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Journal of Nutritional Biochemistry 20 (2009) 1021 – 1027
Journal of Nutritional Biochemistry

Green tea polyphenols down-regulate caveolin-1 expression via ERK1/2 and p38MAPK in endothelial cells☆

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Received 2 March 2008; received in revised form 26 November 2008; accepted 1 December 2008

Abstract

Caveolin-1 (Cav-1), a negative regulator of endothelial nitric oxide synthase (eNOS), influences various aspects of the cardiovascular functions. We had reported that a high-fat diet up-regulated aortic Cav-1 expressions in rats. In this study, we investigated the effects of green tea polyphenols (GTPs) on endothelial Cav-1 expression and phosphorylation *in vitro*. Bovine aortic endothelial cells (BAECs) were treated with 4 μg/ml GTPs for 0, 4, 8, 12, 16 and 24 h, and with 0, 0.04, 0.4, 4 and 40 μg/ml GTPs for 16 h, respectively. Cav-1 protein and mRNA were detected using Western blot and reverse transcriptase polymerase chain reaction. Cav-1 protein expression was down-regulated after treatment of BAECs with 4 μg/ml GTPs for 12, 16 and 24 h. And decrease in the level of Cav-1 mRNA was observed after GTP treatment for 4 and 8 h. GTPs (0.04–4 μg/ml) down-regulate Cav-1 protein expressions and mRNA levels dose dependently. PD98059, an inhibitor of extracellular signal-regulated kinase 1/2 (ERK1/2), up-regulated Cav-1 expression in BAECs alone and abolished the down-regulation effects of GTPs in BAECs while pretreatment with it. Inhibition of p38 mitogen-activated protein kinase (p38MAPK) with SB203580, which down-regulates Cav-1 expression in BAECs alone, deteriorated the Cav-1 down-regulating effects by GTPs. In addition to the effects on expression of Cav-1, GTP treatment inhibited phosphorylation of Cav-1 [tyrosine 14 (Tyr14)]. These data indicate that GTPs down-regulate gene expression of Cav-1 time- and dose- dependently via activating ERK1/2 and inhibiting p38MAPK signaling.

Keywords: Green tea polyphenols; Endothelial cell; Caveolin-1; ERK1/2; p38MAPK

1. Introduction

Tea, an infusion of the leaves of *Camellia sinensis*, is the most ancient and widely consumed beverage in the world [1]. Green tea, popular in Asia, is rich in green tea polyphenols (GTPs), which are commonly known as flavanols or catechins and comprise 30–40% of the extractable solids of dried green tea leaves. GTPs have demonstrated significant antioxidant, anti-carcinogenic, anti-inflammatory and anti-atherosclerotic properties [2,3]. Epidemiologic studies suggested that green tea consumption is associated with reduced mortality due to cardiovascular disease (CVD) [4]. Short- and long-term tea consumption has been shown to reverse endothelial dysfunction in patients

☆ This work was supported by grant 30471461 (Ying C.) from the National Natural Science Foundation (NNSF) of China; grant JX1A01 from the Health Department of Hubei Province; grant 20051136 from the Education Department of Zhejiang Province (Ye X.); and also in part by the Japan-China Sasagawa Medical Fellowship.

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doi:10.1016/j.jnutbio.2008.12.001

with documented coronary heart disease [5], healthy smokers [6,7]and renal transplant recipients [8]. Although the exact mechanisms of endothelial dysfunction are extremely complicated and have not been clarified until recently, accumulating evidence has suggested that diminished bioavailability of NO is one of the key events in the pathogenesis of endothelial dysfunction [9].

Caveolae, flask-shaped invaginations (50–100 nm in size) of the plasma membrane, were described first by Palade [10] from electron micrographs of endothelial cells in 1953. Caveolae have been identified in a wide variety of cell types, such as endothelial cells, muscle cells, adipocytes and pulmonary epithelial cells. Biochemically, caveolae have a distinctive lipid composition that is rich in glycosphingolipids, sphingomyelin and cholesterol [11]. Caveolin-1 (Cav-1), a 21- to 24-kDa integral membrane protein within caveolae, is the product of the caveolin-1 gene that belongs to the caveolin gene family which is composed of three different genes, i.e., *cav1*, *cav2* and *cav3* [12]. Cav-1 in caveolae has many diverse functions in the regulation of various aspects of endothelial cell functions, including transcytosis [13], potocytosis [14], signal transduction, proliferation and differentiation, as well as vascular permeability [15]. Many signaling cascades, including receptor tyrosine kinases, Src family tyrosine kinases and members of the p42/44 MAP kinase cascade (MEK1/2 and ERK1/2), are held in check by direct molecular interactions with the Cav-1 scaffolding domain located between amino acids 82 and 101. The majority of these interactions serve to hold a given signaling molecule in an inactive or repressed state, although in some cases the scaffolding domain stimulates activity or has no effect at all [16]. Endothelial nitric oxide synthase (eNOS) binding to Cav-1 scaffold domain *in vitro* holds it in an inactive state. It is believed that Cav-1 negatively regulates eNOS activities via protein–protein interactions [17]. We had reported that the enhanced Cav-1 expression in high-fat diet-fed rat aorta was responsible for the decreasing eNOS activity via negatively regulating eNOS activities [18].

A previous report showed that epigallocatechin-3-gallate (EGCG), the major constituent of GTPs, enhanced eNOS activity [19]. However, to our knowledge, no study has yet been conducted to explore the effects of GTPs on Cav-1 expression. The objective of this study was to investigate the effects of GTPs on Cav-1 expression in endothelial cells *in vitro* and the signaling cascades involved.

2. Materials and methods

2.1. Reagents and materials

Dulbecco's Modified Eagle's Medium (DMEM; containing phenol red, 25 mM HEPES, 1.0 g of dextrose/ml and 2 mM L-glutamine) and fetal bovine serum (FBS) were purchased from Gibco. SB203580 (inhibitor of p38 MAPK) and PD98059 (inhibitor of ERK1/2 signaling pathway) were obtained from Alexis (Switzerland). Polyclonal antibodies against Cav-1 and phospho-Cav-1 [tyrosine 14 (Tyr14)] were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA), and mouse anti-α-tubulin was from Sigma (St. Louis, MO, USA). Green tea polyphenols (catechins 70%, flavonols 10% and polymeric flavonoids 20%) were kindly provided by Unilever Health Institute (Vaardingen, The Netherlands). Trizol reagent was from Invitrogen; murine leukemia virus retranscribed (M-MLV) and oligo (dT) were obtained from Promega (Southampton, UK).

2.2. Cell culture

Primary bovine aortic endothelial cells (BAECs; no. C-003-5C) obtained from Health Science Research Resources Bank (Osaka, Japan) were maintained at 37°C in 5% CO_2 in DMEM containing 10% FBS as described previously [20]. Confluent cultures were detached using trypsin/EDTA and plated on 100-mm-diameter dishes for protein expression and phosphorylating signal analysis.

2.3. Electrophoresis and immunoblotting

Whole cell extracts were prepared [20] by lysing the cells in extraction buffer containing 50 mmol/L Tris/HCl, pH 8.0, 150 mmol/L NaCl, 1% Nonidet-P40, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 0.1 mmol/L DTT, 0.05 mmol/L PMSF, 0.002 mg/ml aprotinin, 0.002 mg/ml leupeptin and 1 mmol/L NaVO3 after stimulation. The protein concentration was quantified with BIO-RAD Dc protein assay reagent (Bio-Rad, Hercules, CA, USA). Equal amounts of protein were mixed with SDS sample buffer and incubated for 3 min at 98°C before loading. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunological blotting were performed according to the method of Amersham Biosciences. Immunoreactive bands were detected by means of an ECL plus Western Blotting Detection System (Amersham Biosciences, Little Chalford, UK) according to the manufacturer's instructions. The chemiluminescent signals were scanned from autoradiographic films (Nippon Polaroid KK, Tokyo, Japan). Quantitative analysis was performed by Gel Pro 3.0 software (Biometra, Goettingen, Germany).

2.4. Reverse transcription-polymerase chain reaction

Total RNA was extracted from endothelial cells following a modification of the guanidinium thiocyanate method [18] by using TRIzol (Invitrogen) following the manufacturer's instructions and quantified by UV spectrophotometry. Total RNA (1.0 μg) from each sample was reverse transcribed to cDNA using oligo (dT) primers and M-MLV. After 30 cycles of polymerase chain reaction (PCR; Platinum Taq, Invitrogen) with cDNA, amplified fragments were electrophoresed through 2% agarose/Tris-boric acid-EDTA (TBE) gels in 1× TBE buffer and visualized under UV light. The primers used in the PCR were as follows: caveolin 5′-primer: ATG ACC ACT GTC CAC GCC AT, and caveolin 3′-primer: GCC TGC TTC ACC ACC TTC TT; glyceraldehyde-3-phosphate

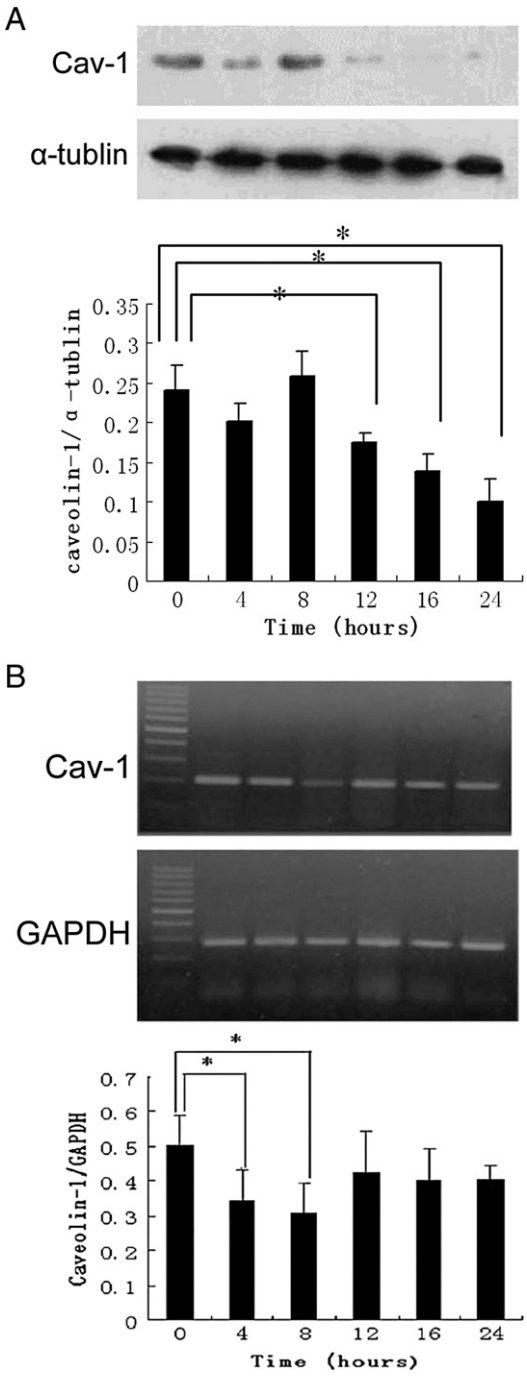


Fig. 1. Time course of effects of green tea polyphenols on Cav-1 protein and mRNA expression in BAECs. BAECs were incubated with 4 μg/ml GTPs for the indicated time intervals. (A) Representative Western blot analysis of Cav-1; the bar graph represents three combined experiments. (B) Representative RT-PCR analysis of Cav-1; the bar graph represents five combined experiments. Data represent the results of the densitometric analysis (mean±S.E.M.) of three to five independent experiments. *P<.05 vs. with the bars indicated.

dehydrogenase (GAPDH) 5′-primer: TAC AAG CCC AAC AAC AAG G, and 3′-primer: ACA GTG AAG GTG GTG AAG C. Quantitative analysis was performed by a Biometra

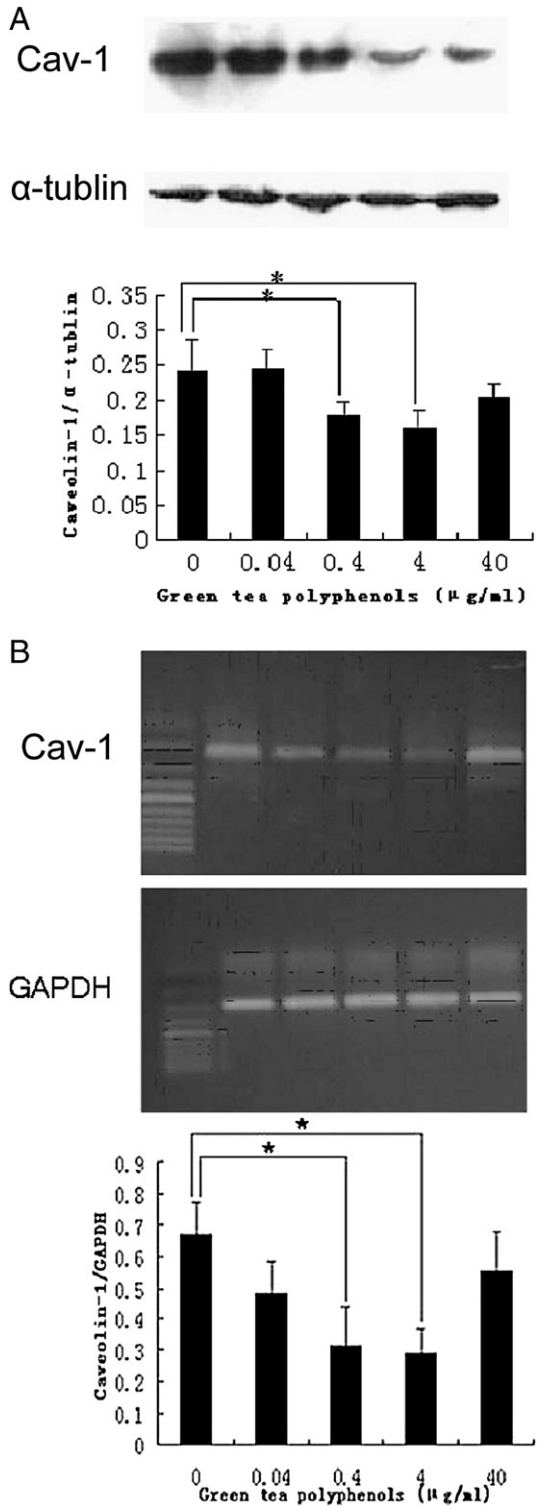


Fig. 2. Dose-response effects of Cav-1 protein and mRNA expressions treated with GTPs in BAECs. BAECs were incubated with different doses of GTPs for 16 h. (A) Representative Western blot analysis of Cav-1; the bar graph represents three combined experiments. (B) Representative RT-PCR analysis of Cav-1; the bar graph represents five combined experiments. Data represent the results of the densitometric analysis (mean±S.E.M.) of three to five independent experiments; *P<.05 vs. with the bars indicated.

densitometer (Biometra, Goettingen, Germany) and Gel Pro 3.0 software.

2.5. Statistical analysis

All quantitative data are presented as means±S.E. Western blots and reverse transcription (RT)-PCR shown are representative of three or more independent experiments. Data were compared by ANOVA-SNK followed by Newman–Keuls statistical comparisons evaluated with a Student's *t* test. Differences were considered significant when $P<.05$.

3. Results

3.1. Time course of effects of green tea polyphenols on Cav-1 protein and mRNA expression in BAECs

Fig. 1 depicts the effects of GTPs (4 μg/ml) on Cav-1 gene expressions by the BAECs. Compared with control values, Cav-1 protein expressions were down-regulated with GTP treatment for 12, 16 and 24 h ($P<05$, Fig. 1A), and GTPs inhibited Cav-1 expression by 28%, 43% and 59% for 12, 16 and 24 h of treatment, respectively. Decreasing mRNA levels of *cav-1* gene were also observed with GTPs treated for 4 and 8 h ($P<.05$, Fig. 1B), with maximum inhibition by 40% for 8 h of treatment.

3.2. Dose–response effects of Cav-1 protein and mRNA expressions

Fig. 2 depicts the dose–response effects of GTPs (16 h) on Cav-1 gene expressions in BAECs. Compared with control values, Cav-1 protein expressions were down-regulated after treatment with 0.4 and 4 μg/ml GTPs for 16 h ($P<05$, Fig. 2A), and GTPs inhibited Cav-1 expression by 27% and 35%, respectively. Decreasing mRNA levels of cav-1 gene were also observed in BAECs treated with 0.4 and 4 μg/ml GTPs for 8 h ($P<05$, Fig. 2B), with maximum inhibition by 56% in the 4 μg/ml GTP group.

3.3. The role of ERK1/2 cascade in Cav-1 expressions

When BAECs were incubated with PD98059, an inhibitor of ERK1/2 cascade, increasing expression of Cav-1 became visible by Western blotting and RT-PCR (Fig. 3A and B, $P<05$). Compared with control values, Cav-1 expression in BAECs treated with 4 μg/ml GTPs was down-regulated, which can be reversed by co-treating with PD98059.

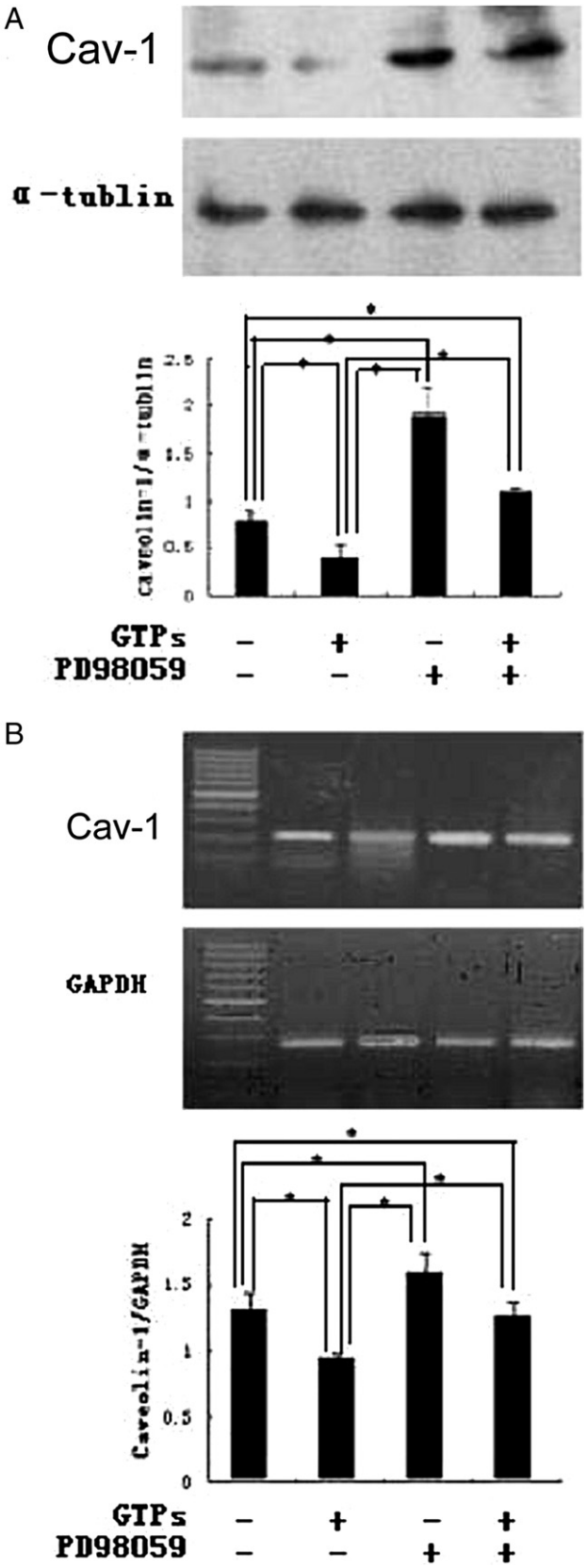
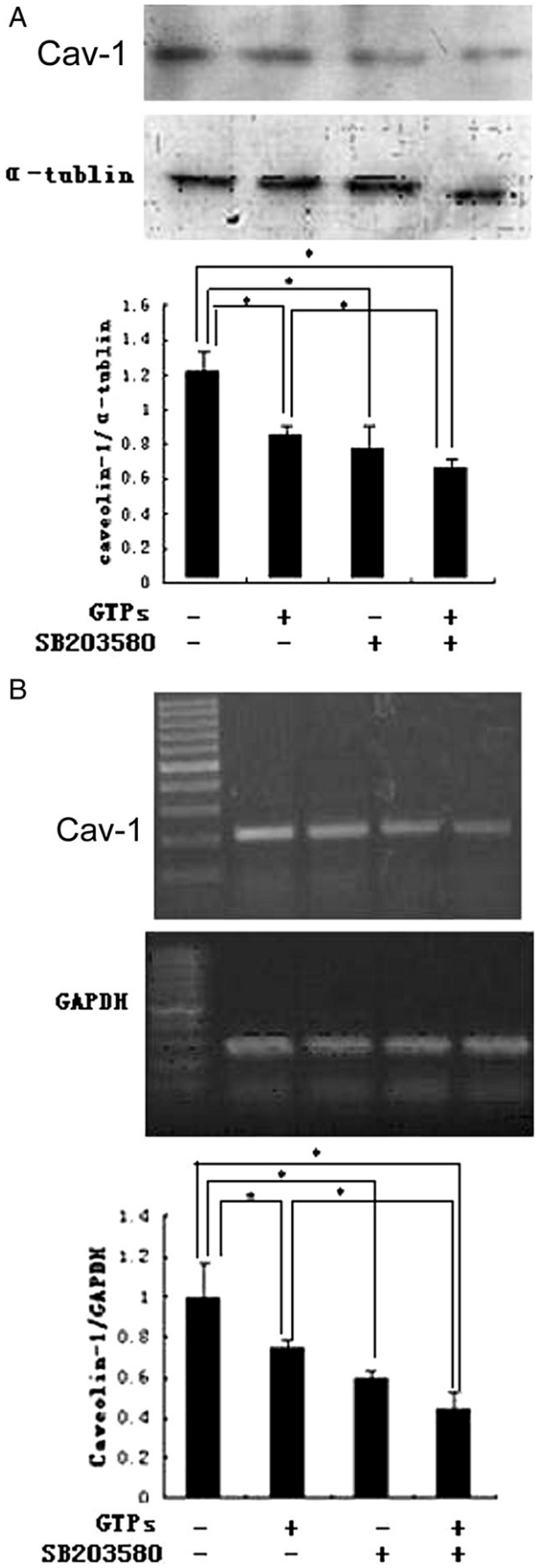


Fig. 3. Treatment with PD 98059 up-regulates Cav-1 expression and ameliorates GTPs' inhibition on Cav-1 expression. Cav-1 protein and mRNA expressions treated with 4 μg/ml GTPs with or without PD 98059 for 16 or 8 h, respectively. (A) Representative Western blot analysis of Cav-1; the bar graph represents three combined experiments. (B) Representative RT-PCR analysis of Cav-1; the bar graph represents three combined experiments. Data represent the results of the densitometric analysis (mean±S.E.M.) of independent experiments; *$P<.05$ vs. with the bars indicated.



3.4. The role of p38MAPK cascade in Cav-1 expressions

When BAECs were incubated with SB203580, an inhibitor of p38MAPK pathway, decreasing expression of Cav-1 became visible by Western blotting and RT-PCR (Fig. 4A and B, $P<.05$). Compared with control values, Cav-1 expression in BAECs treated with 4 μg/ml GTPs was down-regulated, which can be deteriorated by co-treating with SB203580.

3.5. Tyrosine phosphorylation of Cav-1 in GTP-treated BAECs

GTPs (4 μg/ml) inhibited tyrosine phosphorylation of Cav-1 (Tyr14) at 5, 10, 15, and 30 min after treatments ($P<.05$ vs. control); the phosphorylating signal inhibitions lasted for 30 min and then resumed. At 60 min after treatment with GTPs, no difference in tyrosine phosphorylation signal was observed compared with control ($P>.05$, Fig. 5).

4. Discussion

The effects of green tea polyphenols on Cav-1 protein and mRNA expressions in cultured endothelial cells were examined in this study. Green tea polyphenols dose- and time-dependently down-regulated cav-1 expression by activating ERK1/2 and inhibiting p38MAPK in cultured endothelial cells. The inhibition of cav-1 (Tyr14) phosphorylation by GTPs was also observed.

Because of the high rates of tea consumption in the global population (about 3 billion kilograms of tea are produced each year worldwide), even small effects of tea polyphenols in humans could have considerable benefits to public health [21]. GTPs have been extensively studied as CVD and cancer chemopreventive agents. A recent report from the Ohsaki study showed that reduced mortality due to all causes and due to CVD is associated with GTP consumption [22]. In animal and *in vitro* studies, evidence was devoted to the roles of GTPs in protecting against CVD; however, the exact mechanism underlying this phenomenon has not been fully elucidated.

Endothelial NOS was observed to be abundant in caveolae where it associates with Cav-1 by interacting with Cav-1 scaffold domain. And an inverse relationship between binding of eNOS to Cav-1 and eNOS activity was found. Fivefold increase in plasma NO levels was shown in $CAV1^{-/-}$ mice [23]. It is suggested that Cav-1 inhibits eNOS activity in unstimulated endothelial cells via protein–protein interactions. Moreover, in Cav-1-deficient mice, the acetylcholine-

Fig. 4. Treatment with SB203580 down-regulates Cav-1 expression and deteriorates GTPs' inhibition on Cav-1 expression. Cav-1 protein and mRNA expressions treated with 4 μg/ml GTPs with or without SB203580 for 16 or 8 h, respectively. (A) Representative Western blot analysis of Cav-1; the bar graph represents three combined experiments. (B) Representative RT-PCR analysis of Cav-1; the bar graph represents three combined experiments. Data represent the results of the densitometric analysis (mean±S.E.M.) of independent experiments; *$P<.05$ vs. with the bars indicated.

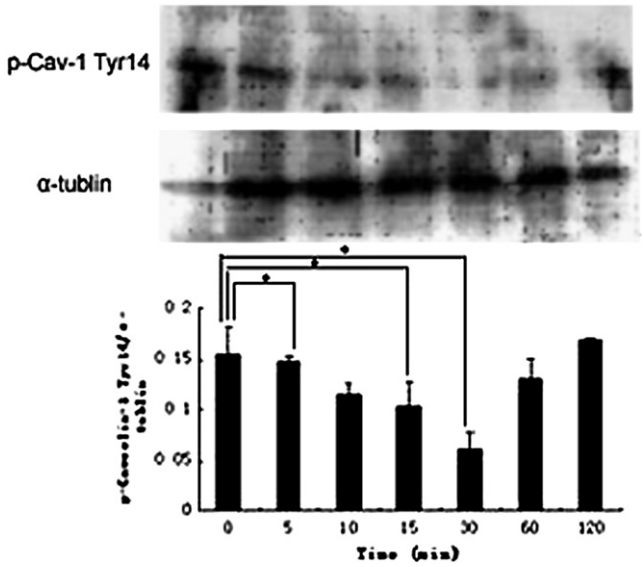


Fig. 5. The effects of GTPs on Cav-1 tyrosine phosphorylation (Tyr14) in BAECs. Serum-deprived BAECs were incubated with different doses of GTPs for 0, 5, 10, 15, 30, 60 and 120 min. A representative Western blot analysis of Cav-1; the bar graph represents three combined experiments. Data represent the results of the densitometric analysis (mean±S.E.M.); **P*<.05 vs. with the bars indicated.

stimulated relaxations and NO-dependent microvascular leakage are largely enhanced [24,25]. These results clearly show that Cav-1 negatively regulates NO synthesis. Therefore, alteration in Cav-1 abundance would modulate eNOS activity and, consequently, vascular function. We recently reported that there were consistent elevations in aortic Cav-1 mRNA and protein levels in high-fat diet-fed rats. The decrease in both total NOS and constitutive NOS activities in rats provided further evidence that the up-regulation of Cav-1 by high-fat diet inhibited cNOS (mainly eNOS) activities in the aorta [18]. The observed down-regulating effects of GTPs on Cav-1 expressions in this study indicated that GTP or green tea consumption is beneficial for the NO production in endothelial cells and thereby for CVDs.

Cav-1 gene expression was shown to be directly regulated by activation of the Ras-p42/44 MAP kinase cascade, although p42/44 MAP kinase-independent pathway also existed [26]. Down-regulation of Cav-1 expression in NIH3T3 cells and the activity of Cav-1 promoter had been evaluated by inhibition of ERK1/2 (p42/44MAPK) with PD98059 [27]. And EGCG, the major constituent of green tea, had been exhibited to activate ERK1/2 cascades in endothelial cells [28]. From the above evidence and from our data, GTPs down-regulated Cav-1 expression via activating ERK1/2 cascade.

Kim et al. [29] demonstrated that CO, by activating p38MAPK, up-regulates Cav-1 in smooth muscle cells, and Lei et al. [30] suggested that Cav-1 up-regulation in response to LPS was triggered through activating p38 kinase pathway in murine macrophages. A recent study showed that subcytotoxic oxidative stress generated by hydrogen peroxide application stimulated the activity of a Cav-1 promoter reporter gene constructed in fibroblasts which can be prevented by an antioxidant quercetin [31]. These evidences provided strong support for the role of p38MAPK in the up-regulation of Cav-1 in different types of cells. GTPs are also antioxidants with the ability to inhibit NADPH oxidase subunit p67phox and p22phox expressions in endothelial cells [20], although EGCG or GTPs had been reported to activate or inhibit the p38MAPK cascades [32,33].

Cav-1 possesses several tyrosine residues in which Tyr14 represents the main site of phosphorylation by Src family kinases. Although the exact function remains largely undefined, phosphorylated Cav-1 was reported to play an important role in endothelial migration [34]. There has been no report on the effects of dietary polyphenols on tyr14 phosphorylation of Cav-1 till now. The route and the role of GTP-induced inhibition of tyr14 phosphorylation will be of further interest.

Green tea polyphenols are complex compounds including flavonols, flavandiols, flavonoids and phenolic acids. The exact chemical characterization and bioavailability are poorly known [35]. It is known that (−)-EGCG has predominated in scientific scrutiny. *In vivo*, bioavailability and metabolism issues should be considered. Meng et al. [36] reported that unchanged EGCG represented only approximately 16% of the product excreted in human urine, with the remainder being methylated species. The effects of GTPs on Cav-1 expression *in vivo* will be further studied.

Taken together, GTPs down-regulate the gene expression of Cav-1 time- and dose-dependently via both activating ERK1/2 and inhibiting p38MAPK signaling.

Acknowledgments

We thank Dr. Z. Ye and Dr. C. Tian for reviewing the manuscript and for providing many helpful suggestions.

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