Analysis of Induction Mechanisms of an Insulin-Inducible Transcription Factor SHARP-2 Gene by (−)-Epigallocatechin-3-gallate

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ABSTRACT: The rat enhancer of split- and hairy-related protein-2 (SHARP-2) is an insulin-inducible transcription factor. In this study, we examined the mechanisms involved in the regulation of the rat SHARP-2 gene expression by (−)-epigallocatechin-3-gallate (EGCG). The induction of SHARP-2 mRNA by EGCG was repressed by pretreatments with inhibitors for either phosphoinositide 3-kinase (PI3K) or RNA polymerase II. Then, we examined a biological relationship between EGCG and transcription factor NF-κB interfering with the insulin action. The protein levels of the NF-κB were rapidly decreased by an EGCG treatment. Finally, the mechanism(s) of transcriptional activation of the rat SHARP-2 gene by both NF-κB and EGCG was analyzed. While overexpression of the NF-κB p65 protein decreased the promoter activity of the SHARP-2 gene, EGCG did not affect it. Thus, we conclude that EGCG induces the expression of the rat SHARP-2 gene via both the PI3K pathway and degradation of the NF-κB p65 protein.

KEYWORDS: SHARP-2, transcription factor, insulin, (−)-epigallocatechin-3-gallate, phosphoinositide 3-kinase, nuclear factor-kappa B

INTRODUCTION

The diabetes mellitus, a lifestyle disease, causes complications such as neuropathy, retinopathy, and nephropathy, leading to not only the decrease of quality of life, but also death. The International Diabetes Federation reported that the number of the diabetic patients in the world would be assumed to reach over 522 million in 2030. Feeding on a high energy diet such as a high carbohydrate diet to normal rats or an insulin administration to diabetic rats. We also reported that the overexpression of SHARP-2 in both primary cultured rat hepatocytes and rat H4IIIE highly differentiated hepatoma cells decreased the level of phosphoenolpyruvate carboxykinase (PEPCK) mRNA, a gluconeogenic enzyme gene, and that SHARP-2 decreased the promoter activity of the rat PEPCK gene. Therefore, we hypothesize that SHARP-2 is an important transcription factor involved in the regulation of the level of blood glucose. Some compounds which can increase the expression of the SHARP-2 gene may be useful for prevention and treatment of diabetes mellitus. (−)-Epigallocatechin-3-gallate (EGCG), a green tea polyphenol, is one of the candidates. It has been reported that EGCG decreased the level of PEPCK mRNA both in vivo and in vitro and that it had antiobesity and antidiabetic effects.

We also reported that EGCG as well as insulin rapidly induced the level of SHARP-2 mRNA. However, the signaling pathway(s) remains to be determined. The 67 kDa laminin receptor is a cell membrane protein binding to laminin which is a major constituent of basal membrane. It has been reported that the 67 kDa laminin receptor is an EGCG receptor. However, there are controversial reports.

Nuclear factor-kappa B (NF-κB) is a transcription factor that regulates expression of many genes. In the cytoplasm, an inactive NF-κB protein consists of three subunits, p65, p50, and I-κB. By extracellular stimuli, I-κB kinase (Ikk) phosphorylates I-κB and I-κB dissociates from an inactive NF-κB protein. An active form of NF-κB, a p65/p50 heterodimer, then translocates into the nucleus, binds to the specific nucleotide sequence within the transcriptional regulatory region of the target genes, and regulates the transcription rate of them. Subacute hepatocellular activation of NF-κB in constitutively active form of human Ikk-β-expressed transgenic mice caused hepatic insulin resistance. In addition, it has been reported that obesity and a feeding with a high fat diet caused activation of NF-κB in both adipocytes and hepatocytes and led to insulin resistance. Therefore, an activation of NF-κB interferes with the insulin effects.

In the present study, we analyzed of a mechanism(s) of up-regulation of the SHARP-2 gene expression by EGCG and...
found an involvement of NF-κB degradation in the EGCG-regulation.

MATERIALS AND METHODS

Materials. Dulbecco’s modified Eagle’s medium (DMEM) was purchased from Wako (Osaka, Japan). Fetal bovine serum (FBS), EGCG, LY294002, lipopolysaccharide (LPS), and horseradish peroxidase conjugate-rabbit anti-mouse IgG antibody were purchased from Sigma Aldrich (Saint Louis, MO). Streptomycin and penicillin G were purchased from Meijiseika (Tokyo, Japan). Compound-C, rapamycin, okadaic acid, JNK inhibitor II, staurosporin, PD98059, and actinomycin D were purchased from Merck chemicals (Darmstadt, Germany). TRIZol reagent was purchased from Invitrogen (Groningen, The Netherlands). High capacity RNA-to-cDNA kit and Big Dye Terminator v1.1 Cycle Sequencing kit were purchased from Applied Biosystems (Foster City, CA). FastStart Universal SYBR Green Master (RoX) and Genopure Plasmid Maxi Kit were purchased from Roche Diagnostics (Indianapolis, IN). Bio-Rad Protein Assay was purchased from Bio-Rad Laboratories (Hercules, CA). Polyvinylidene difluoride (PVDF) membrane and Immobilon Western chemiluminescent HRP substrate were purchased from MILLIPORE (Bedford, MA). Rabbit anti-rat NF-κB p65 (A) antibody (SC-109), rabbit anti-rat NF-κB (H-209) antibody (SC-10779), or mouse anti-rat β-actin (C4) antibody (SC-47778) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Horseradish peroxidase conjugate-goat anti-rabbit IgG antibody was purchased from BIOSOURCE (Camarillo, CA). Dual-Luciferase Reporter Assay System were purchased from Promega (Madison, WI). The pSG5 was purchased from Stratagene (San Diego, CA).

Cells and Cell Culture. Rat H4IIE hepatoma cells were a generous gift from Dr. Daryl K. Granner (Vanderbilt University). Cells were grown in DMEM supplemented with 10% FBS, 100 μM streptomycin, and 100 units/ml penicillin G at 37°C in a 5% CO2 incubator.

One million of H4IIE cells were seeded in a 6-cm dish. After 24 h, the medium was replaced with serum-free DMEM and then cultured for 24 h. At 2 h after the medium was replaced with the same medium, cells were treated with 25 μM EGCG for 2 h. To analyze a signal transduction pathway(s), H4IIE cells were pretreated with various inhibitors for the indicated times. LY294002 (50 μM), compound-C (1 μM), rapamycin (0.1 μM), okadaic acid (10 nM), JNK inhibitor II (10 μM), staurosporin (0.1 μM), PD98059 (25 μM), and actinomycin D (0.8 μM) were used as the inhibitors.

Preparation of Total RNA and Real-Time Polymerase Chain Reactions (PCR). Total RNA was prepared from various cultured cells by using the TRIzol reagent. Total RNA (1 μg) was reverse-transcribed with the High capacity RNA-to-cDNA kit. Procedures were performed according to the manufacturer’s recommended protocol. Quantitative real-time PCR was carried out using the cDNA, primers, and FastStart Universal SYBR Green Master (RoX) by means of an ABI 7300 Real Time PCR System. Combinations of SHARP-2 forward 5'-GGGTGAGGCCCAAGAAATG-3' and reverse 5'-CTAGATTAGGAGGACCTTTCCAGGGC-3' and NF-κB antisense, 5'-CTAGGGCTTTGGAAGTCCCTAACGTGCTGGAATGCCCCTCAACTC-3', respectively, were used as the primers. Relative amounts of both SHARP-2 and 36B4 mRNA levels were determined. The level of SHARP-2 mRNA was normalized by that of 36B4 mRNA.

Western Blot Analysis. H4IIE cells were treated with 25 μM EGCG or 100 ng/ml LPS for the indicated times, then harvested in phosphate-buffered saline. Preparation of the nuclear and cytoplasmic fractions were according to the method of Schreiber et al. The protein concentration was determined using the Bio-Rad Protein Assay. Each extract (50 μg/lane) was resolved with 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a PVDF membrane. Rabbit anti-rat NF-κB p65 (A) antibody (SC-109), rabbit anti-rat NF-κB (H-209) antibody (SC-10779), or mouse anti-rat β-actin (C4) antibody (SC-47778) was used as the primary antibody. Horseradish peroxidase conjugate-goat anti-rabbit IgG antibody or horseradish peroxidase conjugate-rabbit anti-mouse IgG antibody was employed as the secondary antibody. The proteins were visualized using the Immobilon Western chemiluminescent HRP substrate and Hyperfilm ECL.

RESULTS

Analysis of a Signal Transduction Pathway(s) of an Induction of SHARP-2 mRNA by EGCG. We previously reported that the level of SHARP-2 mRNA was rapidly induced by EGCG as well as insulin. However, the signaling pathway(s) remained unclear. To determine a signaling pathway(s) involved in the induction of SHARP-2 mRNA by EGCG, H4IIE cells were treated with various inhibitors. First, to examine whether the induction of the level of SHARP-2 mRNA by EGCG was mediated by the phosphoinositide 3-kinase (PI3K) pathway as well as insulin, H4IIE cells were treated with LY294002, a PI3K inhibitor. The induction of the level of SHARP-2 mRNA by insulin was completely inhibited by a treatment with LY294002. In contrast, the induction of SHARP-2 mRNA by EGCG decreased to approximately 70% (Figure 1A).

Next, to examine whether an induction of SHARP-2 mRNA by EGCG was mediated by the AMPK pathway, H4IIE cells were treated with compound-C, a AMPK inhibitor. The induction of SHARP-2...
mRNA by EGCG was not inhibited by an addition of compound-C, indicating that the AMPK signaling pathway was not involved in the induction of SHARP-2 mRNA by EGCG (Figure 1B).

To identify another signaling pathway(s) in the SHARP-2 mRNA induction by EGCG, H4IIE cells were treated with various inhibitors: rapamycin, a p70S6K inhibitor; okadaic acid (OA), 10 μM JNK inhibitor II (JN), 0.1 μM staurosporin (St), 25 μM PD98059 (PD), or 0.8 μM actinomycin D (AD) indicated on the bottom and then treated with (+) or without (−) 25 μM EGCG for another 2 h. **p < 0.001.

EGCG occurred at the transcriptional level of the rat SHARP-2 gene.

**Effect of EGCG on NF-κB Expression.** NF-κB is a transcription factor that regulates expression of many genes.15-17,30 It has been reported that obesity and a feeding with a high fat diet caused an activation of NF-κB in liver, induced the insulin resistance, and interfered with the insulin effect.20-23

We then determined an issue of whether the level of NF-κB protein was down-regulated in H4IIE cells by a treatment with EGCG. Western blot analysis was performed using antibodies against NF-κB p65 subunit, β-actin, or A subunit of nuclear factor Y (NF-YA). The levels of the NF-κB p65 in both the cytoplasm and the nucleus decreased at 5 min by a treatment with EGCG and almost disappeared at 60 min. In contrast, the levels of cytoplasmic β-actin and nuclear NF-YA proteins remained unchanged (Figure 3). By treating with LPS, a NF-κB activator, a decrease of the levels of the NF-κB p65 in the cytoplasm and a concomitant increase in the nucleus were observed in H4IIE cells (Figure 3).

These results indicated that EGCG specifically promoted the degradation of the NF-κB p65 protein.

**Analysis of the Mechanism(s) of Transcriptional Regulation of the Rat SHARP-2 Gene by NF-κB.** To analyze the mechanism(s) of transcriptional regulation of the rat SHARP-2 gene by NF-κB, we prepared two reporter plasmids, p(xB)κ-tk/Luc and pGL4.20-F3Nhe. The p(xB)κ-tk/Luc harbors six copies of the consensus NF-κB-binding sites just upstream of the thymidine kinase gene promoter.27 The pGL4.20-F3Nhe contains a 3.7 kb upstream region from a transcriptional initiation site of the rat SHARP-2 gene linked to the firefly luciferase gene. This region contains multiple elements mediating the transactivation by various extracellular stimuli.2 Two effector plasmids, the pSG5 and the pSG5-p65s, were employed. The pSG5 is an empty vector and the pSG5-p65s is an active form of NF-κB p65 subunit expression vector. When the p(xB)κ-tk/Luc and an effector plasmid were
experiments were performed and a representative result was shown. Figure 3. Western blot analysis of NF-κB p65 protein level in H4IIE cells treated with EGCG. The cytoplasmic and nuclear fractions were prepared from H4IIE cells treated with 25 μM EGCG or 100 ng/mL LPS for the indicated time. Each extract (50 μg/lane) was resolved using a 10% SDS-PAGE gel and transferred onto a PVDF membrane for Western blot analysis. The rabbit anti-rat NF-κB p65 (A) antibody (1:400 dilution), mouse anti-rat β-actin (C4-1) antibody (1:400 dilution), or rabbit anti-rat NF-YA (H-209) antibody (1:2000 dilution) was used as the primary antibody. Horseradish peroxidase conjugate-goat anti-rabbit IgG antibody (1:20 000 dilution) or horseradish peroxidase conjugate-rabbit anti-mouse IgG antibody (1:20 000 dilution) was used as second antibody. The proteins were visualized using the chemiluminescent method. Three independent experiments were performed and a representative result was shown.

Figure 4. Analysis of a NF-κB-responsive region of the rat SHARP-2 gene. H4IIE cells were transfected with 8 μg of a luciferase reporter plasmid indicated on the bottom, 2 μg of an effector plasmid which is either the pSG5 (−) or the pSG5-p65 plasmid (+), and 0.5 μg of the phRLuc-CMV using the calcium phosphate method. Firefly luciferase activities were normalized by sea pansy luciferase activities. A value of 100 was assigned to the level of each promoter activity in the absence of EGCG. Each column and bar represents the mean and standard error of four independent experiments. *p < 0.05.

These results indicated that a 3.7 kb upstream region of the rat SHARP-2 gene was not sufficient for the transcriptional activation by EGCG.

Figure 5. Analysis of an EGCG-responsive region of the rat SHARP-2 gene. H4IIE cells were transfected with 10 μg of a luciferase reporter plasmid shown on the bottom and 0.5 μg of the phRLuc-CMV using the calcium phosphate method. Twenty-four hours after transfection, cells were treated for 2 h with (+) or without (−) 25 μM EGCG. Firefly luciferase activities were normalized by sea pansy luciferase activities. A value of 100 was assigned to the level of each promoter activity in the absence of EGCG. Each column and bar represents the mean and standard error of four independent experiments.

These results indicated that a 3.7 kb upstream region of the rat SHARP-2 gene was negatively regulated by NF-κB.

### DISCUSSION

We analyzed the mechanism(s) of up-regulation of the SHARP-2 gene expression by EGCG. We previously reported that insulin induced the expression of the rat SHARP-2 gene via the PI3K pathway. When H4IIE cells were treated with PI3K inhibitor, the induction of SHARP-2 mRNA level by EGCG was partially blocked (Figure 1A). It has been reported that some downstream signaling molecules of PI3K in the insulin signaling pathway were atypical PKC (aPKC) and p70S6 kinase. We have previously reported that EGCG activated aPKC in H4IIE cells. However, the induction of SHARP-2 mRNA by EGCG was not suppressed by pretreatments with inhibitors such as staurosporin and rapamycin (Figure 2). Therefore, these signaling molecules were not downstream signaling molecules of PI3K involved in the induction of SHARP-2 mRNA by EGCG. These results coincide with the case of the induction of SHARP-2 mRNA level by insulin, indicating that both insulin and EGCG increase the level of SHARP-2 mRNA via a common PI3K pathway.

The induction of SHARP-2 mRNA by EGCG was completely blocked by a pretreatment with actinomycin D, suggesting that the induction was controlled at the transcriptional level of the rat SHARP-2 gene (Figure 2). Expression of the rat SHARP-1 gene, another member of the SHARP family, was also induced by EGCG. Signal transduction pathways of the EGCG-induction of the rat SHARP-1 gene were PI3K/aPKC-, AMPK-, and NF-κB-signaling pathways. At least PI3K/aPKC- and AMPK-signaling pathways were different from the mechanism(s) of the induction of the rat SHARP-2 gene.
gene since these inhibitors did not affect the induction of SHARP-2 mRNA level by EGCG.

It has been reported that NF-κB activation in liver induced the insulin resistance. We then examined whether EGCG actually affects the expression of NF-κB in the H4IIE cells. The levels of the NF-κB p65 protein in both the cytoplasmic and nuclear fractions were decreased in EGCG-treated H4IIE cells (Figure 3). It has been reported that EGCG decreased the level of nuclear NF-κB protein in the mouse liver fed with EGCG for 6 weeks and that the level of cytoplasmic NF-κB p65 protein was decreased by an EGCG treatment for 3 h in human hepatocellular carcinoma HLE cells. Thus, we hypothesize that EGCG promotes an inhibition of NF-κB activity by protein degradation and prevents insulin resistance.

The NF-κB regulates expression of several genes. Co-transfection experiments into H4IIE cells revealed that a 3.7 kb upstream region of the rat SHARP-1 gene was repressed by NF-κB (Figure 4). In our previous study, a co-transfection of p65 with a reporter plasmid also decreased promoter activity of the rat SHARP-1 gene. Thus, the p65 subunit could act as a repressor of both genes. It has been reported that both tumor necrosis factor-α and LPS activated NF-κB and it suppressed promoter activity of the murine cytokine P (cpp) gene in murine hepatoma Hepa1c1c7 cells. In addition, the activated NF-κB suppressed the cyp3a4 gene expression by binding to a pregnane X receptor-retinoid X receptor α heterodimer in primary cultured human hepatocytes and human HepG2 cells.

Finally, to identify an EGCG-responsive cis-acting element of the rat SHARP-2 gene, a 3.7 kb region upstream from a transcription initiation site of the rat SHARP-2 gene was examined. However, EGCG did not enhance the promoter activity of the rat SHARP-2 gene in H4IIE cells (Figure 5). Same results were obtained when the reporter plasmid-transfected H4IIE cells were treated with EGCG for 4, 6, and 8 h (data not shown). Therefore, a 3.7 kb upstream region of the rat SHARP-2 gene responded to NF-κB but was not sufficient for the transcriptional activation by EGCG. Thus, we concluded that EGCG can increase the transcription activity of the rat SHARP-2 gene through the PI3K pathway as well as insulin and that EGCG promotes degradation of the NF-κB p65 protein as a repressor of the rat SHARP-2 gene.

Further studies will be required for an identification of a NF-κB- and an EGCG-responsive element(s) of the rat SHARP-2 gene to fully understand the transcriptional regulatory mechanism by EGCG. It remains to be determined an issue of whether protein degradation of NF-κB by EGCG is dependent on an ubiquitin-proteasome pathway.

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**ABBREVIATIONS USED**

SHARP-2, enhancer of split- and hairy-related protein-2; PEPCK, phosphoenolpyruvate carboxykinase; EGCG, (−)-epigallocatechin-3-gallate; NF-κB, nuclear factor-kappa B; Ik, I-kB kinase; DMEM, Dulbecco’s modified Eagle’s medium; FBS, fetal bovine serum; LPS, lipopolysaccharide; PVDF, polyvinylidene difluoride; PCR, polymerase chain reaction; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; PI3K, phosphoinositide 3-kinase; AMPK, AMP-activated protein kinase; PKC, protein kinase C; NF-YA, A subunit of nuclear factor Y; aPKC, atypical PKC; cyp, cytochrome P.

**REFERENCES**

(15) Kucharczak, J.; Simmons, M. J.; Fan, Y.; Gélinas, C. To be, or not to be: NF-kappaB is the answer—role of Rel/NF-kappaB in the regulation of apoptosis. Oncogene 2003, 22, 8961–8982.


