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SYNERGISTIC STIMULATION OF NITRIC OXIDE HEMOGLOBIN PRODUCTION IN RATS BY RECOMBINANT INTERLEUKIN 1 AND TUMOR NECROSIS FACTOR

Hiroaki Kosaka, Noboru Harada, Manabu Watanabe, Harumasa Yoshihara, Yasuo Katsuki, and Takeshi Shiga

Department of Physiology, Medical School, Osaka University, 2-2 Yamadaoka, Suita, Osaka 565, JAPAN

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SUMMARY: Nitric oxide (NO) is formed from arginine in *Escherichia coli* lipopolysaccharide (LPS) treated rat; however, none of specific cytokine inducing NO generation is yet determined. We studied the effect of interleukin 1 (IL-1) and tumor necrosis factor (TNF) on NO production in rats by detecting NO-hemoglobin in their blood, using electron spin resonance. Either IL-1 or TNF alone stimulated NO-hemoglobin formation. Combined administration of IL-1 and TNF markedly enhanced NO-hemoglobin generation, demonstrating the synergistic character of both stimuli on NO production. Further, LPS and TNF in combination were more potent stimulator of NO-hemoglobin production in rats than each alone. © 1992 Academic Press, Inc.

Nitric oxide (NO) is one of the physiological regulator, endothelium derived relaxing factor (1-3). The NO mediates a variety of cell functions such as relaxation of vascular smooth muscle, inhibition of platelet aggregation, and neurotransmission. Recently it was found that *Escherichia coli* lipopolysaccharide (LPS) caused an increase of inducible type of NO synthase in vascular smooth muscle (reviewed in 4).

LPS induced NO_2^-/NO_3^- generation *in vitro* and *in vivo* after a time delay of 6 h (5-8) and administration of LPS to rats caused nitrosation of amines (9, 10). *In vivo* production of NO had been shown by ESR using CO-hemoglobin as a trapping agent of NO in the peritoneal cavity of rats treated with LPS (11). NO binds to hemoglobin with extremely high affinity, and nitric oxide hemoglobin (HbNO) gives characteristic electron spin resonance (ESR) signal (12-14). HbNO was detected in the blood of rats treated with LPS (11, 15, 16). A portion of NO is oxidized to NO_2^-/NO_3^-

ABBREVIATIONS: ESR, electron spin resonance; HbNO, nitric oxide hemoglobin; LPS, *Escherichia coli* lipopolysaccharide; IL-1, interleukin 1; TNF, tumor necrosis factor.

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by the reaction with O_2 in plasma and the NO_2^- is converted to NO_3^- in the presence of oxyhemoglobin (17-21).

Interleukin 1 (IL-1) was first described as a lymphocyte-activating factor (reviewed in 22). Tumor necrosis factor (TNF) is known to mediate hemorrhagic necrosis of tumors, a biologic effect originally associated with endotoxemia (reviewed in 23). IL-1 and TNF are cytokines produced by macrophages and monocytic cells exposed to the same stimuli, such as LPS (reviewed in 24), and have many similarities in their activities, although IL-1 and TNF neither share any apparent structural homology or recognize the same cellular receptor (25, 26).

The present study concerns whether these cytokines are involved in the production of NO *in vivo*. To evaluate NO release in rats, the detection of HbNO in blood by ESR is a simple and elegant method reflecting the pathophysiological state. On the other hand, for the determination of NO_2^-/NO_3^- to evaluate NO release, high baseline level of NO_3^- due to food and drinking water must be excluded as possible (8, 9). We find that IL-1 is as effective as LPS to induce HbNO in rats. Our present results further show synergism between IL-1 and TNF, for the first time, i.e., simultaneous administration of IL-1 and TNF to rats markedly induced generation of HbNO in the blood compared with that of IL-1 or TNF alone.

MATERIALS AND METHODS

Male Wistar rats (4 weeks old, about 60-70 g) were treated intraperitoneally (i.p.) with LPS, IL-1 and/or TNF. After 6 h, blood (0.4 ml) was collected from the abdominal aorta, transferred to ESR tube, and frozen immediately under liquid nitrogen. The rats were anaesthesized by pentobarbital (50 mg/kg b. w., i.p.) 15 minutes prior to the sampling.

Recombinant human TNF was a gift of Suntory Co., Ltd. (Osaka, Japan). TNF had a specific activity of 2.8×10^6 U/mg protein and contained less than 0.12 ng endotoxin/mg protein. Recombinant human IL-1β was a gift of Otsuka Pharmaceutical Co. Ltd. (Osaka, Japan). IL-1β had a specific activity of 2×10^7 U/mg protein and the contamination of endotoxin was less than 1 ng/mg protein.

ESR spectra were recorded with a Varian E-12 spectrometer at 110 K. ESR spectrometer settings were as follows; incident microwave power, 10 mW; modulation frequency, 100 kHz; modulation amplitude, 0.5 mT; response time, 1 s; and sweep rate, 12.5 mT/min. The concentrations of HbNO were determined by double integrations of the spectra, using $CuSO_4$-EDTA as a standard.

RESULTS

Rats were treated i.p. with LPS. The LPS concentration (60 μg/rat) was about 1 mg/kg, enough to induce nitric oxide synthase as reported (8, 9, 11). After 6 h, blood sample was collected from the abdominal aorta, transferred to ESR tube, and frozen immediately under liquid nitrogen. An ESR signal of HbNO was detected. A three-line hyperfine structure characteristic to HbNO of T-type is slightly present in the ESR signal

(Fig. 1-1A). Injection of LPS and TNF in combination strongly enhanced the HbNO generation (Fig. 1-1B). IL-1 also increased LPS-induced HbNO production (Fig. 1-1C).

Next, rats were treated i.p. with IL-1 and/or TNF. Injection of either IL-1 or TNF alone produced HbNO (Fig. 1-2A, B). HbNO induced by IL-1 was similar extent as that by LPS. TNF was less potent than IL-1 to induce HbNO. IL-1 and TNF are cytokines produced by macrophages and monocytic cells exposed to LPS and have many similarities in their activities. But, administration of IL-1 and TNF in combination markedly accelerated HbNO formation than that of either IL-1 or TNF alone (Fig. 1-2C). With untreated rat, HbNO was not detected in the blood.

To know which cytokine is efficient in the synergistic action for HbNO production, each cytokine was diluted. When concentration of IL-1 was the same and TNF solution was diluted, the concentration of HbNO generated in rats after 6 h decreased dependent on TNF concentration (Fig. 2). Conversely, under the same concentration of TNF, the HbNO produced in rats diminished with the decrease of IL-1 concentration. When both IL-1 and TNF solution were diluted, the formation of HbNO in rats after 6 h was markedly decreased.

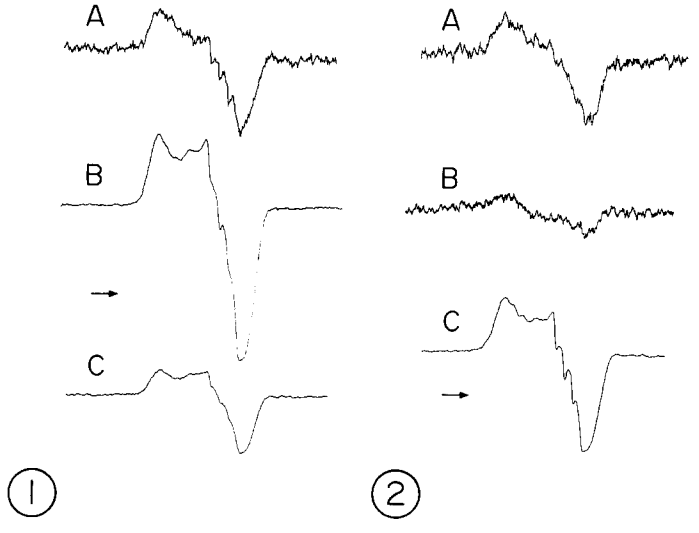

Figure 1-1. Effect of IL-1 and TNF on LPS-induced formation of HbNO in rats. Rats were treated i.p. with LPS alone (60 μg/rat) (A), LPS (60 μg/rat) plus TNF (200 μg/rat) (B), or LPS (60 μg/rat) plus IL-1 (10 μg/rat) (C), for 6 h. Span of arrow corresponds to 5 mT. Receiver gains were: (A), 10 x 10^3; (B) and (C), 2.5 x 10^3. **Figure 1-2.** Formation of HbNO in rats by IL-1 and TNF alone and in combination. Rats were treated i.p. with IL-1 alone (10 μg/rat) (A), TNF alone (200 μg/rat) (B), or IL-1 (10 μg/rat) plus TNF (200 μg/rat) (C), for 6 h. Span of arrow corresponds to 5 mT. Receiver gains were: (A) and (B), 10 x 10^3; (C), 2.5 x 10^3.

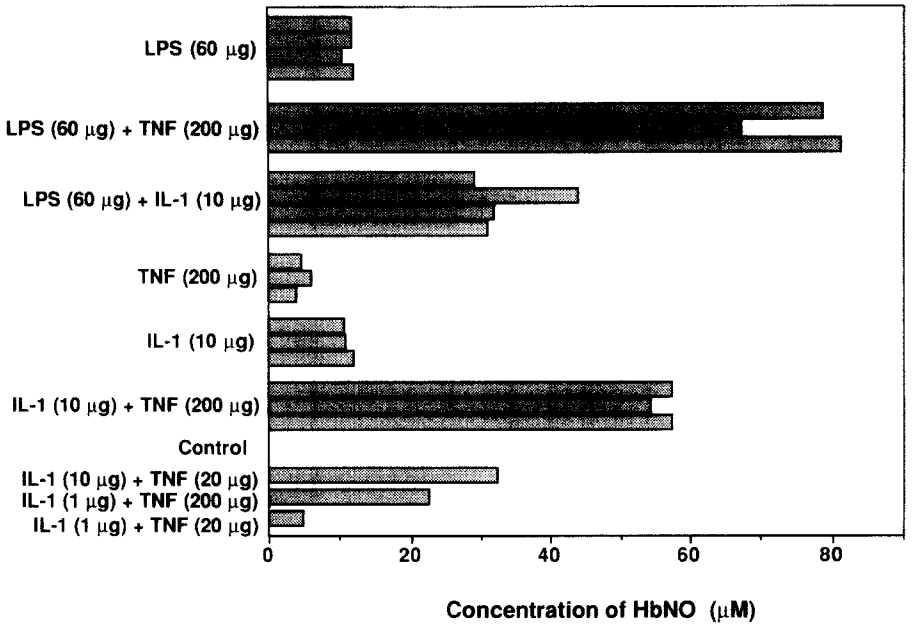

Figure 2. Comparison of HbNO generation by LPS, IL-1 and/or TNF. One column represents an experiment with one rat.

DISCUSSION

The present study first demonstrated that TNF and IL-1 synergistically induced NO production in rats (Fig. 1-2). Concentration of the formed HbNO was far greater than that produced by LPS alone. TNF and IL-1 lack signal peptide sequences that are characteristic for secretory proteins, suggesting that they typically remain cell-associated and that their release may occur mainly as a result of damage to the producing cell (reviewed in 24). Thus, the amount of IL-1 and/or TNF released by the concentration of LPS in the present study must be lower than the concentration of IL-1 and TNF injected simultaneously. One of the reasons of septic shock may be the induction of nitric oxide synthase by IL-1 and TNF as demonstrated here, because NO dilatates blood vessels and HbNO in the circulating blood interferes oxygen delivery to the tissue. IL-1 could act synergistically with TNF to cause hypotention (27), and lethality (28).

Our study also showed that LPS and TNF in combination were more potent stimulator of HbNO production than each alone (Fig. 1 and 2). TNF and LPS acted synergistically to induce shock and bowel necrosis (29). LPS may release high levels of IL-1 and low levels of TNF. In fact, LPS and TNF in combination induced more HbNO generation than LPS and IL-1 in combination (Fig. 1-1). IL-1 was as potent as LPS to generate HbNO (Fig. 2).

Several lines of evidence indicates that TNF can induce the production of IL-1 in various types of cells (reviewed in 24). On the other hand, the addition of IL-1 to cultures of human monocytes enhanced their cytotoxicity for tumor target cells. The IL-1-induced cytotoxicity was inhibited in the presence of antibodies specific for TNF, suggesting that TNF induction by IL-1 was required (30). The synergistic production of NO by IL-1 and TNF shown here may be consistent to this cross-induction of TNF and IL-1, and may be involved in the cytotoxicity of tumors.

A few studies have investigated the effect of these purified cytokines in combination. Both IL-1 and TNF have been shown to synergize for the production of prostaglandin E_2, the cytotoxicity to human tumor cell lines (31), the induction of bone resorption (32) and the arrest of the growth of melanoma cells *in vitro* (33).

N^G-Monomethyl-L-arginine, an inhibitor of nitric oxide synthase, inhibited HbNO formation in the blood of rats stimulated with LPS (11). LPS generates IL-1 and TNF, both of which control the induction of nitric oxide synthase in macrophages, liver and smooth muscle of blood vessel.

The present study first demonstrated that TNF and IL-1 synergistically induced NO production in rats. The concentration of HbNO formed was far greater than that produced by LPS. Our study also demonstrates that LPS and TNF in combination were more potent stimulator of HbNO production by rats than each alone.

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