Isolation of a renal function-facilitating constituent from the Oriental drug, Salviae Miltiorrhizae Radix

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Key words: renal failure, renal function, Salviae Miltiorrhizae Radix, magnesium lithospermate B

Abstract

An attempt was made to isolate the active component which exhibits an improving effect on renal function from Salviae Miltiorrhizae Radix (Chinese crude drug). Systematic isolation from aqueous extract of Salviae Miltiorrhizae Radix was carried out, and Compound 1 was found to be more effective than any of the other constituents in improving renal functional parameters; that is, a marked reduction of glomerular filtration rate following adenine ingestion was improved by administration of this substance. The renal plasma flow and renal blood flow were also increased in renal failure rats. On the basis of chemical and spectroscopic data, Compound 1 was shown to be identical with magnesium lithospermate B.

Introduction

The prognosis of chronic renal failure has been markedly improved due to the establishment of hemodialysis therapy and advances in the technology of medical care. However, continuation of maintenance dialysis places a great burden on the patient from both the mental and physical standpoints, and social problems including financial issues have arisen because of the increase of dialysis patients. Under these circumstances, various conservative therapies are available for chronic renal failure, such as a low protein-high calorie diet, essential amino acid therapy, and administration of activated charcoal or lactulose [1–3]. We have been studying the actions of crude drugs in rats with experimental renal failure for several years, as a part of our research on drug therapy. Through such studies, we have shown that administration of Salviae Miltiorrhizae Radix (a traditional Chinese medicinal herb known as “Dan Shen”) can improve uremic symptoms significantly by decreasing the serum levels of urea nitrogen, creatinine, methylguanidine and guanidinosuccinic acid, and by improving hyperphosphatemia as well as the pattern of free amino acids in the blood [4, 5]. Salviae Miltiorrhizae Radix was also found to improve the renal excretory functions, exhibiting a characteristic effect which was not observed with other medicinal herbs such as Rhei Rhizoma (Da Huang) or the prescription of Chinese medicine, Onpi-tō (Wen Pi Tang) [6, 7].

In China, Salviae Miltiorrhizae Radix has been widely employed in clinics by experience, to relieve pain due to coronary insufficiency, to eliminate blood stasis and to facilitate vasodilation. In addition, intravenous drip of this drug has recently been used in the treatment of uremic patients with chronic renal failure [8]. In the present study, Salviae Miltiorrhizae Radix extract was purified in order to elucidate its active constituents, taking parameters of the renal function as indices, and a new compound, magnesium lithospermate B, was isolated.

Materials and Methods

Animals and treatments

Male rats of the LWH: Wistar strain with a
body weight of 200-210 g, were placed in metabolic cages and kept at a temperature of 23 ± 1°C under a 12-h dark-light cycle. They were allowed an adaptation period of several days and were fed on commercial feed (type CE-2, CLEA Japan Inc., Tokyo, Japan) during the adaptation period. They were then fed ad libitum on an 18% casein diet containing 0.75% adenine to produce experimental renal failure. In rats with adenine-induced renal failure, the renal impairment is known to become aggravated as the period of adenine feeding increases. It was confirmed previously by histological and biochemical procedures that renal failure was present after 6 days of ingestion [9-15]. The administration of the adenine diet to the rats for 6 days was followed by the administration of a fraction or pure constituent obtained from Salviae Miltiorrhizae Radix. These samples were dissolved in saline and given intraperitoneally to the rats. The dose of each fraction was decided on the basis of its yield from Salviae Miltiorrhizae Radix. Pure constituents were administered at the same molar dose. Control rats were treated with an equal volume of saline. The levels of various serum constituents in the rats used in this experiment were as follows. In rats fed on an adenine diet for 6 days, the urea nitrogen value was 2.4 times (38.3 ± 0.6 mg/dl) that of normal rats, while the creatinine value reached a significantly increased level of 1.5 times (1.35 ± 0.02 mg/dl) that of normal rats. Six rats were employed in each experimental group. Values are expressed as the means ± S.E.

Purification of active components from Salviae Miltiorrhizae Radix

Roots of Salviae Miltiorrhizae Radix (Salvia Miltiorrhiza BUNGE) grown in China, and supplied by Tochimoto Tenkaido Co., Ltd., Osaka, Japan, were finely powdered and successively extracted with water (fraction A) and acetone (fraction B) according to Chart 1. Fraction A was chromatographed on MCI-gel CHP-20P (7.5 cm i.d. × 35 cm) and the column was eluted successively with water (fraction I), 50% aqueous MeOH (fraction II) and a mixture of

**Chart 1.** Extraction and separation of active components from Salviae Miltiorrhizae Radix.

Salviae Miltiorrhizae Radix (1 kg)

extd. with H$_2$O

H$_2$O ext. (450 g)
(Fraction A)

extd. with acetone

Acetone ext. (43 g)
(Fraction B)

MCI-gel CHP-20P eluted with H$_2$O-MeOH-acetone

H$_2$O eluate (340 g)
(Fraction I)

50% MeOH eluate (62 g)
(Fraction II)

MeOH-acetone eluate (4 g)
(Fraction III)

MCI-gel CHP-20P eluted with H$_2$O-EtOH

H$_2$O-soluble
(Fraction II-1)

EtOH-soluble
(Fraction II-2)

Sephadex LH-20 eluted with H$_2$O

(Compound 2) (2.36 g)

Sephadex LH-20 eluted with n-PrOH

(Compound 1) (9.19 g)

(Compound 3) (0.09 g)

(Compound 1) (1.86 g)

(Compound 4) (0.03 g)
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MeOH-acetone (fraction III). Fraction II was then chromatographed again on MC1-gel CHP-20P using a water-EtOH mixture as the eluent to afford fractions II-1 and II-2. Fraction II-1 was further subjected to Sephadex LH-20 chromatography with water to afford Compounds 1 and 2. Fraction II-2 was column-chromatographed on Sephadex LH-20, eluting with n-PrOH, to give Compounds 1, 3 and 4.

Examination of renal functions

The glomerular filtration rate (GFR), renal plasma flow (RPF), hematocrit value (Ht) and renal blood flow (RBF) were measured, at 5.5 to 6.0 h after intraperitoneal administration of the fraction or constituent purified from Salviae Miltiorrhizae Radix. GFR and RPF were estimated by means of the renal clearance test using a single intravenous administration of sodium thiosulfate or sodium para-aminobipiperurate, respectively, as an indicator [16, 17]. At 25 min after intravenous administration of the sodium thiosulfate or sodium para-aminobipiperurate, the bladder was allowed to empty by reflex action by making each rat inhale ether for 3–5 sec. The urine thus voided was discarded. During the next 30 min, the urine was collected, and the collection was terminated after further reflex emptying of the bladder through ether inhalation. Blood samples were taken from the conscious rats by heart puncture in the middle of the period allocated for the clearance test. Thiosulfate and para-aminobipiperurate were determined by titrimetry and colorimetry, respectively. RBF was calculated on the basis of RPF and Ht using the equation shown below. Ht was determined with a hematocrit measurement apparatus, model KH-120A (Kubota Co., Tokyo, Japan).

\[
RBF = \frac{RPF}{1 - Ht} \text{ (ml/min)}
\]

Statistics

All data were analyzed statistically by Student’s t-test.

Results

Effect of various fractions from Salviae Miltiorrhizae Radix on renal function

As shown in Table 1, intraperitoneal administration of fraction A induced a significant increase in GFR (a 23% change), whereas fraction B induced an increase of 16% (no significant difference). Fractions I, II and III separated further from fraction A increased the GFR by 18%, 31% and 15%, respectively, but only fraction II reached the statistically significant range when compared with the control value. The administration of fraction A also caused a significant increase in RPF (a 26% change), while fraction B increased the

<table>
<thead>
<tr>
<th>Exp. No.</th>
<th>Material</th>
<th>Dose (mg/kg)</th>
<th>GFR (ml/min/kg)</th>
<th>RPF (ml/min/kg)</th>
<th>RBF (ml/min/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control</td>
<td>—</td>
<td>3.56 ± 0.51</td>
<td>16.11 ± 1.33</td>
<td>26.86 ± 2.21</td>
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<tr>
<td></td>
<td></td>
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<td>(100)</td>
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<td>(100)</td>
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<tr>
<td></td>
<td>Fraction A</td>
<td>100</td>
<td>4.38 ± 0.15*</td>
<td>20.28 ± 1.66*</td>
<td>33.82 ± 2.84*</td>
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<td></td>
<td>(123)</td>
<td>(126)</td>
<td>(126)</td>
</tr>
<tr>
<td></td>
<td>Fraction B</td>
<td>25</td>
<td>4.13 ± 0.40</td>
<td>18.65 ± 1.18</td>
<td>31.10 ± 1.99</td>
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<tr>
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<td></td>
<td>(116)</td>
<td>(116)</td>
<td>(116)</td>
</tr>
<tr>
<td>2</td>
<td>Control</td>
<td>—</td>
<td>3.36 ± 0.31</td>
<td>19.57 ± 2.08</td>
<td>32.43 ± 3.45</td>
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<td>(100)</td>
<td>(100)</td>
</tr>
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<td></td>
<td>Fraction I</td>
<td>75</td>
<td>3.97 ± 0.39</td>
<td>24.75 ± 3.67</td>
<td>41.25 ± 6.18</td>
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<tr>
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<td></td>
<td>(118)</td>
<td>(126)</td>
<td>(127)</td>
</tr>
<tr>
<td></td>
<td>Fraction II</td>
<td>40</td>
<td>4.41 ± 0.26*</td>
<td>27.57 ± 1.86*</td>
<td>46.20 ± 3.18*</td>
</tr>
<tr>
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<td></td>
<td></td>
<td>(131)</td>
<td>(141)</td>
<td>(142)</td>
</tr>
<tr>
<td></td>
<td>Fraction III</td>
<td>20</td>
<td>3.85 ± 0.61</td>
<td>23.67 ± 4.24</td>
<td>38.57 ± 6.94</td>
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<td></td>
<td></td>
<td></td>
<td>(115)</td>
<td>(121)</td>
<td>(119)</td>
</tr>
</tbody>
</table>

Table 1. Effect of various fractions from Salviae Miltiorrhizae Radix on renal function parameters. GFR, glomerular filtration rate; RPF, renal plasma flow; RBF, renal blood flow. Figures in parentheses indicate percentages of the control value. *Significantly different from the control value, p<0.05.
RPF by 16%. Fraction II significantly increased the RPF by 41%, and fractions I and III produced increases of 26% and 21%, respectively; these variations were not statistically significant. Ht remained unchanged following treatment with the various fractions as compared with each respective control. Thus, RBF almost paralleled RPF; RBF was significantly increased by 26% in the fraction A-treated group, and was increased by 16% in the fraction B-treated group. Fraction II caused a significant increase in RBF (a 42% change), while fractions I and III produced increases of 27% and 19%, respectively.

**Effect of various constituents from fraction II on renal function**

As shown in Table 2, administration of Compounds 1 and 2 caused a significant elevation of GFR (a 43% change), and administration of Compounds 3 and 4 increased the GFR by 22%, respectively. Compound 1 also significantly elevated the RPF by 36%, and Compounds 2, 3 and 4 increased the RPF by 25%, 21% and 16%, respectively. RBF paralleled RPF while Ht showed no changes; RBF was increased by 37% in the Compound 1-treated group and was increased by 27%, 18% and 11% in the groups treated with Compounds 2, 3 and 4, respectively.

**Effect of Compound 1 on renal function**

The rats of the Compound 1-treated group exhibited a dose-dependent increase in GFR. As shown in Fig. 1, a single intraperitoneal administration of 5 mg Compound 1 caused a 22% increase in GFR as compared with the control rats. A further increase in the dose to 10 mg produced a further increase of 31% in the GFR value. An examination of the effect of intraperitoneal administration of Compound 1 on the RPF demonstrated significant increases at the 5 mg and 10 mg levels. As shown in Fig. 1, the RPF value was increased from 15.69 ml/min/kg to 18.90 ml/min/kg at the 5 mg level (a 20% change, \( p < 0.05 \)) and from 15.69 ml/min/kg to 20.87 ml/min/kg at the 10 mg level (a 33% change, \( p < 0.05 \)). The RBF value also revealed a direct correlation with the amount of Compound 1 administered. Intraperitoneal administration of 5 mg Compound 1 increased the RBF from 27.09 ml/min/kg to 32.54 ml/min/kg (a 20% change). Compound 1 at a dose of 10 mg produced a significant rise in RBF from 27.09 ml/min/kg to 35.34 ml/min/kg (a 30% change, \( p < 0.05 \)).

Additional experiments were carried out to clarify these renal effects further; that is, Compound 1 was administered successively at a dose of 10 mg/kg body weight once a day for 6 days (from the 6th to 11th day) to rats with renal failure induced with an adenine diet, and its effects on the renal function parameters were examined. As shown in Table 3, the GFR in control rats administered with adenine was significantly decreased by 68% of the level in normal rats. The RPF and RBF values also exhibited a significant decrease when compared with the respective normal values. In contrast, the rats given Compound 1 revealed an 82% increase in GFR as compared with the control rats. An examination of the effect of consecutive intraperitoneal administration of Compound 1 on the RPF demonstrated a significant

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**Table 2. Effect of various constituents from fraction II on renal function parameters.** Other details are as in the legend to Table 1.

<table>
<thead>
<tr>
<th>Material</th>
<th>Dose (mg/kg)</th>
<th>GFR (ml/min/kg)</th>
<th>RPF (ml/min/kg)</th>
<th>RBF (ml/min/kg)</th>
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<tbody>
<tr>
<td>Control</td>
<td>—</td>
<td>3.27 ± 0.27</td>
<td>15.23 ± 1.74</td>
<td>25.78 ± 2.83</td>
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<tr>
<td></td>
<td></td>
<td>(100)</td>
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<td>(100)</td>
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<tr>
<td>Compound 1</td>
<td>20</td>
<td>4.69 ± 0.42*</td>
<td>20.64 ± 2.22*</td>
<td>35.30 ± 3.81*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(143)</td>
<td>(136)</td>
<td>(137)</td>
</tr>
<tr>
<td>Compound 2</td>
<td>20</td>
<td>4.66 ± 0.44*</td>
<td>19.00 ± 1.26</td>
<td>32.64 ± 2.56</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(143)</td>
<td>(125)</td>
<td>(127)</td>
</tr>
<tr>
<td>Compound 3</td>
<td>10</td>
<td>3.99 ± 0.84</td>
<td>18.38 ± 2.53</td>
<td>30.50 ± 5.05</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(122)</td>
<td>(121)</td>
<td>(118)</td>
</tr>
<tr>
<td>Compound 4</td>
<td>5</td>
<td>3.99 ± 0.62</td>
<td>17.74 ± 1.65</td>
<td>28.72 ± 2.88</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(122)</td>
<td>(116)</td>
<td>(111)</td>
</tr>
</tbody>
</table>
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Fig. 1. Effect of single intraperitoneal administration of Compound 1 on renal function parameters. Other details are as in the legend to Table 1.

Table 3. Effect of Compound 1 on renal function parameters in rats treated for 6 consecutive days. Other details are as in the legend to Table 1.

<table>
<thead>
<tr>
<th>Material</th>
<th>GFR (ml/min/kg)</th>
<th>RPF (ml/min/kg)</th>
<th>RBF (ml/min/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.41 ± 0.35</td>
<td>7.44 ± 2.56</td>
<td>12.82 ± 4.58</td>
</tr>
<tr>
<td>(100)</td>
<td>(100)</td>
<td>(100)</td>
<td></td>
</tr>
<tr>
<td>Compound 1</td>
<td>2.57 ± 0.33*</td>
<td>13.14 ± 1.06*</td>
<td>22.38 ± 2.14*</td>
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<tr>
<td>(182)</td>
<td>(177)</td>
<td>(175)</td>
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</table>

Fig. 2. $^{13}$C-NMR spectra of Compound 1 (in acetone-$d_6$ + $D_2O$).

increase. As shown in Table 3, the RPF value was increased from 7.44 ml/min/kg to 13.14 ml/min/kg (a 77% change, $p<0.05$) in rats treated for 6 consecutive days. The change in RBF calculated from the RPF and Ht was almost parallel to RPF because Ht remained almost unchanged when Compound 1 was given; 6 days of Compound 1 administration resulted in a significant rise in RBF.
Characterization of Compound 1

Compound 1 was the major compound isolated in a 1.1% yield by a combination of MCI-gel CHP-20P chromatography and Sephadex LH-20 chromatography. A dark green coloration with ferric chloride reagent indicated a phenolic nature for this compound. $^{13}$C-Nuclear magnetic resonance ($^{13}$C-NMR) spectra of this compound showed signals attributable to four carboxyl carbons, four aromatic rings, a double bond and six aliphatic carbons (Fig. 2). On methylation with diazomethane, Compound 1 gave a product whose electron-impact mass spectrum (EI-MS) exhibited an M$^+$ ion peak at m/z 844. These findings suggested that Compound 1 was a tetramer of caffeic acid, being consistent with the lithospermic acid B isolated previously from an intravenous drip preparation of Salviae Miltiorrhizae Radix [18]. In addition, the fact that Compound 1 was less soluble in acetone and ethyl acetate, and that this aqueous solution was almost neutral suggested that the compound was a salt of lithospermic acid. On treatment with cation exchange resin, or with dilute acids followed by extraction with ethyl acetate, Compound 1 afforded lithospermic acid B. Negative fast atom bombardment mass spectroscopy (FAS-MS) provided useful information for assessment of the cation. In the spectra, Compound 1 revealed a peak of a much higher mass number at m/z 739. On this basis, it was deduced that Compound 1 was a sodium or magnesium salt of lithospermic acid. The cationic metal was unambiguously confirmed by energy dispersing X-ray analysis; that is, the X-ray signal specific for magnesium ion was observed in spectra of Compound 1 (Fig. 3). Furthermore, the magnesium salt prepared by treatment of lithospermic acid B with magnesium hydroxide gave an IR spectrum and negative FAB-MS identical with those of Compound 1. Thus, Compound 1 was determined as magnesium lithospermate B (Fig. 4). The detailed results will be reported elsewhere in the near future.

Discussion

As described previously [10], renal function underwent progressive deterioration as kidney damage increased due to extended administration of adenine. Intraperitoneal administration of Salviae Miltiorrhizae Radix extract produced a significant rise in GFR, RPF and RBF in rats with mild or moderately impaired renal function such as was evident on days 6 and 12, while Salviae Miltiorrhizae Radix did not exhibit any clearly beneficial effects on severely impaired renal function [6]. Results for nephrectomized rats have been reported by Li et al. [19], and their data support the findings of our studies. However, no previous report has indicated which of the components of Salviae miltiorrhizae Radix might be the effective one. The present experiments were therefore...
carried out to test the activities of various fractions and pure constituents obtained from Salviae Miltiorrhizae Radix on renal function.

We first investigated two fractions (fractions A and B) separated from Salviae Miltiorrhizae Radix in adenine-fed rats for their renal effects in order to evaluate any possible bioactivity which they might display. Intraperitoneal administration of fraction A increased the GFR, RPF and RBF. Using an MCI-gel CHP-20P column, fraction A was further separated into fractions I–III. These fractions were also tested for their effects on the renal functional parameters, and fraction II was found to exert the most pronounced effect on the GFR, RPF and RBF. Although the administered doses were different, their levels were decided on the basis of the fraction yields from Salviae Miltiorrhizae Radix. Furthermore, we separated pure constituents (Compounds 1–4) from fraction II using a Sephadex LH-20 column. When these four constituents were compared at the same molar dose (28 μM/kg body weight), Compound 1 was found to be the most effective compared to any of the other constituents on the renal function parameters (GFR, RPF and RBF). Although the administered doses were different, their levels were decided on the basis of the fraction yields from Salviae Miltiorrhizae Radix. However, the role of Compound 1 in the change from increased glomerular filtration and renal blood flow to the terminal state, glomerulosclerosis, remains to be elucidated.

This active component, tentatively named Compound 1, was identified as magnesium lithospermate B on the basis of its chemical and spectroscopic data. The constituents of Salviae Miltiorrhizae Radix so far reported include naphthoquinone (phenanthrene quinone) derivatives, such as tanshinone I, tanshinone II-A, tanshinone II-B, cryptotanshinone, isotanshinone I, isotanshinone II, isocryptotanshinone, tanshinonic acid, hydroxytanshinone and miltirone [22]. The purified substance obtained in the present experiments, magnesium lithospermate B, is a newly found constituent of Salviae Miltiorrhizae Radix. Compounds 2 to 4, which are thought to be the stereoisomer, geometric isomer of magnesium lithospermate B and caffeic acid, respectively, displayed no extensive ability to activate renal function. It has been noted previously that aqueous extract of Salviae Miltiorrhizae Radix can improve the renal function of rats with adenine-induced renal failure [6]. The present data clearly suggest that one of the active components responsible for this action is magnesium lithospermate B. The underlying mechanism of this effect remains to be elucidated. However, we have confirmed so far that similarly to the renal function parameters, administration of magnesium lithospermate B to rats with renal failure can induce an increase in urinary excretion of prostaglandin E₂, and that pretreatment with the cyclooxygenase inhibitor indomethacin eliminates the effect of magnesium lithospermate B. These observations suggest that cyclooxygenase metabolites of arachidonic acid might play an important role in the improvement of renal failure caused by treatment with magnesium lithospermate B. Also, the fact that the increase in urinary excretion of prostaglandin E₂ was associated with a significant in-
crease in GFR, RPF and RBF suggests the involvement of prostaglandins in the changes in renal hemodynamics. Further investigations along these lines are currently in progress.

Part of this work was reported at the 31st Annual Meeting of the Japanese Society of Nephrology, Nara, October 1988.

References