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Effects of physiological quercetin metabolites on interleukin-1β-induced inducible NOS expression

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Abstract

Cytokines released by inflammatory cells around the pancreatic islets are implicated in the pathogenesis of diabetes mellitus. Specifically, interleukin-1β (IL-1β) is known to be involved in islet β-cell damage by activation of nuclear factor-κB (NF-κB)-mediated inducible nitric oxide synthase (iNOS) gene expression. Though most flavonoids are shown to have various beneficial effects, little is known about the anti-inflammatory effects of their metabolites. Therefore, we investigated the effects of quercetin and its metabolites quercetin 3′-sulfate, quercetin 3-glucuronide and isorhamnetin 3-glucuronide on IL-1β-stimulated iNOS gene expression in RINm5F β-cells. The nitrite level, iNOS protein and its mRNA expression levels and iNOS promoter activity were measured. In addition, IκBα protein phosphorylation, nuclear translocation of nuclear factor-κB (NF-κB) and NF-κB DNA binding activity were determined. Adenosine 5′-triphosphate disodium salt-induced insulin release was also measured. Quercetin significantly reduced IL-1β-induced nitrite production, iNOS protein and its mRNA expression levels, and it also inhibited IL-1β-induced IκBα phosphorylation, NF-κB activation and iNOS promoter activity. Additionally, quercetin significantly restored the inhibition of insulin secretion by IL-1β. Meanwhile, quercetin metabolites did not show any effect on IL-1β-induced iNOS gene expression and also on insulin secretion. Therefore, in terms of iNOS expression mechanism, dietary ingestion of quercetin is unlikely to show anti-inflammatory effects in rat islet β-cells exposed to IL-1β.

Keywords: Quercetin; Quercetin metabolites; Interleukin-1β; iNOS; NF-κB; RINm5F β-cells

1. Introduction

The characteristic feature of type 1 diabetes mellitus is an autoimmune insulitis, namely, the infiltration of inflammatory cells within and around the pancreatic islets [1]. Various proinflammatory agents such as cytokines and free radicals are implicated in autoimmune insulitis [2]. Specifically, interleukin-1β (IL-1β) induces the expression of inducible nitric oxide synthase (iNOS) and overproduction of NO in islet β-cells, which lead to insulin secretion defect and β-cell injury [3,4].

Accumulating evidences suggest that the β-cells are main source of cytokine-mediated free radical formation [5,6]. Since β-cells have low expression of antioxidant enzymes, β-cells are prone to be a target of free radicals which lead to β-cell dysfunction [7]. In addition, inflammatory cytokines such as IL-1β, tumor necrosis factor-α (TNF-α) and interferon-γ are known to induce numerous target genes including iNOS in β-cells. Among these genes, almost 50% is NO-dependent, which implies that NO plays a major role in cytokine-induced β-cells damage [8]. Accordingly, the agents to inhibit iNOS gene expression may be helpful for the protection of β-cells.

Quercetin is a flavonoid which is found in various fruits, vegetables and red wine [9]. There are growing evidences that flavonoids may have antioxidant and anticarcinogenic activities [10,11]. Also, quercetin is known to have many beneficial effects in pancreatic islet β-cells. Quercetin protected β-cells against oxidative stress and recovered insulin secretion in streptozotocin-induced diabetic rats [12]. Quercetin was shown to reduce the disturbance of hepatic gene expression in streptozotocin-treated mice and to potentiate glucose-induced insulin secretion in insulin 1 (INS-1) β-cells [13,14]. Recently, we reported that quercetin induced catalytic subunit of γ-glutamyl-cysteine ligase in INS-1 β-cells and inhibited IL-1β-induced iNOS protein expression in RINm5F cells [15].

Meanwhile, quercetin is quickly absorbed from the gastrointestinal tract and is metabolically transformed to sulfate and glucuronide conjugates, whereas quercetin metabolites are slowly excreted via kidney [16]. To our knowledge, there is no report about the effect of quercetin metabolites on pancreatic islet β-cells. Therefore, in this study, we compared the effects of quercetin and its metabolites quercetin 3′-sulfate, quercetin 3-glucuronide and isorhamnetin 3-

Abbreviations: IL-1β, interleukin-1β; iNOS, inducible nitric oxide synthase; NO, nitric oxide; NF-κB, nuclear factor-κB; ATP, adenosine 5′-triphosphate disodium salt; PMSF, phenylmethylsulfonyl fluoride.

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glucuronide in terms of IL-1β-induced iNOS gene expression using RINm5F β-cells.

2. Materials and methods

2.1. Materials

Fetal bovine serum (FBS), RPMI 1640 medium and Lipofectamine 2000 reagent were purchased from Gibco BRL. Human IL-1β was from R&D systems (Minneapolis, MN, USA). Griess Reagent System, pGEM-T Easy Vector, pGL3 Luciferase Reporter Vector, Dual-Luciferase Reporter Assay System, pRL-TK and T4 polynucleotide kinase were from Promega (Madison, WI, USA). Top-Pfu DNA polymerase was from Bioonline. Anti-mouse iNOS antibody was from BD Transduction Laboratories (Palo Alto, CA, USA). Western Blotting Luminol Reagent, NF-κB oligonucleotides probe, and antibodies to p50 and p65 were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-rabbit inhibitor of κB (IκBα) and anti-mouse phospho-IκBα antibodies were from Cell Signaling Technology (Beverly, MA, USA). RNA STAT-60 was from TEL-TEST (Friendswood, TX, USA). AccuPrep genomic DNA extraction kit was from Bioneer (Daejeon, Korea). First Strand cDNA Synthesis Kit for reverse transcriptase polymerase chain reaction (PCR), alkaline phosphatase-conjugated anti-digoxigenin antibody, CDP-Star and 4′,6-diamidino-2′-phenylindole dihydrochloride (DAPI) were from Roche Diagnostics (Mannheim, Germany). Rat/Mouse insulin enzyme-linked immunosorbent assay (ELISA) kit was from Millipore (Billerica, MA, USA). All other reagents including quercetin, peroxidase-conjugated anti-mouse antibody and adenosine 5′-triphosphate disodium salt (ATP) were purchased from Sigma (St. Louis, MO, USA). Quercetin 3′-sulfate, quercetin 3-glucuronide and isorhamnetin 3-glucuronide were synthesized as previously described [17] (see Fig. 1 for structures).

2.2. Cell culture

RINm5F (CRL-11605) cell, a rat insulinoma cell line, was purchased from American Type Tissue Collection (Manassas, VA, USA). RINm5F cells were cultured in RPMI-1640 medium supplemented with 10% FBS, 2 mM L-glutamine and antibiotics (100 U/ml penicillin and 100 U/ml streptomycin) and maintained at 37°C in humidified air containing 5% CO_2. Cells were subcultured weekly, and cells of passages 19–30 were used in this study. Cells were plated at a concentration of 2×10^5 per ml. At 70% confluency, the cells were incubated with serum-free RPMI medium for 16 h prior to the incubation with IL-1β (100 pg/ml).

2.3. Measurement of NO as nitrite

RINm5F cells were treated with quercetin, quercetin 3′-sulfate, quercetin 3-glucuronide and isorhamnetin 3-glucuronide (10 μM) for 1 h and then incubated with IL-1β (100 pg/ml) for an additional 24 h. Released nitrite, a stable product of NO in aqueous medium, was measured using Griess Reagent System (Promega, Madison, WI, USA) as described previously [18]. Briefly, the culture medium was mixed with an equal volume of sulfanilamide solution (1% in 5% phosphoric acid) and of *N*-1-naphthyl ethylenediamine dihydrochloride solution (0.1% in water). The absorbance was measured at 540 nm on MR700 Microplate Reader (Dynatech Laboratories Inc., Chantilly, VA, USA). Nitrite concentrations were determined based on a calibration curve of standard $NaNO_2$ concentrations against absorbance.

2.4. Immunofluorescent staining

The immunofluorescent staining was performed as described previously [18,19]. RINm5F cells were treated with IL-1β (100 pg/ml) for 30 min following pretreatment of quercetin, quercetin 3′-sulfate, quercetin 3-glucuronide and isorhamnetin 3-glucuronide for 10 min. Then, the cells were fixed with 2% paraformaldehyde in phosphate-buffered saline (PBS), and the cells were subsequently treated with 0.1% Triton X-100 in PBS for 30 s to permeabilize cell membrane. After blocking nonspecific reaction using normal goat serum, the cells were incubated with anti-rabbit p65 IgG (1:400). Subsequently, the cells were incubated with fluorescein isothiocynate (FITC)-conjugated anti-rabbit IgG as a secondary antibody. Following the incubation of secondary antibody, the cells were rinsed with methanol and then incubated with 4′,6-diamidino-2′-phenylindole dihydrochloride (DAPI, 1 μg/ml) in methanol for 15 min at 37°C to observe nucleus. Green (FITC-p65) and blue (DAPI) images were acquired by Olympus 1X71 fluorescent microscope equipped with CCD camera (Olympus Corporation, Tokyo, Japan). Negative control was stained with the secondary antibody alone.

2.5. Western blot analysis

RINm5F cells were treated with quercetin, quercetin 3′-sulfate, quercetin 3-glucuronide and isorhamnetin 3-glucuronide (10 μM) for 1 h and then incubated with IL-1β (100 pg/ml) for 16 h. Western blot analysis was performed as described previously [18,19]. The cells were harvested and solubilized with lysis buffer [25 mM Tris-HCl, pH 7.4, 0.1% sodium dodecyl sulfate (SDS), 0.1% Triton X-100, 1% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl

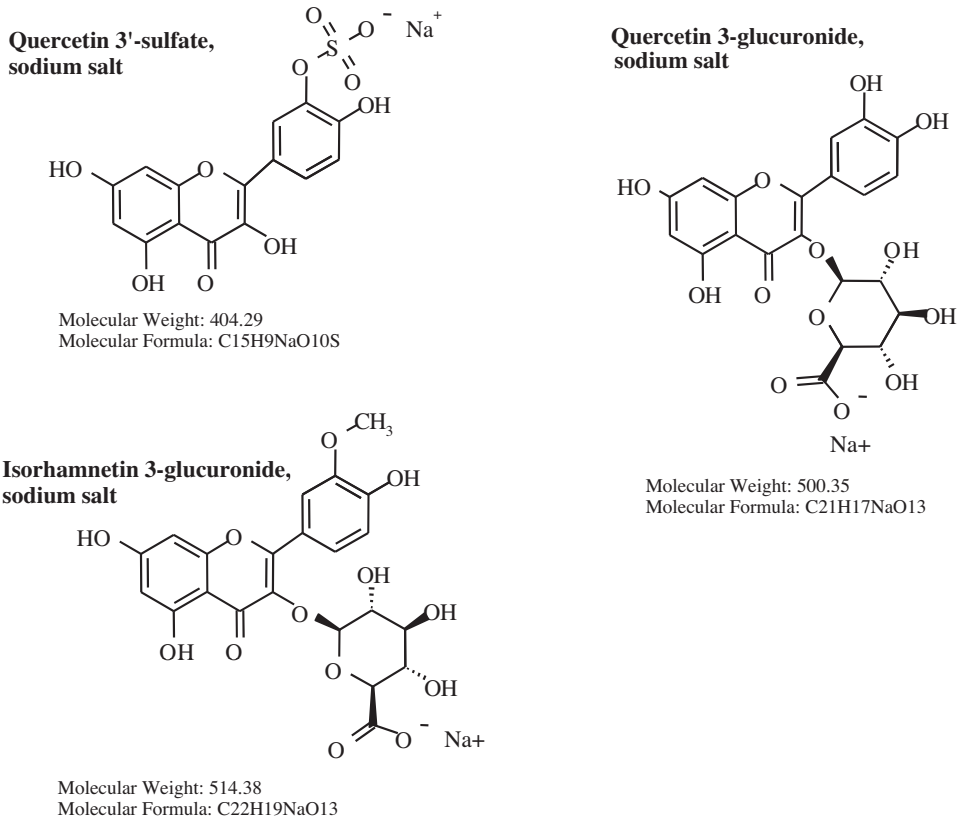

Fig. 1. Molecular structures of quercetin metabolites.

fluoride (PMSF), 10 μg/ml aprotinin and 5 μg/ml leupeptin]. The soluble fraction was collected, and protein content was determined by bovine serum albumin (BSA) assay. Thirty micrograms of total protein was separated on an 8% SDS-polyacrylamide gel electrophoresis (PAGE) and transferred onto nitrocellulose membrane. After blocking with 5% skimmed milk, the membrane was incubated with anti-mouse iNOS IgG [0.5 μg/ml of TTBS (20 mM Tris-HCl, pH 7.4, 150 mM NaCl and 0.1% Tween 20)]. Then, the membrane was probed with peroxidase-conjugated anti-mouse IgG (0.5 μg/ml of TTBS). The immunoreactive band was detected by enhanced chemiluminescence using Western Blotting Luminol Reagent (Santa-Cruz Biotechnology, Santa-Cruz, CA, USA). For the measurement of IκBα protein phosphorylation, the cells were pretreated with quercetin, quercetin 3′-sulfate, quercetin 3-glucuronide and isorhamnetin 3-glucuronide for 30 min and then incubated with IL-1β for 30 min. Equal loading and transfer of samples were verified by Ponceau S staining or the band intensity of β-tubulin (52 kDa). The immunoreactive band intensity was determined by densitometry using Scion Image program (Scion Corporation). Each value was normalized by the ratio of iNOS or phospho-IκBα band intensity to β-tubulin band intensity.

2.6. Northern blot analysis

RINm5F cells were treated with quercetin, quercetin 3′-sulfate, quercetin 3-glucuronide and isorhamnetin 3-glucuronide (10 μM) for 1 h and then incubated with IL-1β (100 pg/ml) for 6 h. Total RNA isolation and the method for Northern blot analysis were described previously [20,21]. The preparation of iNOS probe was demonstrated in the previous study [18]. Equal loading of sample was verified ribosomal 18S and 28S bands. Each band intensity was determined by densitometry using Scion Image program. Each value was normalized by the ratio of iNOS mRNA band intensity to 18S band intensity.

2.7. Preparation of nuclear extracts and electrophoretic mobility shift assay (EMSA)

RINm5F cells were treated with IL-1β (100 pg/ml) for 30 min following pretreatment of quercetin, quercetin 3′-sulfate, quercetin 3-glucuronide and isorhamnetin 3-glucuronide for 10 min. Nuclear extracts were isolated according to a modified procedure described previously [18,21]. The cells were rinsed with PBS, suspended in hypotonic buffer A [10 mM KCl, 10 mM HEPES, pH 7.9, 1 mM PMSF, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol (DTT), 0.2 mM Na_3VO_4, 1 μg/ml leupeptin, 1 μg/ml aprotinin and 0.5% Nonidet P-40] for 15 min on ice. Nuclei were pelleted by centrifugation at 12,000*g* for 15 s at 4°C. Then, the pellets were suspended in hypertonic buffer B (400 mM NaCl, 20 mM HEPES, pH 7.9, 1 mM PMSF, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.2 mM Na_3VO_4, 1 μg/ml leupeptin 1 μg/ml aprotinin) for 30 min on ice. The nuclear proteins in the supernatant were recovered after centrifugation at 12,000*g* for 10 min and stored at −80°C. For DNA binding activity of NF-κB, the oligonucleotide probe was described in a previous study [18]. Two complementary oligonucleotides were end-labeled with [γ-^{32}P]dATP using T4 polynucleotide kinase. Nuclear extracts (10 μg) were incubated with the ^{32}P-labeled probe (30,000 cpm) in binding reaction buffer [10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM DTT, 1 μg poly (dI–dC) and 5% glycerol] for 20 min at 4°C. The reaction product was resolved on a 6% nondenaturing polyacrylamide gel in Tris/borate/EDTA buffer. The gel was dried and exposed at −80°C. For supershift EMSA experiments, 0.2 μg of specific antibodies against NF-κB proteins p50 or p65 was applied to the reaction mixture for 30 min before the addition of ^{32}P-labeled probe.

2.8. Rat iNOS promoter preparation

Rat genomic DNA was prepared from RINm5F cells using AccuPrep genomic DNA extraction kit according to the manufacturer's instruction. The rat iNOS promoter region ranging from −1173 to +33 was amplified by PCR using Top-Pfu DNA polymerase. Primers used were as follows: forward, from −1173, 5′-CCGGTACCAAGGCAAGCACTTTGACGACTC with *Kpn*I site (underlined); reverse, from +33, 5′-CCGCTCGAGAGAGTCTCAGTCTTCAACTCCCTG with *Xho*I site (underlined). The PCR product was amplified from agarose gel, digested and cloned into *Kpn*I and *Xho*I sites of pGL3 basic vector to obtain piNOS-LUC plasmid construct [18]. The construct was verified by DNA sequencing using An ABI PRISM 310 genetic analyzer (Perkin Elmer Ltd. Co., Seoul, Korea).

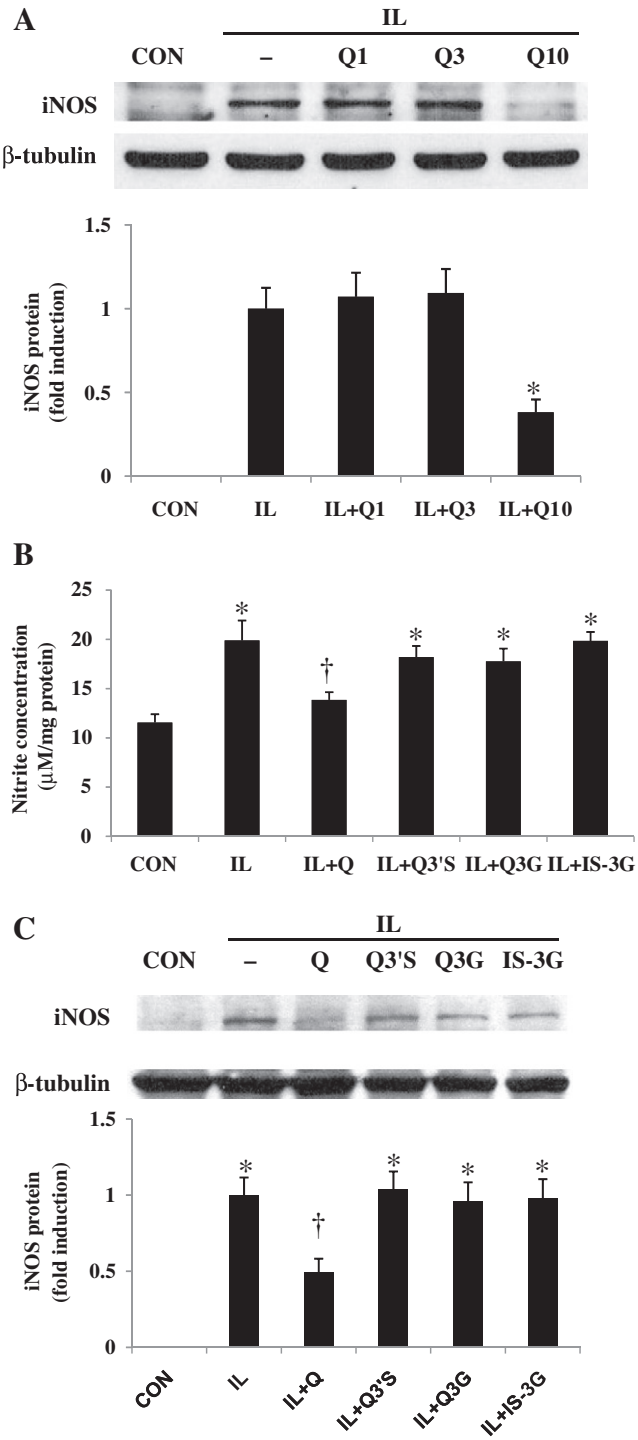

Fig. 2. Effects of quercetin and its metabolites on IL-1β-induced nitrite production and iNOS protein in RINm5F cells. (A) Dose-dependent effects of quercetin on iNOS protein expression by IL-1β. Whole cell lysates were collected from RINm5F cells treated with quercetin (1, 3 and 10 μM) for 1 h prior to exposure of IL-1β (100 pg/ml) for 16 h. The cellular protein (30 μg) was resolved by 8% SDS-PAGE and was subjected to Western blot using iNOS antibody as described in Materials and Methods. Data are expressed as mean±S.D. (n=3), and each value is expressed relative to IL-1β value. *P<.05 vs. IL. CON, control without any treatment; IL, IL-1β; Q1, Q3 and Q10, quercetin (1, 3 and 10 μM). (B) Effects of quercetin and its metabolites on nitrite production by IL-1β. RINm5F cells were pretreated with quercetin (10 μM) and its metabolites (10 μM) for 1 h prior to incubation with IL-1β (100 pg/ml) for 24 h. The culture supernatants (50 μl) were used for measurement of nitrite concentration using Griess reagent. Data are expressed as mean±S.D. (n=3). *P<.05 vs. CON; †P<.05 vs. IL. CON, control without any treatment; IL, IL-1β; Q, quercetin; Q3′S, quercetin 3′-sulfate; Q3G, quercetin 3-glucuronide; IS-3G, isorhamnetin 3-glucuronide. (C) Effects of quercetin and its metabolites on iNOS protein expression by IL-1β. Whole cell lysates were collected from RINm5F cells treated with quercetin (10 μM) and its metabolites (10 μM) for 1 h prior to exposure of IL-1β (100 pg/ml) for 16 h. The cellular protein (30 μg) was resolved by 8% SDS-PAGE and was subjected to Western blot using iNOS antibody as described in Materials and Methods. Data are expressed as mean±S.D. (n=3), and each value is expressed relative to IL-1β value. *P<.05 vs. IL. CON, control without any treatment; IL, IL-1β; Q, quercetin; Q3′S, quercetin 3′-sulfate; Q3G, quercetin 3-glucuronide; IS-3G, isorhamnetin 3-glucuronide.

2.9. Transient transfection and iNOS promoter luciferase expression assay

Transient transfection was performed using Lipofectamine 2000 reagent as described previously [21]. Briefly, RINm5F cells (0.5 μg/3×10^5 cells) were transiently transfected with piNOS-LUC plasmid construct. After transfection for 16 h, the cells were pretreated with 10 μM of quercetin, quercetin 3′-sulfate, quercetin 3-glucuronide and isorhamnetin 3-glucuronide for 30 min, and then IL-1β (100 pg/ml) was added for 8 h. The cells were harvested for determination of the firefly and Renilla luciferase activities. All data were normalized by the ratio of firefly to Renilla luciferase activities.

2.10. Insulin secretion test

Measurement of insulin release analysis was performed as described previously [18]. Briefly, following the treatment of IL-1β (100 pg/ml) for 16 h with the pretreatment of 10 μM of quercetin, quercetin 3′-sulfate, quercetin 3-glucuronide and isorhamnetin 3-glucuronide for 1 h, the cells were washed with Krebs–Ringer bicarbonate (KRB) buffer. Following the treatments of ATP (100 μM) for 30 min, the media were collected, and the insulin content was determined by rat insulin ELISA kit. Data are expressed as ng per mg protein to adjust for the differences in the cell mass among the groups. Also, we performed insulin secretion test to observe dose-dependent effects of quercetin and its metabolites (3, 10 and 30 μM) according to the same procedure as we mentioned above.

2.11. Statistical analysis

The relative band densities were quantified using Scion Imaging software (Scion Corporation, Frederick, MD, USA). All data obtained from each experiment were expressed as mean±S.D. The data were analyzed using one-way analysis of variance (ANOVA) with Origin 7.0 software (Microcal Software, Northampton, MA, USA). Statistical comparisons among the groups were done by Bonferroni's multiple-range *t* test after the ANOVA. $P<.05$ was accepted as statistically significant.

3. Results

3.1. Effects of quercetin metabolites on IL-1β-induced nitrite production and iNOS protein

First, we examined the dose-dependent effects of quercetin on IL-1β-induced iNOS protein expression. As shown in Fig. 2A, quercetin did not inhibit IL-1β-induced iNOS protein expression at concentrations of 1 and 3 μM; however, quercetin of 10 μM significantly inhibited IL-1β-induced iNOS protein expression. Based on this result, we set the concentrations of quercetin and its metabolites at 10 μM in all the experiments. Subsequently, we examined the effects of quercetin and its metabolites on IL-1β-induced nitrite production in RINm5F cells. As shown in Fig. 2B, pretreatment of quercetin significantly decreased nitrite production by IL-1β. However, the quercetin metabolites tested did not reduce IL-1β-induced nitrite production. Also, we observed whether quercetin or its metabolites affected IL-1β-induced iNOS protein expression (Fig. 2C). Quercetin significantly inhibited IL-1β-induced iNOS protein, while the quercetin metabolites had no effect on iNOS protein by IL-1β.

3.2. Effects of quercetin metabolites on IL-1β-induced iNOS mRNA expression and iNOS promoter activity

To investigate whether quercetin and its metabolites inhibit IL-1β-induced iNOS at the mRNA level, Northern blot analysis was performed. Results showed that quercetin significantly reduced iNOS mRNA expression by IL-1β, while its metabolites did not affect it (Fig. 3A). We then investigated whether IL-1β-induced NF-κB binding to iNOS promoter mediates NF-κB-dependent iNOS gene transcription. After transient transfection of iNOS promoter containing two κB binding sites into RINm5F cells, promoter activity was measured. As shown in Fig. 3B, pretreatment of quercetin significantly inhibited IL-1β-induced iNOS promoter activity. In contrast, none of its metabolites affected iNOS promoter activity.

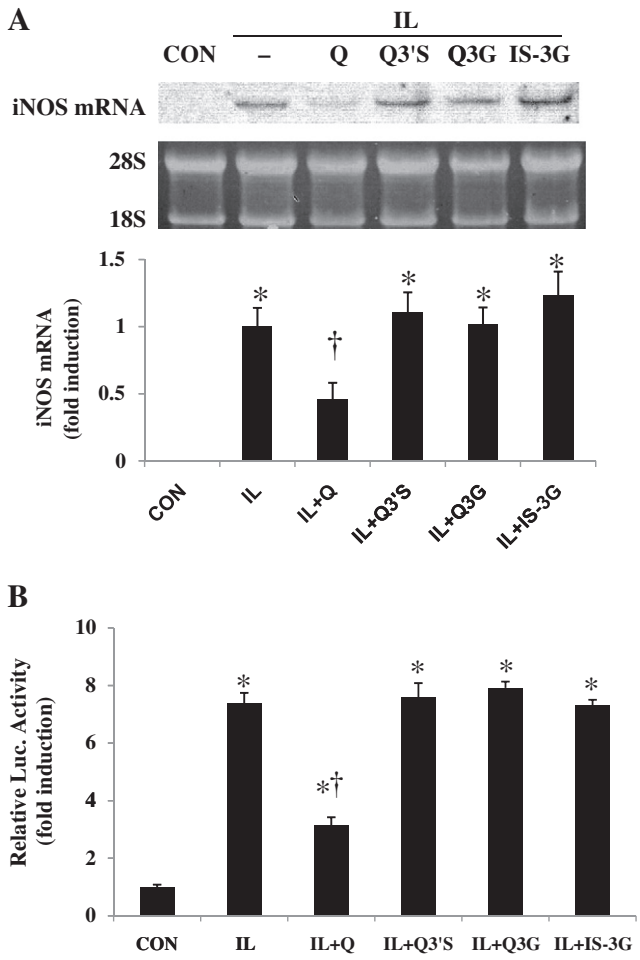

Fig. 3. Effects of quercetin and its metabolites on IL-1β-induced iNOS mRNA expression and iNOS promoter luciferase activity. (A) Effects of quercetin and its metabolites on iNOS mRNA levels induced by IL-1β. RINm5F cells were pretreated with quercetin (10 μM) and its metabolites (10 μM) for 1 h, and then the cells were incubated with IL-1β (100 pg/ml) for 8 h. Equal loading of sample was verified by ribosomal 18S and 28S bands. Data are expressed as mean±S.D. ($n=3$), and each value is expressed relative to IL-1β value. *$P<.05$ vs. IL. CON, control without any treatment; IL, IL-1β; Q, quercetin; Q3′S, quercetin 3′-sulfate; Q3G, quercetin 3-glucuronide; IS-3G, isorhamnetin 3-glucuronide. (B) Effects of quercetin and its metabolites on relative iNOS promoter activity. After transient transfection of iNOS promoter, the cells were pretreated with quercetin (10 μM) and its metabolites (10 μM) for 30 min and then incubated with IL-1β (100 pg/ml) for 8 h. The cells were collected and measured by dual luciferase assays as described in Materials and Methods. The efficiencies were normalized by the ratio of firefly to Renilla luciferase activity. Data are expressed as mean±S.D. ($n=3$), and each value is expressed relative to the control. *$P<.05$ vs. CON; †$P<.05$ vs. IL. CON, control without any treatment; IL, IL-1β; Q, quercetin; Q3′S, quercetin 3′-sulfate; Q3G, quercetin 3-glucuronide; IS-3G, isorhamnetin 3-glucuronide.

3.3. Effects of quercetin metabolites on IL-1β-induced activation of NF-κB binding activity, IκBα phosphorylation and nuclear translocation of p65

Since the activation of NF-κB is a critical step for the IL-1β-induced iNOS expression, we tested whether quercetin and its metabolites affect the binding of NF-κB to the iNOS promoter by EMSA. As shown in Fig. 4A1, pretreatment of quercetin markedly inhibited IL-1β-induced binding activity of NF-κB with κB sites, whereas its metabolites did not inhibit it. Next, we characterized proteins in DNA–protein complex using specific antibodies against NF-κB subunits p65 and p50. The supershifted band was strongly detected by the addition of p65 antibody and was also mildly detected by p50 (Fig. 4A2). Subsequently, we examined whether quercetin and its

metabolites decrease IL-1β-induced IκBα phosphorylation in the cells. The result demonstrated that quercetin significantly inhibited the IL-1β-induced IκBα phosphorylation, while its metabolites did not inhibit it (Fig. 4B). Next, we observed nuclear translocation of p65 in the cells using immunofluorescent staining. In control group, diffuse cytoplasmic staining was observed, while in IL-1β-treated group, clear nuclear staining was observed in all the cells, indicating nuclear translocation of p65 (Fig. 4C). Meanwhile, pretreatment of quercetin partially inhibited IL-1β-induced nuclear translocation of p65 in some cells (Fig. 4C, white arrows), but not in other cells (Fig. 4C, red arrows). Unlike quercetin, all quercetin metabolites tested did not affect IL-1β-induced nuclear translocation of p65 (Fig. 4C).

3.4. Effects of quercetin metabolites on IL-1β-induced inhibition of insulin release

Insulin release is significantly inhibited at a high concentration of and at a long exposure to cytokines such as IL-1β. To determine

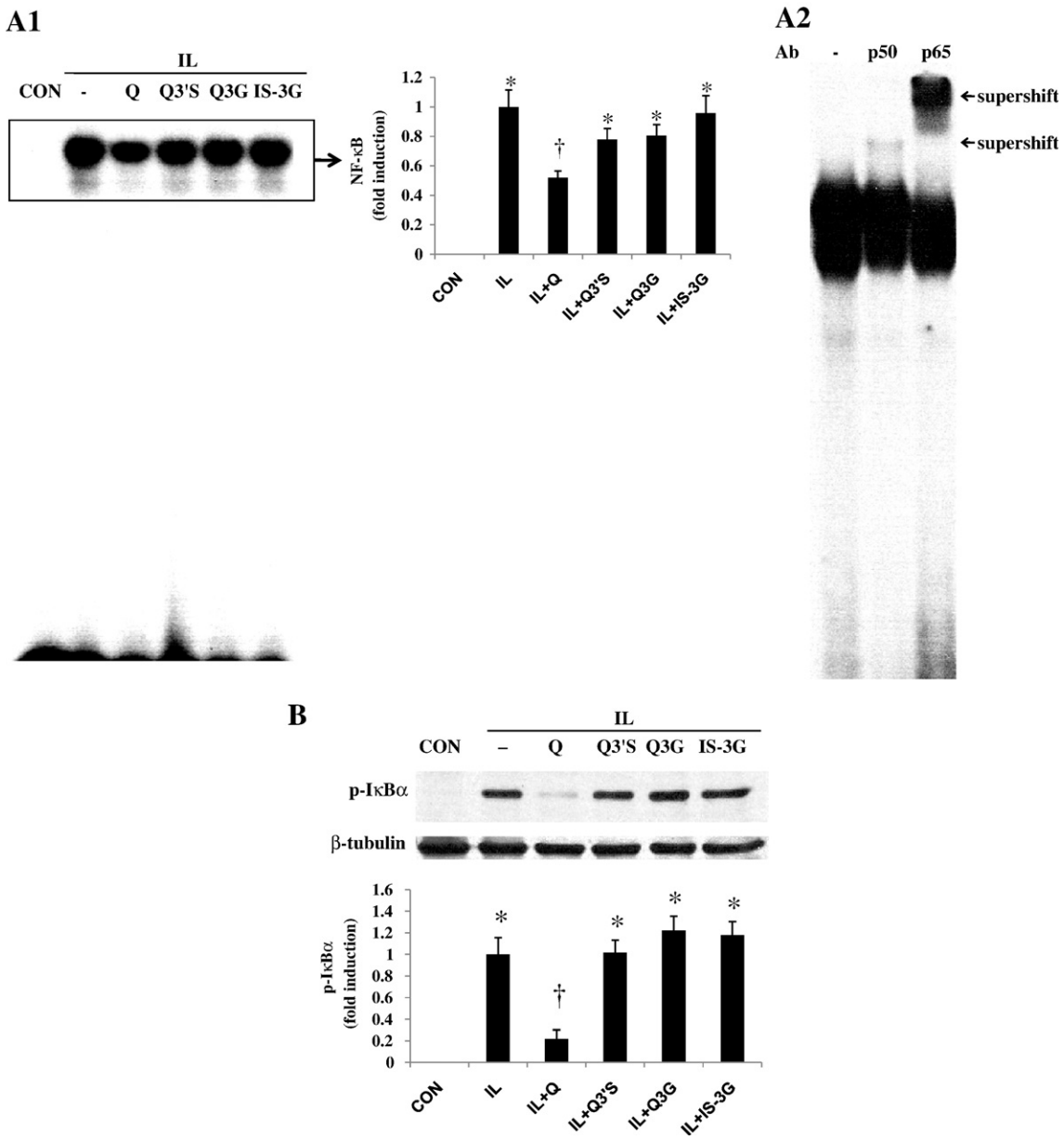

Fig. 4. Effects of quercetin and its metabolites on IL-1β-induced NF-κB activation. (A1) EMSA of NF-κB binding site on rat iNOS promoter. RINm5F cells were pretreated with quercetin (10 μM) and its metabolites (10 μM) for 30 min and then incubated with IL-1β (100 pg/ml) for 30 min. Nuclear extracts were analyzed on EMSA using NF-κB consensus probe. Data are expressed as mean±S.D. (n=3), and each value is expressed relative to IL-1β value. *P<.05 vs. IL. CON, control without any treatment; IL, IL-1β; Q, quercetin; Q3′S, quercetin 3′-sulfate; Q3G, quercetin 3-glucuronide; IS-3G, isorhamnetin 3-glucuronide. (A2) Immune-supershift EMSA. For the supershift assay, specific antibodies (0.2 μg) against NF-κB subunits (p50 and p65) were incubated with nuclear extracts for 30 min before the addition of probe. The arrows indicate specific band supershifted by anti-p50 (lower) and anti-p65 (upper) NF-κB subunit. (B) Effects of quercetin and its metabolites on IκBα phosphorylation following pretreatment of quercetin (10 μM) and its metabolites (10 μM) for 30 min and then stimulation with IL-1β (100 pg/ml) for 30 min. Then, IκBα phosphorylation was measured by Western blot analysis. Equal loading of samples was verified by reprobing the same blot with β-tubulin. Data are expressed as mean±S.D. (n=3), and each value is expressed relative to IL-1β value. *P<.05 vs. IL. CON, control without any treatment; IL, IL-1β; Q, quercetin; Q3′S, quercetin 3′-sulfate; Q3G, quercetin 3-glucuronide; IS-3G, isorhamnetin 3-glucuronide. (C) Immunofluorescent staining for p65 NF-κB subunit. RINm5F cells were pretreated with quercetin (10 μM) and its metabolites (10 μM) for 30 min and then incubated with IL-1β (100 pg/ml) for 30 min. Subsequently, the cells were processed to detect NF-κB localization (green color) and DAPI nuclear signal (blue color). CON, control without any treatment; IL, IL-1β; Q, quercetin; Q3′S, quercetin 3′-sulfate; Q3G, quercetin 3-glucuronide; IS-3G, isorhamnetin 3-glucuronide.

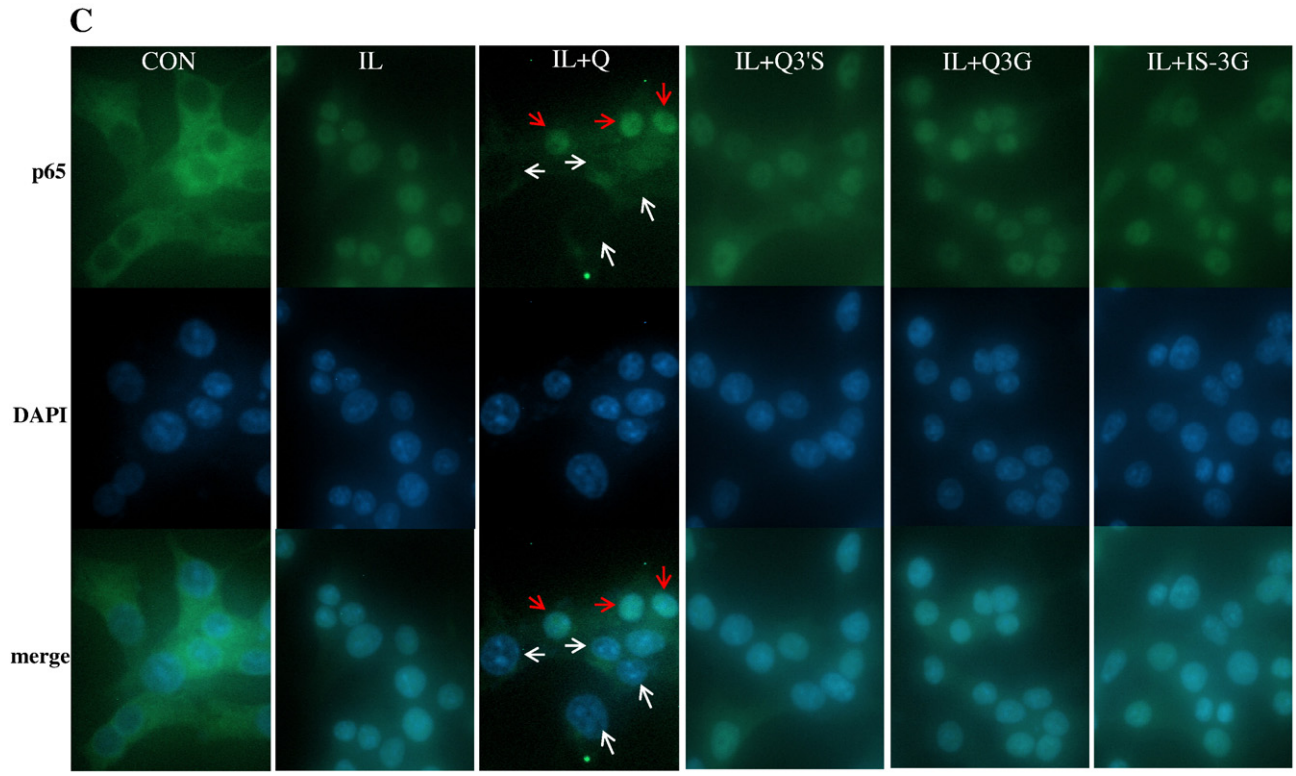

Fig. 4 (*continued*).

whether quercetin and its metabolites protect IL-1β-induced decrease in insulin secretion, insulin concentration was measured in RINm5F cells. Since RINm5F cells do not have type 2 glucose transporter, glucose cannot stimulate insulin secretion [22]. Therefore, ATP was applied to stimulate insulin secretion because RINm5F cells have purinergic P2Y receptor [23]. As shown in Fig. 5A, quercetin significantly restored IL-1β-induced decrease in insulin secretion toward control level, while its metabolites did not affect it. We further examined the dose-dependent effects of quercetin and its metabolites on insulin secretion. As shown in Fig. 5B, quercetin metabolites did not prevent IL-1β-induced decrease in insulin secretion at all concentrations tested. Also, there was no difference in insulin secretion according to concentration difference of quercetin metabolites. Meanwhile, quercetin did not inhibit IL-1β-induced decrease in insulin secretion at 3 μM, and there was no further inhibitory effect of quercetin at 30 μM than at 10 μM.

4. Discussion

This is the first study to evaluate the effect of quercetin metabolites on IL-1β-induced iNOS gene expression mechanism using islet β-cells. The excessive NO production via IL-1β-mediated iNOS induction was known to be involved in β-cell dysfunction of type 1 diabetes mellitus [24,25]. Since NO is very reactive, it is readily converted to highly toxic peroxynitrite [26]. Peroxynitrite was found to induce DNA strand breaks in islet β-cells, and this was accompanied by mitochondrial damage leading to the β-cell death [27].

The clinical association of NO in type 1 diabetes incidence was demonstrated in the epidemiologic studies. In the study using Colorado, USA, children with type 1 diabetes, nitrate exposure through drinking water may be related with the etiology of type 1 diabetes incidence [28]. In the study using Finnish children, higher dietary intake of nitrites was closely associated with the development of type 1 diabetes [29]. These findings have evoked a lot of interest in the protective effects of free radical scavengers such as natural and synthetic antioxidants on the incidence of type 1 diabetes.

Pancreatic islet β-cells are well known to have very low antioxidant enzyme activities compared with the liver and kidney [30,31]. The RINm5F β-cell line is comparable to primary rat islet β-cells in terms of IL-1β-mediated iNOS expression, NO production and β-cell cytotoxicity [32–34]; especially, IL-1β-induced iNOS expression was dependent on p38 mitogen-activated protein kinase (MAPK) and extracellular signal-regulated kinase (ERK) activation in both RINm5F cell and primary rat islets [34]. We previously demonstrated that the response of RINm5F β-cell to IL-1β was similar to that of the primary isolated islets in nitrite formation and iNOS protein expression [18,35]. These facts suggest that RINm5F β-cell may be an appropriate substitute for the primary rat islet β-cells. Therefore, we aimed to compare the effects of quercetin and its metabolites on IL-1β-mediated iNOS expression mechanisms employing RINm5F β-cell.

During absorption from the gastrointestinal tract, dietary flavonoids such as quercetin glycosides are modified as follows: deglycosylation in the small intestine, and rapid and extensive phase-2 conjugation in the small intestine and liver [36,37]. Through this structural modification, quercetin metabolites quercetin 3′-sulfate, quercetin 3-glucuronide and isorhamnetin 3-glucuronide (3′-methylquercetin 3-glucuronide) make up 93% of total plasma quercetin, while quercetin aglycone and the dietary quercetin glycosides exist at low concentrations in the plasma [38]. Accordingly, quercetin metabolites are structurally different from their parent aglycones, and their effects on cell function are presumed to be different from the parent aglycones depending on their extent of conjugation [39]. It is generally known that the maximal plasma concentration of quercetin can reach between 2 and 7 μM [16]. In our previous study, 3 μM of quercetin aglycone did not significantly inhibit IL-1β-induced iNOS protein expression [15]. In addition, quercetin aglycone up to 100 μM did not show any effect on cell viability and cell morphology, whereas some flavonoids — apigenin and resveratrol —

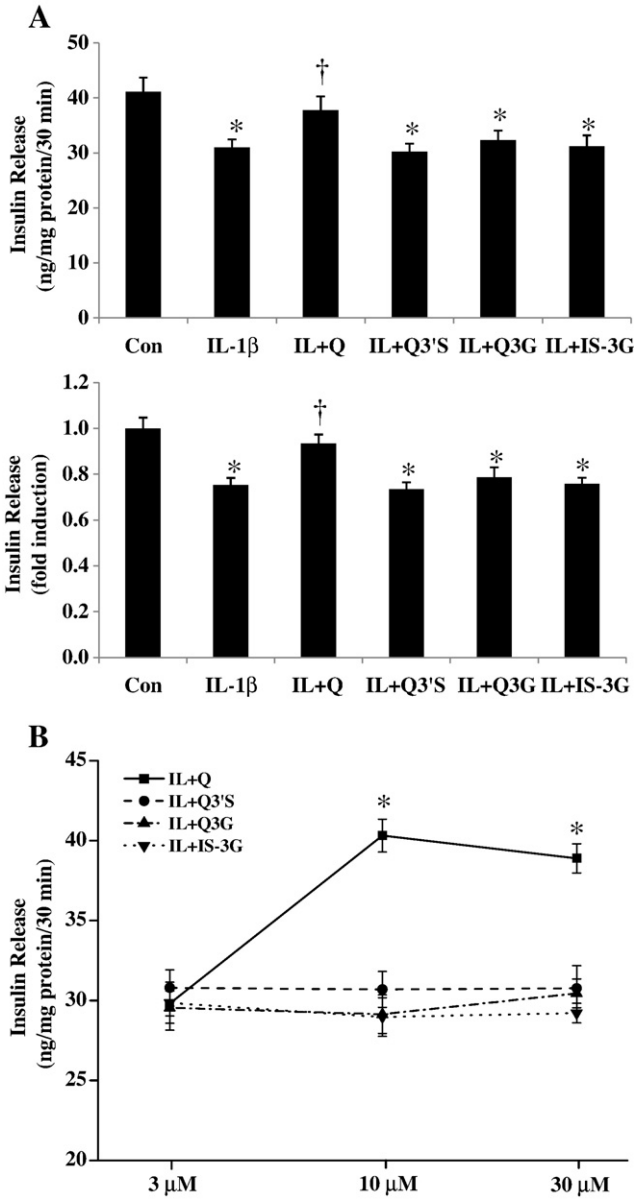

Fig. 5. Effects of quercetin and its metabolites on IL-1β-induced inhibition in insulin secretion. (A) RINm5F cells were pretreated with quercetin (10 μM) and its metabolites (10 μM) for 1 h and then stimulated with IL-1β (100 pg/ml) for 16 h. The cells were washed with KRB buffer and incubated with ATP (100 μM) for 30 min. Following incubation, the media were collected, and then insulin content was measured using rat insulin ELISA kit. Data are expressed as mean±S.D. (n=5). The lower graph is demonstrated as fold induction of control value based on the data of the upper graph. *P<.05 vs. CON; †P<.05 vs. IL. CON, control without any treatment; IL, IL-1β; Q, quercetin; Q3′S, quercetin 3′-sulfate; Q3G, quercetin 3-glucuronide; IS-3G, isorhamnetin 3-glucuronide. (B) Dose-dependent effects of quercetin and its metabolites on insulin secretion. RINm5F cells were pretreated with quercetin (3, 10 and 30 μM) and its metabolites (3, 10 and 30 μM) for 1 h and then stimulated with IL-1β (100 pg/ml) for 16 h. The cells were washed with KRB buffer and incubated with ATP (100 μM) for 30 min. Data are expressed as mean±S.D. (n=3). *P<.05 vs. quercetin metabolites. CON, control without any treatment; IL, IL-1β; Q, quercetin; Q3′S, quercetin 3′-sulfate; Q3G, quercetin 3-glucuronide; IS-3G, isorhamnetin 3-glucuronide.

reduced cell viability at 100 μM (our unpublished data). Therefore, in this study, we tested the effects of quercetin and its metabolites at a supraphysiological concentration of 10 μM.

In our previous study, IL-1β significantly induced nitrite production at a concentration of 100 pg/ml; however, no further increase in nitrite production was observed at concentrations higher than 100 pg/ml [18]. The plasma concentration of IL-1β in normal humans was less than 10 pg/ml; even in acute myocardial infarct patients, IL-1β concentration was less than 30 pg/ml [40,41]. Also, IL-1β of 100 pg/ml significantly induced nitric oxide in RIN cells or rat islets [42,43]. Therefore, we set the concentration of IL-1β at 100 pg/ml in this study.

In the present study, quercetin significantly inhibited IL-1β-induced nitrite production, iNOS protein and its mRNA expressions, whereas quercetin metabolites did not inhibit them. To further examine these results, we compared the effects of quercetin and its metabolites on the rat iNOS promoter activity since the iNOS gene induction is primarily regulated by the transactivation of iNOS promoter through primary transcription factors including NF-κB [44]. Consistent with the result of iNOS mRNA, quercetin suppressed the iNOS promoter activity induced by IL-1β; meanwhile, the quercetin metabolites did not suppress IL-1β-induced iNOS promoter activity.

NF-κB is well known to be the main transcription factor in the regulation of iNOS gene transcription [33,45]. In a resting condition, NF-κB is bound to the inhibitory protein IκB in the cytosol. However, on its activation, IκB becomes phosphorylated at serine residues (Ser-32 and Ser-36), which triggers a proteolytic degradation of IκB leading to the release and translocation of the NF-κB dimer to the nucleus, where binding of NF-κB to the κB binding sites on the promoter region occurs, and this finally induces gene transcription [46]. In this study, quercetin significantly inhibited IL-1β-induced IκBα phosphorylation, whereas quercetin metabolites did not inhibit it. Also, quercetin prevented IL-1β-induced nuclear translocation of p65 in some cells, but not in other cells. At present, we do not explain why quercetin changed nuclear translocation of p65 in some cells but not in all cells. However, a similar finding was also reported in our previous study in which epicatechin, one of the flavonoids, prevented IL-1β-induced nuclear translocation of p65 in some cells but not in other cells [18]. In addition, EMSA showed that quercetin inhibited IL-1β-induced binding of NF-κB to iNOS promoter region, whereas the quercetin metabolites did not inhibit it.

IL-1β has been shown to inhibit mitochondrial enzymes activities such as aconitase and NADH-ubiquinone oxidoreductase and to inhibit the oxidation of glucose to CO_2, which leads to the reduction of cellular ATP concentrations in the pancreatic islets. Additionally, NO mediates IL-1β-induced decrease in insulin secretion through mitochondrial dysfunction [47,48]. So we then examined the effects of quercetin and its metabolites on IL-1β-induced inhibition of insulin secretion. In this study, quercetin significantly restored IL-1β-induced inhibition of insulin secretion, whereas quercetin metabolites did not restore it.

Like our results in the quercetin metabolites, our previous study demonstrated that quercetin induced catalytic subunit of γ-glutamylcysteine ligase (GCLC) expression in INS-1 β-cells, whereas quercetin 3′-sulfate and quercetin 3-glucuronide did not induce GCLC expression [15]. Also, another study showed that quercetin metabolites had no effect on the expressions of vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule 1 (ICAM-1) and monocyte chemoattractant protein-1 in TNF-α-activated human vascular smooth muscle cells [49]. However, quercetin metabolites showed various effects depending on cells or experimental conditions. Quercetin and quercetin 3′-sulfate were shown to inhibit cyclooxygenase-2 activity in Caco-2 colon cancer cells, while quercetin 3-glucuronide and 3′-methylquercetin 3-glucuronide did not inhibit it [50]. In human neutrophils, quercetin and quercetin 3′-sulfate did not affect *N*-formyl-methionyl-leucyl-phenylalanine (fMLP)-stimulated elevation of intracellular calcium concentrations, whereas quercetin 3-glucuronide reduced fMLP-induced calcium response [51].

In the present study, different actions in IL-1β-stimulated iNOS expression between quercetin and its metabolites may be due to the

structural distinction of these chemicals. That is, quercetin is rather hydrophobic and can easily diffuse through the cell membrane and enter the cell interior. Meanwhile, structural modification by conjugation of quercetin tends to decrease hydrophobicity and limits passive diffusion into the cell [52]. Accordingly, unlike quercetin, quercetin metabolites may not enter the β-cells easily and may be unable to inhibit IL-1β-mediated inflammatory process.

Collectively, quercetin significantly inhibited IL-1β-induced iNOS gene expression by inhibition of NF-κB activation, and it restored the inhibition of insulin secretion by IL-1β, whereas all quercetin metabolites tested had no effect on iNOS gene expression and insulin secretion. Since the RINm5F cells used in this study are very similar to primary rat islet β-cells in terms of IL-1β-induced iNOS expression, dietary intake of quercetin aglycone in human appears to be ineffective in preventing cytokine-induced NO-mediated β-cell damage. Therefore, this study infers that the products of quercetin metabolism have no biological activity in pancreatic islet cells, and the use of quercetin in the treatment of inflammatory diabetic state is not indicated.

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