Antinociceptive Mechanism of *Gosha-jinki-gan* in Streptozotocin-Induced Diabetic Animals: Role of Nitric Oxide in the Periphery

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ABSTRACT—Using streptozotocin-induced diabetic mice and rats, we evaluated the antinociceptive mechanism of *Gosha-jinki-gan*. The antinociceptive effect of *Gosha-jinki-gan* (0.3 g/kg, p.o.) in diabetic mice, as determined by the tail-pressure test, was inhibited by N^G-nitro-L-arginine methyl ester (L-NAME; 2, 5 mg/kg, i.p.). When L-NAME (10 μg) or methylene blue (500 μg) was topically administered to the intraplantar area of the hind paw, the region used for the paw-pressure test, the antinociceptive activity of *Gosha-jinki-gan* (0.3 g/kg, p.o.) in diabetic rats was decreased. These results suggested that the antinociceptive effect of *Gosha-jinki-gan* partly resulted from the peripheral action of increasingly produced nitric oxide.

Keywords: *Gosha-jinki-gan*, Antinociception, Nitric oxide

It was reported that *Gosha-jinki-gan*, a *Kampo* medicine, is especially useful for reducing subjective symptoms in diabetic patients (1). In our previous report, we suggested that the increased antinociceptive effect of *Gosha-jinki-gan* observed in streptozotocin (STZ)-induced diabetic mice was partly due to stimulation of spinal κ-opioid receptors via dynorphin release since the effect of *Gosha-jinki-gan* was inhibited by intrathecal (i.t.) injection of anti-dynorphin A (1–13) antiserum or s.c. injected nor-binaltorphimine, a κ-opioid antagonist (2). However, the antinociceptive activity of *Gosha-jinki-gan* persisted after these treatments. Thus, the mechanism of *Gosha-jinki-gan*-induced antinociception, which could not be explained by dynorphin release, remained to be clarified by further studies.

We previously reported that *Gosha-jinki-gan* showed vasodilatory and anti-platelet aggregatory effects in diabetic rats via increased production of nitric oxide (NO) (3, 4). It was suggested that NO induces peripheral antinociception (5, 6). Therefore, in the present study, we evaluated the involvement of NO in the antinociceptive effect of *Gosha-jinki-gan*. Furthermore, we also evaluated the antinociceptive effects of aqueous extracts of *Alismatis* rhizoma and *Dioscoreae* rhizoma, crude drugs considered to be involved in increased NO production after *Gosha-jinki-gan* administration (4).

In the present study, we used male ddY mice (Japan SLC, Shizuoka) initially weighing 20–25 g and Sprague-Dawley rats (Charles River Japan, Kanagawa) initially weighing 140–190 g. Animals had free access to solid food and water in the animal room, which was maintained at 22±2℃ with a 12-hr light/dark cycle. Evaluation using diabetic mice was initiated 2 weeks after i.v. administration of STZ (150 mg/kg), which was dissolved in 33.3 mM citrate buffer solution (pH 4.5). Diabetes was induced in rats by i.v. administration of STZ (60 mg/kg), and then the experiment was performed 4 weeks thereafter. Age-matched control animals were administered vehicle alone. Animals with blood glucose levels above 400 mg/dl were considered diabetic.

Antinociceptive responses were evaluated by a tail-pressure test in mice or a paw-pressure test in rats using an analgesimeter (Ugo-Basile, Milan, Italy). In the tail-pressure test, the tail of the mouse was pressed at 1–1.5 cm from the root using a 1.5-mm-thick plate with a loading rate of 16 g/sec. In the paw-pressure test, the right hind paw of rat was subjected to pressure using a stylus (tip diameter of 1.5 mm, loading rate of 32 g/sec). The weight (g) at which animals withdrew or struggled was considered the nociceptive threshold. The results were expressed as means±S.E.M. Statistical analysis was performed by one way analysis of variance (ANOVA)

followed by Tukey's test. In all cases, $P<0.05$ was considered significant.

Gosha-jinki-gan is composed of 10 crude drugs in fixed proportions: *Rehmanniae* radix 5.0 g, *Achyranthis* radix 3.0 g, *Corni* fructus 3.0 g, *Moutan* cortex 3.0 g, *Alismatis* rhizoma 3.0 g, *Dioscoreae* rhizoma 3.0 g, *Plantaginis* semen 3.0 g, *Hoelen* 3.0 g, processed *Aconiti* tuber 1.0 g and *Cinnamomi* cortex 1.0 g. The drug was prepared as a spray-dried powder from a hot-water extract and obtained from Tsumura (Tokyo). Powdered extracts of *Alismatis* rhizoma and *Dioscoreae* rhizoma were prepared as follows: A twenty-fold volume of water was added to each crude drug, and then the solution was boiled down to half the previous volume. Subsequently, the condensed solution was centrifuged at $1,500\times g$ for 5 min, and the supernatant was freeze-dried. Doses of each crude drug extract were established as the equivalent of 1.0 g/kg of *Gosha-jinki-gan* based on the weight-ratio of each crude component in *Gosha-jinki-gan* and the yield of each powder extracted. These drugs were administered p.o. after suspending in distilled water. N^G-Nitro-L-arginine methyl ester (L-NAME) was purchased from Research Biochemicals International (RBI, Natick, MA, USA). Methylene blue trihydrate and L-arginine were purchased from Sigma Chemical (St. Louis, MO, USA). According to the method described by Hylden and Wilcox (7), anti-dynorphin A (1-13) antiserum (Peninsula Laboratories, Belmont, CA, USA) was dissolved in saline and injected intrathecally. As with anti-dynorphin antiserum, 5 μl of non-immunized rabbit serum (Chemicon International, Temecula, CA, USA), which was used as the control for anti-dynorphin antiserum, was injected intrathecally.

In diabetic mice, *Gosha-jinki-gan* (0.3 g/kg, p.o.) showed an antinociceptive effect that lasted for 180 min after administration. L-NAME (2, 5 mg/kg, i.p.), a NO synthase inhibitor, dose-dependently inhibited the antinociceptive effect of *Gosha-jinki-gan* in diabetic mice (Fig. 1A). However, the antinociceptive activity of *Gosha-jinki-gan* observed in non-diabetic mice was not significantly influenced by L-NAME (5 mg/kg, i.p.) administration (data not shown). *Gosha-jinki-gan*-induced antinociception, which persisted in diabetic mice after administering L-NAME (5 mg/kg, i.p.) or anti-dynorphin A (1-13) antiserum (5 μg, i.t.), was eliminated by the concomitant use of these two agents (Fig. 1B). *Gosha-jinki-gan* (0.3 g/kg, p.o.) showed a more potent antinociceptive effect in diabetic rats with a decreased nociceptive threshold (Fig. 2B) compared to that in non-diabetic rats (Fig. 2A). The antinociceptive effect of *Gosha-jinki-gan* observed in non-diabetic rats was not influenced L-NAME (10 μg), which was injected into the

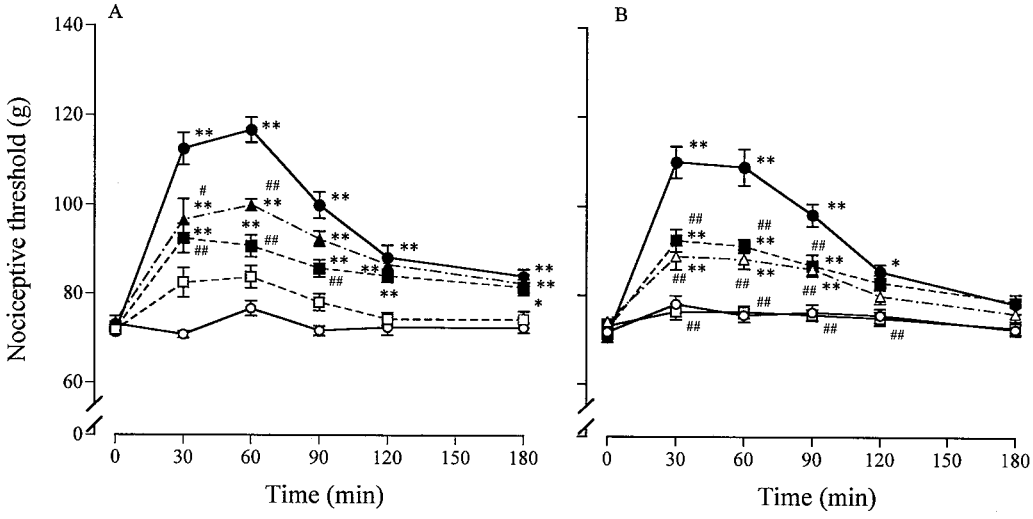

Fig. 1. Effects of L-NAME and anti-dynorphin A (1-13) antiserum on *Gosha-jinki-gan*-induced antinociception in diabetic mice. The nociceptive threshold was determined by the tail-pressure test. L-NAME or saline was i.p. injected 30 min before *Gosha-jinki-gan* administration. Anti-dynorphin A (1-13) antiserum or non-immunized rabbit serum was i.t. injected immediately after *Gosha-jinki-gan* administration. A: ○, Distilled water (10 ml/kg, p.o.)+saline (10 ml/kg); □, Distilled water + L-NAME (5 mg/kg); ●, *Gosha-jinki-gan* (0.3 g/kg, p.o.)+saline; ▲, *Gosha-jinki-gan* + L-NAME (2 mg/kg); ■, *Gosha-jinki-gan* + L-NAME (5 mg/kg). B: ○, Distilled water + saline + anti-dynorphin A (1-13) antiserum (5 μg); ●, *Gosha-jinki-gan* + saline + non-immunized rabbit serum (5 μl); ■, *Gosha-jinki-gan* + L-NAME (5 mg/kg)+ non-immunized rabbit serum; △, *Gosha-jinki-gan* + anti-dynorphin A (1-13) antiserum; □, *Gosha-jinki-gan* + L-NAME + anti-dynorphin A (1-13) antiserum. Each point and vertical bar represents the mean±S.E.M. of 6 to 7 animals. $^*P<0.05$, $^{**}P<0.01$, compared with the value of distilled water plus saline-treated animals (○) (Tukey's test). $^{\#}P<0.05$, $^{\#\#}P<0.01$, compared with the value of *Gosha-jinki-gan* plus saline-treated animals (●) (Tukey's test).

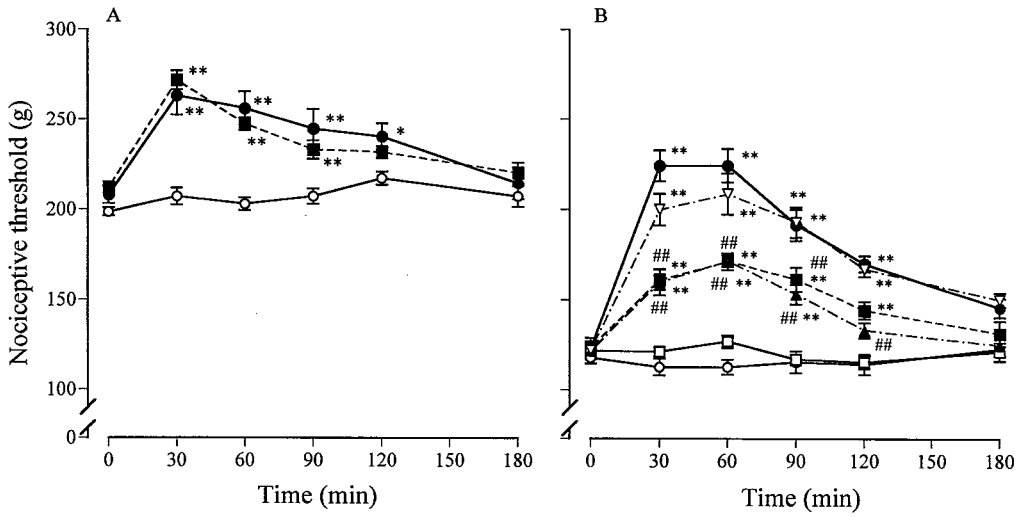

Fig. 2. Antinociceptive effects of *Gosha-jinki-gan* in non-diabetic (A) and diabetic (B) rats and effects of L-NAME or methylene blue on *Gosha-jinki-gan*-induced antinociception. The nociceptive threshold was determined by the paw-pressure test. L-NAME or saline was i.pl. injected immediately after *Gosha-jinki-gan* administration. Methylene blue was i.pl. injected 1 hr before *Gosha-jinki-gan* adminisration. ○, Distilled water (10 ml/kg, p.o.)+ saline (50 μl/paw); ●, *Gosha-jinki-gan* (0.3 g/kg, p.o.)+ saline; □ Distilled water + L-NAME (10 μg/paw); ■, *Gosha-jinki-gan* + L-NAME; ▲, *Gosha-jinki-gan* + methylene blue (500 μg/paw); ▽, *Gosha-jinki-gan* + L-NAME + L-arginine (500 μg/paw). Each point and vertical bar represents the mean±S.E.M. of 7 animals. $^{*}P<0.05$, $^{**}P<0.01$, compared with the value of distilled water plus saline-treated animals (○) (Tukey's test). $^{\#\#}P<0.01$, compared with the value of *Gosha-jinki-gan* plus saline-treated animals (●) (Tukey's test).

intraplantar (i.pl.) of the right hind paw, the region pressed during the paw pressure test (Fig. 2A). However, the antinociceptive effect of *Gosha-jinki-gan* in diabetic rats was reduced by administration of L-NAME (10 μg, i.pl.) or methylene blue (500 μg, i.pl.), a guanylate cyclase inhibitor. The *Gosha-jinki-gan*-induced antinociception, which was inhibited by L-NAME, was recovered by combination with L-arginine (100 μg, i.pl.) (Fig. 2B). The same dose of L-NAME injected s.c. into the rostral back of diabetic rats did not influence the antinociceptive

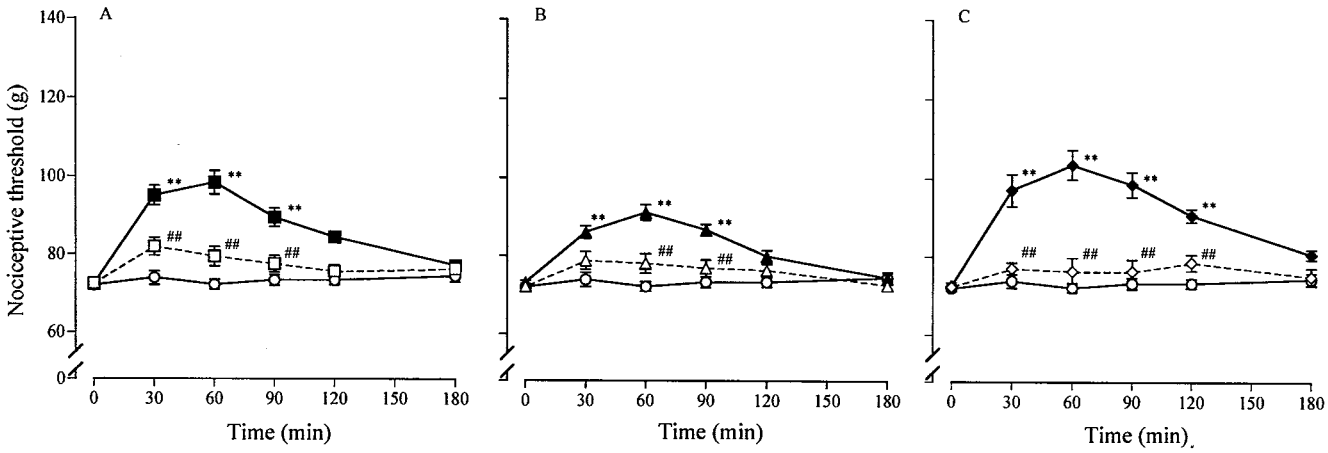

Fig. 3. Antinociceptive effects of aqueous extracts of *Alismatis* rhizoma (A), *Dioscoreae* rhizoma (B) and mixture of these two crude drugs (C) in diabetic mice. The nociceptive threshold was determined by the tail-pressure test. L-NAME or saline was i.p. injected 30 min before administration of each crude drug. ○, Distilled water (10 ml/kg, p.o.)+ saline (10 ml/kg); ■, *Alismatis* rhizoma (80 mg/kg, p.o.)+ saline; □, *Alismatis* rhizoma + L-NAME (5 mg/kg); ▲, *Dioscoreae* rhizoma (60 mg/kg, p.o.)+ saline; △, *Dioscoreae* rhizoma + L-NAME; ◆, *Alismatis* rhizoma + *Dioscoreae* rhizoma + saline; ◇, *Alismatis* rhizoma + *Dioscoreae* rhizoma + L-NAME. Each point and vertical bar represents the mean±S.E.M. of 8 to 9 animals. $^{**}P<0.01$, compared with the value of distilled water plus saline-treated animals (○) (Tukey's test). $^{\#\#}P<0.01$, compared with the value of extract(s) plus saline-treated animals (■, ▲, ◆) (Tukey's test).

activity of *Gosha-jinki-gan* (data not shown). Extracts of both *Alismatis* rhizoma (80 mg/kg, p.o.) and *Dioscoreae* rhizoma (60 mg/kg, p.o.), crude drugs which are components of *Gosha-jinki-gan*, significantly increased the nociceptive threshold of diabetic mice, but the antinociceptive effects of these two crude drugs were inhibited by pretreatment with L-NAME (5 mg/kg, i.p.) (Fig. 3: A and B). The antinociceptive activity of *Alismatis* rhizoma extract tended to increase by the concomitant administration of *Dioscoreae* rhizoma extract (Fig. 3C).

The present study indicated that the antinociceptive effect of *Gosha-jinki-gan* was inhibited by L-NAME administration in diabetic mice. This finding suggested that *Gosha-jinki-gan*-induced antinociception was partly due to NO. It was reported that systemically administered L-NAME shows an antinociceptive effect by itself (8). Therefore, in this study, the dose of L-NAME was established at 2 or 5 mg/kg, which did not show any antinociceptive effect. Our previous study suggested that the processed *Aconiti* tuber included in *Gosha-jinki-gan* was responsible for the antinociceptive effect of *Gosha-jinki-gan* induced via dynorphin release in the spinal cord. However, processed *Aconiti* tuber accounts for approximately half (40−60%) of the antinociceptive effect of *Gosha-jinki-gan* in diabetic mice (2). The results of the present study suggested that the antinociceptive activity of *Gosha-jinki-gan* that persisted after anti-dynorphin antiserum administration was due to increased NO production. This observation supported the possibility of *Gosha-jinki-gan*-induced antinociception based on two independent mechanisms including increased NO production and the release of dynorphin, an endogenous κ-opioid ligand, resulting from the action of processed *Aconiti* tuber.

Processed *Aconiti* tuber was not involved in the action of *Gosha-jinki-gan* related to NO production (3, 4). Among 10 crude drugs comprising *Gosha-jinki-gan*, only *Alismatis* rhizoma and *Dioscoreae* rhizoma showed an anti-platelet aggregatory effect in diabetic rats as shown by administering *Gosha-jinki-gan*. This effect was eliminated when administered in combination with L-NAME. Based on these observations, we suggested that *Alismatis* rhizoma and *Dioscoreae* rhizoma played major roles in increasing NO production after *Gosha-jinki-gan* administration (4). In the present study, extracts of *Alismatis* rhizoma and *Dioscoreae* rhizoma, which were equivalent to 1.0 g/kg of *Gosha-jinki-gan*, showed an antinociceptive effect. However, this effect was antagonized by L-NAME. Furthermore, a mixture of *Alismatis* rhizoma extract and *Dioscoreae* rhizoma extract showed an antinociceptive effect thought to explain the antinociceptive activity of *Gosha-jinki-gan* based on increased NO production. Thus, it was suggested that most of the *Gosha-jinki-gan*-induced antinociception related to NO production was caused by these two crude drugs.

The present results obtained in diabetic rats suggested the possibility of peripheral expression of *Gosha-jinki-gan*-induced antinociception resulting from increased NO production. The possibility of peripheral NO-induced antinociception was demonstrated in several previous reports. It was reported that when acetylcholine or L-arginine was injected i.pl. in rats, in which hyperalgesia was induced by carrageenin, antinociception was observed. However, this effect was inhibited by an NO synthase inhibitor or methylene blue (5, 6). Moreover, when non-enzymatic NO donors such as sodium nitroprusside and 3-morpholino-sydnonimine were topically administered to hyperalgesic rats, the development of antinociception was observed. This effect was inhibited by methylene blue (5, 9). These observations suggested that NO increased the cGMP level, thus preventing sensitization of the nociceptors. The mechanism of controlling the sensitivity of the nociceptors by NO remains to be clarified. However, Duarte et al. (10) indicated that Ca^{2+}-dependent reactions, which were based on changes in a balance between the levels of cGMP and cAMP, might play a key role. At the same time, it was suggested that the NO-cGMP system played a promotive or positive role in supraspinal and spinal transmission of nociceptive information (11, 12). We observed that i.t. administration of L-NAME (3 μg) at a dose that would not influence the nociceptive threshold increased the antinociceptive effect of *Gosha-jinki-gan* in diabetic mice (Y. Suzuki et al., unpublished data). This observation suggested that the increased NO production by *Gosha-jinki-gan* induced enhancement of pain perception at least at the spinal level. It was speculated that supraspinal and spinal level of hyperalgesia induction by NO, which increased after *Gosha-jinki-gan* administration, was thought to be canceled by the effect of NO to decrease the sensitivity of nociceptors.

The antinociceptive effect of *Gosha-jinki-gan* observed in non-diabetic animals was not influenced by L-NAME administration. This result suggested that NO-related antinociceptive activity of *Gosha-jinki-gan* was specifically increased under diabetic conditions. We observed that i.pl. administration of sodium nitroprusside showed a more potent antinociceptive effect in diabetic rats than in non-diabetic rats (Y. Suzuki et al., unpublished data). Conversely, this observation suggested that the peripheral expression of antinociceptive effect was less intense in non-diabetic animals even though NO production was increased. Therefore, it was considered that the role of NO in *Gosha-jinki-gan*-induced antinociception was reduced in non-diabetic animals, since *Gosha-jinki-gan* mainly expressed the antinociceptive effect based on the stimulation

of spinal κ-opioid receptors via dynorphin release. Under diabetic conditions, there are many factors that inhibit NO production or accelerate NO elimination such as activation of aldose reductase and increase in advanced glycosylation endproducts (13, 14). It was thought that decreased NO activity might influence deterioration of sciatic nerve conduction velocity, decrease nerve (Na^+, K^+)-ATPase activity and interfere with endothelium-dependent relaxation, all of which have been observed in diabetic rats (13, 15). Deficiency of NO under diabetic conditions may partly explain the decreased nociceptive threshold in diabetic animals and increased antinociceptive effect of *Gosha-jinki-gan* produced via NO production in diabetic animals.

In conclusion, it was suggested that the increased antinociceptive effect of *Gosha-jinki-gan* in diabetic animals partly resulted from the peripheral action of NO, the production of which was increased by the action of *Alismatis* rhizoma and *Dioscoreae* rhizoma. Further evaluations are necessary to clarify which components contained in *Alismatis* rhizoma and *Dioscoreae* rhizoma accelerate NO production.

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