Lipopolysaccharides Do Not Alter Metabolic Disturbances in Hippocampal Slices of Fetal Guinea Pigs after Oxygen-Glucose Deprivation

RICHARD BERGER, YVES GARNIER, DORIS PFEIFFER, AND ARNE JENSEN

Department of Obstetrics and Gynecology, Ruhr-Universität Bochum, In der Schornau 23–25, D-44892 Bochum, Germany

ABSTRACT

The aim of the present study was to clarify whether endotoxins [lipopolysaccharides (LPS)] have a toxic effect on fetal brain tissue after cerebral ischemia, while excluding their effect on the cardiovascular system. Experiments were therefore performed on hippocampal slices prepared from mature fetal guinea pigs. In particular, we studied the influence of LPS on nitric oxide production, energy metabolism, and protein synthesis after oxygen-glucose deprivation (OGD). Incubating hippocampal slices in LPS (4 mg/L) for as long as 12 h did not alter cGMP tissue concentrations significantly. However, 10 min after OGD of 40-min duration, cGMP tissue concentrations were substantially increased in relation to controls, and this increase was almost completely blocked by the application of 100 μ M *N*^ω-nitro-L-arginine, indicating that nitric oxide synthase was activated after OGD in fetal brain tissue. Again, LPS did not have any effect on cGMP tissue concentrations after OGD. Furthermore, addition of LPS altered neither protein synthesis nor energy metabolism

measured 12 h after OGD. We therefore conclude that, apart from their well-known influence on the cardiovascular system, LPS do not alter metabolic disturbances in hippocampal slices of fetal guinea pigs 12 h after OGD. A direct toxic effect of LPS on immature brain tissue within this interval does not therefore seem to be very likely. However, delayed activation of LPS-sensitive pathways that may be involved in cell death, or damage limited to a small subgroup of cells such as oligodendrocyte progenitors, cannot be fully excluded. (*Pediatr Res* 48: 531–535, 2000)

Abbreviations

aCSF, artificial cerebrospinal fluid
AEC, adenylate energy charge
OGD, oxygen-glucose-deprivation
LPS, lipopolysaccharides
L-NNA, *N*^ω-nitro-L-arginine
NO, nitric oxide

Hypoxic-ischemic cerebral damage is an important contributor to perinatal mortality and morbidity, including long-term neurologic sequelae in term and preterm fetuses. On the other hand, there is increasing evidence that perinatal brain damage is caused not only by hypoxic-ischemic insults, but also by ascending intrauterine infection before or during birth (1). Infants whose amnion is acutely inflamed are at a much greater risk of developing brain injury than control subjects (2, 3). However, it remains unclear whether fetal brain damage is the result of cerebral hypoperfusion caused by circulatory decentralization owing to severe endotoxemia or is caused by a direct effect of endotoxins on cerebral tissue (4). The present study was therefore set up to clarify whether endotoxins (LPS) have a direct toxic effect on fetal brain tissue after cerebral ischemia, while excluding their effects on the cardiovascular system. For this purpose we used the *in-vitro* system of OGD in hippocam-

pal slices prepared from mature guinea pig fetuses (5, 6). In particular, we studied the influence of LPS on NO production, energy metabolism, and protein synthesis after OGD. Prolonged inhibition of protein synthesis after OGD seems to be an especially sensitive early marker of ischemic cell injury (7).

METHODS

The present study was performed on guinea pigs, a precocious species, at 0.9 gestation (term, 68 d). The dams were anesthetized with halothane and decapitated, and fetuses were delivered by cesarean section. The fetal hippocampi were dissected out and cut into 500- μ m-thick transverse slices. The tissue slices were transferred to an incubation chamber, containing aCSF (standard). To prevent bacterial contamination, 10 mg/L each of streptomycin and erythromycin were added to the aCSF. Additionally, aCSF was filtered (pore diameter, 0.1 μ m) into sterilized containers, and CuSO₄ was added to the incubation chamber reservoir. The aCSF was pumped through the incubation chamber at a rate of 1 mL/min (5, 8). The aCSF was equilibrated with a gas mixture of oxygen and carbon

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Correspondence: Priv.-Doz. Dr. R. Berger, Universitätsfrauenklinik Bochum, Knapp-schaftskrankenhaus, In der Schornau 23–25, D-44892 Bochum, Germany.

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dioxide (95% O₂/5% CO₂), and the incubation temperature was held at 37°C. After a period of 90 min, during which the tissue was allowed to recover from preparation stress, the glucose concentration of the standard aCSF was lowered from 10 mM to 2 mM for 30 min. This was done to accelerate the breakdown of high-energy phosphates in tissue slices during exposure to OGD (5, 6).

The experimental protocol included a 120-min preincubation phase (90 min in 10 mM glucose and 30 min in 2 mM glucose aCSF), an ischemic phase (10–40 min), and a recovery phase (12 h starting from the end of OGD). A separate incubation chamber, equilibrated with 95% N₂/5% CO₂, was used for the induction of OGD. In contrast to the standard aCSF, the ischemic aCSF contained no glucose or HEPES. HEPES was omitted because its buffering capacity can influence the fall in pH accompanying OGD. Before the tissue slices were transferred to the anoxic incubation chamber, they were washed in aglycemic aCSF to lower the glucose concentrations in the tissue still further. During OGD, the tissue slices were completely submerged in the aCSF. No additional aCSF was pumped through the chamber during this period (flow rate, 0 mL/min). In the postischemic phase, the tissue slices were transferred back to standard aCSF (flow rate, 1 mL/min) and equilibrated with 95% O₂/5% CO₂.

To prove whether the hippocampal slice model has the power to detect effects of LPS on neuronal tissue, we measured tissue concentrations of TNF- α in hippocampal slices incubated for 12 h in standard aCSF containing 4 mg LPS/L aCSF (*Escherichia coli*; O127:B8; Sigma Chemical Co., Deisenhofen, Germany). Intraperitoneal injection of LPS at this dosage (4 mg/kg body weight) killed newborn guinea pigs ($n = 3$) within 6 h. Moreover, this dosage caused cardiac failure in adult guinea pigs within 4 h (8, 9). At various intervals, six groups (control group, $n = 3$; study group, $n = 3$) consisting of four slices each were sampled. Tissue slices from each group were pooled and homogenized by ultrasonification for 5 s in 100 μ L of ice-cold Tris-citrate (pH 7.4), with 10 mM 4-(2-aminoethyl)-benzene sulfonyl fluoride (Sigma Chemical Co.) as described previously (10). The homogenates were centrifuged (12,000 $\times g$, 10 min, 4°C), the supernatants were retained, and the pellet was homogenized again. The supernatants from the two extractions were pooled, and aliquots (100 μ L, approximately 0.5 mg protein/aliquot) were removed for the quantification of TNF- α using a commercially available ELISA (R&D Systems, Wiesbaden, Germany).

To further elucidate the effects of endotoxins on cellular metabolism, we studied NO production in hippocampal slices incubated in standard aCSF containing 4 mg LPS/L aCSF for 12 h. As a measure of NO production, we determined the tissue concentration of cGMP at various intervals using an RIA (NEN, Bad Homburg, Germany) (6, 11). For this purpose, tissue slices were frozen in liquid nitrogen and extracted with perchloric acid. Moreover, we tested whether the activation of NO synthase in immature brain tissue after OGD is altered by LPS. In this set of experiments, hippocampal slices were incubated in aCSF containing LPS (4 mg/L) 2 h before, during, and 10 min after OGD. Ten minutes after OGD, the tissue concentration of cGMP was measured as described above. To

confirm that elevated tissue concentrations of cGMP really reflected increased NO production, we simultaneously blocked NO synthase in a portion of the slices with 100 μ M L-NNA (6).

A further series of experiments was set up to test the effect of LPS on the recovery of energy metabolism and protein synthesis in tissue slices 12 h after OGD (duration, 20–40 min). For this purpose, a set of hippocampal slices was incubated in aCSF containing LPS (4 mg/L) 2 h before, during, and 12 h after OGD.

Tissue slices were frozen in liquid nitrogen for subsequent measurement of tissue concentrations of adenine nucleotides. ATP, ADP, and AMP were measured by HPLC after extraction with perchloric acid (5). The AEC, a measure of the relation of energy consumption to energy production, was estimated from the following formula (12):

$$\text{AEC} = ([\text{ATP}] + 0.5[\text{ADP}])/([\text{ATP}] + [\text{ADP}] + [\text{AMP}])$$

The protein content of the tissue slices was measured by the method of Lowry *et al.* (13).

Protein synthesis was assessed from the incorporation rate of ¹⁴C-leucine into tissue proteins. After 30 min of incubation in standard aCSF, to which 5 μ Ci/mL L-[1-¹⁴C]leucine (specific activity, 54 mCi/mmol; Amersham Buchler, Braunschweig, Germany) had been added, the tissue slices were homogenized in trichloroacetic acid. The radioactivity of the trichloroacetic acid-precipitated material was then measured by liquid scintillation counting (5).

All data are given as means $\pm$ SD. Except for the experiments on TNF- α concentration, test groups consisted of five tissue slices each. The statistical significance of differences among groups was assessed by a two-way ANOVA, followed by the Scheffé *post hoc* test. The experimental protocols were approved by the appropriate institutional review committee and met the guidelines of the governmental agency responsible.

RESULTS

The effect of LPS on TNF- α tissue concentration is shown in Figure 1. During an incubation period of 12 h, TNF- α tissue concentrations did not change in the control group, whereas in the study group LPS caused a significant rise in the tissue concentration of this cytokine. In contrast, incubating hippocampal slices in LPS (4 mg/L) for as long as 12 h did not alter NO production as measured by cGMP tissue concentrations (Fig. 2A). However, cGMP concentrations were dramatically altered by inducing OGD in hippocampal slices. Twenty minutes after OGD, cGMP tissue concentrations were significantly increased compared with controls (Fig. 2B), and this increase was almost completely blocked by the application of 100 μ M L-NNA (Fig. 2C). Again, LPS had no effect on cGMP tissue concentrations even after OGD (Fig. 2B).

As already shown in earlier studies, prolonged OGD in this experimental model leads to disturbances of energy metabolism and protein synthesis during the subsequent recovery phase (5, 6). In the same way, a reduction of ATP concentration (Fig. 3A) and protein synthesis (Fig. 3B) in slices of the control group to 36% and 63% of initial values, respectively, was observed here in tissue slices after 40 min OGD. Incubat-

Figure 1. Concentration of TNF- α in hippocampal tissue slices incubated in standard aCSF containing LPS (4 mg/L) for 12 h. Values are given as mean $\pm$ SD. The statistical significance of differences within groups was assessed by ANOVA and Scheffé *post hoc* test. b, $p < 0.05$ (with LPS vs without LPS).

ing slices in LPS (4mg/L) altered neither the postischemic recovery of tissue concentrations of ATP nor the rates of protein synthesis. Similar results were obtained for the total adenylate pool and AEC (Table 1).

DISCUSSION

Recently various clinical studies have shown that fetuses suffering from intrauterine infection have a higher incidence of cerebral palsy in later life (1, 14). However, the exact pathophysiologic mechanisms underlying this observation are still poorly understood. As we know from studies in fetal sheep, endotoxemia may cause circulatory decentralization and thus lead to cerebral hypoperfusion and brain damage (4). Besides this indirect effect on brain tissue, LPS may also have direct toxic effects. To clarify this point, we performed experiments on hippocampal tissue slices (5, 6). In this way, any effect of LPS on the microcirculation could be excluded. Using this setup, hippocampal slices of mature guinea pig fetuses remain metabolically intact for periods of up to 12 h, even when the temperature of the bath is set at 37°C.

As shown in Figure 1, application of LPS to the incubation medium did significantly increase tissue concentrations of TNF- α . Thus, the hippocampal slice model seems to be a suitable experimental setup for detecting effects of LPS on fetal neuronal tissue. Furthermore, because TNF- α concentration increased substantially in the hippocampal tissue after application of endotoxins, it is very likely that considerable amounts of LPS penetrated the slices during incubation. This view is supported by *in vivo* studies in which a significant expression of cytokines in the brain tissue of adult animals was observed after injection of LPS into the cerebral ventricles (15–17). However, application of LPS to the incubation medium did not increase cGMP concentrations in hippocampal slices prepared from fetal guinea pigs, indicating that NO synthase was not

Figure 2. Concentration of cGMP in hippocampal tissue slices after exposure to LPS and OGD. A, Time course of concentration of cGMP in hippocampal tissue slices after different periods of incubation in standard aCSF with and without LPS (4 mg/L). B, Concentration of cGMP in hippocampal tissue slices after different durations of OGD (10–40 min) and a recovery period of 10 min. A set of these tissue slices was incubated in LPS (4mg/L) for 2 h before, during, and 10 min after OGD. C, In addition to the experimental protocol described in B, all slices were incubated in 100 μ M L-NNA for 30 min before, during, and 10 min after OGD. Values are given as mean $\pm$ SD. The statistical significance of differences within and between groups was assessed by ANOVA and the Scheffé *post hoc* test. a, $p < 0.05$ (OGD vs controls). Significant differences between groups could not be detected.

Figure 3. Concentration of ATP (A) and rate of protein synthesis (B) in hippocampal tissue slices 12 h after application of LPS and OGD. OGD lasted 20–40 min. A set of these tissue slices was incubated in LPS (4 mg/L) for 2 h before, during, and 12 h after OGD. The ATP concentration in controls was 16.4 ± 3.2 nmol/mg protein (without LPS) and 15.0 ± 3.1 nmol/mg protein (with LPS). Protein synthesis in controls amounted to 181 ± 22 dpm/ μ g protein per 30 min (without LPS) and 173 ± 34 dpm/ μ g protein per 30 min (with LPS). Values are given as mean $\pm$ SD. The statistical significance of differences within and between groups was assessed by ANOVA and the Scheffé *post hoc* test. a, $p < 0.05$ (OGD vs controls). Significant differences between groups could not be detected.

activated by this procedure (Fig. 2A). This seems to contradict some *in vitro* (18) and *in vivo* (19) studies on adult animals in which an increase in cerebral NO release was measured after stimulation with LPS. However, because the LPS signal cascade depends on the expression of various membrane-bound receptors, *e.g.* CD14, TLR2/4 (20, 21), this discrepancy may possibly arise from ontogenetic differences in the maturation of

Table 1. Total adenylate pool and AEC 12 h after OGD and application of LPS

Time	TAN		AEC	
	Without LPS	With LPS (4 mg/L)	Without LPS	With LPS (4 mg/L)
Control	100 $\pm$ 19	92 $\pm$ 19	100 $\pm$ 1	100 $\pm$ 11
OGD 20 min	77 $\pm$ 10*	85 $\pm$ 13	97 $\pm$ 1	98 $\pm$ 1
OGD 30 min	55 $\pm$ 3*	54 $\pm$ 14*	96 $\pm$ 3*	94 $\pm$ 2*
OGD 40 min	38 $\pm$ 6*	53 $\pm$ 10*†	95 $\pm$ 1*	95 $\pm$ 1*

Values are given as percent of control (mean $\pm$ SD). The absolute values of total adenylate pool (TAN) in the control groups were 17.9 ± 3.5 nmol/mg protein (without LPS) and 16.5 ± 3.4 nmol/mg protein (with LPS), and those of AEC were 0.94 ± 0.01 (without LPS) and 0.94 ± 0.00 (with LPS), respectively. OGD lasted between 20 and 40 min. Statistically significant differences within and between groups were assessed by ANOVA and the Scheffé *post hoc* test. * $p < 0.05$ (OGD vs control); † $p < 0.05$ (LPS vs without LPS).

these proteins that link LPS stimulus to the activation of NO synthase. One might speculate that the application of LPS together with interferon- γ would have been more effective in stimulating NO synthase (22). However, as demonstrated in a variety of studies, NO production can easily be induced by sole application of LPS (18, 19, 23, 24). Thus, the presence of interferon- γ is not an absolute prerequisite for LPS-mediated NO activation.

In contrast to the effect of adding LPS to the incubation medium, OGD caused a marked rise in cGMP concentrations in fetal tissue slices, and this increase was almost completely inhibited by blocking NO synthase with L-NNA (Fig. 2, B and C). This implies that NO synthase is activated by OGD in our model, producing significant amounts of NO. However, the activation of NO synthase after OGD was not additionally influenced by LPS. NO does not therefore seem to play a major role in mediating direct toxic effects of LPS on fetal brain tissue.

The effect of LPS on metabolic disturbances of hippocampal slices after prolonged periods of OGD was investigated by measuring tissue concentrations of high-energy phosphates and protein synthesis. Incubating hippocampal slices in aCSF containing LPS modulated neither ATP tissue concentrations nor rates of protein synthesis (Fig. 3). Nor did this treatment modify changes in adenylate concentrations or the AEC (Table 1). In vulnerable brain areas such as the hippocampal CA1 subfield, protein synthesis is already markedly suppressed shortly after ischemia, *i.e.* before cell damage is morphologically manifested. Prolonged inhibition of protein synthesis can therefore be viewed as a sensitive early marker of ischemic cell injury, and this is probably also true for LPS-mediated damage of neuronal tissue (7). Furthermore, cells cannot survive if their energy metabolism is severely disturbed. Although the indicators used in our experiments have sufficient power to detect damaging effects on hippocampal slices, we cannot exclude the possibility that LPS may injure neuronal tissue *via* delayed activation of pathways that are involved in apoptotic cell death. However, inasmuch as intraperitoneal injection of LPS at a dosage of 4 mg/kg kills newborn guinea pigs within 6 h, such mechanisms seem to be of minor importance in the present study. Nevertheless, delayed cell injury may explain some

discrepancies between our results and studies in adult animals and completely differentiated cell cultures in which LPS-induced injury was observed (25–30). In addition, if LPS is exclusively toxic to a certain cell type, *e.g.* oligodendrocyte progenitors, and the portion of this cell type contributing to total energy metabolism and protein synthesis is very small, possible deleterious effects of LPS on hippocampal slices may have been overlooked. However, inasmuch as clinical observations have shown that bacterial infection of the immature brain not only induces white matter but also cortical injury (31), a differentiation between cell type-specific lesions was not the main question of the present paper. A further reason why some experiments in adult animals and fully differentiated cell cultures revealed toxic effects of LPS on neuronal cells that were not found in the present study may be that LPS did not stimulate NO production in fetal hippocampal slices owing to ontogenetic differences in the maturation of proteins that link LPS stimulus to the activation of NO synthase. Because NO as a radical can substantially damage neuronal tissue, this discrepancy would seem to be of biologic importance.

Despite the fact that the dose of LPS used in the present study killed newborn guinea pigs within 6 h, but did not have any effect on protein synthesis and energy metabolism, one might argue that increasing the LPS concentration would still have a direct toxic effect on fetal hippocampal slices. However, even doubling the LPS dose failed to affect the recovery of ATP tissue concentrations and protein synthesis in the slices 12 h after OGD lasting between 20 and 40 min (data not shown). Furthermore, the lack of any effect of LPS on cell metabolism in the present study is unlikely to result from species differences, because the pathways by which LPS activates various cellular mechanisms are ubiquitous and probably highly preserved in the genome of mammals. Differences in electrical activity between neurons in the slice and in the brain that might explain discrepancies in cellular activation after application of LPS are not very likely, because the fetal brain consumes much less energy than the adult owing to a lack of environmental stimuli (32).

From the present study, we conclude that, apart from their influence on the cardiovascular system, LPS do not alter metabolic disturbances in hippocampal slices of fetal guinea pigs 12 h after OGD. A direct toxic effect of LPS on immature brain tissue within this interval does not therefore seem very likely. However, delayed activation of LPS-sensitive pathways that may be involved in cell death, or damage limited to a small subgroup of cells such as oligodendrocyte progenitors, cannot be fully excluded.

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