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Water-Soluble Genistin Glycoside Isoflavones Up-Regulate Antioxidant Metallothionein Expression and Scavenge Free Radicals

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Genistin has antioxidant activities; however, its insolubility in water often limits its biological availability in vivo. Using a novel transglycosylation process, the solubility of genistin glycosides was increased 1000 to 10000-fold, but it was not known whether these modified genistin glycosides maintained antioxidant activity. We found that both genistin and its glycosides similarly up-regulated the transcription of several metallothionein (MT) antioxidant genes (MT1A, MT2A, MT1E, and MT1X), as well as the glucose 6-phosphate dehydrogenase (G6PD) gene in HepG2 cells. This gene induction was mediated by the sequestration of zinc in the cytosol, which up-regulated the metal-responsive transcription factor-1 (MTF-1) that induced MT gene expression. Although not as effective as ascorbic acid, genistin glycosides possessed slightly greater reducing power than genistin. We concluded that genistin and genistin glycosides have a direct antioxidant effect and an indirect antioxidant effect, perhaps via induction of MT by activity of MTF-1.

KEYWORDS: Genistin; genistin glycosides; metallothioneins; metal-responsive transcription factor-1

INTRODUCTION

Isoflavones are polyphenolic phytochemicals with strong antioxidant activities that are notably found in soybeans. Both glycone and aglycone isoflavones are effective antioxidants. One of the natural glycone isoflavones, genistin, is found in high levels in soybeans and protects against the oxidative DNA damage induced by hydroxyl radicals (*1*), has superoxide anion scavenging capacity (*1*), and reduces the oxidation of low-density lipoprotein (*2*). One disadvantage of isoflavones is a low water solubility that limits their application in the food industry and may also impede its bioavailability in aqueous systems in vivo. Thus increasing water solubility of genistin glycosides may enhance their industrial application and biological functions.

We developed a novel method for the enzymatic transglycosylation of genistin using *Thermus scotoductus* 4-α-glucanotransferase (TS4αGTase) to enhance water solubility of genistin (*3*). In the presence of genistin and soluble starch, the enzyme transfers a glucosyl unit from soluble starch to genistin and produces a series of transglycosylated genistin glycosides. The resulting genistin glycosides showed a dramatic 1000 to 10000-fold increase in water solubility. Water solubility of glucosyl- and maltosyl-α(1→4)-genistin were dramatically increased by 3.7×10^3- and 4.4×10^4-fold, respectively, compared with genistin. The increase of water solubility was proportional to the degree of glycosylation (*3*). However, no studies have examined whether genistin glycosides retain functional antioxidant activities. Therefore, we compared the antioxidant activities of genistin and its glycosides in this study.

Genistin and its glycosides may exert their antioxidant activity either directly by reducing reactive oxygen species or indirectly by activating other antioxidative proteins. Metallothioneins (MTs) are potent antioxidative proteins that bind to Zn^{2+} and Cu^{2+} and can protect cells from oxidative stress in vitro (*4*, *5*). MT gene transcription is also rapidly induced by Cd and oxididized (*6*, *7*) and by hormones (*8*).

Human hepatocyte cell line, HepG2, displays the MT induction by zinc ion (*9*), and there is evidence that the MT levels were increased in the cultured cell line treated with genistein of dietary flavonoids (*10*). Therefore, HepG2 cells were the appropriate model system for testing the effects of genistin and its glycosides on the human liver. We found that four MT isoforms (MT1A, MT2A, MT1E, and MT1X) were all significantly up-regulated by both genistin and its glycosides. In addition, their direct antioxidant activities were assessed using four different conventional methods: the superoxide dismutase

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(SOD) activity assay, 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging assay, reducing power activity assay, and deoxyribose (DR) assay.

MATERIALS AND METHODS

Chemicals. Dulbecco's minimum essential medium (DMEM), fetal bovine serum (FBS), liquid gentamicin reagent solution, penicillin and streptomycin (PEST), and trypsin-ethylenediaminetetraacetic acid (T-EDTA) were purchased from Join Bio-Innovation (Seoul, Korea). Falcon tissue culture plates were obtained from Becton Dickinson (Franklin Lakes, NJ). FluoZin-3 AM was obtained from Molecular Probes (Eugene, OR). PowerScript reverse transcriptase was obtained from Clontech (Palo Alto, CA). The oligo$(dT)_{15}$ primer and random hexamers were obtained from Promega (Madison, WI). AccuPower PCR PreMix was purchased from Bioneer (Seoul, Korea). All other reagents used were purchased from Sigma Chemical (St. Louis, MO). The isoflavone mixture was donated by Pulmuone, South Korea. HepG2 cells were obtained from the Korean Cell Line Bank (Seoul, Korea).

Preparation and Purification of Genistin and Genistin Glycosides. The cloning, bacterial overexpression, and purification of TS4αGTase were described previously (*3*). This enzyme is capable of transferring a glucose moiety from soluble starch to the OH group at the C4 position in the genistin glucose moiety. Genistin was purified from an isoflavone mixture using recycling preparative high-performance liquid chromatography (HPLC; LC-918, JAI, Tokyo, Japan) equipped with a refractive index detector (RI-50) and a polymeric gel filtration column (W-251) for HPLC. The mobile phase was 80% MeOH at a flow rate of 2 mL/min, and the injection volume was 1.5 mL. The fraction containing genistin was collected and was concentrated using a rotary vacuum evaporator. A solution containing 0.03% genistin and 2.1% soluble starch was preincubated at the optimum temperature (75 °C) for 10 min. Then, TS4αGTase (0.5 U/mL) was added. After 4 h of incubation at 70 °C, the reaction was stopped by boiling for 10 min, and the sample was filtered through a 0.45 μm pore membrane (Waters, Milford, MA). A Waters 600E HPLC system connected to a Nova-Pak C_{18} column (150 × 3.9 mm i.d., Waters), and a UV detector (SLC 200, Samsung, Seoul, Korea) operating at 245 nm was used to purify the genistin derivatives. The gradient mobile phase consisted of solvent A (water:formic acid, 100:0.1, v/v) and solvent B (MeOH:water:formic acid, 50:50:0.1, v/v/v). The gradient started at 80% solvent A with 20% solvent B, and solvent B was increased gradually from 20% to 100% over 40 min. The flow rate was 5 mL/min. The fraction containing the genistin glycosides (from G2-genistin to G6-genistin together) was collected and was concentrated using a rotary vacuum evaporator. The fraction was spotted on K5F silica gel plates (Whatman, Kent, U.K.), and the results were analyzed using the method of Li et al. (*3*) for confirmation of five transferred genistin products. These appeared as spots, and the fraction was injected into the above-HPLC under the same conditions for confirmation of the five peaks.

Cell Culture and Viability Test. HepG2 cells were seeded in 6-well Falcon plates at 1×10^6 cells/mL in DMEM supplemented with 10% FBS, 1% liquid gentamicin reagent solution, and 1% PEST. The cells were cultured at 37 °C in a humid atmosphere containing 5% CO_2 until 60−80% confluent and were then used in the reverse transcription-polymerase chain reaction (RT-PCR) or real-time RT-PCR assays. For the cell viability assay, HepG2 cells were seeded in 24-well Falcon plates at 1×10^4 cells/mL and grown for 48 h. The culture medium was replaced on alternate days, and the cells were kept in medium free of serum and antibiotics during treatment. Cell viability was measured using mitochondrial dehydrogenase activity assays with the substrate MTT [3-(4,5-dimethylthiazole-2-yl)2,5-diphenyltetrazolium bromide] according to the method of Chung et al. (*11*).

Treatments. In experiments examining the effects of genistin and the genistin glycosides on cell viability using the MTT assay, HepG2 cells were incubated in fresh DMEM with or without experimental additives. Cells were exposed to 2 mM sodium nitroprusside (SNP), which is a potent NO donor, and genistin (0, 0.05, 0.1, and 0.2 mM) or genistin glycosides (0, 0.05, 0.1, 0.25, 0.5, 1.25, 2.5 and 5.0 mM) for 24 h. They were also exposed to genistin with 2 mM SNP simultaneously for 24 h. In the cultures used in the RT-PCR assays, HepG2 cells were treated with 0.05 mM genistin in DMEM medium for 12, 24, or 48 h. The cells in 0.025−0.1 mM genistin were incubated for 24 h. The control consisted of untreated cells in the 12, 24, and 48 h groups. A stock solution of genistin was prepared in dimethyl sulfoxide (DMSO). Control cultures received DMSO alone. Genistin glycosides (0, 0.1, and 0.2 mM) were prepared directly in DMEM.

Quantification of Antioxidant Activity. *(1) Determination of SOD Activity*. The activities for disproportionation of the superoxide anion, an important scavenger of intracellular free radicals, were examined using the method of Marklund and Marklund (*12*) with minor modification.

(2) DPPH Free Radical Scavenging Activity. The free radical scavenging capacity of genistin and the genistin glycosides was analyzed using the DPPH, as previously described (*13*) with minor modification. The percentage inhibition of DPPH was calculated using the following equation

$$\text{radical scavenging activity (\%)} = [A_{\text{sample(517nm)}}/A_{\text{control(517nm)}})] \times 100$$

where $A_{\text{sample(517nm)}}$ is the absorbance of the sample and $A_{\text{control(517nm)}}$ is the absorbance of the control at 517 nm.

(3) Reducing Power Activity. The reducing power was measured using $K_3Fe(CN)_6-FeCl_3$ (*13*) with minor modification.

(4) DR Assay of Non-Site-Specific·OH Radical Scavenging Activity. The non-site-specific ·OH radical scavenging activity of genistin and the genistin glycosides was determined as described by Halliwell et al. (*14*) with minor modification. The percent inhibition of hydroxyl radical was calculated as follows:

$$\%\ \text{inhib} = [(A_{\text{control(532nm)}} - A_{\text{sample(532nm)}})/A_{\text{control(532nm)}}] \times 100$$

RNA Extraction, RT-PCR, and Real-Time RT-PCR. Total RNA was extracted from cells using a TRI Reagent LE kit from Sigma (St. Louis, MO) according to the manufacturer's protocol and suspended in diethylpyrocarbonate (DEPC)-treated water. For cDNA synthesis, 2 μg of total RNA was reverse transcribed using the PowerScript reverse transcriptase (Clontech) according to the Clontech Laboratories protocol, using a combination of oligo$(dT)_{15}$ primer and random hexamers. RT-PCR was performed by using the Bioneer AccuPower PCR PreMix kit according to the manufacturer's instructions. PCR primers were designed using published nucleotide sequences for MT1A, MT2A, MT1E, MT1X, the metal-responsive transcription factor-1 (MTF-1), zinc transporter-1 (ZnT-1), and 18s rRNA from Hasumi et al. (*15*) and the sequences for human glutathione-S-transferase A1 (hGSTA1) and glucose-6-phosphate dehydrogenase (G6PD) from Xiang et al. (*16*) and Riganti et al. (*17*), respectively.

First, the expression of MTs, MTF-1, and ZnT-1 were measured by RT-PCR, and then the expression of MTs, which was our primary interest, was confirmed with real-time RT-PCR, a much more sensitive method than RT-PCR. Real-time PCR allows quantification of small changes in gene expression. The same MT primers were used for both RT-PCR and SYBR real-time PCR assay. We avoided long amplicon sizes (best 100−300 bp, must be < 500 bp) to get a high amplification efficiency for real-time PCR analysis. The real-time PCR was performed for MTs detection by using 12.5 μL of iQ SYBR Green Supermix from Bio-Rad (Hercules, CA), 0.5 μL of each primer (15 μM), 1 μL of cDNA, and 10.5 μL of sterile water. The final volume of the reaction mixture was 25 μL. Real-time PCR using the MT1A, MT2A, MT1E, and MT1X templates was performed in one cycle of 3 min at 95 °C followed by 40 cycles of 30 s at 94 °C, 30 s at 60 °C, and 30 s at 72 °C. A final extension was carried out for 5 min at 72 °C. Following amplification, a melting curve of amplified DNA was analyzed at temperatures between 54 and 95 °C with a heating rate of 0.2 °C per second. All real-time PCR were performed in iCycler iQ (Bio-Rad, Hercules, CA). During the primer extension step, the increase in the fluorescence from the amplified DNA was recorded by using the SYBR Green optic channel set at a wavelength of 490 nm. Data were collected and viewed using the iCycler iQ optical system software version 3.1 (Bio-Rad, Hercules, CA).

RT-PCR using templates for ZnT-1 began with a 10 min incubation at 95 °C, followed by 30 cycles of 30 s at 94 °C, 30 s at 60 °C, and 30 s at 72 °C. A final extension occurred for 5 min at 72 °C. RT-PCR using the 18s rRNA and MTF-1 templates was performed similarly, except that 10 cycles were used (18s rRNA) or different annealing (30 s of 62 °C, MTF-1) was used. RT-PCR using templates G6PD and GSTA1 began with a 15 min incubation at 95 °C, followed by 25 cycles at 94 °C for 30 s and 60 °C for 30 s, with a final a extension of 30 s at 72 °C.

The primers of MT-1A were as follows: F, 5′-CTCGAAATGGACCCCAACT, and R, 5′-ATATCTTCGAGCAGGGCTGTC; MT-2A: F, 5′-CCGACTCTAGCCGCCTCTT, and R, 5′-GTGGAAGTCGCGTTCTTTACA; MT-1E: F, 5′-GCTTGTTCGTCTCACTGGTG, and R, 5′-CAGGTTGTGCAGGTTGTTCTA; MT-1X: F, 5′-TCTCCTTGCCTCGAAATGGAC, and R, 5′-GGGCACACTTGGCACAGC; ZnT-1: F, 5′-AATACCAGCAACTCCAACGG, and R, 5′-ATCACTGAACCCAAGGCATC; MTF-1: F, CTTTCTGTTGTTGCTGGGGC, and R, ATGCCTCTT CTTGTTTGATGATG; hGSTA1: F, 5′-TCCATATGGCAGAGAAGCCCAAGC, and R, 5′-CCGAATTCTCCATGACTGCGTTATTA; G6PD: F, 5′-CCGGATCGACCACTACCTGGGCAAG, and R, 5′-GTTCCCCACGTACTGGCCCAGGACCA; and 18s rRNA: F, 5′-CGGCTACCACATCCAAGGAA and R, 5′-GCTGGAATTACCGCGGCTGC).

The intensities of the RT-PCR bands were analyzed and quantified with Sigma gel software (Jandel Scientific, San Rafael, CA) using 18s rRNA as internal controls. The identities of the PCR products were verified using DNA sequencing.

Detection of Labile Intracellular Zinc Using FluoZin-3 AM Fluorescent Indicators. The cells were seeded at 1×10^5 cells/mL in 6-well Falcon plates (2 mL/well) and were grown for 7 days. The cells were serum-starved for 6 h and then treated with the cell-permeant Zn^{2+}-selective probe FluoZin-3 AM (4 μM; Molecular Probes, Eugene, OR) at 37 °C for 30 min in DMEM (*18*). The cells were washed in DMEM to remove nonspecifically bound FluoZin-3 and then incubated for an additional 40 min to complete the intracellular cleavage of the AM ester. During this time, genistin and genistin glycosides were added. The cells were harvested from the 6-well plates by trypsinization and were collected by centrifugation at 500*g* for 10 min. The supernatant was removed, and the cells were resuspended in 135 mM NaCl, 1.1 mM EGTA, and 20 mM HEPES, pH 7.4. The cells were homogenized and centrifuged at 12000*g* for 10 min. The level of intracellular labile zinc was measured using a spectrofluorometer (Kontron Instruments, MD SFM-25) with excitation at 494 nm and emission at 516 nm.

Statistical Analyses. Each experiment was repeated at least three times. Statistical analyses were performed using Student's *t*-test. Data are reported as the mean ± SD. A value of $p < 0.05$ was considered statistically significant.

RESULTS

Transglycosylation Reaction and the Purification of Genistin Glycosides. The purified genistin was transglycosylated using TS4αGTase to obtain genistin glycosides (**Figure 1A**), which had dramatically increased water solubility (*3*).

The transglycosylated products contained five genistin derivatives from G2- to G6-genistin, and these fractions were separated using preparative liquid chromatography (**Figure 1B**). Each genistin glycoside peak showed a single spot on TLC and a single peak on the HPLC chromatogram (data not shown). Since the enzymatic reaction produced limited amount of each genistin glycoside, we mixed equal moles of G2- to G6-genistin [α-D-maltosyl-(1→6)-genistin, α-D-maltotriosyl-(1→6)-genistin, α-D-maltotetraosyl-(1→6)-genistin, α-D-maltopentaosyl-(1→6)-genistin, and α-D-maltohexaosyl-(1→6)-genistin, respectively]. Each genistin glycoside was added according to its molecular weight so that the mixture contained the same moles of G2- to G6-genistin. We calculated the concentration of genistin glycosides according to the average molecular weight calculated as follows: G2 molecular weight, 756 g/mol; G3 molecular weight, 918; G4 molecular weight, 1080; G5 molecular weight, 1242; G6 molecular weight, 1404. The average molecular weight of genistin glycosides were (756 + 918 + 1080 + 1242 + 1404)/5 = 1080 g/mol.

In the present study, the mixture of genistin glycosides was used for their antioxidant activity and antioxidant gene expression measurements.

The Antioxidant Activities of Genistin and Its Glycosides. We examined whether oxidative stress affected cell viability with SNP treatment. SNP (2 mM) produced NO free radicals and caused significant cytotoxicity, reducing cell viability by 35%. However, when the cells were treated with genistin, cell viability was restored in a concentration-dependent manner (**Figure 2A**). A similar effect was found with genistin glycoside treatment. These results suggested that genistin and its glycosides directly quench free radicals. Next, the effect of genistin and its glycosides on SOD activity was tested (**Table 1**). The treatment of genistin and its glycosides similarly increased SOD activity. At 0.1 mM, genistin and its glycosides showed 61% and 65% of the SOD activity compared with ascorbic acid treatment. The non-site-specific hydroxyl radical scavenging activity of genistin and the genistin glycosides were quantified using the deoxyribose assay (**Table 1**). Both genistin and its glycosides were effective at quenching the hydroxyl radicals, but genistin was significantly more effective than its glycosides.

The antioxidant activities of genistin and its glycosides were further investigated using the DPPH hydroxyl radical scavenging activity and reducing power activity assay (**Figure 2B**,**C**). **Figure 2B** shows that both genistin and its glycosides had concentration-dependent DPPH radical scavenging activity. Genistin was slightly more effective than its glycosides (**Figure 2B**), but both had lower radical scavenging activity than ascorbic acid (**Figure 2B**). At high concentration (10 mM) where genistin is hardly dissolved, genistin glycoside showed radical scavenging activity similar to that of genistin at 0.25 mM. In the reducing power assay (**Figure 2C**), the genistin and genistin glycosides showed some activity but much weaker than that of ascorbic acid.

Effects of Genistin and Genistin Glycosides on the Expression of Antioxidant Genes. Recent studies have reported that some polyphenolic antioxidants could increase intracellular zinc, which induces the expression of MT antioxidant proteins (*19*). Accordingly, we tested the effect of genistin on MT gene expression. After the treatment of cells with 0.1 mM genistin for 24 h, the expression of MT1A, MT2A, MT1E, and MT1X mRNA increased 19.4-, 13.4-, 41.3-, and 18.3-fold, respectively (**Figure 3A**). After incubation for 48 h, the induction disappeared, and the MT expression was returned to baseline levels ($p < 0.05$) (data not shown).

After treatment with 0.025, 0.05, and 0.1 mM genistin for 24 h, G6PD mRNA levels increased moderately in a concentration-dependent manner, by 1.2-, 1.4-, and 1.8-fold, respectively. The induction disappeared after treatment with 0.05 mM genistin for 48 h (**Figure 3B**). The hGSTA1 showed similar induction with genistin treatment, although hGSTA1 expression was reduced by half after treatment with 0.05 mM genistin for 48 h (**Figure 3B**).

Genistin glycosides significantly induced MT genes, as did genistin. Treatment with 0.1 mM genistin glycosides induced MT1A and MT1E mRNA up to 17.4- and 13.7-fold, similar to the genistin treatment. However, lower levels of MT1E and MT1X mRNA were obtained when the cells were treated with 0.1 mM genistin glycosides for 24 h than when they were exposed to genistin. Genistin glycosides significantly induced

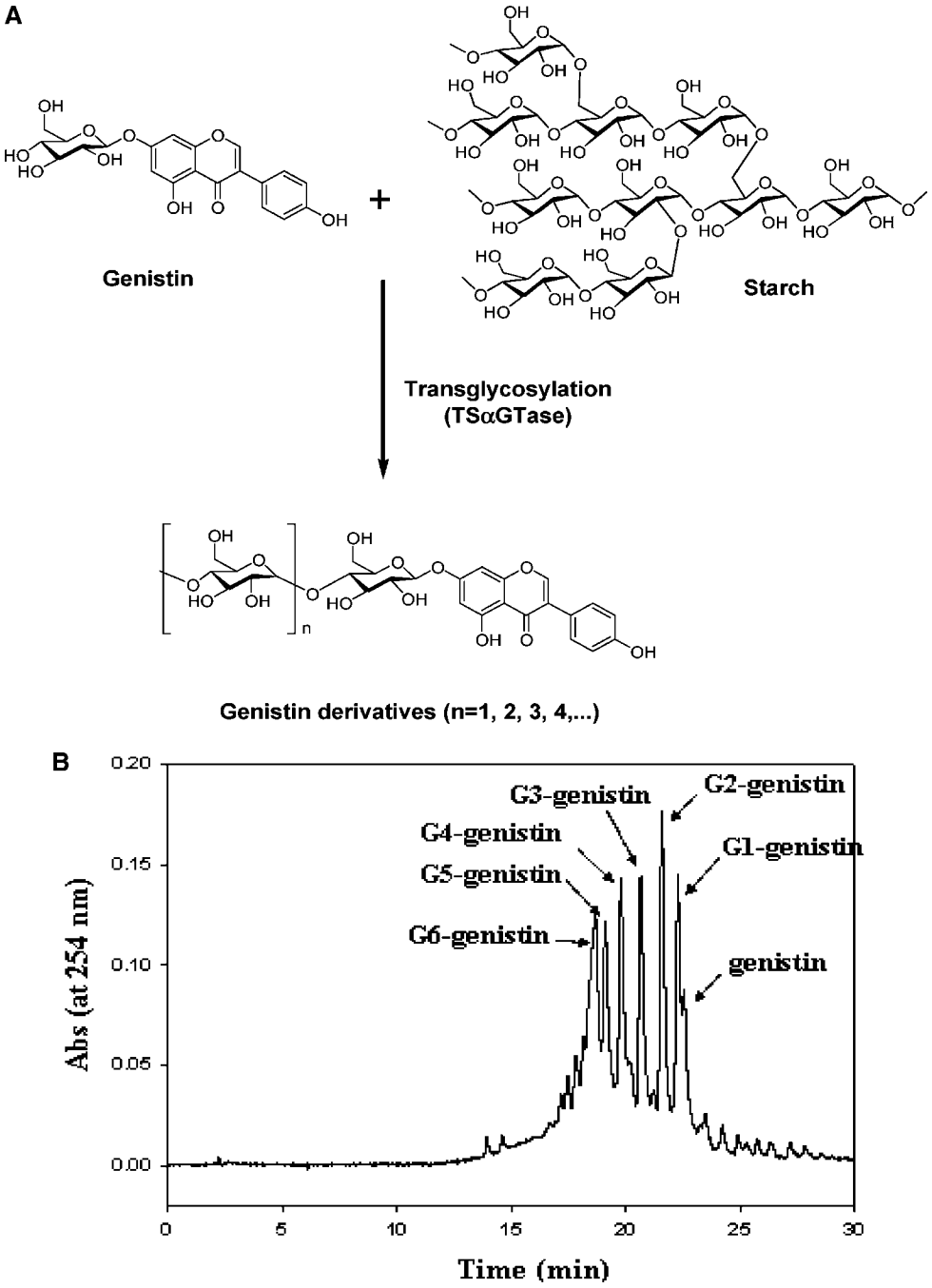

Figure 1. Schema of the enzymatic synthesis of genistin glycosides from genistin by TS4αGTase (**A**) and preparative HPLC chromatogram of genistin and its glycosides (**B**). G1-genistin, α-D-glucosyl-(1→6)-genistin; G2-genistin, α-D-maltosyl-(1→6)-genistin; G3-genistin, α-D-maltotriosyl-(1→6)-genistin; G4-genistin, α-D-maltotetraosyl-(1→6)-genistin; G5-genistin, α-D-maltopentaosyl-(1→6)-genistin; G6-genistin, α-D-maltohexaosyl-(1→6)-genistin.

MT1A, MT2A, MT1E, and MT1X mRNA levels in a concentration-dependent manner. After treatment with 0.2 mM genistin glycosides for 24 h, the MT1A, MT2A, MT1E, and MT1X mRNA levels were increased by 28.9-, 35.3-, 57.9-, and 26.6-fold, respectively (**Figure 3A**). The G6PD mRNA level was also increased after 24 h with 0.1 and 0.2 mM genistin glycosides (**Figure 3B**). There was no obvious change in the hGSTA1 mRNA level in cells treated with 0.1 and 0.2 mM genistin glycosides for 24 h (**Figure 3B**).

MTF-1 Induction by Labile Zinc Plays a Central Role in the Transcriptional Activation of MT Genes. Zinc stimulates transcription of MT via the activation of metal-sensitive transcription factors such as MTF-1 (*19*, *20*). Therefore, we tested whether genistin and its glycosides could alter intracellular zinc concentrations followed by MTF-1 induction. The cytoplasmic labile zinc concentration was measured using the fluorescence intensity of FluoZin-3 AM for cytoplasmic Zn^{2+}, and it should be useful for detecting low intracellular Zn^{2+} levels (*18*). Treatment of cells with 0.1 mM genistin and genistin glycoside (0.1 mM, 0.2 mM) significantly increased the cytoplasmic fluorescence (**Figure 4A**).

A transient increase in the labile zinc concentration can upregulate MTF-1, which transactivates MT expression (*19*, *20*). Accordingly, we examined the effects of genistin and its glycosides on the induction of MTF-1 mRNA expression (**Figure 4B**). Cells treated with 0.05 mM genistin did not show altered MTF-1 mRNA levels after 12 h treatment; however, after a 24 h incubation with both 0.05 mM genistin and 0.2 mM genistin glycosides, MTF-1 mRNA levels were significantly increased ($p < 0.05$) (**Figure 4B**).

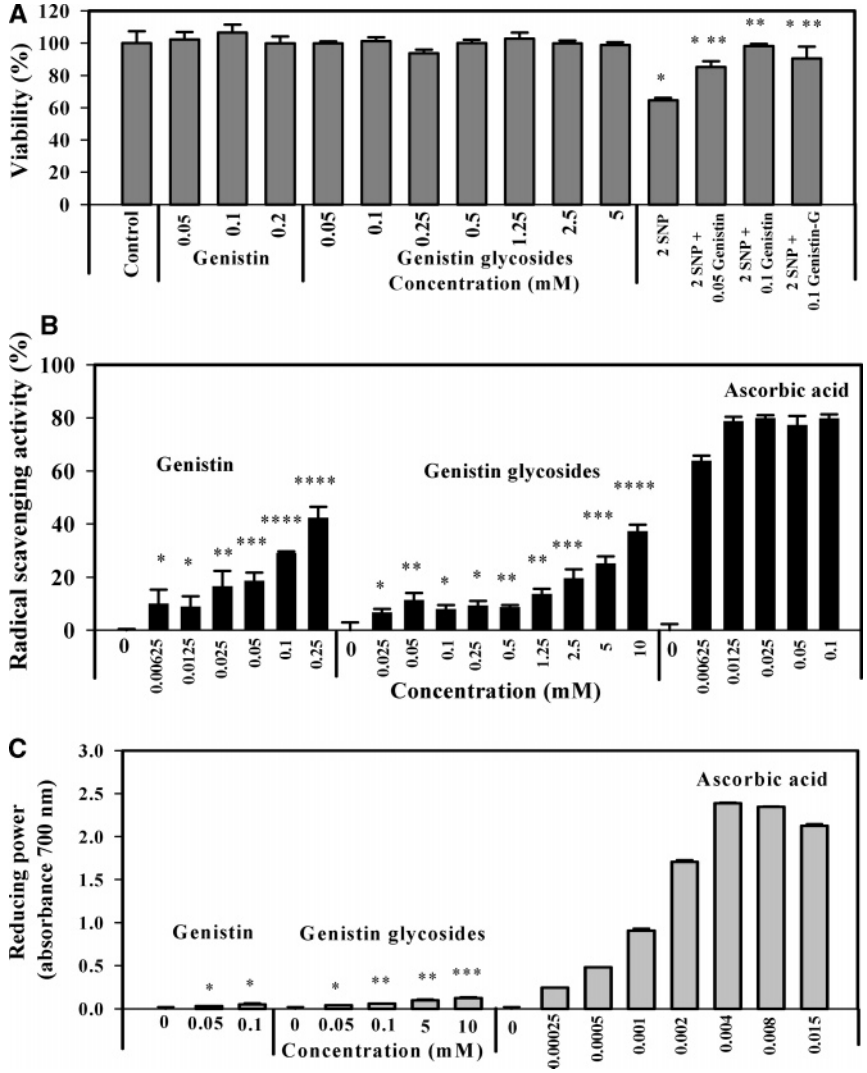

Figure 2. Cell viability (**A**), DPPH radical scavenging activity (**B**), and reducing power (**C**) of genistin and genistin glycosides (genistin-G). Data are the mean values ± standard deviation ($n = 4$). **A**: *$p < 0.05$ vs control; **$p < 0.05$ vs 2 mM SNP. **B**: *$p < 0.05$ vs control; **$p < 0.01$ vs control; ***$p < 0.001$ vs control; ****$p < 0.0001$ vs control. **C**: *$p < 0.05$ vs control; **$p < 0.01$ vs control; ***$p < 0.001$ vs control.

Table 1. Effects of Genistin and Its Glycosides on Superoxide Dismutase Activity and Non-Site-Specific Hydroxyl Radical Scavenging Activity[a]

concentration (mM)	% of SOD activity[b]			% inhibition of non-site-specific hydroxyl radical scavenging activity[c]		
	ascorbic acid	genistin	genistin-G[d]	vitamin E	genistin	genistin-G
0	0	0	0	0	0	0
0.05	58.1 ± 1.6	33.3 ± 1.17	35.5 ± 3.6	53.1 ± 0.7	57.6 ± 0.5	37.9 ± 0.3
0.1	62.3 ± 1.6	38.2 ± 1.0	40.5 ± 1.4	54.1 ± 0.8	58.8 ± 0.5	39.4 ± 0.5
0.5	71.3 ± 2.1	61.1 ± 1.9	54.8 ± 4.7	69.8 ± 2.5	60.0 ± 0.1	45.3 ± 2.0

[a] Values represent the mean ± SD, $n = 3$. [b] % Activity $= 100 - (A_{sample(420nm)}/A_{control(420nm)} \times 100)$. [c] Non-site-specific hydroxyl radical scavenging in the presence of EDTA. [d] Genistin glycosides.

Genistin-Promoted Increase in Intracellular Labile Zinc Induces the Expression of ZnT-1 mRNA. In human cells, the seven homologous zinc tranporter proteins have been identified (ZnT-1 to ZnT-7) that belong to the SLC30 gene family (*21*). High intracellular zinc levels are strictly regulated by zinc channels, zinc-efflux transporters (e.g., ZnT1), and zinc-binding proteins (e.g., MTs) as a cellular protection mechanism against free radical attack (*9*). ZnT1 is abundant in cell membranes, and its gene expression is regulated by MTF-1 (*9*, *21*); therefore, we also examined whether genistin treatment could induce ZnT-1. We found that the ZnT-1 was similarly induced compared with MTs after genistin and its glycoside treatment. After treatment with 0.025, 0.5, or 0.1 mM genistin for 24 h, the ZnT-1 mRNA levels increased significantly compared with the control levels. In HepG2 cells, ZnT-1 mRNA increased 1.5-fold after 0.1 mM genistin treatment (**Figure 4C**).

DISCUSSION

Genistin has a variety of health benefits, but the low water solubility of genistin limits their application in the food industry (*22*) and may also hinder its bioavailability in vivo since genistin is most often delivered in an aqueous media.

Dr. Park's group (*3*) developed a novel enzymatic transglycosylation method that improved the water solubility of genistin glycosides up to 1000- to 10000-fold compared with genistin. However, the modification of chemical structure of genistin may change the functional properties. In the current study, we determined that genistin glycosides have a level of antioxidant activity similar to that of genistin. At the same concentration, genistin glycosides showed slightly lower radical scavenging activity, but their activity was further increased at high concentration where genistin can hardly dissolve.

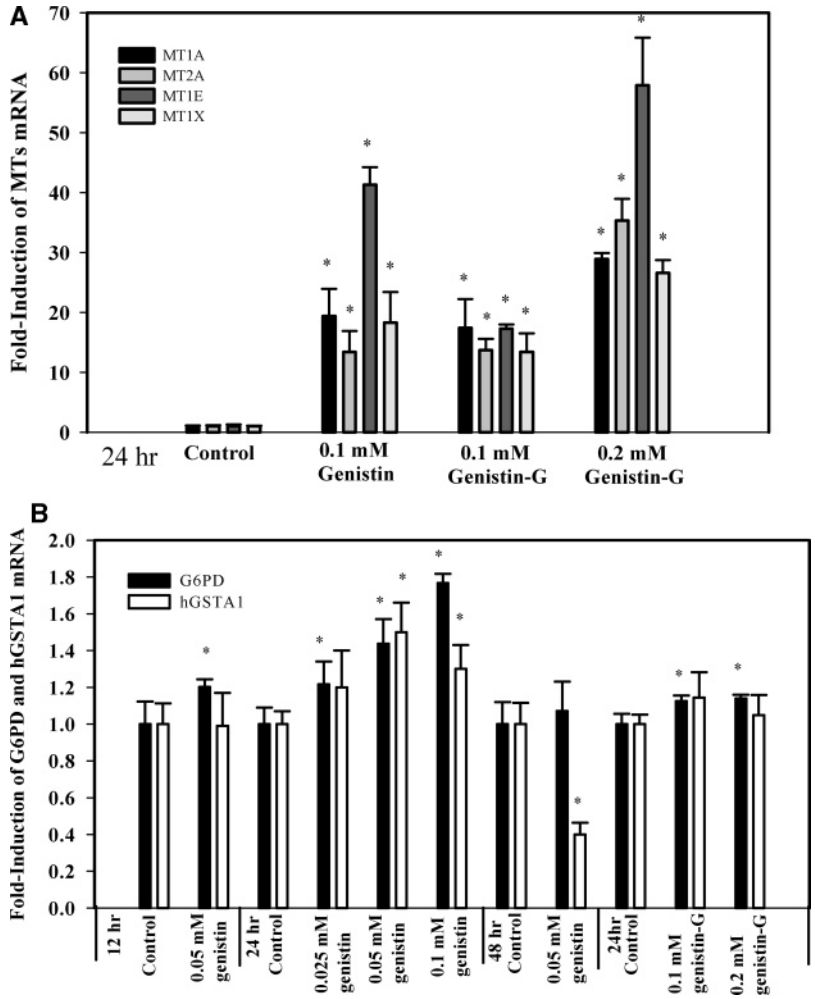

Figure 3. Effect of genistin and its glycosides (genistin-G) on the expression of MTs (MT1A, MT2A, MT1E, and MT1X) (**A**), G6PD, and hGSTA1 (**B**). *$p < 0.05$ vs control (24 h treatment). The data are the means ± standard deviation ($n = 3$). The mRNA levels of the MTs, G6PD, and hGSTA1 in each sample were normalized using 18s rRNA as a reference. The fold induction of G6PD and hGSTA1 mRNA in treated cells was calculated as the ratio of the corresponding mean value in the control cells. Fold induction of MTs mRNA in real-time PCR result: d Threshold cycle (dCt) = (Ct of MTs mRNA) – (Ct of 18s mRNA); ddCt = (dCt of mRNA in treated cells) – (dCt of mRNA in untreated cells); fold induction = 2^{-ddCt}.

Genistin glycosides scavenged free radicals directly, induced SOD activity, and showed reducing power ability, although they were not as effective as ascorbic acid. These results were not surprising and were in agreement with those of Michell et al. (*23*), who demonstrated that genistein and daidzein did not strongly scavenge DPPH or galvinoxyl free radicals compared with ascorbic acid, and also in agreement with those of Lee et al. (*2*), who suggested that genistin was a much weaker antioxidant than green tea epicatechin or α-tocopherol.

Cells are able to reduce oxidative damage from reactive oxygen species (ROS) through various antioxidant defense systems including MTs, GST, and G6PD (*11*). Especially, MTs are small metal-binding proteins that have important cellular functions: controlling intracellular divalent metal concentrations (*6*) and neutralizing toxic metals such as cadmium and mercury (*24*). In addition, a role for MT as direct antioxidant has become increasingly more accepted since MTs have 300−800 times higher antioxidative activity compared with glutathione (GSH) on a molar basis (*5*).

In addition, phenolic and quione compounds have shown to induce MT synthesis in murine cells through activation of MTF-1 (*6*, *19*), thus it was of interest to us to investigate if genistin and its glycosides also induced synthesis of antioxidant genes including MTs. HepG2 cells normally express MTs (*9*), thus the HepG2 was an appropriate model system for testing the effects of genistin and its glycosides on MT expression.

In addition to their direct ROS scavenging antioxidant activity, genistin and its glycosides, in fact, were able to induce antioxidant genes including several MTs. In humans, four subgroups of MT proteins (MT-1, MT-2, MT-3, and MT-4) are encoded by a family of genes located at 16q13 that includes 10 functional and seven nonfunctional MT isoforms (*25*). The known functional MT-1 isoforms are MT1A, 1B, 1E, 1F, 1G, 1H, and 1X, and the known functional MT-2 isoform is MT2A (*26*). In our experiment, three of the functional MT-1s (MT1A, MT1E, and MT1X) and one functional MT-2 were tested, and the transcription of all four MTs were significantly increased by the treatment of both genistin and its glycosides. The ability of genistin glycosides to induce MT genes was similar to that of genistin. Our results are the first to demonstrate the effects of genistin and genistin glycosides on antioxidant gene expression, including that of MTs and G6PD.

We further studied the mechanism of MT gene induction by genistin treatment. In line with the previously known mechanism of MT gene regulation, we also found that the treatment of genistin and its glycosides increased cytosolic zinc concentration followed by the up-regulation of MTF-1, which is supposed to bind to the metal response element (MRE) promoter of MT

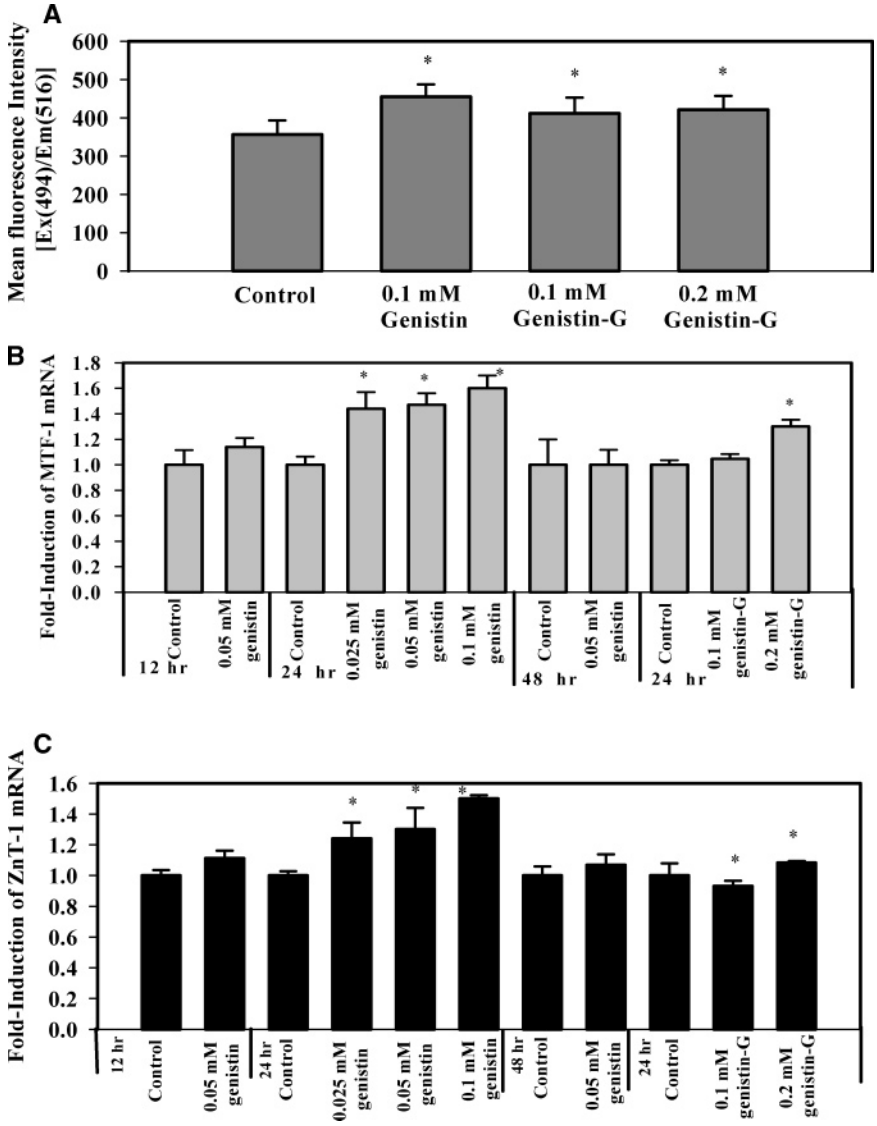

Figure 4. Effect of genistin and genistin glycosids on cytoplasmic labile zinc levels (**A**) and on the accumulation of MTF-1 (**B**) and ZnT-1 (**C**). Cytoplasmic zinc in HepG2 cells was detected using the fluorescent indicator FluoZin-3 AM. HepG2 cells were incubated in serum-free medium for 6 h before the experiment (**A**). *$p < 0.05$ vs control. The data are the means ± standard deviation ($n = 3$). The MTF-1 and ZnT-1 mRNA levels in each sample were normalized to the quantity of 18s rRNA as a reference. The fold induction of MTF-1 and ZnT-1 mRNA in treated cells was calculated as the ratio of the corresponding mean value in the control cells.

genes for transactivation. The up-regulation of MTF-1 and MTs was confirmed using both RT-PCR or real-time PCR. The induction of another MTF-1-induced gene, ZnT-1, was also confirmed. Currently, it is not clear how genistin and its glycosides increased intracellular zinc concentration in a non-oxidative stress pathway. Hypothetically, the increase in labile zinc could have been caused by increased influx, decreased efflux, or release from organelles.

Interestingly, tBHQ and other redox-cycling phenolic compounds have been found to induce synthesis of MT through release of zinc from cellular stores and subsequent activation of MTF-1 (*6*, *10*, *19*). Due to structual resemblance to tBHQ and other polyphenols, it is possible that genistin and its glycosides might increase cytosolic zinc concentration by the similar mechanism. Further studies are required to elucidate the detailed mechanism.

Our data suggest that genistin and its glycosides protect against ROS-induced cytotoxicity in two ways: direct antioxidant activity and indirect induction of antioxidant gene expression. Genistin and its glycosides are directly effective in reducing free radicals. In addition, our results indicate that induction of MTs and other antioxidant genes synthesis may act as an indirect antioxidant effect of genistin and its glycosides.

The major advantage of the glycosylation of genistin in this study is that genistin glycosides greatly improve the water solubility (*3*) while maintaining the antioxidant activities. The newly formed α(1,4)-glycosidic linkage of the transglycosylation products was easily hydrolyzable in the human body by intestinal microorganisms that use glycosyl hydrolases (*3*, *27*) and digestive enzymes, implying that a high concentration of genistin glycosides may be metabolized in the same way as genistin.

In conclusion, genistin glycosides and genistin have similar antioxidant activity levels, both directly quenched ROS, and both induced the antioxidant MT genes. Considering their excellent water solubility, genistin glycosides may have potential as a healthy nutraceutical and a functional food ingredient.

ABBREVIATIONS USED

MTs, metallothioneins; tBHQ, *tert*-butylhydroquinone; MTF-1, metal-responsive transcription factor; SOD, superoxide dis-

mutase; DPPH, 1,1-diphenyl-2-picrylhydrazyl; DMEM, Dulbecco's minimum essential medium; DR, deoxyribose; FBS, fetal bovine serum; PEST, penicillin and streptomycin; T-EDTA, trypsin-ethylenediaminetetraacetic acid; TS4αGTase, *Thermus scotoductus* 4-α-glucanotransferase; HPLC, high-performance liquid chromatography; RT-PCR, reverse transcription-polymerase chain reaction; MTT, 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide; SNP, sodium nitroprusside; DMSO, dimethyl sulfoxide; DEPC, diethylpyrocarbonate; ZnT-1, zinc transporter-1; hGSTA1, human glutathione-S-transferase A1; G6PD, glucose-6-phosphate dehydrogenase; MRE, metal response element; ROS, reactive oxygen species; GSH, glutathione.

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