Stimulation of production of glial cell line-derived neurotrophic factor and nitric oxide by lipopolysaccharide with different dose-responsiveness in cultured rat macrophages

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ABSTRACT
To understand the molecular basis of inflammation-induced neurotrophic influences, we investigated the effects of lipopolysaccharide (LPS) on production of glial cell line-derived neurotrophic factor (GDNF) in the injured rat spinal cord or in cultured rat macrophages in comparison with the effects on synthesis/secretion of inducible nitric oxide synthase (iNOS) and nitric oxide (NO). We found that GDNF mRNA expression lasted longer than that of iNOS mRNA in the injured spinal cord after injection of the high-dose LPS that had improved locomotor function, suggesting that the GDNF expression and its balance with NO generation were critical for injury regeneration. Therefore, we next investigated the effects of LPS on cultured macrophages. Levels of iNOS mRNA and secreted NO were enhanced by LPS at lower concentrations (10 ng/mL and above), whereas mRNA expression and secretion of GDNF were elevated only at higher concentrations (100 ng/mL and above). The culture medium of macrophages treated with 10 ng/mL of LPS was actually neurotoxic against cultured cortical neurons, whereas that conditioned at 1000 ng/mL was not. These observations suggest that neurotoxicity partly based on NO is induced by a lower degree of inflammation, whereas neurotrophic effects based on GDNF are manifested at a higher degree of inflammatory activity.

Activated microglia/macrophages are involved in inflammatory reactions after nerve injury, and may have harmful effects on surrounding neurons. Actually, anti-inflammatory treatment is advantageous to minimize the primary injury and/or protect against secondary injury following spinal cord injury (1, 5, 21). Harmful actions are predominantly due to the production of nitric oxide (NO), reactive oxygen species, and inflammatory cytokines (15, 20, 24). NO is generated by inducible NO synthase (iNOS) in the activated microglia/macrophages, and the released NO leads to neuronal death (11, 19). On the other hand, these cells also have the ability to produce neurotrophic factors such as neurotrophins and glial cell line-derived neurotrophic factor (GDNF) (2, 4, 16), all of which are protective against neuronal death and advantageous for nerve regeneration.

We recently examined the relationship between lipopolysaccharide (LPS)-induced inflammation and improvement of locomotor function after spinal cord injury in rats, and surprisingly found that a local injection of high-dose LPS (10 or 100 μg) improved locomotor function to a greater extent than low-dose LPS (1 μg) (7). Experiments using GDNF gene mutant mice confirmed that the restoration of locomotor function could be correlated with an increase in the GDNF mRNA level rather than with a reduction...
in the mRNA level of iNOS (7). GDNF and its mRNA were limitedly expressed in microglia/macrophages around the injury site of the spinal cord (8), and the locomotor function improvement was in parallel with the increase in the GDNF mRNA level in these cells (7).

These previous observations suggest that a higher degree of inflammation leads to a higher degree of repair activity of central nervous system injuries through GDNF produced by activated microglia/macrophages. In the present study, to clarify the molecular mechanisms that characterize activated microglia/macrophages, we examined the effects of LPS on GDNF synthesis/secretion in vitro in cultured macrophages in comparison with the expression of iNOS mRNA and secretion of NO by these cells.

**MATERIALS AND METHODS**

**Surgery for spinal cord injury and administration of LPS.** Adult male Wistar rats (7–8 weeks old, 150–200 g; Nippon SLC, Shizuoka, Japan) were cared for according to the Guidelines of Experimental Animal Care issued from the Office of the Prime Minister of Japan. Animals were anesthetized with pentobarbital (30 mg/kg), and the left side of the spinal cord was hemitransected at the level of the 9th thoracic vertebra. Then, 10 μg of LPS in 10 μL of phosphate-buffered saline (PBS) was injected into the injury site. For the reverse transcription-polymerase chain reaction (RT-PCR) experiment, the spinal cords were dissected out; and the segments just rostral or caudal to the injury site (5-mm length each) were collected.

**Macrophage cultures.** Macrophages were obtained from the peritoneal cavity of adult male Wistar rats (7–8 weeks old, 150–200 g) 2 days after an intraperitoneal injection of 5 mL of thioglycolate medium (Sigma, Saint Louis, MO, USA). The cells were washed with PBS and plated in RPMI 1640 medium (Sigma) containing 100 units/mL penicillin, 100 μg/mL streptomycin (Invitrogen, Carlsbad, CA, USA), and 10% fetal bovine serum (Cansera Int., Rexdale, Canada); and after 1 h the medium was replaced to remove nonadherent cells. Macrophages were left unstimulated or stimulated with LPS (Sigma) at 1, 10, 100 or 1000 ng/mL for 3, 12 or 2 h.

**RT-PCR.** RT-PCR was performed as described earlier (8). The following primers were used: up-primer 5’-GAGAGGAATCGGCAGGCTGCAGCTG-3’ and down-primer 5’-CAGATAATCCACATCGGTTTAGCGG-3’ for GDNF (product size: 337 bases); up-primer 5’-CAGAGGACCCAGAGACGAC-3’ and down-primer 5’-ACTGGTGAACCTCAAGGTG-3’ for iNOS (product size: 488 bases); and up-primer 5’-CGGAGTCAGGATTTGTCGTAT-3’ and down-primer 5’-AGCCTTCTTCCATGTGGTGAAGAC-3’ for glyceraldehyde-3-phosphate dehydrogenase (GAPDH, product size: 309 bases). The GAPDH gene was used as an internal control. PCR products were applied to 2% agarose gels. The bands corresponding to the target cDNAs were densitometrically quantified, and the ratio of the band intensity of GDNF or iNOS cDNA products to that of GAPDH cDNA products was calculated. The values are expressed as the means ± S.E. (n = 3) of the fold-increase over the value of the control group in which total RNA samples were prepared immediately after the administration of LPS or vehicle.

**Enzyme immunoassay.** The amount of GDNF secreted into the medium was measured by an enzyme immunoassay (EIA) specific for GDNF, as described previously (18, 23).

**Measurement of NO secretion.** The NO concentration in the medium was measured as the nitrite (NO3-) concentration by using the Griess reagent according to a previous report (22). Briefly, 100 μL of medium was incubated with an equal volume of the Griess reagent (1% sulfanilamide, 0.1% naphthylethylene diamine dihydrochloride, 2.5% phosphoric acid) at room temperature for 10 min. The absorbance at 550 nm was measured with a microplate reader (Model 550; Bio-Rad, Hercules, CA, USA), and the concentration was calculated by using sodium nitrite as a standard.

**Neuron-rich cultures.** Neuron-rich cultures were prepared from the cerebral cortices of 17-day-old rat embryos as described previously (13). After a 4-day culture, the medium was exchanged for that composed of equal volumes of B27-supplemented Neurobasal medium containing 100 units/mL penicillin and 100 μg/mL streptomycin (Invitrogen) and RPMI 1640 medium containing LPS (10 or 1000 ng/mL) or LPS-containing RPMI 1640 medium that had been conditioned by macrophages. Then, the cells were cultured for another 2 days, and subsequently stained for microtubule-associated protein 2 (MAP-2) by using its specific antibody as described earlier (12) to discriminate neuronal cells from other cell types.
RESULTS

Induction of GDNF and iNOS mRNA in the spinal cord

Our previous study suggested that LPS injection into the injury site of the spinal cord enhanced the mRNA expression of both GDNF and iNOS, the latter a key enzyme for the generation of NO (7). We performed this experiment again to determine the detailed time-dependent changes in the levels of both mRNAs. GDNF mRNA was significantly enhanced at day 0.5 and 1 and returned to the control level by day 3 after the LPS injection (10 μg), whereas a significant increase in the iNOS mRNA was found only at day 0.5 after the injury (Fig. 1). Namely, the LPS caused an increase in mRNA expression of GDNF that lasted much longer than that of iNOS, suggesting that the neurotrophic effects of GDNF would exceed the neurotoxic effects evoked by reactive oxygen species including NO and/or inflammatory cytokines. A previous study by us demonstrated that activated microglia/macrophages were responsible for the synthesis/secretion of GDNF and NO in the injury site of the spinal cord (7), which prompted us to examine in detail the effects of LPS on peripheral macrophages in vitro.

Time-courses of GDNF and NO production after LPS stimulation

Time-dependent changes in GDNF and NO production in cultured macrophages were examined after treatment with LPS at 10 or 1000 ng/mL. Upregulation of GDNF mRNA expression was observed 12 or 24 h after exposure to LPS when the cells were treated with 1000 ng/mL of LPS, whereas no change was found when the cells were treated with vehicle or 10 ng/mL of LPS (Fig. 2A). The secretion level of GDNF was significantly increased 24 h after treatment with LPS only at 1000 ng/mL, but no change was detected when vehicle or 10 ng/mL of LPS was used (Fig. 2B). These results demonstrate that GDNF synthesis was facilitated in a time-dependent manner up to 24 h after high-dose LPS stimulation.

The expression of iNOS mRNA in LPS-treated cells rapidly rose to its maximal level at 3 h, and then gradually decreased up to 24 h, although the level was still significantly high even at 24 h.

Fig. 1 Effect of LPS administration on the expression of GDNF and iNOS mRNAs in the injury site of the rat spinal cord. LPS (10 μg) or vehicle was injected into the hemitranssection site of the spinal cord immediately after the operation, and the spinal cord was removed at the indicated times. Total RNA was prepared from combined tissues of both rostral and caudal areas adjacent to the injury site, and subjected to RT-PCR for mRNAs of GDNF (A) and iNOS (B). Significant differences from the value of the corresponding vehicle group were determined by means of Student’s t-test. Significance, *p < 0.05.

A. GDNF mRNA

B. iNOS mRNA
The pattern of change in iNOS mRNA expression with time was similar when the cells were treated with LPS at either 10 or 1000 ng/mL. In spite of such a rapid elevation of the level of iNOS mRNA, the secretion of NO showed only a small but significant increase at 3 h, and then a marked increase at 24 h when the cells were treated with either 10 or 1000 ng/mL of LPS (Fig. 2D). Although the difference in time when the maximal values were elicited for iNOS mRNA expression and NO secretion is unknown at present, NO secretion proceeded in a time-dependent manner up to 24 h after LPS treatment (Fig. 2D).

**Dose-dependent effects of LPS on the synthesis/secretion of GDNF and NO**

Next the dose-dependent effects of LPS on the level of GDNF and iNOS mRNAs and on the secretion of GDNF and NO were examined (Fig. 3). The GDNF mRNA increased with an increase in the LPS dose from 10 to 1000 ng/mL, whereas the iNOS mRNA level was significantly high even at a dose as low as 1 ng/mL, increased dose-dependently up to 100 ng/mL, and was sustained at a high level at 1000 ng/mL of LPS (Fig. 3A). The secretion of GDNF and NO showed dose-dependences similar to those for the expression of GDNF mRNA and iNOS mRNA, respectively. The GDNF content in the me-
LPS decides macrophage nature

The basal level of NO secretion was 10.4 ± 1.8 μM in the absence of LPS. The NO level was significantly increased (21.9 ± 3.3 μM) after stimulation with LPS at a dose as low as 1 ng/mL, and remarkably facilitated by LPS at 10 ng/mL (150 ± 8.9 μM), 100 ng/mL (168 ± 8.9 μM) or 1000 ng/mL (174.7 ± 10.6 μM). These results demonstrate clearly that high-dose LPS enhanced the production of both NO and GDNF but that low-dose LPS stimulated only NO production.

Neurotoxicity of the LPS-treated macrophages

Finally, the neurotoxicity of the medium conditioned by LPS-treated macrophages was examined by using cultures of rat neuron-rich cortical cells. Neuronal survival was constant (over 90%) when the cortical cells were cultured in the non-conditioned medium even if fetal bovine serum and 10 or 1000 ng/mL of LPS were present, demonstrating that neither serum nor LPS affected neuronal survival.

For the experiment, the cortical cells were cultured for 2 days in medium containing the conditioned medium (test sample), and stained with the antibody against MAP2, a structural protein that exists in the cell body and dendrites/axons of differentiated neurons (3). As MAP2-positive cells were considered as surviving neurons, their number was counted. The medium conditioned by the cells stimulated with 100 ng/mL of LPS significantly caused a much greater neuronal loss than the non-conditioned medium with the same concentration of LPS, whereas the conditioned medium from cultures stimulated with 1000 ng/mL of LPS did not cause such neuronal loss (Fig. 4). This neuronal toxicity can be explained by the fact that the activation with 10 ng/mL of LPS was sufficient for production of NO but not for that of GDNF, as was shown in Fig. 3. Furthermore, the cells treated with 1000 ng/mL of LPS still induced a high level of NO, but simultaneously elicited substantial production of GDNF, which would be expected to antagonize the NO toxicity.

DISCUSSION

Inflammatory reactions have opposing roles via production of neurotoxic or neurotrophic molecules that respectively include cytokines, reactive oxygen species, and NO or include neurotrophic factors such as GDNF produced by activated microglia/macrophages (15, 20, 24). Other investigators described the activation or increase in the number of macrophages by LPS and inhibition of secondary injury after spinal cord injury by a daily LPS injection (6,
Effect of the medium conditioned by LPS-treated macrophages on the survival of cultured cortical neurons. Rat cortical cells were cultured for 2 days in medium composed of equal volumes of Neurobasal medium and non-conditioned RPMI1640 medium containing 10 or 1000 ng/mL of LPS or none (control group) or in equal volumes of Neurobasal medium plus medium conditioned by macrophages that had been incubated with 10 or 1000 ng/mL of LPS or without LPS (CM group). Then, the number of MAP2-positive cells was counted. The values are expressed as the number of MAP2-positive cells ± S.E. per cm² (n = 6). Statistically significant differences between groups are indicated by brackets: #p < 0.05 (ANOVA with Tukey’s post hoc test), *p < 0.05 (Student’s t-test), NS, not significant.

Our previous finding that a high degree of activation of microglia/macrophages elicited by high-dose LPS induced GDNF expression and improved locomotor function after spinal cord injury (7) provides a molecular basis for the neurotrophic effects of LPS. Furthermore, as revealed in this study, GDNF and NO synthesis/secretion by cultured macrophages stimulated with LPS showed a different dose responsiveness to the LPS (Fig. 3). A concentration of 10 ng/mL was sufficient to fully induce NO production, but concentrations over 100 ng/mL were needed to induce a substantial level of GDNF production. Therefore, the monitoring of only GDNF and NO production would be insufficient to characterize macrophages as being neurotrophic or neurotoxic. DNA or protein microarrays and other high-throughput transcription profiling technology may be better for this purpose, because they would be expected to detect a large number of genes, the expression of which would be changed by LPS in cultured macrophages (10, 17). However, such data are sometimes too complicated and not practical to determine the properties of macrophages. Therefore, we considered biological methods using cultured cells to be more practical and reliable for characterization of macrophages. Indeed, the present results demonstrate that the macrophages activated by low-dose LPS were toxic against cultured cortical neurons (Fig. 4), which finding is apparently consistent with the upregulation of NO production and lack of effect on GDNF secretion at the low dose. These findings suggest that macrophages in a low state of activation would be neurotoxic, unlike those in the highly activated state.

As GDNF enhances the phagocytic activity of cultured macrophages (8), GDNF synthesized by macrophages would activate macrophages in an autocrine manner. Therefore, not only neuroprotection but also activation of macrophages would result from macrophage-secreted GDNF in the injured spinal cord. Such GDNF might further upregulate GDNF synthesis in the macrophages, which would promote more and more restorative macrophage function. GDNF may be involved in an activation loop to establish an adequate neurotrophic environment for nerve regeneration. Interestingly, the expression of mRNAs for Gfα1, which is the glycosylphosphatidylinositol-anchored specific binding site of GDNF, and for Ret, the tyrosine kinase that transduces GDNF signals, was confirmed in the cortical neurons by RT-PCR analysis (9).

Inflammatory reactions are sometimes considered to be harmful and anti-inflammatory therapies are therefore used for spinal cord injury. However, our present results suggest that such therapies are not always advantageous for establishing neurotrophic circumstances to achieve nerve regeneration. Thus, the quality of inflammation and the nature of activated macrophages are critical for the establishment of neurotrophic circumstances, and are thus new aspects for consideration for our goal of facilitating nerve regeneration.

REFERENCES

LPS decides macrophage nature