Depressive Effect of a Traditional Chinese Medicine (Sho-Saiko-To) on Endotoxin-Induced Nitric Oxide Formation in Activated Murine Macrophage J774A.1 Cells

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The present study investigated whether or not Sho-saiko-to (crude powder extract, TJ-9) can suppress nitric oxide (NO) generation by endotoxin-activated J774A.1 cells in order to study the preventive mechanism of Sho-saiko-to against endotoxemia. In this experiment, we estimated the NO₂⁻ in the murine macrophage cell line J774A.1 using the Griess method. Our results clearly demonstrated that J774A.1 cells stimulated with endotoxin (0.01–10 µg/ml) can effectively produce NO, and the production was dependent on the dose of endotoxin. On the other hand, we investigated the suppressive effect of TJ-9 (10–100 µg/ml) on NO generation by endotoxin (0.1 µg/ml)-activated J774A.1 cells. The NO level when the cells were incubated with endotoxin and TJ-9 (10–20 µg/ml) was slightly lower than that in cells treated with endotoxin alone. In contrast, treatment with TJ-9 (50–100 µg/ml) significantly inhibited endotoxin-activated NO generation in J774A.1 cells, whereas the treatment with TJ-9 (10–100 µg/ml) alone was ineffective in inducing NO formation and in inhibiting cell viability in the J774A.1 cells. These findings suggest that a Kampo prescription of Sho-saiko-to shows a suppressive effect on NO generation in macrophages stimulated with endotoxin, and that it may be useful in improving endotoxin-shock symptoms.

Key words Kampo medicine; Sho-saiko-to; endotoxin; suppressive effect; nitric oxide formation

Endotoxins of gram-negative bacteria possess a very large number of biological activities. Therefore, endotoxin induces shock states in humans and animals which is characterized by fever, hypotension, intravascular coagulation, and finally multi-organ failure. It has recently been reported^{1–4)} that nitric oxide (NO) may play a role as an important regulator in many cellular functions in endotoxemic animals. The biosynthesis of NO occurs in a number of non-neuronal, mammalian tissues including vascular endothelial cells, platelets, leukocytes, fibroblasts, Kupffer cells and as yet unidentified cells types within the adrenal cortex and lung.¹⁾ Thus, it was shown that the NO radical functions efficiently as a mediator, a messenger, or a regulator of cell function in a number of physiological systems and pathophysiological states. The metabolism of arginine to NO in macrophages is mediated by a Ca²⁺/calmodulin-insensitive NO synthase enzyme, which is induced following the activation of these cells with bacterial endotoxin and/or cytokines.¹⁾

Sho-saiko-to is one of the most frequently prescribed Kampo medicines, and has primarily been used to treat chronic hepatitis. We recently reported^{5–8)} that Sho-saiko-to protects the tumor necrosis factor (TNF)-induced lethality in galactosamine-hypersensitized mice, and that Sho-saiko-to-pretreated mice are protected against oxygen toxicity, Ca²⁺ overload in cytoplasm or mitochondria and carbohydrate metabolic disorders during endotoxemia. Therefore, the present study was conducted to discuss the role of NO in order to clarify the defense mechanism of Sho-saiko-to against endotoxemia.

MATERIALS AND METHODS

Materials *Salmonella typhimurium* lipopolysaccharide

(endotoxin, Westphal obtained from Difco Laboratories, Detroit, Mich., U.S.A.) was used throughout this study. The traditional Chinese preparation, Sho-saiko-to, was obtained from Tsumura Co., Tokyo. Tsumura-Sho-saiko-to (crude powder extract, TJ-9) contains spray-dried aqueous extracts of seven crude drugs in the following proportions: 7.0 g Bupleuri Radix, 5.0 g Pinelliae Tuber, 3.0 g Scutellariae Radix, 3.0 g Zizyphi Fructus, 3.0 g Ginseng Radix, 2.0 g Glycyrrhizae Radix, and 1.0 g Zingiberis.

Cell Culture The murine monocyte-macrophage cell line, J774A.1, was obtained from the Japan Cancer Research Resources Bank (JCRB, Tokyo). J774A.1 cells were maintained continuously in 75 cm² plastic culture flasks (Falcon) in RPMI (Roswell Park Memorial Institute)-1640 medium supplemented with penicillin (100 units/ml), streptomycin (100 µg/ml) and 10% fetal calf serum. Cells were harvested by gentle scraping and passaged every 3–6 d by dilution of a suspension of the cells 1:10 in fresh medium.

Measurement of Nitrite Production as an Assay of NO Release in J774A.1 Cells NO production by J774A.1 cells was assayed by measuring the accumulation of nitrite in the culture medium using the Griess reaction.⁹⁾ J774A.1 cells were removed from culture flasks by vigorous pipetting and centrifuged, then resuspended in the medium to a concentration of 1 × 10⁶ cells/ml. Cells were plated in 24 well culture plates (Falcon) and allowed to adhere for 2 h. Thereafter, the medium was replaced with fresh medium or medium containing endotoxin and/or the presence of TJ-9, and was incubated at 37 °C in 5% CO₂ in air for up to 48 h. Briefly, 300 µl of culture supernatant from control cells or cells stimulated with endotoxin were mixed with an equal volume of Griess

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reagent (1% sulfanilamide, 0.1% naphthylethylenediamine dihydrochloride, 2.5% H_3PO_4) at room temperature for 10 min. Absorbance was read at 560 nm using an ELISA analyzer (Model ETY-96, Toyo Sokki Co., Ltd., Kanagawa). The nitrite concentration in the medium was determined with sodium nitrite used as a standard. Data are expressed as the total μM nitrite production by 10^6 cells for 48 h, as indicated in the figures. Data are expressed as the mean $\pm$ S.E. Statistical significance of the data was evaluated using the Student's *t*-test.

Cell Viability Cell viability was assessed by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma)-based colorimetric assay.¹⁰ Cells (5×10^3 cells/well) in 96 well plates were exposed to TJ-9 at 37°C in 5% CO_2 in air for up to 48 h. After completion of the drug treatment, 10 μl of MTT (5 mg/ml) solution was added to each well, followed by 100 μl of complete medium. After incubation for 4 h at 37°C , MTT solution in the medium was removed. The incorporated formazan crystals in viable cells were solubilized with 100 μl of dimethylsulfoxide (Sigma). The absorbance of each well was then read at 540 nm using an ELISA analyzer.

RESULTS

Generation of NO by J774A.1 Cells Stimulated with Endotoxin We examined the effect of the dose response of endotoxin-stimulation on the generation of NO by cultured J774A.1 cells. J774A.1 cells were incubated with endotoxin (0.01–10 $\mu\text{g}/\text{ml}$) at 37°C for up to 48 h. The amount of stable nitrite (NO_2^-), the end product of NO generation by activated macrophages under various conditions, was determined by the Griess method.⁹ As shown in Fig. 1, unstimulated J774A.1 cells did not produce detectable amounts of NO_2^- in the absence of endotoxin. However, treatment with endotoxin (0.01–10 $\mu\text{g}/\text{ml}$) led to high levels of NO_2^- production by cultured J774A.1 cells. The effect of endotoxin on NO production by activated J774A.1 cells was observed to be in a dose

Fig. 1. Effect of Dose-Dependence on the Generation of NO by J774A.1 Cells Stimulated with Endotoxin

J774A.1 cells were cultured to confluence in 24 well plates (10^6 cells/well) and treated for 48 h in the presence of endotoxin (0.01–10 $\mu\text{g}/\text{ml}$). NO production by J774A.1 cells was assayed by measuring the accumulation of nitrite in the culture medium using the Griess reaction.⁹ Each bar represents the mean $\pm$ S.E. of 3 experiments.

dependent fashion.

Effect of TJ-9 on NO Production in Endotoxin-Activated J774A.1 Cells As can be seen in Fig. 1, incubation of J774A.1 cells in culture for 48 h with endotoxin (0.1 $\mu\text{g}/\text{ml}$) increased the level of NO_2^- in the media. Therefore, we observed the effect of TJ-9 (10–100 $\mu\text{g}/\text{ml}$) on NO production by endotoxin (0.1 $\mu\text{g}/\text{ml}$)-activated J774A.1 cells (Fig. 2). J774A.1 cells, activated in the presence of endotoxin alone (0.1 $\mu\text{g}/\text{ml}$), produced a high level of NO_2^- . However, at a range of 10–20 $\mu\text{g}/\text{ml}$ of TJ-9, the level following the incubation of cells in the presence of endotoxin was slightly lower than that in cells treated with endotoxin alone. In contrast, treatment with TJ-9 (50–100 $\mu\text{g}/\text{ml}$) showed a significantly inhibitory effect on endotoxin-activated NO production in J774A.1 cells. On the other hand, cell viability in the presence of TJ-9 (10–50 $\mu\text{g}/\text{ml}$) was indicated to be almost 100%, while the addition of 100 $\mu\text{g}/\text{ml}$ of TJ-9 showed 91% cell viability (Fig. 3). Treatment with TJ-9 (10–100 $\mu\text{g}/\text{ml}$) alone,

Fig. 2. Effect of TJ-9 on Generation of NO by J774A.1 Cells Stimulated with Endotoxin (0.1 $\mu\text{g}/\text{ml}$)

Cells were incubated with combinations of endotoxin (0.1 $\mu\text{g}/\text{ml}$) and TJ-9 (10–100 $\mu\text{g}/\text{ml}$) for 48 h. Nitrite accumulation in the culture medium was determined as described in Fig. 1. Each bar represents the mean $\pm$ S.E. of 3 experiments. *a)* Significant difference from the value of cells treated with endotoxin alone at $p < 0.05$.

Fig. 3. Effect of TJ-9 on Viability of J774A.1 Cells

Cell viability was assessed by the method described in Materials and Methods. Cells in 96 well plates were exposed to TJ-9 for 48 h at 37°C in 5% CO_2 in air for up to 48 h. Each point represents the average of 8 experiments.

however, was ineffective in inducing NO formation in J774A.1 cells (data was not shown).

DISCUSSION

In this report, using activated murine macrophage J774A.1 cells, we described the role of the NO in the preventive effects of Sho-saiko-to against endotoxemia. Either as a single dose or in a combination of endotoxin, interleukin-1 (IL-1), TNF and γ -interferon (IFN), NO synthase (iNOS) was induced in macrophages, resulting in the formation of large quantities of NO. NO also contributes to the cytotoxic or cytostatic actions of macrophages activated by various immunological stimuli.¹¹⁾ Endothelium-derived NO plays¹⁾ a role in the physiological regulation of vascular tone and blood pressure. In addition, it has been implicated in the pathogenesis of vascular injury, hypotension and shock induced by endotoxin and TNF. Sho-saiko-to improved endotoxin shock,⁵⁻⁸⁾ based on our series of studies on metabolic pharmacological effects. Therefore, we investigated whether or not TJ-9 can suppress NO generation by endotoxin-activated J774A.1 cells.

In the present study, our results clearly demonstrate that endotoxin (0.1–10 μ g/ml) can effectively produce NO from activated J774A.1 cells, and NO production by endotoxin-activated J774A.1 cells showed an increase in a dose dependent manner. Nitric oxide^{1,12)} produced by activated macrophages has been shown to be involved in TNF-induced shock, hypotension, and vasodilatation, and in addition, NO, a highly reactive free radical produced by activated macrophages, has emerged as another important mediator of inflammatory responses. Furthermore, NO may react with superoxides resulting in the formation of peroxynitrite, which can lead to the iron-independent generation of hydroxyl radicals.¹³⁾ We have previously reported^{14,15)} that endotoxin injection resulted in lipid peroxide formation and membrane damage in experimental animals, causing decreased levels of scavengers or quenchers of free radicals. On the other hand, our present result demonstrated that TJ-9 (50–100 μ g/ml) showed clear inhibition in the production of NO from endotoxin-activated J774A.1 cells (Fig. 2). Previously, we observed that the administration of TJ-9 prevented the peroxidation of membrane lipid by superoxide-free radicals generated in endotoxemia.⁶⁾ Thus, the ischemic state of tissues in endotoxemia may result, at least in part, in a potentiality of NO radical generation. It is possible that the preventive effects of TJ-9 on oxygen toxicity in endotoxemia are caused, at least in part, by the inhibition of NO production as described above. Sho-saiko-to

may, therefore, prove to be important in gram-negative bacteria-induced shock. Moreover, Sho-saiko-to is known to show a glucocorticoid-like action.¹⁶⁾ Glucocorticoids have been used in the treatment of endotoxin shock. Induction of NO synthase in macrophages is strongly inhibited by glucocorticoids such as dexamethasone and hydrocortisone.¹⁷⁾ It is, therefore, of interest that endotoxin-induced NO is inhibited by Sho-saiko-to, having a glucocorticoid-like action.

We believe that iNOS is responsible for the excess production of NO in sepsis, which leads to the development of shock.¹⁾ Some L-arginine analogs reversibly inhibit iNOS and restore the endotoxin-induced loss of catecholamine-vasomotor responsiveness *in vivo*.¹⁸⁾ From the findings described above, the suppression of NO generation from endotoxin-activated macrophages by Sho-saiko-to may also prove useful in improving these endotoxin-induced shock symptoms.

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