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In vitro modifying effect of erythropoietin upon thymic lymphocytes: an inhibitor analysis

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Summary

Erythropoietin (EPO) is a physiological stimulator of erythropoiesis. One of the main effects of EPO is to prevent apoptosis of erythroid progenitor cells in the bone marrow. These properties of EPO are widely used for treatment of various hematopoietic disorders including posttransplant conditions. Previously, it was found that activating EPO-effect on T-lymphocytes (TLC) accompanied by an increase in the number of fluorescent mitochondria ($n_{m/c}$) and an increase in the total transmembrane potential on plasmatic (Δφp) and mitochondrial membranes (Δφm). However, it remains unclear which membrane potential is responsible for the EPO effect. Hence, we used specific inhibitors of oxidative phosphorylation in the respiratory chain. The aim of the present work was to assess the role of mitochondrial functions in EPO effects upon thymic lymphocytes.

Materials and methods

We studied EPO (Eprex, Cilag) influence on fluorescence of rat TLC after short-term incubation and treatment with some inhibitors: dinitrophenol (DNP-uncoupler of oxidative phosphorylation and inhibitor of respiratory chain), pentachlorphenol (PCP- uncoupler of oxidative phosphorylation), N,N -dicyclohexylcarbodiimide (DCCD- inhibitor of Ca^{2+}- dependent mitochondria ATP-ase). The cells were then tested by electrical field gradient sensitive probe DSM [4-(p-dimethylaminostyryl)-1-methylpyridinium]. Rat TLC were isolated according to the standard method. The microfluorimetric studies of DSM-stained TLC were performed by means of fluorescent microscope "Lumam R-8", "LOMO", Russia) with thermostatic plate. Fifty to 70 single cells were measured per each specimen the mean fluorescence intensity of TLC was calculated ($\tilde{F}$), as well as nm/c values. Statistical evaluation of the data was performed by the Spearmen range correlation.

Results

In a series of experiments with TLC, we have registered a decrease in $\tilde{F}$ and $n_{m/c}$ after incubation with all used inhibitors. It was found that the difference in decrease of nm/c rates and $\tilde{F}$ values depends on the type of inhibitor and on the duration of incubation. Maximal irreversible reduction of the TLC energy potential ($\tilde{F}$ and $n_{m/c}$) after incubation was seen with DNP being not restored by EPO. After incubation with PCP, EPO restores $n_{m/c}$ and $\tilde{F}$ by ca. 20-23%. The reaction of TLC on the DCCD confirms the important role of the ATP-ase for maintenance of mitochondrial membrane potential. After de-energization of TLC by DCCD, EPO has the maximum rescuing effect, i.e. recovery by approx. 42% for $n_{m/c}$ and ~38% for $\tilde{F}$ values.

Conclusion

EPO is able to partially recover the damage and polarization of the mitochondria membranes in TLC disturbed after exposure to specific ATP-ase inhibitor (DCCD). This *in vitro* approach may be used for screening other growth factors.

Keywords

Erythropoietin, T-lymphocytes, energy activity, inhibitors, electrical field gradient sensitive probe DSM [4-(p-dimethylaminostyryl)-1-methylpyridinium].

Introduction

Erythropoietin (EPO) was initially known as a physiological growth factor which is produced, mainly, by renal glomeruli, macrophages and some other cell types. EPO is shown to support survival and mitotic activity, as well as inhibit apoptosis of late erythroid precursor cells in bone marrow [1]. Therefore, recombinant erythropoietins-alpha (Eprex, Epobioicrine, Epostim etc.) are widely used to correct anemias in different diseases and posttransplant conditions [2].

However, biological mechanisms of EPO action upon immune cells are still not clear. E.g., antioxidant *in vivo* effects of EPO (reduced lipid peroxidation in blood lymphocytes) are shown by Osikov et al. [3]. Immunotropic effects of EPO are recently studied, due to its immunomodulatory effects under clinical and experimental conditions [4-6]. Response of lymphocytes and macrophages to EPO seems to be mediated by the EPO receptors on their surface [7]. *In vitro* biological effects of EPO towards T-lymphoid cells were previously shown by Hisatomi et al. [8] who demonstrated suppression of IL-2 gene expression in TLC cells after their short-term (6 h) incubation with EPO. Hence, EPO is able to exert fast immediate action upon T-lymphoid cells over short incubation terms. Indeed, we have also revealed modulatory effect of EPO upon rat thymocytes using a specific potential-sensitive chemical probe [9]. EPO was found to exert activating effect upon the TLC by changing total transmembrane potential of plasmatic (Δφp) and mitochondrial membranes (Δφm). This activation correlated with increased numbers of the probe-labeled fluorescent mitochondria in exposed cells [10]. To discern these mechanisms, we used specific inhibitors of oxydative phosphorylation, in order to assess the type of potential responsible for these EPO effects. Hence, the aim of this work was to evaluate the role of mitochondrial functions in EPO effects upon thymic lymphocytes in order to specify this response to EPO, either Δφm, or Δφp. To address this issue, we used specific inhbitors of phosphorylation in the respiratory chain which serve as important tools for studying energy supply in the living cells.

To address this issue, we used specific inhibitors of oxydative phosphorylation. In order to evaluate the role of mitochondrial functions for energy supply in thymic lymphocytes exposed to EPO. Hence, the aim of this study was to assess a restoring EPO effect upon the *in vitro* response of thymocytes after treatment with different specific inhibitors of oxidative phosphorylation.

Materials and Methods

We have studied lymphoid cells isolated from thymuses of white Wistar rats (200-300 g), after gentle mincing of thymus glands [9]. The cells were resuspended in standard Hank's solution at 2 to $3x10^7$ cells/mL. Percentage of viable (dye-excluding) cells was determined by routine Trypan Blue staining.

To determine possible points for EPO actions, we used different inhibitors of the oxidative phosphorylation, i.e., dinitrophenol (DNP, Sigma, USA), an inhibitor of respiratory chain and uncoupler of oxidative phosphorylation; pentachlorophenol (PCP, Sigma USA), an uncoupler of oxidative phosphorylation; dicyclohexyl carbodiimide (DCCD, Sigma, USA), an inhibitor of mitochondrial membrane-bound ATP-ase domain. Erythropoietin (EPO) was purchased from Cilag (Eprex) was dissolved in Hank's solution. A potential-sensitive fluorescent probe [4-(p-dimethylaminostyryl)-1-methylpyridinium] (DSM) was used to test the energy potential of cells. DSM was produced and purchased from the Latvian Institute of Organic Synthesis [9]. The thymocyte suspensions in Eppendorf-type tubes were pre-incubated with inhibitors for 10…20…40 min at 37°C, then EPO was added at the final concentration of 2U/ml), followed by incubation for 30 min, addition of the DSM probe (1.5 μM), and post-incubated for 20 min. Final concentrations of inhibitors were as follows: DNP, 0.1 mM; PCP, 1.5 μM; DCCD, 0.1 mM, at a v/v ratio of <5% to the initial suspension volume. The control samples of cells were supplied with equal volumes of Hanks' solution, and, after 10…30 min. at 37°C, were incubated with DSM and EPO, as described above. Control and experimental TLC samples were then studied at the luminescent microscope LUMAM – R8 (LOMO, St. Petersburg, Russia) at a 900x magnification, using a temperature-controlled stage for the count chamber. The fluorescence was excited by a mercury lamp (λ= 405-436 nm). To measure light emission, a FMEL-1 photometric device was used, with an interference filter with a maximum transmission at 585 nm. Fluorescence intensity was manually measured for single cells localized in the vision field, then transformed to a digital signal. The numbers of DSM-stained bright mitochondria, looking as intracellular yellow granules, were counted per each single cell (the $n_{m/c}$ values) [9]. Fifty to seventy cells were studied *per* sample, and the mean fluorescence intensity was calculated as conventional units ($\tilde{F}$, arbitrary units). The fluorescent signal did not quench over the measurement time. Photomicrographs of the DSM-stained cells were performed with a TSA 5.0 camera mounted in the LOMO R8 microscope, and analyzed by a Microanalysis View software (from the same manufacturer). Statistical evaluation of the data was performed by the Spearmen range correlation criterion. A total of 8,000 cells have been studied in 160 samples. Each independent experiment included 3 to 5 measure points.

Results

Initial amount of intact thymic lymphoid cells in cell suspensions was 92 to 96%. Several control experiments with have shown a decrease of $n_{m/c}$ and $\tilde{F}$ values for these cells after incubation with either inhibitor. The degree of such decrease depended on the type of inhibiting substance, and incubation terms (Table 1, Fig. 1 A, B, C). The most pronounced and faster effect was observed with DNP, i.e., fluorescent mitochondria became virtually absent in DNP-treated cells as soon as after 10 min of exposure. $\tilde{F}$ values were also decreased by 20 min, with both $n_{m/c}$ and $\tilde{F}$ reduction. Fluorescent mitochondria disappeared by 40 min, with $\tilde{F}$ at the background levels. EPO addition did not restore $\tilde{F}$ and $n_{m/c}$- parameters. The dynamics of thymocytes with absent mitochondrial fluorescence after DNP exposure shown in Fig. 1A ($N_{C\text{-}EM}$,%), like as absence of recovery after EPO addition. This effect may be caused by classical protonophore properties of DNP which irreversibly reduces both mentioned components of electrochemical gradient, thus causing the mitochondrial membrane depolarization.

Table 1. Time-dependent changes of mean fluorescence (F̃, arb. units) and number of fluorescent mitochondria *per* one cell ($n_{m/c}$) upon short-term exposure of rat TLC to respiratory chain inhibitors followed by EPO treatment

	Control		+DNP		+PCP		+DCCD	
t,min	$n_{m/c}$	F̃	$n_{m/c}$	F̃	n m/c	F̃	$n_{m/c}$	F̃
10	10,8±0,8	27,6±2,6	0,5±0,1*	5,7±06**	4,4±0,8*	9,6±0,8*	3,3±0,3*	9,2±0,6*
20	9,0±0,7	25,5±2,5	0,4±0,1*	4,5±0,5*	2,8±0,3**	7,0±0,6*	1,9±0,3**	5,5±0,5*
40	8,8±0,6	23,0±2,5	0	2,3±0,3*	0	2,5±03*	0	2,8±0,4**
+ EPO								
30	20,5±1,0	48,3±4,7	0	2,3±0,3*	4,7±0,6*	9,7±0,7*	8,7±0,8**	18,4±0,8**

Note: * – P< 0.01; ** – P< 0.025; $n_{m/c}$ – mean number of fluorescent mitochondria per cell; F̃, arb. units – mean fluorescence levels for all the cell measured at the given time points. Controls: TLC incubated without inhibitors at 37°C, with EPO supplied after 40 min. of incubation. Experimental samples: cells with addition of DNP, or PCP, or DCCD followed by incubation with EPO at 37°C. Mean values (M) and mean error (m) are shown for each time point

Similar, but less pronounced decrease was observed 10 min after treatment with PCP, i.e., n m/c dropped by 37%, and F̃, by ~ 35%. Following 20-min incubation, we observed only ~ 26% $n_{m/c}$ and ~ 25% F̃ of control values. At 40 min., no fluorescent mitochondria are seen.

Meanwhile, Fig. 1B shows increase of non-fluorescent cell numbers ($N_{C\text{-}EM}$, %) induced by PCP, followed by a recovery induced by EPO supplement. The inhibitory effect of PCP upon thymocytes proved to be partially reversible after addition of EPO ($n_{m/c}$ recovery by ~23%, and F̃ values by ~20%).

A 10-min. incubation of thymocytes with DCCD again retains only a part of fluorescent cells ($n_{m/c}$, 30%, and ~ 33% F̃). Only 18% $n_{m/c}$ and ~ 20% F̃ remain after 20 min. with DCCD, a mitochondrial membrane-bound ATP-ase. At 40 min. with DCDD, no fluorescence was observed in the cells, with background F̃ values. However, 30-min. incubation with EPO has resulted into recovery of mitochondria-associated fluorescence to 42% for $n_{m/c}$ and 38% for F̃ levels, as compared with control samples. Fig. 1C illustrates the dynamics ($N_{C\text{-}EM}$, %) of de-energized thymocytes induced by DCCD followed by restoration of potential-coupled fluorescence after EPO treatment.

Typical patterns of DSM-stained cells before and after treatment with mitochondrial ATP-ase inhibitor (DCCD) are shown in Fig. 2 A-C.

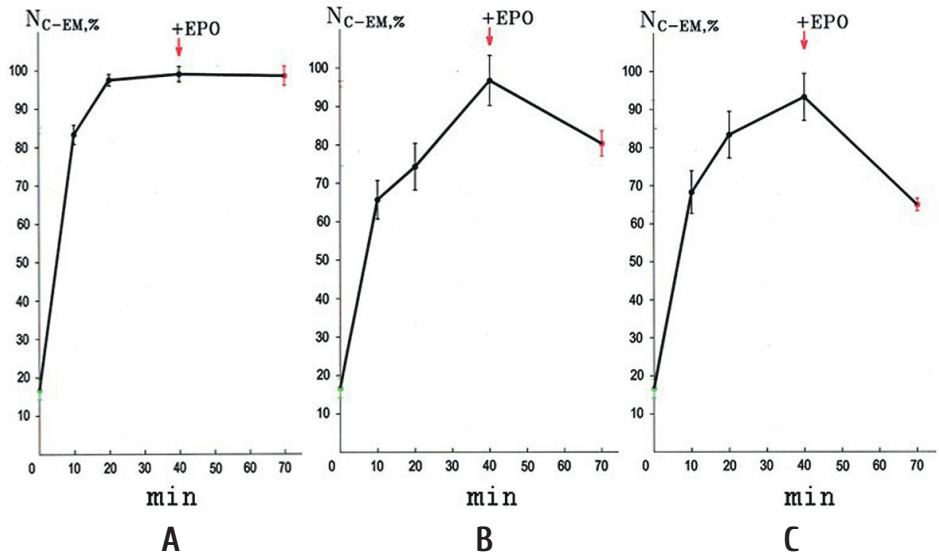
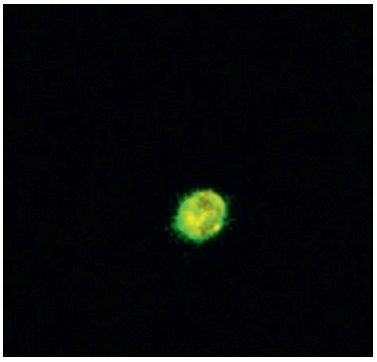
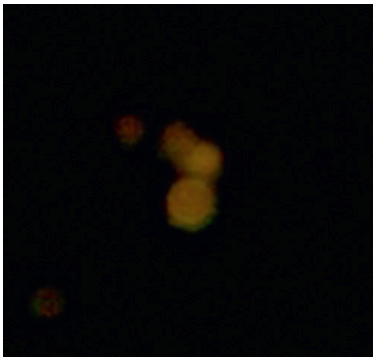


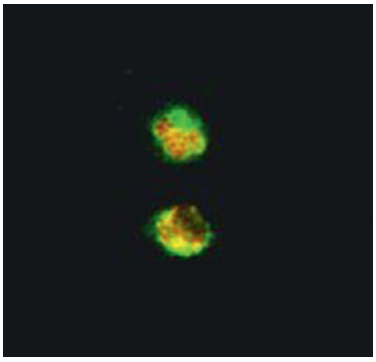
Figure 1. Changing amounts of rat thymic lymphocytes devoid of fluorescent mitochondria ($N_{C\text{-}EM}$, %) from initial time points (0 min.), following incubation with different inhibitors, and after EPO addition. Incubation at 37°C with DNP (Fig. 1A); PCP (Fig. 1B); DCCD (Fig. 1C) was followed by uniform exposure to EPO. Abscissa: incubation terms (min); ordinate, mean values of TLC without fluorescent mitochondria ($N_{C\text{-}EM}$, %) for each time point. Vertical bars show appropriate confidence intervals for P<0.05 as compared to initial values



2A



2B



2C

Figure 2. Rat thymocytes stained with a fluorescent DSM probe. 2A, an original sample after incubation with EPO, F=52,0 arb. units; 2B, cells after 40 min of incubation with DCCD, F=3,0 arb. units; 2C, thymocytes after exposure of DCCD-treated cells to EPO, F=20,0 arb. units

Discussion

In our works, we have used inhibitor analysis, in order to assess the mechanisms which regulate anti-apoptotic effects of erythropoietin. The transmembrane potential of membranes in thymocytes was determined as fluorescence intensity of DSM probe. Total DSM fluorescence depends on a summary potentials of plasmatic and mitochondrial membranes. Earlier we have found that EPO acts upon thymic lymphoid cells by changing electric charge of cellular membranes. The EPO stimulatory effect is accompanied by increased $n_{m/c}$, due to proton potential ($\Delta\varphi m$), and/or external membrane potential ($\Delta\varphi p$) [9].

Our data suggest that some metabolic effects of EPO are exerted via mitochondrial respiratory pathways. EPO was shown to reverse the de-energizing effects of DCCD, thus presuming functional changes of F0F1 membrane ATP which is specifically inhibited by the DCCD.

The mitochondrial F0F1-ATP-ase is a complex lipoprotein containing of hydrophilic catalytic center (F1), and a membrane domain (F0) [11]. DCDD used in this work is a specific proton translocation inhibitor in the F0F1 ATP-ase [12], causing decrease in $n_{m/c}$ and general $\tilde{F}$ shown in our experiments, thus reflecting a critical role of ATP-ase for sustaining the mitochondrial membrane potential. The reduced fluorescence of DSM-induced mitochondria could be considered as mitochondrial de-energization, which proved to be reversible by EPO treatment. This finding may reflect ability of EPO to restore functional integrity of mitochondrial membranes as a component of their effects upon immune system. The restored mitochondrial functions in the target cells for EPO allow to perform regulatory signaling in lymphoid cells, e.g., *via* phosphorylation of some transcription factors, e.g., STAT5 in lymphoid cells and tissues [13]. Further studies in other lymphoid cell models could further elucidate the role of EPO as a regulator of mitochondrial function.

Conclusions

1. Thymocytes may represent a non-usual, but suitable model for evaluation of growth factor effects upon dividing, apoptosis-prone immune cells.

2. The *in vitro* testing of modifying EPO effects in thymocytes exposed to inhibitors of mitochondrial functions has revealed irreversible deletorious effect upon energetic potential of these cells. Erythropoietin (EPO) does not reverse the DNP-induced damage, but partially restores mitochondrial damage induced by DCDD, a specific inhibitor of the F0F1 mitochondrial membrane ATP-ase.

3. More extended studies are required, in order to screen positive effects of EPO in other non-erythroid cell types.

Conflict of interests

No potential conflicts of interests are reported.

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In vitro модифицирующий эффект эритропоэтина на лимфоциты тимуса: ингибиторный анализ

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Резюме

Эритропоэтин (ЭПО) является физиологическим стимулятором эритропоэза. Одним из основных эффектов ЭПО является снижение частоты апоптоза эритроидных клеток-предшественниц в костном мозге. Эти свойства ЭПО широко применяются при лечении различных заболеваний системы крови, в том числе – после трансплантации стволовых клеток. Ранее было установлено, что ЭПО оказывает активирующее воздействие на Т-лимфоциты (ТЛЦ), сопровождающееся увеличением количества флуоресцирующих митохондрий в клетке ($n_{m/c}$) и увеличением суммарного трансмембранного потенциала на плазматической (Δφp) и митохондриальных мембранах (Δφm). Однако остается неясным, какой именно мембранный потенциал реагирует на воздействие ЭПО: Δφm, или (и) Δφp. Для ответа на этот вопрос мы использовали специфические ингибиторы окислительного фосфорилирования. Цель настоящего исследования – оценка роли митохондриальных функций в воздействии ЭПО на лимфоциты тимуса.

Материалы и методы

Исследовалось влияние ЭПО (“Eprex”, Cilag) на флуоресценцию ТЛЦ крыс *in vitro* после краткосрочной инкубации и воздействия несколькими ингибиторами: динитрофенолом (ДНФ) – ингибитором дыхательной цепи и разобщителем окислительного фосфорилирования; пентахлорфенолом (ПХФ) – разобщителем окислительного фосфорилирования; дициклогексилкарбодиимидом (ДЦКД) – ингибитором мембрансвязанной части АТФ-азы митохондриальной мембраны с помощью зонда DSM [4-(p-диметиламиностирилил)-1-метил пиридиний], определяющего трансмембранный градиент электрического поля. ТЛЦ выделяли из тимусов по стандартной методике. Окрашенные ДСМ клетки исследовали на люминесцентном микроскопе («Люмам – Р 8», ЛОМО, Россия) с использованием термостатированного столика. В каждом препарате измеряли флуоресценцию 50-70 клеток и рассчитывали среднюю интенсивность флуоресценции ТЛЦ ($\tilde{F}$). В каждой флуоресцирующей клетке подсчитывали $n_{m/c}$. Статистическую обработку данных экспериментов проводили по коэффициенту корреляции рангов Спирмена.

Результаты и обсуждение

В серии экспериментов с ТЛЦ зарегистрировано снижение $n_{m/c}$ и $\tilde{F}$ после инкубации со всеми использованными ингибиторами, причем степень и скорость снижения этих параметров зависела от типа ингибитора и длительности инкубации. Максимальное снижение энергетики ТЛЦ достигалось при инкубации с ДНФ, после которого ЭПО не восстанавливает $\tilde{F}$ и $n_{m/c}$. После инкубации с ПХФ ЭПО восстанавливает ~20-23% $n_{m/c}$ и $\tilde{F}$. Реакция ТЛЦ на ДЦКД подтверждает важную роль АТФ-азы в поддержании мембранного митохондриального потенциала. После деэнергизации ТЛЦ под действием ДЦКД, ЭПО восстанавливает ~42% $n_{m/c}$ и ~38% $\tilde{F}$.

Заключение

ЭПО способен частично восстанавливать поляризацию мембран митохондрий в ТЛЦ, нарушенную в результате воздействия ингибитора АТФ-азы (ДЦКД).

Ключевые слова

Эритропоэтин, Т-лимфоциты, энергетическая активность, ингибиторы, потенциалчувствительный витальный флуоресцентный зонд-катион 4-(п-диметиламиностирил)-1-метилпиридиния (ДСМ).