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Molecular Mechanisms of Early Growth Response Protein-1 (EGR-1) Expression by Quercetin in INS-1 Beta-Cells

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ABSTRACT

Early growth response-1 (EGR-1), one of immediate early response genes, is involved in diverse cellular response. We recently reported that quercetin increased catalytic subunit of γ-glutamylcysteine ligase (GCLC) via the interaction of EGR-1 to GCLC promoter in INS-1 beta-cells. Therefore, this study investigated molecular mechanisms of quercetin-induced EGR-1 expression in INS-1 cells. Quercetin significantly induced EGR-1 protein and its mRNA expressions. This induction of EGR-1 was completely blocked by pretreatment with a PKA inhibitor, H89 and partially blocked by a p38 inhibitor, SB203580. Additionally, the siRNA-mediated inhibition of PKAα and p38 resulted in significant reduction of quercetin-induced EGR-1 promoter activity. Also, quercetin-induced EGR-1 protein expression was significantly decreased in the cells transfected with PKAα siRNA. Study using truncated EGR-1 promoter constructs showed that serum response element (SRE) sites, not cAMP response element site, were essential for EGR-1 transcription. However, electrophoretic mobility shift assay showed that quercetin did not affect the band intensity of DNA-protein complex on SRE site of EGR-1 promoter. Also, immune-shift assay using serum response factor (SRF) and phospho-SRF antibodies showed no difference between control and quercetin-treated groups. Collectively, quercetin-induced EGR-1 expression is largely dependent on PKA and partly on p38 MAPK pathway, and SRE sites of EGR-1 promoter are involved in quercetin-induced EGR-1 transcriptional activity. J. Cell. Biochem. 113: 1559–1568, 2012.

KEY WORDS: EGR-1; QUERCETIN; PKA; SRE; INS-1 BETA-CELLS

The main pathological features of diabetes mellitus are significant beta-cell loss and insulin resistance in target tissues, which leads to severe metabolic imbalances in many organs [Leahy, 1990]. Some flavonoids have been recognized to protect beta-cells against oxidative stress or cytokine-induced damage [Lapidot et al., 2002; Myhrstad et al., 2002; Coskun et al., 2005]. Quercetin, one of the major flavonols, was known to protect beta-cells against oxidative stress and to restore insulin release in streptozotocin-induced diabetic rats [Coskun et al., 2005]. Recently, quercetin was shown to promote hepatocyte proliferation by inhibiting the expression of the cyclin-dependent kinase inhibitor p21 in streptozotocin-treated mice and to potentiate glucose-induced insulin secretion in INS-1 beta-cells [Kobori et al., 2009; Youl et al., 2010].

The zinc finger transcription factor early growth response-1 (EGR-1, known as zif268, NGF I-A, and Krox24) was known to be induced in response to diverse stimuli including growth factors and cytokines [Gashler and Sukhatme, 1995; Kang et al., 2007]. The induction of EGR-1 was primarily known to be regulated at the transcriptional level through *cis*-regulatory elements such as serum response element (SRE) and cAMP-response element (CRE) [Sakamoto et al., 1991; Thiel and Cibelli, 2002]. For SRE-mediated EGR-1 transcription, two kinds of transcription factors are required: Serum response factor (SRF) and ternary complex factors (TCFs).

Abbreviations: EGR-1, early growth response-1; GCLC, γ-glutamylcysteine ligase; SRE, serum response element; SRF, serum response factor; PKA, protein kinase A; MEK, MAPK/ERK kinase; ERK, extracellular signal-regulated kinase; CRE, cAMP-responsive element; RT-PCR, reverse time-polymerase chain reaction; EMSA, electrophoretic mobility shift assay; PMSF, phenylmethylsulfonyl fluoride.

Seo-Yoon Chang and Jae Min Cho contributed equally to this study.

Additional supporting information may be found in the online version of this article.

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Activated SRF induces gene transcription by binding to SRE sites and by recruiting TCFs [Treisman, 1992; Thiel and Cibelli, 2002]. Also, CRE site was known to activate EGR-1 transcription via the interaction of transcription factor CRE-binding protein (CREB) [Sakamoto et al., 1994; Russell et al., 2003; Kang et al., 2007]. Meanwhile, EGR-1 induction was known to be involved in several kinase pathways such as ERK, p38 MAPK, PKA, and PI-3 kinase [Rolli et al., 1999; Bernal-Mizrachi et al., 2000; Guha et al., 2001; Shin et al., 2006].

Several reports showed that EGR-1 plays a key role in beta-cell function. EGR-1 expression was implicated in beta-cell proliferation and transactivation of insulin gene [Eto et al., 2006; Kang et al., 2006]. Moreover, the inhibition of EGR-1 expression may contribute to beta-cell dysfunction in Zucker Diabetic Fatty rats [Garnett et al., 2005]. In addition, EGR-1 expression in pancreatic islets was significantly higher than other tissues including brain and lung [Garnett et al., 2005]. Recently, we reported that quercetin rapidly induced EGR-1 protein expression and induced the expression of catalytic subunit of γ-glutamylcysteine ligase (GCLC) by the interaction of EGR-1 to EGR-1 binding site of GCLC promoter in INS-1 beta-cells [Kang et al., 2009a]. Accordingly, to characterize the expression mechanism of EGR-1 by quercetin in INS-1 beta-cells, we analyzed signaling pathways and the EGR-1 promoter regions involved in EGR-1 gene expression.

MATERIALS AND METHODS

REAGENTS

Cell culture media, quercetin, and poly (dI-dC) were purchased from Sigma (St. Louis, MO). SP600125, forskolin, SB203580, PD98059, H89, LY294002, Akt inhibitor, and wortmanin were from Calbiochem (La Jolla, CA). Fetal bovine serum (FBS) and Lipofectamine™ 2000 reagent were from Gibco BRL Life Technologies (Grand Island, NY). [γ-^{32}P] dATP were from Perkin Elmer Life Sciences (Boston, MA). QuickChange® Site-Directed Mutagenesis Kit was from Stratagene (La Jolla, CA). RNA STAT-60 was obtained from Tel-Test Inc. (Friendswood, TX). First Strand cDNA Synthesis Kit, alkaline phosphatase-conjugated anti-digoxigenin (DIG) antibody, and CDP-Star were from Roche Diagnostics (Mannheim, Germany). PKAα siRNA (sc-156094), p38 siRNA (sc-156091), polyclonal anti-EGR-1, and anti-SRF antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-TATA-binding protein antibody was from Abcam (Cambridge, UK). Anti-phospho-p38, anti-p38, anti-phospho-ERK, anti-phospho-JNK, and anti-phospho-Akt antibodies were from Cell Signaling Technology, Inc. (Beverly, MA). Anti-ERK antibody was from Stressgen (Victoria, BC, Canada). Anti-JNK antibody was from Millipore (Billerica, MA). Anti-Akt antibody was from Epitomics (Burlingame, CA). pGL3 basic vector, rabbit anti-(mouse IgG)-peroxidase conjugate, Dual-Luciferase® Reporter Assay System and pRL-TK were from Promega (Madison, WI). Plasmid for PKAα catalytic subunit (Addgene plasmid 15310: pCalpha EV) was from Addgene (Cambridge, MA). Bio-Rad protein assay kit was from BIO-RAD (Richmond, CA). RIPA Lysis Buffer was from Millipore (Temecula, CA). Nitrocellulose membrane was from Schleicher and Shuell (Dassel, Germany). SuperSignal® West Dura Extended Duration Substrate was from Pierce Biotechnology (Rockford, IL).

CELL CULTURE AND QUERCETIN TREATMENT

INS-1 cells 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. In this study, we tested the effect of quercetin at concentration of 25 μM based on our previous study [Kang et al., 2009a] and the finding that quercetin up to 100 μM did not show any effect on cell viability and cell morphology (unpublished data).

WESTERN BLOT ANALYSIS

Western blot analysis was performed essentially as we previously described [Kim et al., 2004, 2006]. Briefly, cells were washed twice with ice-cold PBS and then lysed with RIPA lysis buffer. Lysates were cleared by centrifugation and total protein concentrations were determined using Bio-Rad protein assay kit. Thirty microgram of total protein for each sample was separated on an 8% SDS-polyacrylamide gel. Following electrophoresis, proteins were transferred to nitrocellulose membranes. Blocking was performed in 5% milk, 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.5% Tween-20 for 1 h. The membranes were incubated with primary antibody. The detection was performed by SuperSignal® West Dura Extended Duration Substrate. The immunoreactive band intensity was determined by densitometry using Scion Image program (Scion Corporation, Frederick, MD). Each value was normalized by the ratio of EGR-1 band intensity to TATA-binding protein band intensity. Data are expressed as mean ± S.D. of three independent experiments and each data is expressed relative to control value.

NORTHERN BLOT ANALYSIS

Total RNA isolation and the method for northern blot analysis were performed as we previously described [Kim et al., 2006, 2008; Kang et al., 2009b]. 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 digoxigenin-labeled EGR-1 cDNA probe 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 incubated with alkaline phosphatase-conjugated anti-digoxigenin antibody, and then EGR-1 mRNA level was detected by chemiluminescence using CDP-Star as substrate. Equal loading of sample was verified by house keeping gene GAPDH.

REAL TIME-POLYMERASE CHAIN REACTION (RT-PCR)

Total RNA isolation and cDNA synthesis were conducted as we previously described [Kang et al., 2006; Kim et al., 2008]. RT-PCR was performed with a KAPA SYBR FAST QPCR kit (Kapa Biosystems, Inc., Woburn, MA) and a Stratagene Mx3000P QPCR System (Stratagene). The specific primers used for the amplification of

TABLE I. Primers Used in Real Time-Polymerase Chain Reaction

Gene	Forward primer	Reverse primer
β-actin	5′-CACCCGCGAGTACAACCT TC-3′	5′-CATGCCGGAGCCGTTGTC-3′
PKA	5′-GCAGTGGAGTTCCCGTTCC-3′	5′-AGCATGGGGTTCACTGAACC-3′
p38	5′-CATCGTGTGGCAGTGAAGAAGC-3′	5′-GACCTTGCAGGTGTAAACACA TCC-3′

β-actin, PKA catalytic subunit, and p38 MAPK are listed in Table I. The PCR reaction was carried out for 40 cycles.

PLASMIDS

The EGR-1 expression vector (HA-EGR-1) was prepared as we previously described [Kang et al., 2009a]. Rat EGR-1 promoter ranging from −462 to +26 was generated by PCR amplification method and cloned into pGL3 basic vector, then designated as *p*-462 (GenBank accession no: J041540) [Kang et al., 2007]. Using *p*-462 as a template, serial deletion constructs (*p*-375, *p*-325, *p*-124, *p*-73, and *p*-46) were prepared. The forward primers with *KpnI* site at the 5′ end (underlined) were as follows: For *p*-375, 5′-TCAGGTACCAACAGACCTTATTTGGGCAGCGC-3′; *p*-325, 5′-TCAGGTACCTGCCGCTTCCGGCTCTG-3′; *p*-124, 5′-TCAGGTACCGTCCTCCCGGTCGGTCC-3′; *p*-73, 5′-TCAGGTACCCCATGTACGTCACGGCGGAG-3′; *p*-46, 5′-TCAGGTACCCGTGCTGTTTCAGACCCTTG-3′. The reverse primer with *XhoI* site (underlined) was as follows: 5′-TCACTCGAGCCAAGTTCTGCGCGCTGG-3′. To synthesize SRE site (GG→TT)- and CRE site (AC→TG)-mutated promoter constructs, QuickChange® Site-Directed Mutagenesis Kit was employed, and then several mutated promoter constructs were prepared such as *p*-462 mSRE4, *p*-462 mSRE5, *p*-462 dmSRE, *p*-124 mSRE4, *p*-124 mSRE5, *p*-124 dmSRE, and *p*-73 mCRE. The sequence of construct was verified by sequencing using an ABI PRISM 310 genetic analyzer (PerkinElmer Ltd. Co., Seoul, Korea).

TRANSIENT TRANSFECTION AND LUCIFERASE ACTIVITY ASSAY

Transient transfection was performed using Lipofectamine™ 2000 reagent as we previously described [Kim et al., 2006, 2008]. Briefly, INS-1 cells were transiently transfected with various constructs (0.5 μg/3 × 10^5 cells) of EGR-1 promoter. After transfection of 8 h, the medium of transfected cells were treated with or without 25 μM quercetin, incubated for additional 16 h and then harvested for determination of the firefly and Renilla luciferase activities. All data were normalized by the ratio of firefly to Renilla luciferase activities. To evaluate the effect of PKA on EGR-1 promoter activity in the basal condition, PKAα expression vector and *p*-124 or *p*-73 EGR-1 promoter were cotransfected into the cells, and then promoter activity was analyzed in the same manner.

PREPARATION OF NUCLEAR EXTRACTS AND ELECTROPHORETIC MOBILITY SHIFT ASSAY (EMSA)

Following the serum starvation for 16 h, the cells were treated with quercetin (25 μM) for indicated times to examine DNA binding activity to SRE site of rat EGR-1 promoter. Nuclear extracts were prepared as we previously described [Dignam et al., 1983]. EMSA was performed as we previously described [Kim et al., 2006]. The complementary oligonucleotide used as probes or competitors was as follows: Wild type (GenBank accession no: J04154, from −109 to −69, 5′-CCTTCCATATTAGGGCTTCCTGCTTCCCATATATGGCCATG-3′) from Bioneer (Daejon, Korea). The antibodies against SRF, phospho-SRF, Elk-1, and phospho-Elk-1 were used for immune-supershift assay. To verify equal loading of nuclear extract, EMSA was performed using Sp1 consensus oligonucleotide.

siRNA-MEDIATED KNOCKDOWN STUDY

Both PKAα and p38 gene silencing by siRNA was performed as we previously described [Kim et al., 2008]. Briefly, INS-1 cells were seeded in 12-well plates in regular medium and were grown to reach 50–60% confluence. The day of the experiment, cells were changed to fresh medium without antibiotics and FBS. The siRNA complex was prepared and transfection was performed according to the manufacturer's instructions. The transfection efficiency of siRNA was determined by mRNA level using RT-PCR. Based on the result of RT-PCR, we set the concentration of siRNA complex at 50 nM. The cells were transiently transfected with PKAα, p38, or control scramble siRNA (which corresponds to a nontargeting 20–25 nucleotide siRNA as a negative control). After 24 h, the cells were cotransfected with *p*-462 EGR-1 promoter construct (0.5 μg) and pRL-TK plasmid (50 ng) for 8 h. Subsequently, the cells were incubated with or without 25 μM quercetin for additional 16 h. Then, firefly and Renilla luciferase activities were measured using Dual Luciferase Assay system (Promega). All data were normalized by the ratio of firefly to Renilla luciferase activities.

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 ± S.D. 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

EGR-1 PROTEIN AND ITS mRNA EXPRESSION BY QUERCETIN IS MEDIATED BY PKA AND p38 MAPK PATHWAYS

Quercetin increased EGR-1 protein expression in rat beta-cell line RINm5F and mouse beta-cell line MIN6 as well as INS-1 cells (Fig. 1A). We investigated the involvements of PKA, MAPK and PI3-kinase pathway in quercetin-induced EGR-1 protein expression using specific inhibitors. We tested the effectiveness of these inhibitors in our experimental conditions (see Supplementary Fig. 1). As shown in Figure 1B,C, the induction of EGR-1 by quercetin was completely blocked by H89, a PKA inhibitor and significantly inhibited by SB203580, a p38 MAPK inhibitor. However, an MEK inhibitor PD98059, a JNK inhibitor SP600125,

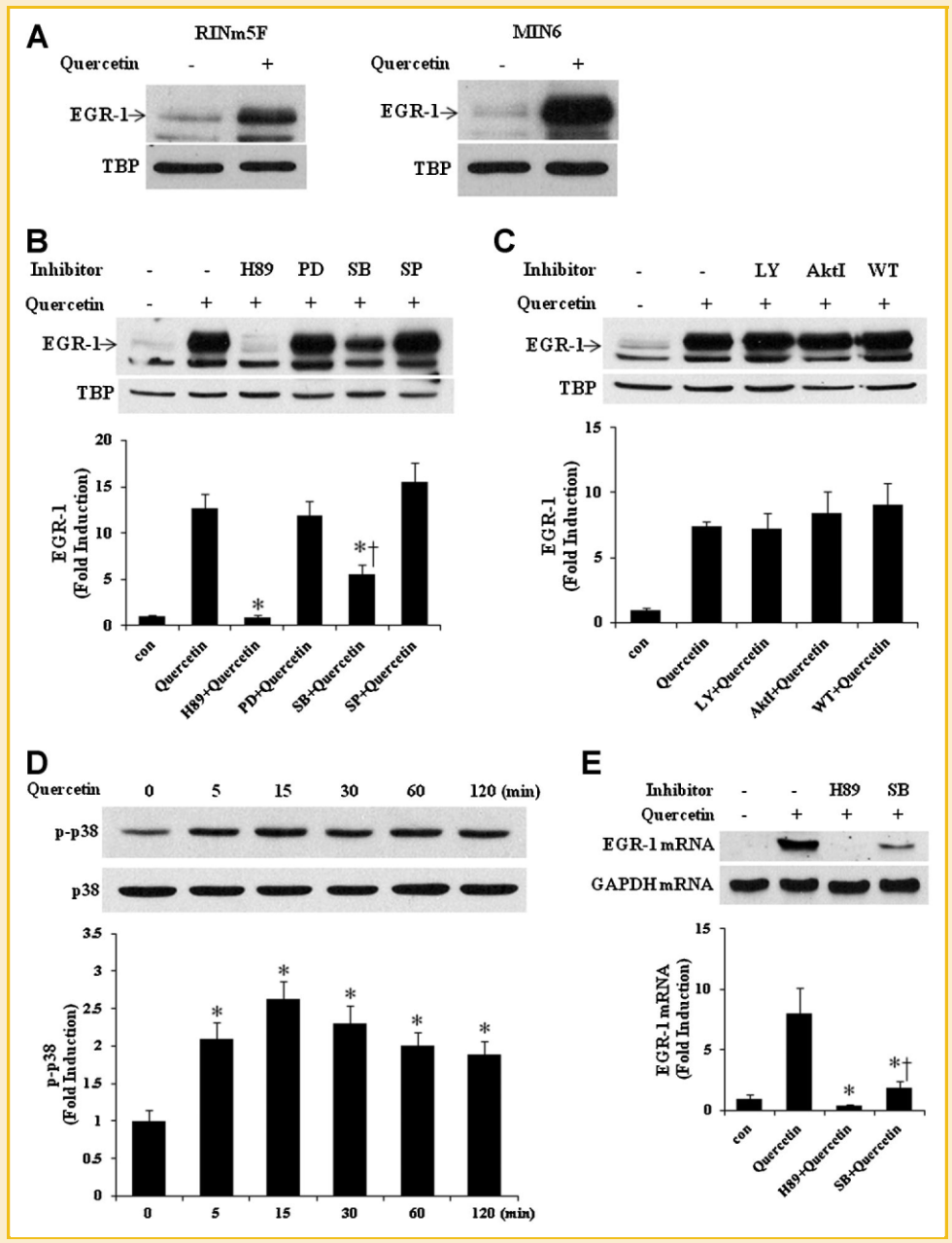

Fig. 1. Quercetin induces expression of the EGR-1 gene by cAMP/PKA and p38 MAPK signaling in INS-1 cells. A: EGR-1 protein expression by quercetin in RINm5F cells and MIN6 cells. B: INS-1 cells were pretreated with either H89 (10 μM) or PD98059 (PD, 10 μM) or SB203580 (SB, 10 μM) or SP600125 (SP, 10 μM) for 30 min, and then incubated with quercetin (25 μM) for 2 h. Western blot analysis for EGR-1 was performed. Equal loading of protein was verified by probing the same blot for TATA-binding protein (TBP). Data are expressed as mean ± S.D. of three independent experiments. $^{*}P < 0.05$ versus quercetin only; †$P < 0.05$ versus H89 and quercetin. C: INS-1 cells were pretreated with either LY294002 (LY, 10 μM) or Akt inhibitor (AktI, 2 μM) or wortmanin (WT, 100 nM) for 30 min, and then incubated with quercetin (25 μM) for 2 h. Western blot analysis for EGR-1 was performed. Equal loading of protein was verified by probing the same blot for TATA-binding protein (TBP). Data are expressed as mean ± S.D. of three independent experiments. D: INS-1 cells were treated with quercetin (25 μM) for the indicated time. Western blot analysis for p38 and phospho-p38 was performed. Data are expressed as mean ± S.D. of three independent experiments. $^{*}P < 0.05$ versus untreated control. E: INS-1 cells were pretreated with H89 (10 μM) or SB203580 (SB, 10 μM) for 30 min, then incubated with quercetin (25 μM) for 1 h. Northern blot analysis for EGR-1 mRNA was performed. Equal loading of sample (10 μg) was verified by GAPDH. Data are expressed as mean ± S.D. of three independent experiments. $^{*}P < 0.05$ versus quercetin only; †$P < 0.05$ versus H89 and quercetin.

PI3-kinase inhibitors wortmanin and LY294002 and Akt inhibitor did not exert any effect on EGR-1 expression. We also found that quercetin rapidly induced the phosphorylation of p38 MAPK (Fig. 1D). We next examined the effects of H89 and SB203580 on quercetin-induced EGR-1 expression at mRNA level. Consistent with the result of EGR-1 protein expression, H89 completely inhibited quercetin-induced EGR-1 mRNA and SB203580 significantly inhibited it (Fig. 1E).

EGR-1 PROMOTER ACTIVITY BY QUERCETIN IS REGULATED BY PKA AND p38 MAPK PATHWAYS

As shown in Fig. 2A, the rat EGR-1 promoter sequences are illustrated based on the transcription start site (GenBank accession number: J041540) and SRE and CRE sites, which are important *cis*-elements for EGR-1 transcription, are marked on the promoter. In addition, we depicted forward regions of the serially-deletion constructs (*p*-462, *p*-375, *p*-325, *p*-124, *p*-73, and *p*-46) (arrows in Fig. 2A). First, we measured rat EGR-1 promoter activity using p-462 construct whether the induction of EGR-1 mRNA by quercetin is regulated at the transcriptional level. As shown in Fig. 2B, quercetin significantly increased EGR-1 promoter activity compared with untreated control group. We also examined EGR-1 promoter activity using PKA and p38 inhibitors. H89 completely inhibited quercetin-induced EGR-1 promoter activity and SB203580 significantly inhibited it (Fig. 2B). To further explore the effects of PKA and p38 MAPK on EGR-1 promoter activity, the gene silencing study was performed. As shown in Fig. 2C, each transfection of small interfering duplexes of PKAα and p38 MAPK significantly decreased PKA and p38 MAPK mRNA expression, respectively. In PKAα or p38 MAPK siRNA-transfected INS-1 cells, EGR-1 promoter activity by quercetin was significantly reduced compared with control siRNA-transfected cells (Fig. 2D). To further evaluate the role of PKA on EGR-1 expression, EGR-1 protein level was examined following either PKAα gene silencing or overexpression of PKAα catalytic subunit. In PKAα siRNA-transfected cells, quercetin-induced EGR-1 protein expression was significantly decreased compared with scramble siRNA-transfected cells (Fig. 2E). We

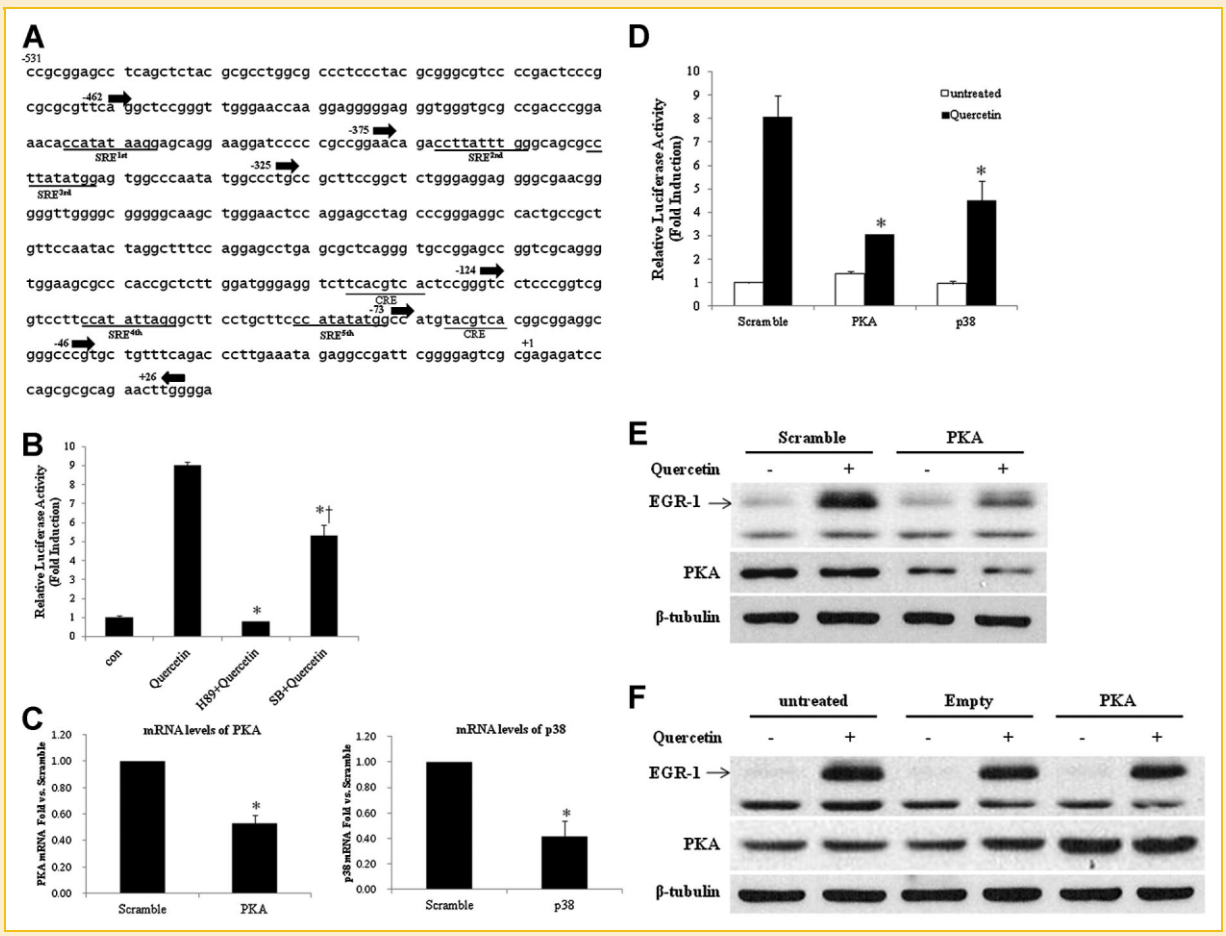

Fig. 2. Quercetin-induced EGR-1 promoter activity is dependent on PKA and p38 MAPK signaling. A: Schematic distribution of *cis*-elements serum response element (SRE) and cAMP response element (CRE) on the rat EGR-1 promoter. B: INS-1 cells were pretreated with H89 (10 μM) or SB203580 (SB, 10 μM) for 30 min, next incubated with quercetin (25 μM) for 16 h, and then promoter activities of *p*-462 EGR-1 construct were measured. Each value represents mean ± S.D. of five independent experiments. $^{*}P < 0.05$ versus quercetin only; †$P < 0.05$ versus H89 and quercetin. C, D: INS-1 cells transfected with control siRNA scramble or siRNA against PKAα or p38 were cotransfected with *p*-462 construct, and next were treated with quercetin (25 μM) for 16 h, and then promoter activities were measured. C: The mRNA expressions of PKA or p38 following gene silencing. Each value represents mean ± S.D. of five independent experiments. $^{*}P < 0.05$ versus scramble siRNA group. D: Promoter activity of *p*-462 EGR-1 construct was presented as a fold induction relative to untreated value of scramble siRNA group. Open bars and closed bars represent untreated group and quercetin-treated group, respectively. Each value represents mean ± S.D. of five independent experiments. $^{*}P < 0.05$ versus quercetin-treated value of scramble siRNA group. E: INS-1 cells transfected with control siRNA scramble or PKAα siRNA (PKA) were treated with quercetin (25 μM) for 2 h. Western blot analyses for EGR-1 and PKA were performed. Similar results were obtained in three independent experiments. F: INS-1 cells were transfected with PKAα catalytic subunit expression vector (PKA) or empty vector (Empty) for 16 h, and then the cells were treated with quercetin (25 μM) for 2 h. Western blot analyses for EGR-1 and PKA were performed. Similar results were obtained in three independent experiments.

examined the effect of exogenous PKAα overexpression on quercetin-induced EGR-1 expression. As shown in Fig. 2F, there was no difference in quercetin-induced EGR-1 expression between untreated control and empty vector-transfected cells. In the exogenous PKAα-transfected cells, the expression of PKA protein was significantly increased compared with either untreated control cells or empty vector-transfected cells. Contrary to our expectation, exogenous overexpression of PKA failed to further increase quercetin-induced EGR-1 expression compared with both untreated control and empty vector-transfected cells. Based on the results of PKA gene silencing and overexpression, endogenous PKA expression itself may be sufficient for EGR-1 induction by quercetin.

EGR-1 PROMOTER ACTIVITY BY QUERCETIN IS NOT DEPENDENT ON CRE SITE

To investigate the role of *cis*-element on EGR-1 promoter, serially-deleted EGR-1 promoter constructs were employed (Fig. 3A). In basal condition, promoter activities were not significantly different except for *p*-46 construct. However, the response to quercetin in *p*-375 (devoid of 1st SRE site) and *p*-73 (devoid of 4th and 5th SRE sites) constructs was significantly decreased compared with *p*-462 and *p*-124 constructs, respectively (Fig. 3B). Meanwhile, the responses of *p*-124 (devoid of distal CRE site) and *p*-46 (devoid of proximal CRE site) constructs by quercetin were not reduced compared with *p*-325 and *p*-73 constructs, respectively (Fig. 3B). To confirm this finding, we examined the promoter activity using CRE site-mutated *p*-73 construct (transcription factor CREB-binding core sequence was mutated: AC→TG). As shown in Fig. 3C, though the promoter activity of mutated *p*-73 construct was significantly decreased in basal condition, the response by quercetin was intact compared with wild type. These findings suggest that CRE site on EGR-1 promoter may not be involved in quercetin-induced EGR-1 promoter activity.

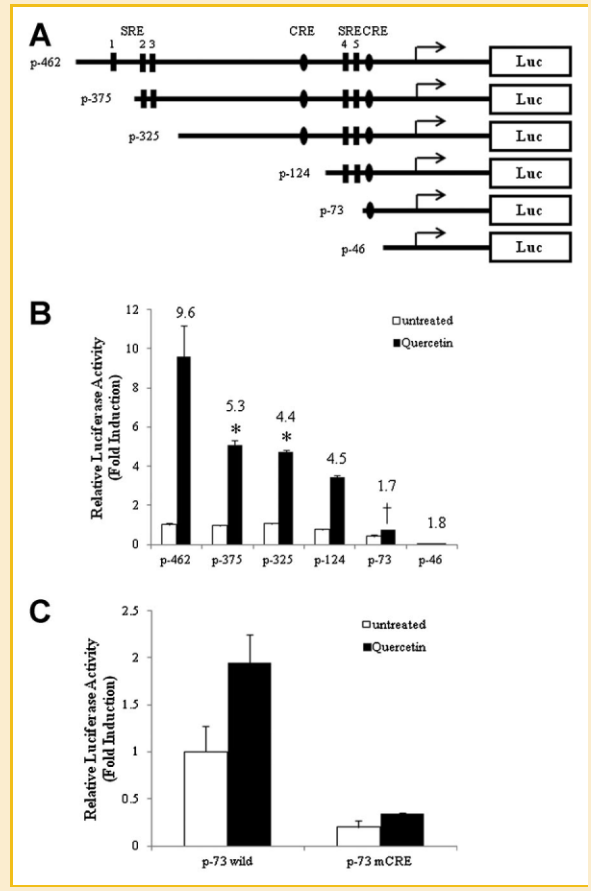

Fig. 3. Functional significance of cAMP response element (CRE) in quercetin-induced EGR-1 promoter activity. A: Schematic description of six deletion constructs of luciferase/rat EGR-1 promoter. The known SRE and CRE sites are serially numbered from 5′ to 3′ position. B: Promoter activities of each deletion construct in the absence or presence of quercetin. Luciferase activity was presented as a fold induction relative to untreated *p*-462 EGR-1 construct. Open bars and closed bars represent untreated group and quercetin-treated group, respectively. Each value represents mean ± S.D. of five independent experiments. Figures on the error bar mean fold change relative to untreated value of each promoter construct. $^{*}P < 0.05$ versus quercetin-treated value of *p*-462; $\dagger P < 0.05$ versus quercetin-treated value of *p*-124. C: INS-1 cells transfected with wild or mutant *p*-73 EGR-1 promoter constructs were treated with quercetin (25 μM) for 16 h, and then promoter activities were measured. Each promoter activity was presented as a fold induction relative to untreated value of *p*-73 wild type, respectively. Open bars and closed bars represent untreated group and quercetin-treated group, respectively. Each value represents mean ± S.D. of five independent experiments.

EGR-1 PROMOTER ACTIVITY BY QUERCETIN IS DEPENDENT ON SRE SITE

To evaluate the functional significance of SRE site in EGR-1 promoter activity, transcription factor SRF-binding core sequence of 4th and 5th SRE site in *p*-462 and *p*-124 constructs was mutated (GG→TT). In *p*-462 construct, single mutation of either 4th SRE or 5th SRE site did not affect the promoter activity compared with wild type; however, both mutations of 4th and 5th SRE sites significantly reduced quercetin-induced promoter activity (Fig. 4A). Meanwhile, in case of *p*-124 construct, single or both mutation of SRE site significantly decreased quercetin-induced promoter activity (Fig. 4B). To evaluate whether SRE site is relevant for PKA-regulated EGR-1 transcription, we observed the effect of PKA on EGR-1 promoter activity following the overexpression of PKAα catalytic subunit. As shown in Fig. 4C, exogenous PKAα induced the increase in promoter activity of *p*-124 (with SRE site), however, did not increase promoter activity of p-73 (devoid of SRE site). These findings confirmed the importance of the SRE site in quercetin-induced EGR-1 gene transcription. Subsequently, to test the binding activity of nuclear proteins to the SRE site on EGR-1 promoter, EMSA was performed using a radiolabeled oligonucleotide covering 4th SRE and 5th SRE site. The relative intensity of each band was similar irrespective of quercetin treatment (Fig. 4D). Next, nuclear factors binding to SRE sites were identified employing relevant antibodies. As shown in Fig. 4E, the incubation with antibodies against SRF or phospho-SRF resulted in supershifted band formation; however, there was no change in supershifted band intensity between control and quercetin-treated groups. Also, the addition of Elk-1 and phospho-Elk-1 did not affect the mobility and intensity of DNA-protein complex (Fig. 4E).

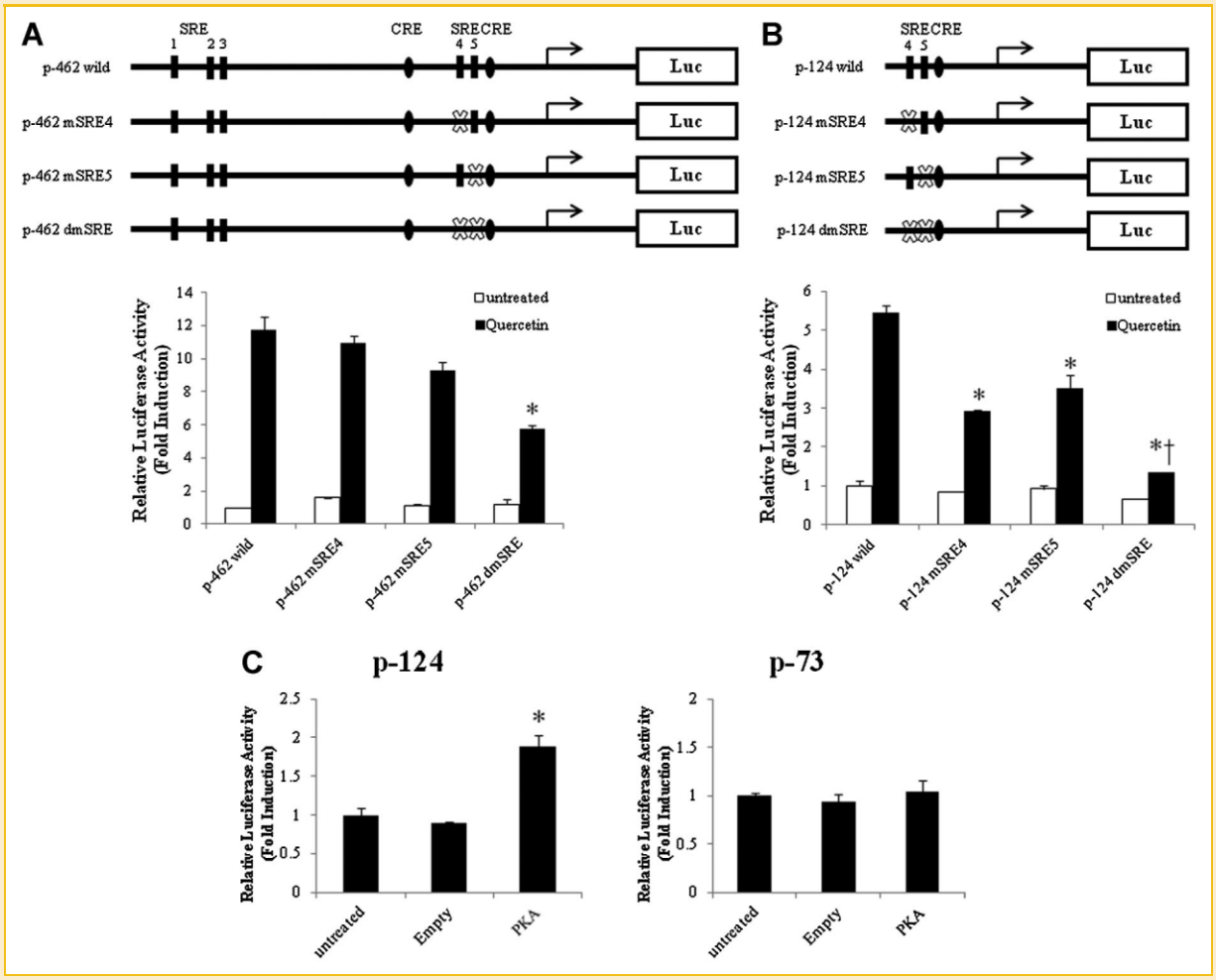

Fig. 4. Functional significance of serum response element (SRE) in quercetin-induced EGR-1 promoter activity. A, B: INS-1 cells transfected with wild or mutant *p*-462 (A) or *p*-124 (B) EGR-1 promoter constructs were treated with quercetin (25 μM) for 16 h, and then promoter activities were measured. Each promoter activity was presented as a fold induction relative to untreated value of *p*-462 or *p*-124 wild type, respectively. Open bars and closed bars represent untreated group and quercetin-treated group, respectively. Each value represents mean ± S.D. of five independent experiments. In (A), $^{*}P < 0.05$ versus quercetin-treated value of any other group. In (B), $^{*}P < 0.05$ versus quercetin-treated value of wild *p*-124; †$P < 0.05$ versus quercetin-treated value of *p*-124 mSRE4 and mSRE5. C: INS-1 cells were cotransfected with EGR-1 promoter (*p*-124 or *p*-73) and PKAα catalytic subunit expression vector (PKA) or empty vector (Empty) for 16 h, and then promoter activities were measured. Each promoter activity was presented as a fold induction relative to untreated value of *p*-124 or *p*-73, respectively. Each value represents mean ± S.D. of five independent experiments. $^{*}P < 0.05$ versus untreated value or empty vector. D: Gel mobility shift assay of SRE on rat EGR-1 promoter. INS-1 cells were treated with quercetin (25 μM) for the indicated times. Nuclear extracts were analyzed on mobility shift assay with radiolabeled probe covering 4th SRE and 5th SRE binding sites (5′-CCTTCCATATTAGGGCTTCCTGCTTCCCATATATGGCCATG-3′). Arrow means DNA-protein complex. E: INS-1 cells were treated with quercetin (25 μM) for 30 min. Then, immune-supershift assay was performed using specific antibody against SRF, phospho-SRF, Elk-1, and phospho-Elk-1. The arrowheads indicate band supershifted by anti-SRF or phospho-SRF antibody.

DISCUSSION

The roles of EGR-1 in pancreatic beta-cells have been revealed through in vivo and in vitro studies. The reduction in EGR-1 expression may contribute to decreased beta-cell proliferation and to the development of diabetes mellitus [Garnett et al., 2005]. The expression of EGR-1 in beta-cells was associated with beta-cell proliferation and transactivation of insulin and PDX-1 genes [Eto et al., 2006, 2007; Kang et al., 2006]. We recently reported that quercetin-induced EGR-1 was involved in the transcriptional activation of GCLC, which plays an important role in intracellular redox balance system [Kang et al., 2009a]. The present study demonstrates molecular mechanisms of quercetin-induced EGR-1 expression in INS-1 beta-cells.

In the absence of external stimuli, EGR-1 expression is mostly negligible in many cell types [Thiel and Cibelli, 2002]. In this study, quercetin significantly induced EGR-1 protein in beta-cell lines RINm5F and MIN6 cells as well as INS-1 cells, which implies that quercetin induces EGR-1 in various pancreatic beta-cells.

EGR-1 is rapidly induced by many extracellular stimuli such as cytokines and growth factors and EGR-1 induction was known to be regulated via several kinase pathways including ERK, p38 MAPK, PKC, and PI-3 kinase [Rolli et al., 1999; Guha et al., 2001; Duan et al., 2002; Shin et al., 2006]. Lipopolysaccharide induced EGR-1

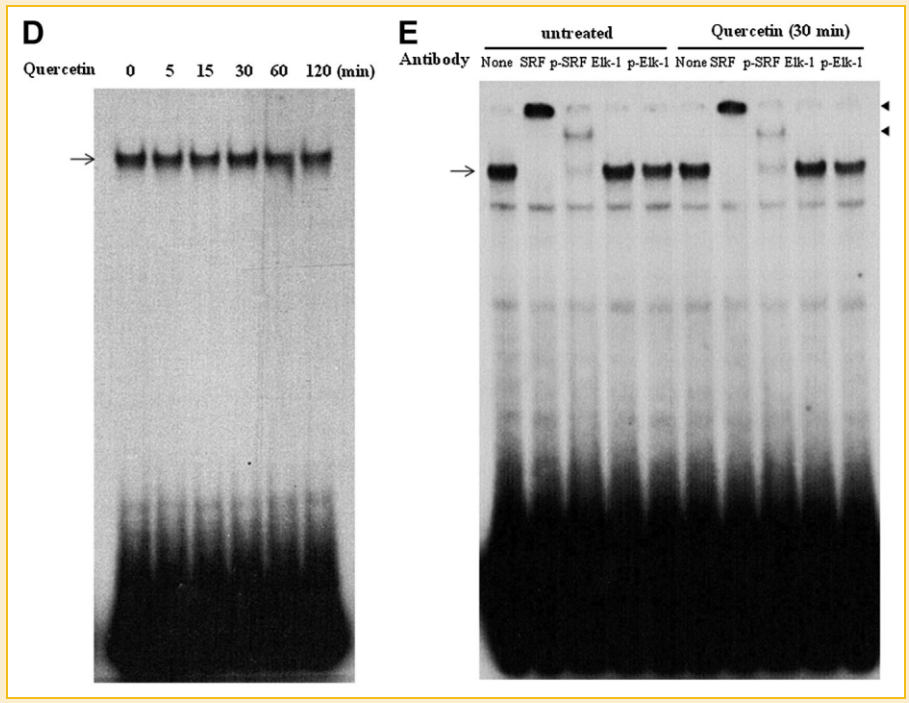

Fig. 4. (*Continued*)

expression via the activation of MEK-ERK pathway in human monocytic cells [Guha et al., 2001]. Gonadotropin releasing hormone-induced EGR-1 was dependent on PKC/ERK pathways in pituitary gonadotrope cells [Duan et al., 2002]. p38 MAPK pathway mediated an antibiotic anisomycin-induced EGR-1 expression in human Jurkat T-cells [Rolli et al., 1999]. Also, PI-3 kinase was involved in oncogenic H-Ras-mediated reduction of EGR-1 expression [Shin et al., 2006].

In this study using specific inhibitors, quercetin-induced EGR-1 protein expression was largely mediated by PKA and partly by p38 MAPK pathways, but not by ERK, JNK, and PI-3 kinase pathways. To further evaluate the role of PKA in EGR-1 expression, PKA gene silencing study was conducted. In a previous report, PKA isoforms α, β, and γ catalytic subunits were identified in pancreatic beta-cells. Among these isoforms, glucose and glucagon-like peptide-1 caused translocation of PKAα to the nucleus and translocation of PKAβ to the plasma membrane and nucleus, but did not affect translocation of PKAγ [Gao et al., 2002]. Based on this fact, we performed gene silencing study using siRNA duplex against PKAα. In PKAα siRNA-transfected cells, quercetin-induced EGR-1 protein expression and EGR-1 promoter activity was significantly decreased. Previous studies using beta-cells demonstrated that exendin-4 increased EGR-1 expression in PKA- and ERK-dependent manners [Kim et al., 2008], and PKA pathway was involved in EGR-1 induction under high glucose condition [Bernal-Mizrachi et al., 2000]. Taken together, EGR-1 expression in beta-cells appears to be largely regulated by PKA signaling pathway.

The EGR-1 promoter possesses several *cis*-elements for binding of CREB, SRF, and E twenty-six (Ets) [Bernal-Mizrachi et al., 2000; Thiel and Cibelli, 2002]. First, we examined the involvement of CRE site in EGR-1 transcription since transcription factor CREB binds to CRE site and stimulates gene transcription following PKA-mediated phosphorylation of Ser^{133} residue [Rosenberg et al., 2002; Sands and Palmer, 2008]. Unexpectedly, our promoter study showed that CRE site was not involved in quercetin-induced EGR-1 transcription, though promoter activities of *p*-46 construct or CRE site-mutated *p*-73 construct were significantly reduced in basal condition. These findings are contrary to the result that PKA inhibitor H89 and PKA gene silencing inhibited quercetin-induced EGR-1 promoter activity. In addition, overexpression of PKAα catalytic subunit did not increase promoter activity of *p*-73 construct (with CRE site, without SRE site). Therefore, quercetin-mediated PKA pathway may not affect the activation of CRE site on EGR-1 promoter though further study about the role of CRE site will be required.

Our promoter studies showed that SRE sites were implicated in quercetin-induced transcriptional activation of EGR-1 gene and SRE site was also relevant for PKA-regulated EGR-1 gene transcription. Such an involvement of SRE site in EGR-1 expression was well demonstrated in different conditions [Treisman, 1992; Bernal-Mizrachi et al., 2000; Cibelli et al., 2002]. The SRE sites of rat EGR-1 promoter were involved in forskolin- or KCl-induced EGR-1 expression in MIN6 beta-cells [Bernal-Mizrachi et al., 2000], and the distal SRE site of human EGR-1 promoter were responsible for nitric oxide-induced EGR-1 synthesis in SH-SY5Y neuroblastoma cells [Cibelli et al., 2002].

Meanwhile, the SRE site is continuously occupied in vivo by SRF and Ets proteins of the TCFs Elk-1, Net, and Sap-1 [Treisman, 1992]. Therefore, we examined nuclear proteins bound to SRE site using EMSA. SRF and phospho-SRF were bound to SRE site in both basal and quercetin-treated conditions, though there was no difference in the supershifted band density between these groups. Meanwhile, Elk-1, one of TCF, did not bind to SRE site in basal and quercetin-treated conditions. The similar findings to our result were reported in other studies [Clarkson et al., 1999; Wu et al., 2002]. In 3T3-F442A preadipocytes, SRE site of EGR-1 promoter was constitutively bound by SRF but not by TCF [Clarkson et al., 1999]. Also, Elk-1 and Sap-1 did not bind to SRE site of EGR-1 promoter in primary human endothelial cells [Wu et al., 2002]. Therefore, SRE sites are likely to transduce quercetin response by SRF-dependent, but not TCF-dependent mechanism in INS-1 beta-cells, though further research on the precise role of SRE sites will be necessary.

Collectively, quercetin increases EGR-1 expression largely by PKA and partly by p38 MAPK pathway, and SRE sites on EGR-1 promoter are involved in quercetin-induced EGR-1 transcription.

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