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Quercetin-Induced Upregulation of Human GCLC Gene Is Mediated by *cis*-Regulatory Element for Early Growth Response Protein-1 (EGR1) in INS-1 Beta-Cells

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ABSTRACT

The catalytic subunit of γ-glutamylcysteine ligase (GCLC) catalyses the rate-limiting step in the de novo synthesis of glutathione (GSH), which is involved in maintaining intracellular redox balance. GSH is especially important for antioxidant defense system since beta-cells show intrinsically low expression of antioxidant enzymes. In the present study, we investigated the regulatory mechanisms by which quercetin, a flavonoid, induces the expression of the GCLC gene in rat pancreatic beta-cell line INS-1. Promoter study found that the proximal GC-rich region (from −90 to −34) of the GCLC promoter contained the quercetin-responsive *cis*-element(s). The quercetin-responsive region contains consensus DNA binding site for early growth response 1 (EGR1) at -67 (5′-CGCCTCCGC-3′) which overlaps with a putative Sp1 binding site. Electrophoretic mobility shift assay showed that an oligonucleotide containing the EGR1 site was bound to nuclear factors EGR1, Sp1, and Sp3. In the promoter analysis, mutation of EGR1 site significantly reduced the quercetin response, whereas mutation of Sp1 site decreased only the basal activity of the GCLC promoter. Additionally, the transient overexpression of EGR1 significantly increased basal activity of the GCLC promoter. Finally, we showed that quercetin potently induced both EGR1 mRNA and its protein levels without affecting the expression of Sp1 and Sp3 proteins. Therefore, we concluded that EGR1 was bound to GC-rich region of the GCLC gene promoter, which was prerequisite for the transactivation of the GCLC gene by quercetin. J. Cell. Biochem. 108: 1346–1355, 2009.

KEY WORDS: GCLC; EGR1; QUERCETIN; INS-1 β-CELLS

Glutathione (L-γ-glutamyl-L-cysteine-glycine, GSH), a ubiquitous cellular non-protein sulfhydryl compound, plays an important role in maintaining intracellular redox homeostasis and in protecting cells against oxidative stress [Reed, 1990; Moskaug et al., 2005]. GSH is synthesized in two sequential enzymatic reactions catalyzed by γ-glutamylcysteine ligase (GCL) and GSH synthetase. The reaction catalyzed by GCL is the primary and rate limiting step in de novo synthesis of GSH. GCL is composed of two subunits, catalytic (GCLC, 73 kDa) and regulatory (GCLR, 28 kDa) chains [Gipp et al., 1995; Hamilton et al., 2003].

The expression of GCLC is regulated primarily at the transcriptional level, but a post-transcriptional mechanism such as mRNA stability was also reported to be involved in its expression [Song et al., 2005]. The 5′-flanking regulatory region of the human GCLC gene contains multiple putative binding sites for transcription factors. These sites include antioxidant responsive elements (AREs),

Abbreviations used: GCLC, γ-glutamylcysteine ligase; GSH, glutathione; EGR1, early growth response 1; AREs, antioxidant responsive elements; Sp1, specificity protein 1; EMSA, electrophoretic mobility shift assay.

Jung-Hoon Kang and Seo-Yoon Chang contributed equally to this work.

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activator protein 1 (AP1) site, and specificity protein 1 (Sp1) site [Mulcahy et al., 1997; Tomonari et al., 1997]. Among these, ARE has been clearly shown to function as a major element in mediating the transcriptional activation of both GCLC and GCLR through interaction with their cognate transcription factor NF-E2-related factor 1/2 (Nrf1/2) [Li et al., 1996; Mulcahy et al., 1997; Wild et al., 1999]. In addition, the transcription factors (c-Jun/c-Fos) of AP1 family were reported to enhance the transactivation of GCLC gene via directly binding to AP1 site or association with Nrf1/2 [Yang et al., 2002, 2005]. However, some studies using GCLC promoter devoid of AREs or/and AP1 site suggested the involvement of another *cis*-elements. Tumor necrosis factor-α, cellular inducer of oxidative stress, also activated the GCLC gene through the heat shock and stress response elements without activating ARE [Takamura et al., 2006]. The proximal GC-rich region located between −108 and −28 was shown to regulate constitutive activity of the GCLC gene [Tomonari et al., 1997]. These studies indicate that ARE and AP1 sites are not the only *cis*-elements in regulating the GCLC gene.

Diabetes mellitus is characterized by absolute loss or dysfunction of the islet beta-cells, which result ultimately in severe metabolic imbalances and non-physiological changes in many tissues of the body [Robertson et al., 2003]. Persistent and chronic hyperglycemia causes oxidative stress which in turn reduces the capacity of antioxidant defense system and accelerates the progress of diabetes mellitus [Ihara et al., 1999; Robertson et al., 2003]. Flavonoids, naturally occurring polyphenolic compounds, have been known to prevent and reduce the risk of developing various diseases [Sharma et al., 2005; Perez-Vizcaino et al., 2006]. That is, flavonoids decrease oxidative stress by scavenging reactive oxygen radicals or increase the capacity of endogenous antioxidant defense system by modulating cellular antioxidant-related genes including GCL [Myhrstad et al., 2002; Kim et al., 2004; Moskaug et al., 2005]. Some flavonoids have been demonstrated to have properties of protective effects on pancreatic beta-cells against oxidative stress or cytokine-induced damage [Lapidot et al., 2002; Myhrstad et al., 2002; Coskun et al., 2005]. In particular, quercetin has been shown to protect beta-cells against oxidative stress and to restore insulin release in streptozotocin-induced diabetic rats [Coskun et al., 2005]. Additionally, adenoviral overexpression of the GCLC increased intracellular GSH levels and protected beta-cells from adverse effects of interleukin-1β or ribose, inducers of intracellular oxidative stress [Tran et al., 2004]. However, the regulatory mechanisms for the induction of the GCLC gene by quercetin have not been studied in beta-cells. In this study, we investigated the regulatory mechanisms whereby quercetin induces the GCLC gene using INS-1 beta-cells.

MATERIALS AND METHODS

REAGENTS

Cell culture media, quercetin, and poly (dI-dC) were purchased from Sigma (St. Louis, MO). Fetal bovine serum (FBS) and Lipofectamine™ 2000 reagent were obtained from Gibco BRL Life Technologies (Grand Island, NY). [α-^{32}P] rUTP and [γ-^{32}P] dATP were purchased from Perkin Elmer Life Sciences (Boston, MA). QuickChange® Site-Directed Mutagenesis Kit was purchased from Stratagene (La Jolla, CA). RNA STAT-60 was obtained from Tel-Test Inc. (Friendswood, TX). Polyclonal anti-GCLC was obtained from Abcam (Cambridge Science Park, Cambridge, UK). Polyclonal anti-EGR-1, -AP2, -Sp1, -Sp3, -YY1, and -β-tubulin antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Rabbit anti-(mouse IgG)-peroxidase conjugate and pSV-β-gal was from Promega (Madison, WI). Bio-Rad protein assay kit was from BIO-RAD (Richmond, CA). Chemiluminescence detection system (ECL) kit was from Amersham Corp. (Arlington Height, IL). pCMV-HA was from Clontech Laboratories, Inc. (Mountain View, CA). Quercetin 3-glucuronide and quercetin 3′-sulfate were synthesized as previously described [Needs and Kroon, 2006].

CELL CULTURE

INS-1 cells (between passages 10 and 27) were cultured in RPMI 1640 medium supplemented with 10% FBS, 2 mM L-glutamine, 50 μM 2-mercaptoethanol, 1 mM pyruvate, streptomycin (100 μg/ml), and penicillin (100 units/ml) at 37°C under an atmosphere of 95% air/5% CO_2. Cells were subcultured weekly and passages 19–30 were used exclusively. INS-1 cells were firstly isolated by Asfari and coworkers [Asfari et al., 1992]. These cells have important biological features of the pancreatic islet beta-cells including a relatively high insulin content and glucose-stimulated insulin secretion with physiological range of glucose concentration. Therefore, these cells have been widely used as a good model of beta-cells.

WESTERN BLOT ANALYSIS

Western blot analysis was performed essentially as described previously [Kim et al., 2004, 2006a]. Briefly, cells were washed twice with ice-cold PBS and then lysed with lysis buffer (20 mM HEPES, pH 7.4, 150 mM NaCl, 10% glycerol, 1.5 mM $MgCl_2$, 1 mM EGTA, 1 mM sodium vanadate, 10 mM sodium pyrophosphate, 1 mM NaF, 1% Triton X-100, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 1 mM PMSF). Lysates were cleared by centrifugation and total protein concentrations were determined using Bio-Rad protein assay kit. Twenty microgram of total protein for each sample was separated on a 10% SDS–polyacrylamide gel. Following electrophoresis, proteins were transferred to nitrocellulose membranes (Schleicher and Shuell, Dassel, Germany). Blocking was performed in 5% milk, 10 mM Tris–HCl, pH 7.4, 150 mM NaCl, 0.5% Tween-20 for 1 h. The blots were incubated with anti-GCLC, -Sp1, -Sp3, -EGR1, or β-tubulin antibodies. The detection was performed by ECL detection kit. Equal loading and transfer of samples were verified by Ponseau S staining or the band intensity of β-tubulin (52 kDa). The relative band densities were quantified using Scion Imaging software (Scion Corporation, Frederick, MD).

NORTHERN BLOT ANALYSIS

Total RNA isolation and the method for Northern blot analysis were described previously [Kang et al., 2006; Kim et al., 2008]. RNA samples (10 μg) were fractionated in a 1% formaldehyde agarose gel and transferred onto a Hybond-N nylon membrane. The UV cross-linked membrane was hybridized in a rapid hybridization buffer with rat EGR1 or GCLC riboprobe labeled with [α-^{32}P] rUTP and washed under high stringency conditions: once with 2× SSC, 0.1%

SDS at room temperature for 20 min, once with 2× SSC, 0.1% SDS at 65 °C for 15 min, and once again with 0.1× SSC, 0.1% SDS at 65°C for 15 min. The membrane was subjected to autoradiography. Equal loading of sample was verified by the ribosomal 18S band.

PREPARATION OF EGR1 EXPRESSION VECTOR

The cDNA for rat EGR1 mRNA (GenBank/EMBL accession number: NM_012551) was amplified with the primers: the forward primer with *Eco*R I sites at their 5′ end (underlined), 5′-CCGAATTCGGATGGACAACTACCCCAAACTG-3′, and the reverse primer with *Xho* I at 5′ end (underlined), 5′-TCTAGACTCGAGCTTAGCAAATTTCAATTGTCCTAGGAGA-3′. The polymerase chain reaction (PCR)-amplified EGR1 cDNA was restricted and inserted into pCMV-HA. The sequence of construct was verified by sequencing using An ABI PRISM 310 genetic analyzer (Perkin Elmer Ltd. Co., Seoul, Korea).

CONSTRUCTION OF THE SERIAL-DELETION GCLC PROMOTERS

A 649 bp fragment spanning from −609 to +40 of the human GCLC promoter was amplified by PCR using 0.1 μg of the human genomic DNA (Promega) as the template. The PCR primers used were as follows: forward primer with *Kpn* I site at 5′ end (underlined), 5′-ACTCTCGAGCTAGATCAGAGAACATAGGTACCAG-3′, and reverse primer with *Hin*d III at 5′ end (underlined), 5′-CTGAAGCTTCAGTCTTTGCGTCCGCTAGC-3′. The PCR-amplified product was digested and inserted into pGL3-basic vector, designated as pGCL-609. And we also prepared serially-deleted promoter constructs using PCR. The forward primers with *Kpn* I sites at their 5′ ends (underlined) were as follows: pGCL-328: sense primer (5′-ACTCTCGAGGCTCTCACTCTAAGTGTGAGG-3′); pGCL-147, sense primer (5′-ACTCTCGAGAGCTGCTCCCCTCAACTG-3′); pGCL-90, sense primer (5′-ACTCTCGAGGTTCCCACCGGGCTTCAG-3′); pGCL-34, sense primer (5′-ACTCTCGAGCCTTCGCCCGTAGTCTATAAAACC-3′). The reverse primer was same as that used for the construction of the pGCL-609. The nucleotide sequence of the construct was confirmed by sequencing using an ABI PRISM 310 genetic analyzer (Perkin Elmer Life Sciences).

TRANSIENT TRANSFECTION AND LUCIFERASE ACTIVITY ASSAY

Transient transfection was performed using Lipofectamine™ 2000 reagent as described previously [Kim et al., 2006a, 2008]. Briefly, INS-1 cells (1 μg/2 × 10^5 cells) were transiently transfected with various constructs. After transfection of 24 h, the medium of transfected cells were changed to fresh one with or without 25 μM quercetin, incubated for additional 8 h and then harvested for determination of the luciferase and β-galactosidase activities. Luciferase activity was normalized to β-galactosidase activity.

PREPARATION OF NUCLEAR EXTRACTS

The INS-1 cells (5 × 10^5 cells/ml) were washed with ice-cold PBS and then scraped into 1.5 ml of PBS. Cells were pelleted by centrifugation at 4°C. The pellet was resuspended in 500 μl of ice-cold hypotonic solution consisting of 10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5% Nonidet P-40, 50 mM NaF, 1 mM Na_3VO_4, 1 mM dithiothreitol, 1 μg/ml leupeptin, 1 μg/ml pepstatin and incubated for 15 min on ice. The nuclei were collected by centrifugation at 4°C and then the nuclei were further incubated for 30 min on ice in 30 μl of ice-cold hypertonic solution consisting of 20 mM HEPES, pH 7.9, 25% glycerol, 500 mM NaCl, 1.5 mM $MgCl_2$, 0.5 mM EDTA, 50 mM NaF, 1 mM Na_3VO_4, 5 mM dithiothreitol, 1 μg/ml leupeptin, 1 μg/ml pepstatin. The nuclear extract was clarified by centrifugation at 4°C. The supernatant fraction was stored at 80°C prior to use. Protein concentrations were measured using the Bio-Rad protein assay kit.

ELECTROPHORETIC MOBILITY SHIFT ASSAY (EMSA)

EMSA was performed to examine the binding activity of nuclear proteins to human GCLC promoter by the method reported previously [Kim et al., 2006a, 2008]. Binding reactions were performed in 20 μl of 10 mM HEPES, pH 8.0, 0.1 mM EDTA, 50 mM NaCl, 50 mM KCl, 5 mM $MgCl_2$, 4 mM spermidine, 2 mM dithiothreitol, 0.1 mg/ml bovine serum albumin, 10% glycerol, 1 μg of poly(dI-dC), ^{32}P-labeled oligonucleotide probe (30,000 cpm) and 5 μg of nuclear extract. The reaction was allowed to continue for 20 min at 4°C. The radiolabeled probe contains the sequence from −74 to −50 of the human GCLC promoter (5′-CAGGCCACGCCTCCGCCGCTGCACC-3′). In supershift studies, 1 μg of the appropriate antibody was preincubated with the nuclear extract for 10 min at 4°C before addition of the labeled probe. In competition experiments, the nuclear extract was incubated with a 100-fold molar excess of appropriate unlabeled competitor oligonucleotides. Electrophoresis was carried out on 6% nondenaturating polyacrylamide gels with 0.5× TBE (45 mM tris (hydroxymethyl) aminomethane, 45 mM boric acid, 1 mM EDTA, pH 8.0) and run at 15 V/cm for ∼2 h. The gel was dried under vacuum and subjected to autoradiography. Oligonucleotides used in competition assays were as follows (underlines mean mutated sites): EGR1 mutant, 5′-CAGGCCACGCCTCTACCGCTGCACC-3′; Sp1 mutant, 5′-CAGGATACGCCTCCGCCGCTGCACC-3′; EGR1/Sp1 mutant, 5′-CAGGCCAATCCTCCGCCGCTGCACC-3′; EGR1 consensus, 5′-GGATCCAGCGGGGGCGAGCGGGGGGCGA-3′; and Sp1 consensus, 5′-TGAAGCCCCGCCCCAACGGA-3′.

SITE-DIRECTED MUTAGENESIS

The mutant EGR1 and Sp1 constructs were prepared using QuickChange Site-Directed Mutagenesis Kit. The top strand of the primer set was as follows: for mEGR1, 5′-CAGGCCACGCCTCTACCGCTGCACCGC-3′ and for mSp1, 5′-GGCTTCAGGATACGCCTCCGCCGCTGC-3′. The two underlined nucleotides were changed from CG (wild type) to TA for EGR1 site and from CC (wild type) to AT for Sp1 site. The mutation was verified by sequencing using An ABI PRISM 310 genetic analyzer (Perkin Elmer Ltd. Co.).

STATISTICAL ANALYSIS

The relative band densities were quantified using Scion Imaging software (Scion Corporation, Frederick, MD). All data obtained from each experiment were expressed as mean ± SD. The data were analyzed using one-way ANOVA with Origin 7.0 software (Microcal Software, Northampton, MA). Statistical comparisons among the groups were done by Bonferroni's multiple range *t*-test after the ANOVA. $P < 0.05$ was accepted as statistically significant.

RESULTS

QUERCETIN INDUCES THE GCLC GENE IN TIME- AND DOSE-DEPENDENT MANNERS

Initially, we determined whether quercetin might induce the expression of GCLC in a pancreatic beta-cell line INS-1. Quercetin induced GCLC mRNA in time- and dose-dependent manners. The GCLC mRNA was detectable at 2 h, peaked at 8 h and declined afterward (Fig. 1A,D). However, until 12 h, a significant amount of GCLC mRNA still sustained. In dose-response experiments, a marked induction of GCLC mRNA was observed at doses of over 25 μM (Fig. 1B). The production of GCLC protein started to increase at 4 h and sustained until 24 h (Fig. 1C,D), which was well correlated with the kinetics of GCLC mRNA induction. In this experiment, treatment with various concentrations (1–100 μM) of quercetin for 24 h did not induce the cytotoxicity of INS-1 cells by MTT assay (data not shown). Meanwhile, we observed the quercetin-induced GCLC expression in another beta-cell RINm5F cells. Like the INS-1 cells, the expression of GCLC protein was peak at 12 h and declined afterward (Fig. 1E). Contrary to quercetin, quercetin metabolites (quercetin 3-glucuronide and quercetin 3′-sulfate) did not induce GCLC protein expression (Fig. 1F).

THE REGION FROM −90 TO −34 OF GCLC PROMOTER CONTAINS *cis*-ELEMENTS RESPONSIVE TO QUERCETIN

To investigate the *cis*-elements responsible for the GCLC gene induction by quercetin, five 5′-terminal deleted fragments of the human GCLC promoter ranging from −609/+40 to −34/+40 were subcloned into promoterless luciferase reporter gene vector pGL3 basic (Fig. 2A). Luciferase activity was measured after transient transfection into INS-1 cells with these constructs. Constitutive and quercetin-induced luciferase activities were not significantly altered until deletion to −90, which eliminates AP1 site and CAAT box (Fig. 2B). However, further deletion up to −34 (pGCL-34), which eliminates a high GC rich sequences, showed a significant reduction of both basal and quercetin-induced luciferase activities compared to other longer constructs (Fig. 2B). These results indicate that the region located between −90 and −34 is responsible for the constitutive and the quercetin-induced activation of the GCLC gene.

Sp1 AND EGR1 TRANSCRIPTION FACTORS SPECIFICALLY BIND TO THE QUERCETIN-RESPONSIVE REGION

The quercetin-responsive sequence between −90 and −34 contains a consensus EGR1 binding site (at −67, CGCCTCCGC), which overlaps with a putative Sp1 site (Fig. 3A). Therefore, to test the functional properties of the EGR1 motif within the quercetin-responsive region, EMSAs were performed using a probe which contains oligonucleotide sequence spanning from −74 to −50 within the quercetin-responsive region. Several nuclear protein/DNA complexes were detected when the probe was incubated with nuclear extracts from untreated INS-1 cells (Fig. 3B, bands a, c, d, and e). However, a unique nuclear protein/DNA complex (Fig. 3B, band b) was observed only in nuclear extracts from quercetin-treated INS-1 cells. The protein/DNA complex induced by quercetin was detectable at 1 h and sustained until 4 h with its maximum intensity at 2 h. This quercetin-induced complex was not

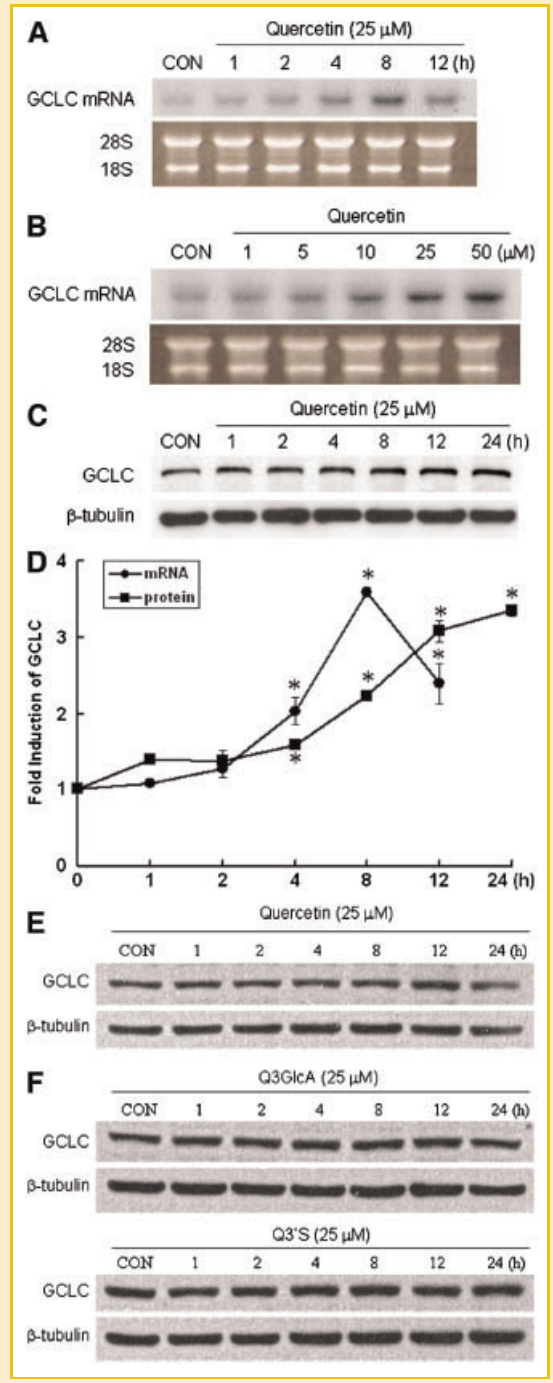

Fig. 1. Quercetin induces expression of the GCLC gene in pancreatic beta-cell lines. INS-1 and RINm5F cells were serum-starved overnight, treated with quercetin for the indicated time points in time-dependent experiments and for 8 h in dose-dependent experiments, and then harvested for determination of the GCLC mRNA and protein levels. A,B: Effect of quecertin on GCLC mRNA expression in INS-1 cells. C: Effect of quecertin on GCLC protein in INS-1 cells. Total 28S and 18S RNAs were used for loading control in Northern blot, and β-tubulin in Western blot, respectively. CON, untreated control. D: Results of A and B are expressed as mean (±SD) of relative band density from three independent experiments. $^{*}P < 0.05$ versus untreated control. E: Effect of quercetin on GCLC protein expression in RINm5F cells. F: Effect of quercetin metabolites, quercetin 3-glucuronide (Q3GlcA) or quercetin 3′-sulfate (Q3′S) on GCLC protein expression in INS-1 cells.

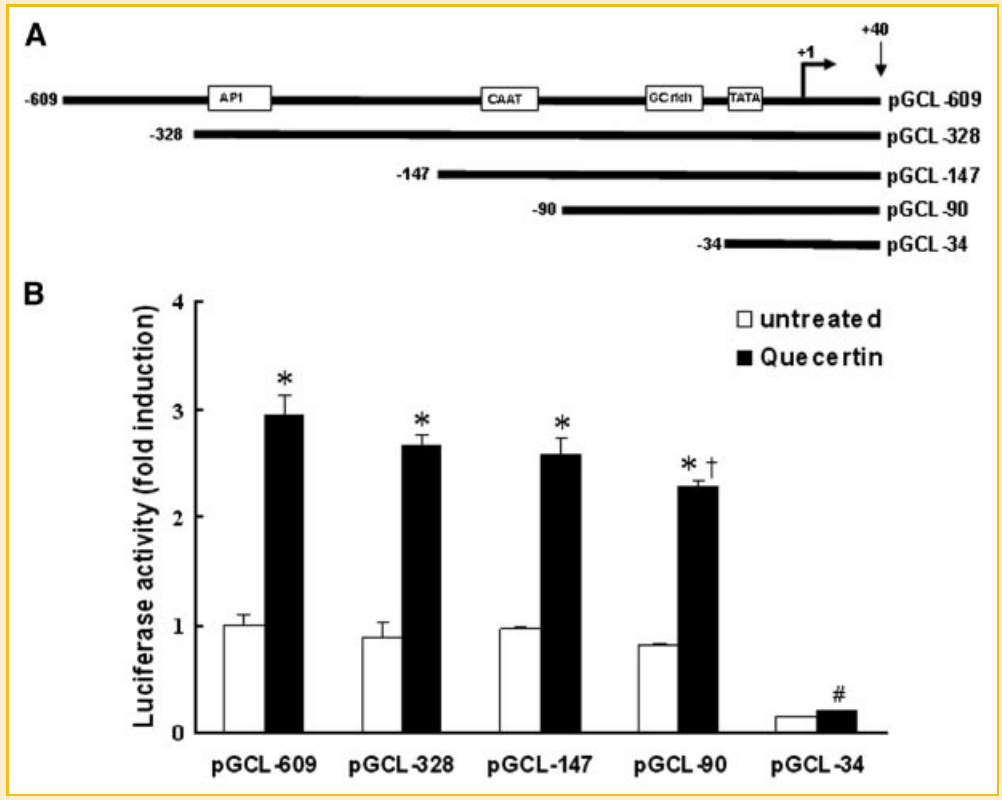

Fig. 2. The proximal region from −90 to −34 of the human GCLC promoter is responsible for the transcriptional activation of GCLC gene by quercetin. A: Schematic description of five deletion constructs of luciferase/human GCLC promoter used in this experiment. The known putative AP1 site and GC rich region along with CAAT and TATA boxes are indicated. B: Promoter activities of each deletion construct in the absence or presence of quercetin. Luciferase activity was presented as a fold induction relative to untreated pGCL-609 construct. Open bars and closed bars represent untreated group and quercetin-treated group, respectively. Each value represents mean ± SD of five experiments. $^{*}P < 0.05$ versus untreated value of respective group; $^{\dagger}P < 0.05$ versus pGCL-609, −328 and −147 constructs; $^{\#}P < 0.05$ versus pGCL-609, −328, −147, and −90 constructs.

detectable in nuclear extracts from untreated or from 8 or 12 h quercetin-treated INS-1 cells. Next, to determine nuclear factors bound to the oligonucleotide probe, antibodies against EGR1, Sp1, Sp3, Wilms' tumor 1 (WT1), and AP2 were used. Especially, the binding motifs for WT1 and AP2 are known to contain high GC rich sequence and to be very similar to those of EGR1 and Sp1. Yin Yang 1 (YY1) antibody was used as a negative control. EGR1, Sp1 and Sp3 antibodies reacted noticeably with complexes b, a, and c, respectively, whereas WT1, AP2, and YY1 did not react with any complex (Fig. 3C). Finally, to examine the binding specificities of the nuclear protein/DNA complexes, a competition assay was performed by using various unlabeled oligonucleotides. The quercetin-induced complex (Fig. 3D, band b) was completely competed away by wild, EGR1 consensus, and Sp1 mutant oligonucleotides, but not by Sp1 consensus, EGR1 mutant, and EGR1/Sp1 mutant oligonucleotides. Sp1 and Sp3-containing complexes (bands a and c) were also substantially competed away with Sp1 consensus and EGR1 mutant oligonucleotides. These results indicate that EGR1, Sp1, and Sp3 are involved in the transcriptional activity of the GCLC gene in response to quercetin.

SITE-DIRECTED MUTATION OF THE EGR1 OR Sp1 SITE REDUCES QUERCETIN RESPONSE

To investigate the functional role of the EGR1 or Sp1 site in regulating GCLC transcriptional activity, site-specific mutations were introduced into the EGR1 or Sp1 site of the human GCLC promoter. Wild and mutant promoter/luciferase reporter constructs were transfected into INS-1 cells, treated with quercetin and reporter activities of each construct were measured. In Figure 4, mutation of the EGR1 site caused a great reduction in promoter activity by quercetin but did not affect basal transcriptional activity compared with wild construct. In contrast, mutation of Sp1 site decreased the basal activity by 50% compared with wild construct but the response by quercetin was still intact. These results suggested that Sp1 and EGR1 sites are required for the basal and quercetin-induced transcriptional activity of the GCLC gene, respectively.

QUERCETIN INDUCES EGR1 EXPRESSION

Protein levels of Sp1, Sp3, and EGR1 were examined in an attempt to correlate their expression with the induction of the GCLC gene in INS-1 cells. Treatment with quercetin clearly induced the expression of EGR1 protein (Fig. 5A). EGR1 protein was detected at 1 h, peaked at 2 h, and declined after 4 h. However, quercetin did not affect the protein expressions of Sp1 and Sp3 (Fig. 5A). Interestingly, the induction kinetics of EGR1 protein was in parallel to those of protein/DNA complex b (Fig. 3B) and the GCLC mRNA (Fig. 1A). The effect of quercetin on the steady-state level of EGR1 mRNA was also examined (Fig. 5B). EGR1 mRNA was detected with its peak at 1 h, but a significant amount of the transcript was sustained until 2 h and

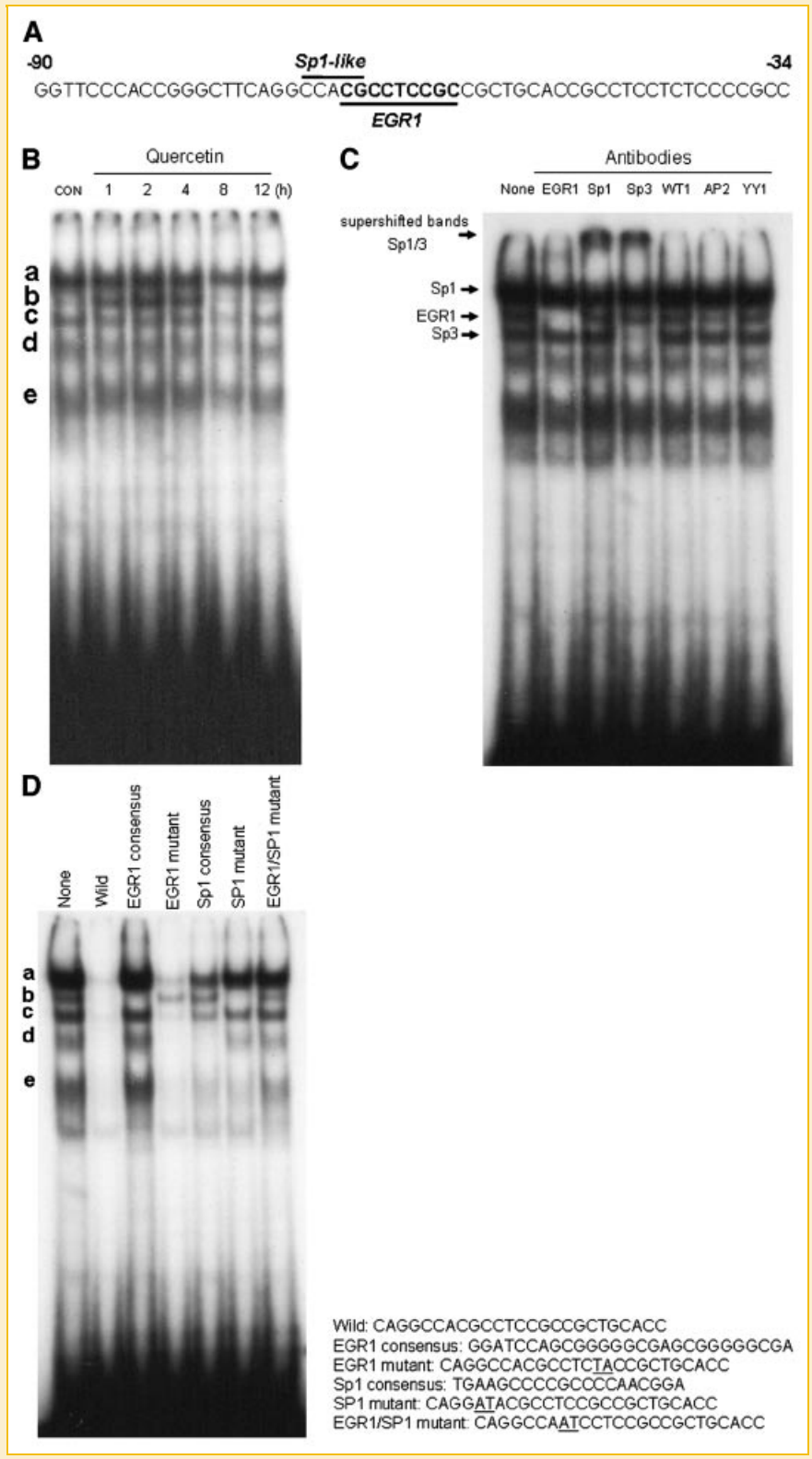

Fig. 3. EGR1, Sp1, and Sp3 bind to the quercetin-responsive region of the human GCLC promoter. A: Nucleotide sequence of the quercetin-responsive region. The sequence is numbered relative to the transcriptional start site. The consensus EGR1 site overlapping with a putative Sp1 site is indicated in italic and underlined. B: Assay for DNA/protein complex formation. INS-1 cells were serum-starved overnight and then were treated with quercetin (25 μM) or not for the indicated time points. Nuclear extracts (10 μg) were obtained and EMSAs were performed as described in Materials and Methods Section. Five nuclear protein/DNA complexes were indicated by complex bands a, b, c, d, and e. C: Immune supershift assay. Nuclear extract from INS-1 cells treated with quercetin for 2 h was incubated with the indicated antibodies (1 μg) and applied to EMSA. Arrows point to specific complexes that reacted with the respective antibodies. D: Competition assays. The same nuclear extract used for immune supershift assay was incubated with a 100-fold molar excess of unlabeled oligonucleotides containing wild or mutated sequences for EGR1 or/and Sp1 binding sites.

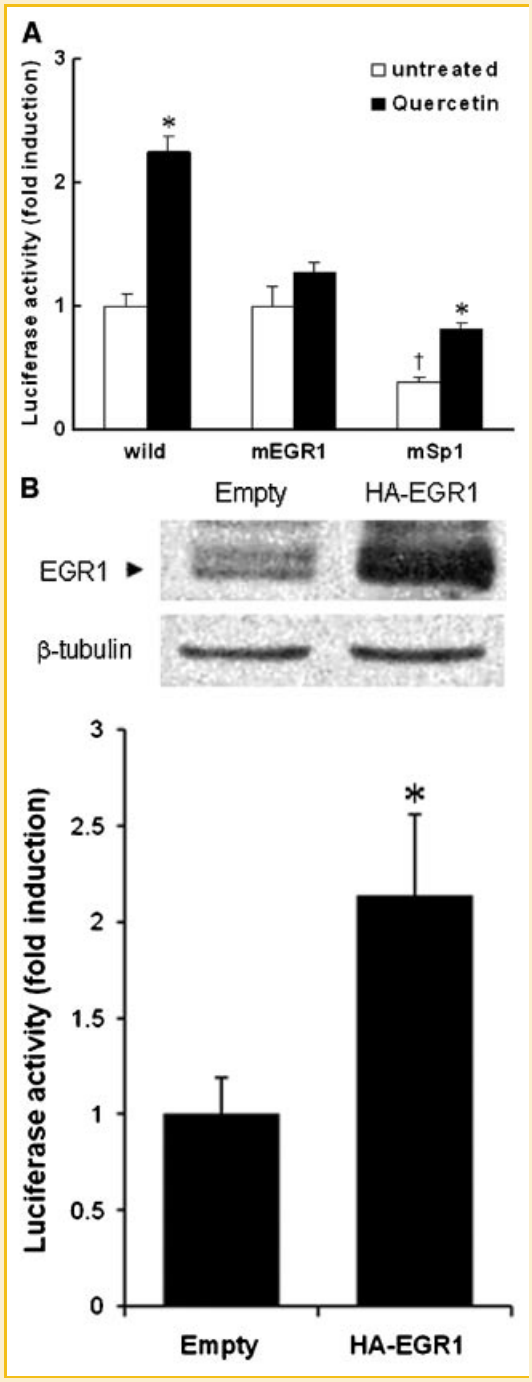

Fig. 4. Point-mutational analysis of the GCLC promoter. A: The effect of mutated EGR1 or Sp1 site on the GCLC promoter. Luciferase activity was presented as a fold induction relative to untreated pGCL-609 construct. Open bars and closed bars represent untreated group and quercetin-treated group, respectively. Each value represents mean ± SD of three experiments. $^{*}P < 0.05$ versus untreated value of respective group; $^{\dagger}P < 0.05$ versus untreated wild value. B: Effect of EGR1 overexpression on the GCLC promoter activity. INS-1 cells transiently co-transfected with pGCL-90 construct (wild) and/or EGR1 expression vector (HA-EGR1) were incubated without any treatment for 24 h and then were harvested for determination of the luciferase and β-galactosidase activities. Luciferase activity was presented as a fold induction relative to empty vector alone. Each value represents mean ± SD of three experiments. $^{*}P < 0.05$ versus empty vector alone.

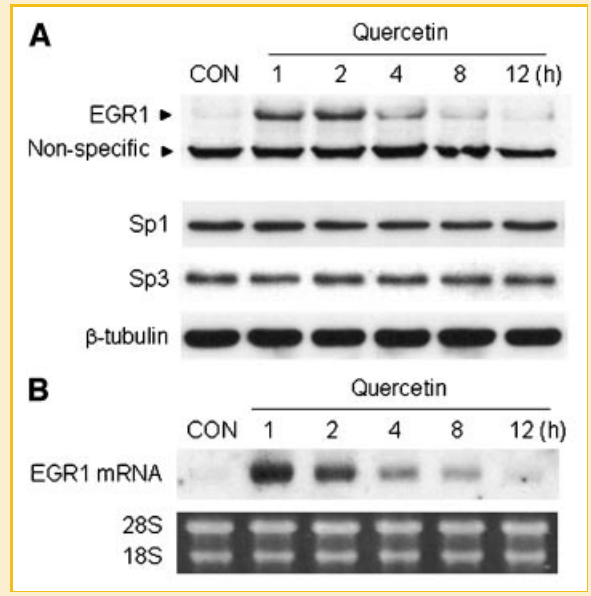

Fig. 5. Quercetin induces the expression of endogenous EGR1 but does not affect the expression level of Sp1 or Sp3. The cells were serum-starved overnight, treated with quercetin (25 μM) and then harvested for determination of EGR1 mRNA. A: Effect of quecertin on EGR1 mRNA expression. B: Effect of quecertin on EGR1 protein expression. Total 28S and 18S RNAs were used for loading control in Northern blot, and β-tubulin in Western blot. CON, untreated control.

declined afterward, which was well correlated with the induction of its protein.

PARALLEL EXPRESSIONS OF EGR1 AND GCLC

We examined the relationship between EGR1 and GCLC expression using other flavonoids by Northern blotting analysis. As shown in Figure 5C, curcumin, quercetin, and gallic acid increased EGR1 mRNA expression compared to other flavonoids. These flavonoids also increased GCLC mRNA expression. This finding suggests that there is a parallel correlation between EGR1 and GCLC expression in INS-1 beta-cells.

PROTECTIVE EFFECT OF QUERCETIN ON CYTOKINE-INDUCED iNOS EXPRESSION

Though the direct protective effect of GCLC under radical stress or toxic condition was not observed, we examined the protective effect of quercetin under free radical stress condition. Following the exposure to the cytokines (IL-1β and IFN-γ), the cells were treated with quercetin. As shown in Fig. 5D, quercetin inhibited cytokine-induced iNOS expression dose-dependently.

DISCUSSION

The present study investigated the functional role of the proximal GC-rich sequence within GCLC promoter in constitutive and quercetin-induced activation of the GCLC gene using a pancreatic beta-cell line INS-1, and provided evidence that the EGR1 binding

site on the GC-rich region largely contributed to the transcriptional activation of GCLC.

Induction of the GCLC gene has been an important adaptive response to numerous cellular insults [Reed, 1990]. Differences in the ability of cells to induce the GCLC gene are believed to contribute to the susceptibility of different cell types to toxic insults. Beta-cells were vulnerable to oxidative stress due to unusually low levels of antioxidant enzymes such as superoxide dismutase, catalase, and peroxidase [Robertson et al., 2003]. Our study showed that quercetin treatment potently induced the GCLC in a pancreatic beta-cell line INS-1, in which the time point (8 h) and dose (>25 μM) for maximum induction of the GCLC gene were well consistent with the previous study [Myhrstad et al., 2002]. The induction of the GCLC protein was also observed in another beta-cell line RINm5F cell. In addition, we observed that quercetin inhibited both IL-1β and IFN-γ-induced iNOS expression dose-dependently (Fig. 7), though the direct protective effect of GCLC under radical stress condition was not evaluated. The overproduction of NO by iNOS in islet beta-cells is well known in mediating the cytotoxicity of beta-cells [Southern et al., 1990]. Therefore, the up-regulation of the GCLC expression by quercetin appears to play an important role in protecting the beta-cells against oxidative stress and in restoring insulin release in streptozotocin-induced diabetic rats.

The transcription of the GCLC gene was independently regulated and displayed cell-type specific differences in both basal and inducible conditions [Dahl and Mulcahy, 2001]. Our promoter analysis revealed that the proximal GC-rich region from −90 to −34 contained the *cis*-elements for transactivation of the GCLC gene in response to quercetin as well as for the maintenance of basal activity of the gene. In consistent with our study, others' studies also indicated importance of the GC rich region to be necessary for transcriptional activity [Tomonari et al., 1997]. Although this study did not assessed the ARE and its binding factor Nrf1/2 which have been demonstrated in the induction of GCLC gene by a wide range of stimuli including quercetin [Myhrstad et al., 2002; Dickinson et al., 2003], it is unlikely that the only interaction of ARE sites with Nrf1/2 is sufficient to drive full activation of the gene promoter because GCLC promoter without ARE motifs still significantly responded to various stimuli [Kim et al., 2006b; Kimura et al., 2009]. In addition, cells deficient in Nrf1 or Nrf2 still retained ability to activate GCLC promoter by *tert*-butylhyroquinone [Yang et al., 2005]. And trivalent arsenite, a known human carcinogen, induced GCLC gene independently of Nrf1/2 [Thompson et al., 2009]. On the other hand, it could be assumed that the predominant action of ARE and Nrf1/2 might mask the contributive effect of other regulatory factors on the activation of GCLC gene. Therefore, it is possible that *cis*-elements within the proximal GC-rich region contribute to quercetin-induced activation of GCLC gene in concert with ARE or other regulatory motifs.

The zinc finger transcription factor EGR1, also named zif268, NGF I-A, and Krox24, is a member of immediate early response gene group that is induced in response to diverse stimuli including growth factors and cytokines [Gashler and Sukhatme, 1995; Kang et al., 2007]. The exact role of EGR1 has not been defined in beta-cells. However, some studies showed that EGR1 expression in beta-cells was implicated in the proliferation and transactivation of insulin gene [Eto et al., 2006; Kang et al., 2006]. Moreover, the inhibition of EGR1 induction was associated with the development of diabetes [Garnett et al., 2005]. The consensus sequences for EGR1 is 5′-C(G/A/T)CC(C/T)(A/C)CGC-3′. The GC rich region of the human GCLC promoter identified in this study contains a consensus EGR1 binding motif located at −67 (5′-CGCCTCCGC-3′) which is well conserved in rat GCLC gene. In this study, when an oligonucleotide harboring the consensus EGR1 motif as a probe was employed in EMSA, quercetin induced a nuclear protein/DNA complex which clearly reacted with specific EGR1 antibody, as confirmed by an immune shift study. Moreover, mutation of the EGR1 site significantly reduced the quercetin response without affecting basal activity, which confirmed the functional role of the EGR1 site in the induction of the GCLC by quercetin. Through this study, both EGR1 mRNA and protein expressions were also found to be induced by quercetin in a time-dependent manner. Interestingly, the kinetics for EGR1 induction was well correlated with the generation of quercetin-induced protein/DNA complex in our EMSA. In addition, we evaluated the relationship between EGR1 and GCLC expression using other flavonoids such as apigenin, curcumin, and gallic acid. Like the finding shown in quercetin, both curcumin and gallic acid increased EGR1 mRNA expression compared to other flavonoids. These flavonoids significantly increased GCLC mRNA expression (Fig. 6). This finding implies that there is a parallel pattern between EGR1 and GCLC expression in INS-1 beta-cells. Therefore, our findings suggest that quercetin induces EGR1 expression that could bind to the EGR1 binding site within proximal GC rich region of the GCLC promoter leading to the GCLC gene induction.

Transcription factors Sp1 and Sp3 are ubiquitously and constitutively expressed in mammalian cells and involved in the transcriptional regulation of numerous housekeeping, tissue-specific, viral, and inducible genes. Sp1 and Sp3 bind GC box (5′-CCCGCCC-3′) of gene promoter with similar affinity and act as positive or negative regulator of gene expression [Li et al., 2004]. The binding sites for Sp1, Sp3 and EGR1 are very similar and often

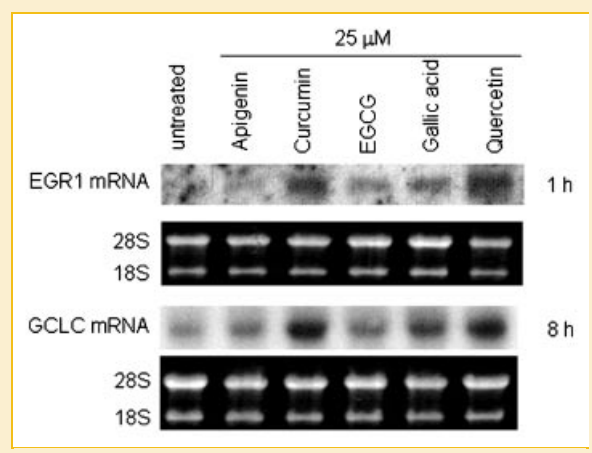

Fig. 6. Parallel expressions of EGR1 and GCLC mRNA by other flavonoids. The cells were serum-starved overnight, treated with each flavonoid (25 μM) for 1 h or 8 h and then harvested for determination of EGR1 mRNA or GCLC mRNA, respectively. Total 28S and 18S RNAs were used for loading control.

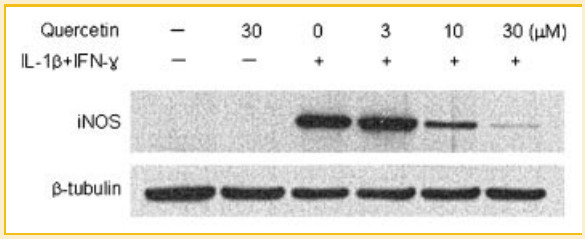

Fig. 7. The effect of quercetin on interleukin-1β (IL-1β) and interferon-γ (IFN-γ)-induced iNOS protein expression in RINm5F cells. The cells were serum starved overnight, then were pretreated with quercetin (3, 10, 30 μM) for 1 h, next were incubated with IL-1β (1 ng/ml) and IFN-γ (10 ng/ml) for 16 h. Then, the cells were harvested for determination of the iNOS protein level. β-Tubulin was used for equal loading control.

overlap in GC rich region of some gene promoters, which could trigger the competition for DNA binding between those factors [Huang et al., 1997; Al-Sarraj et al., 2005]. Our competition assay revealed that a putative binding site for Sp1 and Sp3 was located at −70 (5′-CCACGCCT-3′) and overlapped with quercetin-responsive EGR1 site. The binding activities of Sp1 and Sp3 to the overlapping sequence were also confirmed in immune supershift assay. Because the band intensities of nuclear protein/DNA complexes in our EMSA, except for that of the complex induced by quercetin, were not significantly affected irrespective of quercetin treatment, binding competition between Sp1, Sp3, and EGR1 for the overlapping DNA binding sequence might not be involved in our system. In addition, our mutational analysis confirmed that the overlapping Sp1 site was required for the regulation of basal activity of the GCLC promoter.

In summary, we provided evidences that EGR1 also participated in the regulation of the GCLC gene induction in response to quercetin. Specifically, this study showed that the proximal GC-rich region (from −90 to −34) of the GCLC promoter was involved in quercetin-induced transcriptional activation of the GCLC gene in a pancreatic beta-cell line INS-1. EGR1 site within the GC rich region was required for quercetin induction of the GCLC gene, whereas Sp1/3 binding site overlapping with the EGR1 site was involved in the maintenance of basal activity of the gene.

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