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Shengmai San, a Chinese Herbal Medicine Protects Against Rat Heat Stroke by Reducing Inflammatory Cytokines and Nitric Oxide Formation

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Abstract. The aim of the present study was to ascertain whether the possible occurrence of overproduction of inducible nitric oxide synthase (iNOS)-dependent nitric oxide (NO) in the brain and inflammatory cytokines in the peripheral blood exhibited during heat stroke can be reduced by prior administration of *Shengmai San*, a Chinese herbal medicine. Aminoguanidine, an iNOS inhibitor, was evaluated at the same time as a reference (positive control). Urethane-anesthetized rats were exposed to heat stress (ambient temperature of 43°C) to induce heat stroke. Control rats were exposed to 24°C. Mean arterial pressure and cerebral blood flow after the onset of heat stroke were all significantly lower than in control rats. However, cerebral iNOS immunoreactivity and NO levels were all greater after the onset of heat stroke. The serum levels of interleukin-1β, interleukin-6, and tumor necrosis factor-α were all increased after the onset of heat stroke. *Shengmai San* (1.2 g/ml per rat) or aminoguanidine (30 μmol/ml per rat) was administered orally, daily, and consecutively for 7 days before the initiation of heat stress; and this significantly attenuated the heat stress-induced arterial hypotension, cerebral ischemia, and increased levels of brain iNOS-dependent NO production and serum cytokines formation. *Shengmai San* shared with the aminoguanidine almost the same efficacy in reducing iNOS-dependent NO and cytokines overproduction during heat stroke. These results suggest that *Shengmai San* or aminoguanidine protects against heat stroke-induced arterial hypotension and cerebral ischemia by inhibition of iNOS-dependent NO overproduction in the brain and excessive accumulation of several inflammatory cytokines in the peripheral blood stream.

Keywords: *Shengmai San*, aminoguanidine, heat stroke, nitric oxide, cerebral ischemia, striatum

Introduction

When rodents are exposed to a hot environment, both increased metabolic demand and reduced splanchnic circulation produce hypoxia in the visceral organs; the hypoxia generates highly reactive oxygen and nitrogen species that accelerate mucosal injury (1, 2) and cerebral ischemia and injury (3). Intestinal mucosal permeability to endotoxin increases in heat stressed rats (4) and lead to production of inflammatory cytokines that induce release of nitric oxide (NO) (3) and endothelins (5). Both pyrogenic cytokines and endothelin-derived factors can precipitate arterial hypotension, hyperthermia, and cerebral ischemia during heatstroke (5 – 7).

Shengmai San (SMS), a Chinese herbal medicine, is routinely being used for treating coronary heart disease (8, 9). In addition, SMS effectively suppressed the ischemia-reperfusion injury during middle cerebral artery ligation (10) as well as the cerebral ischemia and damage during heat stroke (11) by reducing brain oxidative stress. However, it is not known whether prior administration of SMS is able to attenuate inflammatory cytokines and NO formation that occurred during heat stroke.

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Therefore, in order to validate the matter, the present experiments were performed to assess the effects of heat stress on the extent of inducible nitric oxide synthase (iNOS)-dependent NO in rat brain as well as the plasma levels of tumor necrosis factor (TNF)-α, interleukin (IL) 1β, and IL-6 in rats with or without prior administration of SMS. After the onset of heat stroke, ischemic injury was noted to occur in different brain structures including the striatum, hypothalamus, cortex, and thalamus (12 – 14). In the present study, the striatum was chosen as a representative region for measurement of local cerebral blood flow (CBF) as well as iNOS-dependent NO production. In fact, both the plasma levels of TNF-α, and IL-1β (15, 16) and cerebral levels of iNOS-dependent NO (3) are shown to be well related to the severity of heat stroke.

Materials and Methods

Materials

Constituents herbs of *Shengmai San* (SMS), *Panax ginseng* C.A. MEY (PG), *Ophiopogon japonicus* KER-GAWL (OJ), and *Schisandra Chinensis* (TURCZ) Baill (SC) were the products of *Jilin Sheng*, *Sichuan Sheng*, and *Jilin Sheng* of P. R. China, respectively. The SMS preparation used in this study was kindly prepared by Sun Ten Pharmaceutical Co., Ltd. (Taipei, Taiwan). Briefly, the three component herbs of SMS, OG (48 g), OJ (48 g), and SC (24 g) were suspended in 1200 mL distilled water, soaked for 1 h, and then decocted for 1 h. The supernatant was filtered through gauze, and the filtrate was then freezedried. The dried filtrate was mixed with Neusilin FL2 as an additive to produce a granulated SMS preparation. The SMS granules were stored at −80°C until used. For each experiment, the SMS granules were weighed precisely and solubilized in distilled water to make the final concentrations indicated. The specimen number (091515) of SMS used in this study was recorded and stored for 10 years at Sun Ten Pharmaceutical Co., Ltd. The HPLC profile indicated that the chemical components contained at least Schizandrin and Ginsenoside. To evaluate the pharmacological efficacy of SMS, an iNOS inhibitor aminoguanidine (AG) (Sigma Chemical Co., St. Louis, MO, USA) was evaluated at the same time as a reference (positive control).

Experimental groups

Adult male Sprague-Dawley rats weighing 280 and 340 g were obtained from the Animal Resource Center of the Chi-Mei Medical Center (Tainan, Taiwan). Between experiments, the animals were housed in group cages at an ambient temperature (Ta) of 24 ± 1°C with a 12-h eight/dark cycle, with the lights being switched on at 6:00 AM.

Animal chow and water were allowed ad libitum

Four groups of animals were designated for experiments: a) normothermic control group, b) vehicle-treated heat stroke group, c) SMS-treated heat stroke group, and d) AG-treated heat stroke group. In the normothermic group, the rats were treated with an oral dose of distilled water (DW) (1 ml per rat daily and consecutively for 7 days) and exposed to room temperature (24°C). In the vehicle-treated heat stroke group, the animals were treated with an oral dose of DW (1 ml per rat daily and consecutively for 7 days) and exposed to heat stress (43°C). At a certain point, when MAP (mean arterial pressure) began to decrease from its peak level, this moment was arbitrarily defined as the onset of heat stroke (17, 18). Immediately after the onset of heat stroke, heat stress was terminated and the animals were allowed to recover at room temperature (24°C). Our pilot results revealed that the latency for the onset of heat stroke (i.e., the interval between the start of heat stress and the onset of heat stroke) was 70 ± 2 min (n = 8) for vehicle-treated heat stroke rats. In the SMS-treated and AG-treated heat stroke groups, the animals were treated respectively with an oral dose of SMS (1.2 g/ml per rat) and AG (30 μmol/ml per rat) daily and consecutively for 7 days and then exposed to heat stress (Ta 43°C for exactly 70 min). Again, immediately after the onset of heat stroke, the drug-treated heat stroke animals were allowed to recover at room temperature (Ta 24°C).

Physiological parameter monitoring

Different groups of animals were used for different sets of experiments: a) measurements of MAP, CBF, and serum concentrations of TNF-α, IL-1β, and IL-6; b) measurements of MAP, CBF, and striatal levels of NO; or c) determination of immunoreactivity of iNOS in the striatum.

All experiments were approved by the Animal Research Committee of the Chi-Mei Medical Center. Adequate anesthesia was maintained to abolish the corneal reflex and pain reflexes induced by tail pinch throughout the course of all experiments (about 4 h) following a single dose of urethane (1.4 g per kg body weight, Sigma Chemical Co.).

The right femoral artery and vein of rats, under urethane, were cannulated with polyethylene tubing (PE 50) for blood pressure monitoring and blood sampling. Colonic temperature (Tco) was monitored continuously by a thermocouple, while MAP was continuously monitored with a pressure transducer.

CBF monitoring

Animals, under urethane anesthesia, were positioned in a stereotoxic apparatus (model 1460; David Kopf Instruments, Tujunga, CA, USA) to insert a probe for measurement of local CBF in the striatum (5). A 100-μm-diameter thermocouple and two 230-μm fibers were attached to the oxygen probe. This combined probe measures oxygen, temperature, and microvascular blood flow. OxyLite™ (Oxford Potronix, Oxford, UK) is a laser Doppler flow meter whose primary purpose is to measure real-time microvascular red blood cell perfusion. Laser Doppler signals were recorded in BPU (blood perfusion units), which are a relative unit scale defined using a carefully controlled motility standard.

Extracellular NO monitoring

A microdialysis probe (CMA 20; Carnegie Medicine, Stockholm, Sweden) with a 4-mm-long dialysis membrane was vertically implanted into the left striatum (5). A Ringer's solution (0.860 g NaCl, 0.030 g KCl, 0.033 g $CaCl_2$ per 100 ml) was perfused through the microdialysis probe at a constant flow (2.0 μl/min). After 2 h of stabilization, the dialysates from the striatum were collected at 10-min intervals. The NO concentrations in the dialysates were measured with the Eicom ENO-20 NO analysis system (Eicom, Kyoto) (19). After histological verification of the probe's path, all the data obtained were included in our results.

Immunohistochemical staining

Rats were killed with intravenous urethane (2.8 g/kg) and were transcardially perfused with heparinized 0.05 mol/l phosphate-buffered saline (PBS) followed by ice-cold 15% sucrose in PBS. The brains were rapidly removed and frozen in liquid nitrogen. Coronal brain sections (5-μm-thick) were cut on a cryostat and were thaw-mounted on gelatin-coated slides. The techniques for immunohistochemical staining of iNOS were detailed previously (3). The extent of immunoreactivity was scored on a scale of 0 – 3, in which 0 is no staining, 1 indicates weak staining, 2 indicates moderate staining, and 3 indicates strong staining.

Determination of cytokines

For the determination of serum levels of TNF-α, IL-6, and IL-1β, animals from each group were killed 15 min after the onset of heat stroke. For measurement of serum cytokines, 5 ml blood was withdrawn from the femoral vein of each rat. Blood samples were centrifuged at 1400 × *g* for 15 min at 4°C. The serum was collected in polyethylene tubes and stored at −70°C until assay. The concentration of cytokines, including TNF-α, IL-6, and IL-1β, in the serum was determined by double-antibody sandwich ELISA (R & D Systems, Minneapolis, MN, USA), according to the manufacturer's instructions. Recombinant rat TNF-α, mouse IL-1β, or rat IL-6 were the standards for calibration, and the detection limit of all assays was 2 pg/ml.

Statistical analyses

Data are presented as means ± S.E.M. Comparisons between groups were performed by one-way analysis of variance (ANOVA) for data in Table 1. Post-hoc comparisons were performed by Duncan's test. For the Table 2 data, Wilcoxon signed rank test was used when only two groups were compared. The Wilcoxon tests convert the scores or values of a variable to ranks, require calculation of a sum of the ranks, and provide critical values for the sum necessary to test the null hypothesis at a given significant level. The data were treated by "median", followed by first and third quartile. A P value less than 0.05 was considered as statistical significance.

Results

Table 1 summarizes the effects of heat exposure (43°C Ta) on MAP; CBF; serum concentrations of TNF-α, IL-6, and IL-1β; and striatal levels of NO in different groups of rats. As shown in the table, 15 min after the termination of heat stress in the vehicle-treated group, both the MAP and CBF values were significantly lower than in those of the pre-heat or time "0" min controls (P<0.05). On the other hand, the values of TNF-α, IL-6, and IL-1β in the serum as well as the extracellular levels of NO in the striatum of the vehicle-treated heatstroke group were all significantly higher in rats 15 min after the termination of heat exposure (or at the onset of heatstroke) than in those of the pre-heat or time "0" min controls (P<0.05). Heatstroke-induced arterial hypotension, cerebral ischemia, and increased levels of serum cytokines and cerebral NO formation were significantly attenuated by prior administration of SMS (1.2 g in 0.3 ml per rat, orally, daily, and consecutively for 7 days) or AG (30 μmol in 0.3 ml per rat, orally, daily, and consecutively for 7 days) before initiation of heat stress.

Table 2 summarizes the effects of heat exposure (43°C for 70 min) plus 15 min room temperature (24°C) exposure on iNOS immunoreactivity of the striatum from vehicle-treated, SMS-treated, or AG-treated rats. In vehicle-pretreated rats killed 15 min after the termination of 70-min heat exposure (or the onset of heatstroke), the iNOS immunoreactivity of the striatum were greater than those in the normothermic controls. However, the heatstroke-induced increase of the parameter observed

Table 1. Effects of heat exposure (43°C for 70 min) on MAP, CBF, striatal NO, and serum levels of TNF-α, IL-6, and IL-1β in rats treated with distilled water (DW, 0.3 ml), SMS (1.2 g in 0.3 ml), or AG (30 μmol in 0.3 ml) orally, daily, and consecutively for 7 days before initiation of heat exposure

Groups of animals	parameters	Time after initiation of heat exposure		
		0 min	70 min	85 min
DW-treated rats	MAP (mmHg)	93 ± 2	115 ± 4*	31 ± 3*
	CBF (BPU)	298 ± 28	457 ± 42*	176 ± 28*
	Striatal NO (μM)	2.6 ± 0.4	6 ± 1*	11 ± 2*
	Serum TNF-α (pg/ml)	2 ± 1	27 ± 2*	35 ± 3*
	Serum IL-6 (pg/ml)	79 ± 18	396 ± 64*	482 ± 89*
	Serum IL-1β (pg/ml)	47 ± 9	205 ± 48*	251 ± 71*
SMS-treated rats	MAP (mmHg)	92 ± 3	120 ± 3	87 ± 2†
	CBF (BPU)	303 ± 27	465 ± 45	434 ± 32†
	Striatal NO (μM)	2.2 ± 0.3	4 ± 1†	5 ± 1†
	Serum TNF-α (pg/ml)	3 ± 1	5 ± 1†	12 ± 2†
	Serum IL-6 (pg/ml)	82 ± 19	126 ± 22†	186 ± 35†
	Serum IL-1β (pg/ml)	51 ± 10	78 ± 6†	110 ± 9†
AG-treated rats	MAP (mmHg)	91 ± 2	116 ± 3	80 ± 2†
	CBF (BPU)	301 ± 29	459 ± 40	364 ± 45†
	Striatal NO (μM)	2.5 ± 0.3	3 ± 1†	5 ± 2†
	Serum TNF-α (pg/ml)	3 ± 1	9 ± 2†	17 ± 3†
	Serum IL-6 (pg/ml)	81 ± 17	159 ± 37†	215 ± 37†
	Serum IL-1β (pg/ml)	49 ± 8	101 ± 39†	126 ± 8†

Data represent means ± S.E.M. of eight rats per group. For the determination of striatal NO or serum cytokines, samples were obtained 0, 70, and 85 min after the initiation of heat exposure. The heat exposure was terminated at 70 min, and the ambient temperature was restored to room temperature (24°C). *P<0.05, compared with time "0" min; $^{\dagger}P$<0.05, compared with DW-treated rats (ANOVA followed by Duncan's test).

Table 2. Effects of heat exposure (43°C for 70 min) plus 15 min room temperature (24°C) exposure on iNOS immunoreactivity of the striatum from distilled water-treated, SMS-treated, or aminoguanidine (AG)-treated rats

Treatment	iNOS immunoreactivity (0 – 3)
Normothermic controls	0 (0, 0.75)
Distilled water-treated heatstroke controls	2 (2, 2)*
SMS-treated heatstroke rats	1 (0, 1)†
AG-treated heatstroke rats	1 (0, 1)†

Values represent the median with the first and third quartile in parentheses of eight rats per group. For determination of iNOS immunoreactivity, animals were killed 70 min after termination of heat exposure plus 15 min room temperature (24°C) exposure or at the equivalent time for normothermic controls. The data were evaluated by a Wilcoxon signed test followed by the Duncan's test when appropriate. *P<0.05, significance of difference from the corresponding control values (distilled water-treated heatstroke controls, group 2).

in the striatum were greatly attenuated by SMS or AG pretreatment. A typical example for iNOS staining of the striatum of a normothermic control rat, a heat stroke rat receiving distilled water, and a heat stroke rat receiving SMS is depicted in Fig. 1.

Discussion

As mentioned in the Materials and Methods section, the material medica of SMS used in the study was purchased from Sun Ten Pharmaceutical Co., Ltd. The HPLC profile indicated that the chemical components contained at least Schizandrin and Ginsenoside. To evaluate the pharmacological efficacy of SMS, aminoguanidine (an iNOS inhibitor) was evaluated at the same time as a reference (positive control). It was found that prior administration of SMS shared with the AG almost the same pharmacological efficacy in attenuating the overproduction of inflammatory cytokines (including IL-1β, IL-6, and TNF-α) in the peripheral blood stream, arterial hypotension, cerebral ischemia, as well as the overproduction of iNOS-dependent NO in the striatum during heat stroke. An oral dose of SMS or AG administered daily and consecutively for 7 days before the

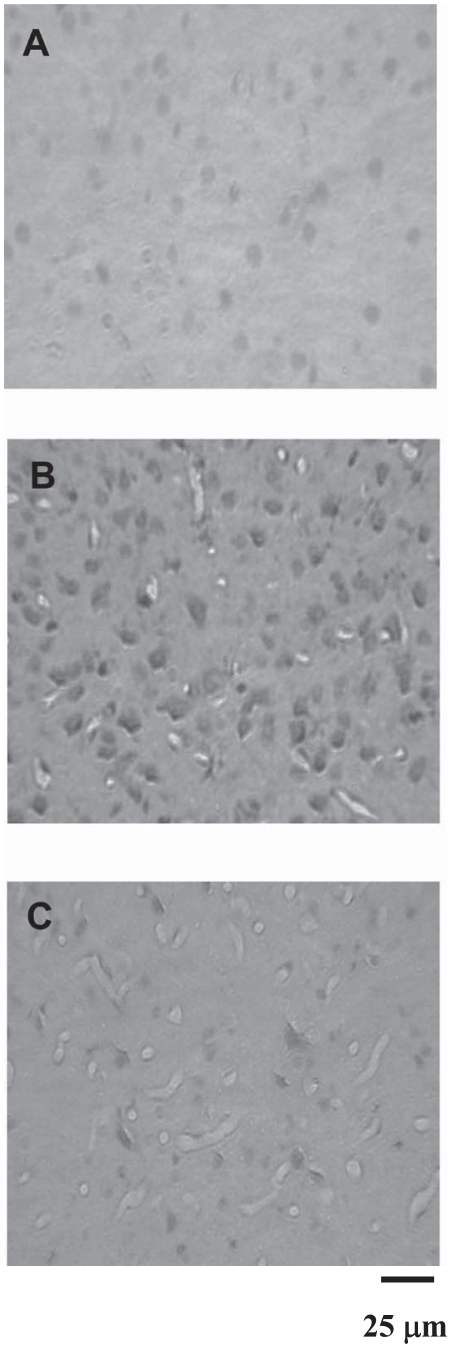

Fig. 1. Photomicrographs of iNOS staining of the striatum of a normothermic control rat (A); a heatstroke rat receiving distilled water (0.3 ml per rat orally, daily, and consecutively for 7 days before initiation of heat exposure) (B); and a heat stroke rat receiving SMS (1.2 g in 0.3 ml per rat orally, daily, and consecutively for 7 days before initiation of heat exposure) (C). Bar, 25 μm.

initiation of heat stress significantly reduced the overproduction of iNOS-dependent NO and inflammatory cytokines, hypotension, and cerebral ischemia exhibited during heat stroke. The results are in part consistent with several previous results. For example, it has been shown that an endotoxin given systemically induces an increase of tissue cytokines and iNOS-dependent NO production in the brain and lead to arterial hypotension (20). The plasma levels of both TNF-α and IL-1β are greatly elevated during heat stroke in both rats and rabbits (5, 7, 15, 16, 21). The increased levels of these inflammatory cytokines in the peripheral blood stream are associated with the heat stroke-induced arterial hypotension and cerebral ischemia and damage (5, 15; present results). Furthermore, prior administration of IL-1β-receptor antagonists (14) significantly protects against arterial hypotension and cerebral ischemia and damage during heat stroke.

In fact, iNOS can be expressed in most tissues including neurons and astrocytes, under the pathological condition (22). Our previous (3) and present results have demonstrated that inhibition of the iNOS-dependent NO formation in the brain with aminoguanidine (an iNOS inhibitor) or SMS alleviates arterial hypotension as well as cerebral ischemia exhibited during the onset of heatstroke by reducing production of inflammatory cytokines. In a recent report (23), the appearance of reactive glial cells in the brain was observed after the onset of heat stroke as measured by the increased number of GFAP (glial fibrillary acidic protein)-reactive cells. Prior administration of SMS or aminoguanidine might reduce gliosis and NO release in rat brain associated with heatstroke. It has been shown that endotoxin can induce expression of iNOS mRNA and protein in the brain (24). Glial cells may contribute to iNOS expression under inflammatory conditions (25). The iNOS-dependent NO formation in the brain can be antagonized by pretreatment with aminoguanidine (26). The production of NO by iNOS may signal the formation of peroxynitrite; and subsequently, hydroxyl radical formation can damage lipids, proteins, and DNA and lead to cell death (27). As shown in our previous results, indeed, both circulatory shock and cerebral ischemia during heatstroke are associated with increased production of free radicals (specifically, hydroxyl radicals and superoxide), increased lipid peroxidation, and decreased enzymatic anti-oxidant defences in the brain (17, 28). Preatment with magnolol (29), α-tocopherol, mannitol (21), or SMS (11) significantly attenuated arterial hypotension, cerebral ischemia, neuronal damage, and the increased free radical formation and/or lipid peroxidation in the brain.

It has been shown that heatstroke triggers the increase in extracellular glutamate (3), IL-1, and TNF-α (30) in brain. The formation of reactive oxygen species may be triggered by glutamate, IL-1, TNF, or NO as aforementioned. Hall and colleagues (2) have stated that hyperthermia stimulates xanthine oxidase formation of

reactive oxygen species that activate metals and limit heat tolerance by promoting circulatory dysfunction. Overproduction of NO may contribute to the splanchnic vasodilation that precedes vascular collapse (2) or cerebral ischemia (3, present results) during heatstroke. Accordingly, in the present results, SMS pretreatment may attenuate the excessive accumulation of inflammatory cytokines as well as reactive nitrogen and oxygen species in the peripheral blood stream and/or several brain structures and result in attenuation of arterial hypotension as well as cerebral ischemia and damage during heatstroke.

Another line of evidence has accumulated to suggest that overproduction of nitric oxide may contribute to the hypotensive states associated with circulatory shock models of different etiologies, including endotoxemia, septicemia, and hemorrhage (31 – 33). It is, therefore, plausible that administration of SMS at any time may be beneficial in either preventing or reversing circulatory shock.

The data shown in the present study indicate that SMS could be a potential herbal medicine for preventing overproduction of both iNOS-dependent NO and inflammatory cytokines during heat stroke. As described in the aforementioned section, many biochemical and physiological processes are involved in the pathogenesis of heat stroke. SMS is an attractive formula for preventing heatstroke because it consists of three herbal constituents having different physiological and pharmacological functions (10).

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