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Glucosylation of Resveratrol Improves its Immunomodulating Activity and the Viability of Murine Macrophage RAW 264.7 Cells

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Effects of resveratrol glucosylation on the immunomodulation properties of resveratrol and on the viability of macrophage cells have been studied by using murine macrophage RAW 264.7 cells. Nitric oxide (NO) and interleukin 6 (IL-6) expression in macrophages in vitro were studied after treatment with different concentrations of (*E*)-resveratrol, (*E*)-resveratrol 3-*O*-β-D-glucoside (R-3-G), or (*E*)-resveratrol 4'-*O*-β-D-glucoside (R-4'-G). In vitro viability of RAW 264.7 cells after treatment with the aforementioned three compounds was also studied. As demonstrated by macrophage cell viability assays, two different resveratrol monoglucosides, R-3-G and R-4'-G, exhibited 50–80% reduced cytotoxicity in comparison to (E)-resveratrol in A549 and HepG2 cells. Compared to the resveratrol aglycon, both glucosylated resveratrol derivatives positively modulated NO and IL-6 production in macrophages positively via transcriptionally up-regulating IL-6 and iNOS expression. Conjugation of a glucose moiety on resveratrol was found to enhance the immunomodulating activity of resveratrol and the viability of RAW 264.7 cells.

Keywords: Resveratrol, resveratrol glucosides, RAW 264.7 cells, cell viability, immunomodulation

Introduction

(*E*)-resveratrol has been demonstrated to possess wide range of important potential health beneficial effects by modulating physiological and pathological processes [1,2], such as anti-cancer, anti-mutagenesis, antioxidant, anti-inflammatory and cardioprotective effects [3–5]. Since the initial report on possible cardioprotective benefits of resveratrol in the red wine, the production and application of resveratrol as therapeutic agents have become increasingly important topics of intensive research [6]. However, it has been reported that resveratrol has low water solubility, poor bioavailability and is chemically unstable, which may limit its pharmacological and biological activities in vivo [7]. Similarly, quercetin, one of the flavonols with numerous biological functions has limited applications as therapeutic agents because of its low water solubility and bioavailability. It has less than 1% of bioavailability when administered in capsule form to human [8]. Makino *et al.* [9] demonstrated that glycosylation of quercetin with specific sugar motifs is an effective strategy to improve the biological activity of quercetin in vivo by greatly improving its bioavailability, which may lead to enhanced pharmacological activity [9]. In this regards, we prepared glucosylated resveratrol derivatives, (*E*)-resveratrol 4'-*O*-β-D-

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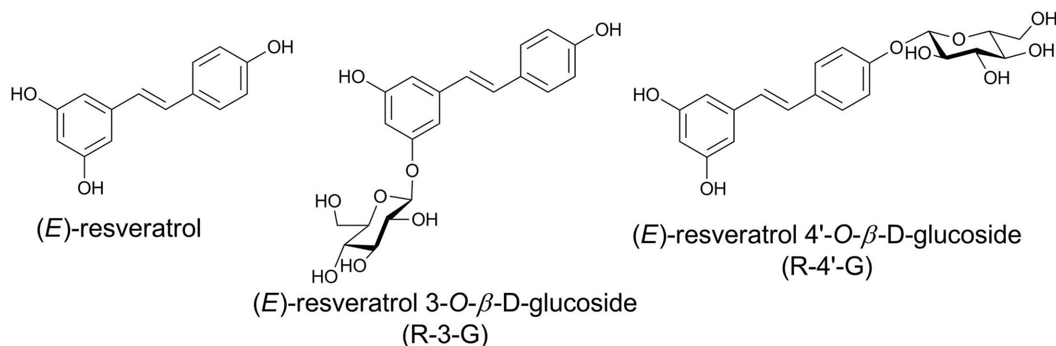

Fig. 1. Chemical structures of compounds used in this study.

glucoside (R-4'-G) and (*E*)-resveratrol 3'-*O*-β-D-glucoside (R-3-G) by enzymatic glycosylation.

In immune system, the resveratrol, at relatively low concentrations (0–10 μg/ml), has been reported to have anti-inflammatory properties. It is found to inhibit nitric oxide (NO) and pro-inflammatory cytokines production such as tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) from macrophages [1,10,11]. Although the anti-proliferative and anti-inflammatory activities of resveratrol have been documented in many studies, the effects of resveratrol and its glycosylated derivatives on the development of immune responses have not been extensively investigated [4,12]. In the present study, we evaluated and compared the effects of resveratrol and its glucosylated derivatives (Fig. 1) on the macrophage cell viability and immunomodulating activity using RAW 264.7 cells.

Materials and Methods

Chemicals and reagents

(*E*)-resveratrol was purchased from Sigma-Aldrich (St. Louis, USA). R-3-G and R-4'-G were prepared in laboratory by enzymatic glucosylation approach as described in our previous reports [13–15]. R-3-G and R-4'-G were dissolved in dimethyl sulfoxide (DMSO). All other chemicals and reagents were of standard grade.

Cell culture

The murine macrophage cell line (RAW 264.7, KCLB # 40071) and several cell lines such as A549 (human lung carcinoma, KCLB # 10185), PC-3 (human prostate carcinoma, KCLB # 21435), HepG2 (human hepatoma carcinoma, KCLB # 88065) HeLa (human cervical carcinoma, KCLB # 10002) and HT-29 (human colon carcinoma, KCLB # 30038) were purchased from the Korea Cell Line Bank (Seoul, Korea) and grown in RPMI-1640 medium (Gibco-Invitrogen, USA) supplemented with 10% (v/v) fetal bovine serum (FBS), 2 mM L-glutamine and 100 U/ml penicillin/streptomycin (PEST). Unless stated otherwise, the cells were incubated with (*E*)-resveratrol, R-3-G or R-4'-G (50–150 μg/ml) for 24 h.

MTT cytotoxicity assay

The cytotoxicity of (*E*)-resveratrol and its glycosylated derivatives (R-4'-G and R-3-G) was studied using the 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT) microculture tetrazolium viability assay [16]. Precultured RAW 264.7 cells (5×10^4 cells/well) in RPMI-1640 media were plated on a 96-well microplate. Different concentrations of the (*E*)-resveratrol, R-3-G or R-4'-G were prepared with dilutions ranging from 25 μg/ml (219.06 μM)–150 μg/ml (384.23 μM). After 24 h of incubation, 20 μl of MTT (5 mg/ml) was added to each well. The plates were continued to incubate for additional 4 h. After the removal of media, 200 μl dimethyl sulfoxide (DMSO) was added to solubilize the formazan crystals. The absorbance was then read at 595 nm using a microplate reader (Molecular Devices Co., USA).

Reverse transcriptase-polymerase chain reaction (RT-PCR) analyses

To evaluate the messenger ribonucleic acid (mRNA) expression levels of inducible nitric oxide synthase (iNOS) and IL-6, total RNA from resveratrol or its glucosylated derivatives-treated RAW 264.7 cells were prepared using a Total RNA Extraction kit (Intron Biotechnology, Korea). The total RNA samples were stored at −80℃ for

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further use. RT-PCR was performed using the ONE-STEP RT-PCR PreMix kit (Intron Biotechnology, Korea) with sense and antisense primers for β-Actin (β-Actin, 5'-TGGAATCCTGTGGCATCCATGAAAC-3' (sense) and 5'-TAAAACGCAGCTCAGTAACAGTCCG-3') (antisense)); iNOS (5'-CTGCAGCACTTGGATCAGGAACCTG-3' (sense) and 5'-GGGAGTAGCCTGTGTGCACCTGGA-3' (antisense)); and IL-6 (5'-CTGGAGTCACAGAAGGAGTGGC-3' (sense) and 5'-GGCATAACGCACTAGGTTTGCCG-3' (antisense)), under incubation conditions of 95℃ predenaturation for 5 min and 35 cycles of '95℃ denaturation for 30 s, 55℃ annealing for 30 s, 72℃ extension for 40 s,' and a final elongation period of 10 min at 72℃. The products obtained by RT-PCR were analyzed by 1.5% agarose gel electrophoresis and visualized with ethidium bromide under UV transillumination. The relative levels of the mRNA were quantified using ImageJ software from the National Institute of Health (Bethesda, USA).

Assay for IL-6 secretion

The concentration of IL-6 released from murine RAW 264.7 cells treated with the (*E*)-resveratrol, R-3-G or R-4'-G was determined, using a mouse IL-6 enzyme-linked immunosorbent assay (ELISA) kit (BD Biosciences, USA), according to the manufacturer's protocol. Briefly, RAW 264.7 cells subcultured on 12-well culture plates in RPMI-1640 media were incubated for 24 h in the presence of various concentrations of the (*E*)-resveratrol, R-3-G or R-4'-G. Supernatant from each dish was harvested and IL-6 concentrations were measured by optical density following the manufacturer's instructions.

Measurement of NO production

The RAW 264.7 cells were cultured for 24 h in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (PEST). The cells were then treated with different concentrations (25 μg/ml (219.06 μM))–(150 μg/ml (384.23 μM)) of the (*E*)-resveratrol, R-3-G or R-4''-G for 24 h. The levels of NO in the culture supernatants were determined using Griess reagent (Sigma-Aldrich). Briefly, nitrite in the culture supernatants was mixed with an equal volume of Griess reagent (0.1% naphthyl ethylenediamine [w/v] and 1% sulfanilamide [w/v] in 5% phosphoric acid [v/v]), and the absorbance at 540 nm was then measured. For reference study, sodium nitrite ($NaNO_2$) (Sigma-Aldrich) was used.

Statistical analysis

Data were reported as mean ± standard error of the mean (SEM). Comparisons between two groups were performed with unpaired Student's *t*-tests. A *p* value of less than 0.05 was considered statistically significant.

Results

Cell viability of RAW 264.7 cells

The effects of natural (*E*)-resveratrol and its glucosylated derivatives on cell viability were evaluated using a MTT assay and RAW 264.7 cells. In these assays, resveratrol exhibited significant cytotoxicity following treatment with resveratrol (Fig. 2A). In contrast, glucosylated derivatives of (*E*)-resveratrol, R-3-G and R-4'-G, did not affect cell viability up to 100 μg/ml (256.16 μM) and 150 μg/ml (384.23 μM), respectively. These results suggest that glucosylation of resveratrol at either the 3 or 4' position can significantly reduce the cytotoxicity of (*E*)-resveratrol towards macrophage cells. Furthermore, glucosylation at the 4'-position versus the 3-position was more effective at reducing resveratrol cytotoxicity.

IL-6 expression and secretion by RAW 264.7 cells

(*E*)-resveratrol has been shown to modulate lymphocyte proliferation at lower concentrations [e.g., 2.73 μg/ml (< 12 μM)] [1], and IL-6 has been shown to modulate immunological responses [17]. Therefore, it was investigated whether (*E*)-resveratrol and its glucosylated derivatives (R-3-G and R-4'-G) could induce IL-6 expression and stimulate IL-6 secretion using RT-PCR and ELISAs. (*E*)-resveratrol at 50 μg/ml (219.06 μM) and 75 μg/ml (328.5 μM) slightly stimulated IL-6 expression and secretion compared with DMSO-treated cells (control), while relatively higher concentrations of (*E*)-resveratrol [e.g., 100 μg/ml (438.12 μM) and 150 μg/ml (657.20 μM)] significantly decreased IL-6 expression and secretion. In contrast, both R-3-G and R-4'-G were associated with a significant increase in IL-6 expression and secretion (Figs. 2B, C, D), although the pattern of IL-6 secretion differed according to the glucosylation position. Moreover, while treatment with R-4'-G resulted in a slight increase in IL-6 expression and secretion levels, treatment with R-3-G dramatically increased IL-6 expression

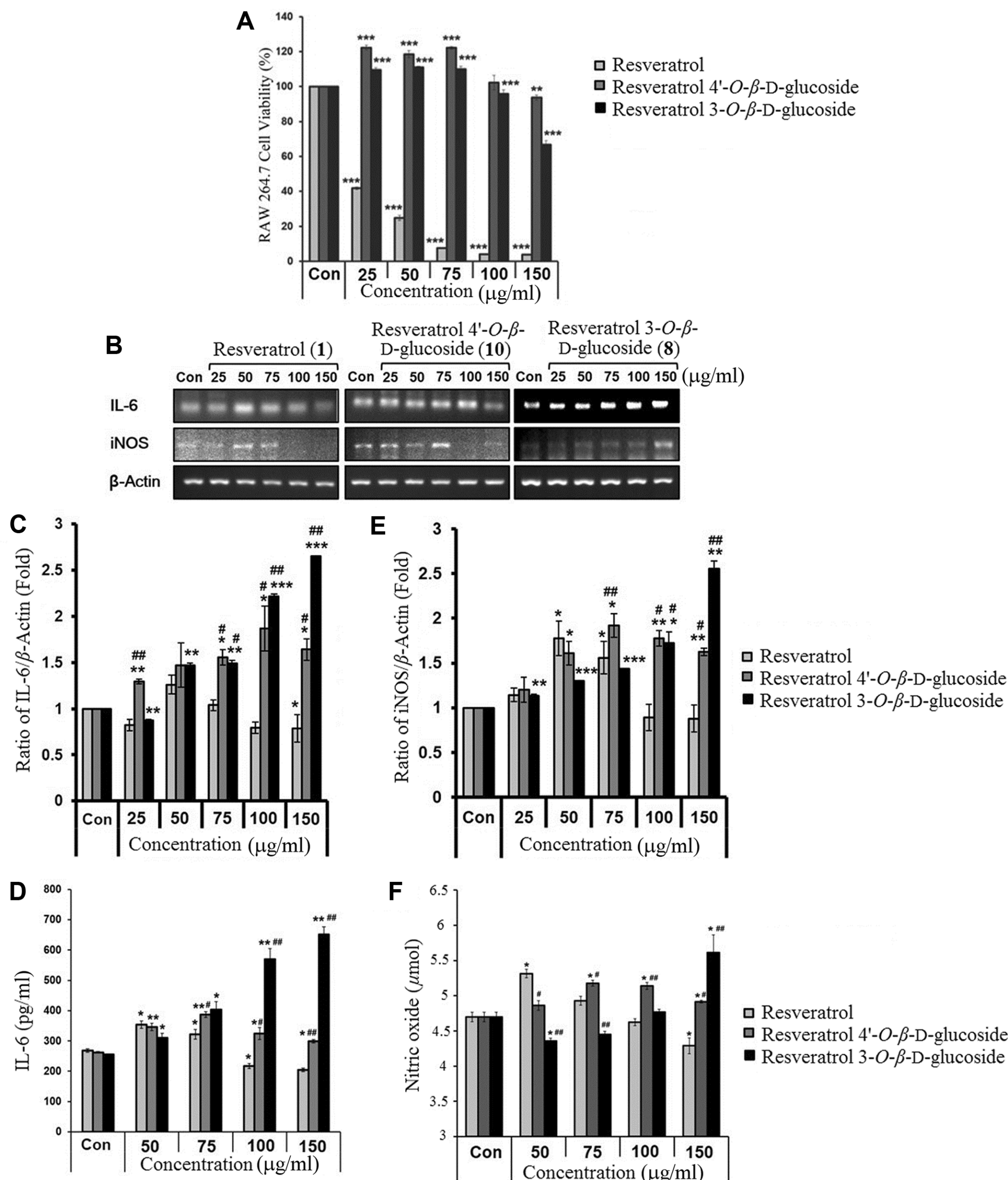

Fig. 2. (A) Cytotoxicity associated with different concentrations and glucosylated derivatives of resveratrol that were used to treat RAW 264.7 cells. Data are normalized to control (Con) data with mean ± standard error (SE) vales reported, n = 3. $^{**}p < 0.01$; $^{***}p < 0.001$, Student's *t*-test compared to control. (B) Effects of resveratrol and its glucosylated derivatives on mRNA expression of IL-6 and iNOS in RAW 264.7 cells. The amount of IL-6 (C) and iNOS (E) mRNA was expressed as the ratio of densitometric measurement of mRNA in the cells treated with resveratrol and its glucosylated derivatives to the corresponding internal standard (β-actin). Data represent the mean ± standard deviation (SD), n = 3. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$, Student's *t*-test compared to control. $^{\#}p < 0.05$; $^{\#\#}p < 0.01$, Student's *t*-test compared to the supernatant of cells treated with resveratrol. (D) Concentration-dependent effects of resveratrol and its glucosylated derivatives on the secretion of IL-6 by RAW 264.7 cells. Data represent the mean ± standard deviation (SD), n = 3. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$, Student's *t*-test compared to control. $^{\#}p < 0.05$; $^{\#\#}p < 0.01$, Student's *t*-test compared to the supernatant of cells treated with resveratrol. (F) Effects of different concentrations of resveratrol and its glucosylated derivatives on nitric oxide (NO) production by RAW 264.7 cells. Results are the mean ± SE of three independent experiments. $^{*}p < 0.05$, Student's *t*-test compared to control. $^{\#}p < 0.05$; $^{\#\#}p < 0.01$, Student's *t*-test compared to the supernatant of cells treated with each concentration of resveratrol. Data represent the mean ± standard error of the mean (SEM). Comparisons between two groups were made using unpaired Student's *t*-tests. A *p*-value less than 0.05 was considered statistically significant.

and secretion in a dose-dependent manner (Figs. 2B, C, D). In combination, these results suggest that glucosylation of resveratrol positively modulates IL-6 production in macrophage compared to aglycon resveratrol, and glucosylation at the 3-position of resveratrol is associated with greater immunostimulating activity compared to that associated with 4'-position glucosylation.

iNOS expression and NO production by RAW 264.7 cells

Expression of iNOS results in production of NO, a signaling molecule which is involved in various physiological and pathological processes in cells. In activated macrophage, NO mediates immunomodulating effects [18]. Therefore, the effect of resveratrol and its glucosides on iNOS expression and NO production by RAW264.7 macrophage cells was investigated (Figs. 2B, E, F). For the concentrations tested (e.g., 50 μg/ml, 75 μg/ml, 100 μg/ml, and 150 μg/ml), 50 μg/ml (219.06 μM) aglycon (*E*)-resveratrol induced production of NO more effectively than the glucosylated derivatives at the same concentration (Fig. 2F). However, for resveratrol concentrations ranging from 75 μg/ml (328.5 μM) to 150 μg/ml (657.20 μM), higher iNOS expression and NO production levels were detected (Figs. 2B, E, F), consistent with the IL-6 secretion results (Fig. 2D). Considering the effect of resveratrol on cell viability (Fig. 2A), we hypothesize that the dose-dependent decrease in IL-6 and NO levels is due to RAW 264.7 cell cytotoxicity. For the glucosylated derivatives, these were more effective at expressing iNOS and producing NO at relatively higher concentrations [e.g., 75 μg/ml (192.11 μM) to 150 μg/ml (384.23 μM)] compared to (*E*)-resveratrol (Figs. 2B, E, F). Moreover, at concentrations ranging from 192.11 μM to 384.23 μM, R-4'-G was more effective at expressing iNOS and producing NO than R-3-G, although these effects were reversed at 384.23 μM (Figs. 2B, E, F). This is in contrast with the IL-6 expression and secretion results (Figs. 2B, C, D), although the effects of R-3-G and R-4'-G on NO and IL-6 levels were more significant than those of resveratrol. Cumulatively, these results demonstrate that resveratrol glucosides positively modulate expression and production of NO and IL-6 in macrophage cells compared with resveratrol aglycon. Furthermore, the significant dose-dependent increase in IL-6 and NO expression following treatment with resveratrol glucosides suggests that these derivatives mediate early immunostimulating activities by transcriptionally upregulating IL-6 and iNOS expression, yet the natural anti-inflammatory activity of resveratrol is compromised.

In this study, glucosylation of resveratrol reduced the cytotoxicity of resveratrol, while glycosylated resveratrol derivatives positively modulated NO and IL-6 production in macrophage cells compared to aglycon resveratrol via transcriptionally upregulatiing IL-6 and iNOS expression.

Effects of resveratrol and its glucosylated derivatives on the cancer cell death

We also evaluated in vitro and compared the anticancer effects of resveratrol and its glucosylated derivatives. Several cancer cell lines, including A549, PC-3, HepG2, HeLa and HT-29 cells, were treated with 100 μg/ml of resveratrol and its glucosylated derivatives for 24 h, and then cell viability was determined in each cell type. In accordance with the previous study, resveratrol showed significant reduction in the viability of A549, HepG2, HeLa and HT-29 cells, but not PC-3 cells, indicating that it could induce cancer cell death (Fig. 3). However, both R-3-G and R-4'-G neutralized the resveratrol-induced cytotoxicity in several cancer cells. Furthermore, the glucosylated derivatives could rather accelerate the cell proliferation up to 50–80% in A549 and HepG2 cells. These results suggested that the glycosylation of resveratrol could significantly reduce its anticancer activity.

Discussion

In this study, we studied and compared the effects of (*E*)-resveratrol and its glucosylated derivatives, R-3-G and R-4'-G, on cell viability and immunomodulating activities using RAW 264.7 cells. Whereas the natural resveratrol exhibited significant cytotoxicity on RAW 264.7 cells in a dose-dependent manner (Fig. 2A), its glucosylated derivatives, R-3-G and R-4'-G, did not reduce the cell viability up to 100 μg/ml, although, at 150 μg/ml, the viability started to decrease. The results demonstrated that glucosylation of resveratrol at either 3 or 4'-position significantly abolished the cytotoxicity of resveratrol on macrophage cells and that glucosylation at 4'-position was shown to be more effective than 3-position in reducing the cytotoxicity of resveratrol. Although

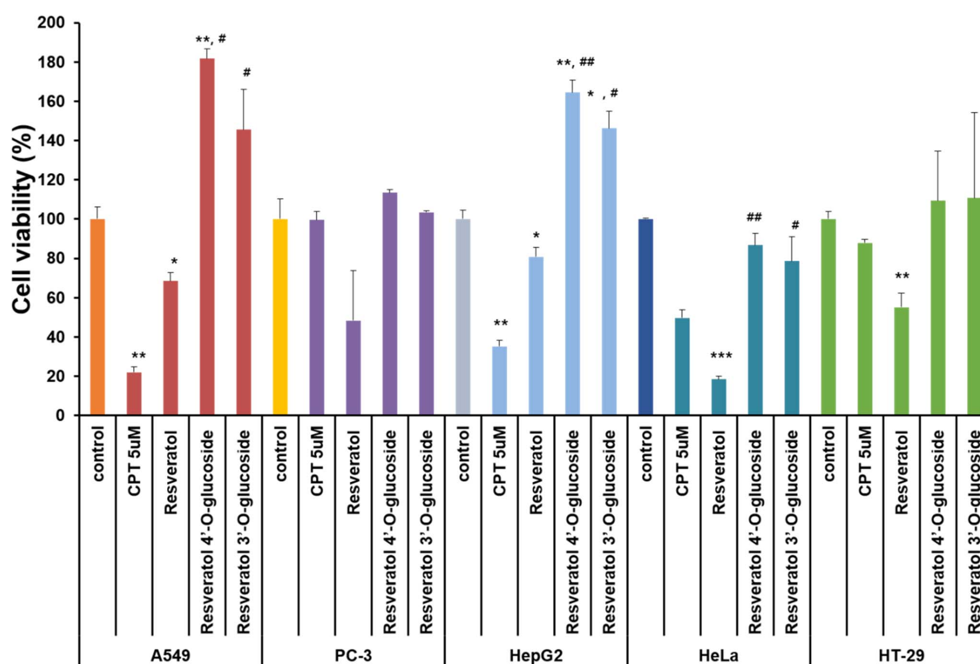

Fig. 3. Cell viability was assessed by MTT assay in a variety of human cancer cell lines including lung cancer cells (A549) (red colored bar), prostate cancer cells (PC-3) (purple colored bar), liver cancer cells (HepG2) (light blue colored bar), cervical cancer cells (HeLa) (dark blue colored bar), and colon cancer cells (HT-29) (green colored bar) after 24 h exposure with 100 μg/ml of (*E*)-resveratrol, (*E*)-resveratrol 3-*O*-β-D-glucoside (R-3-G) and (*E*)-resveratrol 4'-*O*-β-D-glucoside (R-4'-G). Data are expressed as percent normalized to control of each cell line. Data = mean ± SD, n = 3. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$, Student t-test compared to control of each cell line. #, $p < 0.05$; ##, $p < 0.01$, Student t-test compared to supernatant of cells treated with each concentration of resveratrol.

there might be some controversy, it could be possible that the improved cell viability by glucosylation of resveratrol may result from increased water solubility of resveratrol glucosides and thereby reduced membrane permeability of these glucosides comparing to the natural form as observed in other polar molecules [19].

The ELISA assay (Fig. 2D) showed that glucosylation of resveratrol positively modulated the IL-6 production in macrophage compared to natural aglycon form as R-3-G exhibited more effective IL-6 secretion than R-4'-G. The resveratrol was revealed to modulate lymphocytes proliferation at lower concentration [1]. It was reported that the resveratrol inhibits the IL-6 secretion from mouse peritoneal macrophages, showing its anti-inflammatory activities [20]. Similarly, the concentration-dependent decrease in IL-6 secretion by natural resveratrol in our study as shown in Fig. 2D suggested its anti-inflammatory action. Interestingly, however, that the glucosylated resveratrol derivatives, R-3-G and R-4'-G, significantly increased the IL-6 secretion in a dose-dependent manner may suggest that, in the contrary to the anti-inflammatory activity of the natural form, these glucoside derivatives newly gained early immunostimulating activities via up-regulation of IL-6 secretion while losing the natural resveratrol's anti-inflammatory activity.

On the other hand, immune response is characterized by the production of reactive nitrogen species as well as inflammatory cytokines [21]. In activated macrophages, adequate NO production is very important for the immune responses as it is involved in numerous physiological and pathological processes. In the present study, it was shown in the Fig. 2F that the natural resveratrol aglycon stimulated the production of NO at relatively lower concentration (50 μg/ml) but the NO levels started to significantly decrease with at higher concentrations (75–150 μg/ml), which was similar to the results of IL-6 secretion (Fig. 2D). The suppression of IL-6 secretion and NO production by resveratrol is consistent with the results of the previous reports in which resveratrol have anti-inflammatory effects suppressing the lymphocyte

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proliferation, cytokine production [12] and NO production [22].

At lower concentration (50 μg/ml), both glucosylated derivatives were less effective in NO production than the natural resveratrol of the same concentration. Interestingly, however, the glucosylated derivatives were shown to become more effective in NO production than the natural resveratrol at relatively higher concentrations (75–150 μg/ml) (Fig. 2F), indicating that glucosylation of resveratrol positively modulated the NO production in macrophage cells compared to resveratrol standard. In a similar way to the results of IL-6, that the glucosylated resveratrol derivatives significantly increased the NO peroduction in a dose-dependent manner may suggest that these glucoside derivatives newly gained early immunostimulating activities via up-regulation of NO production while losing the natural resveratrol's anti-inflammatory activity. However, since the cytotoxicity of natural resveratrol aglycon was shown to be significant, the dose-dependent decrease in IL-6 and NO levels could be due to its cytotoxicity on the RAW 264.7 cells, questioning its anti-inflammatory activity at relatively higher concentrations. In the meantime, it cannot be ruled out that the dose-dependent increase in NO production, as well as IL-6 secretion, with increasing concentrations of these glycosylated derivatives could be primarily due to their reduced cytotoxicity on macrophage cells comparing to that by natural resveratrol.

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국문초록

당화된 레스베라트롤의 대식세포 RAW 264.7 세포의 생존능력과 레스베라트롤의 면역제어 활성을 증가

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레스베라트롤의 면역제어 성질과 대식세포의 생존능력과 관련하여 당화된 레스베라트롤의 효과를 확인하기 위해 대식세포 RAW 264.7에서 연구하였다. 인비토로에서 대식세포에서 총 4개의 레스베라트롤 및 당화된 유도체 (E)-resveratrol, (E)-resveratrol 3-*O*-β-D-glucoside (R-3-G), 및 (E)-resveratrol 4'-*O*-β-D-glucoside (R-4'-G)를 여러 가지 농도로 처리한 후 일산화질소 (NO)와 인터루킨 6 (IL-6) 발현을 연구하였다. 앞서 언급한 물질로 처리한 후 인비토로에서 RAW 264.7 세포의 생존능력도 연구하였다. 대식세포 생존능력 평가분석 결과를 보면, 두 개의 레스베라트롤 모노글루코사이드인 R-3-G와 R-4'-G은 (E)-resveratrol와 비교하여 A549 and HepG2 세포에서 50–80% 감소된 독성을 보여준다. 당이 없는 레스베라트롤과 비교하면, 당화된 레스베라트 유도체는 긍정적으로 전사적으로 IL-6 및 iNOS 발현이 높아지는 방향으로 NO 및 대식세포에서 IL-6의 생산이 조절된다. 레스베라트롤의 당의 역할은 RAW 264.7 세포의 생존능력과 레스베라트롤의 면역제어 활성을 증가시켜 주는 것으로 보여주고 있다.

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