Synergistic effects of Sho-saiko-to and interferon α/β on *in vivo* nitric oxide generation in mice

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Abstract

Nitric oxide (NO) is a multifunctional mediator essential to various biological systems. Examination was made of the effects of the herbal medicine Sho-saiko-to (SST) and interferon α/β (IFN α/β) on *in vivo* NO generation. SST (0.92 g/kg of body weight) was orally administered to mice for 3 weeks with or without intraperitoneal injection of IFN α/β (10^5 unit/kg of body weight, 2 times per week). Serum nitrite/nitrate was measured as an index of *in vivo* NO generation. Significant increase in serum nitrite/nitrate was observed in mice treated with SST and IFN α/β, but not with either alone. SST and IFN α/β are thus shown to synergistically increase NO generation *in vivo*.

Key words nitric oxide, Sho-saiko-to (Xiao-Chai-Hu-Tang), 小柴胡湯, interferon α/β, synergistic effect.

Abbreviations SST, Sho-saiko-to ; IFN α/β, interferon α and β ; IL-1, interleukin-1 ; TNF-α, tumor necrosis factor-α ; NO, nitric oxide ; NOS, nitric oxide synthase ; iNOS, inducible nitric oxide synthase ; cNOS, constitutive nitric oxide synthase ; VSMC, vascular smooth muscle cell ; PBS, phosphate buffered saline ; LPS, lipopolysaccharide ; NADPH, reduced nicotinamide adenine dinucleotide phosphate ; FAD, flavin adenine dinucleotide.

Introduction

The herbal medicine Sho-saiko-to (SST) is widely used for treating various infectious diseases such as chronic viral hepatitis, bronchitis and the common cold.[1,3] SST has many pharmacological effects, especially on immunological response.[2,3] Its immunopotentiating action includes activation of macrophages, enhancement of antibody production and induction of inflammatory cytokines such as interleukin-1 (IL-1), tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ).[3,6]

Interferons (IFNs) induce many diverse biological activities especially in the immune system.[7] IFN α and β (IFN α/β) have potent anti-viral activities and are now widely used for treating viral hepatitis.[8,9] SST is used for treatment of chronic viral hepatitis, and effectiveness of combined administration of SST and IFN α/β has previously been suggested in Japan.[10,11] However, the therapeutic potential of this combination for viral hepatitis has recently been denied because interstitial pneumonia develops as an adverse effect especially when SST is administered with much exogenous IFNs.[12,13] The mechanism for the onset of interstitial pneumonia by these drugs is little understood. Allergic reactions to the drugs and/or synergistic effects of these drugs on immunological and inflammatory responses may possibly be involved.[12,13]

Study was thus made to determine the mechanism for synergism between SST and IFNs in regard to various biological effects. This paper reports that SST and IFN α/β act synergistically to increase nitric oxide (NO) generation *in vivo*. NO is a multifunctional mediator which can be induced in a variety of cell types and is involved in various biologi-

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cal functions such as inflammatory reactions.[14] The biological significance of the synergism of SST with IFN α/β on NO generation is discussed, particularly in relation to immunomodulatory and adverse effect.

Materials and Methods

Preparation of spray-dried aqueous extracts of Sho-saiko-to : SST was prepared as a spray-dried powder of hot water extracts from seven species of the medicinal herbs, Bupleuri Radix (7.0 g), Pinelliae Tuber (5.0 g), Scutellariae Radix (3.0 g), Zizyphi Fructus (3.0 g), Ginseng Radix (3.0 g), Glycyrrhizae Radix, (2.0 g) and Zingiberis Rhizoma (1.0 g). Spray-dried powder of Sho-saiko-to was obtained from Tsumura & Co. (lot NO ; 240009020).

Experimental protocols : Six-week-old male Balb/c mice, obtained from Charles River Japan Inc., were housed five per cage in standard facilities, with free access to water and standard mice chow. Mouse IFN α/β was obtained from Paesel GmbH & Co. (Frankfurt, Germany). The animals were divided into 4 groups which underwent the following treatments (Fig. 1) :

1) Control group (n=10) ; the mice received *i.p.* PBS (0.12 ml per animal) 2 times per week for 3 weeks, with free access to tap water.

2) SST group (n=10) ; the mice received *i.p.* PBS (0.12 ml per animal) 2 times per week for 3 weeks, with free access to tap water containing 0.5 % SST.

3) IFN α/β group (n=10) ; the mice received *i.p.* IFN α/β (10^5 units/kg of body weight in 0.12 ml of PBS) 2 times per week for 3 weeks, with free access to tap water.

4) SST+IFN α/β group (n=10) ; the mice received *i.p.* IFN α/β (10^5 units/kg of body weight in 0.12 ml of PBS) 2 times per week for 3 weeks, with free access to tap water containing 0.5 % SST.

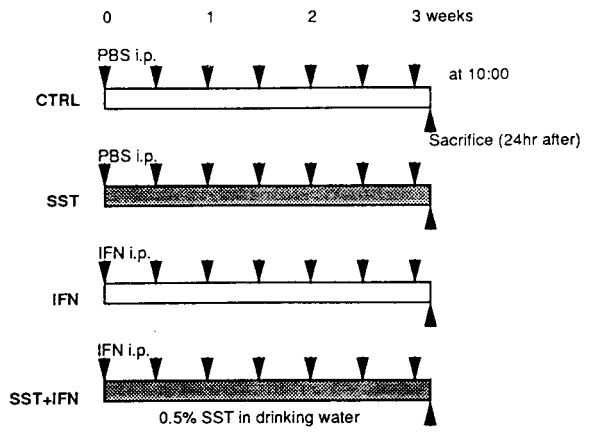

Fig. 1 Experimental procedure.
The animals were divided into 4 groups ; control (CTRL), Sho-saiko-to (SST), interferon α/β (IFN) and SST+IFN. Each group consisited of 10 mice. See Materials and Methods for treatment.

During each experiment, the average of daily water intake was 4.4 ml / mouse (23 - 24 g body weight). The daily intake of SST from drinking water was thus around 22 mg/mice, or 0.92 g/kg of body weight. Intraperitoneal injections of PBS or IFN α/β were conducted at 10 : 00 a.m. on days 1, 4, 8, 11, 15, 18 and 22. The animals were sacrified 24 hr after the last injection. Blood was obtained at the time of sacrifice and serum was separated by centrifugation after clot formation.

NO measurement : NO is an extremely unstable molecule and rapidly undergoes oxidative degradation to stable inorganic nitrogen oxides nitrite (NO_2^-) and nitrate (NO_3^-). The concentrations of serum NO_2^- and NO_3^- (NO_2^-/NO_3^-) were thus used here as indices of *in vivo* NO generation. Serum NO_2^-/NO_3^- was measured by the method of Schmidt *et al.*.[15] Briefly, serum was diluted 1 : 1 with distilled water and NO_3^- was converted to NO_2^- with 0.4 u/ml nitrate reductase (Boehringer Mannheim) in the presence of 50μM NADPH and 5μM FAD. The samples were mixed with 1/5 volume 60 % $ZnSO_4$ and centrifuged to precipitate plasma proteins. A 100-μl of aliquot of each deproteinized sample was mixed with 100μl Griess reagent (1 % sulfanilamide/0.1 % naphthylene diamine dihydrochrolide/5 % H_3PO_4) in a 96-well microplate for 10 min at room temperature and absorbance at 540 nm was determined with a microplate reader (Titertek Multiskan MCC/340).[16] Background absorbance was measured in the absence of sulfanilamide and naphthylethylene diamine dihydrochrolide and subtracted from that of a test sample. Nitrite concentration was determined using $NaNO_2$ as the standard.

Statistical analysis : The results are presented as means±S.E.M.. Significance of difference was assessed by the paired Student's t test. P less than 0.05 was

considered statistically significant.

Results

Serum nitrite and nitrate in mice treated with SST and/or IFN α/β (Fig. 2)

Serum NO_2^-/NO_3^- in the control group was 13.6±1.6μM (n=10). Treatment of the mice with SST or IFN α/β failed to cause significant change in this parameter. In mice receiving SST or IFN α/β for 3 weeks, it was 15.2±1.2 μM (n=10) and 12.9±1.7 μM (n=10), respectively. Serum NO_2^-/NO_3^- in mice receiving both SST and IFN α/β was 20.3±1.6 μM (n=10), this value being nearly 50 % higher than that of the control group ($p<0.05$).

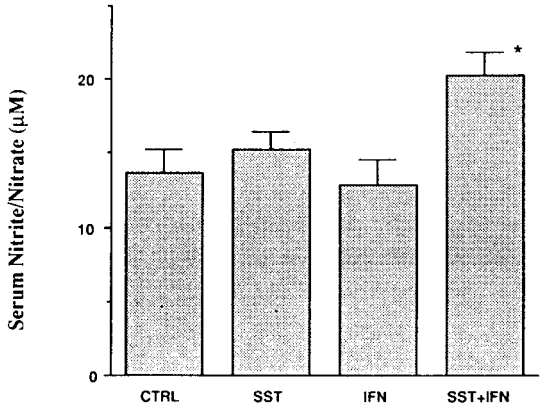

Fig. 2 Effects of SST and/or IFN α/β on serum nitrite and nitrate in mice.
Serum NO_2^-/NO_3^- was measured in mice treated with SST and/or IFN α/β following the procedure shown in Fig. 1. Values represent means±S.E.M.(n=10). *$p<0.05$ compared with control.

Discussion

SST is administered orally as treatment for various infectious diseases.[1, 2, 10, 11] The pharmacological action of SST has been studied with attention directed to increased nonspecific resistance of the host to pathogenic microorganisms due to immunomodulation.[3] The immunomodulatory action of SST include immunopotentiation such as activation of macrophages, enhancement of antibody production and induction of IL-1, TNF-α and IFN production.[3, 6] Macrophage activation appears quite likely involved in the immunostimulatory effect of SST, since macrophages serve as important effector cells in the host defense system to eliminate microbial invaders and tumor cells.[5]

Recent work shows NO to be essential in macrophage-induced cytotoxicity against microorganisms and tumor cells.[14, 17] Karupiah *et al.* found the antiviral activity of IFN-γ to be due mainly to the induction of NO production.[18] Lander *et al.* reported NO to possibly have immuno-stimulatory properties.[19] Serum NO_2^-/NO_3^- increases in various inflammatory diseases in which NO is generated by activated macrophages and neutrophils.[14]

The results of the present study demonstrate the oral administration of SST to stimulate NO production *in vivo* when given in combination with IFN α/β. IFN α/β are endogenously produced in various infectious diseases and expresses potent anti-viral activity.[7] Results clearly suggest the effectiveness of SST for treating various infectious diseases in which IFNs are produced endogenously at sites of infection.

A crude drug is comprised of many ingredients, and its effects are quite complicated and not necessarily attributable to just one component. In addition to its immunopotentiating effects, SST exerts anti-inflammatory and anti-allergic action.[1, 3] An aqueous extract of Scutellariae Radix was previously shown to inhibit NO production from lipopolysaccharide (LPS)-stimulated macrophages.[16] However, the present study clearly demonstrates enhanced *in vivo* NO production when administered in combination with IFN α/β. These conflicting data may be due to component complexicity and the diverse effects of SST on immunological response. Although the mechanisms for immunomodulatory effects remains to be clarified, some components in SST appear to stimulate the reticuloendothelial system with consequent NO-inducing activity. Kondo and Takano recently reported glycyrrhizin, a major ingredient of Glycyrrhizae Radix, to activate macrophages *in vivo* and stimulate NO production in response to LPS.[20] We investigated the effects of herbal drugs on NO production *in vitro*, and found aqueous extracts of Ginseng Radix to be capable of inducing NO production from macrophage cells.[21] SST may possibly be involved indirectly in *in*

vivo synergism with IFN α/β, since SST induces the expression of inflammatory cytokines such as TNF-α, IL-1β and IFN-γ.[3,4,6] These proinflammatory cytokines may enhance iNOS induction when present with IFN α/β.

The present results thus appear to be evidence for enhancement of the defence system by SST in combination with endogenous IFNs and may provide clues to explain the mechanism by which SST acts as an immunodulatory drug, especially in infectious diseases. However it is also possible that this synergism may be associated with adverse effects such as interstitial pneumonia occasionaly induced when SST is administered with much exogenous IFN α/β.[12,13] Combined administration may augment inflammatory reactions by potentiating immunostimulatory effects. In spite of its therapeutic potential, NO has cytotoxic and genotoxic effects as evident from the damage it causes to proteins and DNA.[22] Abundant NO production from inflammatory cells may thus damage tissue components in a manner similar to other chemical oxidants.[22] NO is an important mediator of physiologic and inflammatory processes in the lung.[23] It is reported that stimulation by cytokines and LPS causes alveolar macrophages and lung epithelial cells to produce large amount of NO and superoxide which react to form peroxynitrite, a potent oxidant capable of damaging the alveolar epithelium and pulmonary surfactant.[23] It is therefore possible that increased NO production from epithelial and parenchymal cells in the lung tissue is associated with the pathogenesis of interstitial pneumonia in which inflammatory reactions occur in interstitial tissue of the lung.

NO is derived from L-arginine by isoforms of nitric oxide synthase (NOS): constitutive (cNOS) and inducible (iNOS).[14] Although the iNOS pathway was first characterized in macrophages activated by LPS and IFN-γ, NO production is not limited to the reticuloendothelial system.[14,22] Hepatocytes also express iNOS following exposure to LPS together with TNF-α, IL-1, and IFN-γ.[24] NOS is also induced in vascular smooth muscle cells (VAMC). Ginseng Radix and Scutellariae Radix, constituents of SST, were previously shown to induce iNOS in VSMC.[25] Further study should be made to elucidate the source of *in vivo* NO production and mechanism for the synergistic effects induced by the simultaneous administration of SST and IFNs.

和文抄録

マウスの生体内における一酸化窒素（NO）産生に対する小柴胡湯（SST）とインターフェロン α/β（IFN α/β）の作用を検討した。飲水に混じたSST（0.92 g/kg体重）を経口的に3週間連続投与し，IFN α/β（10^5 unit/kg体重）は週2回（計7回），腹腔内投与を行った後，血中の NO_2^-/NO_3^- を測定して生体内でのNO産生の指標とした。SSTあるいはIFN α/β を単独で投与した場合の血中 NO_2^-/NO_3^- レベルはコントロール群と有意差を認めなかったが，SSTとIFN α/β を併用した群では NO_2^-/NO_3^- レベルは，約50％の有意な増加を示した。この結果より，SSTとIFN α/β がマウス生体内でのNO産生に対して相乗的に作用することが明らかとなった。

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