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ENDOTOXIC LIPOPOLYSACCHARIDES STIMULATE STEROIDOGENESIS AND ADENYLATE CYCLASE IN ADRENAL TUMOR CELLS

J. WOLFF and G. HOPE COOK

National Institutes of Arthritis, Metabolism and Digestive Diseases, National Institutes of Health, Bethesda, Md. 20014 (U.S.A.)

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SUMMARY

Lipopolysaccharides (endotoxins) from *Escherichia coli*, *Serratia marcescens* and *Salmonella typhosa* stimulated steroid production in Y-1 adrenal tumor cells in culture with a latent period of 3–4 h. Lipid A, derived from *Escherichia coli* lipopolysaccharide, also stimulated steroidogenesis. Lipopolysaccharides and lipid A also stimulate adenylate cyclase activity and cause rounding of the cells. In contrast, lipopolysaccharides do not stimulate steroidogenesis in receptor-deficient adrenal tumor cells (OS-3) or Leydig tumor cells (I-10). This tends to rule out contamination by enterotoxin to which these lines respond. Although both hormone and lipopolysaccharide responses are lost in these lines, there was no interaction between these sites as judged by the failure of lipopolysaccharides to block, during their latency, the response to corticotropin in Y-1 cells. The possibility that the lipopolysaccharide effect is one on membrane conformation is discussed.

INTRODUCTION

The bacterial exo-enterotoxins obtained from *Vibrio cholerae* and *Escherichia coli* exhibit widespread biological effects that are thought to operate through the adenylate cyclase system [1–4]. In contrast to these protein toxins, the endotoxins or lipopolysaccharides, which are derived from the cell walls of gram-negative organisms, are thought to act by a variety of other mechanisms [5]. It was of great interest, therefore, when Bitensky et al. [6] showed that the increase in tissue sensitivity toward catecholamines seen with injected endotoxin might be due to activation of adenylate cyclase. *E. coli* endotoxin produced a selective stimulation of the epinephrine-sensitive adenylate cyclase of liver membranes from mice. It has also been shown that endotoxin-treated fat cells have an increased cyclic AMP content, especially, after norepinephrine stimulation [7]. To investigate whether this property of the lipopolysaccharides could be expressed in other membranes, we investigated their effect on steroidogenesis of Y-1 mouse adrenal tumor cells in culture [8]. The response to crude lipopolysaccharides from *E. coli*, *Serratia marcescens* or *Salmonella typhosa*

Abbreviation: ACTH, corticotropin.

[8] was half-maximal at 5, 10 and 30 ng/ml of incubation medium, respectively. In the present study we report the nature of activation of steroidogenesis obtained with these agents in Y-1 cells as well as similar studies in a mutant derived from it called OS-3, and a receptor-deficient Leydig tumor cell line, I-10.

METHODS

The bacterial lipopolysaccharides, obtained from Difco, were *E. coli* (0111 : B_4) (B) or (0-128 : B12) (W), *S. marcescens* T, and *S. typhosa* (0901) (B). They were suspended in protein-free F-10 medium by brief, low-energy, sonication (1–2 s). Sonication was not necessary but facilitated formation of uniform suspensions. The corticotropin (ACTH) used was either ACTH (ACTHAR-Armour) or Synacthen ($^{1-24}$ACTH), the 1-24 N-terminal polypeptide of ACTH. Mixed gangliosides were the gift of Dr. E. G. Trams. Lipid A was prepared from *E. coli* 055 : B5 lipopolysaccharide by the method of Andersson et al. [12]. We should like to thank Dr. John L. Ryan for his generous gift of this material.

Cell lines were obtained and maintained as described [13]. Steroid output, measured fluorimetrically against standards of 20α-dihydroprogesterone, and protein were measured as described in the accompanying paper [13].

Adenylate cyclase was determined by the method of Salomon et al. [14]. The cells were exposed to stimulants for 15–16 h, harvested in 0.25 M sucrose, 3 mM Tris–HCl, pH 7.4, 1 mM dithiothreitol, sedimented, and homogenized in a tight-fitting Dounce homogenizer. The $11000 \times g$ pellet was resuspended, the DNA gel was removed and the suspension was again centrifuged at $11000 \times g$. The pellet was resuspended and was assayed as previously described [15], but in the presence of 30 μM 5′-guanylylimidophosphate.

RESULTS

A characteristic of the steroidogenic response of Y-1 cells to choleragen is a latent period of approx. 1 h [8]. It was, therefore, important to see if a similar delay

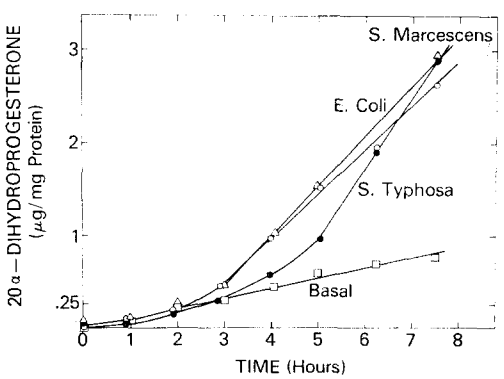

Fig. 1. Time curve for the steroidogenic response to lipopolysaccharides in Y-1 cells. Dishes were washed twice with medium at 37 °C and were then incubated with 50 μg/ml of lipopolysaccharide. 1-ml samples of the media were withdrawn at intervals and assayed for steroid fluorimetrically. Results are expressed as 20α-dihydroprogesterone. All results are means of triplicate dishes.

TABLE I

EFFECT OF LIPID A FROM *E. COLI* LIPOPOLYSACCHARIDE ON STEROIDOGENESIS

Y-1 cells incubated for 15 h (not corrected for latent period). Lipid A (1.0 mg/ml) was sonicated briefly in Dulbecco's phosphate-buffered saline just before addition to the media.

Lipid A (μg/ml)	Steroid output (μg/mg per h of 20α-dihydroprogesterone)
Control	0.044 $\pm$ 0.001
0.01	0.11 $\pm$ 0.002
0.1	0.18 $\pm$ 0.004
1.0	0.19 $\pm$ 0.002
10.0	0.25 $\pm$ 0.014
$^{1-24}$ACTH (1 μM)	0.41 $\pm$ 0.010

occurred in the response to the bacterial lipopolysaccharides. As shown in Fig. 1, while there are slight differences in the response pattern with the *Salmonella* lipopolysaccharide, all three substances exhibited a latent period of 3–4 h; this is considerably longer than required under the same growth conditions, etc., for choleragen [8].

Since most, if not all, of the effects of the lipopolysaccharides are apparently caused by the lipid moiety (lipid A) of these compounds [16–18], it was of interest to see if steroidogenesis could also be stimulated by lipid A, the lipid derived from lipopolysaccharide. As can be seen in Table I, sonicated suspensions of lipid A from *E. coli* were able to increase the steroid output of Y-1 cells, although on a weight basis, this material was less active than lipopolysaccharide itself as is true when other biological properties are measured [18].

That these polysaccharides may stimulate steroidogenesis by means of the adenylate cyclase present in the Y-1 cells is shown in Table II. Although stimulation

TABLE II

THE EFFECT OF LIPOPOLYSACCHARIDES OR LIPID A ON ADENYLATE CYCLASE ACTIVITY OF Y-1 ADRENAL CELLS

Cells were harvested in 0.25 M sucrose, 3 mM Tris buffer, 1 mM dithiothreitol containing 2 μg/ml of the appropriate concentration of lipopolysaccharide or lipid A and washed and pooled in the same medium. Cells were homogenized and the 11000 $\times g$ pellet was resuspended in the above medium, sonicated for 1 s at low energy, recentrifuged, resuspended, and used immediately. Incubation time 16 h in Expt A and 13 h in Expt B.

Experiment	Stimulator	Concentration	Adenylate cyclase activity (nmol cyclic AMP/mg protein per 10 min)
A	Control	—	0.32
	$^{1-24}$ACTH	1.6 μM	1.18
	F^-	10 mM	0.92
	E. coli lipopolysaccharide	2 μg/ml	0.97
	S. marcescens lipopolysaccharide	2 μg/ml	1.03
	S. typhosa lipopolysaccharide	2 μg/ml	0.55
B	Control	—	0.35
	E. coli lipid A	10 μg/ml	0.67

TABLE III

THE EFFECT OF LIPOPOLYSACCHARIDES ON THE RESPONSE TO ACTH IN Y-1 CELLS

Lipopolysaccharides added 10 min before ACTH. Incubation was for 1.0 h. All values are means of duplicate determinations on each of three dishes. Steroid secretion is expressed as fluorescence equivalent to stated amounts of 20α-dihydroprogesterone.

Lipopolysaccharide (50 μg/ml)	Steroid secretion (μg/mg per h)	
	Control	ACTH (50 munits/ml)
—	0.25±0.011	3.37±0.038
E. coli	0.29±0.027	3.70±0.089
S. marcescens	0.31±0.020	3.33±0.047
S. typhosa	0.37±0.033	3.64±0.153

in the crude preparations was never as intense as after choleragen treatment of the cells [8], stimulation persisted through the preparative procedure when the cells were continuously exposed to high concentrations (2 μg/ml) of the lipopolysaccharides or lipid A. There was some variation in the relative response to the three different lipopolysaccharides especially when ITP was used as the nucleoside triphosphate; *S. typhosa* endotoxin tended to be less active. Addition of *E. coli* lipopolysaccharide or lipid A to control membrane preparations only during the assay period for adenylate cyclase did not increase basal activity (unpublished results).

The steroidogenic response to ACTH during the latent period (1 h incubation in this experiment) was in no way altered by the presence of excess concentrations of the lipopolysaccharides as shown in Table III. There was no cell rounding during the latent period in response to lipopolysaccharides, and the rounding response to ACTH was not altered. This suggested the possibility that these ACTH receptors were independent of the surface domain with which the lipopolysaccharides interact.

As a further test of the relation between these receptor domains, we tested the effect of these substances in the two receptor-deficient lines OS-3 and I-10. As shown in Table IV, while these cells responded sensitively to choleragen, they did not respond to very large concentrations of lipopolysaccharides with an increased output of steroids. This is in marked contrast to the Y-1 cell line. Moreover, the OS-3 cells did not show rounding in response to lipopolysaccharides as do the Y-1 cells. Although adenylate cyclase is not stimulated in OS-3 or I-10 cells, this was not investigated

TABLE IV

THE EFFECT OF CHOLERAGEN AND LIPOPOLYSACCHARIDES ON STEROID SECRETION BY Y-1, OS-3 AND I-10 CELLS

Agent	Concentration for half-maximal secretion (ng/ml)		
	Y-1	OS-3	I-10
Choleragen	1.3	3.0	0.3
E. coli (0111 : B_4)B	5.0	> 50000	> 50000
S. marcescens (T)	10.0	> 50000	> 50000
S. typhosa (0901) (B)	30.0	> 50000	> 50000

TABLE V

EFFECT OF LIPOPOLYSACCHARIDES ON THE STEROIDOGENIC RESPONSE OF I-10 AND OS-3 CELLS TO CHOLERAGEN AND CYCLIC AMP

The results are expressed as μg steroid/mg per h.

Cell line	Lipopolysaccharide (50 μg/ml)*	Control	Cyclic AMP (0.5 mM)	Choleragen (0.36 nM)
I-10	—	0.11	0.57	0.47
	S. marcescens	0.11	0.53	0.47
	S. typhosa	0.10	0.54	0.49
OS-3	—	0.03	—	0.34
	E. coli	0.02	—	0.34
	S. typhosa	0.02	—	0.31

* Added 5 min before cyclic AMP or choleragen. Incubation time 16.0 h. Steroid production is expressed as fluorescence equivalent to stated amounts of 20α-dihydroprogesterone.

extensively, since steroidogenesis is a much more sensitive response. That the resistance to the lipopolysaccharides was not due to a general poisoning of lines OS-3 or I-10 is depicted in Table V where we show that the steroidogenic response to cyclic AMP on choleragen in I-10 is in no way influenced by the presence in the culture dish of large concentrations of *S. marcescens* or *S. typhosa* lipopolysaccharides. Similar results were obtained with OS-3 cells.

DISCUSSION

The effect of endotoxins on the Y-1 adrenal tumor cells bears a certain resemblance to the effect of the enterotoxin, choleragen. Both products exhibit a latent period for steroidogeneses (unlike ACTH), although that for the lipopolysaccharides is longer than that for choleragen. Both compounds stimulate the adenylate cyclase of Y-1 cells, although the effect with the lipopolysaccharides is less marked. Since enterotoxins similar to choleragen occur in *E. coli*, it was possible that the observed effects with lipopolysaccharides were the result of contamination of these preparations with such enterotoxins. Moreover, *E. coli* enterotoxin stimulates steroidogenesis in OS-3 cells [19]. The results with the OS-3 and I-10 lines of the present study effectively rule out such contamination (Table IV) and suggest that the ability to stimulate steroid production and adenylate cyclase are intrinsic to the lipopolysaccharides. Furthermore, large concentrations of mixed gangliosides, which completely prevent the steroidogenic response to choleragen [8], had no effect on the steroidogenic response of Y-1 cells to the lipopolysaccharides (unpublished observations). It seems possible that the high sensitivity of the Y-1 cell will permit the development of an assay for lipopolysaccharides that is more convenient than those currently available.

The simultaneous loss of responsiveness to hormones and endotoxins in OS-3 and I-10 cells might suggest that the lipopolysaccharides could share a portion of the receptor domain of ACTH or lutropin but not that of choleragen. Nevertheless, during their latent period, endotoxins from *E. coli*, *S. marcescens* or *S. typhosa* failed

to block the ACTH response of Y-1 cells (Table III). Thus the receptors appear to be different. Since both the F^--activated and choleragen-stimulated cyclases of the deficient cell lines is intact, the simultaneous loss of both responses may imply loss of a commonly shared connection (allosteric regulator) or transducer, between receptor and cyclase, or a more massive membrane deficit affecting both receptor domains.

It is tempting to propose that these changes in adenylate cyclase activity may result from conformational changes produced in the membranes of lipopolysaccharides. In addition to their well-known role in passive hemagglutination reactions [16], it is known that lipopolysaccharides bind to such membranes as shown by labelled endotoxin [20–22] or electron microscopy [23]. Moreover, a homogeneous membrane receptor for lipopolysaccharide has been isolated from erythrocytes [22]. The compounds also destabilize lysosomal membranes [24, 25] and phospholipid bilayers [16, 26], and penetrate phospholipid monolayers. These effects are presumed to occur by virtue of the lipid moiety of the lipopolysaccharides [15–17]. While most of these effects require much higher concentrations of lipopolysaccharide than necessary to activate adenylate cyclase or steroidogenesis in Y-1 cells, it is possible that the conformational changes required are of a much subtler sort and would not necessarily be seen by the above physical techniques. Evidence has previously been supplied to suggest that hormones [27] and polycations [28] may stimulate adenylate cyclase through conformational changes produced in membranes. Because of the low concentrations of lipopolysaccharide required to elicit a steroidogenic response, we cannot, at present, rule out a more specific effect on the membrane. However, the conformational interpretation developed above is otherwise consistent with the information available on lipopolysaccharides. It would rationalize both enhancement of hormone-induced activity [6, 7] and direct stimulation of adenylate cyclase.

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