Effective Isolation of Magnesium Lithospermate B and Its Inhibition of Aldose Reductase and Fibronectin on Mesangial Cell Line

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We developed an effective isolation method of magnesium lithospermate B from *Salviae miltiorrhizae* Radix and found for the first time that magnesium lithospermate B shows strong *in vitro* inhibition (IC_{50}=0.04 μM) of aldose reductase (AR), 2.5 times than that of clinically used epalrestat (IC_{50}=0.1 μM) and accumulation of fibronectin dose dependantly.

Key words magnesium lithospermate B; aldose reductase; fibronectin; *Salviae miltiorrhizae* Radix; antidiabetics

Many patients die of diabetes mellitus each year and the prevalence of all types of diabetes is estimated to be 2.3% of the world's population.[1)] Although several drugs are known as antidiabetics, few drug for the treatment of diabetic nephropathy is known. In the course of our search for drugs for diabetic nephropathy, we found that salvianoric acid A, a caffeic acid derivative[2)] and several abietane-type diterpenoids,[3)] isolated from *Salviae miltiorrhiza*, were reported to have aldose reductase (AR) inhibitory activity. Magnesium lithospermate B isolated from *S. miltiorrhizae* Radix, is known to have the vasodilating and antihypertensive effect.[4)] Recent study reported that aldose reductase, the enzyme that converts glucose to sorbitol, may be involved with another signal transduction in the pathogenesis of diabetic nephropathy.[5)] Thus, the aim of this study is to investigate the inhibitory effect of magnesium lithospermate B on mouse mesangial cell line in the reference to the activity of aldose reductase and the accumulation amount of fibronectin causing the development of diabetic nephropathy.

Magnesium lithospermate B[6)] (Fig. 1) was isolated from 80% MeOH extract of *Salviae miltiorrhizae* Radix, and subsequently purified by normal silica gel column chromatography with polar-eluent (isopropylalcohol/H_2O/ethylacetate=3/1/3) in 1.01% yield. Although yield was almost the same as that of the known procedure,[7)] expensive MCI-gel and Sephadex was replaced by silica gel for chromatography. Therefore, this method is an improved one for cutting cost of isolation of magnesium lithospermate B. Acidification of magnesium lithospermate B with 0.5 N-HCl afforded lithospermic acid B[6)] in 84% yield. The sodium and barium salts of lithospermic acid B were prepared in 96% and 94% yield from lithospermic acid B by treatment of sodium and barium hydroxides, respectively.

After treatment of the magnesium lithospermate B on the mouse mesangial cell for 24 h, we evaluated the activity of aldose reductase in the cell lysate by spectrophotometry and the amount of fibronectin in the medium by western blot. The value of IC_{50} was calculated using the computer software GraphPad Prism™ 2.0.[8)]

High glucose (30 mM) media significantly increased the activity of aldose reductase, compared to normal (5.5 mM) media (control: 100±0.03%, high glucose without lithospermate B: 150.13±0.21%). There was a tendency to decreased activity of aldose reductase in accordance with the increasing dosage of magnesium lithospermate B (Fig. 2). The magnesium lithospermate B showed significant inhibition of aldose reductase activity in mouse mesangial cell (10 μg lithospermate B: 122.14±0.02%, 25 μg: 95.72±0.23%, 50 μg: 84.79±0.15%, 100 μg: 81.32±0.16%, all $p<0.05$). This result indicates magnesium lithospermate B (IC_{50}=0.04 μM) shows 2.5 times more *in vitro* inhibition of aldose reductase than that of clinically used epalrestat (IC_{50}=0.10 μM). The production of fibronectin was decreased similar pattern to the activity of aldose reductase (control: 100±0.02%, high glucose without lithospermatic acid: 136.22±0.05%, 5 μg lithospermate B: 115.65±0.09%, 10 μg: 78.66±0.13%, 40 μg: 28.17±0.18%, all $p<0.05$) (Fig. 3).

Sodium and barium lithospermates B show no differences with the magnesium lithospermate B against to aldose reductase activity and fibronectin production. The IC_{50} of lithospermic acid B on the AR was 12.16 μM, and that of lithospermic acid B on the fibronectin was 23.33 μM. This result

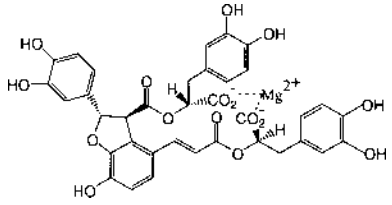

Fig. 1. The Structure of Magnesium Lithospermate B

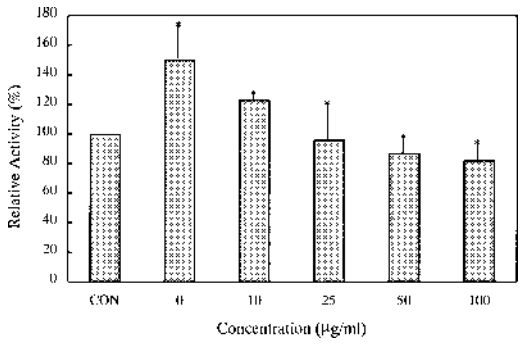

Fig. 2. Inhibitory Effect of Magnesium Lithospermate B on Aldose Reductase on the Mouse Mesangial Cell

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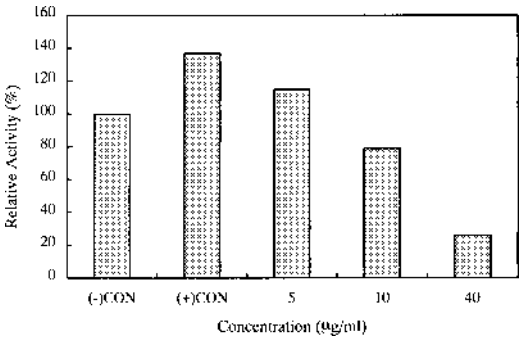

Fig. 3. Inhibitory Effect of Magnesium Lithospermate B on Fibronectin Accumulation on the Mouse Mesangial Cell

implies that alkali (Group 1) or alkaline earth (Group 2) metal salts of lithospermate B are essential for the activities and a steric conformation as shown in Fig. 1 for alkaline earth metal salts may further contribute for high activities.

In conclusion, these results for the first time indicated that magnesium lithospermate B inhibits the activity of aldose reductase 2.5 times than that of clinically used epalrestat and accumulation of fibronectin dose dependantly. Therefore, the magnesium lithospermate B can be a candidate drug for treatment of diabetic nephropathy.

Experimental

General Optical rotations were measured on a Rudolph AP III-589 polarimeter. IR spectra were measured on a Nicolet Impact 400 spectrometer. NMR spectra were recorded on a Bruker AC250 spectrometer. Chemical shifts were given as δ (ppm) with tetramethylsilane (TMS) as an internal standard. Direct mass and HRMS were obtained on a Trio 2000 (VG-Biotec) and a JMS-700 Mstation (JEOL) spectrometer in FAB mode. Column chromatography was carried out on Merck Kieselgel 60 (230—400 mesh).

Plant Material The dried and powered roots of *Salviae miltiorrhizae* Radix were obtained in Seoul, Korea in March 1998, and a voucher specimen is available in the Yonsei University.

Extraction and Isolation The dried and powdered roots of *Salviae miltiorrhizae* Radix (100 g) were mixed with 80% methanol (800 ml) and placed at room temperature for 3 d. After removal of the insolubles by gravity filtration through gauze, the methanol was removed from the liquid *in vacuo*. The water layer of the filtrate was sequentially extracted with hexane, diethyl ether, CH_2Cl_2 and ethyl acetate (300 ml×4 each). The resulting aqueous layer was lyophilized to yield a solid material (30 g). The magnesium salt of lithospermic acid was obtained from the solid by chromatography with a SiO_2 matrix employing isopropanol/H_2O/EtOAc (3 : 1 : 3, v/v/v, Rf=0.5) as an eluent. The yield of the magnesium lithospermate B as amorphous powder was 1.01 g from 100 g of *Salviae miltiorrhizae* Radix.

$[\alpha]_D^{24}$=+147.3° (c=0.7, CH_3OH): all spectral data (NMR, ^{13}C-NMR, IR, HR-MS) of magnesium lithospermate B are identical to those of the literature.[7]

Preparation of Sodium and Barium Salts of Lithospermic Acid Acidification of magnesium lithospermate B with 0.5 N-HCl afforded lithospermic acid B[6] in 84% yield. The sodium and barium salts of lithospermic acid B were prepared in 96% and 94% yield from lithospermic acid B by treatment of sodium and barium hydroxides in methanol, respectively.

Sodium Lithospermate B: IR (KBr, cm^{-1}) 3400, 1606, 1403, 1263, 1168: ^{1}H-NMR (250 MHz, acetone-d_6+D_2O) δ_H 7.52 (1H, d, J=16 Hz), 7.15 (1H, d, J=4 Hz), 6.44—6.90 (10H, m), 6.30 (1H, d, J=16 Hz), 5.62 (1H, J=4 Hz), 5.00 (2H, m), 4.49 (1H, d, J=4 Hz), 2.70—3.30 (4H, m): ^{13}C-NMR (63 MHz, acetone-d_6+H_2O) δ_C 177.3, 176.6, 172.5, 169.2, 142.8, 145.9 (×3), 145.4, 144.5, 144.3 (×2), 143.2, 133.4, 130.7, 130.5, 126.5, 124.4, 121.5 (×3), 118.0, 117.6, 117.2, 116.9, 116.5, 116.2, 116.0 (×3), 113.3, 87.2, 78.3, 78.1, 56.7, 37.7.

Barium Lithospermate B: IR (KBr, cm^{-1}) 3407, 1597, 1396, 1019, 928, 652: ^{1}H-NMR (250 MHz, acetone-d_6+D_2O) δ_H 7.53 (1H, d, J=16 Hz), 7.14 (1H, d, J=4 Hz), 6.82—6.15 (11H, m), 5.63 (1H, d, J=4 Hz), 4.87 (2H, m), 4.15 (1H, d, J=4 Hz), 2.71—3.30 (4H, m): ^{13}C-NMR (63 MHz, acetone-d_6+H_2O) δ_C 177.2, 176.6, 172.5, 169.1, 142.8, 145.8 (×3), 145.4, 144.5, 143.5 (×2), 143.3, 133.4, 130.7, 130.5, 126.6, 124.4, 121.5 (×3), 118.0, 117.6, 117.2, 116.9, 116.6, 116.2, 116.0 (×3), 113.3, 87.2, 78.3, 78.1, 56.7, 37.6.

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