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RESEARCH ARTICLE

Stabilization of small heterodimer partner mRNA by grape seed procyanidins extract in cultured hepatocytes

Jun Inoue, Minako Tanaka, Manami Nanmoku, Takuya Yashiro and Ryuichiro Sato

Department of Applied Biological Chemistry, Graduate School of Agricultural and Life Sciences, The University of Tokyo, Tokyo, Japan

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Scope: Consumption of dietary grape seed procyanidins extract (GSPE) has improved the plasma lipid profile in humans and experimental animals. The effect of GSPE on the reduction of the postprandial plasma triglyceride (TG) levels has been attributed to the activation of the small heterodimer partner (SHP). GSPE increases SHP gene expression in rat liver and the TG-lowering effect of GSPE is abolished in SHP-deficient mice. However, the mechanism by which GSPE increases SHP mRNA levels remains unclear. This study addressed the effect of GSPE on SHP mRNA stability.

Methods and results: The present study shows for the first time that SHP mRNA is rapidly degraded, as measured by actinomycin D-based mRNA chase experiments, and GSPE transiently stabilizes SHP mRNA in HepG2 cells. This degradative effect was completely abolished with 2 h of prolonged treatment with GSPE. However, treatment of fresh HepG2 cells with a pretreated GSPE-containing medium also stabilized SHP mRNA, indicating that GSPE inactivation is not responsible for the transient effects that GSPE has on SHP mRNA stability.

Conclusion: SHP expression is intricately controlled by mRNA stabilization, which is transiently increased by GSPE, along with at the transcriptional and posttranslational levels.

Keywords:
Grape seed procyanidins extract / mRNA stability / Plasma triglyceride / Red wine / Small heterodimer partner

1 Introduction

Naturally occurring compounds proanthocyanidins are abundant in apples, pears, and grapes [1, 2]. They belong to a group of flavonoids that take the form of oligomers or polymers that consist of polyhydroxy flavan-3-ol units. Several in vitro and animal studies have demonstrated the beneficial effects of proanthocyanidins on the prevention and amelioration of atherosclerosis [3]. This beneficial effect is believed to be because of their antioxidative effects. In addition, modulation of intracellular signaling pathways and the transcriptional activities of proanthocyanidins are also believed to mediate these beneficial effects. Recently, Del Bas *et al.* reported that dietary grape seed procyanidin extract (GSPE) decreases the plasma levels of triglyceride (TG) and apolipoprotein B in rat along with the concomitant induction of the small heterodimer partner (SHP) gene expression in the liver [4]. The importance of the SHP gene in the TG-lowering effects of GSPE has been confirmed by an experiment using SHP-deficient mice [5].

SHP is an atypical nuclear receptor that lacks a DNA-binding domain. SHP regulates the activity of certain nuclear receptors, including liver receptor homolog 1 (LRH-1), liver X receptor α, and hepatocyte nuclear factor 4α, through direct interaction, thereby controlling the expression of a variety of genes that regulate several cellular

Correspondence: Dr. Ryuichiro Sato, Department of Applied Biological Chemistry, Graduate School of Agricultural and Life Sciences, The University of Tokyo, 1-1-1 Yayoi, Bunkyo, Tokyo 113-8657, Japan
E-mail: aroysato@mail.ecc.u-tokyo.ac.jp
Fax: +81-3-5841-8029

Abbreviations: ABCB1, ATP-binding cassette transporter B1; **AREs,** A+U-rich elements; **ARE-BPs,** ARE-binding proteins; **CDCA,** chenodeoxycholic acid; **FXR,** farnesoid X receptor; **GSPE,** grape seed procyanidins extract; **LRH-1,** liver receptor homolog 1; **SHP,** small heterodimer partner; **TG,** triglyceride

processes, including bile acid metabolism [6, 7], lipogenesis [8], and glucose homeostasis [9]. Regulation of SHP expression occurs at the transcriptional and posttranslational levels. Several transcription factors, including LRH-1 and farnesoid X receptor (FXR), regulate SHP expression [6, 7, 10]. At the same time, SHP protein is rapidly degraded via the ubiquitin–proteasome pathway [11]. Elevation of the level of the SHP protein suppresses LRH-1 activity, thus exerting a negative feedback control on its own transcription [6, 7].

Currently, the reasons behind the observed increases in the SHP mRNA levels by GSPE treatment are largely unknown. Recently, FXR was shown to play a pivotal role in GSPE induction of SHP gene expression [12]. In FXR-driven luciferase expression assays, GSPE has enhanced FXR activity only in the presence of chenodeoxycholic acid (CDCA), a known FXR ligand. However, treatment with GSPE increases the SHP mRNA levels in HepG2 cells even in the absence of CDCA [12]. Therefore, it is speculated that an FXR-independent pathway contributes to GSPE induction of the SHP mRNA levels. Thus, for the first time, we report that the SHP mRNA levels rapidly degraded and subsequently stabilized by GSPE in HepG2 cells.

2 Materials and methods

2.1 Materials

GW4064 and actinomycin D were purchased from Sigma. GSPE was provided by Kikkoman. The GSPE extract contained flavanol (99.2% pure) and proanthocyanidins (86.4%). The average degree of polymerization was between 4.0 and 5.6.

2.2 Cultured cells

HepG2 cells were maintained in medium A (DMEM supplemented with 100 units/mL penicillin, 100 μg/mL streptomycin, and 10% fetal bovine serum) at 37°C in a 5% CO_2 atmosphere.

2.3 Real-time PCR

The total RNA was extracted from HepG2 cells using an RNeasy mini kit (Qiagen, Tokyo, Japan) according to the manufacturer's instructions. cDNA was synthesized and amplified from 2 μg of total RNA using a high-capacity cDNA reverse transcription kit (Applied Biosystems, Tokyo, Japan). Quantitative real-time PCR (TaqMan probe and SYBR green) analysis was performed on an Applied Biosystems 7000 sequence detection system. Expression was normalized for GAPDH control. The TaqMan ID numbers for the genes analyzed are SHP, Hs00222677_m1; LDL receptor Hs00181192_m1; GAPDH, 4352934. The sequences of the primer sets used were as follows: ABCB1 (ATP-binding cassette transporter B1), 5′-GTCTGGACAAGCA CTGAAAGATAAGA-3′ and 5′-CAACGGTTCGGAAGTTTT CTATTGC-3′; p21Cip1, 5′-GGAGACTCTCAGGGTCGAA AA-3′ and 5′-GGCGTTTGGAGTGGTAGAAAT-3′.

2.4 Determination of SHP mRNA level

HepG2 cells were seeded in 6-well plates at a density of 1.0×10^6 cells/well, cultured with medium A for 20 h, and subsequently treated with 50 mg/L GSPE, 100 mg/L GSPE, or 30 μM GW4064 for 1 h, respectively. GSPE was dissolved in distilled water and GW4064 was dissolved in DMSO. The final DMSO concentration of the cultured medium was 0.1% in this study. The cells were harvested and the SHP mRNA levels were analyzed by real-time PCR. Relative mRNA levels (SHP/GAPDH) were determined after being normalized to GAPDH.

2.5 Determination of SHP mRNA stability

HepG2 cells were seeded in 6-well plates at a density of 1.0×10^6 cells/well, cultured with medium A for 20 h, and subsequently treated with 100 mg/L GSPE dissolved in distilled water or the 4h-precultured medium containing 100 mg/L GSPE for the indicated period of time. After preincubation with 5 μg/mL actinomycin D for 30 min to inhibit transcription, cells were harvested at the indicated time and the SHP mRNA levels were analyzed by real-time PCR. Actinomycin D was dissolved in ethanol and the final ethanol concentration of the cultured medium was 0.1% in this study. Relative mRNA levels (SHP/GAPDH) were determined after being normalized to GAPDH.

2.6 Statistical analysis

All data are presented as the mean ± SD. Effects were assessed using Student's *t*-test.

3 Results

3.1 Effect of GSPE on the SHP mRNA levels

To evaluate the effect of GSPE on SHP gene expression, HepG2 cells were treated with the indicated concentration of GSPE for 1 h and were then harvested. The extracted RNA was used as a template for cDNA synthesis, and SHP mRNA was quantitated by real-time PCR. Consistent with the results of a previous study [5], GSPE significantly increased the SHP mRNA levels after 1 h of treatment

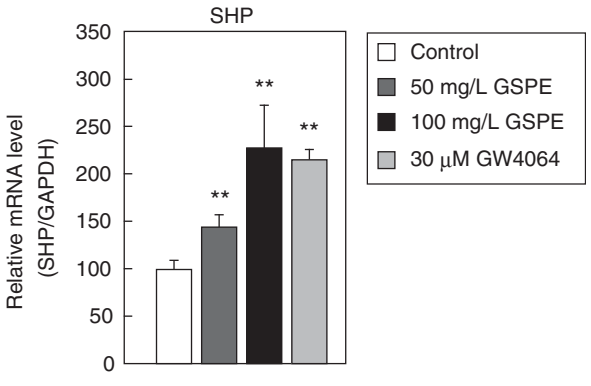

Figure 1. Effect of GSPE on SHP mRNA expression in HepG2 cells. HepG2 cells were cultured in the indicated concentration of GSPE or 30 μM GW4064 for 1 h. Relative mRNA levels (SHP/GAPDH) were determined by real-time PCR after being normalized to GAPDH mRNA. All data are presented as the mean ± SD and represent at least three independent experiments performed in triplicate. **Significantly different from control, $p<0.01$.

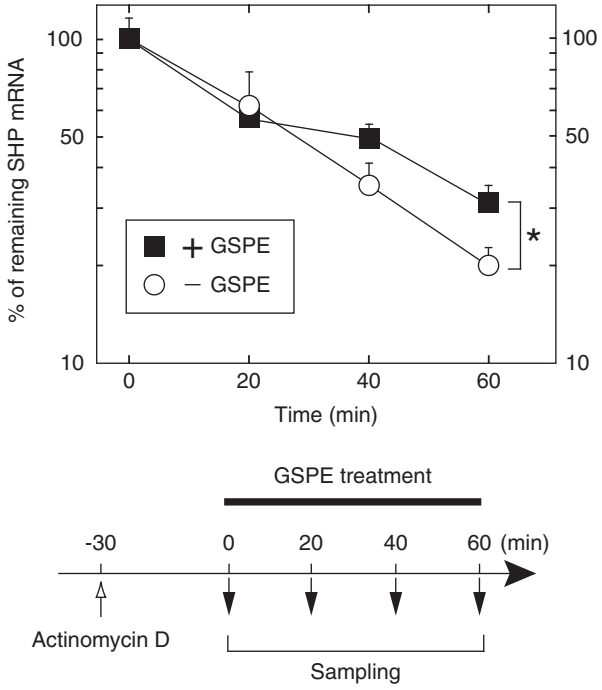

Figure 2. Effect of GSPE on SHP mRNA stability in HepG2 cells. After preincubation with 5 μg/mL actinomycin D for 30 min, 100 mg/L GSPE was added to the medium and the cells were further incubated for the indicated period of time. Relative mRNA levels (SHP/GAPDH) were determined by real-time PCR after being normalized to GAPDH mRNA. The mRNA level at the 0-min time point was set at 100%. The percentage of mRNA remaining is plotted over time. All data are presented as the mean ± SD. The same results were obtained in three separate experiments. *$p<0.05$.

(Fig. 1). Del Bas *et al.* previously reported that GSPE slightly enhances FXR activity only in the presence of CDCA in FXR-driven luciferase expression assays. They also speculated that GSPE stimulates SHP gene expression through FXR activation [12]. However, in the present study, GSPE stimulated the SHP gene expression in the absence of CDCA to the same extent as the potent FXR ligand GW4064, suggesting that GSPE can increase the SHP mRNA levels through mechanisms other than FXR activation. This observation suggests that GSPE may stabilize SHP mRNA.

3.2 GSPE transiently stabilizes SHP mRNA and reused GSPE retains the SHP mRNA stabilizing effect

To determine whether GSPE stabilizes SHP mRNA in HepG2 cells, actinomycin D-based mRNA chase experiments were performed. In the presence of actinomycin D, an RNA synthesis inhibitor, the SHP mRNA levels decreased in a time-dependent manner with a half-life of 30.9 ± 4.1 min (mean ± SD) (Fig. 2). When HepG2 cells were treated with 100 mg/L GSPE, SHP mRNA was stabilized after 40 min of treatment and the SHP mRNA levels remained significantly elevated after 60 min of treatment (Fig. 2), indicating the involvement of GSPE in SHP mRNA stability. It should be noted that GAPDH mRNA, which was used for the normalization of RNA in this study, was stable during the time-course experiment and GSPE treatment did not affect the stability of the GAPDH mRNA. To further analyze the effect of GSPE on the stabilization of SHP mRNA, HepG2 cells were pretreated with 100 mg/L GSPE for the indicated period of time and then the SHP mRNA levels were determined. As shown in Fig. 3, 30 min of GSPE pretreatment completely stabilized SHP mRNA after a lag time of 40 min, while 60 min of GSPE pretreatment completely stabilized SHP mRNA without any time delay and the state lasted for 60 min. It should be noted that pretreatment with GSPE for 30 or 60 min accompanied by treatment with actinomycin D for 30 min before the 0-min time point resulted in an increase in the SHP mRNA levels by 0.97 ± 0.21 or 1.27 ± 0.19-fold compared with control, respectively (data not shown). Treatment with 100 mg/L GSPE for 60 min resulted in approximately a two-fold increase in the SHP mRNA levels (Fig. 1). These data reflect the effect actinomycin D has on the inhibition of RNA synthesis. Based on these results, it was concluded that GSPE treatment stabilizes SHP mRNA after 40 min of treatment and that SHP mRNA degradation is completely inhibited for 60 min after approximately 60 min GSPE treatment, indicating that the effect of GSPE on SHP mRNA stability may be transient. Therefore, HepG2 cells were pretreated with GSPE for 120 min and the SHP mRNA levels were determined. Pretreatment with GSPE for 120 min did not stabilize SHP mRNA but resulted in an increase in the SHP mRNA levels at the 0-time point

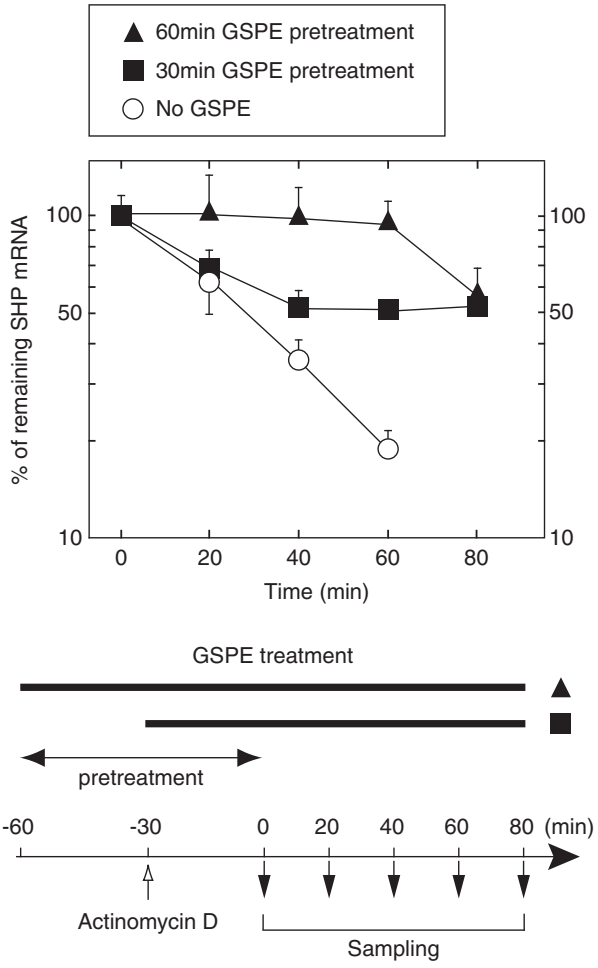

Figure 3. Effect of GSPE pretreatment on SHP mRNA stability in HepG2 cells. HepG2 cells were pretreated with 100 mg/L GSPE for 30 or 60 min and 5 μg/mL actinomycin D for 30 min. Cells were then incubated with GSPE for the indicated period of time. Relative mRNA levels (SHP/GAPDH) were determined by real-time PCR after being normalized to GAPDH mRNA. The mRNA level at the 0-min time point was set at 100%. The percentage of mRNA remaining is plotted over time. All data are presented as the mean ± SD. The same results were obtained in three separate experiments.

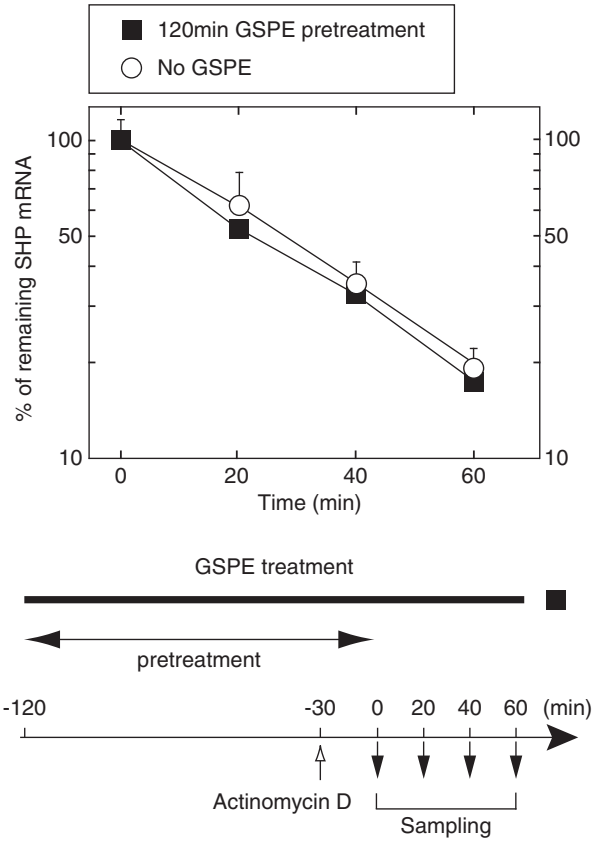

Figure 4. GSPE pretreatment for 120 min does not stabilize SHP mRNA. HepG2 cells were pretreated with 100 mg/L GSPE for 120 min and 5 μg/mL actinomycin D for 30 min. Cells were then incubated with GSPE for the indicated period of time. Relative mRNA levels (SHP/GAPDH) were determined by real-time PCR after being normalized to GAPDH mRNA. The mRNA level at the 0-min time point was set at 100%. The percentage of mRNA remaining is plotted over time. All data are presented as the mean ± SD. The same results were obtained in three separate experiments.

(1.75 ± 0.23-fold increase compared with control) (Fig. 4 and data not shown), indicating that GSPE can transiently stabilize SHP mRNA. Since the transient effect of GSPE on SHP mRNA may depend on the stability of the effective component of GSPE, the effect of reused GSPE on SHP mRNA stability was evaluated. HepG2 cells were cultured in the GSPE-containing medium used to incubate a separate set of HepG2 cells for 4 h. As shown in Fig. 5, SHP mRNA was stabilized following 30 min of pretreatment with both the reused and fresh GSPE, indicating that the effective component of GSPE that affects SHP mRNA stability is not depleted or inactivated after prolonged incubation.

3.3 GSPE does not stabilize general mRNA

Finally, to determine whether GSPE stabilizes mRNA other than the SHP gene, we examined the effect of GSPE on the stability of various mRNAs, including ABCB1, p21Cip1, and LDL receptor. We have previously reported that the LDL receptor mRNA is rapidly degraded and CDCA treatment results in the stabilization of LDL receptor mRNA through the activation of the extracellular signal-regulated kinase pathway [13]. In addition, mRNA levels not normalized by GAPDH mRNA levels were expressed in this study, indicating that the effect GSPE has on SHP mRNA stability is a direct effect of GSPE on SHP mRNA rather than an effect of GSPE on GAPDH mRNA. As shown in Fig. 6, although SHP mRNA was stabilized following 60 min of pretreatment with GSPE, ABCB1 mRNA was not stabilized. Moreover, the LDL receptor and p21Cip1 mRNA were slightly stabilized by GSPE. It is important to note that the LDL

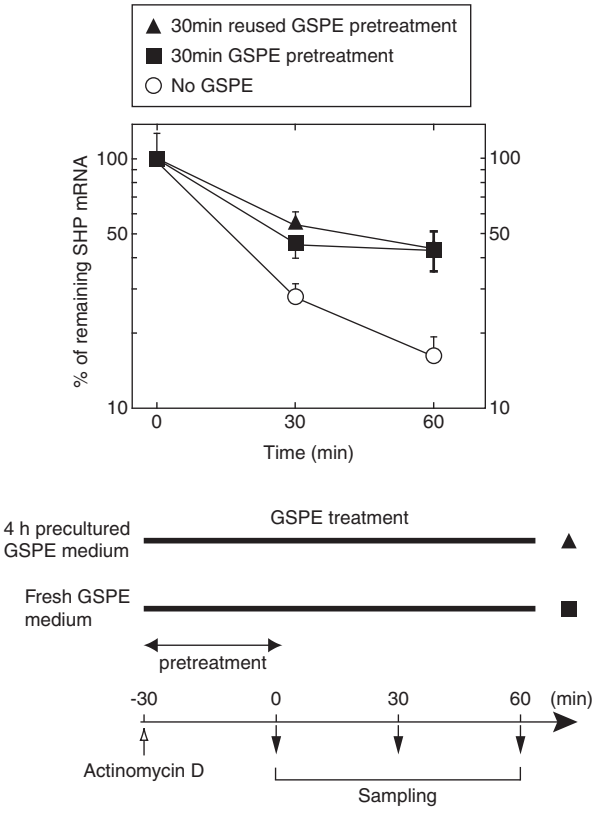

Figure 5. Reused and fresh GSPE stabilize SHP mRNA. HepG2 cells were pretreated with 100 mg/L reused or fresh GSPE for 30 min and 5 μg/mL actinomycin D for 30 min. Cells were then incubated with GSPE for the indicated period of time. Relative mRNA levels (SHP/GAPDH) were determined using real-time PCR after being normalized to GAPDH mRNA. The mRNA level at the 0-min time point was set at 100%. The percentage of mRNA remaining is plotted over time. All data are presented as the mean ± SD. The same results were obtained in three separate experiments.

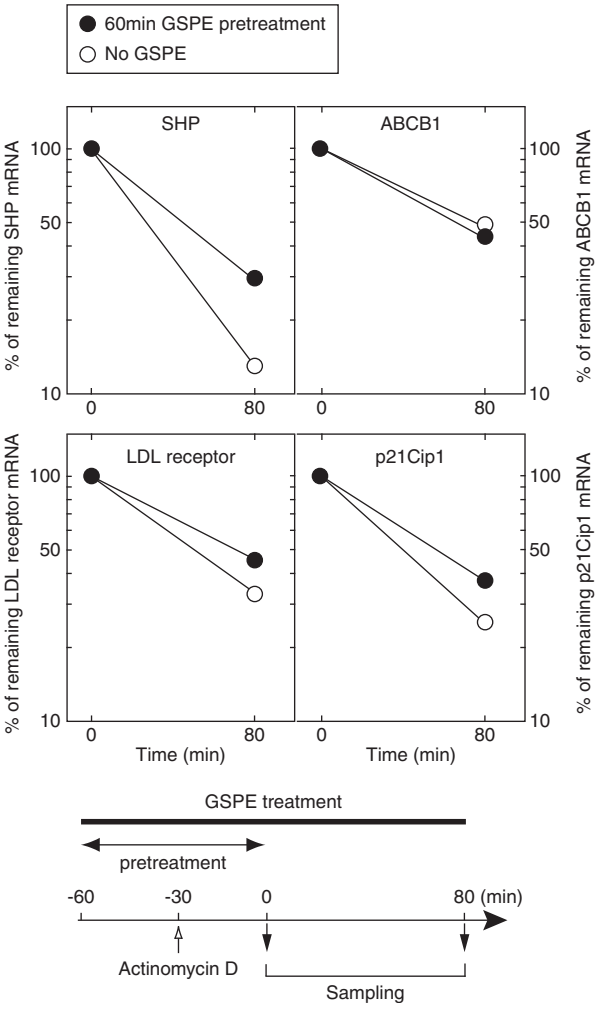

Figure 6. GSPE pretreatment for 60 min does not stabilize general mRNA. HepG2 cells were pretreated with 100 mg/L GSPE for 60 min and 5 μg/mL actinomycin D for 30 min. Cells were then incubated with GSPE for the indicated period of time. The mRNA level at the 0-min time point was set at 100%. The percentage of mRNA remaining is plotted over time. The same results were obtained in three separate experiments.

receptor and p21Cip1 mRNA levels were not induced by GSPE at the 0-time point (0.85 ± 0.02 and 1.01 ± 0.01-fold increases compared with the control, respectively). These results indicate that GSPE does not generally stabilize mRNA.

4 Discussion

The French have one of the lowest incidences of coronary heart disease in the Western world despite consuming a traditional diet with a relatively high fat content. This phenomenon is called the ''French paradox'' and is attributed to the high consumption of red wine in France [14]. Red wine is rich in polyphenols such as anthocyanins, catechins, proanthocyanidins, flavonols, stilbenes, and other phenolics. Proanthocyanidins are considered potential candidates responsible for the protective effects of red wine against coronary heart disease [2, 15].

It has been shown that hypertriglyceridemia is one of the risk factors for the development of coronary heart disease. Dietary GSPE decreases plasma TG levels in rodents. GSPE-mediated decreases in the plasma TG levels are attributed, at least in part, to the activation of SHP because dietary GSPE increases SHP gene expression and the TG-lowering effects of GSPE are not observed in SHP-deficient mice. However, the mechanism by which GSPE modulates SHP gene expression is unknown. The steady-state level of mRNA is determined by the rates of synthesis and degradation. It has been shown that mRNA instability is determined by a number of factors that bind to AU-rich elements (ARE) located on the 3′-untranslated region [16]. ARE sequences control mRNA degradation via interactions with specific binding proteins (ARE-BPs), such as HuR. Exposure to

certain stimuli such as heat shock or ultraviolet light results in changes in the way ARE-BPs bind to the target mRNA, thereby affecting the stability of the target mRNA. Currently, the specific ARE-BPs that interact with SHP mRNA and the method by which they modulate its stability are unknown. In this study, GSPE treatment was shown to stabilize SHP mRNA in HepG2 cells. Thus, GSPE treatment leads to alterations in certain ARE-BPs that bind to and subsequently stabilize SHP mRNA. Flavonoid-mediated alteration of mRNA stability has also been previously reported. Treatment with a naturally occurring flavonoid found in a variety of fruits and leafy vegetables, apigenin [17, 18], stabilizes cyclooxygenase-2 mRNA by increasing the localization of HuR into the cytoplasm of keratinocytes [19]. This study also demonstrated that GSPE exerts an effect on SHP mRNA stability for a short period of time. This conclusion is based on the finding that pretreatment with GSPE for 120 min did not have an additional stabilizing effect on SHP mRNA. Since the reused GSPE still had an SHP mRNA-stabilizing effect, this transient effect may not have been due to degradation of the effective component of GSPE, but instead due to the desensitization of cells that were pretreated with GSPE for relatively long periods of time. Further studies are required to determine if ARE-BPs contribute to SHP mRNA stabilization and the role GSPE plays in this process.

It has been shown that SHP expression is tightly regulated at the transcriptional and posttranslational levels. SHP gene expression is enhanced by FXR and LRH-1. Elevation of SHP protein levels inactivates LRH-1 activity through direct interaction, thereby decreasing SHP gene expression [6, 7]. SHP protein is rapidly degraded by the ubiquitin–proteasomal pathway with a half-life of <30 min. CDCA treatment, which stimulates SHP gene expression through FXR activation, results in the activation of extracellular signal-regulated kinase pathway, thereby stabilizing SHP protein levels through the inhibition of ubiquitination [11]. In the present study, SHP mRNA was also rapidly degraded with a half-life of 30.9 ± 4.1 min. Therefore, it is important to emphasize that SHP expression is regulated at the posttranscriptional level in addition to the transcriptional and posttranslational levels. Because SHP functions as a critical regulator of various metabolic pathways through the regulation of certain transcription factors, the fine tuning of SHP expression may be required for the maintenance of lipid homeostasis. It has been reported that SHP gene expression and protein stability are elevated in the liver of *ob/ob* mice [11, 20]. These results provide a potential link between chronically elevated SHP expression and metabolic disease states. It is presently unclear whether SHP mRNA stability is related to the development of certain diseases. Recently, Zhou *et al.* reported the presence of single nucleotide polymorphisms in the SHP gene of cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy-like patients, including mutations in exon 1 and 3′-untranslated regions [21]. They also reported that two exonic mutations, 113G→A and 512G→C (nucleotide +1 corresponding to the A of the ATG start codon), of the SHP gene increase the mRNA levels when they are exogenously expressed using the CMV promoter in mouse hepatoma Hepa-1 cells, suggesting a possibility that these exonic mutations could result in the stabilization of SHP mRNA. Further studies are required to determine whether these mutations affect SHP mRNA stability and whether the perturbation of SHP mRNA stability causes certain diseases.

The effective component of GSPE that stabilizes SHP mRNA remains unknown. It has been reported that only proanthocyanidins with low degrees of polymerization, such as from monomers to pentamers, are absorbed in the gastrointestinal tract [22–24]. Because the consumption of dietary GSPE results in an increase in SHP gene expression in mouse liver, the low degree of polymerization of certain proanthocyanidins may contribute to this regulation. Thus, the effective component of GSPE on SHP mRNA stabilization needs to be determined.

In conclusion, the present study shows that the SHP mRNA level is rapidly degraded and treatment with GSPE stabilizes SHP mRNA in HepG2 cells. Furthermore, the effect of GSPE on the stabilization of SHP mRNA is transient and reused GSPE still retains its stabilizing effect, indicating that the effective component of GSPE that stabilizes SHP mRNA is stable. The effective component of GSPE and the manner in which it stabilizes SHP mRNA still needs to be determined. GSPE may alter the functions of certain ARE-BPs that modulate SHP mRNA stability. ARE-BPs that respond to GSPE treatment are currently being investigated.

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The authors have declared no conflict of interest.

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