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A novel mechanism of soluble guanylate cyclase stimulation: time-dependent activation by bacterial lipopolysaccharide in rat fetal spleen cells

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Small amounts of bacterial lipopolysaccharide (LPS) greatly increase cGMP levels in short term cultures of rat fetal liver and spleen cells in a dose and time dependent manner. To determine the role of guanylate cyclase in this response, a series of experiments was undertaken using either intact or broken fetal spleen cells, the most sensitive tissue evaluated to date. The phosphodiesterase inhibitor, 1-methyl-3-isobutylxanthine, potentiated the LPS-cGMP effect in cultures of these cells even at maximal doses of LPS. Moreover, after incubation of intact cells with LPS for 4 h, soluble guanylate cyclase (EC 4.6.1.2) activity was increased 2-fold, whereas particulate activity was unchanged. This increase in soluble activity was proportional to the dose of LPS, was synchronous with the elevation of cGMP levels, and was not associated with any change in cGMP-phosphodiesterase (EC 3.1.4.17) activity. In contrast to intact cells, neither total nor soluble guanylate cyclase activity was increased by the addition of LPS to spleen cell whole sonicate or cytosol for various times from 10 min to 3.5 h. These results suggest that the LPS-cGMP response is due to a persistent indirect stimulation of soluble guanylate cyclase activity that is both dose and time dependent.

Introduction

Bacterial lipopolysaccharide (LPS) greatly increases cGMP levels in short term cultures of rat fetal liver cells and to a lesser extent in similar cultures of rat adult spleen cells without affecting the levels of cAMP. This effect is produced by very small (1 ng/ml) concentrations of LPS, is both dose and time dependent, is abolished by inhibitors of RNA and protein synthesis, and is potentiated by the phosphodiesterase inhibitor, 1-methyl-3-isobutylxanthine (MIX) [1–6]. Since this potentiation by MIX occurs with near maximum doses of LPS, it seems possible that the elevation of cGMP produced by LPS is due to an effect of LPS on guanylate cyclase (EC 4.6.1.2) activity. In an effort to evaluate this hypothesis, a series of experiments was undertaken in which guanylate cyclase activity was measured in cells cultured for various periods in the presence or absence of different concentrations of LPS and in broken cell preparations treated with LPS. Fetal spleen tissue was used for these experiments, be-

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Abbreviations: LPS, lipopolysaccharide; MIX, 1-methyl-3-isobutylxanthine; HBSS, Hank's balanced salt solution.

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cause it was found to be even more responsive to LPS than fetal liver and because larger quantities of tissue could be obtained from a given number of animals. The results reported here indicate that LPS stimulates soluble guanylate cyclase activity in an indirect but persistent manner that is both time and dose dependent.

Materials and Methods

Methods

The methods were modified slightly from those previously described [3–5]. To minimize contamination by exogenous LPS, all glassware and instruments were wrapped in aluminum foil and baked for 4 h at 180°C prior to use and sterile technique was rigorously followed wherever possible. Cell suspensions in *Limulus* lysate absorbed Waymouth's MB752-1 medium (Gibco, Grand Island, NY) were prepared from fetal spleens obtained aseptically from timed pregnant rats (Holtzman Animal Labs, Madison, WI) on the 20th day of gestation. Spleens were washed three times in Hank's balanced salt solution (HBSS) and placed in a sterile filter bag obtained from a standard platelet recipient set (Fenwal, Deerfield, IL). The filter containing the spleens was transferred to a sterile plastic petri dish (Fisher Scientific, Memphis, TN) that contained 10 ml of absorbed Waymouth's medium and the spleens were then disaggregated by gently forcing them through the filter with a sterile plastic 1 ml syringe handle. The concentration of nucleated cells was determined on a Coulter counter and adjusted to $(3–10) \cdot 10^6$ cells per ml.

In a single experiment, 1 ml aliquots of fetal spleen cells were cultured with increasing concentrations of LPS with and without MIX for 4 h at 37°C in Waymouth's medium containing 0.5% fetal calf serum. Cultures were terminated with 1 ml of 10% trichloroacetic acid and cGMP was measured by radioimmunoassay.

All other experiments were designed to measure the effect of LPS on guanylate cyclase or phosphodiesterase (EC 3.1.4.17) activity. In one group of these experiments, the effect on guanylate cyclase activity of incubating intact cells with LPS was evaluated. Twenty to 25 ml of fetal spleen cell suspension in Waymouth's medium were cultured in 250-ml sterile polypropylene centrifuge tubes (Corning Glass Works, Corning, NY) for varying periods up to 4 h at 37°C in a 5% CO_2/95% air atmosphere with or without varying amounts of LPS. The LPS was added in a 500 μl volume of HBSS that contained 100–125 μl of fetal calf serum (Hyclone, Logan, UT). Control cultures received an equal volume of HBSS that contained only fetal calf serum. At the time specified for each experiment, the cultures were removed from the incubator, mixed gently, and 1-ml aliquots were added directly to 1 ml of 10% trichloroacetic acid. This sample was used to determine whole culture cGMP levels. The remainder of the culture was immediately centrifuged at $1000 \times g$ for 5 min at 4°C. The supernatant was discarded, and 2 ml of 2 mM triethanolamine buffer were added to the pellet. The 250 ml centrifuge tube was placed in ice water, and the cells were disrupted by sonication using a microprobe with three 5-s bursts of 40 watts each separated by 10-s intervals (Branson Instruments, Danbury, CT). This resulted in disruption of greater than 99% of the cells. A portion of the whole sonicate was then centrifuged at $48000 \times g$ for 20 min at 4°C. The supernatant was removed and used in various dilutions to assay soluble guanylate cyclase activity. The pellet was resuspended in 1 ml of 2 mM triethanolamine buffer with two 3-s bursts using a Polytron homogenizer (Brinkman Instruments, Westbury, NY). This preparation was used to assay particulate guanylate cyclase activity.

In a second group of experiments, the effect on guanylate cyclase activity of adding LPS directly to a broken cell preparation was evaluated. Twenty to 25-ml volumes of the fetal spleen cell suspension were transferred to 250-ml centrifuge tubes which were immediately spun at $1000 \times g$ for 5 min at 4°C. Supernatants were discarded and whole sonicate, soluble and particulate enzyme preparations were made exactly as described above. Aliquots of these preparations were incubated in individual tubes with various combinations of HBSS, LPS, fetal calf serum and/or dithiothreitol in a shaking water bath at 30°C. At various times from 10 min to 3.5 h, guanylate cyclase activity was determined by adding 50 μl of reaction mixture to each tube and continuing the incubation for 3 min. The reaction was then stopped with 1 ml of cold 10% trichloroacetic acid.

Guanylate cyclase was assayed by incubating each enzyme preparation (whole sonicate, soluble fraction or particulate fraction) with GTP in a shaking water bath at 30 °C for various periods and measuring the amount of cGMP formed using a radioimmunoassay [7]. Unless otherwise specified, a 50 μl volume of enzyme was mixed with 50 μl of reaction mixture consisting of final concentrations for the 100 μl of 0.5 mM GTP, 50 mM triethanolamine buffer pH 7.7, 1 mM MIX, 2 mM magnesium chloride, and 5 mM dithiothreitol. The presence of a GTP regenerating system (3.75 mM creatine phosphate and 10 μg of creatine kinase) did not significantly affect the assay, while replacing Mg^{2+} with Mn^{2+} increased activity several-fold but decreased test to control ratios. Thus, neither a regenerating system nor Mn^{2+} was routinely used. Assays were initiated by either adding enzyme to reaction mixture or the opposite as specified for each experiment and were terminated by the addition of 1 ml of 10% trichloroacetic acid. For blanks, the trichloroacetic acid was added just prior to the addition of reaction mixture or enzyme. Under these conditions, the amount of cGMP generated was linear with time for at least 4 min (Fig. 2) and enzyme concentration (Fig. 3).

cGMP was measured by radioimmunoassay of diethyl ether-extracted samples [8,9]. After centrifugation, the supernatants containing trichloroacetic acid were extracted three times with 5 vol. of water-saturated diethyl ether, heated to 50 °C for 25 min to remove residual diethyl ether, diluted as necessary in pH 4.0 sodium acetate buffer to a final concentration of 50 mM, acetylated and assayed for cGMP. Fresh standards were acetylated along with the samples for each assay. Dilution analysis of a pooled sample with or without a fixed amount (8 fmol) of added standard showed correlation coefficients greater that 0.98 for both cases and y intercepts of 8.87 and 0.88 fmol, respectively. The protein concentration of all enzyme preparations was measured by a micro-modification of the Biuret method [10] and activity was expressed as fmol of cGMP per μg of protein per unit of time.

cGMP-phosphodiesterase activity was measured in the presence or absence of purified calmodulin and/or 1 mM EGTA as previously described [11].

The data were evaluated by standard statistical methods including the unpaired t test and linear regression analysis [12].

Reagents and chemicals

Salmonella minnesota 9700 LPS prepared by the phenol extraction method of Westphal and Jann [13] was purchased from Difco Laboratories, Detroit, MI and used as supplied. Because occasional lots of tissue culture medium were found to contain significant quantities of LPS (up to 0.5 ng per ml), all Waymouth's medium was absorbed with *Limulus* lysate [2,5,14]. One vial (50 mg) of *Limulus* lysate (Whittaker, Walkersville, MD) dissolved in pyrogen free water (4 ml) was added to each 100 ml bottle of Waymouth's. After incubation at 37 °c for 4 h and room temperature for 20 h, the medium was passed through a 0.45 micron filter (Nalge Company, Rochester, NY) and then heated to 56 ° C for 30 min to destroy and remaining *Limulus* activity. The 2 mM triethanolamine buffer, pH 7.1, used for sonication, resuspension and dilution of enzyme preparations contained final concentrations of 10 mM NaCl, 10 mM KCl, 0.1 mM dithiothreitol and 5 μM EDTA. GTP, MIX, and dithiothreitol were purchased from Sigma Chemical Co., St. Louis, MO. All other chemicals including $MgCl_2$, $MnCl_2$, NaCl, KCl, sodium acetate, acetic acid, trichloroacetic acid and hydrochloric acid were purchased from either Sigma or Fisher Scientific. Guanosine 3′,5′-cyclic phosphoric acid 2′-*O*-succinyl [^{125}I]iodotyrosine methyl ester (New England Nuclear, Boston, MA) diluted in 50 mM acetate buffer, pH 4.0, was used in the radioimmunoassay for cGMP.

Results

The results of incubating rat fetal spleen cells obtained on day 20 of gestation with increasing amounts of LPS with or without 0.1 mM MIX for 4 h are shown in Fig. 1. LPS alone produced a dose dependent increase in cGMP levels which reached 44-fold (0.41–18.2 pmol/ml) at an LPS concentration of 10 ng/ml. MIX by itself raised the basal concentration of cGMP 15-fold to 6.0 pmol/ml and LPS in the presence of MIX further increased cGMP levels to a maximal value of 47

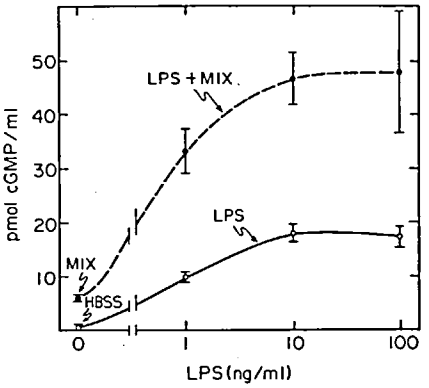

Fig. 1. Effect of increasing concentrations of LPS, with or without 0.1 mM MIX, on cGMP levels in cultures of 20 day rat fetal spleen cells. The incubation period was 4 h, the culture volume was 1 ml, and the cell concentration was $3 \cdot 10^6$ per ml. Values equal the mean of three replicate cultures ± 1 S.D.

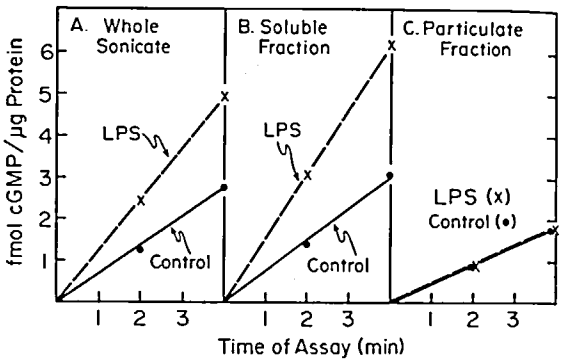

Fig. 2. Effect of culturing fetal spleen cells with LPS on whole sonicate, soluble and particulate guanylate cyclase activity expressed as a function of assay time. The enzyme preparations were made immediately after 4 h of culture in the presence or absence of 20 ng of LPS per ml of medium. The guanylate cyclase assay was initiated by adding 50 µl of undiluted enzyme to 50 µl of reaction mixture and was terminated 2 or 4 min later with 1 ml of 10% trichloroacetic acid. Values equal the mean of two measurements.

pmol/ml. This effect of LPS in fetal spleen cells is 4–5 times greater than with 13 day fetal liver [4], the next most sensitive tissue. Furthermore, a much larger amount of splenic tissue could be obtained from a given number of animals. Thus, fetal spleen tissue obtained on day 20 of gestation was used in all subsequent experiments.

In order to evaluate the effect of LPS on guanylate cyclase, total (whole sonicate), soluble and particulate enzyme activities were measured in fetal spleen cell suspensions cultured in the presence or absence of 20 ng of LPS per ml for 4 h. Enzyme activity as a function of time of assay or of enzyme concentration is shown in Figs. 2 and 3, respectively. In both cases, the activity was linear under the conditions of the assay and in both cases whole sonicate and soluble cyclase activities were about 2-fold greater in the preparations derived from LPS treated cells, whereas particulate enzyme activity was identical in LPS treated and control cells. Thus, cells incubated with LPS appeared to have a clear elevation of guanylate cyclase activity which resided entirely within the soluble fraction.

In an effort to determine the optimal assay system, the effect of varying the conditions of assay was evaluated using soluble enzyme prepared from cells cultured for 4 h in the presence or absence of 10 ng/ml of LPS. Including a GTP generating system or deleting dithiothreitol from the reaction mixture had no significant effect on total activity or on the LPS to control ratio of activities. Substituting Mn^{2+} for Mg^{2+} increased

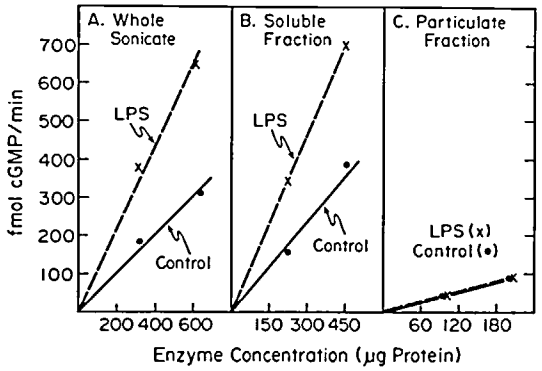

Fig. 3. Effect of culturing fetal spleen cells with LPS on whole sonicate, soluble and particulate guanylate cyclase activity expressed as a function of enzyme concentration. The enzyme preparations were made immediately after 4 h of culture in the presence or absence of 20 ng of LPS per ml of medium. The guanylate cyclase assay was initiated by adding 50 µl of undiluted or 1:2 dilution of enzyme to 50 µl of reaction mixture and was terminated 2 min later with 1 ml of 10% trichloroacetic acid. Values equal the mean of two measurements.

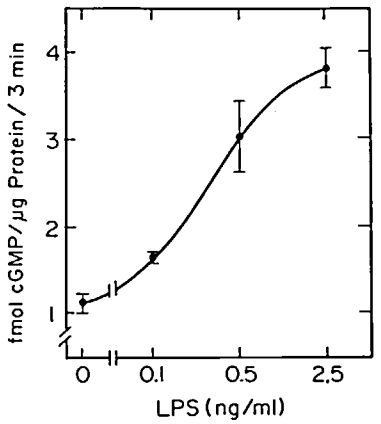

Fig. 4. Effect of culturing fetal spleen cells with increasing concentrations of LPS on soluble guanylate cyclase activity. The culture period was 4 h. Values equal the mean of four measurements ± 1 S.D.

soluble guanylate cyclase activity about 7-fold; however, the ratio between LPS and control activities decreased from 2.2 to 1.2. Time and protein concentration curves confirmed that the cyclase reaction was linear with Mn^{2+}. Thus, since changes in the reaction mixture did not improve the fold effect of LPS, all experiments were done with the standard reaction mixture, i.e., without Mn^{2+} or a regenerating system.

In an effort to more firmly link the increase in soluble guanylate cyclase activity to the LPS-cGMP response, the effects of varying the dose of LPS and the duration of culture on both guanylate cyclase activity and cGMP levels were studied. When fetal spleen cells were incubated with increasing amounts of LPS from 0.5 to 2.5 ng/ml for 4 h, there was a dose dependent increase in guanylate cyclase activity from 1.4 to 3.4 times control values (Fig. 4). Moreover, this increase in soluble guanylate cyclase activity was proportional to the cGMP levels in the cultures. This is shown in Fig. 5, where soluble guanylate cyclase activity is compared to corresponding cGMP levels in the cultures from three separate experiments. Linear regression analysis of this data showed a strong correlation ($r = 0.91$) between soluble guanylate cyclase activity and the cGMP concentration. The time course of the cGMP response to LPS in culture is characterized by a lag period of 60–120 min followed by a rapid and persistent elevation of the cGMP concentration. When fetal spleen cells were cultured for varying times from 30 min to 4 h in the presence of a constant amount of LPS (5 ng/ml), both soluble guanylate cyclase activity and cGMP levels rose in a concomitant fashion between 2 and 4 h of culture (Fig. 6). Thus, the increase of soluble guanylate cyclase activity in cells cultured with LPS is proportional to the dose of LPS used and synchronous with the elevation of cGMP levels.

In order to determine whether LPS could directly increase guanylate cyclase activity, the effect of adding LPS to a broken cell preparation was evaluated (Table I). Fresh whole sonicate or cytosol made from fetal spleen cells was incubated in the presence or absence of LPS from 10 min to 3.5 h with or without fetal calf serum. Fetal calf serum was included because very small amounts (1–20 µl/ml) markedly increase the ability of LPS to elevate cGMP levels in whole cell cultures [5]. There was no significant difference ($P > 0.1$) between LPS and control samples with or without fetal calf serum with both enzyme preparations at all times tested. With whole sonicate, a substantial increase in guanylate cyclase activity did occur with time primarily between 1 and 3.5 h of incubation. This effect was partially blocked by serum and did not appear to be related to LPS. With cytosol, a marked increase in soluble guany-

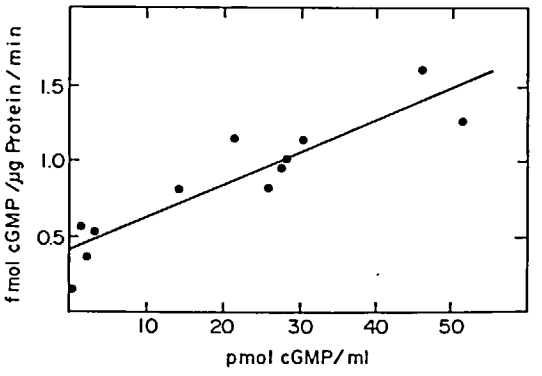

Fig. 5. Relationship between whole culture cGMP levels and soluble guanylate cyclase activity in fetal spleen cells cultured for 4 h with various concentrations of LPS. Each point represents the cGMP level and soluble guanylate cyclase activity from a single culture. Data are from three separate experiments of four cultures each.

TABLE I

EFFECT OF ADDING LPS DIRECTLY TO FETAL SPLEEN WHOLE SONICATE OR CYTOSOL ON TOTAL AND SOLUBLE GUANYLATE CYCLASE ACTIVITY

Thirty μl of fetal spleen whole sonicate or cytosol were incubated for varying periods at 30 °C in a shaking water bath with 20 μl containing either buffer, LPS, fetal calf serum or fetal calf serum + LPS. The final concentrations of LPS and serum were 10 ng and 20 μl/ml, respectively. At 10 min, 1 h and 3.5 h, guanylate cyclase activity was assayed by adding 50 μl of standard reaction mixture to each tube and continuing the incubation for 3 min. Values are expressed as fmol cGMP/μg protein per 3 min and equal the mean of four replicate samples ± 1 S.D.

Enzyme preparation	Additives	Guanylate cyclase activity after various times of incubation with additives (fmol cGMP/μg protein per 3 min)		
		10 min	1 h	3.5 h
Whole sonicate	buffer	1.10 ± 0.50	1.31 ± 0.09	6.13 ± 0.77
Whole sonicate	buffer + LPS	1.14 ± 0.07	1.46 ± 0.14	5.70 ± 0.47
Whole sonicate	serum	1.13 ± 0.08	1.55 ± 0.05	4.13 ± 0.43
Whole sonicate	serum + LPS	1.17 ± 0.06	1.63 ± 0.09	3.71 ± 0.42
Cytosol	buffer	2.32 ± 0.42	19.2 ± 2.0	12.3 ± 0.80
Cytosol	buffer + LPS	2.31 ± 0.29	18.3 ± 1.8	11.0 ± 0.40
Cytosol	serum	1.42 ± 0.21	2.48 ± 0.34	4.57 ± 0.64
Cytosol	serum + LPS	1.42 ± 0.17	2.56 ± 0.67	3.97 ± 0.25

late cyclase activity also occurred with time between 10 min and 1 h of incubation, which in contrast to whole sonicate was almost entirely blocked by serum and by 5 mM dithiothreitol (values at 1 and 3.5 h with dithiothreitol without LPS were 2.90 ± 0.62 and 4.36 ± 0.46 fmol cGMP/μg protein per 3 min, respectively). This time dependent activation of soluble guanylate cyclase activity and prevention by dithiothreitol and bovine serum albumin have been described with other soluble cyclase preparations [15,16]. Thus, under the conditions of the experiments, LPS did not activate soluble guanylate cyclase activity in broken cell preparations.

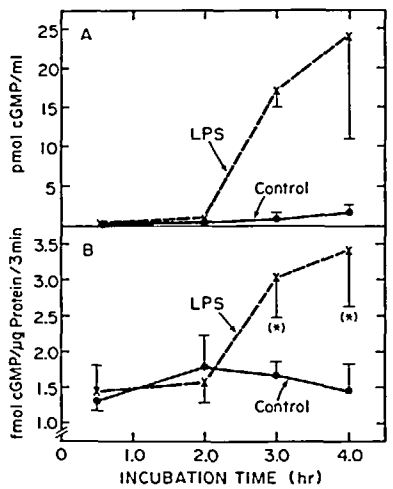

Fig. 6. Effect of culturing fetal spleen cells with LPS for increasing periods of time on (A) cGMP levels and (B) soluble guanylate cyclase activity. The LPS concentration was 5 ng per ml. Each point represents the mean of all samples obtained from six separate experiments ± 1 S.D. Values indicated by an asterisk are significantly different ($P < 0.05$) from their corresponding control values and from the 0.5 and 2 h LPS values.

Since it is possible that the elevation of cGMP levels produced by LPS could be due at least in part to a decrease in the rate of destruction of this nucleotide, cGMP phosphodiesterase activity was measured in fetal spleen cells cultured with or without LPS for 4 h. The activities of these preparations were similar (27 vs. 30 fmol/μg protein per min) and were not affected by 100 μM EGTA or 0.75 μg of calmodulin. In contrast, soluble guanylate cyclase activity from these same cultures was more than 2.5-fold greater in LPS treated than control cells (5.28 vs. 2.08 fmol/μg protein per 3 min). Thus, under these conditions only an increase in soluble guanylate cyclase could be detected in cells incubated with LPS.

Discussion

An elevation of cellular cGMP levels could result from either increasing synthesis or retarding breakdown of the nucleotide. Several findings support the concept that the elevation of cGMP levels in this system is due to an effect of LPS on soluble guanylate cyclase activity. First, the phosphodiesterase inhibitor, MIX, produced an increase in cGMP levels by itself which was further enhanced in a dose dependent fashion by LPS. Since MIX at the dose used (0.1 mM) would likely have produced nearly complete inhibition of all phosphodiesterases present, the additional effect of LPS implies an action on synthesis of cGMP, i.e., on guanylate cyclase. Second, in cells incubated with LPS for 4 h, total (whole sonicate) guanylate cyclase activity is increased and this increase is entirely accounted for by an elevation of soluble guanylate cyclase activity. Third, the increase in soluble guanylate cyclase activity in whole cultures is both dependent on the dose of LPS added to the cultures and proportional to the whole culture level of cGMP. Fourth, the time courses of the increase in soluble guanylate cyclase activity and the elevation of whole culture cGMP levels are very similar. Fifth, no significant change in cGMP-phosphodiesterase activity could be detected in LPS treated cultures in comparison to control cultures. On the other hand, since breaking cells destroys subcellular anatomical relationships and may release or activate proteolytic enzymes that could alter measured results, other mechanisms may be involved in raising cGMP levels in this system that are not readily apparent from our experiments. Nevertheless, the data taken together are consistent with the notion that LPS produces at least part and perhaps all of its effect on the cGMP concentration by increasing the activity of soluble guanylate cyclase in the cells.

Although the mechanism by which LPS increases soluble guanylate cyclase activity in this system is unknown, several findings suggest that the effect represents a novel indirect action involving protein synthesis rather than a direct effect of LPS on the enzyme. First, LPS failed to activate soluble guanylate cyclase when added directly to either fetal spleen cell cytosol or whole sonicate. Second, the increases in soluble guanylate cyclase activity and cGMP levels produced by LPS are preceded by a lag of approximately 120 min but once initiated are very persistent. Third, RNA and protein synthesis appear to be a necessary component of the LPS-cGMP response in that the cGMP elevation is blocked by inhibitors of RNA and protein synthesis but not by inhibitors of DNA synthesis [6]. Fourth, simple increased synthesis of soluble guanylate cyclase seems an unlikely mechanism in that sodium nitroprusside, a known activator of soluble guanylate cyclase [15–17], produces large increases of cGMP levels in LPS responsive cells that are even greater than those produced by LPS [5], i.e., the level of this enzyme does not appear to be rate limiting. Fifth, including Mn^{2+} in the reaction mixture in place of Mg^{2+} increased soluble guanylate cyclase activity 7-fold but decreased the LPS to control ratio of activity. Since newly synthesized enzyme should be activated just as well by Mn^{2+} as old enzyme, this observation also favors activation of soluble guanylate cyclase by LPS rather than synthesis of additional enzyme. While there may be several possible explanations for these findings, the data do support an indirect stimulation of soluble guanylate cyclase activity by LPS and are consistent with the idea that incubation of the cells with LPS leads to synthesis of a protein or proteins which then results in the activation of the soluble cyclase. Whether or not this putative protein acts directly on the cyclase or through another hypothetical intermediate remains to be determined. Furthermore, since fetal spleen tissue consists of several cell types, it is possible that more than one class of cells may be involved in this proposed sequence of events.

In summary, the present data indicate that LPS increases cGMP levels in fetal spleen cells by activation of soluble guanylate cyclase. This activation apparently does not involve a direct effect of LPS on or a de novo synthesis of soluble guanylate cyclase. However, it is dependent on incubation time and on protein synthesis [6] and appears to be due to de novo production of a protein or proteins that subsequently induce(s) a persistent activation of the enzyme. Therefore, this effect is very different from the known mechanisms of soluble guanylate cyclase activation that involve either direct interaction of an activator

with the heme moiety of the enzyme [17] or the release of membrane fatty acids that subsequently activate the enzyme [18].

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