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Endotoxin-Induced Reduction of β -Adrenergic Binding Sites on Splenic Lymphocytes In Vivo and In Vitro

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ENDOTOXIN-INDUCED REDUCTION OF β -ADRENERGIC BINDING SITES ON SPLENIC
LYMPHOCYTES IN VIVO AND IN VITRO:
ITS MODULATION BY ANTERIOR HYPOTHALAMIC LESIONS

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Summary

Bacterial endotoxin induced a 38 % decrease in the number of β -adrenergic binding sites (Bmax) on splenic lymphocytes, four days after intraperitoneal administration to guinea pigs. No change in the affinity (Kd) for [125-I]-cyanopindolol ([125-I]-CYP) binding was observed. Incubation of guinea pig splenocytes in vitro with different concentrations of bacterial endotoxin for 24 hours resulted in an increased incorporation of [³H]-thymidine, a parameter for lymphocyte activation. Activation of splenic lymphocytes with the optimal endotoxin concentration of 100 μ g/ml for 24 hours induced a 27 % decrease in the Bmax whereas the Kd for [125-I]-CYP binding was not changed. Based on these findings, we speculate that activation of lymphocytes with endotoxin in vitro and in vivo is associated with a reduction in the number of β -adrenergic binding sites on these cells. Anterior hypothalamic (AHA) lesions protected against the endotoxin-induced reduction in the number of β -adrenergic binding sites on lymphocytes. The protective effect of these lesions could not be related to alterations in the plasma levels of cortisol, triiodothyronine (T3), thyroxine (T4), adrenaline and noradrenaline or to splenic noradrenaline content. Since AHA lesions have been shown to inhibit several lymphocyte functions, it is suggested that these lesions prevent lymphocyte activation after in vivo endotoxin administration and through this abrogate the reduction of the β -adrenergic binding sites.

A fundamental or acquired β -adrenoceptor impairment might contribute to the pathogenesis of bronchial asthma, since both non-specific bronchial hyper-reactivity and IgE antibody production can be promoted by β -adrenergic blockade (1, 24, 35). Previously, it has been demonstrated that in guinea pigs a decreased function of the β -adrenoceptors in the trachea in vitro and the number of binding sites in peripheral lung tissue developed, four days after intraperitoneal administration of dead Gram-negative bacteria like Bordetella pertussis and Haemophilus influenzae or the common cell wall component endotoxin (32, 38). The modulation of the β -adrenergic system by bacterial

endotoxin is not limited to the respiratory airways since the coronary vessels and lymphocytes are also shown to be affected (39, 40). These data suggest that an endotoxin-elicited factor(s) circulates in the blood which affects the β -adrenergic system. Such a factor may be of neurohumoral origin since anterior hypothalamic (AHA) lesions prevent the decrease in the number of β -adrenergic binding sites in lung tissue after bacterial endotoxin injection (41). The hypothalamus is involved in the regulation of the autonomic and endocrine systems (20). Gram-negative bacteria and endotoxin have been shown to affect the neuroendocrine system probably through a central mechanism (13, 19, 25, 27, 43). The modulation of the β -adrenergic system may therefore be related to an altered hormonal or autonomic outflow. Indeed, the β -adrenergic system can be influenced by several hormones, of which glucocorticoids and thyroid hormones are the most important (16, 22). Furthermore, β -adrenoceptors can be desensitized by the catecholamines adrenaline and noradrenaline (22).

A second mechanism by which AHA lesions offer protection against the deleterious effect of endotoxin may be related to the immunosuppressive nature of these lesions. AHA-lesions have been shown to suppress several immunological parameters like anaphylactic reactions, antibody production, delayed type hypersensitivity reactions and lymphocyte proliferation (33), whereas endotoxin activates immunocytes (21). Moreover, a pivotal role for the immune system, in particular the spleen, in the endotoxin-induced modulation of the β -adrenergic system has already been demonstrated (40).

In the present study, the effect of AHA lesions on the endotoxin-induced reduction in the number of β -adrenergic binding sites on splenic lymphocytes was determined. To unravel a putative influence of hormones i.e. glucocorticoids, thyroid hormones or catecholamines i.e. adrenaline and noradrenaline, their plasma levels and splenic noradrenaline level were measured as well. Furthermore the influence of splenic lymphocyte activation in vitro with bacterial endotoxin was investigated in relation to the β -adrenergic binding sites on these cells.

Methods

Animals:

The animals used in this study were male Dunkin Hartley guinea pigs (Olac Ltd., Bicester, England) weighing 270-320 g at the time of the operations. Bilateral electrolytic lesions were made in the anterior hypothalamus (AHA) as described previously (41), according to an atlas of the guinea pig forebrain (2). Ten days after the operation and four days prior to experiment the animals were injected (i.p.) with 1 mg/kg endotoxin (L.P.S.; E. coli 0111:B4) in a volume of 1 ml/kg. Control animals were injected with 1 ml/kg sterile saline.

The guinea pigs were killed by an i.p. injection of 180 mg pentobarbitone sodium (Nembutal). Blood samples were collected into heparinized tubes by heart puncture and plasma prepared at 4 °C. Plasma samples were stored at - 70 °C until hormones and catecholamines were determined. Subsequently, splenic lymphocyte membranes were prepared as described elsewhere (40). The membrane preparations were stored at - 70 °C until binding assays were performed. In a parallel experiment, the spleens were removed, immediately frozen on dry ice and stored at - 70 °C until catecholamines were measured.

[125-I]Cyanopindolol binding assay:

In a previous study, the β -adrenergic binding sites on splenic lymphocytes of the guinea pig have been characterized (40). Lymphocyte membranes were incubated in triplicate in a final assay volume of 250 μ l which contained approximately 50 μ g protein with increasing concentrations of [125-I]cyanopin-

dolol ([125-I]-CYP; 40, 80, 150, 300 and 500 pM). Incubations were carried out at 37 °C for 60 min and were terminated by rapid dilution with 5 ml ice-cold assay buffer, followed by vacuum filtration through Whatman GF/B glass fiber filters. The tubes were then rinsed with 5 ml cold buffer and the contents were again filtered. The filters were then transferred into vials (Packard, Downers Grove, IL, USA), picofluor-30 scintillation fluid (Packard, Downers Grove, IL, USA) was added and the radioactivity was counted in a liquid scintillation counter (Philips PW 4700, Eindhoven, The Netherlands) at an efficiency of 60 %. Separate incubations were carried out in each assay in the presence of 10 μ M timolol to assess the non-specific binding to the membranes. Specific binding was defined as total radioactivity bound minus the non-specific binding. Binding data were analyzed by the method of Scatchard (31) with linear regression analysis. The maximal number of binding sites (Bmax) and the affinity (Kd) for the receptors were determined.

Catecholamines:

To determine noradrenaline and adrenaline levels the spleen was homogenized in 0.1 N perchloric acid using a polytron PT10 and plasma was diluted 10:1 with 2 N perchloric acid. After centrifugation at 4 °C for 15 min at 15000 x g, 20 μ l of the supernatant was used in a radioenzymatical assay for catecholamines according to Van der Gugten et al. (37). Data of respectively plasma and spleen are expressed as respectively nmol/l and nmol/g.

Glucocorticoids:

Glucocorticoid concentrations were measured in duplicate according to a competitive protein binding assay (23) after dichloromethane extraction of 25 μ l plasma. Human plasma (0.6 %) was used as corticosteroid-binding globulin (transcortin) source, [3H]-cortisol as tracer and dextran-coated charcoal as absorbent for the unbound fraction. Standard cortisol was supplied by Sigma (St. Louis, USA). The intra-assay coefficient of variation was less than 8 %. Recovery of added tracer was >92 %. Fifty percent displacement of tracer steroid was obtained at a concentration of 800 nmol/l.

Thyroid hormones:

The concentrations of the thyroid hormones triiodothyronine (T3) and thyroxine (T4) in the plasma samples were measured with immunoassays. Plasma T4 levels were determined with a polarization fluorescence assay (34) (TDX, Abbott Diagnostics, Chicago, IL, USA), with a between assay coefficient of variation of 5.0 % (n=75). T3 concentrations were measured in duplicate with a direct radio immunoassay (Diagnostic Products Corporation, Los Angeles, CA, USA) with a coefficient of variation of 7.0 % (n=25). The cross reactivity with other thyroid hormones was negligible.

Lymphocyte activation in vitro:

The guinea pigs were killed by an i.p. injection of 180 mg pentobarbitone sodium (Nembutal). The spleens were removed under sterile conditions, placed in Eagle's minimal essential medium (Eagle's MEM, Flow Laboratories, Irvine, Scotland) and teased apart with two pairs of tweezers to make cell-suspensions. The cell-suspensions were passed through sterile cotton-wool and after centrifugation of the suspensions at room-temperature at 400 x g for 10 min, the pellets were resuspended in Eagle's MEM. After another centrifugation, the splenocytes were resuspended in Iscove's medium (Gibco, Paisley, Scotland) enriched with 0.25 % bovine serum albumin; 36 μ g/ml transferrin; 5 μ g/ml insulin; 1 μ g/ml linoleic acid; 1 μ g/ml oleic acid; 1 μ g/ml palmitic acid; 290

TABLE I

Treatment	N	Bmax (fmol/mg protein)	Kd (pM)
Sham control	10	115 $\pm$ 13*	180 $\pm$ 20
Sham endotoxin	10	71 $\pm$ 16	229 $\pm$ 49
Lesion control	10	108 $\pm$ 12	178 $\pm$ 33**
Lesion endotoxin	6	137 $\pm$ 15	338 $\pm$ 39

Maximal number of binding sites (Bmax) and the affinity (Kd) for [125-I]-cyanopindolol on splenic lymphocyte membranes, four days after i.p. endotoxin administration (1 mg/kg) to sham-operated and AHA-lesioned guinea pigs. * $p < 0.05$ as compared to the value of the sham control group and ** $p < 0.01$ as compared to the value of the lesion control group.

TABLE II

Concentration (nmol/l)	TREATMENT			
	Sham control (n = 8)	Sham endotoxin (n = 7)	Lesion control (n = 9)	Lesion endotoxin (n = 9)
PLASMA:				
cortisol	1042.5 $\pm$ 127.3	914.1 $\pm$ 88.1	641.2 $\pm$ 130.9*	1007.7 $\pm$ 125.4
T3	0.39 $\pm$ 0.03	0.36 $\pm$ 0.04	0.40 $\pm$ 0.03	0.35 $\pm$ 0.04
T4	41.75 $\pm$ 4.32	40.0 $\pm$ 3.09	40.56 $\pm$ 2.48	40.78 $\pm$ 2.45
adrenaline	49.7 $\pm$ 10.9	42.6 $\pm$ 11.5	35.0 $\pm$ 7.1	57.9 $\pm$ 10.9
noradrenaline	17.2 $\pm$ 2.5	17.7 $\pm$ 4.1	14.8 $\pm$ 2.4	21.9 $\pm$ 4.7
SPLEEN:				
noradrenaline [†]	6.6 $\pm$ 0.6	6.8 $\pm$ 1.0	5.6 $\pm$ 0.4	6.6 $\pm$ 0.6

†: content in nmol/g.

* $P < 0.05$ as compared to the value of the sham control group.

Plasma levels of hormones and catecholamines and splenic noradrenaline content, four days after i.p. endotoxin administration (1 mg/kg) to sham-operated and AHA-lesioned guinea pigs.

significant increase could be observed (Table I). However, the Kd-value in AHA-lesioned guinea pigs was significantly ($p < 0.01$) increased after endotoxin injection.

Hormones and catecholamines:

In both sham-operated and AHA-lesioned guinea pigs, no significant changes in the plasma levels of cortisol, T3, T4, adrenaline and noradrenaline could be observed at four days after endotoxin injection (Table II). Also no difference was found in the splenic noradrenaline content between endotoxin-pretreated and saline-pretreated guinea pigs. AHA lesioned saline-pretreated guinea pigs showed a decreased ($p < 0.01$) plasma cortisol level as compared to sham-operated saline-pretreated animals. However, no difference in plasma cortisol level could be observed between sham-operated and AHA-lesioned guinea pigs after endotoxin treatment. AHA-lesioned saline-pretreated guinea pigs did not differ from sham-operated saline-pretreated animals concerning the levels of thyroid hormones and catecholamines.

$\mu\text{g/ml}$ glutamine (all from Sigma Chemical Company, St. Louis, USA); 1.8 $\mu\text{g/ml}$ ethanolamine (Merck Nederland B.V., Amsterdam, The Netherlands); 100 IU/ml penicillin and 100 $\mu\text{g/ml}$ streptomycin. To determine the conditions for optimal activation of the splenocytes, 200 μl of splenocyte-suspensions with different cell-densities (10^5 - 10^7 cells/ml) were incubated in triplicate with different endotoxin concentrations (10^{-2} - 10^{-4} $\mu\text{g/ml}$) in 96-wells microtitration-plates (Greiner GmbH, Nürtingen, West-Germany) for 24 hours at 37 °C in a humidified atmosphere of 5 % CO_2 and 95 % O_2 (Heraeus incubator). Before harvesting, a 4 hour pulse with [^3H]-thymidine (1 μCi in 10 μl) was given. The cells were collected on Titertek glass fiber filters with a Titertek Cell-harvester (Flow Laboratories) and washed extensively with distilled water. Incorporated radioactivity was determined by liquid scintillation counting and was expressed as mean D.P.M. $\pm$ S.E.M. In later experiments, two tissue culture flasks (Falcon 25 cm^2 , Becton Dickinson, N. J., USA) were filled with each 15 ml of 5×10^6 splenocytes/ml suspension from one spleen. One of these splenocyte-cultures was activated with 100 $\mu\text{g/ml}$ endotoxin, the other culture was used as control. These cultures were incubated for 24 hours in an incubator (as described above). At the end of this period, the splenocytes were resuspended and separated on a percoll gradient (1.077 g/ml), the interface collected and washed once with Krebs bicarbonate buffer. Accordingly, lymphocyte membranes were prepared as described above.

Materials:

Nembutal, containing 60 mg/ml pentobarbitone sodium was acquired from Abbot Laboratories, North Chicago, IL, USA. Hypnorm, containing 10 mg/ml fluanison and 0.2 mg/ml fentanyl base was from Duphar B.V., Amsterdam, The Netherlands. Endotoxin, E. coli O111:B4 lipopolysaccharide (Phenolic extraction) was acquired from Sigma Chemical Company, St. Louis, USA.

Timolol maleate was obtained from Merck, Sharp & Dohme, Haarlem, The Netherlands. The radioactive β -adrenoceptor antagonist [^{125}I]cyanopindolol ([^{125}I]-CYP, 2200 Ci/mmol) and [^3H]-cortisol (80-90 Ci/mmol) were from NEN, Boston, MA, USA. S-adenosyl-L-[methyl- ^3H]-methionine ([^3H]-SAM, 5-10 Ci/mmol) which was used in the radioenzymatical determination of catecholamines and [^3H]-thymidine (5 Ci/mmol) were purchased from Amersham, Great Britain.

Statistics:

The unpaired Student's t-test was used to compare the B_{max} and the K_d values of the β -adrenergic binding sites on lymphocytes after in vivo endotoxin administration and the hormone and catecholamine levels. The paired Student's t-test was used to compare the B_{max} and K_d values of the β -adrenergic binding sites on lymphocytes after in vitro incubation with endotoxin. A value of $P < 0.05$ was considered as significant. Results are expressed as means $\pm$ S.E.M.

Results

Lymphocyte β -adrenergic binding sites:

Four days after the administration of endotoxin (1 mg/kg, i.p.) to sham-operated guinea pigs, the number of β -adrenergic binding sites (B_{max}) on splenic lymphocyte membranes was significantly ($p < 0.05$) reduced by 38.3 ± 13.9 % as compared to the number of binding sites on lymphocytes from saline-pretreated animals (Table I). The affinity (K_d) of the binding sites for [^{125}I]-CYP was not affected after the endotoxin injection of sham-operated guinea pigs. Endotoxin administration was unable to induce a decrease in the number of β -adrenergic binding sites in AHA-lesioned guinea pigs and even a small, non-

Lymphocyte activation and β -adrenergic binding sites:

Incubation of splenocytes for 24 hours with increasing concentrations of endotoxin resulted in an increased incorporation of $[3H]$ -thymidine. The optimal endotoxin concentration for the activation of lymphocytes was 100 $\mu\text{g/ml}$ (Fig. 1). No differences were found in $[3H]$ -thymidine incorporation per cell between different densities i.e. 10^6 , 5×10^6 and 10^7 cells/ml (results not shown). Incubation of splenocyte cultures (5×10^6 cells/ml) with 100 $\mu\text{g/ml}$ endotoxin for 24 hours, resulted in a $27.5 \pm 7.6\%$ decrease ($P < 0.02$, paired t-test) in the number of β -adrenergic binding sites on splenic lymphocyte membranes as compared to control cultures (Table III). The affinity (K_d) of the binding sites for $[125\text{-I}]\text{-CYP}$ was not affected after incubation of the cultures with endotoxin.

FIG. 1

The effect of incubation of guinea pig splenocytes (5×10^6 cells/ml) for 24 hours with different endotoxin concentrations on the $[3H]$ -thymidine incorporation.

TABLE III

GROUP	N	Bmax (fmol/mg protein)	Kd (pM)
Control	13	$131 \pm 15^*$	256 ± 36
Endotoxin	13	95 ± 10	176 ± 19

Maximal number of binding sites (Bmax) and the affinity (Kd) for $[125\text{-I}]\text{-cyanopindolol}$ on lymphocyte membranes, 24 hours after incubation of guinea pig splenocytes (5×10^6 cells/ml) with endotoxin (100 $\mu\text{g/ml}$) in vitro. * $P < 0.02$ as compared to the value of the sham control group.

Discussion

In this report, we have shown that the number of β -adrenergic binding sites on splenic lymphocytes is reduced at four days after i.p. endotoxin administration to sham-operated guinea pigs, which is in accordance with earlier findings (40). AHA lesions, which prevent the endotoxin-induced reduction in the number of β -adrenergic binding sites in lung tissue (41), are shown to act similarly with respect to splenic lymphocytes β -adrenoceptor number. However, in AHA-lesioned guinea pigs an increased K_d -value for [125 I]-CYP binding to splenic lymphocytes is observed after endotoxin injection. This observation is not in line with our previous study in which no change in affinity could be observed in lung tissue (41). At present, no reason for this discrepancy can be given. An explanation concerning the mechanism of action of AHA-lesions may be that they abrogate a loop in which endotoxin, either directly or through an endotoxin-elicited factor(s) activates the AHA. Since the hypothalamus is involved in the regulation of the autonomic and endocrine systems (20), the modulation of the β -adrenergic system by bacterial endotoxin injection may be related to alterations in hormonal and autonomic outflow. It has been shown that glucocorticoids and thyroid hormones can influence the β -adrenergic system (16, 22). β -adrenoceptors can also be desensitized by circulating catecholamines or by catecholamines released directly in the vicinity of the lymphocytes in the spleen (18, 22).

Bacterial endotoxin injection rapidly elevates glucocorticoid levels, probably through an effect on the neural control of adrenocorticotrophic hormone (19, 43). Thyroid hormone levels may also be affected by endotoxin since an increased release of thyrotrophic hormone was demonstrated after administration to rats (13). Recently, interleukin-1, which can be synthesized by macrophages and monocytes after endotoxin administration, has been shown to be a likely mediator for the endotoxin-induced rise in plasma glucocorticoid and thyrotropin (5, 6, 30). A second cause for elevated glucocorticoid and thyroid hormone levels after endotoxin injection can be the production of adrenocorticotropin and thyrotropin by activated lymphocytes (4). Acute changes in the sympathetic outflow have also been described to occur after endotoxin injection (25, 27). In the present study, however, no influence of endotoxin administration to sham-operated guinea pigs on the plasma levels of thyroid hormones, cortisol and catecholamines can be shown. Splenic noradrenaline content is also not affected by endotoxin pretreatment of sham-operated guinea pigs. However, it remains possible that alterations in these hormones and catecholamines take place before day four after endotoxin administration to guinea pigs. The plasma and splenic catecholamine data in the present study are comparable with previous findings (38). The plasma cortisol levels are intermediate to those reported in literature (15, 17, 28) and are much lower than the reported value of 1990 nmol/l in stressed guinea pigs (12). Thyroid hormone levels which are insensitive to stress-effects, are alike the values from other groups (7, 15, 26). Evaluating these data, it has to be considered that the blood-samples are not taken under complete stress-free circumstances, which may mask possible differences in plasma catecholamines and cortisol. On the bases of these findings, it can be suggested that the reduction in the number of β -adrenergic binding sites on splenic lymphocytes is not related to major alterations in the plasma levels of cortisol, thyroid hormones, adrenaline and noradrenaline nor to splenic noradrenaline content four days after endotoxin injection.

Recently, it has been demonstrated that splenectomy of guinea pigs, prior to endotoxin administration prevented the reduction in the function of the β -adrenoceptors in the trachea and the number of binding sites in lung tissue (40). A pivotal role for the spleen, in particular lymphocytes was suggested in the deleterious effect of endotoxin on the β -adrenergic system. This hypothesis is strengthened by the present data in which it is shown that activation of lymphocytes in vitro with endotoxin is accompanied by a reduction in the number

of β -adrenergic binding sites. This reduction resembles the decrease in the number of β -adrenergic binding sites on splenic lymphocyte membranes after in vivo endotoxin administration (40). Since the modulation of the β -adrenergic system after bacterial endotoxin injection extends to the respiratory airways and coronary vessels (39, 40), it is tempting to speculate that during the activation of lymphocytes with endotoxin, factors are produced which are involved in the deterioration of the β -adrenoceptors in lymphocytes as well as in other cells and tissues. Although the cultures consisted predominantly of lymphocytes, it cannot be excluded that such a factor is derived from other cells like macrophages. Moreover, lymphocytes may act in concert with macrophages leading to the detrimental effect of endotoxin on the β -adrenergic system.

Assuming that the mechanisms of endotoxin-induced β -adrenoceptor deterioration in vivo and in vitro are similar events, the protective influence of AHA lesions may be related to their immunosuppressive action (33). Since it has been shown that hormones and neurotransmitters interact with the immune system (3, 4), the effect of AHA lesions on these factors may give further credence to this hypothesis. Literature data about the influence of these lesions on plasma glucocorticoids are contradictory (14, 29, 36) but a decrease of plasma thyrotropin level and thyroid function has been observed (9, 10). Furthermore, it has been suggested that AHA lesions produce an imbalance in the autonomic nervous system resulting in an increased sympathetic outflow (11). Our data however show no difference in the plasma level of the sympathetic mediators adrenaline and noradrenaline and the splenic noradrenaline content nor in the plasma levels of thyroid hormones between sham-operated and AHA-lesioned control guinea pigs. However, the plasma cortisol level in AHA-lesioned control guinea pigs is diminished as compared to the level in sham-operated controls. Since these data do not explain the immunosuppressive action of AHA lesions, other hormones or neural influences may play a role. In line with these findings, Cross et al. (8) was also unable to relate the immunosuppressive action of AHA lesions with elevated glucocorticoid levels.

It can be concluded that a reduction in the number of β -adrenergic binding sites on lymphocytes occurs after in vivo administration of and in vitro incubation with bacterial endotoxin. These changes in the β -adrenergic system of lymphocytes seem to occur parallel with activation of lymphocytes by endotoxin. AHA lesions prevent the endotoxin-induced decrease in the number of β -adrenergic binding sites on lymphocytes. This effect of AHA lesions may be due to immunosuppression, although the mechanism of the immunosuppression remains unexplained.

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