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Fibroblast Growth Factor-2 Upregulates Reactive Sulfur Species Production *via* ERK1/2 Signal-Mediated Cystathionine γ-Lyase Induction in Cultured Bovine Aortic Endothelial Cells

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Fibroblast growth factor-2 (FGF-2) regulates several vascular endothelial cell functions, including proliferation. It has been suggested that the regulation may be modulated by reactive sulfur species (RSS), which are hydrogen sulfide and biomolecules containing persulfide/polysulfide groups. Since RSS promote vascular endothelial cell proliferation, we hypothesized that FGF-2 regulates the levels of RSS-producing enzymes in the cells. Bovine aortic endothelial cells were cultured and treated with FGF-2, and intracellular RSS levels were determined. The expression of RSS-producing enzymes, cystathionine γ-lyase (CSE), cystathionine β-synthase, 3-mercaptopyruvate sulfurtransferase, and cysteinyl-tRNA synthetase 2, was evaluated, and the intracellular signaling pathway that mediates FGF-2 regulation of RSS accumulation was investigated. We revealed that FGF-2 upregulates the expression of RSS by selectively inducing CSE *via* the ERK1/2 signaling pathway in vascular endothelial cells. The effect of FGF-2 on the function of vascular endothelial cells may be modulated by intracellular RSS, especially higher-molecular-mass RSS such as protein persulfide, the levels of which are increased by the growth factor.

Key words cystathionine γ-lyase, fibroblast growth factor-2, reactive sulfur species, vascular endothelial cell

INTRODUCTION

Vascular endothelial cells form monolayers that cover the luminal surface of blood vessels and serve as a barrier between the blood and subendothelial matrix. These cells are also involved in the regulation of the blood coagulation-fibrinolytic system by synthesizing and secreting prostacyclin,[1] anticoagulant heparan sulfate proteoglycans,[2] plasminogen activators,[3] and plasminogen activator inhibitor-1.[4] Severe or repeated damage of the vascular endothelial cell monolayer initiates vascular lesions such as atherosclerotic lesions.[5] Therefore, a better understanding of cellular defense mechanisms in vascular endothelial cells can contribute to the development of new therapeutics of vascular lesions.

Reactive sulfur species (RSS), including hydrogen sulfide, cysteine persulfide, and glutathione persulfide, have been reported to be cytoprotective molecules against oxidative stress[6] and heavy metal cytotoxicity[7,8] because of their high nucleophilic properties.[9] We have reported that a copper complex, copper diethyldithiocarbamate, causes transcriptional induction of cystathionine γ-lyase (CSE), an RSS-producing enzyme, in the cells.[10] Additionally, RSS may modulate vascular endothelial cell functions such as proliferation. We recently revealed that sodium trisulfide, a sulfane sulfur donor, stimulates the proliferation of vascular endothelial cells.[11]

Fibroblast growth factor-2 (FGF-2) regulates vascular endothelial cell functions, including proliferation,[12] proteoglycan synthesis,[13] and fibrinolytic activity.[14] FGF-2 does not contain a specific signal sequence for secretion; thus, it may stimulate cell proliferation near the damaged site when it is leaked from nearby damaged endothelial cells and repair the monolayer.[15] Because of the stimulatory effect of sodium trisulfide on vascular endothelial cell proliferation,[11] we hypothesized that FGF-2 regulates the expression of RSS by regulating RSS-producing enzyme(s) when vascular endothelial cell monolayers are damaged.

Using a culture system of bovine aortic endothelial cells, the present study was undertaken to explore the regulation of RSS expression by FGF-2 in vascular endothelial cells. Additionally, since our previous study using a copper complex as a molecular probe showed that endothelial CSE expression can be mediated by multiple pathways, including ERK1/2, p38 MAPK, and hypoxia-inducible factor-1α/β (HIF-1α/β) pathways,[10] the pathways that mediate the FGF-2 regulation of endothelial RSS expression were also determined.

MATERIALS AND METHODS

Cell Culture and Treatment Vascular endothelial cells from bovine aorta (Cell Applications, San Diego, CA, USA) were cultured in Dulbecco's modified Eagle's medium (DMEM; Nissui Pharmaceutical, Tokyo, Japan), supplemented with

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10% heat-inactivated fetal bovine serum (FBS, Fujifilm Wako Pure Chemical, Osaka, Japan), in a humidified 5% CO_2 atmosphere at 37°C until they formed confluent monolayers. After removing the medium, cells were washed twice with serum-free DMEM and then incubated with either 5, 10, 20, or 50 ng/mL recombinant human FGF-2 (Fujifilm Wako Pure Chemical) for 8 or 24 h; 50 ng/mL for 1, 2, 3, 4, 6, 8, 12, 24, or 48 h; or 50 ng/mL for 8 or 24 h, with or without pretreatment for 1 h with 1, 5, and 10 μM FGF receptor (FGFR) inhibitor PD161570 (Santa Cruz Biotechnology, Dallas, TX, USA), with 5, 10, and 20 μM ERK1/2 pathway inhibitor PD98059 (Cayman Chemical, Ann Arbor, MI, USA), or with 0.1 and 1 μM ERK1/2 pathway inhibitor SCH772984 (Cayman Chemical).

Measurement of RSS Cellular RSS imaging was performed using the specific probe SSP4. Vascular endothelial cells were seeded onto glass-bottom dishes. After incubation with FGF-2, the culture medium was removed and the cell layer was washed twice with calcium- and magnesium-free phosphate-buffered saline (CMF-PBS, Nissui Pharmaceutical), and the cells were fixed with 4% paraformaldehyde phosphate buffer solution (Nacalai Tesque, Kyoto, Japan) in CMF-PBS for 25 min at room temperature. The cell layer was washed twice with CMF-PBS and incubated with 20 μM SSP4 (Dojindo, Kumamoto, Japan) in serum- and phenol red-free DMEM containing 500 μM cetyltrimethylammonium bromide (Nacalai Tesque) at 37°C for 1 h. The medium was removed, and the cells were washed with CMF-PBS and incubated in serum- and phenol red-free DMEM for 30 min. Fluorescence images were then acquired using a Keyence BZ-9000 fluorescence microscope (Keyence, Osaka, Japan) at an excitation wavelength of 482 nm and an emission wavelength of 515 nm. The fluorescence intensity was measured using the ImageJ software (US National Institutes of Health, Bethesda, MD, USA). The fluorescence intensity of 4',6-diamidino-2-phenylindole (DAPI) (Nacalai Tesque), which stains DNA, was used to normalize that of SSP4. The low-molecular-mass RSS within the cells was quantitatively analyzed as described previously,[16] using a liquid chromatography–electrospray ionization–tandem mass spectrometry (LC-ESI-MS/MS) with β-(4-hydroxyphenyl)ethyl iodoacetamide (HPE-IAM) (Molecular Biosciences, Boulder, CO, USA). Briefly, the cells were collected with methanol containing 1 mM HPE-IAM, sonicated, and incubated at 37°C for 30 min to yield HPE-AM adducts of sulfur nucleophiles. After centrifugation, aliquots of the supernatant were diluted with 0.1% formic acid containing known amounts of isotope-labeled internal standards, which were then analyzed by LC-ESI-MS/MS. The pellets were lysed with PBS containing 1% sodium dodecyl sulfate (SDS) and incubated at 95°C for 15 min, followed by the determination of protein concentration by BCA protein assay using bovine serum albumin as a standard.

Western Blot Analysis Confluent cultures of vascular endothelial cells were treated with FGF-2 in serum-free DMEM. After incubation, the cell layers were washed twice with cold CMF-PBS and lysed with SDS sample buffer [50 mM Tris-HCl buffer (pH 6.8) containing 2% sodium dodecyl sulfate and 10% glycerol]. After collection, the lysate was incubated at 95°C for 5 min. The protein concentration was determined using the Protein Assay BCA kit (Nacalai Tesque). 2-Mercaptoethanol and bromophenol blue were added to the samples and incubated at 95°C for 3 min. The protein samples were then separated by SDS-polyacrylamide gel electrophoresis, transferred to an Immobilon-P membrane (Merck Millipore, Billerica, MA, USA), and blocked for 1 h with 5% skim milk in 20 mM Tris-HCl buffer (pH 7.5), containing 15 mM NaCl and 0.1% Tween 20 or 2% bovine serum albumin in Tris-HCl buffer solution containing Tween 20 (20 mM Tris-HCl, 15 mM NaCl, and 0.1% Tween 20), and then incubated with primary antibody at 4°C overnight. After washing with the buffer solution, the membranes were incubated with secondary antibody for 1 h at room temperature. Immunoreactive bands were visualized by enhanced chemiluminescence using Chemi-Lumi One L (Nacalai Tesque, Kyoto, Japan) and scanned with an LAS 3000 Imager (Fujifilm, Tokyo, Japan). The antibodies used in this study were as follows. Polyclonal anti-CSE antibody was prepared as described previously.[17] Anti-cystathionine β-synthase (CBS) antibody (M01) was purchased from Abnova Corporation (Taipei, Taiwan). Anti-3-mercaptopyruvate sulfurtransferase (3-MST; D-8) antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-cysteinyl-tRNA synthetase 2 (CARS2; ARP68165_P050) antibody was purchased from Aviva Systems Biology (San Diego, CA, USA). Anti-phospho-p44/42 MAPK (Erk1/2; #9101), anti-p44/42 MAPK (#9102), anti-phospho-p38 MAPK (#9211), anti-p38 MAPK (#9212), anti-phospho-SAPK/JNK (#9255), and anti-SAPK/JNK (#9252) antibodies, and horseradish peroxidase conjugated anti-rabbit and mouse IgG secondary antibodies (#7074 and #7076, respectively) were purchased from Cell Signaling Technology (Danvers, MA, USA). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH; #5A12, Fujifilm Wako Pure Chemical) was used as an internal standard. The band intensities were analyzed using ImageJ software. The list of primary antibodies used in this study is shown in Table 1.

Real-Time RT-PCR Analysis After treatment and incubation, the culture medium was removed and the cell layer was washed twice with CMF-PBS and then lysed with QIAzol lysis reagent (QIAGEN, Venlo, Netherlands). The cells were collected by scraping and homogenized by pipetting. A quarter volume of chloroform was mixed with each lysate. Lysates were then centrifuged at 15,000 × *g*, and supernatants were harvested, followed by the addition of an equal volume of 70% ethanol. These suspensions were centrifuged at 15,000 × *g*, and the supernatants were discarded. The precipitates were resuspended in 70% ethanol and centrifuged at 15,000 × *g*, followed by the collection and drying of the precipitates containing the total RNA. The RNA quality was determined by spectrophotometric analysis ($OD_{260/280}$) using a NanoDrop Lite spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). The complementary DNA was synthesized from each mRNA sample using a high-capacity cDNA reverse transcription kit (Thermo Fisher Scientific). RT-PCR was performed using the GeneAce SYBR qPCR mix α (Nippon Gene, Tokyo, Japan) with 10 ng of cDNA and 0.2 μM primers in a StepOnePlus real-time PCR system (Thermo Fisher Scientific). The sense and anti-sense primer sequences were 5′-TCTCTTGGAGCAGTTCCATCTCCTA-3′ and 5′-GCAGCCCAGGATAAATAACCTTTTC-3′ for the bovine CSE gene; 5′-GGACTCGGTGCGGAACTACA-3′ and 5′-GGCAACACGGTCAGCGG-3′ for the bovine CBS gene; 5′- GCAGTGGGTGGCTGAGGC-3′ and 5′-CGATGTCAAAGAAGGCGGC-3′ for the bovine 3-MST gene; 5′- GAGGCGACAGGTACGGCAAG-3′ and 5′- CAGACTGGCGATGGTGGAAC-3′ for the bovine CARS2 gene; and 5′-CCATCCAGCGTCCTCCAAA-

Table 1. Primary Antibodies Used in the Present Study

Antibody	Species	Clonality	Supplier	Code No.	Dilution
CSE	Rabbit	Polyclonal	-	-	2,000
CBS	Mouse	Monoclonal	Abnova Corporation	M01	2,000
MPST (3-MST)	Mouse	Monoclonal	Santa Cruz Biotechnology	D-8	1,000
CARS2	Rabbit	Polyclonal	Aviva Systems Biology	ARP68165_P050	2,000
p44/42 MAPK (Erk1/2)	Rabbit	Polyclonal	Cell Signaling Technology	#9102	1,000
p-p44/42 MAPK(p-Erk1/2)	Rabbit	Polyclonal	Cell Signaling Technology	#9101	1,000
p38 MAPK	Rabbit	Polyclonal	Cell Signaling Technology	#9212	1,000
p-p38 MAPK	Rabbit	Polyclonal	Cell Signaling Technology	#9211	1,000
SAPK/JNK	Rabbit	Monoclonal	Cell Signaling Technology	#9252	1,000
p- SAPK/JNK	Mouse	Polyclonal	Cell Signaling Technology	#9255	1,000
GAPDH	Mouse	Monoclonal	Fujifilm Wako Pure Chemical	#5A12	10,000

GA-3′ and 5′- TTCAATCTGGGGTGGATGGAA-3′ for the bovine β_2-microglobulin (B2M) gene. The fold change for each gene was assessed after normalization of the intensity value to that of B2M.

Statistical Analysis Statistical analyses were performed using Student's *t*-test or analysis of variance with Dunnett's or Tukey-Kramer's test for multiple comparisons using the Excel software (Microsoft, Redmond, WA, USA). Data are expressed as the mean ± S.E., and the differences between groups were considered significant at $p < 0.05$.

RESULTS

FGF-2 Increases RSS in Vascular Endothelial Cells Figure 1 shows the RSS levels in vascular endothelial cells treated with FGF-2. The cells were stained with a fluorescent probe SSP4 and DAPI to visualize sulfane sulfur and nuclei, respectively (Fig. 1A, left panels) and the fluorescence intensity was quantified (Fig. 1A, right graph). The total sulfane sulfur levels within the cells were significantly elevated by FGF-2 at 10 ng/mL, and FGF-2 increased the intracellular RSS levels in the vascular endothelial cells in a concentration-dependent manner. There are two types of RSS; one is the low-molecular-mass RSS such as hydrogen sulfide, cysteine persulfide/polysulfide, and glutathione persulfide/polysulfide and the oth-

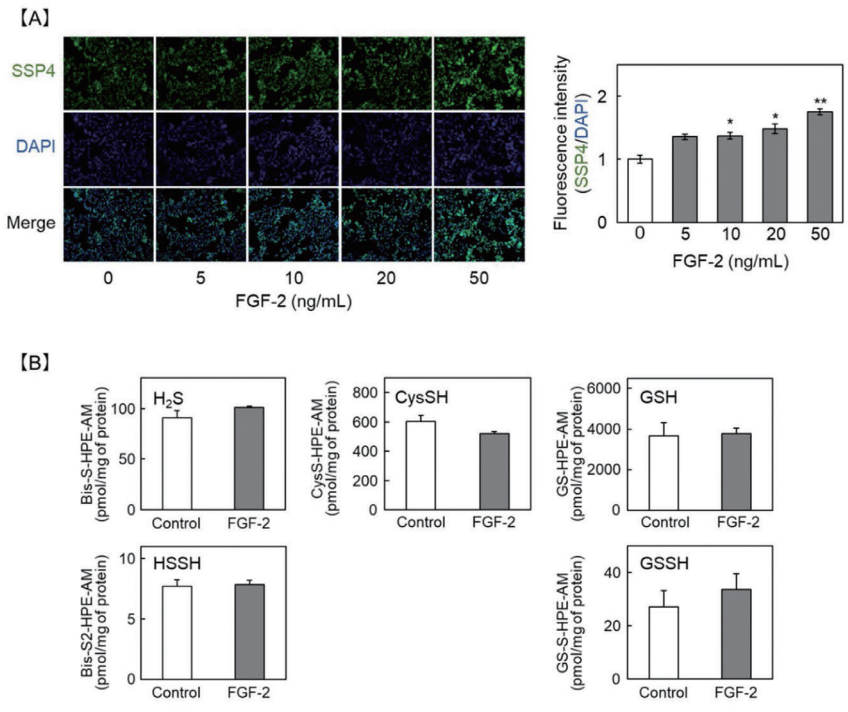

Fig. 1. The RSS Levels in Vascular Endothelial Cells After Treatment with FGF-2

[A] Fluorescence microscope images of vascular endothelial cells treated with FGF-2 (left panel). Green, SSP4 to stain sulfane sulfur; blue, DAPI to stain nuclei. Confluent cultures of bovine aortic endothelial cells were treated with FGF-2 (5, 10, 20, and 50 ng/mL) for 24 h in serum-free DMEM, then the cellular sulfane sulfur levels were determined using the fluorescence probe SSP4. Quantification of fluorescence intensity of sulfane sulfur stained with SSP4 (right panel). The intensity of SSP4 at three arbitrarily selected areas in the left panel was normalized to that of DAPI in the corresponding areas. Fluorescence intensity was measured using ImageJ software. The data are represented as means ± S.E. of three technical replicates. Significance from the control: $*p < 0.05$; $**p < 0.01$. [B] Quantitative analysis of low-molecular-mass RSS within vascular endothelial cells treated with FGF-2. Confluent cultures of bovine aortic endothelial cells were treated with FGF-2 (50 ng/mL) for 24 h in serum-free DMEM, then hydrogen sulfide, hydrogen persulfide, cysteine, cysteine persulfide, reduced glutathione, and reduced glutathione persulfide within the cells were quantitatively analyzed by liquid chromatography–electrospray ionization–tandem mass spectrometry. Cysteine persulfide was not detected. The data are represented as means ± S.E. of three experimental replicates.

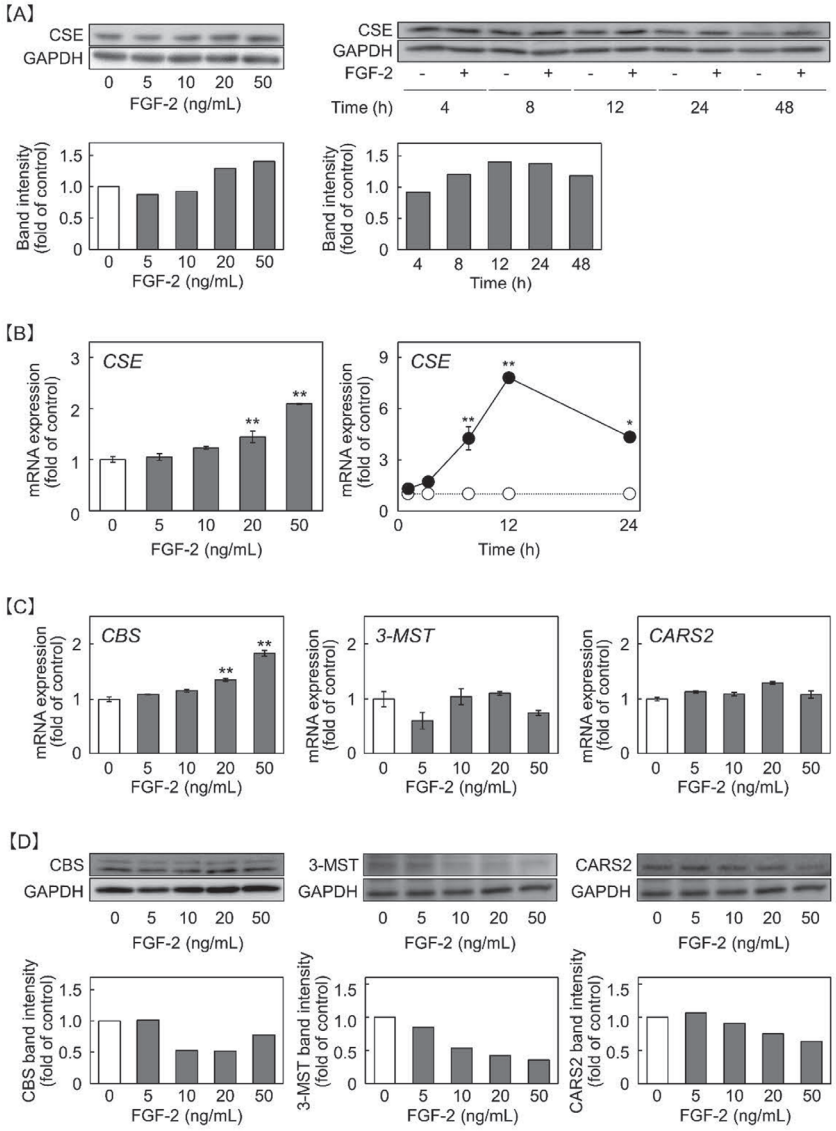

Fig. 2. The Expression of RSS-Producing Enzymes in Vascular Endothelial Cells After Treatment with FGF-2

[A] The expression of CSE protein. Confluent cultures of bovine aortic endothelial cells were treated with FGF-2 (5, 10, 20, and 50 ng/mL) for 24 h (left panels) or with FGF-2 (50 ng/mL) for 4, 8, 12, 24, and 48 h (right panels) in serum-free DMEM. Quantification of band intensities of CSE (lower panels). [B] The expression of CSE mRNA. Confluent cultures of bovine aortic endothelial cells were treated with FGF-2 (5, 10, 20, and 50 ng/mL) for 8 h (left panel) or with FGF-2 (50 ng/mL) for 2, 4, 8, 12, and 24 h (right panel) in serum-free DMEM. The mRNA data are represented as means ± S.E. of three biological replicates. Significance from the corresponding "Absence of FGF-2": $*p < 0.05$; $**p < 0.01$. [C] The expression of mRNA coding for CBS, 3-MST, and CARS2. Confluent cultures of bovine aortic endothelial cells were treated with FGF-2 (5, 10, 20, and 50 ng/mL) for 8 h. The mRNA data are represented as means ± S.E. of three biological replicates. Significance from the control: $*p < 0.05$; $**p < 0.01$. [D] The expression of CBS, 3-MST, and CARS2 protein. Confluent cultures of bovine aortic endothelial cells were treated with FGF-2 (5, 10, 20, and 50 ng/mL) for 24 h in serum-free DMEM. Quantification of band intensity of CBS, 3-MST, and CARS2 (lower panels).

er is the high-molecular-mass RSS such as protein persulfide/polysulfide.[18] Next, the intracellular content of low-molecular-mass RSS, hydrogen sulfide, cysteine persulfide, and glutathione persulfide, was quantitatively determined (Fig. 1B). FGF-2 failed to increase the low-molecular-mass RSS, hydrogen sulfide, and glutathione persulfide whereas cysteine persulfide levels were below the detection limit (less than 0.5 pmol/mg protein). Taken together, these results suggest that FGF-2 may increase the levels of only the high-molecular-mass RSS such as protein persulfide/polysulfide, in vascular endothelial cells.

FGF-2 Selectively Induces CSE among RSS-Producing Enzymes in Vascular Endothelial Cells Figure 2 shows the expression of RSS-producing enzymes (CSE, CBS, 3-MST, and CARS2) in vascular endothelial cells treated with FGF-2. First, we investigated CSE expression, because this enzyme was specifically induced by copper diethyldithiocarbamate in our previous study.[10] The expression of CSE protein was elevated by FGF-2 at 20 ng/mL or higher after 24 h of treatment in a concentration-dependent manner (Fig. 2A, left panels). The increase in CSE protein expression by FGF-2 at 50 ng/mL occurred after 8 h in a time-dependent manner (Fig. 2A, right panels). Additionally, CSE mRNA levels were elevated by FGF-2 in a concentration- and time-dependent manner (Fig. 2B, left and right panels, respectively). The expression of CBS mRNA was increased (Fig. 2C, left panel), but that of CBS protein was unchanged by FGF-2 (Fig. 2D, left panels), suggesting that induction of CBS protein requires a high-

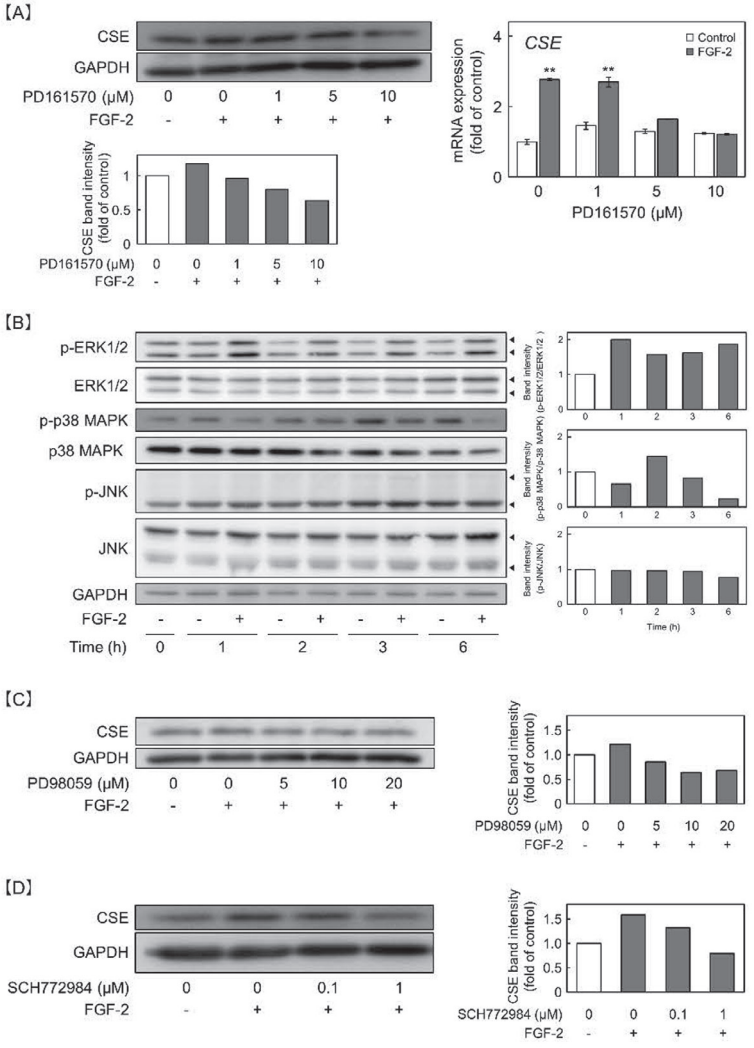

Fig. 3. Possible Involvement of FGFR and MAPKs in the Induction of CSE by FGF-2 in Vascular Endothelial Cells

[A] Expression of CSE protein (left panels) and mRNA (right panel) in the cells pretreated with an FGFR inhibitor PD161570. Confluent cultures of bovine aortic endothelial cells were pretreated with PD161570 (1, 5, and 10 μM) for 1 h, and then treated with FGF-2 (50 ng/mL) for 24 h (left panels) or 8 h (right panel) in serum-free DMEM. Quantification of band intensity of CSE (lower left panel). The mRNA data are represented as means ± S.E. of three biological replicates. **Significance from the corresponding control (without FGF-2): $p < 0.01$. [B] Phosphorylation of ERK1/2, p38 MAPK, and JNK in the cells after treatment with FGF-2. Confluent cultures of bovine aortic endothelial cells were treated with FGF-2 (50 ng/mL) for 1, 2, 3, or 6 h in serum-free DMEM. Quantification of band intensity of phosphorylated MAPKs (right panels). [C] Expression of CSE protein in the cells pretreated with an ERK1/2 pathway inhibitor PD98059. Confluent cultures of bovine aortic endothelial cells were pretreated with PD98059 (5, 10, and 20 μM) for 1 h, and then treated with FGF-2 (50 ng/mL) for 24 h in serum-free DMEM. Quantification of band intensity of CSE (right panel). [D] Expression of CSE protein in the cells pretreated with an ERK1/2 pathway inhibitor SCH772984. Confluent cultures of bovine aortic endothelial cells were pretreated with SCH772984 (0.1 and 1 μM) for 3 h, and then treated with FGF-2 (50 ng/mL) for 24 h in serum-free DMEM. Quantification of band intensity of CSE (right panel).

er elevation of the CBS mRNA level. The mRNA (Fig. 2C) and protein (Fig. 2D) levels of the other RSS-producing enzymes, 3-MST and CARS2, were not elevated by FGF-2. These results suggest that FGF-2 selectively induced CSE expression in vascular endothelial cells.

FGF-2-Induced Endothelial CSE Expression is Mediated by the FGFR-ERK1/2 Pathway Because the biological activities of FGF-2 can be mediated by the downstream signaling cascade of FGFR, we investigated whether FGFR is involved in the induction of CSE expression by FGF-2 in vascular endothelial cells. Induction of CSE protein (Fig. 3A, left panels) and mRNA (Fig. 3A, right panel) by FGF-2 at 50 ng/mL was suppressed by pretreatment with the FGFR inhibitor PD161570 at 5 and 10 μM, indicating that FGFR is involved in endothelial CSE induction by FGF-2.

The mitogen-activated protein kinases (MAPKs, *i.e.*, ERK1/2, p38 MAPK, and JNK) pathways that can be phosphorylated by the activation of FGFR[19] in vascular endothelial cells treated with FGF-2 were investigated (Fig. 3B). The phosphorylation of ERK1/2 was elevated by FGF-2 at 50 ng/mL treated for 1 h or longer. The activation of p38 MAPK was suppressed by FGF-2 treatment for 1 h or longer, and the phosphorylation of JNK was unaffected by FGF-2. These results were consistent with our previous report[13] and suggested that the ERK1/2 pathway may be involved in the induction of CSE expression by FGF-2. In fact, the induction of endothelial CSE protein expression by FGF-2 was suppressed by PD98059 and SHC772984, inhibitors of the ERK pathway (Figs. 3C and 3D, respectively).

DISCUSSION

Although it has been suggested that RSS are multifunctional molecules, little is known about the specific functions and regulation of RSS expression in vascular endothelial cells. In the present study, we found that FGF-2 induced CSE expression and increased the intracellular RSS accumulation. As the intracellular content of low-molecular-mass RSS was not increased by FGF-2, it is thought that high-molecular-mass RSS, *i.e.* protein persulfide/polysulfide, were increased by the growth factor to account for the overall accumulation of intracellular RSS. Recently, we reported that sodium trisulfide stimulates the proliferation of vascular endothelial cells.[11] Some proteins containing persulfide/polysulfide, which are increased by FGF-2, may contribute to the stimulatory effect of FGF-2 on vascular endothelial cell proliferation,[12] although the proteins have not been identified yet. The possibility that RSS modulates the regulation of vascular endothelial cell functions other than proliferation by FGF-2, including fibrinolytic activity[14] and proteoglycan synthesis,[13] cannot be excluded. The present study supports the hypothesis that RSS are modulators of cytokines/growth factors that regulate vascular endothelial cell function.

We have previously reported that a copper complex, copper diethyldithiocarbamate, induces the gene expression of CSE, but not that of CBS, 3-MST, and CARS2, in vascular endothelial cells.[10] The present study also showed that CSE expression was induced by FGF-2 among RSS-producing enzymes. These results suggest that the expression of intracellular RSS may be constitutively maintained by CSE, CBS, 3-MST, and CARS2, and is upregulated by inducing the CSE expression in vascular endothelial cells. In addition to FGF-2, other factors, such as hypoxia,[20] activation of calcium-sensing receptors,[21] protein restriction,[22] and copper diethyldithiocarbamate, induce endothelial CSE gene expression, thus supporting this hypothesis.

CSE induction is mediated by the ERK1/2 pathway in human macrophages,[23] rat cardiomyocytes,[24] and rat pancreatic β-cells.[25] Additionally, it has been shown that the p38 MAPK pathway can also mediate CSE induction in rat macrophages[26] and rat glioma cells.[27] Taken together, these results and the present data suggest that the MAPK pathway is one of the major pathways that mediates the CSE gene expression. In this study, the vascular endothelial cells activated only the ERK1/2 pathway to induce CSE expression in response to FGF-2. Our previous data showed that endothelial transcriptional induction of CSE was mediated by multiple pathways including the ERK1/2, p38 MAPK, and HIF-1α/β pathways in response to copper diethyldithiocarbamate.[10] These results suggest that the intracellular signaling pathways in vascular endothelial cells upregulate RSS synthesis depending on the external stimuli. The specific ERK1/2 pathway mediates the endothelial CSE expression for endogenous stimuli, whereas the activation of multiple pathways may be required to regulate the CSE expression in response to exogenous ones.

In the present study, we revealed that FGF-2 upregulates the expression of RSS by inducing CSE *via* the ERK1/2 signaling pathway in cultured vascular endothelial cells. Further studies are needed to validate the role of RSS and their specific intracellular pathways that mediate the expression of RSS-producing enzymes as protective mechanisms in vascular endothelial cells.

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Conflict of interest The authors declare no conflict of interest.

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