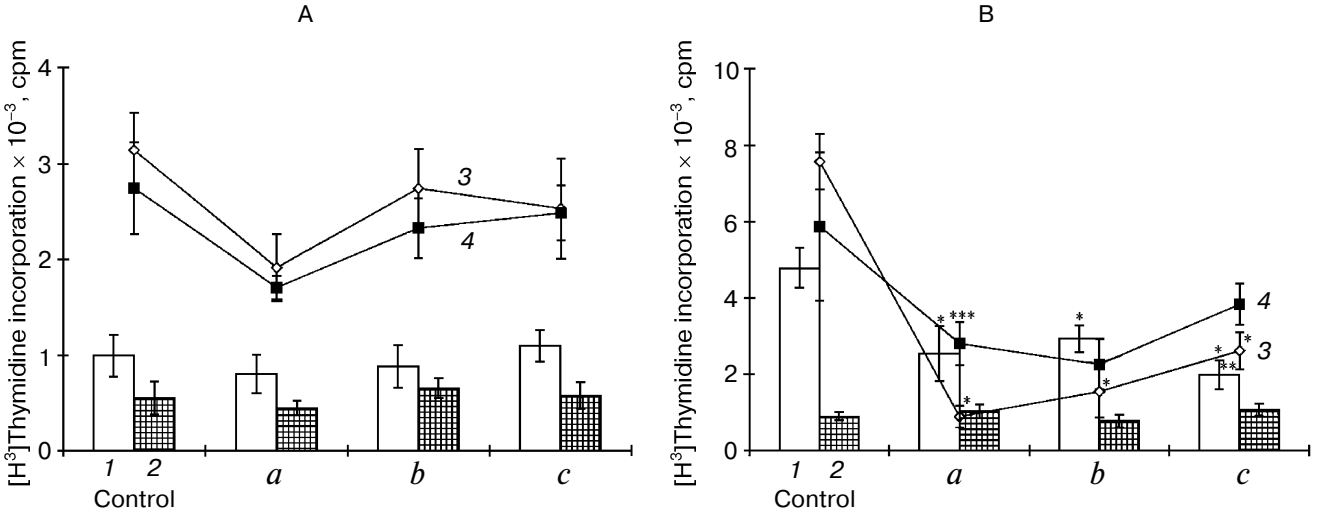

Fig. 1. Effect of CG at doses of 10 (*a*), 50 (*b*), and 100 IU/ml (*c*) on spontaneous (A) and PHA-induced (B) proliferation of PBMC in the presence of autologous neutrophils: *1*) PBMC; *2*) neutrophils; *3*) PBMC + neutrophils; *4*) PBMC + neutrophils + voltaren. * $p < 0.05$ versus control; ** $p < 0.05$ versus CG at 50 IU/ml; *** $p < 0.05$ versus corresponding samples without voltaren.

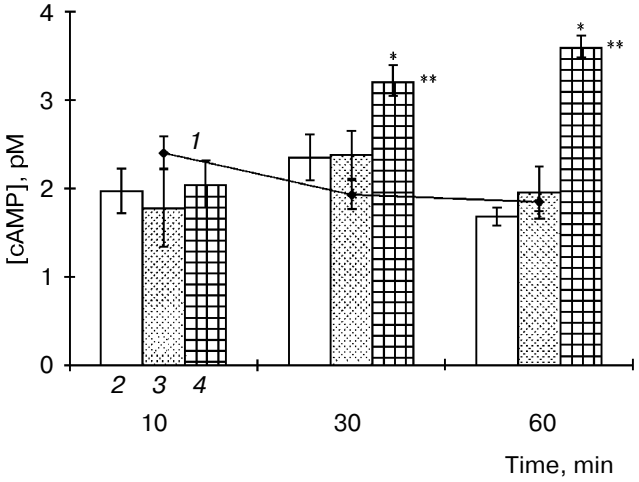

Fig. 2. Effect of CG on cAMP levels in human peripheral blood T lymphocytes: *1*) control; *2*) CG, 10 IU/ml; *3*) CG, 50 IU/ml; *4*) CG, 100 IU/ml. * $p < 0.05$ versus control; ** $p < 0.05$ versus CG at 10 and 50 IU/ml.

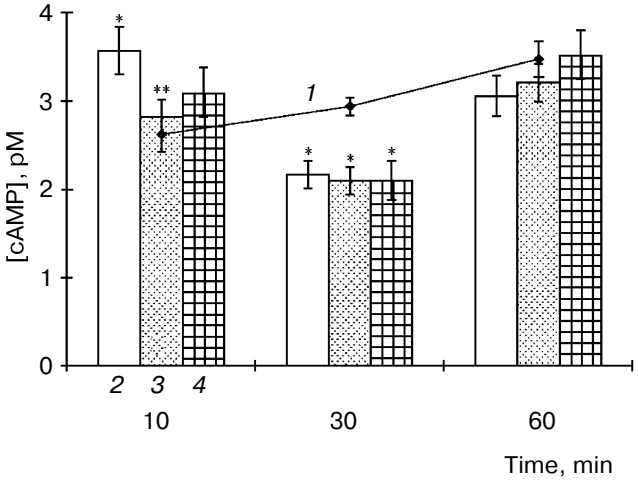

Fig. 3. Effects of CG on cAMP levels in human neutrophils: *1*) control; *2*) CG, 10 IU/ml; *3*) CG, 50 IU/ml; *4*) CG, 100 IU/ml. * $p < 0.05$ versus control; ** $p < 0.05$ versus CG at 10 IU/ml.

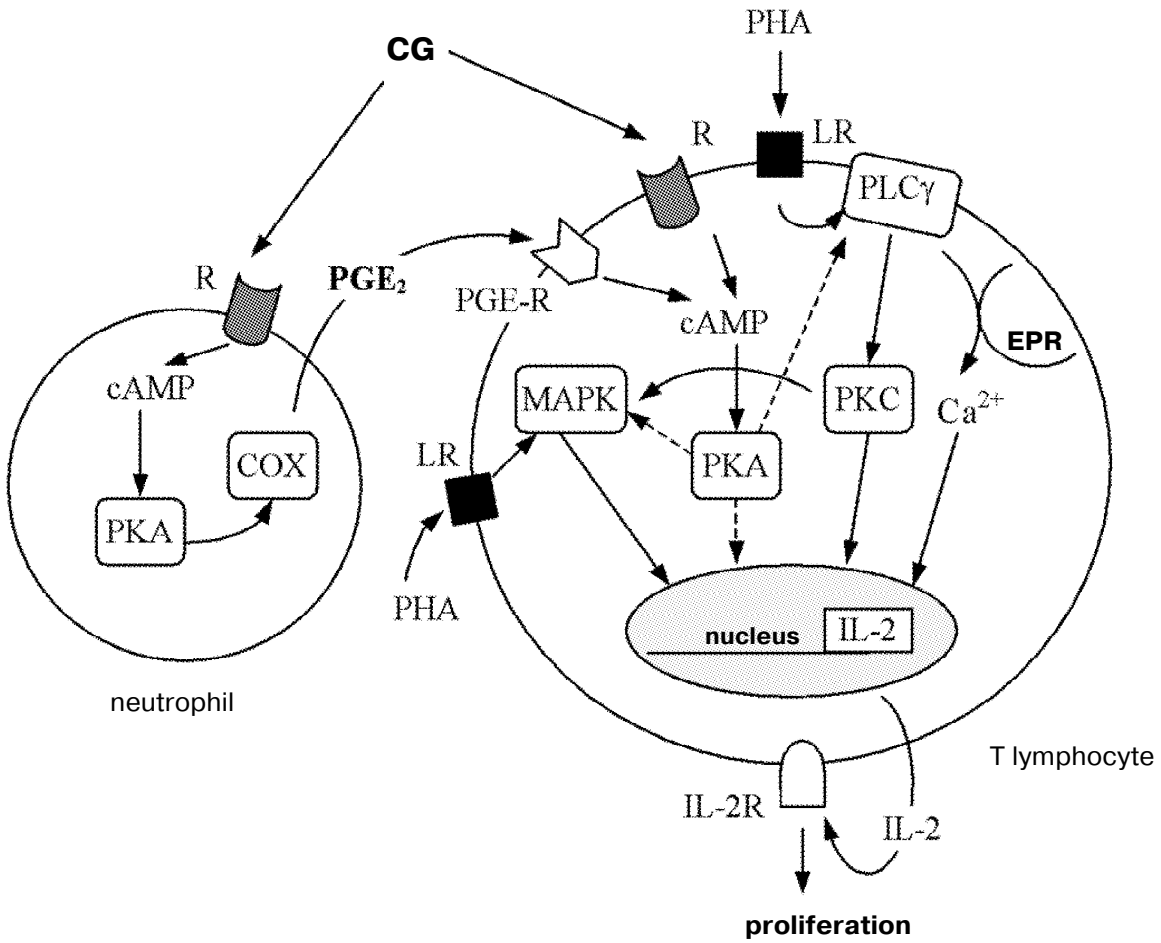

Fig. 4. Cellular and molecular mechanisms of antiproliferative effect of CG (see text for details). COX) cyclooxygenase; IL-2R) interleukin-2 receptor; LR) lectin receptor; PGE-R) PGE_2 receptor; PHA) phytohemagglutinin; R) CG receptor, known for T lymphocytes and hypothetical for neutrophils; EPR) endoplasmic reticulum.

Sensitivity of T lymphocytes and neutrophils to CG can be mediated by certain molecular mechanisms. Gonadotrophic [15] and some immunomodulatory effects of CG [16] are mediated by cAMP; thus, regulation of cAMP by CG should be considered. The data of Fig. 2 indicate that in T lymphocytes the high physiological dose of CG significantly elevated the cAMP levels after incubation for 30 and 60 min. Similar data were obtained in fractionated T splenocytes of mice, and CG-dependent increase in cAMP concentration was associated with decreased functional activity of these cells [16]. This suggests that intracellular mechanisms of CG effects are universal and affect recirculating and stationary pools of T lymphocytes in various microenvironments; also, these effects are not species-specific. Unlike lymphocytes, increase in the cAMP levels in neutrophils was induced by the low dose of CG and was registered at the beginning of culture (Fig. 3). Subsequent incubation of the cells with CG was associated with decreased intracellular cAMP levels independently of the CG dose; in 60 min, cAMP levels returned to the control values excluding the highest does of CG (10 IU/ml; Fig. 3).

Recent studies indicate that CG receptor is expressed on the T lymphocyte membrane [17]. Our data suggest that these receptors in T cells and other similar structures in neutrophils are associated with adenylate cyclase; however, the levels of cAMP are also regulated by a phosphodiesterase that hydrolyzes cAMP into inactive 5′-AMP; hence, the effects of CG can be due to phosphodiesterase inhibition.

It should be noted that in T lymphocytes, antiproliferative and cAMP-increasing effects of CG were detected (cAMP) or were more pronounced (suppression of proliferation) at high CG concentration; this suggests that suppressive action of CG is mediated by cAMP. In neutrophils, cyclooxygenase activation and increase in cAMP concentration were detected only at low concentration of CG. The data indicate that cAMP can be important for CG effects in both cell populations.

Mechanisms of cAMP-dependent suppression of mitogen-mediated T lymphocyte proliferation are well understood. Phytohemagglutinin binds to the lectin receptor and initiates a signaling pathway in the T cells; this pathway is similar to that induced by antigen receptor stimulation. It includes activation of the membrane enzyme phospholipase Cγ (PLCγ) [18] thus enhancing phosphoinositide generation associated with activation of protein kinas C (PKC) and increase in intracellular Ca^{2+} levels [18, 19]; in addition, mitogen-activated protein kinases (MAPK) are mobilized [20, 21]. PKC, Ca^{2+}, and MAPK stimulate certain transcription factors inducing expression of the interleukin-2 (IL-2) gene; IL-2 is an autocrine growth factor of T lymphocytes; IL-2 binds to the high affinity membrane receptors and initiates cell proliferation [22].

cAMP is not directly involved in the activation pathway but efficiently modulates it [23]. cAMP-dependent protein kinase (PKA) inhibits PLCγ [24], PLCγ-associated phosphoinositide metabolism [25], and MAPK activation [26]; cAMP-induced transcription repressor ICER decreases IL-2 gene expression blocking the binding of the corresponding transcription factors with the promotor [27]. Hence, in T lymphocytes cAMP-dependent antiproliferative effects of CG can be due to inhibition of the intracellular enzymes involved in the activation cascade and to direct regulation of transcription.

In neutrophils, CG-dependent activation of cyclooxygenase also can be mediated by cAMP. In another myeloid cell, macrophage, cyclooxygenase mobilization directly depends on cAMP levels [28]. Neutrophils probably secrete PGE_2 in response to CG; PGE_2 can be a paracrine factor that binds to the membrane receptor on T lymphocytes and inhibits T cell proliferation due to activation of cAMP-dependent signaling [14] similar to the mechanism described in the case of gonadotropin. Possible pathways involved in antiproliferative effects of CG are shown in Fig. 4.

Thus, CG can inhibit the mitogen-induced proliferation of T lymphocytes directly and (at low dose) indirectly via cyclooxygenase products of neutrophils. The effect of CG in both cell populations is associated with increased levels of cAMP, and this can be important in mediating the antiproliferative effects of CG.

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