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Polyamine Oxidase Activity in Lymphoid Tissues of Glucocorticoid-Treated Rats

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ABSTRACT. Glucocorticoids are known to negatively affect lymphoid tissues, in which they cause programmed cell death. Polyamine depletion, which occurs in glucocorticoid-treated animals by inhibition of biosynthesis and induction of acetylation, may represent a signal to thymocytes for progression into the apoptotic program. Since catalysis of polyamines by the catabolic pathway produces hydrogen peroxide as a by-product, it has been suggested that the apoptotic process may be, in part, due to oxidative stress as a result of hydrogen peroxide production. In order to verify whether polyamine oxidase (EC 1.5.3.11) may play a role in the process, we examined the activity of the enzyme in the thymus and spleen of glucorticoid-treated rats. We administered dexamethasone (4 mg/kg) or two different doses of corticosterone (4 mg/kg or 30 mg/kg) to rats, which were killed 8 or 24 hr after hormone injection. We found that corticosterone and dexamethasone affected polyamine oxidase activity in both tissues, with an opposite dose-dependent effect of the natural hormone in the thymus. The decrease and increase in polyamine oxidase after the two doses of corticosterone were correlated with the absence and the occurrence of DNA fragmentation, respectively. Moreover, corticosterone affected polyamine oxidase activity earlier (8 hr) than dexamethasone (24 hr), but the synthetic hormone was more efficient than the natural hormone in thymic polyamine depletion. The polyamine oxidase response may represent an important event in lymphoid tissues after glucocorticoid treatment, suggesting a role of the enzyme in the catabolic effects exerted by the two hormones. BIOCHEM PHARMACOL **58**;12:1907–1914, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. Polyamine oxidase; spermidine/spermine N^1-acetyltransferase, enzymes of the catabolic pathway of polyamines; glucocorticoids, effect on polyamine catabolism; thymus; spleen; rat

The naturally occurring polyamines putrescine, spermidine, and spermine are essential for cell proliferation [1, 2]. Their intracellular levels are strictly regulated by the biosynthetic decarboxylases ODC† and *S*-adenosylmethionine decarboxylase, whereas the catabolic pathway is controlled by the activities of SAT (EC 2.3.1.57) and PAO (EC 1.5.3.11) [2–4]. PAO efficiently transforms N^1-acetyl derivatives of spermidine and spermine into putrescine and spermidine, respectively, plus acetamidopropanal and hydrogen peroxide [3, 4], and plays a fundamental role in cell physiology, although its functions have not yet been elucidated [4]. However, current evidence suggests that polyamines play a pivotal role not only in cell proliferation and differentiation but also in apoptosis [reviewed in 5]. Polyamine depletion brings about significant changes in chromatin and DNA structure and leads cells to be more sensitive to DNA-binding antitumor agents [6] and to chemical toxins [7]. The depletion of intracellular polyamine levels and abnormal polyamine metabolic enzyme activity have been reported in cells undergoing programmed cell death [8–12], and a superinduction of SAT by polyamine analogues has been found to be associated with analogue-induced DNA and nuclear fragmentation [2, 13]. Moreover, inhibition of PAO, and subsequent reduced hydrogen peroxide production, significantly reduced apoptosis, thus suggesting a role of PAO in the process [13], although inhibition of PAO has not always been effective in protecting against apoptosis [9, 14]. However, using a different approach, and in another type of cultured cells, a role of PAO in initiation of apoptosis has recently been confirmed [15].

Glucocorticoids have been shown to exert myriad effects on immune system function, in which the thymus gland plays a central role [reviewed in 16]. It has long been known that adrenalectomy results in thymic enlargement and that stress causes thymic atrophy [16], and the thymolytic effects of corticosteroids are among the best described and understood effects of the steroids [17]. Although to a different degree, glucocorticoid effects on polyamine metabolism in lymphoid organs seem to be similar to those generally exerted by polyamine analogues, with a decrease in biosynthesis and an increase in SAT activity. In fact, depletion of the polyamines by down-regulation of the biosynthetic enzymes [18–20] and up-regulation of the catabolic pathway [8, 20] produces an intracellular polyamine depletion

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† *Abbreviations:* ODC, ornithine decarboxylase; PAO, polyamine oxidase; and SAT, spermidine/spermine N^1-acetyltransferase.

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that may result in greater accessibility and susceptibility of DNA to fragmentation, which is an essential part of programmed cell death [13].

It has been shown that PAO plays a role in apoptosis induced by different agents [13, 14], and an induction of SAT activity associated with DNA fragmentation has been observed in dexamethasone-treated thymocytes [8]. The aim of our study was to verify whether glucocorticoid-induced apoptosis in rat thymus may be mediated by changes in PAO activity. In fact, dexamethasone-induced apoptosis is inhibited by interleukin-2 [21]. Since polyamine oxidation down-regulates, and inactivation of PAO enhances interleukin-2 production [22, 23], it has been suggested that PAO may play an important role in the process, together with a general function in cellular immune defense mechanisms [3]. We determined PAO activity in thymus and spleen after administration to rats of the natural glucocorticoid corticosterone or the synthetic hormone dexamethasone, both of which have been reported *in vivo* to inhibit polyamine biosynthetic enzymes [18–20] and induce SAT activity [20, 24] in lymphoid organs. Since low corticosterone concentrations exert stimulatory effects on immune response in contrast to higher concentrations which are suppressive [25], in this study corticosterone was administered at two different doses, the lower dose not being tested for effects on polyamine metabolism. We also determined the changes in SAT activity and polyamine contents after the same treatments. Since antioxidant defences decrease following glucocorticoid treatment [26], catalase activity was also measured in our samples in an attempt to correlate the decrease with changes in polyamine metabolism.

MATERIALS AND METHODS

Chemicals

[acetyl-1-^{14}C]Acetylcoenzyme A (58 mCi/mmol) was purchased from Amersham International. All other chemicals were purchased from Sigma Chemical Co., Merck, or Boehringer-Mannheim.

Animals and Treatments

All experiments were conducted on experimental animals in accordance with guidelines described in the NIH Guide for the Care and Use of Laboratory Animals. Male Wistar rats (130–140 g) (Charles River) were used in all experiments. They were housed under 12-hr light/12-hr dark controlled lighting conditions with free access to water and a standard rat chow diet. Corticosterone-21-acetate (4 mg/kg or 30 mg/kg) or dexamethasone-21-acetate (4 mg/kg), dissolved in saline plus ethanol (30% v/v), was injected i.p. between 9:30 and 10:00 a.m.. The rats were killed 8 or 24 hr after hormone or vehicle administration, and thymus and spleen were rapidly excised, frozen with solid CO_2, and stored at −80° until enzyme or polyamine assays were performed.

Assay of PAO Activity

PAO activity in homogenates was assayed by the method of Suzuki *et al.* [27], which measures the H_2O_2 formed due to the oxidation of N^1-monoacetylspermine by converting homovanillic acid into a highly fluorescent compound in the presence of horseradish peroxidase. Assay blanks were carried out without substrate. The incubations were performed in the presence of pargyline and aminoguanidine to inhibit monoamine oxidases and diamine oxidases present in the homogenate. The reaction was corrected for quenching by the addition of 1–2 nmol of H_2O_2 in place of the substrate. Fluorescence was measured at 323 nm excitation and 426 nm emission. However, according to the recent suggestion by Morgan [28], since aminoguanidine and pargyline are not completely discriminatory nor specific for amine oxidases, in some samples we also performed the enzyme assays without inhibitors to ensure that all the PAO activity present was measured.

Assay of SAT Activity

SAT activity was performed as previously described [29]. In brief, the cytosolic extract resulting from 1-hr centrifugation at 100,000 × *g* was used as a source of enzyme, and the activity was measured at 30° with spermidine as a substrate. The conditions were such that the activity was proportional to the amount of added protein and the time of incubation. Each enzyme determination was performed in triplicate, and the blanks were incubated in the absence of spermidine. Enzyme activity was expressed as units per mg of added protein where 1 unit is equal to the production of 1 pmol/min of acetylated spermidine. Protein content was determined by the method of Geiger and Bessman [30], using BSA as standard.

Polyamine Analysis

Tissue samples were homogenized in 0.2 N perchloric acid and deproteinized. Aliquots of the supernatant were used for the determination of polyamines by HPLC with fluorescence detection according to the method of Löser *et al.* [31]. Polyamine levels were then calculated based on external standard curves run within 24 hr of the sample chromatograms.

Assay of Catalase Activity

The samples were homogenized in 50 mM potassium phosphate buffer, pH 7.0, containing 1% Triton X-100 and centrifuged for 5 min at 2000 × *g* at 4°. Aliquots of the supernatants were adjusted to a final concentration of 1% ethanol and stored for 10 min on ice to ensure full enzyme activation. After dilution to a volume of 2 mL with potassium phosphate buffer, the reaction was started by adding 1 mL of 30 mM H_2O_2. Catalase activity was determined at room temperature by the initial rate of

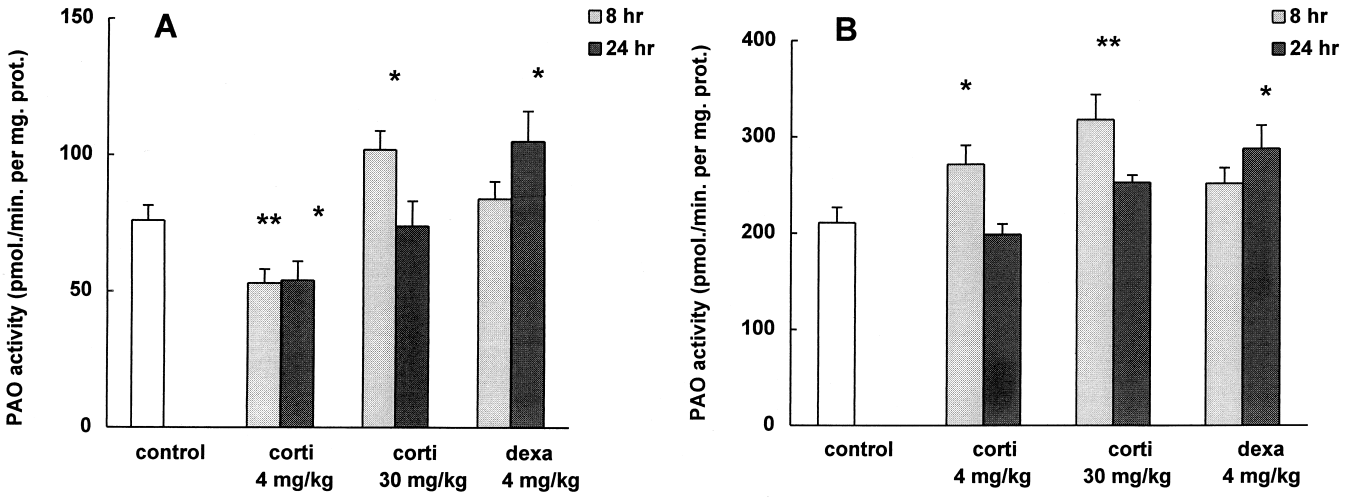

FIG. 1. Effect of corticosterone (corti) and of dexamethasone (dexa) on PAO activity in rat thymus (panel A) and spleen (panel B). Data are the means ± SEM of 4–6 rats per group. *$P < 0.05$; **$P < 0.01$ or less vs control group.

decomposition of H_2O_2 as measured by the decrease in absorbance at 240 nm [32]. Catalase activity was expressed as units per mg of protein, where 1 unit of enzyme decomposes 1 μmol of H_2O_2 per min at pH 7.0 and 25°.

Analysis of DNA Fragmentation

In order to measure the formation of DNA fragmentation ladders, tissues were prepared and analyzed by agarose gel electrophoresis according to the method described by Perego *et al.* [33]. Equal amounts of DNA (40 μg) were subjected to electrophoresis on 2% agarose gels containing 0.1 μg/mL ethidium bromide.

Statistical Analysis

One-way analysis of variance (ANOVA) was employed to determine differences between groups. A value of $P < 0.05$ was considered statistically significant.

RESULTS

The effect of treatment with glucocorticoids on PAO activity in rat lymphoid organs is presented in Fig. 1. At the low dose, the natural hormone decreased thymic PAO activity at 8 and 24 hr after treatment with respect to controls ($P < 0.01$ and $P < 0.05$, respectively) (see Fig. 1, panel A). We were sure to have measured all the enzyme activity present in our samples since the same decrease was obtained when we assayed PAO activity in the absence of the inhibitors pargyline and aminoguanidine, which would inhibit some of the polyamine-oxidizing activity (data not shown). By using a higher dose of corticosterone, i.e. 30 mg/kg, we found the opposite effect on thymic PAO activity, with an increase at 8 hr ($P = 0.01$). The treatment with dexamethasone also increased thymic PAO activity ($P = 0.02$), but the effect was found later (24 hr) (see Fig. 1, panel A). As regards the spleen, in the same Fig. 1 (panel B) we found an increase in PAO activity with all the treatments, but at different times regarding the hormone tested for the low or the high dose of corticosterone ($P < 0.05$ or $P = 0.005$, respectively) and for dexamethasone ($P < 0.05$). Both in thymus and in spleen, the natural hormone affected PAO activity earlier (8 hr) than did dexamethasone (24 hr). The effect of glucocorticoids on PAO activity in lymphoid tissues seemed to be specific, because the activity of the enzyme tested in other organs (such as heart and brain, which were taken concomitantly from the same animals) was not significantly modified by the hormonal administration (data not shown).

To confirm our [24] and other [20] data on the effect of glucocorticoids on the activity of the other enzyme of the interconversion pathway of polyamines in lymphoid organs, SAT activity was measured in the same rats. As shown in Fig. 2, we found an increase in thymic enzyme activity with all the treatments, with dexamethasone and the higher dose of corticosterone more effective than the lower dose of the natural hormone 8 hr after administration (see Fig. 2, panel A). Moreover, the effect of dexamethasone and of the high dose of corticosterone persisted up to 24 hr after treatment (Fig. 2, panel A). In the spleen, we found a marked increase in SAT activity 8 hr after dexamethasone administration, as previously reported [18], and a marked and persistent increase after the high dose of corticosterone ($P < 0.005$) (see Fig. 2, panel B). The low dose of the hormone decreased splenic SAT activity at 24 hr after administration.

The results on enzyme activities were compared with the polyamine levels. As shown in Table 1, administration of corticosterone decreased the thymic level of spermidine and spermine at 8 hr after administration of the low dose ($P < 0.05$), and of spermidine at 8 and 24 hr ($P < 0.002$) when injected at the high dose. Dexamethasone markedly decreased all the polyamines ($P < 0.002$ for putrescine and spermine, $P = 0.01$ for spermidine) at 24 hr after administration. Table 2 reports the effect of glucocorticoids

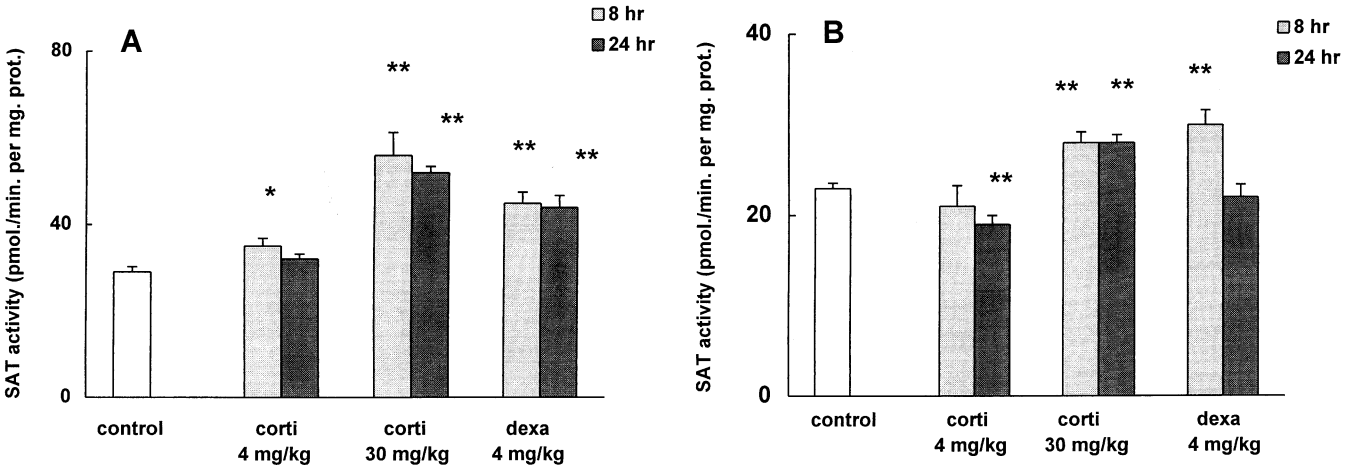

FIG. 2. Effect of corticosterone (corti) and of dexamethasone (dexa) on SAT activity in rat thymus (panel A) and spleen (panel B). Data are the means ± SEM of 4–6 rats per group. *$P < 0.05$, **$P < 0.005$ vs control group.

on polyamine levels in spleen. Neither dose of corticosterone modified polyamine levels at any time tested. In contrast, in the spleen, dexamethasone also decreased putrescine and increased spermidine levels ($P < 0.05$) at 24 hr after administration. Our results on splenic polyamine levels are consistent with the possibility that the hormone also inhibited the key enzyme of the biosynthetic pathway of polyamines, ODC, as was previously reported [20]. The effects of glucocorticoids on PAO activity and polyamine levels were accompanied by a negative effect of the hormones on cell growth of the lymphoid tissues. In fact, the treatment with dexamethasone caused a marked decrease in the weight of the thymus, particularly evident at 24 hr after administration (about 50% of the controls), whereas corticosterone had little, if any, effect on thymus weight (about 20% at the higher dose). A small decrease (about 20%) in the weight of the spleen was observed at 24 hr after dexamethasone treatment. Thus, the values of enzyme activity were similar irrespective of whether they were related to protein or total organ in the case of the spleen. However, since the thymus changes in size due to the treatment, we observed a general decrease in terms of enzyme activity per thymus. A similar pattern of diamine oxidase activity after dexamethasone treatment has been shown in the thymus [34].

In order to confirm the apoptotic effect exerted by glucocorticoids, we performed DNA analysis in thymus of rats treated with hormones, and the results are reported in Fig. 3. The formation of DNA fragmentation ladders was evident after dexamethasone (see Fig. 3, lane 5), and to a lesser degree after corticosterone at the dose of 30 mg/kg (Fig. 3, lane 4), confirming that the treatments were effective in inducing the apoptotic process. In contrast, the low dose of corticosterone did not induce significant DNA fragmentation in our samples (Fig. 3, lane 3). Since treatment of lymphocyte cell lines with dexamethasone lowers the antioxidant defences [26, 35], we also determined catalase activity to demonstrate a possible correlation with changes in PAO activity after glucocorticoid treatment. A decrease in catalase activity, particularly marked after dexamethasone treatment, was found in the thymus and the spleen (see Table 3).

DISCUSSION

The induction of cell death in lymphoid cells by injection of pharmacological doses of exogenous steroids is one of the earliest and most thoroughly studied models of apoptosis [16, 36]. However, there is evidence that apoptosis can also be induced by elevating, with stressors, endogenous corti-

TABLE 1. Polyamine levels in rat thymus after glucocorticoid treatments

	Time after Treatment (hr)	Polyamine levels (nmol/g tissue)		
		Putrescine	Spermidine	Spermine
Vehicle	0	57.1 ± 4.34	104.9 ± 5.40	278.5 ± 5.72
Corticosterone, 4 mg/kg	8	66.6 ± 9.98	86.5 ± 3.33*	209.2 ± 21.89*
Corticosterone, 4 mg/kg	24	63.2 ± 9.81	92.5 ± 4.24	242.4 ± 21.5
Corticosterone, 30 mg/kg	8	52.9 ± 7.00	80.0 ± 2.15†	296.5 ± 25.39
Corticosterone, 30 mg/kg	24	52.1 ± 2.61	83.4 ± 0.40†	265.5 ± 13.19
Dexamethasone, 4 mg/kg	8	66.3 ± 2.68	98.4 ± 6.45	299.7 ± 25.6
Dexamethasone, 4 mg/kg	24	22.4 ± 5.65†	81.3 ± 5.22*	149.1 ± 12.86†

Data are the means ± SEM of triplicate determinations of 4–6 rats per group.
*$P < 0.05$; †$P < 0.002$ versus control group.

TABLE 2. Polyamine levels in rat spleen after glucocorticoid treatments

	Time after Treatment (hr)	Polyamine levels (nmol/g tissue)		
		Putrescine	Spermidine	Spermine
Vehicle	0	14.0 ± 1.27	87.9 ± 3.17	175.9 ± 22.65
Corticosterone, 4 mg/kg	8	16.8 ± 3.89	82.7 ± 4.00	140.3 ± 18.48
Corticosterone, 4 mg/kg	24	11.2 ± 1.04	93.0 ± 5.40	170.6 ± 26.8
Corticosterone, 30 mg/kg	8	13.5 ± 0.62	95.0 ± 6.60	232.7 ± 18.02
Corticosterone, 30 mg/kg	24	15.2 ± 2.52	97.0 ± 4.2	220.0 ± 31.90
Dexamethasone, 4 mg/kg	8	12.3 ± 0.46	101.3 ± 4.70	152.8 ± 20.30
Dexamethasone, 4 mg/kg	24	8.6 ± 1.14*	101.2 ± 1.97*	130.6 ± 7.45

Data are the means ± SEM of triplicate determinations of 4–6 rats per group.
*$P < 0.05$ versus control group.

costerone levels *in vivo* in thymocytes [37] and in the spleen [38].

In agreement with the negative effect exerted by glucocorticoids in lymphoid tissues, the present data provide the first proof that glucocorticoid treatment modifies PAO activity in lymphoid organs of male rats and also confirm that the hormones regulate polyamine biosynthetic [18–20] and catabolic [20, 24] enzymes in an opposite manner. The approach we employed in the study was the same as that used by Lindsay and Wallace [15], since we thought that whether or not PAO plays a role in apoptosis, a treatment known to induce this process [16, 17, 37, 38], may affect enzyme activity. In fact, the relationship between cell death and polyamine catabolism by PAO is complex, and there are conflicting reports on this point. Depending on the specific cell line and evoking stimulus, polyamines, and the products generated enzymatically during their interconversion, may exert a positive or negative control over apoptosis [5, 8, 9, 13, 15, 23, 39–42].

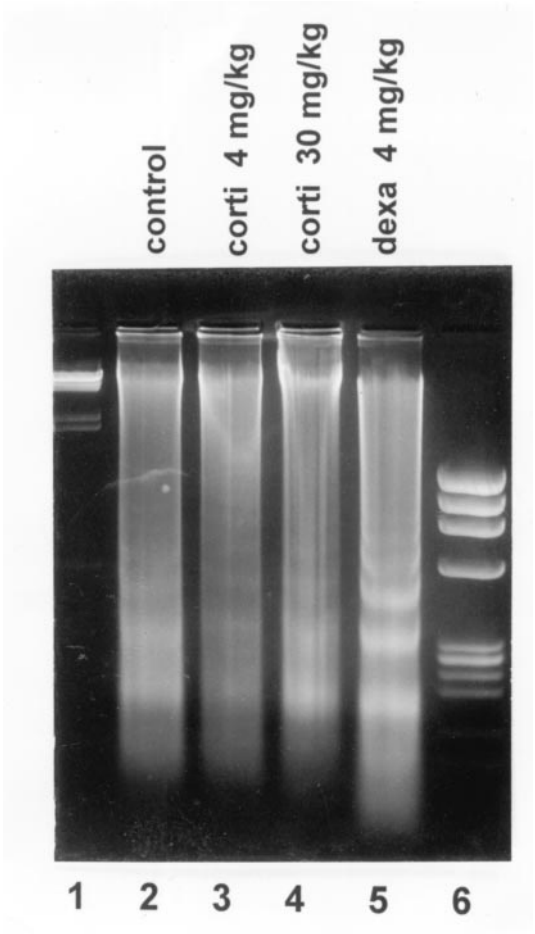

FIG. 3. Effect of corticosterone (corti) and of dexamethasone (dexa) on DNA fragmentation in rat thymus. Fragmented DNA was purified from control or 8 hr-treated rats and analyzed as reported [33]. Samples were subjected to electrophoresis through 2% agarose gel and DNA visualized by EtBr staining. λ/*Hin*dIII DNA molecular weight markers (lane 1); control (lane 2); corticosterone, 4 mg/kg (lane 3); corticosterone, 30 mg/kg (lane 4); dexamethasone, 4 mg/kg (lane 5); ϕ X174/*Hae*III DNA molecular weight markers (lane 6). A representative experiment of three is shown.

An interesting finding of the present investigation was that corticosterone, which caused catabolic changes in thymus and spleen [43] when administered at the low dosage, differently affected PAO activity in the two organs upon different dosages, with a decrease after the low dose and an increase after the high dose in the thymus. Moreover, the thymus-specific decrease in PAO activity after the low dose of corticosterone was accompanied by a slight increase in SAT activity. At the moment, we have no explanation for this observation. We can only stress that the low dose of corticosterone, which decreased PAO activity, did not induce DNA fragmentation (see Fig. 3), thereby suggesting a correlation between the enzyme activity and the apoptotic process. Moreover, it has recently been reported that the physiological role of glucocorticoids is not merely suppressive, but rather immunomodulatory [44]. An opposing dose-dependent influence of corticosterone on blood T-lymphocytes [25] or hydrocortisone on the energetics of rat thymocytes [45] has been shown. Nevertheless, a decrease in PAO activity paralleled by an increase in SAT activity has been reported [46, 47]. One may suppose that the decrease in PAO activity may play a role in preventing the conversion of higher polyamines to putrescine. Such an hypothesis may be in agreement with the protective effect against apoptosis exerted by spermine [8, 41, 48] and suggests that a decrease in spermine may be necessary for the negative effect of glucocorticoids in the thymus, as demonstrated for the thymocyte culture system

TABLE 3. Effect of glucocorticoids on catalase activity

	Time after Treatment (hr)	Catalase activity (units/mg protein)	
		Thymus	Spleen
Vehicle	0	6.4 ± 0.34	24.1 ± 1.05
Corticosterone, 4 mg/kg	8	3.9 ± 0.22†	22.0 ± 0.44
Corticosterone, 4 mg/kg	24	6.9 ± 0.39	25.7 ± 0.90
Corticosterone, 30 mg/kg	8	6.3 ± 0.52	25.2 ± 0.66
Corticosterone, 30 mg/kg	24	6.2 ± 0.46	20.8 ± 0.68*
Dexamethasone, 4 mg/kg	8	3.7 ± 0.08†	21.4 ± 0.55*
Dexamethasone, 4 mg/kg	24	3.8 ± 0.44†	16.6 ± 0.69†

Data are the means ± SEM of triplicate determinations of 4–6 rats per group.
*$P < 0.05$; †$P < 0.005$ versus control group.

[8]. However, when we used a high dose of corticosterone, the effect on PAO activity was the same as obtained after the administration of dexamethasone, i.e. there was an increase in enzyme activity in the thymus and the spleen, with corticosterone effective earlier (8 hr) than dexamethasone (24 hr). Similarly, an increase in SAT activity was observed after administration of the high dose of corticosterone or dexamethasone in both the organs, with the natural hormone more effective in the thymus and the synthetic hormone in the spleen. In the thymus, the increase in PAO activity was accompanied by DNA fragmentation, which was particularly evident after dexamethasone administration (see Fig. 3, lane 5), thus confirming the aforementioned possible correlation between the two effects exerted by glucocorticoids.

As regards polyamines, their levels were not always correlated with the activities of the catabolic enzymes, probably because glucocorticoid treatment at the same time inhibited the biosynthetic decarboxylases [18–20]. Thus, an increase in the interconversion pathway [20, 24, and present study] may on the one hand counteract and on the other stress the negative effects on biosynthesis [18–20], with a decrease in spermine, which exerts a protective effect against apoptosis [8, 41, 48], and an increase in putrescine, which can induce apoptosis by a mechanism that may involve inhibition of hypusine formation *in vivo* [49].

Finally, our results on PAO activity after glucocorticoids are in agreement with recent data demonstrating that such enzyme activity may also be specifically regulated by hormones or toxic treatments in different tissues or cell types [15, 46, 50, 51]. Moreover, in accord with the data demonstrating polyamine depletion as a mediator of apoptosis [5, 8], changes not only in SAT [20, 24, and present study] but also in PAO activity after glucocorticoid treatment may be, at least partially, responsible for the negative effects exerted by the hormones in lymphoid tissues. More interesting is the observation that there was a positive correlation between the increase in PAO activity and DNA fragmentation in the thymus. However, on the basis of the present results, we have not addressed the question as to whether the changes in PAO activity are a necessary step or a consequence of the apoptotic process induced by glucocorticoid treatment. We can only point out that intracellular oxidation is a requisite for glucocorticoid-induced apoptosis in thymocytes [52], and that oxidative stress during hormone-mediated apoptosis is accompanied by a down-regulation of the antioxidant defense [26, 35]. Thus, we can suppose that the hydrogen peroxide formed by the activation of polyamine catabolism in our samples could not be neutralized by the defensive mechanisms, such as catalase, because of their decrease after hormone treatment [26, 35, and this study]. Further studies with the use of PAO inhibitors will clarify this point on the possible role of the enzyme in the negative effect exerted by hormones in lymphoid tissues.

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