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INFLUENCE OF *E. COLI* ENDOTOXIN ON ACTH INDUCED ADRENAL CELL STEROIDOGENESIS

R. GARCIA, M. D. VILORIA and A. M. MUNICIO[1]
Department of Biochemistry, Faculty of Sciences. Universidad Complutense, Madrid (3), Spain

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Summary—The effect of endotoxin (lipopolysaccharide from *E. coli*) on isolated adrenocortical cells was examined. Lipopolysaccharide decreased the ACTH-induced steroidogenesis. This effect was shown by all corticotropin concentrations studied, and the longer the incubation time, the higher the effect produced. The rate of decrease of ACTH-induced steroidogenesis was dependent on the concentration of lipopolysaccharide in the medium.

Binding of [^{125}I]ACTH to adrenocortical cells was modified by lipopolysaccharide; this modification was related to a decrease of the ACTH-induced steroidogenesis. This effect supports the hypothesis of a direct interaction between lipopolysaccharide and the cell membrane with a concomitant distortion of the cell surface affecting the ACTH receptor sites of their environment.

[^{14}C]Lipopolysaccharide binds to isolated adrenocortical cells. Binding specificity was investigated by competitive experiments in the presence of various types of endotoxins, polypeptide hormones and proteins. Unlabelled lipopolysaccharide from the same bacterial strain and isolated under identical conditions than the labelled lipopolysaccharide exerted the strongest inhibitory activity. Unlabelled lipopolysaccharide of various strains different from that originating the labelled lipopolysaccharide exerted the less displacement. It would imply a certain kind of specificity but the decrease in the binding of lipopolysaccharide produced by ACTH and glucagon suggests the existence of non-specific interactions between lipopolysaccharide and cell membrane.

INTRODUCTION

Gram negative bacteria endotoxins trigger a sequence of biological events producing a syndrome of shock, vasoconstriction, disseminated intravascular coagulation and renal cortical necrosis [1]. Extensive cellular damage by endotoxins has been shown in the liver [2, 3], kidneys [4, 5], lungs [6] and tubal mucosa [7]. In the liver, endotoxemia causes changes in hepatocyte mitochondria that can be characterized by different functional alterations [8, 9].

Concerning the *in vivo* effect of lipopolysaccharides on adrenocortical cells, evidence that the hypothalamus mediates endotoxin stimulation of adrenocorticotropic hormone secretion has been reported [10]. The administration of *E. coli* endotoxins causes a large increase in plasma corticosterone concentration through the release of ACTH [11]; however, high concentrations of bacterial lipopolysaccharides added to cultures of adrenocortical cells inhibited the steroidogenic response to ACTH [12].

Some types of interactions between lipopolysaccharide and cell membranes have been reported for red cells [13, 14], lymphocytes [15], granulocytes [16], and platelets [17, 18, 19]. Nevertheless, the mechanism through which bacterial lipopolysaccharides exert their biological activity is not well understood.

The binding properties of ^{14}C-labeled lipopolysaccharide to isolated hepatocytes from rats have been studied, suggesting that the endotoxin binds to non-receptor sites of the membrane bilayer and induces a destabilization of the membrane [20]. This hypothesis is in agreement with data obtained using a lipoprotein enzyme complex as the target structure for the *E. coli* lipopolysaccharide [21, 22].

This paper is concerned with the binding properties of ^{14}C-labeled lipopolysaccharide to isolated adrenocortical cells from rats as well as with the influence of *E. coli* endotoxin on the ACTH function and binding to these cells.

EXPERIMENTAL

Chemicals

Endotoxin labeled with ^{14}C was obtained from *Escherichia coli* 0111a, 0111b:K58:H21 (ATCC) grown in a medium with [^{14}C]glucose as the only carbon source. The extraction of [^{14}C]lipopolysaccharide was carried out according to Romanowska's modification [23] of the general method of Westphal. Specific activity was 4.5 μCi/mg and A_{260}/A_{240} was determined for purity verification. Unlabeled endotoxins were supplied by Difco (Detroit, MI, U.S.A.). [^{3}H]Cortisol was obtained from The Radiochemical Centre (Amersham, Bucks, U.K.) (sp. act. 50 Ci/mmol) and [^{125}I]ACTH was purchased from International CIS (Gif-sur-Yvette, France) [sp. act. 1 μCi/μg]. $ACTH_{1-24}$ was from Ciba-

[1]To whom correspondence should be addressed.

Geigy and cortisol was from Merck. Labeled and unlabeled ACTH were tested previously for biological activity. Trypsin and lima bean trypsin inhibitor were from Worthington.

Isolation of adrenocortical cells

Adrenal glands from male Sprague–Dawley rats (weighing 200–250 g), fasted overnight with water *ad libitum*, were excised, trimmed of fat and quartered. Adrenocortical cells were isolated by trypsin digestion. The tissue was incubated under O_2/CO_2 (95:5, v/v) in 10 ml of modified Eagle's Minimal Essential Medium (MEM) supplemented with 10 mg trypsin and 100 mg bovine serum albumin. Adrenocortical cells were purified by lysis of the red blood cells and further centrifugation in a medium containing 5% bovine serum albumin, according to the method previously described [24]. Cell viability was tested with trypan blue exclusion.

[^{125}I]ACTH binding assays

Isolated adrenal cells were incubated with [^{125}I]ACTH at 37°C under O_2/CO_2 (95:5, v/v) atmosphere. The cell suspension medium was MEM, supplemented with 0.2% bovine serum albumin and 0.05% trypsin inhibitor using 10^5 cells/500 μl of medium. At the end of the incubation, tubes were cooled in ice and then centrifuge in a Microfuge for 2 min. Supernatants were removed and the bottom of the tubes were cut off and then transferred to counting vials. Radioactivity was determined in a LKB-γ counter. [^{125}I]ACTH binding was specific and it was rapid, reversible and linearly related to the number of cells. Differences due to degradation, uptake, etc. were taken into account for calculations. Under the experimental conditions employed, there was no significant degradation of the radioligand.

Competitive binding assays were carried out with [^{125}I]ACTH (20 ng) and unlabeled ACTH (up to 700 μg) in the absence and in the presence of lipopolysaccharide (500 μg). Other competitive binding assays were performed with [^{125}I]ACTH (20 ng) and lipopolysaccharide (up to 1 mg). Incubation time in all competitive incubation assays was 15 min.

For the steroid assay, supernatant aliquots from cell suspensions were extracted with 5 ml of dichloromethane and assayed as described below.

[^{14}C]Lipopolysaccharide binding assays

Isolated adrenocortical cells (10^5 cells/500 μl) were incubated with different amounts of [^{14}C]lipopolysaccharide under atmosphere of O_2/CO_2 (95:5, v/v) at 37°C in MEM-0.25 mM $CaCl_2$ for different times according to the experiment. At the end of the incubation times, 500 μl of 0.9% NaCl were added and the tubes were cooled in ice. Suspensions were filtered through Whatman filters GF/C, previously washed with 0.9% NaCl (10 ml) and then filters were washed with 5 ml 0.9% NaCl.

Non-specific binding was determined in the presence of high concentrations of unlabeled lipopolysaccharide (200-fold).

Radioactivity incorporated was measured in a Packard Tri-Carb 3255 scintillation counter using 15 ml of the mixture: naphtalene, 150 g; PPO, 10.5 g; POPOP, 0.4 g; dioxane to 1.5 l.

Corticosteroid synthesis

Isolated adrenal cells were resuspended in MEM-supplemented with 0.2% bovine serum albumin, 0.05% trypsin inhibitor and 7.5 mM $CaCl_2$. ACTH and lipopolysaccharide were dissolved in MEM and added in 100 μl aliquots to cell suspensions according to experiments. Final volume of incubation, 1 ml. Cell suspensions (10^5 cells/ml) were incubated at 37°C under O_2/CO_2 (95:5, v/v). At the end of the incubation, generally 90 min, corticosteroids were extracted with dichloromethane (5 ml) for the steroid assay.

Corticosteroid production was measured by a competitive binding assay using transcortin from human plasma as the binding system, according to Murphy [25].

Statistics

All values in tables and figures represent the mean values ($\pm$SEM) of at least triplicate determinations of three different experiments. Student's t-test was applied to assess statistical significance. A value of $P < 0.05$ was taken as the criterion of statistical significance.

RESULTS

Influence of E. coli *lipopolysaccharide on the ACTH-stimulated steroidogenesis*

It is known that ACTH promotes steroidogenesis by isolated adrenocortical cells from adrenal tissues [26, 27]. To measure the effect of lipopolysaccharide on the metabolic function of adrenocortical cells, the ACTH-induced steroidogenesis in the presence of lipopolysaccharide was studied.

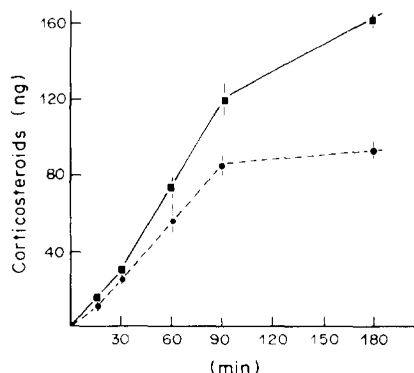

Fig. 1. Time-course of the effect of LPS on ACTH-induced steroidogenesis. Isolated adrenal cells (10^5/ml) were incubated at different times at 37°C with ACTH (1000 μU) (—■—) and ACTH (1000 μU) plus LPS (100 μg) (--●--). Corticosteroids were extracted and measured as described.

Table 1. Effect of lipopolysaccharide on ACTH-induced steroidogenesis

Lipopolysaccharide (μg)	Steroidogenesis variation (%)
—	100
10	88 ± 8
100	56 ± 4
500	53 ± 4
700	32 ± 3
1000	18 ± 2

Cells (10^5/ml) were incubated at 37°C with lipopolysaccharide and ACTH (1 mU) during 90 min. Then, corticosteroids were extracted with dichloromethane and measured as described. Data are the mean values ± SEM calculated from results of four different experiments.

Table 2. Effect of preincubation of adrenal cells with *E. coli* lipopolysaccharide on the biosynthesis of corticosteroids

Preincubation time (min)	Steroidogenesis variation (%)
0	64 ± 5
5	54 ± 4
15	48 ± 4

Cells (10^5/ml) were preincubated (0–15 min) with lipopolysaccharide (100 μg) before addition of ACTH (1 mU). Incubation continued for another 90 min at 37°C. Then, corticosteroids were extracted with dichloromethane and measured as described. Data are the mean values ± SEM calculated from results of four different experiments.

Figure 1 shows the time-course of ACTH-induced steroidogenesis (1000 μU ACTH) and the effect of LPS (100 μg/ml) on this steroidogenesis. The depressed ACTH-induced steroidogenesis by LPS follows a time-course similar to that of the control steroid production by the stimulated adrenal cells. The inhibition was highly dependent on LPS concentration (Table 1). The major effect was observed at the concentration range of 100–1000 μg LPS/ml, which is of the same order of magnitude as that used in *in vitro* experiments with LPS [12, 28, 29]. In further experiments the concentration of LPS used was 100 μg/ml. The inhibitory effect carried out by 100 μg LPS was also dependent on the level of ACTH-induction of the corticosteroid synthesis; this effect was clearly observed at any ACTH concentration. However, the higher the corticosteroid induction by ACTH, the more the effect exerted by LPS (Fig. 2). The effect of *E. coli* LPS was, then, clearly shown on the levels of corticosteroids induced by ACTH; nevertheless, this effect is difficult to evaluate under the basal conditions, due, at least partially, to the low cellular steroid concentrations.

The effect that *E. coli* induces on the synthesis of corticosteroids by the adrenal cells was influenced by its preincubation with the cells prior to the addition of ACTH to the system. Table 2 shows the effect of 100 μg of LPS preincubated with 10^5 cells for different times prior to the addition of ACTH (1000 μU). Thus, preincubation of the cells with the endotoxin provokes a more intense inhibition of the steroidogenesis induced by ACTH.

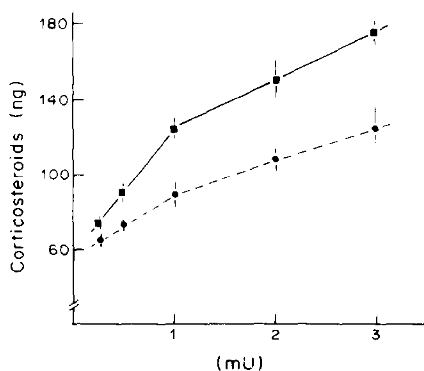

Fig. 2. Effect of LPS on ACTH-induced steroidogenesis. Isolated adrenal cells (10^5/ml) were incubated with increasing amounts of ACTH (250–3000 μU) in the absence (—■—) or presence (--●--) of LPS (100 μg). Corticosteroids were measured as described.

Influence of E. coli *lipopolysaccharide on the binding of ACTH to adrenal cells*

The relationship between binding of ACTH to its specific receptors and the physiological response of the adrenocortical cells is well established [30–33]. In order to elucidate if the decrease on ACTH-induced steroidogenesis exerted by LPS involves a modification on the binding of ACTH to its receptors, the actvity of the *E. coli* LPS on the labelled-hormone binding to the cells was determined. Figure 3 shows the time-course of the specific binding of [^{125}I]ACTH to isolated rat adrenal cells and the

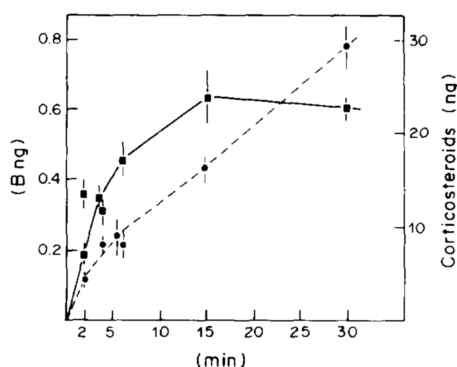

Fig. 3. Time-course of binding of [^{125}I]ACTH (—■—) and corticosteroid response (--●--) of adrenal cells. Isolated adrenal cells (10^5/0.5 ml) were incubated in MEM at 37°C. At each different time the incubation was stopped by cooling in ice and immediate centrifugation in a Beckman microfuge. Aliquots of the supernatants were extracted with dichloromethane for corticosteroid determination. The tube tips were cut and the radioactivity counted. On the left ordinate the amount of [^{125}I]ACTH bound to cells (B) and on the right ordinate the levels of corticosteroids are given. Values were corrected for the non-specific binding of the hormone measured in the presence of excess non-labelled hormone.

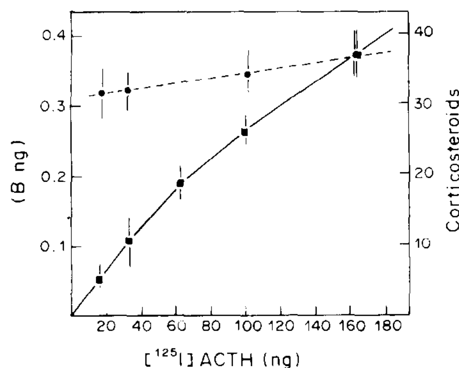

Fig. 4. Effect of increasing concentrations of [^{125}I]ACTH on the steroidogenesis (--●--) and binding of the hormone (—■—). Incubation time was 15 min. On the left ordinate the amount of [^{125}I]ACTH bound to cells (B) and on the right ordinate the levels of corticosteroids are given. Details as in Fig. 3.

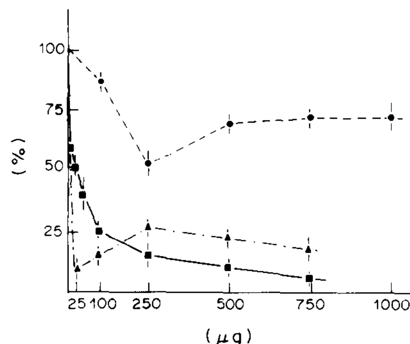

Fig. 5. Percentages of binding of [^{125}I]ACTH to adrenal cells versus unlabelled ACTH and/or LPS. Isolated cells were incubated at 37°C for 15 min (10^5 cells/0.5 ml) in the presence of variable concentrations of either unlabelled ACTH (0–1000 μg; —■—); or LPS (0–1000 μg; --●--) or ACTH (0–1000 μg) plus LPS (500 μg) (-·▲·-).

corticosteroid production. Binding was rapid, being complete within about 12 min; a continuous increase of the [^{125}I]ACTH retention with time was observed up to 12 min, after which no further increase was observed up to 30 min. However, the steroid production exhibited a different pattern with a first rapid phase up to 5–7 min, followed by a linear synthesis during the time of the experiment. In fact, at times of ACTH binding at which there was saturation of hormone binding, steroidogenesis exhibited a linear increase. It appeared, then, that after saturation of a high proportion of the receptors available a maximal effect in the cells could be obtained.

Data shown in Fig. 4 were obtained from saturation analysis and there was a steep increase in the amount bound up to 50–60 ng [^{125}I]ACTH added to the cell suspension with 10^5 cells. Higher concentrations of labelled ACTH resulted in a linear increase in bound radioactivity with a less steep slope. The curve seems to indicate that the binding was a combination of one high affinity process and another one with lower affinity; the later was not saturable with respect to the hormone within the concentration range studied. The simultaneous corticosteroid production (Fig. 4) was maximal even with the lower ACTH concentration used in the assays.

Adrenals cells were exposed to [^{125}I]ACTH and increasing concentrations of either LPS or unlabelled ACTH, alone or in the presence of a fixed amount of LPS (Fig. 5). The simultaneous presence of ACTH plus LPS (500 μg) induced an initial high decrease of the labelled binding and both ligands seem to act in a synergic way at low ACTH concentrations; however, the increase of the unlabelled ACTH concentrations counteracted this effect of LPS. Thus, the addition of 25 μg of ACTH plus 500 μg of LPS to the adrenocortical cells incubated with [^{125}I]ACTH, affected highly the binding of labelled ACTH. On the other hand, when unlabelled ACTH concentration increased, the binding was affected in a smaller extent. Otherwise, the presence of LPS alone modified the hormone binding to its receptors according to a two phase process. With small amounts of LPS there is an important decrease in the binding of the labeled hormone to the receptors while at the higher amounts binding increased to reach a saturation state.

Figure 6 shows the effect of LPS on both [^{125}I]ACTH binding and corticosteroid synthesis. These results indicate a clear decrease of the ACTH-dependent cellular production of corticosteroids in the presence of small amounts of LPS in coincidence with the diminution of the bound hormone; from 500 μg of LPS there was no decrease in the binding of [^{125}I]ACTH although the corticosteroid synthesis still declined gradually.

Binding of [^{14}C]lipopolysaccharide on the isolated adrenal cells

The binding properties of LPS to isolated adrenal cells were studied with [^{14}C]LPS previously isolated from *E. coli* 0111:K58 cultures. The binding saturation assays were performed with [^{14}C]LPS over a

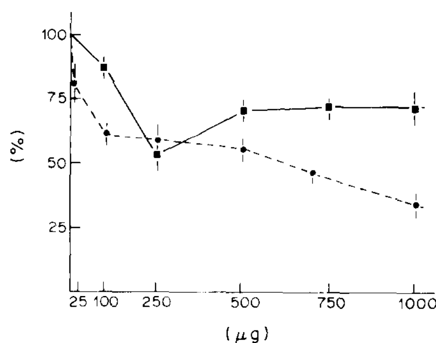

Fig. 6. Percentages of binding of [^{125}I]ACTH to adrenal cells (—■—) and ACTH-induced steroidogenesis (--●--) in the presence of increasing concentrations of LPS (0–1000 μg). Time of incubation, 90 min.

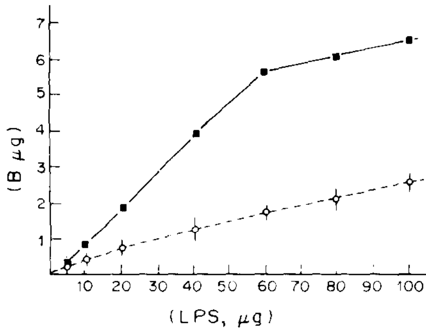

Fig. 7. Binding of [^{14}C]LPS to isolated adrenal cells vs [^{14}C]LPS concentrations. Cells (10^5/0.5 ml) were incubated at 37°C in the presence of increasing concentrations of [^{14}C]LPS (5–100 μg/0.5 ml). Specific activity of [^{14}C]LPS 0.9 μCi/mg. At the end of the incubation period LPS-bound cells were separated by filtration as described under Experimental. Parallel experiments were performed in the presence of a large excess of unlabelled LPS (2 mg/0.5 ml) in order to determine the non-specific binding (--●--). Total binding (—■—).

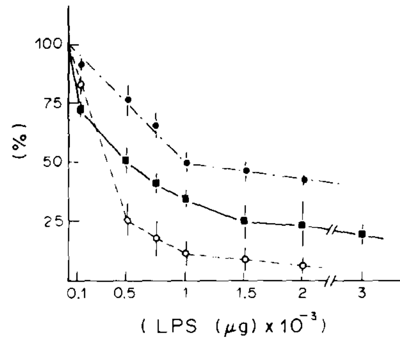

Fig. 9. Effect of different LPS from *E. coli* on binding of [^{14}C]LPS to adrenal cells. Competitive experiments were performed by incubating adrenal cells (10^5/0.5 ml) in the presence of 10 μg [^{14}C]LPS (4.5 μCi/mg) and increasing amounts of unlabelled LPS (*E. coli* 0127:B8, -·●·-; *E. coli* 0111:B4, —■—; *E. coli* 0111:K58, --●--), during 30 min at 37°C. The value of [^{14}C]LPS bound in the absence of unlabelled lipopolysaccharide was taken as 100%.

5–100 μg range (Fig. 7). The non-specific points were determined by incubation with a large excess of unlabeled LPS. The non-specific binding followed a linear saturation in the whole range of [^{14}C]LPS examined; in total binding systems, the concentration of [^{14}C]LPS accumulated followed a linear saturation up to about 60 μg of [^{14}C]LPS and then slopes off as concentration goes higher showing secondary, possibly less specific binding.

The number of cells influenced the total binding of [^{14}C]LPS in dependence of the concentration of [^{14}C]LPS; using 10 μg of [^{14}C]LPS, binding was linear vs cell number up to 10^5 (data not shown).

The time-course of the association of adrenal cells with [^{14}C]LPS for 2–80 min was followed simultaneously to two displacement assays at 10 and 30 min, respectively (Fig. 8). The association of [^{14}C]LPS with

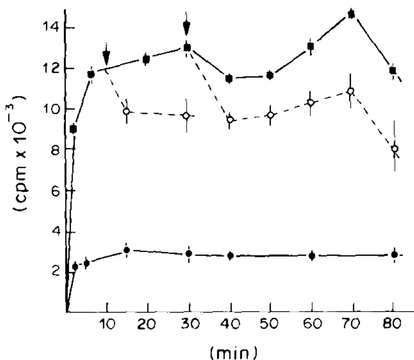

Fig. 8. Time-course of [^{14}C]LPS binding. Cells (10^5/0.5 ml) were incubated at 37°C with [^{14}C]LPS (10 μg/0.5 ml) for various periods of time, in the absence (—■—) or presence (—●—) of a great excess of unlabelled LPS (2 mg/0.5 ml). Arrows show the addition at either 10 or 30 min of unlabelled LPS (2 mg/0.5 ml) to determine the dissociation progress (--●--). Filtration conditions for separation of LPS free and bound to cells were as described.

cells was initially very rapid and at the first minutes started a peculiar pattern of the [^{14}C]LPS bound that has been insistently repeated in a series of similar experiments with different number of cells and [^{14}C]LPS concentration. The discociation of [^{14}C]LPS was initiated by displacement with 2 mg of non-radioactive LPS at two different times and in both cases a clear decline of [^{14}C]LPS was observed. After the displacement, the binding curve also followed a rhythm of the [^{14}C]LPS not displaced from cells (Fig. 8).

Binding experiments with [^{14}C]LPS were also carried out in the simultaneous presence of three different unlabelled LPS preparations from *E. coli*; two preparations were, respectively, from 0127:B8 (different strain from that used for the labelled-LPS) and from *E. coli* 0111:B4 (the same strain as that used for obtaining labelled LPS) and commercially supplied; the another one was obtained by us from *E. coli* 0111:K58 using the same method as for preparing the labelled LPS. [^{14}C]LPS bound to 10^5 cells using 10 μg of labelled LPS was taken as 100% (Fig. 9). It is clear that slight differences in the composition of LPS resulted in different binding influences by the various endotoxins.

Since the effect of diverse endotoxins on the bound [^{14}C]LPS revealed a variation in the interactions between cells and lipopolysaccharides, and taking into account the effect of LPS on the binding of [^{125}I]ACTH to the cells, the analysis of the binding properties of [^{14}C]LPS has been extended to the effect of diverse polypeptide hormones as insulin, glucagon and ACTH. The comparison among the effects induced by these hormones on the percentage of bound [^{14}C]LPS is shown in Fig. 10. A dual effect is clearly observed; the addition of glucagon and ACTH produced a similar decrease in the bound [^{14}C]LPS, whereas insulin had no effect at the same concentration range than the other hormones. It is possible

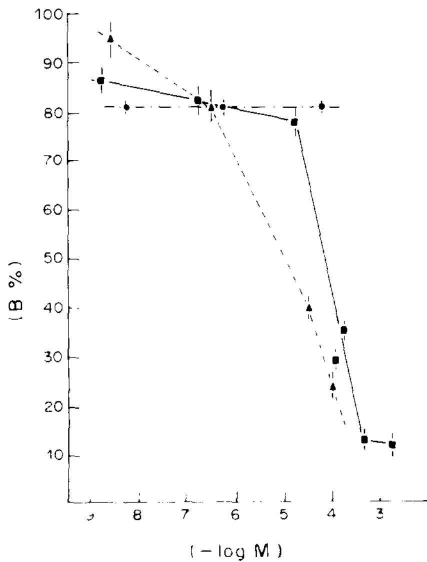

Fig. 10. Effect of different polypeptide hormones on the binding of [^{14}C]LPS (4.5 μCi/mg). Ten μg [^{14}C]LPS were incubated with 10^5 cells in MEM at 37°C with various concentrations of hormones during 30 min: glucagon (--▲--), ACTH (—■—) and insulin (-·●·-). At the end of incubation, [^{14}C]LPS bound to the cells was separated by filtration as described in Experimental. Value for [^{14}C]LPS bound in the absence of any polypeptide hormone was taken as 100%.

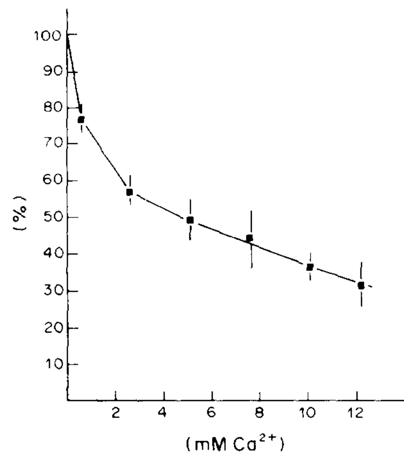

Fig. 11. Effect of calcium concentration on the binding of [^{14}C]LPS (4.5 μCi/mg) to isolated adrenal cells. Binding assays were performed as previously described in MEM containing variable concentrations of $CaCl_2$.

that the different binding mechanisms of the hormones could influence the different ways of displacement of [^{14}C]LPS carried out by the hormones.

Taking into account that most secretory cells, and particularly adrenal cells, have elaborated mechanisms for transducing physiological signals into alterations in cytosolic ionized Ca, adrenal cells were exposed to radioactive LPS under a series of Ca^{2+} concentrations in the medium (Fig. 11). Binding of [^{14}C]LPS was clearly dependent of Ca^{2+} concentration and the higher the presence of this ion in the medium, the lower the ability of LPS to bind adrenal cells.

The presence of diverse proteins in the incubation media, and also under physiological conditions, could influence the binding properties of LPS. In fact, Table 3 shows the notable reduction of LPS-binding by albumin and lima bean trypsin inhibitor in a proportion that is concentration-dependent.

DISCUSSION

Shock caused by burns, trauma, hemorrhage or sepsis eventually acts upon the hypothalamus by means of nerve stimuli from cerebral cortex [34], transformed into humoral messengers specifically corticotropin releasing factor. The release of ACTH from the pituitary into the circulation stimulates the adrenal cortex to release glucocorticoids. This rise in plasma ACTH and glucocorticoid levels has been suggested as a fairly constant characteristic of septic shock [35, 36].

To gain an understanding of the molecular basis of endotoxic shock and since the adrenal cell membrane play a primary role in determining the specificity and the magnitude of the ACTH response, binding of [^{14}C]LPS and the involvement of the endotoxin in the ACTH-binding to isolated rat adrenal cells and their steroidogenic response were investigated in the present study.

Concerning the influence of LPS on the corticosteroid synthesis by adrenal cells, the existence of an inhibitory effect of LPS on the ACTH-induced steroidogenesis is clear, in agreement with results obtained by O'Hare [12] using monolayers of adrenocortical cells. The ACTH-induced steroidogenic response of these cell cultures decreased notably in the presence of high concentrations (1 mg/ml) of bacterial endotoxins. O'Hare interpreted these results as a direct inactivation of ACTH by the endotoxin; however, our results suggest the existence of LPS–cell membrane interactions. The increase of the inhibitory

Table 3. Percentages of binding of lipopolysaccharide to adrenal cells in the presence of various concentrations of albumin and lima bean trypsin inhibitor

Concentration (%)	Albumin	LBTI
0.01	—	73 ± 5
0.05	85 ± 6	34 ± 3
0.10	84 ± 7	26 ± 2
0.20	57 ± 4	–

Cells (10^5/ml) were incubated in MEM at 37°C under O_2/CO_2 with 5 μg of [^{14}C]lipopolysaccharide and either albumin (0.05–0.20%) or lima bean trypsin inhibitor (LBTI) (0.01–0.10%) for 15 min. At the end of incubation, [^{14}C]lipopolysaccharide bound to cells was separated by filtration as described. Data are the mean values ± SEM calculated from results of three different experiments.

effect of LPS on the ACTH-induced steroidogenesis by adrenal cells when the hormone acts after increasing times of preincubation of LPS with the cells, support the hypothesis of a direct interaction of LPS and the cell membrane.

Binding experiments using [^{125}I]ACTH confirmed the presence of a heterogeneous population of ACTH binding sites in agreement with observations of Lefkowitz [30] in mouse adrenal tumor and of McIlhinney and Schulster [32] in rat adrenal cells. The lack of proportionality between the hormone stimulus and the biochemical effect on the cells, suggested a type of amplification of the system [37, 38, 39] similar to that involved in receptor spare capacity.

The initial studies on the influence of *E. coli* endotoxin in the binding of ACTH are in agreement with an influence of LPS on the receptor sites for the hormone. Thus, LPS was able to inhibit about 50%, remaining a fraction of the total binding lower but sufficient for the steroidogenic function. The effect of LPS on the ACTH-stimulated steroidogenesis is consistent with the interpretation of a non-specific process responsible for the binding of LPS to the adrenal cells and, thus, decreasing the ability of ACTH to bind its specific membrane sites as well as the steroidogenic response. The displacement experiments of [^{125}I]ACTH by LPS indicate a modification of the hormone binding to its receptors. This effect supports also the hypothesis of a direct interaction between LPS and the cell membrane with a concomitant distortion of the cell surface affecting the ACTH receptor sites or their environments.

Recent work has implicated the existence in hepatocytes of non-receptors sites for LPS in the cell membrane, although the *E. coli* endotoxins binds to particular ingredients of the membrane [20, 21]. It has been also claimed that LPS binds lymphocytes [19], erythrocytes [14, 19, 40, 41], platelets [19], hepatocytes [41], glia cells [42], etc. with different affinities and effects. However, the results are, effectively, contradictory and they have to be interpreted in terms of the presence of diverse substances as calcium ions, plasma proteins, fetal calf serum, etc.

[^{14}C]LPS binding experiments agree with the effect promoted by the endotoxin on both [^{125}I]ACTH binding and ACTH-stimulated steroidogenesis. In fact, LPS binds to adrenal cells in a significant level. The amount of bound [^{14}C]LPS was influenced by the number of cells and ligand concentration. A 10–15% inhibition of ACTH binding was achieved in the presence of 100 μg of LPS; thus, all the effects on ACTH binding occur at higher concentrations of LPS than the range over which LPS binding is proportional to concentration. These results provide also evidence on the non-specific effect of LPS.

Studies on the inhibition of [^{14}C]LPS binding by the unlabelled LPS and other lipopolysaccharides, performed under identical experimental conditions, indicated that each unlabelled lipopolysaccharide competes with the labelled-LPS throughout similar binding processes; however, marked differences were observed in their effects on the dissociation of [^{14}C]LPS from its initial complex. The interaction displayed clear structural specificity, despite its relative weak nature and the maximal displacement effect was exerted between the endotoxin preparations synthesized by the same procedure from identical *E. coli* strain.

An attempt to elucidate the LPS–cell interaction has been accomplished through the study of binding of [^{14}C]LPS to adrenal cells, assayed in a concentration range, of either polypeptide hormones (ACTH, glucagon and insulin) or proteins usually present in the incubation media as bovine serum albumin and lima bean trypsin inhibitor. ACTH and glucagon, at 10^{-4}–10^{-5} M, were able to reduce in a high extent the interaction of LPS with the cells; however, insulin, at the same concentrations, produces only a low and constant inhibitory effect on the LPS-binding. These results suggest that the hormonal polypeptides may affect the target cell recognition system of LPS in a similar way to the inhibition promoted by LPS on the binding and function of these hormones [43]. The presence of either bovine serum albumin or lima bean inhibitor in the incubation medium produces a concentration-dependent inhibitory effect on the LPS-binding to the adrenal cells. This fact makes it likely that the biological activity of the endotoxin is influenced by the presence in the medium of different concentrations of proteins and it could account for the apparently inconsistent results about either the specific or non-specific nature of the interaction LPS-cell membranes and the inhibitory effect of some proteins on the LPS-binding.

It is worthy to underline the different behaviour of unlabelled LPSs and proteins (albumin, inhibitor, etc.) in the displacement of [^{14}C]LPS. A certain type of specificity was shown by unlabelled LPSs whereas the effect of the proteins was completely non specific; these facts could suggest, firstly, the interaction [^{14}C]LPS-proteins without cell participation, and, secondly, the existence of a physical interaction between LPSs and the cell surface without implication of any specific receptor sites for LPS.

These results agree with those of Galanos *et al.* [44] on the large lamellar structure formed by interaction of LPS with fetal calf serum, that prevents the binding of LPS to the cell surface [45]. The structural models for the amphipathic LPS consider the hydrophobic lipid moieties buried in the middle of a bilayer, while the polar regions of the polysaccharide are exposed to the aqueous spaces [46]. The interaction between cell membranes and LPS seems, then, mediated by hydrophobic associations implying the long chain fatty acids [47]; membrane phospholipids play probably a role in the binding of LPS to the cells because they inhibit the interaction with erythrocyte membranes [48]. Furthermore, using a lipoprotein enzyme [49, 21] as a model for studying

the structural and functional changes under the influence of *E. coli* LPS, we showed that it is reasonable to suppose the involvement of phosphatidylethanolamine in the interaction mechanisms between these macromolecules. This mechanism would serve to support the mode of attachment of LPS to the cell surface, envisaged as a process involving hydrophobic interactions between the hydrophobic groups in the membrane [22]. It is presumable that these facts have to be related to the influence of Ca^{2+} on both the cell membrane structure and the cell functionality [50–58]. We have previously shown that *E. coli* endotoxin affects the mitochondrial membrane involving a significant uptake and mobilization of Ca^{2+} [9]. LPS and Ca^{2+} promote a preferential release of polyunsaturated fatty acids that has been interpreted through a phospholipase activation hydrolysing phospholipids distributed selectively in the mitochondrial membranes [9]. Concerning the presence of Ca^{2+} in the LPS-binding experiments, it is necessary to take into account that 3–4 mM Ca^{2+} in the medium inhibits LPS-binding about 50% and, on the other side, that Ca^{2+} is required for the ACTH-induced steroidogenesis in adrenal cells. These are the reasons for using low Ca^{2+} concentrations in the LPS-binding experiments. Calcium ions play, then, a role in the regulation of mutual ACTH–LPS interactions and high Ca^{2+} levels favours the functionally of the hormone and, simultaneously, load polarly the membrane inhibiting its hydrophobic interactions with LPS. The presence of calcium ions in turn favors the activities of specific systems providing the possibility of time-dependent biochemical alterations [59]. A certain degree of time-dependence in the binding of LPS to adrenal cells has been shown in these experiments albeit other approaches are necessary to dilucidate the molecular events leading to this regulation.

All these facts tend to support the idea of non specific interactions of LPS with the adrenal membrane that, however, modifies the physiological specificity of the cell in order to bind its trophic hormone or to carry out its transport activities. The inhibition of pregnenolone- and dibuyril-cAMP-induced steroidogenesis by LPS can also be interpreted through the membrane modification [43].

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