Мембранная НАД(Ф)Н-оксидаза и гормональная регуляция функциональной активности иммунокомпетентных клеток

Н.Н. Вольский, В.О. Персиянова, В.А. Козлов

Институт клинической иммунологии СО РАМН, Новосибирск, Россия

Действие глюкокортикоидов на иммунокомпетентные клетки обычно объясняется их эффектами на транскрипцию определенных групп генов. Однако, описаны эффекты стероидов, не требующие участия генома и объясняемые прямым влиянием гормонов на клеточные мембраны, органеллы или ферментативные комплексы.

Ранее нами было показано, что глюкокортикоиды обладают прямым активирующим влиянием на НАД(Ф)Н-оксидазу плазматических мембран фагоцитирующих клеток - ферментный комплекс, восстанавливающий кислород с образованием супероксидного радикала (O_2^-) и других активных метаболитов кислорода (АКМ). Роль АКМ в регуляции клеточных функций хорошо известна, и естественно предположить, что обнаруженная нами активация НАД(Ф)Н-оксидазы может быть механизмом, опосредующим некоторые из глюкокортикоидных эффектов в иммунной системе. Одним из них может быть индуцированный глюкокортикоидами апоптоз лимфоцитов, так как установлена важная роль АКМ (особенно перекиси водорода) в процессах апоптоза, вызванного самыми разнообразными агентами. В данной работе исследована роль повышенной активности НАД(Ф)Н-оксидазы в дексаметазон-индуцированном апоптозе лимфоцитов.

Установлено, что при действии на тимоциты мыши дексаметазон вызывает существенное увеличение продукции O_2^- клетками в первые минуты после добавления гормона. С другой стороны, удаление образующейся перекиси водорода из среды инкубации клеток с помощью каталазы приводило к подавлению апоптоза тимоцитов, развивающегося под влиянием дексаметазона и измеренного двумя общепринятыми методами в различные сроки инкубации клеток с гормоном. Снижение концентрации O_2^- в среде инкубации с помощью супероксиддисмутазы такого эффекта не давало. Эти результаты свидетельствуют о необходимости повышенной концентрации перекиси водорода для инициации глюкокортикоид-индуцированного апоптоза лимфоцитов и подтверждаются литературными данными, говорящими об индукции апоптоза лимфоцитов экзогенно добавленной перекисью водорода.

Роль активации НАД(Ф)Н-оксидазы в глюкокортикоид-индуцированном апоптозе лимфоцитов доказывается результатами экспериментов, в которых использовались известные ингибиторы этого фермента. Полученные данные показали, что дифениленіодониум практически полностью подавляет индуцированный дексаметазоном апоптоз тимоцитов при добавлении его к клеткам в концентрации 2 μM, которая вызывает ингибирование активности НАД(Ф)Н-оксидазы. Сходный, хотя и менее выраженный, эффект оказывал и другой ингибитор этого фермента - кромолин.

В совокупности полученные результаты доказывают ключевую роль активации НАД(Ф)Н-оксидазы в развитии глюкокортикоид-индуцированного апоптоза лимфоцитов и обосновывают участие АКМ-зависимых процессов в гормональной регуляции активности иммунокомпетентных клеток.

Ключевые слова: глюкокортикоиды, активные метаболиты кислорода, НАДФН-оксидаза, апоптоз, лимфоциты

Адрес для переписки: Н.Н. Вольский. Институт клинической иммунологии СО РАМН, ул. Ядринцевская 14, Новосибирск 630099, Россия. Эл. почта: ayvo@online.nsk.su

Membrane NADPH Oxidase and Hormonal Regulation of the Immunocompetent Cell Functional Activity

Nikolay N. Volsky, Vera O. Persijanova, Vladimir A. Kozlov

Institute of Clinical Immunology, Siberian Branch of Russian Academy of Medical Sciences, Novosibirsk, Russia

Effect of dexamethasone upon NADPH oxidase activity of murine thymocytes and the role of reactive oxygen species (ROS) were investigated in dexamethasone-induced thymocyte apoptosis. Hormonal stimulation of NADPH oxidase has been demonstrated in cell-free system (homogenate of thymocytes). The depletion of hydrogen peroxide, produced due to NADPH oxidase activity, with catalase addition has been shown to inhibit dexamethasone-induced thymocyte apoptosis. The key role of NADPH oxidase and NADPH oxidase-dependent ROS production in the development of lymphocyte apoptosis was confirmed in experiments with two inhibitors of this enzyme. It has been evidenced that both inhibitors used (diphenylene iodonium and cromolyn) violently inhibited glucocorticoid-induced lymphocyte apoptosis. The possibility of the direct extragenomic action of glucocorticoids upon ROS production in the cells is discussed, as well as the role of this effect in glucocorticoid-induced lymphocyte apoptosis.

INTRODUCTION

Glucocorticoids are natural physiological regulators of immune reactions. It was H. Selye who for the first time described the effect of adrenocortical hormones on lymphoid tissue as one of the major components of a general adaptation syndrome. Further investigations of this phenomenon have led to the emergence of the broad spectrum of natural and synthetic steroid substances that are used now in medical practice as anti-inflammatory and immunoactive drugs.

The mechanisms of glucocorticoid impact upon immunocompetent cells are reviewed in numerous papers. As a rule these mechanisms are pertinent to their thoroughly studied effects on the transcription of certain groups of genes. In line with that classical scheme of steroid hormone influence on cell functions glucocorticoids penetrate into cytoplasm and associate with specific receptor proteins. Then hormone-receptor complexes are transported into cell nucleus where they bind to certain promoter regions of steroid-responsive genes and finally induce/suppress the synthesis of functionally active proteins. This mechanism adequately accounted for various effects of hormones and, as a result, such an explanation nearly completely replaced previously supposed interpretations of glucocorticoid influences on cell functions. However, a great number of experimental evidences have appeared, indicating to the direct extragenomic effects of steroid hormones upon cell membranes, organelles or enzyme complexes.

Previously we have found [1, 2] the activating effects of natural and synthetic glucocorticoids (cortisol, dexamethasone, and prednisolone) on plasma membrane NADPH oxidase of phagocytic cells, an enzyme complex which reduces the oxygen molecules generating superoxide anions (O_2^-) and provides the «oxidative burst» in these cells. It has also been shown that hormonal effect stimulating O_2^- production is not mediated by gene

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Address correspondence to: Dr. Nikolay N. Volsky, Laboratory of Immunopoiesis Regulation, Institute of Clinical Immunology of SB RAMS, Yadrintsevskaya str. 14, Novosibirsk 630099, Russia. E-mail: ayvo@online.nsk.su

transcription and protein synthesis: this effect has been reproduced in free-cell system containing the membrane-bound NADPH oxidase and has been registered without any detectable lag-period during the first few minutes after hormone addition.

The important role of reactive oxygen species (**ROS**) in the regulation of cell functions (including the immunocompetent cell functional activity) is well known. Hence, it appears reasonable that the above-mentioned hormone-induced increase of O_2^- production (and, respectively, another ROS occurring as a sequence of O_2^- metabolism, particularly hydrogen peroxide) could mediate some of the glucocorticoid effects on the immune system. We propose that one of ROS-dependent effects may be the glucocorticoid-induced lymphocyte apoptosis which is now believed to be of primary importance in the lymphoid tissue lysis under stress. The basis for this assumption is a large body of data demonstrating the involvement of ROS (especially, hydrogen peroxide) in apoptosis development in different cell types in response to various agents/conditions (for overviews see [3-5]).

Therefore, the aim of this work is to study the role of the increased NADPH oxidase activity and NADPH oxidase-dependent ROS production in glucocorticoid-induced lymphocyte apoptosis.

MATERIALS AND METHODS

Mice

(CBA x C57Bl)F1 hybrids, males, aged 2 to 4 months old, were used throughout.

Reagents

Horse heart ferricytochrome *c*, bovine erythrocyte superoxide dismutase (**SOD**), 3-[4,5-dimethylthiasol-2-yl]-2,5-diphenyltetrazolium bromide (**MTT**) were obtained from Sigma, USA. Bovine liver catalase, NADPH were obtained from Serva, USA. Propidium iodide (**PI**), fluorescein diacetate (**FDA**), diphenylene iodonium (**DPI**) were obtained from ICN, USA. Dexamethasone sulfate was obtained from KRKA, Slovenia. RPMI 1640, Hanks' solution without phenol red, fetal calf serum were obtained from NPO Vector, Russia. All other reagents were of the best grade commercially available.

Cell Preparation

Murine thymuses were isolated and slightly homogenized in glass homogenizator in Hanks' solution and then filtered through a nylon screen. The cells were washed once with the same solution and resuspended in RPMI 1640 medium supplemented with 5% FCS, 2 mM glutamine and 50 μg/ml gentamicin. Cells were incubated in 96-wells plates (Linbro, UK) at 37°C in a humidified 5% CO_2 atmosphere.

NADPH Oxidase Assay

NADPH oxidase activity was determined by measuring the linear rate of SOD-inhibitable ferricytochrome *c* reduction at 550 nm in Hitachi-340 spectrophotometer [6]. The results were expressed in pmoles/min/10^7 cells, using absorption coefficient 21.1 $mM^{-1} \times cm^{-1}$.

Estimation of Thymocyte Apoptosis

Cells were treated with 100 μM of dexamethasone. Apoptosis intensity was measured during intervals indicated below. Probes without hormone simultaneously incubated under the similar conditions were used as a control. Catalase, SOD, DPI were added to cells simultaneously with dexamethasone, but cromolyn 30 min before the latter. Concentrations are indicated in figures. Apoptotic cells were identified by two standard methods:

1. MTT reduction test as described by Kazansky et al. [7]. MTT was added to cells at a time point, indicated as an estimation time. Then cells were incubated for 4 h and the intensity of color was measured at 570 nm using multiscan Titertek (Finland). Results were expressed in arbitrary units ($OD_{570} \times 10^3$).

2. Lymphocyte apoptosis was estimated by flow cytometric (FACScan, Becton Dickinson) quantification of FDA (0.2 mg/ml) and PI (4 mg/ml) staining [8]. Viable thymocytes were identified as FDA^+PI^-, apoptotic - as FDA^-PI^-, and dead - as FDA^-PI^+ positive cells. The percentage of positive cells was calculated as the number of positive lymphocytes per 2000 lymphocytes counted.

Statistics

Results are presented as mean±s.e.m. The significance of the differences between two sets of

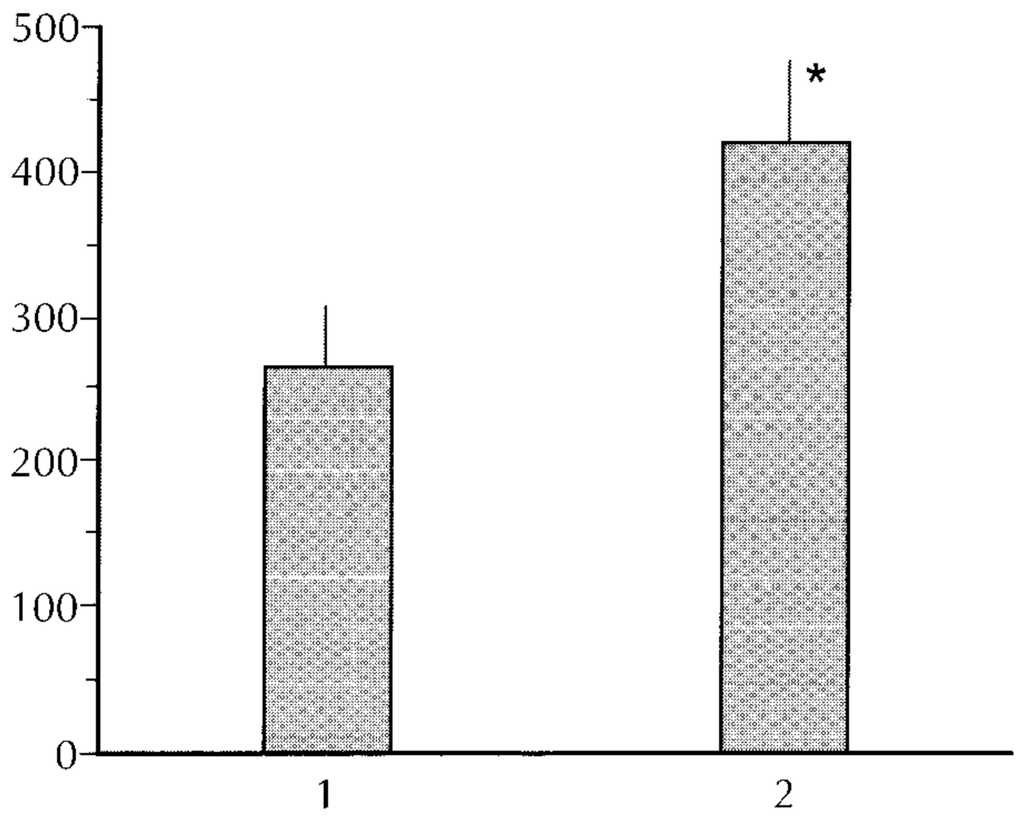


Figure 1. Effect of dexamethasone on NADPH oxidase activity in murine thymocyte homogenates. **Axis Y**: rate of superoxide-dependent cytochrome c reduction (pmoles/min/10^7 cells); (**1**) - control; (**2**) - with dexamethasone (100 μM). (*) difference from control is significant at $p<0.01$.

values was determined by the Mann-Whitney's non-parametric test.

RESULTS

Stimulating Effect of Dexamethasone upon NADPH Oxidase Activity

The addition of dexamethasone caused an increase in the rate of O_2^- production in thymus cell homogenate (**Figure 1**). The magnitude of this stimulating effect on NADPH oxidase activity was similar to that found by us previously in murine peritoneal macrophages and splenocytes (1.5-2.5-fold increase).

Inhibition of Dexamethasone-Induced Lymphocyte Apoptosis by Catalase

To establish the role of ROS in dexamethasone-induced thymocyte apoptosis, O_2^- and hydrogen peroxide were removed from cell incubation medium by means of specific enzyme addition (SOD, catalase). The intensity of thymocyte apoptosis was evaluated by MTT reduction test. MTT test is based on the MTT dye mitochondrial reduction which is sharply dropped in cells at early stages of apoptosis as compared to viable cells. Thus, the rate of MTT reduction is directly proportional to the number of viable non-apoptotic cells. As it is shown in **Figure 2**, the addition of catalase, the enzyme which removes generated hydrogen peroxide, results in a considerable increase of viable cell number at all time-points of incubation (from 4 to 24 h). The number of viable cells in catalase-treated cultures after 4 h of incubation was 2.5 times as much as in the probes incubated with dexamethasone alone. After 6-8 h of incubation the viable cell quantity was as much as 6-7 times as opposed to catalase-untreated cells. However, catalase does not inhibit hormone-induced thymocyte apoptosis completely. At late time-point of incubation (24 h) the number of viable cells in the probes with dexamethasone and catalase was less than in the control probes (without any additions). Besides, addition of catalase to dexamethasone-free probes prevents cells from spontaneous apoptotic death, which is in an agreement with anti-apoptotic effect of catalase reported earlier by other authors [9].

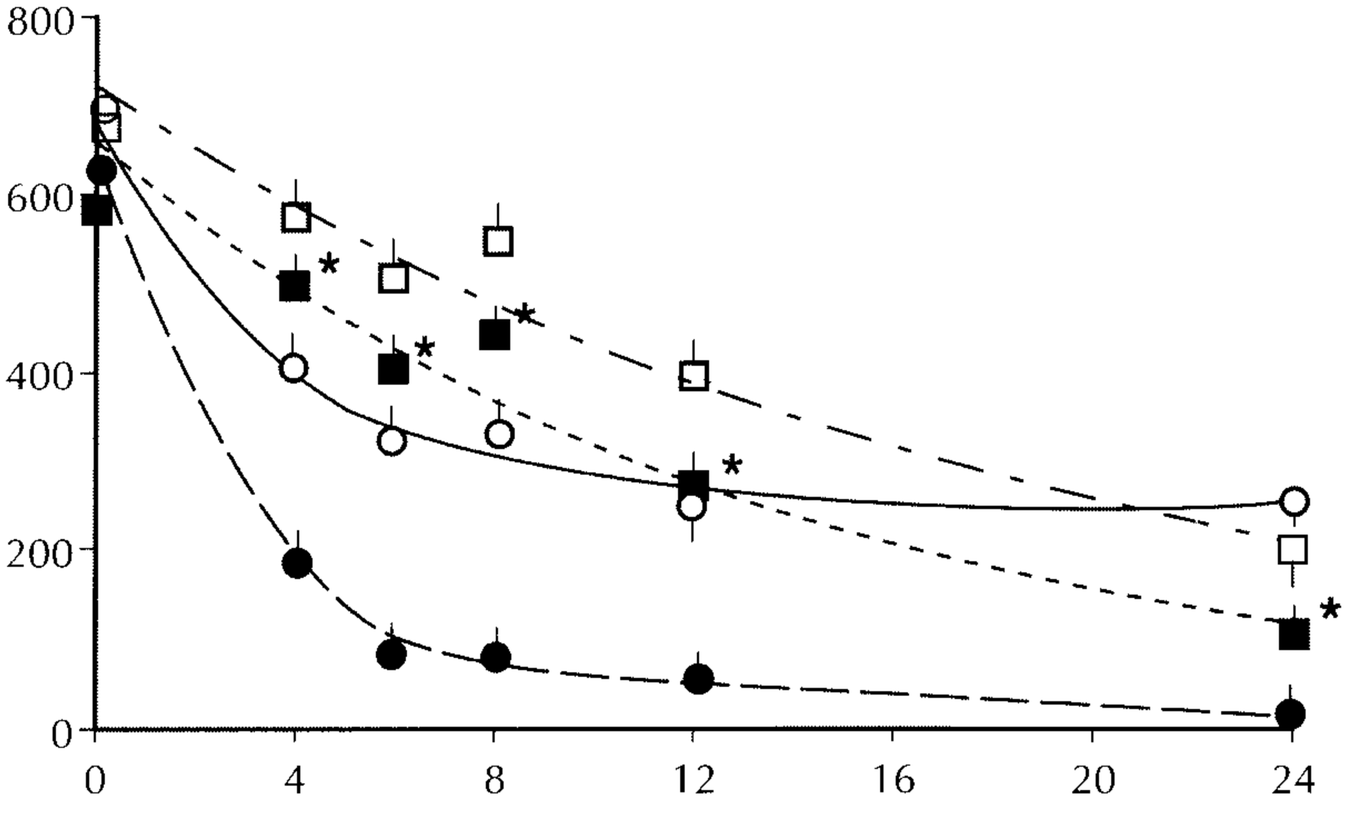
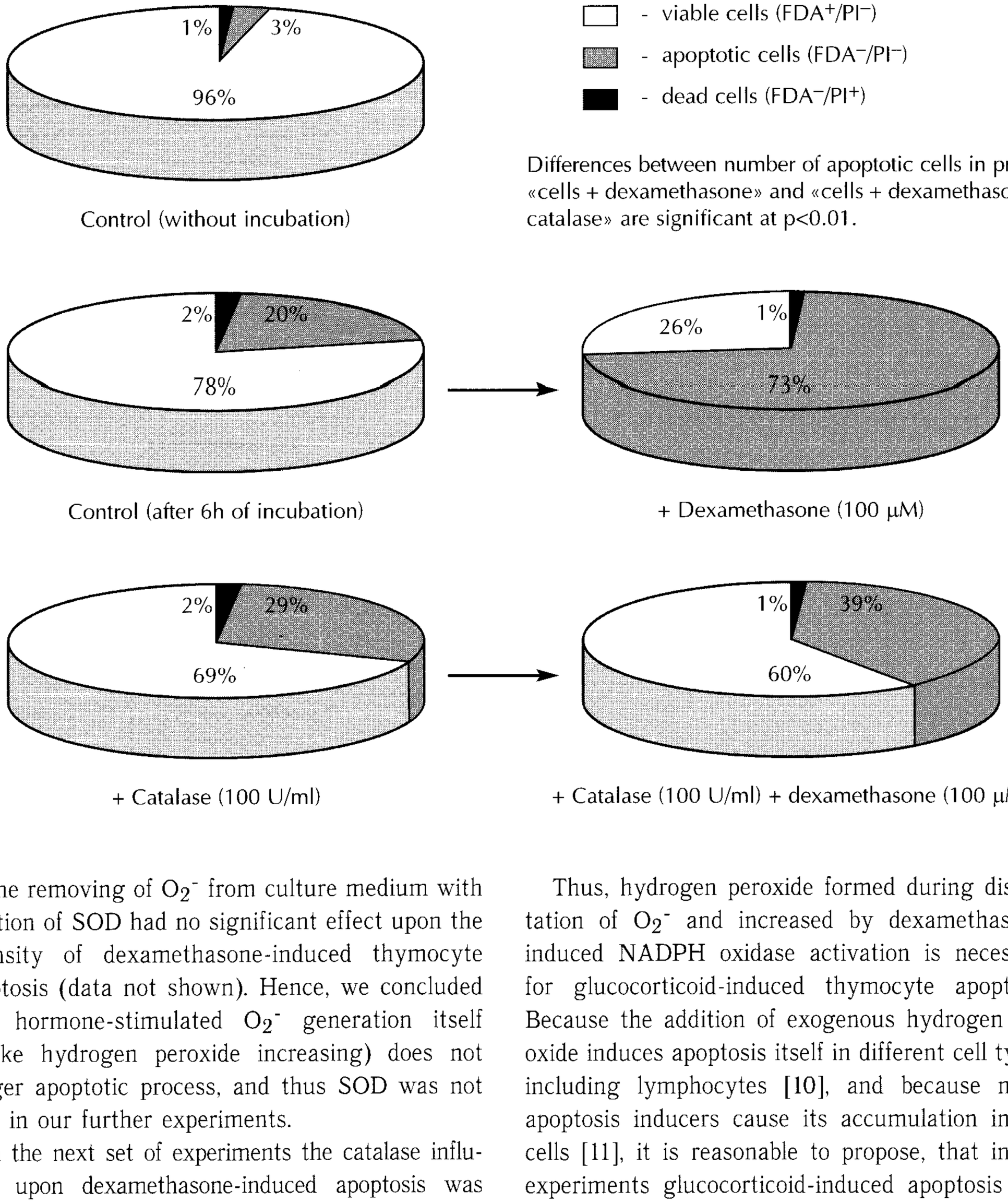


Figure 2. Effects of catalase upon spontaneous and dexamethasone-induced murine thymocyte apoptosis at different time intervals of cell incubation. **Axis X**: time intervals of cell incubation (h). **Axis Y**: rate of MTT reduction (OD x 10^3). (*) difference from corresponding probes with dexamethasone is significant at $p<0.001$.

- control (without addition)
- catalase (100 U/ml)
- dexamethasone (100 μM)
- dexamethasone + catalase

Figure 3. Effect of catalase upon dexamethasone-induced murine thymocyte apoptosis after 6 h of cell incubation



The removing of O_2^- from culture medium with addition of SOD had no significant effect upon the intensity of dexamethasone-induced thymocyte apoptosis (data not shown). Hence, we concluded that hormone-stimulated O_2^- generation itself (unlike hydrogen peroxide increasing) does not trigger apoptotic process, and thus SOD was not used in our further experiments.

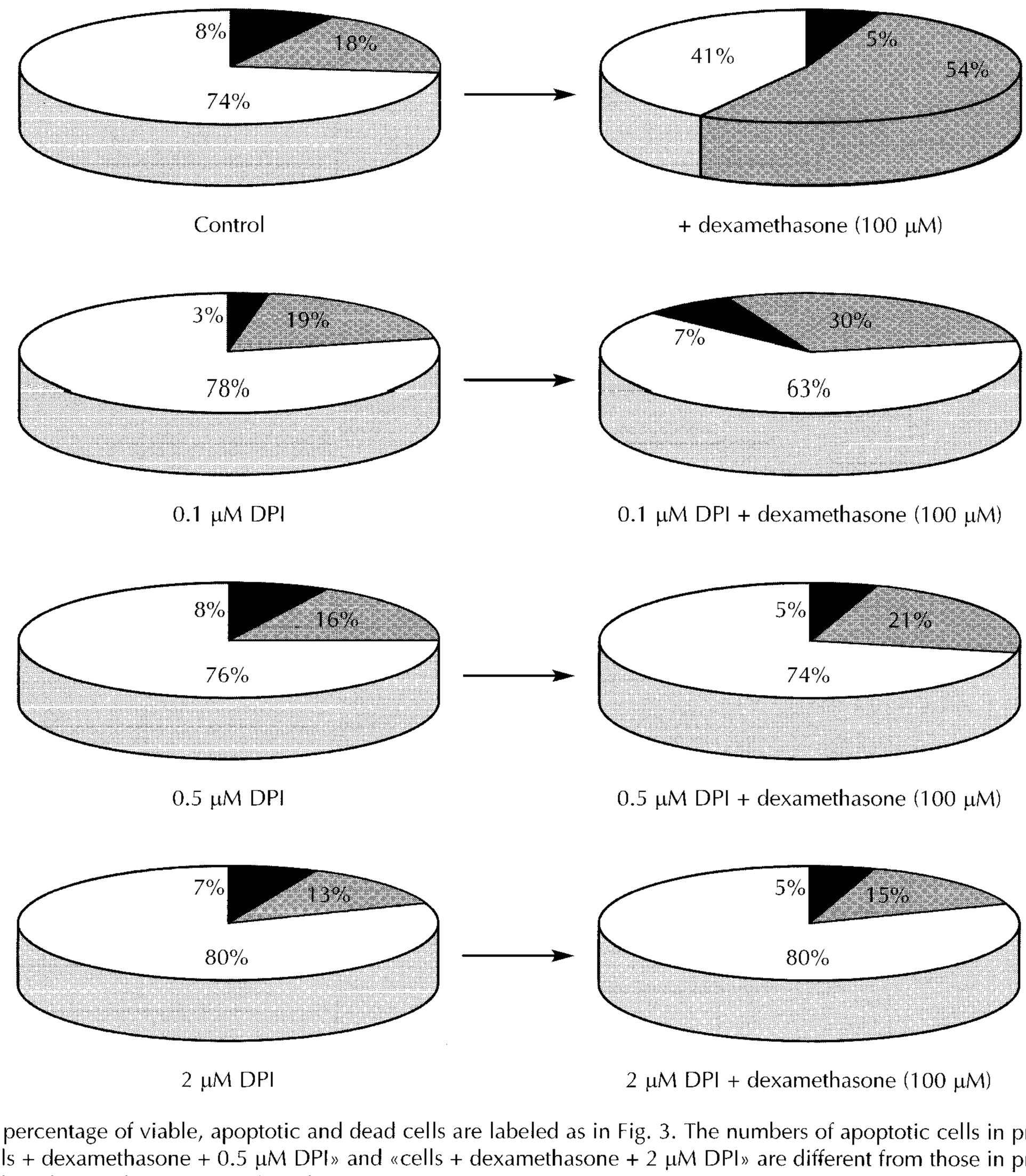
In the next set of experiments the catalase influence upon dexamethasone-induced apoptosis was measured with standard flow cytofluorimeter technique. This method allows to determine the number of viable, apoptotic and dead cells separately. Dexamethasone and catalase effects were assessed after 6 h of cell incubation. As it is demonstrated in **Figure 3** results obtained with this method correspond very closely to the data obtained by MTT technique: due to both methods catalase significantly inhibited hormone-induced apoptosis of thymocytes.

Thus, hydrogen peroxide formed during dismutation of O_2^- and increased by dexamethasone-induced NADPH oxidase activation is necessary for glucocorticoid-induced thymocyte apoptosis. Because the addition of exogenous hydrogen peroxide induces apoptosis itself in different cell types, including lymphocytes [10], and because many apoptosis inducers cause its accumulation in the cells [11], it is reasonable to propose, that in our experiments glucocorticoid-induced apoptosis was caused by hormonal activation of NADPH oxidase and stimulation of NADPH oxidase-dependent hydrogen peroxide accumulation.

Effects of NADPH Oxidase Inhibitors upon Lymphocyte Apoptosis

To confirm this assumption two additional sets of experiments were carried out with inhibitors of NADPH oxidase - DPI and cromolyn. As it is

Figure 4. Effects of various concentrations of DPI upon dexamethasone-induced murine thymocyte apoptosis after 6 h of cell incubation

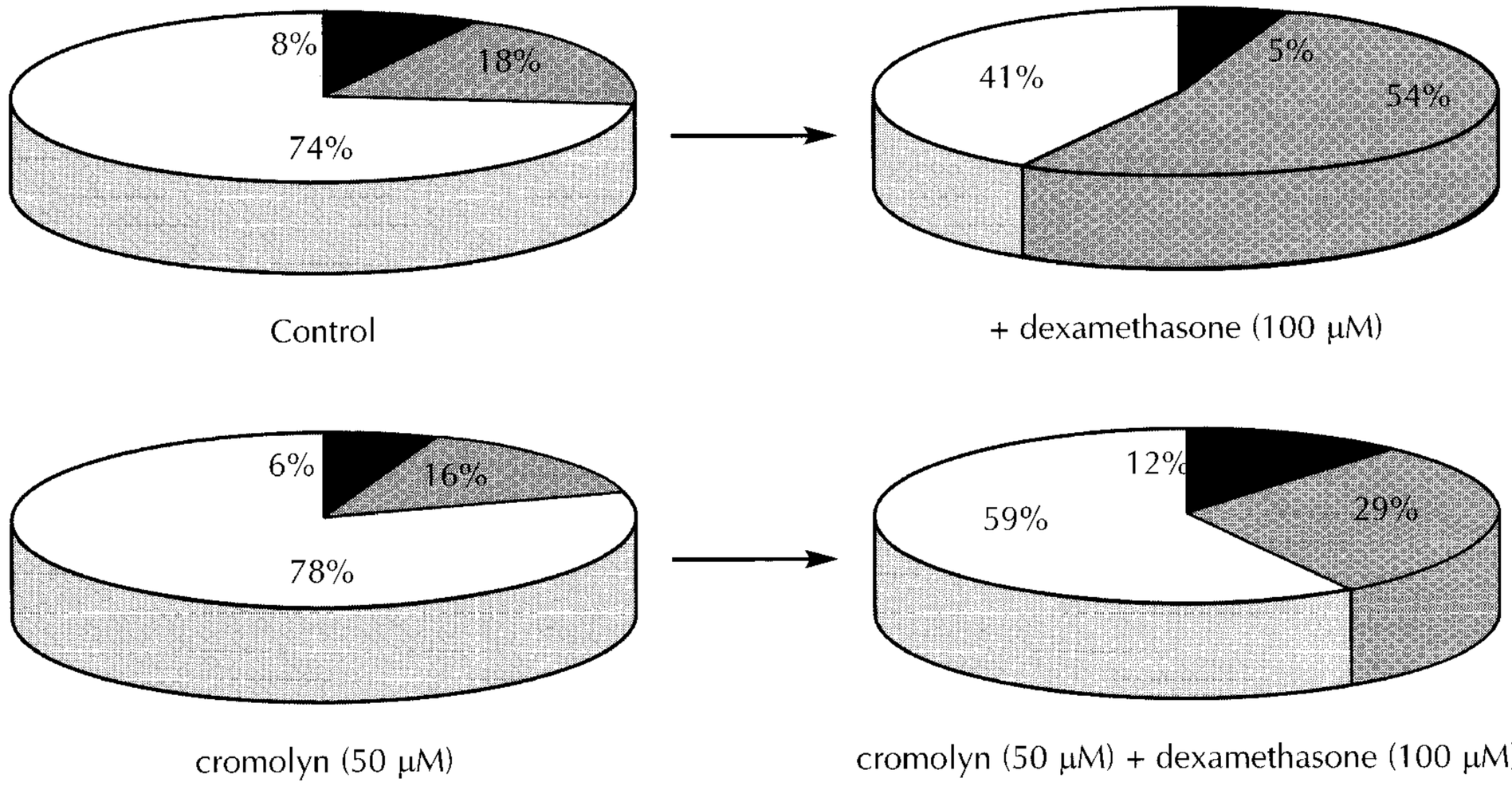


The percentage of viable, apoptotic and dead cells are labeled as in Fig. 3. The numbers of apoptotic cells in probes «cells + dexamethasone + 0.5 μM DPI» and «cells + dexamethasone + 2 μM DPI» are different from those in probes «cells + dexamethasone» significantly at $p<0.01$.

demonstrated in **Figure 4** the data obtained with DPI indicate that 1) DPI does not cause any evident effect on the number of apoptotic cells in hormone-free probes; 2) DPI potently inhibits dexamethasone-induced thymocyte apoptosis, 3) effect of DPI upon apoptosis is dose-dependent, 4) DPI abolishes dexamethasone-induced thymocyte apoptosis completely at 2 μM concentration.

In the 2nd set of experiments with NADPH oxidase inhibitors cromolyn was used which is the drug applied for bronchial asthma treatment. Antiasthmatic curative effect of cromolyn is attributed to its ability to inhibit NADPH oxidase-dependent ROS generation in human neutrophils [12]. In our

Figure 5. Effect of cromolyn upon dexamethasone-induced murine thymocyte apoptosis after 6 h of cell incubation



The percentage of viable, apoptotic and dead cells are labeled as in Fig. 3. Differences between number of apoptotic cells in probes «cells + dexamethasone» and «cells + dexamethasone + cromolyn» are significant at $p<0.01$.

preliminary experiments cromolyn (50 μM) has been shown to inhibit mouse thymus cell NADPH oxidase activity by 70-80% and abolish dexamethasone-induced stimulation of this activity. **Figure 5** shows that cromolyn (like DPI) considerably inhibits the dexamethasone-induced thymocyte apoptosis, although its effect is less pronounced than that of DPI.

Of importance, two different NADPH oxidase inhibitors cause principally the same effect upon dexamethasone-induced lymphocyte apoptosis, confirming the key role of this enzyme in glucocorticoid-mediated programmed cell death.

DISCUSSION

Stimulation of oxygen uptake in phagocytic cells induced by glucocorticoids was described long ago, for the time when «oxidative burst» phenomenon had not yet been bound up with NADPH oxidase activity and ROS generation. One of the earliest evidence of this glucocorticoid effect was obtained in 1968 by I.V. Scards [13] in experiments on guinea pig leukocytes. Today's literature data on this score are contradictory and describe both inhibition and stimulation of ROS production caused by glucocorticoids [4, 14-16]. Some of these discrepancies may be explained by a marked phase character of glucocorticoid effect *in vivo*: as we have shown earlier [1] NADPH oxidase activity was significantly increased in mouse spleen phagocytic cells in 2-3 h after intraperitoneal dexamethasone injection, but 12-20 h later its activity was decreased again to the level lower than that of the appropriate control. As for the works where inhibition of ROS production has been registered during direct addition of glucocorticoids to the cells *in vitro*, these data could possibly be accounted for different experimental models and conditions.

The vast majority of experimental investigations of hormonal effects are based on the assumption that induction of apoptosis by glucocorticoids is mediated through their influence on gene transcription. A set of proteins which synthesis is induced in the cells by hormones has been found. It remains however unclear what of these proteins is responsible for initiation of programmed cell death [17]. It has been suggested that one of such plausible mediators may be calmodulin [17], since glucocorticoids are known to cause a pronounced increase of Ca^{2+} concentration in lymphocytes,

whereas initiation of apoptosis is associated with high Ca^{2+} concentration. On the other hand, it was demonstrated in many works that Ca^{2+}-dependent apoptosis is accompanied by the increase of ROS concentrations in the cells [16]. Thus, the question of the role of Ca^{2+} and ROS in glucocorticoid-induced apoptosis remains open. Likewise is the situation with the another possible mediator - p53 protein. In the cells undergoing p53-mediated apoptosis ROS production is increased concomitantly with p53 overexpression, whereas in the cells resistant to p53 this effect is absent [18].

Similarly to glucocorticoid-induced ROS production, glucocorticoid-induced apoptosis seems to be initiated directly, but not *via* hormone-mediated transcription of certain genes. For instance, the inhibitory effect of RU486 compound upon glucocorticoid-induced apoptosis of lymphocytes is often accounted for some transcriptional events [17]. At the same time considering, that RU486 is a competitive inhibitor of hormone binding to cytoplasmic receptors, it is quite conceivable that it can also inhibit the binding of glucocorticoids with any targets, including those ones that are directly coupled with NADPH oxidase - an enzyme complex involved from our viewpoint in apoptosis initiation.

Indeed, ROS generation appear to be necessary for the development of glucocorticoid-induced apoptosis. For example, the increase of ROS concentration in the cells after exposure to hormone has been reported recently [4, 16]. It was also demonstrated that hyperoxia (95% O_2 atmosphere) increases, while hypoxia (< 5% O_2) inhibits glucocorticoid-induced thymocyte apoptosis [19]. In this model of apoptosis anti-apoptotic effects of the exogenously added antioxidants were observed [20]. The similar effects of Bcl-2 [20] and thioredoxin [21] overexpression demonstrated in the cells transfected with their genes are connected most likely with antioxidant properties of these proteins. It was supposed [20] that initiation of lymphocyte apoptosis by glucocorticoids may be attributed to down-regulation of antioxidant protective enzymes since their activity is decreased at the early stages of hormone-induced apoptosis. Our results allow us to suggest the more direct effect of glucocorticoids upon ROS metabolism in cells.

Two standard techniques to evaluate the quantity of apoptotic lymphocytes were used in most part of our experiments: flow cytofluorimeter method with FDA and PI and MTT reduction method. In preliminary part of this study (estimation of dexamethasone-induced thymocyte apoptosis and of anti-apoptotic effect catalase) [22] the validity of the data was confirmed also with method of DNA electrophoresis in agarose gel.

Dexamethasone was used in our experiments at the concentration of 100 μM. Such a concentration is pharmacological and excessive for thymocyte apoptosis induction, because apoptogenic effect may be obtained at hormone concentration two order magnitude lower. Indeed, as we found in preliminary experiments, and in accordance with literature data, some enhancement of the number of apoptotic cells is already observed at dexamethasone concentration 0.1 μM. Intensity of apoptosis reached its maximum at the concentration of 1 and 10 μM, and further increase of hormone concentration up to 100 μM did not lead to apoptosis enhancement. Nonetheless, due to the relatively low sensitivity of the used method the stable stimulation of NADPH oxidase activity could be only registered with high dexamethasone concentration. So, high concentration of dexamethasone was selected to estimate both apoptosis intensity and NADPH oxidase activity.

Practically all ROS production observed in our experiments may be attributed to the NADPH oxidase activity of macrophages and other phagocytic cells present in the thymus cell suspension. Lymphocytes generate O_2^- in insignificant amounts, and therefore we can not estimate hormone-caused changes of this parameter in lymphocytes. At the same time we register the final effect of hormone, apoptosis induction, exactly in lymphocytes. Actually, on one hand catalase and NADPH oxidase inhibitors decrease accumulation of hormone-induced phagocyte-derived ROS (particularly hydrogen peroxide), and on the other hand; the same agents inhibit hormone-induced lymphocyte apoptosis.

There are two possible suppositions in order to unite these two parallel and linked phenomena. Firstly, all cells but not only professional phago-

cytes and some other cell types, in which ROS production is registered, contain NADPH oxidase-like enzyme complex which is able to reduce oxygen in cell membrane. However, such enzyme activity is so small in most of the cells, including lymphocytes, that it can be registered only by more sensitive special methods [23]. For instance, glucocorticoid-induced enhancement of ROS concentration in lymphocytes has been demonstrated using sensitive cytofluorimetric assay [4, 16]. It is natural to suggest that hormone-induced NADPH oxidase activation and hydrogen peroxide accumulation occur in lymphocytes in the manner principally similar to that in phagocytes. The same holds true for the effects of catalase and NADPH oxidase inhibitors.

Secondly, it may be presumed that hormone-induced increase of hydrogen peroxide concentration in lymphocytes may be inadequate to initiate apoptosis in these cells. If so, macrophages could be the target cells for hormone, that produce enough amount of hydrogen peroxide which in turn affect immediately upon the adjacent lymphocytes initiating their apoptosis. Such a stimulation of T lymphocyte apoptosis by glucocorticoids acting *via* phagocytic cells has been demonstrated in experiments with astrocytes [24].

Our results giving an evidence in favor of the key role of NADPH oxidase and ROS generation in the glucocorticoid-induced apoptosis of lymphocytes are in consistence with the literature reports for the impact of another steroid hormone - dehydroepiandrosterone (**DHEA**) on this process. As well as glucocorticoids, DHEA is considered now to be as physiologic regulator of immunocompetent cell functions. In many actions upon immune system DHEA is a functional antagonist of glucocorticoids. It has long been known that DHEA can inhibit glucocorticoid-induced apoptosis of lymphocytes. Under this DHEA does not compete with glucocorticoids for any target and its addition to cells immediately before glucocorticoids does not influence the intensity of glucocorticoid-induced apoptosis. But DHEA injection into animals one day before lymphocyte isolation leads to their resistance to glucocorticoid-inducing apoptosis [25]. On the other hand, in independent studies [26, 27] DHEA was demonstrated to inhibit the activity of glucose-6-phosphate dehydrogenase (a key enzyme of hexose-monophosphate shunt) and, as a consequence, to decrease NADPH concentration in cells. A deficiency of NADPH leads in turn, to a fall of ROS production in the cells in response to stimulation [27]. These data taken together with ours permit us to suggest that antagonistic effects of DHEA and glucocorticoids may be explained at least in part by the opposite influences of these hormones upon the amount of the NADPH oxidase-generated ROS.

Another particularly interesting example of the functional antagonism between DHEA and glucocorticoids is their opposite influence upon the balance between Th1 and Th2 cells [28]. Glucocorticoids are known to produce shift in this balance to Th2 cells. This shift explains the variety of their hormonal effects on the immune and inflammatory reactions. DHEA prevents these glucocorticoid effects and shifts the Th1/Th2 balance to the opposite pathway leading to a predominance of Th1 cells. It is established now that macrophages are the primary site of DHEA action. In these cells a specific enzyme is located which is able to convert an inactive transport form of hormone (DHEA sulfate) to a hormonal active DHEA [28]. In this case one also can not rule out, that the opposite effects of glucocorticoids and DHEA are due to their influences on the NADPH oxidase activity and on the intensity of ROS-dependent processes.

Thus our results confirm literature data on the involvement of ROS (particularly peroxides) in glucocorticoid-induced lymphocyte apoptosis and account for this hormone effect by direct impact upon NADPH oxidase. One could not exclude, that other steroid and possibly non-steroid hormones (for instance thyroxin) exert their effects upon immunocompetent cells through this mechanism as well.

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