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Regulation of Src homology 2–containing protein tyrosine phosphatase by advanced glycation end products: the role on atherosclerosis in diabetes

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Abstract

Advanced glycation end products (AGEs), among the most important causes of atherosclerosis in diabetes mellitus, stimulate the proliferation of smooth muscle cells (SMCs). Smooth muscle cells are central in the formation of atherosclerotic lesions, where they show both increased migration and accelerated proliferation. In investigating how AGEs stimulate SMC proliferation, we focused on protein tyrosine phosphatase, especially Src homology 2–containing protein tyrosine phosphatase (SHP2), which is considered important in regulating cell proliferation. Advanced glycation end products increased activity of SHP2 in the membrane fraction of rat aortic SMCs compared with control bovine serum albumin ($P < .05$). Upon characterizing the genomic and promoter structure of SHP2, we detected nuclear factor–κB (NF-κB) binding sites in the promoter area. Advanced glycation end product stimulation increased luciferase activity in cells transfected with SHP2 promoter region including NF-κB binding sites ($P < .05$) and increased SHP2 expression ($P < .05$). These data indicate that AGE stimulation appears to activate NF-κB. Activated NF-κB binds to sites on the SHP2 promoter, resulting in increased SHP2 expression, SHP2 activity, and, ultimately, SMC proliferation. It suggests that AGE stimulation induces SMC proliferation via SHP2, underscoring the importance of control of AGE for suppressing macroangiopathy in diabetes mellitus.

1. Introduction

Diabetes mellitus induces micro- and macroangiopathy. Forms of macroangiopathy such as cardiovascular disease or cerebrovascular disease represent the leading cause of mortality in diabetic patients. The primary cause of cardiovascular disease is atherosclerosis, in which multiple factors including hyperglycemia, hyperlipidemia, and hypertension have been implicated. Among atherogenic factors in diabetes, advanced glycation end product (AGE) is important. Hyperglycemia accelerates nonenzymatic glycation of proteins (the Maillard reaction), after which a complex series of rearrangements and oxidative reactions leads to formation of AGE. In diabetes, plasma concentration of AGE increases; and AGE deposits occur in small vessels, renal parenchyma, cardiac muscle, and the crystalline lens as well as in atherosclerotic lesions [1]. Advanced glycation end products are thought to be highly important in diabetic angiopathy [2].

Smooth muscle cells (SMCs) are central in atherosclerotic lesion formation, showing both increased migration and accelerated proliferation [3]; their proliferation is enhanced by AGE stimulation [4-6]. Various pathways have been

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reported to link AGE with cell proliferation, including activation of mitogen-activated protein kinase [5] and nuclear factor–κB (NF-κB) [7].

Growth factors such as insulin-like growth factor I, platelet-derived growth factor (PDGF), and others foster SMC proliferation by binding to specific receptors on the SMC surface, leading to autophosphorylation of tyrosyl residues within their intracellular domains. In turn, this activation induces tyrosine phosphorylation of intracellular substrates of growth factor receptors. Phosphorylation of tyrosine residues has emerged as a key mechanism in signal transduction pathways regulating an extraordinary variety of cellular functions involved in proliferation, differentiation, and metabolism.

Tyrosine phosphorylation signaling is regulated by protein tyrosine phosphatases (PTPases). Accordingly, the degree of tyrosine phosphorylation is determined by a balance between activities of tyrosine kinases and PTPases acting as a regulator of signal transduction in tyrosine kinase–type receptors [8]. In investigating the mechanisms underlying SMC proliferation in response to AGE stimulation, we focused on PTPases, especially Src homology 2–containing PTPase (SHP2). Src homology 2–containing PTPase is believed to regulate intracellular signaling in a positive manner [9-11], but neither regulation of SHP2 activity nor structure of the SHP2 gene has been fully elucidated. We first assayed SHP2 activity with and without AGE stimulation. We then screened a human genomic library and analyzed the SHP2 gene configuration and its promoter region. Finally, we analyzed the relationship between the SHP2 promoter and proliferation of SMCs by AGE stimulation.

2. Methods

2.1. Cell culture

Aortic SMCs were obtained from Wistar Kyoto rats (Charles River, Yokohama, Japan) using an explant method.

The SMC cells and COS7 cells were maintained in Dulbecco modified Eagle medium (DMEM; Gibco BRL, Rockville, MD) containing penicillin (100 U/mL), streptomycin (100 mg/mL), and 10% fetal bovine serum (FBS).

Experiments were performed in compliance with the *Guide for the Care and Use of Laboratory Animals* (National Institutes of Health publication no. 85-23, revised 1996).

2.2. Preparation of AGE–bovine serum albumin

The AGE–bovine serum albumin (BSA) was prepared by incubating BSA (Fraction V; Wako, Tokyo, Japan) in phosphate-buffered saline with 1.67 mol/L glucose for 6 months at 37°C [12].

2.3. Protein extraction for PTPase assay

The PTPase activity after stimulation of 10 μg/mL AGE or BSA for 48 hours was assayed in SMCs cultured in DMEM with 10% FBS. Cells were homogenized in 50 mmol/L 4-(2-hydroxyethyl)-1-piperazine-ethane sulfonic acid (HEPES) at pH 7.6 containing 1000 U/mL aprotinin, 0.002% phenylmethylsulfonyl fluoride, 20 μg/mL leupeptin, 0.1 mmol/L benzamidine, 1 mmol/L dithiothreitol, and 2 mmol/L EDTA (solution A) at 4°C. Homogenates then were centrifuged, and the final supernatant was stored at −80°C as the cytosolic fraction. The pellet was solubilized in solution A containing 1% Triton X-100 at 4°C for 1 hour and centrifuged. This final supernatant was stored at −80°C as the membrane fractions.

2.4. Assay of PTPase activity

A synthetic peptide derived from the autophosphorylated regulatory region of the insulin receptor β-subunit (residues 1154 to 1165) was used as a phosphatase substrate according to a method already described [13].

2.5. Immunoprecipitation for SHP2 activity assay

One microgram of SHP2 antibody (Transduction Laboratories, Lexington, KY) was added to 100 μg of cell lysate for incubation at 4°C for 1 hour. Protein A (Pierce Biotechnology, Rockford, IL) was added to this reaction mixture, which was then rotated gently at 4°C for 1 hour. Agarose beads were centrifuged, washed twice with 50 mmol/L HEPES, and collected.

2.6. Isolation and sequencing of genomic clones

A genomic library (2×10^4 clones) from a human male white subject was cloned into a Lambda FIX II vector (Stratagene, La Jolla, CA) and screened with a ^{32}P-labeled full-length complementary DNA (cDNA) that encoded human SHP2. We obtained this SHP2 cDNA from cultured human mesangial cells (Iwaki, Tokyo, Japan) by RNA extraction and reverse transcriptase polymerase chain reaction (PCR) using an oligo dT primer. The PCR was carried out using 5′-ATGACATCGCGGAGATGGTTTCAC-3′ (primer 1) and 5′-TCTGAAACTTTTCTGCTGTTGCAT-3′ (primer 2). Positive clones were obtained and digested with restriction enzymes. Restriction fragments from human phage clones were subcloned into pBluescript II SK(−) (Stratagene). Sequences were determined by a primer-walking method using an ABI automated sequencer (Perkin-Elmer, Foster City, CA). We isolated more than 50 clones, but all were pseudogenes except for one genomic clone including part of the 3′-portion of the SHP2 gene that contained only the 16th exon and intron. Next, we screened a human bacterial artificial chromosome library (Genome Systems, St Louis, MO) using a part of the sequence of the 16th intron and the full-length cDNA of SHP2 obtained from cultured human mesangial cells. We obtained one clone from the bacterial artificial chromosome library. We also analyzed the DNA sequences using the National Center for Biotechnology Information Basic Local Alignment Search Tool.

2.7. Determination of the transcription initiation site

A cap site hunting method was followed, as described previously by Takada et al [14], using a cap site hunting kit (Cap Site cDNA dT/human liver; Nippon Gene, Tokyo, Japan). The sequence 5′-dGAUGCUAGUCAAGUCGAC-GAAGUGACGG-3′ was ligated to the 5′-end of cap-removed human liver messenger RNA. This linked messenger RNA was used to synthesize the first-strand cDNA by reverse transcriptase in the presence of random primers. Using this fragment, PCR was performed with a forward primer (5′-dGATGCTAGCTGCGAGTCAAGTC-3′) and a reverse primer that hybridized with the cDNA sequence of human SHP2 (5′-GCTCTGGCTCTCTCGTACAAG-3′). A second PCR was performed using the amplified DNA with nested primers; sequences of these forward and reverse primers were 5′-CGAGTCAAGTCGACGAAGTGC-3′ and 5′-GCCAAAGTGGCAAATTTCTCCC-3′. This PCR product was inserted into the pGEM-T Easy Vector (Promega, Madison, WI). We sequenced 5 different clones to identify the initiation site.

2.8. Functional analysis of the SHP2 promoter

2.8.1. Vector construction

Cloned genomic DNA was amplified with 5 different forward primers, −1415 to −1374 (P1, 5′-CTCGAGCTT-CACAGAGTGTCCAAAGTAATAC-3′), −775 to −750 (P2, 5′-CCAGGCTAATTCTTGAATTTTTAGT-3′), −439 to −414 (P3, 5′-GGCTTAAGCGAACCTCTTGCCG-CAGCC-3′), −299 to −274 (P4, 5′-AAGGTTCCACAGC-TAATGAGTGGAGC-3′), and −80 to −58 (P5, 5′-GCTGACGGGAAGCAGGAAGTGGCGG-3′), together with a reverse primer, 25 to 58 (P6R, 5′-CCAGGCCTGGG-GATCCCGGAGACTGTGCAGCTGC-3′). A *Hin*dIII adapter sequence was added to the 5′-end of the last 4 forward primers and reverse primer. The PCR products produced by P2, P3, P4, and P5 with P6R were subcloned into the *Hin*dIII site of the pGL3-Enhancer Vector (Promega). The PCR product from P1 with P6R was subcloned into the *Xho*I and *Hin*dIII sites of the same vector. These sequences were confirmed using an ABI automated sequencer (Perkin-Elmer).

2.8.2. Luciferase assay

The pGL3-Enhancer Vector, including different lengths of the 5′-flanking region of human SHP2, was transfected into COS 7 cells using Fugene 6 (Roche, Indianapolis, IN). According to the experimental procedure of Fugene 6, cells were cultured in DMEM with 10% FBS. Two days later, the cells were lysed. Luciferase was assayed in the lysate (Luciferase Assay System, Promega). This reporter gene activity was quantified by Gene Light spectrophotometry (Microtec-Nichion, Funabashi, Japan).

2.9. RNA preparation.

After stimulation of 10 μg/mL AGE or BSA in DMEM with 10% FBS, SMCs were scraped for RNA extraction. The RNA was isolated using RNeasy Total RNA Isolation Kit (Qiagen, Valencia, CA) according to manufacturer's protocol.

2.10. Real-time PCR analysis

Complementary DNA was synthesized using High-Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA) with 1.5 μg of deoxyribonuclease-treated RNA. Real-time PCR was carried out using an ABI PRISM 7000 and TaqMan Universal Master Mix (Applied Biosystems) according to the manufacturer's recommendations. TaqMan Gene Expression Assay Kit (Applied Biosystems) was used for SHP2 gene. Expression of SHP2 gene was normalized by β-actin result.

2.11. Statistical analysis

Values are expressed as means ± SEM. Sets of data were compared with a 2-tailed unpaired Student *t* test.

3. Results

3.1. Change of total PTPase and SHP2 activities upon AGE stimulation in rat aortic SMCs

In the membrane fractions, PTPase activity increased 1.8 times after stimulation with AGE at 10 μg/mL for 48 hours compared with incubation with control BSA ($P < .05$, n = 8; Fig. 1A). In the cytosolic fraction, however, PTPase activity was not changed significantly by AGE stimulation.

Fig. 1B shows changes in SHP2 activity. In membrane fraction, SHP2 activities were increased significantly by 10 μg/mL for 48-hour AGE stimulation carried out as above compared with control BSA ($P < .05$, n = 4).

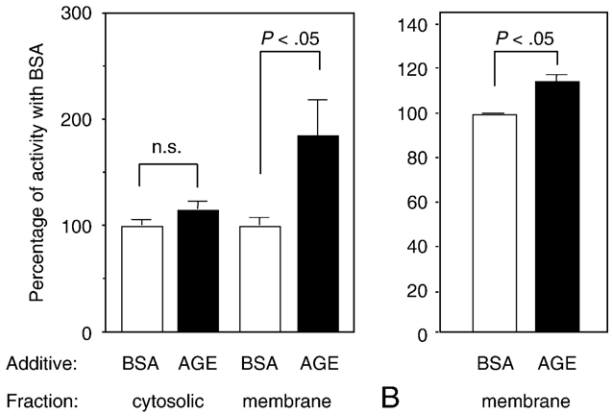

Fig. 1. The PTPase activities upon AGE stimulation in cytosolic and membrane fractions from rat aortic SMCs. Smooth muscle cells were incubated with 10 μg/mL AGE or BSA for 48 hours and then scraped and homogenized for PTPase assay. A synthetic peptide derived from the autophosphorylated regulatory region of the insulin receptor β-subunit (residues 1154 to 1165) was used as a phosphatase substrate (A). Activities of PTPase bound to agarose bead after immunoprecipitation with anti-SHP2 antibody were then studied (B). Beads were centrifuged, washed with HEPES, and assayed for bound SHP2 activity.

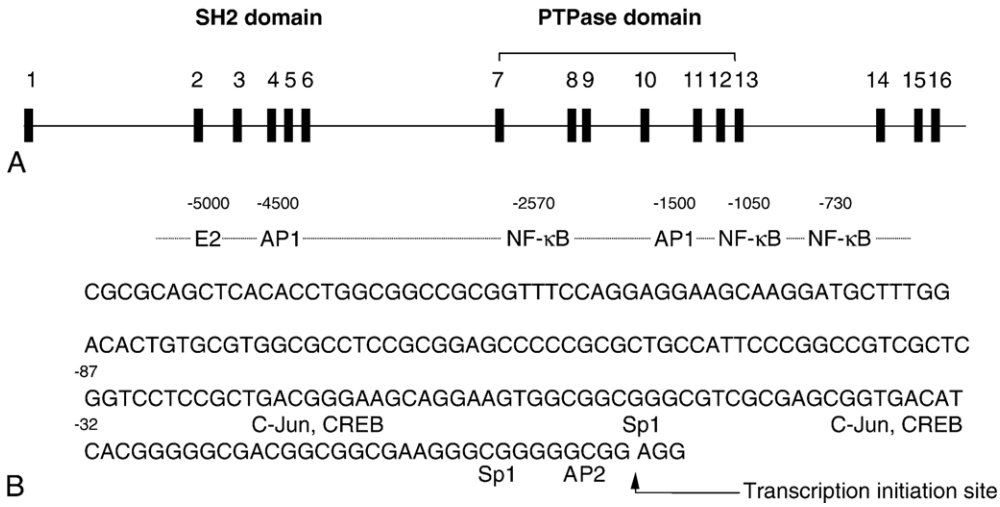

Fig. 2. Scheme of the organization of the human SHP2 gene (A). Black boxes indicate exons. In the nucleotide sequence of the proximal 5′-end of the human SHP2 gene (B), consensus sequences for binding of transcription factors are underlined.

The AGE stimulation for 12 and 24 hours does not change PTPase and SHP2 activities.

3.2. Splice junction sequence of SHP2

The exon/intron organization of the human SHP2 gene is shown in Fig. 2A and Table 1. The splice junction sequence conformed to the GT-AG sequence [15]. The entire protein coding sequence was divided into 16 exons. Exon 1 included the initial methionine codon, and exon 15 included a stop codon. The SHP2 spanned approximately 73 kilobases (kb). The PTPase domain was composed of exons 7 to 13.

3.3. Cloning of 5′-flanking region of SHP2

One positive clone included the 5′-flanking region (Fig. 2B). Sequence analysis of cap site hunting products identified the transcription initiation site. The consensus sequence located in this region is shown.

3.4. Sequence and characteristics of the putative SHP2 promoter region upstream of exon 1

According to the National Center for Biotechnology Information database and our sequencing results, a GC-rich region resembling the consensus binding motif for Sp1 was situated upstream of the predicted transcription initiation site (Fig. 2B). No consensus TATA sequence for RNA polymerase-II–initiated transcription was present. Sp1 is important for basal transcription in many promoters lacking TATA. Motifs in the putative promoter region also included potential binding site for the DNA binding factor cAMP-response element-binding protein (CREB) [16], consensus sequence of C-Jun.

3.5. Functional analysis of the SHP2 promoter

Luciferase activities are represented as percentages of activity for the pGL3 vector including the 5′-flanking region (−439 to +58). As shown in Fig. 3, the region corresponding

Table 1
Exon/intron sequences of the SHP2 gene

Exon	Intron	Size (bp)	3′ splicing site, intron/exon	5′ splicing site, intron/exon
1		213	5′UTR	CATCGCGGAGgtgaggagcc
2	13126	123	tctttttaagATGGTTTCAC	TTTCCGTTAGgtaagttgga
3	3503	195	actattttagAAGAAATGGA	CCTCTGAAAGgtcagtaaca
4	2682	193	aaaactttagGTGGTTTCAT	TCGCTGTCAGgtaaatctcc
5	1176	117	atcttgaaagGAACTGAAA	ACTCAAGCAGgtgagcaga
6	1269	114	actcgatcagCCCCTTAACA	AGAATTTGAGgtaagttatt
7	16881	97	ttcctttccagACACTACAAC	ATCCTGCCCTgtaagtatca
8	4610	80	tttcttctagTTGATCATAC	TATCATCATGgtaagctttg
9	126	159	aaatttctagCCTGAATTTG	GAGAGGAAAGgtaaatcaca
10	4058	132	tctcttccagAGTAAATGTG	GGTTGGACAAgtaagtatat
11	4269	155	tctactccagGGGAATACGG	TGCACTGCAGgtgacagctc
12	1813	68	ctgcccgcagTGCTGGAATT	AGAGAGAAAGgtgggtcatc
13	513	142	tccttcgtagGTGTTGACTG	AGAAGAGCAGgtaccagcct
14	12968	113	caaatttcagAAAAGCAAGA	CCTGTGCAGAgtaagtagtg
15	2438	112	ttctctccagAATGAGAGAA	ACAGAAATAGgtattttaaat
16	1028	426	cttggggagATGTGGACTT	3′UTR

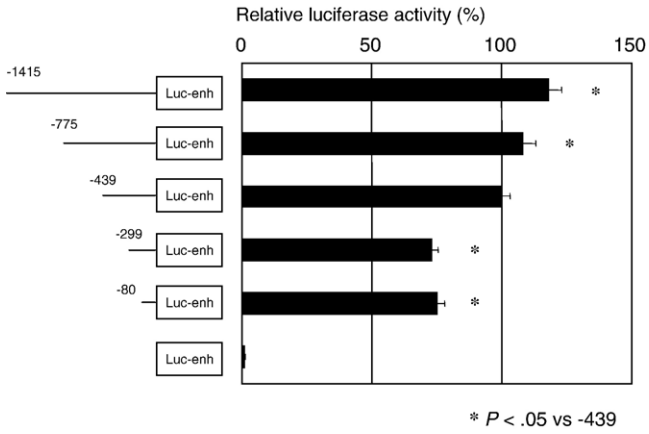

Fig. 3. Reporter constructs with various deletions in the 5′-upstream sequences of SHP2 were assayed for luciferase activity in COS7 cells. Promoterless luciferase vector pGL3-Basic (Promega) was used as a negative control. Luciferase activities are represented as percentages of that obtained with pGL3 vector including the 5′-flanking region (−439 to +58).

to 439 base pairs (bp) upstream of the 5′-end of exon 1 is important for the expression of SHP2.

3.6. Effect of AGE on SHP2 promoter via NF-κB activation

The AGE stimulation at 10 μg/mL for 12 hours increased luciferase activity in COS7 cells transfected with the SHP2 promoter region, corresponding to 1400 bp upstream of the 5′-end of exon 1 and including 2 NF-κB binding sites ($P < .05$, n = 3; Fig. 4). The AGE stimulation for 3, 6, and 24 hours does not change luciferase activities.

3.7. Effect of AGE on SHP2 expression

The AGE stimulation at 10 μg/mL for 24 hours increases SHP2 expression by 1.4 times compared with BSA by

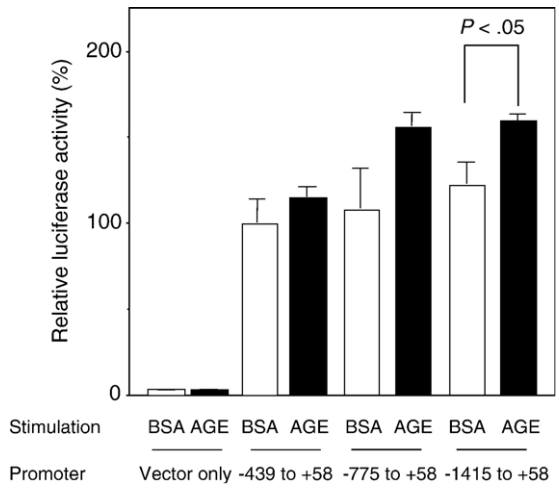

Fig. 4. Effect of AGE on luciferase activity in COS7 cells transfected with SHP2 promoter. COS7 cells were incubated with 10 μg/mL AGE or BSA for 12 hours and then scraped and homogenized for luciferase assay. Reporter constructs with various deletions in the 5′-upstream sequences of SHP2 were assayed for luciferase activity, which is represented as a percentage of construct −439 to +58 stimulated with control BSA.

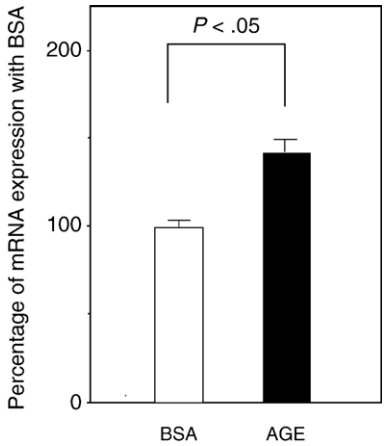

Fig. 5. Effect of AGE on SHP2 expression. Smooth muscle cells were incubated with 10 μg/mL AGE or BSA for 24 hours and then scraped and homogenized for real-time PCR method. Data were normalized by β-actin expression level.

real-time PCR method ($P < .05$, n = 3; Fig. 5) in SMCs. The AGE stimulation for 6 and 12 hours does not change SHP2 expression.

4. Discussion

Protein tyrosine phosphatases participate importantly in regulating intracellular signal transduction with respect to processes such as cell proliferation. Although many PTPases have been studied, we focused on SHP2 (also termed *SYP*, *PTP1D*, *PTP2C*, *SH-PTP3*, *SAP-2*, and *SH-PTP2*), which contains 2 Src homology 2 domains and is expressed widely throughout mammalian tissues [17] including SMCs [11]. Src homology 2–containing protein tyrosine phosphatase, which binds to tyrosine kinase–type receptors via its Src homology 2 domains [18], is activated by tyrosine residue phosphorylation [19]. Activated SHP2 binds to various intracellular proteins associated with cell signaling, as do other adapter protein. Many reports have shown SHP2 to positively influence intracellular signal transduction in processes such as cell proliferation [9-11,20-22].

Proliferation of SMCs is an important factor in atherosclerosis [3]. Smooth muscle cells are activated by various proliferative stimulants. It was reported that AGE stimulates SMC proliferation [4-6]. Because we hypothesized that PTPase activity contributes to SMC proliferation upon AGE stimulation, we assayed PTPase activity with and without AGE stimulation. In our experiments, AGE stimulation at 10 μg/mL increased PTPase activity in membrane fractions in SMCs. This effect of AGE on PTPase activity was observed at relatively low concentrations corresponding to the proliferative effect of AGE [4-6]. Various types of cells, such as endothelial cells [23], macrophages [24], mesangium cells [25], and adipocytes [12], were also stimulated by almost the same concentration of AGE in experiments

that examined the bioactivity of AGE. Moreover, AGE concentration was measured at the microgram per milliliter level in vivo [26,27]. Thus, we thought AGE concentration, which we used, is appropriate for the experiment.

Next, we assayed SHP2 activity upon AGE stimulation. Src homology 2–containing protein tyrosine phosphatase has been reported to be activated by various stimulants such as growth factors or cytokines. In our experiments, AGE stimulation increased SHP2 activity in the membrane fraction. In cell signaling, the importance of SHP2 in membrane fraction has been reported previously [28-30]. It suggests that increased SHP2 activity resulting from AGE stimulation could lead to SMC proliferation, an important event in atherogenesis. Although most PTPases have inhibitory effect on cell proliferation by dephosphorylation of tyrosine kinase–type receptors, SHP2, however, is believed to regulate intracellular signaling in a positive manner. In previous reports that we have searched, no PTPase has been reported to have a positive effect on signal transduction except SHP2. It means that the contribution of SHP2 in the increase of PTPase activity is important in the proliferation of SMCs. In our experiment, AGE has a positive effect on cell growth and SHP2 activity of only 1.2 to 1.3 times for 48 hours. It seems to be comparatively weak. However, its stimulatory effect is consistent with the metabolic environment in diabetes, where exposure to hyperglycemia and consequent AGE is persistent. Generally, the term of the progress of atherosclerosis takes several decades [31]. For example, the annual progression of carotid intima-media thickness, which is a predictor of atherosclerosis, was 0.04 mm/y in diabetes [32]. In the same way, AGE is thought to have an atherogenic effect for a long period in living bodies.

Various studies have found PTPase expression and/or activity to be regulated by various metabolites or stimuli such as glucose [33], nitric oxide [34], free fatty acid [35], or growth factors [13]. We next examined how SHP2 activity is regulated by AGE. At first, we examined whether AGE stimulation regulates PDGF action in SMCs because PDGF is a major growth factor for the proliferation of SMCs and activates SHP2 [19]. We quantified tyrosine phosphorylation of the PDGF receptor because this event is crucial to the first step of signal transduction by growth factors. In our previous study, tyrosine phosphorylation of the PDGFβ receptor upon stimulation by PDGF was not changed by AGE incubation [4]. Next, we examined tyrosine phosphorylation of SHP2 itself by AGE stimulation, as tyrosine phosphorylation of SHP2 is considered an important mechanism of SHP2 activation [19]. The AGE stimulation did not also alter tyrosine phosphorylation of SHP2 (data not shown). These data indicate that tyrosine phosphorylation of PDGF receptor and SHP2 is not involved in SMC proliferation resulting from AGE stimulation.

To elucidate the mechanism of SHP2 regulation by AGE, we therefore characterized the gene structure and promoter region of SHP2. The human SHP2 gene includes 16 exons spanning 73 kb, and their exon-intron boundaries were found to coincide with each cDNA sequence. Introns 1, 6, and 13, respectively, are 13.1, 16.8, and 12.9 kb in length. The translation initiation codon ATG is located 12 codons upstream from the first intron splice site. Src homology 2–containing protein tyrosine phosphatase expression control is directed by a TATA-free promoter, possibly by means of Sp1 as has been reported for many housekeeping proteins expressed ubiquitously in a variety of somatic cells. We identified the SHP2 promoter region, where binding sites were identified for various transcription factors including Sp1, NF-κB, and others. In a luciferase assay, cells transfected with SHP2 promoter had high luciferase activity according to the length of promoter region.

Next, we examined the effect of NF-κB on SHP2 expression. Nuclear factor–κB is a transcription factor known to be activated by AGE stimulation and participates importantly in regulation of gene expression involved in inflammatory and proliferative responses [23]. Among these, NF-κB activation has been detected in atherosclerotic lesions [36]. Nuclear factor–κB is activated by AGE stimulation in SMCs, too [4,7]. In the luciferase assay, AGE stimulation increased luciferase activity in cells transfected with SHP2 promoter regions including NF-κB binding sites. Depending on the number of NF-κB binding site, luciferase activity increased. Furthermore, we examined the effect of AGE on SHP2 expression. Advanced glycation end products increased SHP2 expression compared with BSA by real-time PCR method. It means that AGE stimulation is associated with SHP2 expression through NF-κB activation. Our present analysis of the SHP2 promoter sequence and activity may lead to new ways to identify genetic markers in atherosclerosis. Afterward, increased SHP2 expression leads to increase of SHP2 activity. In our experiments, expression and enzyme activity of SHP2 are increased 24 and 48 hours after AGE stimulation, respectively. These data indicate that the time course from expression to activation is relatively slow in SHP2. It is not special to SHP2. Examples of the time course from enzyme expression to activation needing several hours have been reported previously [37,38].

In summary, at first, we demonstrated that AGE stimulation increases SHP2 activity. Next, to elucidate the mechanism of regulation of SHP2 by AGE stimulation, we isolated and characterized the genomic and promoter structure of SHP2, a phosphatase considered important in regulating cell proliferation. Advanced glycation end product stimulation activated NF-κB in SMCs [4]. Activated NF-κB bound to sites on the SHP2 promoter to begin a sequence including increased SHP2 expression and activity. Increase of SHP2 activity led to SMC proliferation [11]. It suggests that AGE stimulation induces SMC proliferation via SHP2, underscoring the importance of the control of AGE for suppressing macroangiopathy in diabetes mellitus.

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