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Dietary apigenin regulates high glucose and hypoxic reoxygenation-induced reductions in apelin expression in human endothelial cells

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Abstract

The early stages of vascular endothelial dysfunction enhance angiogenic stimulation and strongly influence vascular rearrangement. The aim of this study was to determine whether a short period of high glucose (HG, 30 mM glucose) plus tumor necrosis factor alpha (TNFα) treatment or reoxygenation after hypoxia (H/R) alters the expression levels of apelin in human endothelial cells. In addition, we also examined the effects of the dietary flavonoid apigenin on apelin expression. Human endothelial cell lines were treated with HG plus TNFα or subjected to H/R. The expression levels of genes and proteins were then assessed by the reverse transcriptase polymerase chain reaction, Western blotting and immunofluorescence analyses. The expression level of apelin was significantly higher in the HG group following exposure to reoxygenation or TNFα. Reoxygenation after hypoxia decreased the expression levels of apelin and fatty acid transport protein (FATP) 1 compared with those observed during hypoxia alone and normoxia in a normal glucose concentration. Inversely, apigenin augmented H/R-reduced apelin and FATP1 expression in endothelial cells. Based on our findings, we propose that the early stages of endothelial disorder subtly influence angiogenesis and that HG and H/R stimulate vascular rearrangement and are involved in fatty acid uptake. Furthermore, dietary apigenin might improve the expression of angiogenic genes and fatty acid uptake.

Keywords: Apigenin; Apelin; Endothelial cells; Hypoxia; Angiogenesis

1. Introduction

In vascular endothelial cells, angiogenesis is regulated by hyperglycemia [1], hypoxia [2] and tumor necrosis factor alpha (TNFα) [3], etc. Exposure to hypoxia and reoxygenation (H/R) produces reactive oxygen species (ROS), and so oxidative stress is directly or indirectly associated with angiopoiesis or neovascularization [4,5]. Furthermore, endothelial cell dysfunction caused by acute hyperglycemia is observed during the early stages of diabetes mellitus (DM), and insufficient angiogenesis after ischemia is associated with a worse prognosis of peripheral arterial disease in patients with DM [6]. Apelin is an endogenous ligand for the G-protein-coupled receptor APJ and multiple physiological processes. Apelin/APJ signaling induces retina neovascularization in cooperation with vascular endothelial growth factor (VEGF) or fibroblast growth factor (FGF) 2 and contributes to normal ocular development [7]. Furthermore, apelin enhances superoxide dismutase activity and protects against ischemic heart disease after H/R [8]. Angiogenesis is mainly induced by VEGF and it receptors, and vascular maturation is regulated by the angiopoietin-1/Tei2 system. Although the functions of VEGF are well established, the regulation of apelin expression in H/R and high glucose (HG) conditions is poorly understood.

Dietary polyphenols are considered to induce cardiovascular protection, improve endothelial function, inhibit angiogenesis, and inhibit cell migration and proliferation in blood vessels [9,10]. The dietary flavonoid apigenin inhibits VEGF and erythropoietin gene expression in endothelial cells via the degradation of hypoxia-inducible factor 1 alpha (HIF-1α) protein [11]. Apigenin suppresses the expression of VEGF, an important factor in angiogenesis, in endothelial cells via the degradation of HIF-1α protein. Dietary polyphenols suppress the ROS production induced by stress and might modulate angiogenic induction [12,13].

Deficient levels of angiogenesis after ischemia may contribute to a worse prognosis of peripheral arterial disease in patients with DM. However, the expression levels of apelin, VEGF receptor (VEGFR) and FGF receptor (FGFR) during acute angiogenesis are unknown. Apelin may prevent functional vascular leakage induced by ischemia. A recent study indicated that apelin induces blood vessel formation as a means of achieving functional recovery after ischemia [2]. On the other hand, fatty acid transport proteins (FATPs) are associated with fatty acid uptake and activation [14]. FATP1 promotes the uptake of fatty acids into muscle cells and stimulates the consumption of fatty

Abbreviations: DM, diabetes mellitus; FGFR, fibroblast growth factor receptor; FATP, fatty acid transport protein; HIF-1, hypoxia-inducible factor 1; H/R, reoxygenation after hypoxia; HG, high glucose; ROS, reactive oxygen species; TNF, tumor necrosis factor; VEGFR, vascular endothelial growth factor receptor.

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Table 1
Sequences of primers for RT-PCR

Genes	Forword primer (5′→3′)	Reverse primer (5′→3′)	Amplicon (bp)	Ac no
Apelin	AGTGTGCCTCCCAATACTCCCT	TGCAATGACTCTGAGCAGGTCA	261	NM_017413
FATP1	TCAGATCAACCAACAGGACCC	CGATCTTCTTGCTGGTGGC	74	NM_198580
Tie2	GCCACCATCATTCAGTATCAGC	CTGCAAAAATGTCCACCTGG	70	AB086825

Ac no: Accession number.

acids [15]. FATPs may promote the diffusion of fatty acids in the cytosol of endothelial cells [16]. A recent report showed that VEGF-B regulates FATPs and controls the uptake of fatty acids in endothelial cells [17]. These reports suggest that FATPs are associated with the survival of tissue through the supply of energy during healthy vascular rearrangement [14].

The objective of this study was to determine whether a short period of treatment with high HG plus TNFα, or H/R alters the expression levels of apelin in human endothelial cells. Moreover, we examined the expression of FATP1 as an index of the energy supply for revitalization. In addition, we also examined the effects of the dietary polyphenol apigenin.

2. Materials and methods

2.1. Materials

Human TNFα was purchased from Roche Applied Science (USA). β-Actin and apelin polyclonal antibodies were obtained from Santa Cruz Biotechnology, Inc. (CA, USA). FATP1 polyclonal antibodies were obtained from Abcam, plc (Cambridge, UK). Apigenin was obtained from Sigma-Aldrich, Inc. (St. Louis, MO, USA). All other reagents were purchased from Sigma-Aldrich, unless indicated otherwise.

2.2. Cell culture

The human endothelial cell line ISO-HAS was obtained from the Cell Resource Center for Biomedical Research, Tohoku University (Sendai, Japan). The method used to culture the human vascular endothelial cells was described in a previous report [18]. Namely, the ISO-HAS cells were cultured in 50% Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS, Gibco, Invitrogen, CA, USA) and conditioned medium from the angiosarcoma cell line ISO-1 (DMEM/ISO1) [18,19]. ISO-HAS cells were identified from their characteristic constitutive expression of CD31, von Willebrand factor, Flt-1, KDR and VEGF [20,21]. The cells were seeded at a ratio of 1:1 in 75-cm^2 flasks (Becton Dickinson, Bedford, MA, USA) and grown at 37°C under 5% CO_2 in a humidified atmosphere in DMEM/ISO1. The medium was periodically renewed until the cells had reached 70%–90% confluence, at which point they were treated with 0.25% trypsin (Sigma).

2.3. Treatment of cultures

The ISO-HAS cells were plated in 10-mm cell culture dishes (Sumitomo Bakelite Co., Ltd., Tokyo, Japan) or 96-well plates (Becton Dickinson) and grown at 37°C under 5% CO_2 in a humidified atmosphere until they were confluent, which was typically 24–48 h after seeding. They were then incubated with the control concentration of glucose (5.5 mM) or the test high glucose concentration (30 mM) with or without TNFα (10 ng/ml) or different concentrations of 30 μM apigenin for 0–24 h.

2.4. Hypoxia and reoxygenation

The cells were placed in a sealed chamber (Wakenyaku Co., Ltd.) and exposed to hypoxia (5% CO2, 1% O2 and 94% N2) for 24 h and then to normoxia (5% CO2, 21% O2 and 74% N2) for 30 min to 24 h, as described previously [22].

2.5. Total RNA extraction and cDNA

Total RNA was extracted from the cultured neurons using Trizol reagent (Gibco Invitrogen). DNase I (Gibco, Invitrogen) was used to treat the RNA samples at room temperature for 15 min in order to remove the genomic DNA. DNase I was heated for 15 min at 65°C to inactivate it, and first-strand cDNA synthesis was performed with Superscript II (Gibco, Invitrogen).

2.6. Reverse transcriptase polymerase chain reaction (RT-PCR)

Polymerase chain reaction was performed for apelin and FATP1. The primers used were based on sequences from GenBank and designed using the primer design software Primer Express (Applied Biosystems). Table 1 summarizes the primer sets used. The reaction mixture (50 μl) contained 200 ng of the cDNA sample; 1.25 U of Ampli-Taq DNA polymerase; 1× PCR reaction buffer; 200 mM of each primer; 200 μM dATP, dCTP, dGTP and dTTP; and 1.5 mM $MgCl_2$ with thermal cycler (Applied Biosystems; GeneAmp PCR System 9700). The thermal cycling conditions involved 5 min at 95°C, followed by 35 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 30 s. After amplification, 10 μl of the reaction mixture was electrophoresed on a 2% NuSive: agarose (3:1) (FMC product, Rockland, ME, USA) gel and visualized under UV illumination after being stained with ethidium bromide. Messenger RNA expression was calculated relative to that of 18s ribosomal RNA.

2.7. Western blot analysis

The ISO-HAS cells were lysed in RIPA buffer, and the cell lysates (50 μg protein) were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes, as described previously [23]. The membranes were then incubated with primary antibodies (β-actin, ×3000; apelin, ×800; FATP1, ×1000) against the target protein for 2 h. After being washed twice, the membranes were incubated with a horseradish-peroxidase-conjugated secondary antibody, and the protein levels were detected using an enhanced chemiluminescence system (Invitrogen) with ChemiDoc XRS Plus (Bio-Rad Japan).

2.8. Immunofluorescence

Cells plated on culture slides (Nalge Nunc International K.K., Tokyo, Japan) were rinsed with phosphate-buffered saline (PBS) twice before being fixed in 98% ethanol at room temperature for 10 min. The fixed cells were then washed three times with PBS before being blocked with 10% normal goat serum/PBS for 1 h. The primary antibody against apelin (Santa Cruz Biochemistry, Inc., CA, USA) was diluted to

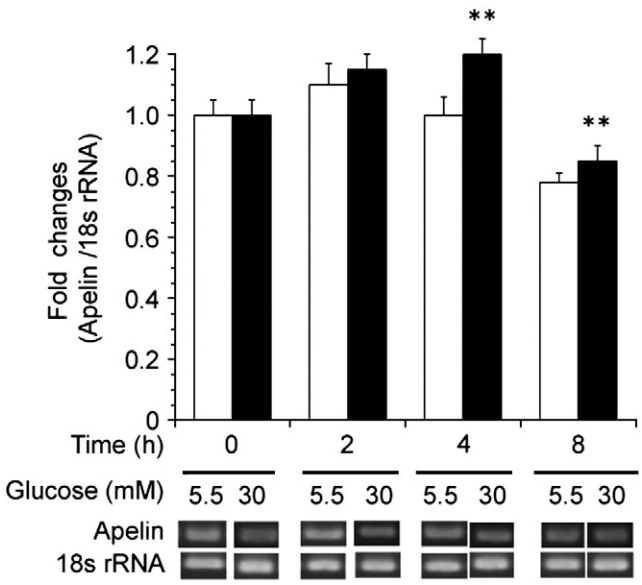

Fig. 1. Effects of high glucose plus TNFα on the gene expression of apelin in human endothelial cells. Human endothelial cells were exposed to 30 mM glucose+TNFα or 5.5 mM glucose+TNFα treatment for 2, 4 or 8 h. Total cellular RNA was isolated from the cultured endothelial cells at the time points indicated. Total cellular RNA was transcribed with reverse transcriptase and amplified by PCR. An 18s ribosomal RNA amplicon was used as an internal control to quantify the total amount of cDNA. Messenger RNA expression levels are shown relative to those of 18s ribosomal RNA. The data shown are mean values (n=4), and the results are presented as change ratios compared with the 5.5 mM glucose at the same time. **P<.01.

10 μg/ml in PBS and applied for 1 h at room temperature. After an additional three washes with PBS, the cells were incubated with Alexa 594-labeled secondary antibody (Molecular Probe, Eugene, OR, USA) for 1 h at room temperature and then washed three times in PBS for 5 min. Images were captured using a fluorescent microscope (BZ-8000; Keyence, Tokyo, Japan). The intensity of fluorescence was calculated in the same area.

2.9. Statistical analysis

The data shown are mean values±S.D. (n=4). The significance of differences was determined using Fisher's protected least significant difference method following an analysis of variance. Results are presented as change ratios compared with the control.

3. Results

3.1. HG plus TNFα-induced apelin gene expression in cultured human endothelial cell lines

Confluent human endothelial cells were exposed to 30 mM glucose (HG) plus TNFα (10 ng/ml) or 5.5 mM glucose plus TNFα for 8 h. The expression of apelin was transiently increased at 2 h and significantly (P<.01) increased in the HG plus TNFα group at 4 h and normal glucose plus TNFα group at 8 h compared with that observed

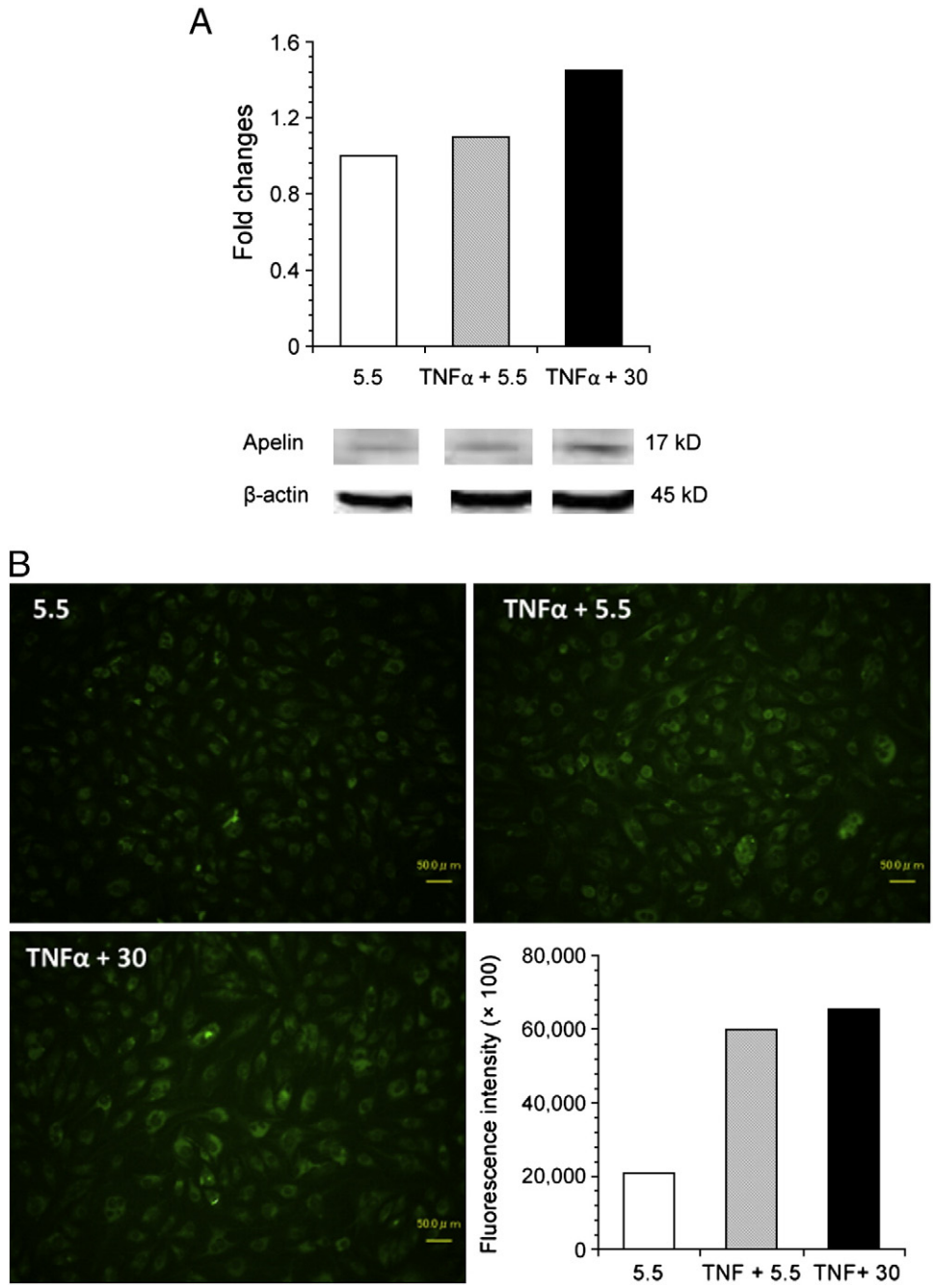

Fig. 2. Effects of high glucose plus TNFα on the protein expression levels of apelin in human endothelial cells. Human endothelial cells were exposed to 5.5 mM glucose+TNFα (10 ng/ml) or 30 mM glucose+TNFα (10 ng/ml) for 24 h. (A) Protein expressions (Western blotting). The cells were lysed, extracts were produced using detergent, and Western blots were carried out as described in "Material and Methods". Total protein (−50 μg) was run, transferred to polyvinylidene difluoride membranes and reacted with apelin antibody (n=4). (B) Protein expressions (immunocytochemical staining). Immunocytochemical staining was carried out with apelin antibody, as described in "Material and Methods". 5.5: 5.5 mM glucose, TNFα+5.5: TNFα (10 ng/ml)+5.5 mM glucose, TNFα+30: TNFα (10 ng/ml)+30 mM glucose. The data shown are mean values (n=2).

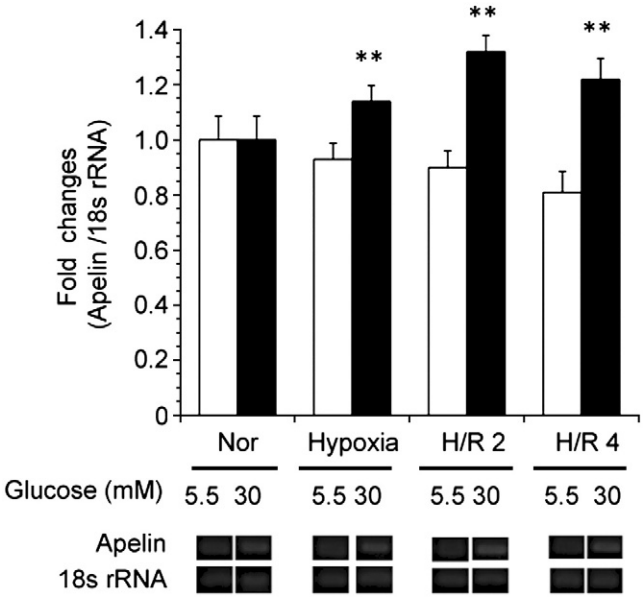

Fig. 3. Effects of high glucose plus H/R on the expression of apelin in human endothelial cells. Human endothelial cells were exposed to treatment with 30 mM glucose or 5.5 mM glucose during 24-h hypoxia and then exposed to reoxygenation for 2 h or 4 h. Total cellular RNA was transcribed with reverse transcriptase and amplified by PCR. An 18s ribosomal RNA amplicon was used as an internal control to quantify the total amount of cDNA. Messenger RNA expression was calculated relative to that of 18s ribosomal RNA. The data shown are mean values (n=4); the results are presented as change ratios compared with the 5.5 mM glucose at the same time. **P<.01.

in the control at 0 h (Fig. 1). In addition, HG plus TNFα treatment induced higher apelin gene expression levels compared with those observed in the control glucose group throughout the 8-h treatment.

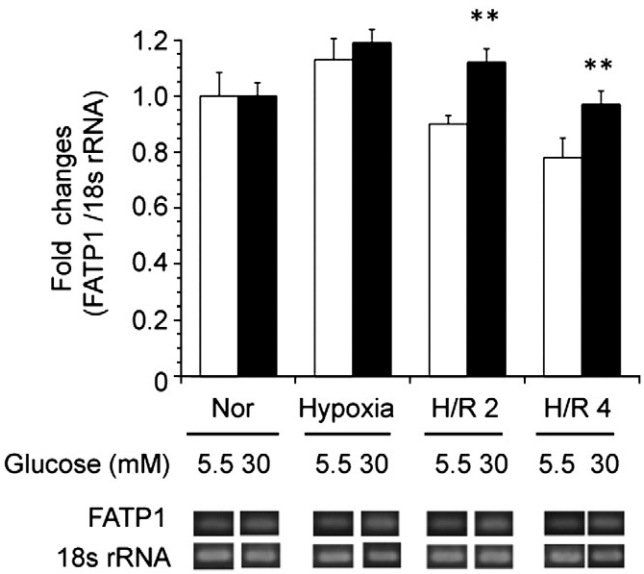

Fig. 4. Effects of high glucose plus H/R on the expression of FATP1 in human endothelial cells. Human endothelial cells were exposed to treatment with 30 mM glucose or 5.5 mM glucose during 24-h hypoxia and then exposed to reoxygenation for 2 h or 4 h. Total cellular RNA was transcribed with reverse transcriptase and amplified by PCR. An 18s ribosomal RNA amplicon was used as an internal control to quantify the total amount of cDNA. Messenger RNA expression was calculated relative to that of 18s ribosomal RNA. The data shown are mean values (n=4), and the results are presented as change ratios compared with the 5.5 mM glucose at the same time. **P<.01.

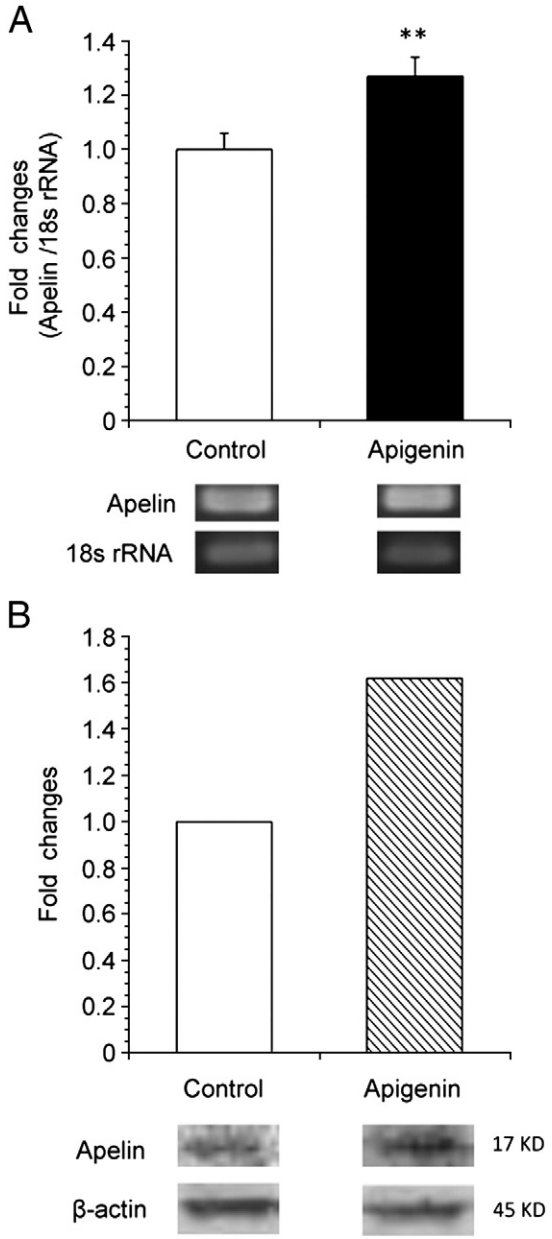

Fig. 5. Effects of apigenin on the expression of apelin in human endothelial cells. (A) Gene expressions. Human endothelial cells were treated without or with 30 μM apigenin for 8 h. Total cellular RNA was isolated from the cultured endothelial cells. Total RNA was transcribed with reverse transcriptase and amplified by PCR. An 18s ribosomal RNA amplicon was used as an internal control to quantify the total amount of cDNA. Messenger RNA expression was calculated relative to that of 18s ribosomal RNA. The data shown are mean values (n=4), and the results are presented as change ratios compared with the control (without apigenin). **P<.01. (B) Protein expressions. ISO-HAS human endothelial cells were treated without or with 30 μM apigenin for 24 h. The cells were lysed, extracts were produced using detergent, and Western blots were carried out as described in "Material and Methods". Total protein (−50 μg) was run, transferred to polyvinylidene difluoride membranes and reacted with apelin antibody.

3.2. Expression of apelin protein in human endothelial cells according to Western blotting and immunofluorescent staining

To confirm the expression levels of apelin, we examined cells that had been treated with HG plus TNFα for 24 h. ISO-HAS cells were incubated with 5.5 mM glucose, 5.5 mM glucose plus TNFα or 30 mM glucose plus TNFα, and their apelin protein expression levels were

studied. As a result, we found that the protein expression levels of apelin were enhanced by treatment with 5.5 mM glucose plus TNFα and 30 mM glucose plus TNFα compared with those of the cells treated with 5.5 mM glucose (Fig. 2A). Furthermore, to investigate whether treatment with TNFα plus 30 mM glucose induces apelin protein expression in the human endothelial cell lines, we performed immunocytochemical staining. We examined cells that had been exposed to 5.5 mM glucose, 5.5 mM glucose plus TNFα or 30 mM glucose plus TNFα for 24 h. As shown in Fig. 2B, the protein expression levels of apelin were strongly enhanced by treatment with 5.5 mM glucose plus TNFα and 30 mM glucose plus TNFα compared with those in the cells treated with 5.5 mM glucose.

3.3. HG and H/R-induced expression of apelin and FATP1 in cultured human endothelial cells

Confluent human endothelial cells were exposed to 30 mM glucose or 5.5 mM glucose during 24-h hypoxia (1% O2) and then subjected to reoxygenation (atmosphere oxygen: 21% O2) for 2 or 4 h, and their apelin gene expression levels were investigated by RT-PCR. As shown in Fig. 3, in the 5.5-mM glucose treatment group, apelin expression was decreased at 2 and 4 h after H/R, but an increase in apelin expression after H/R was detected in the 30-mM treatment group. Thirty-millimolar glucose treatment during hypoxia significantly ($P<.01$) increased the expression levels of apelin compared with those observed during treatment with 5.5 mM glucose. During reoxygenation, the expression levels of apelin were significantly ($P<.01$) increased after 2 and 4 h in the 30-mM glucose group. Similarly, we also examined the gene expression of FATP1 during H/R (Fig. 4). In the 5.5-mM glucose treatment group, the expression of FATP1 was increased during hypoxia compared with normoxia. However, the expression levels observed during reoxygenation were clearly decreased compared with those observed in the 5.5-mM glucose treatment alone group, and the 30-mM glucose treatment group produced the same expression patterns. However, after reoxygenation, the expression levels significantly increased ($P<.01$) in the 5.5-mM glucose treatment group.

3.4. Effects of apigenin on apelin gene expression in human endothelial cells

Confluent human endothelial cells were exposed to 30 μM of apigenin for 8 h. As shown in Fig. 5A, apigenin significantly ($P<.01$) increased the gene expression levels of apelin at 8 h compared with the control. Similar results were confirmed for protein expression at 24 h using Western blotting (Fig. 5B).

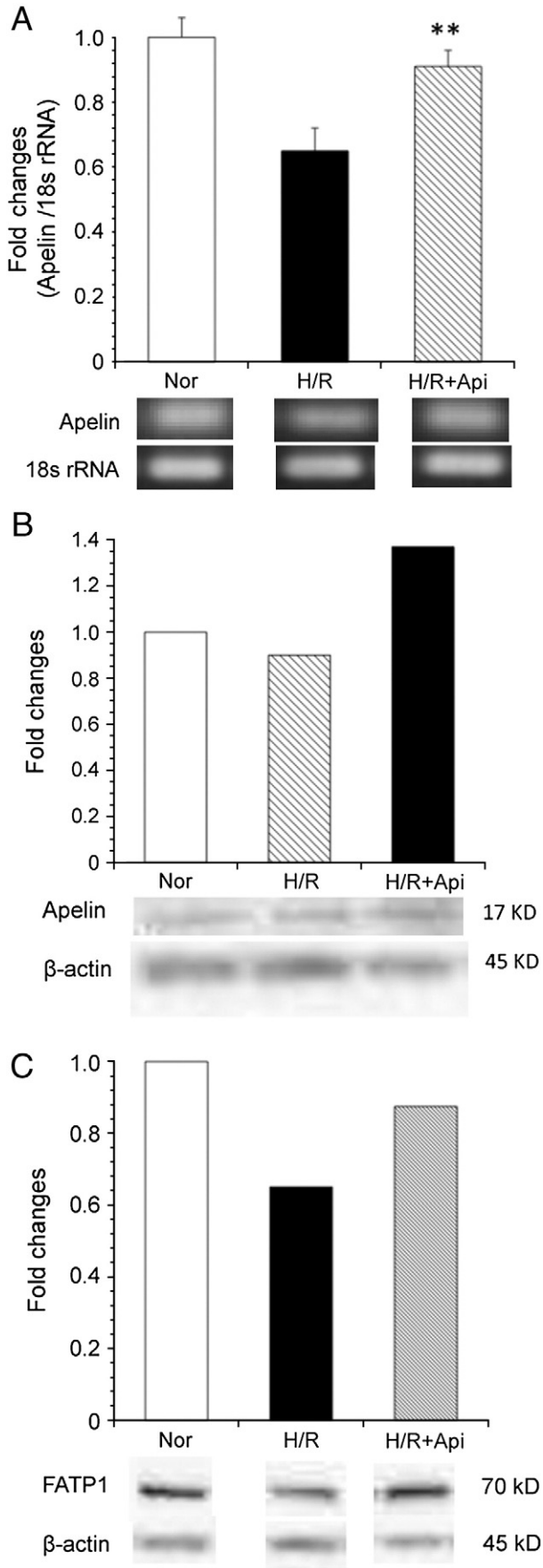

Fig. 6. Effects of apigenin on the H/R-induced expression of apelin in human endothelial cells. (A) Gene expressions. H/R: Human endothelial cells were treated without or with apigenin under hypoxia for 24 h and then exposed to reoxygenation for 4 h. Total cellular RNA was isolated from the cultured endothelial cells. Total RNA was transcribed with reverse transcriptase and amplified by PCR. An 18s ribosomal RNA amplicon was used as an internal control to quantify the total amount of cDNA. Messenger RNA expression was calculated relative to that of 18s ribosomal RNA. The data shown are mean values ($n=4$), and results are presented as change ratios compared with the H/R (without apigenin). **$P<.01$. (B) Protein expressions. Human endothelial cells were treated without or with apigenin under hypoxia for 24 h and then exposed to reoxygenation for 24 h. The cells were lysed, extracts were produced using detergent, and Western blots were carried out as described in "Material and Methods". Total protein (−50 μg) was run, transferred to polyvinylidene difluoride membranes and reacted with apelin or (C) FATP antibody. The data shown are mean values ($n=2$).

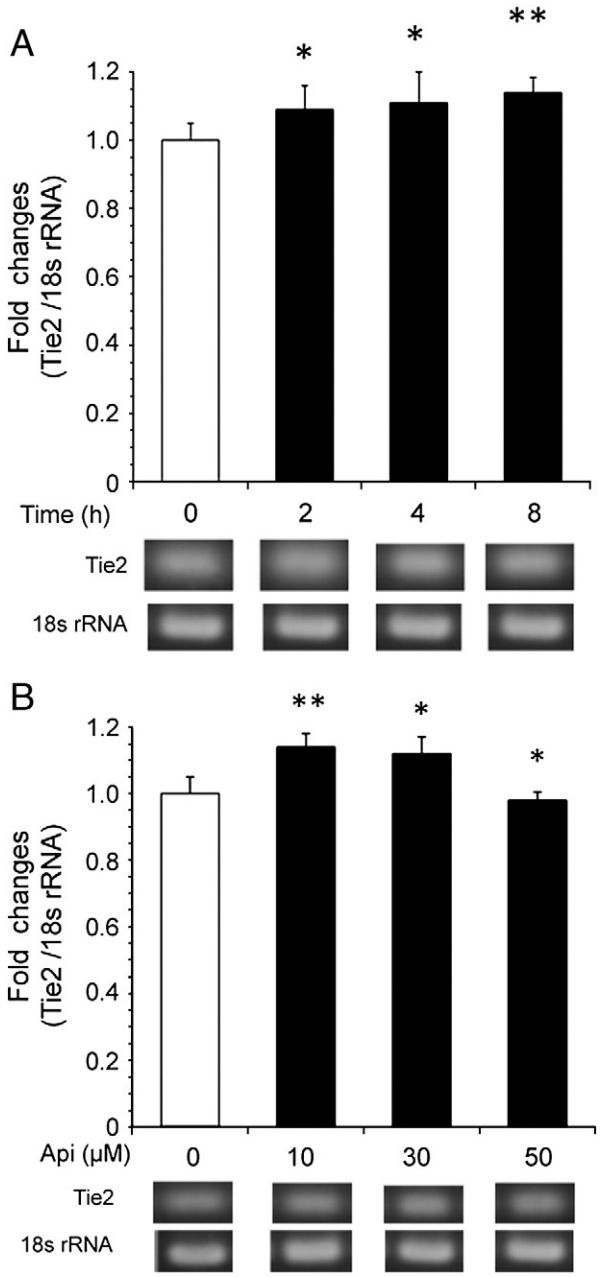

Fig. 7. Effects of apigenin on the expression of Tie2 in human endothelial cells. (A) Confluent human endothelial cells were exposed to 30 μM of apigenin for 8 h. (B) Confluent human endothelial cells were exposed to 10, 30 and 50 μM of apigenin for 8 h. Total cellular RNA was isolated from the cultured endothelial cells. Total RNA was transcribed with reverse transcriptase and amplified by PCR. An 18s ribosomal RNA amplicon was used as an internal control to quantify the total amount of cDNA. Messenger RNA expression was calculated relative to that of 18s ribosomal RNA. The data shown are mean values (n=4), and results are presented as change ratios compared with the control (without apigenin). *P<.05; **P<.01.

3.5. Effect of apigenin on H/R-induced expression of apelin and FATP1 in cultured human endothelial cell lines

Using RT-PCR, we examined the modifying effects of apigenin on the H/R-induced changes in the gene expression levels of apelin. The gene expression levels of apelin were decreased by H/R stimulation in comparison with those observed during normoxia (Fig. 6A). However, the significant decreases in the gene expression levels of apelin induced by H/R (P<.01) were reversed by the addition of apigenin, and the resultant expression levels were high compared with those of the control group. Furthermore, we examined the modifying effects of apigenin on the H/R-induced changes in the expression of apelin protein and FATP1 protein using Western blotting. The expression of apelin was slightly decreased by H/R stimulation in comparison with that observed during normoxia (Fig. 6B). However, apigenin clearly increased the expression levels of apelin protein. In addition, we examined the effects of apigenin on the expression of apelin protein in H/R. The expression of FATP protein was strongly decreased by H/R compared with that observed under normoxia (Fig. 6C). However, the decrease in the protein expression of FATP1 induced by H/R was recovered by the addition of apigenin.

3.6. Effects of apigenin on Tie2 gene expression in human endothelial cells

Confluent human endothelial cells were exposed to 30 μM of apigenin for 8 h. As shown in Fig. 7A, apigenin significantly (P<.01) increased the gene expression levels of Tie2 at 8 h compared with the control. Similarly, the effects of apigenin on the expression of Tie2 mRNA were further investigated by treating human EC with 10, 30 and 50 μM apigenin. Following an 8-h exposure to exogenous Tie2, mRNA levels were significantly increased. The increase in Tie2 mRNA caused by 10, 30 and 50 μM apigenin was significant (Fig. 7B).

4. Discussion

In extensive clinical trials, hyperglycemia was found to be correlated with poor glycemic control and an increased incidence of microvascular and macrovascular disease in diabetic patients [24,25]. Hyperglycemia reduces endothelium-dependent vasodilation in blood vessels, and it has been suggested that high glucose levels cause endothelial deterioration [26–28]. Vascular endothelial dysfunction may trigger loss of vasodilation, vasoconstriction, thrombosis, inflammation and the development of atherosclerosis, as well as exacerbate endothelial dysfunction, causing knock-on effects on angiogenesis [29]. In this study, we focused on an early angiogenesis signal: the apelin expression induced by endothelial cell disorders. Moreover, the effects of the dietary flavonoid apigenin were investigated in human ISO-HAS endothelial cells.

In this study, we clearly indicated that H/R plus TNFα and HG plus TNFα modulate the expression of the angiogenic protein apelin. Our data suggest that the early stages of HG treatment mediate various diseases by subtly influencing the expression of apelin and stimulating angiogenesis and vascular rearrangement. In particular, HG stimulation as well as ischemia and inflammation may induce angiogenesis. Also, we demonstrated that the dietary flavonoid apigenin reverses the H/R-induced attenuation of apelin expression in human endothelial cells. Apelin was demonstrated to actively modulate angiogenesis both *in vitro* and *in vivo*, and it has previously been found to stimulate endothelial cell proliferation, migration and tube formation *in vitro* [30,31]. A previous report demonstrated that apelin is crucial for normal vasculature development in frog embryos [32]. Furthermore, apelin expression is up-regulated during tumor angiogenesis, and its overexpression was found to increase during *in vivo* tumor growth. Our results demonstrate that treatment with high glucose plus TNFα up-regulates the expression of apelin, which plays a key role in hypoxia-induced endothelial angiogenesis in cultured cells.

Treatment with apigenin alone increased the gene expression of apelin. Furthermore, in cells subjected to H/R, apigenin induced the expression of apelin mRNA and protein. Singh et al. [33] demonstrated that increased glucose concentrations suppress vascular protection via angiopoietin-1, but not the expression or phosphorylation of Tie2, and producing these effects may require subtle regulation of the expression levels of apelin and the abovementioned factors.

Furthermore, we demonstrated that apigenin induces the expression of the Tie2 gene. Thus, apigenin may be influencing the expression of apelin by regulating the induction of Tie2. The details of the mechanism of its actions are unknown, but apigenin may regulate angiogenesis by modulating apelin expression. Kidoya et al. [31] showed that apelin is involved in the regulation of blood vessel caliber size during angiogenesis. Apelin gene expression is up-regulated by hypoxia, which agrees with its role in the control of vascular supply, i.e., glucose supply [34]. Thus, during hypoxia, blood vessels increase in size, and the induction of apelin expression may participate in this process. Apelin expression is a pivotal defense mechanism for counteracting hypoxia and may also be essential for plaque regression. High glucose conditions [35] and reoxygenation after hypoxia [36] are strong inducers of oxidative stress. Numerous studies have shown that FGF promotes angiogenesis as well as VEGF expression [37]. FGF2 is a chemotactic factor for endothelial cells that facilitates endothelial cell differentiation *in vitro* [38]. FGF2 has high affinity for FGFR2; however, FGF2 is not necessarily the *in vivo* effector of all of the wide range of angiogenic activities that it has been found to carry out *in vitro*. It has been shown that infarct size, vessel formation and blood perfusion are improved when FGFR is over-expressed after myocardial infarction [39].

Apigenin significantly increased the gene expression levels of apelin. Apigenin also reversed the reductions in apelin expression induced by H/R in human endothelial cells. These results suggest that apigenin subtly affects the expression of apelin. In human endothelial cells, H/R stimuli induce tube formation and angiogenesis [40,41]. This angiogenesis is induced by the signaling of ROS generated simultaneously with H/R [5,42]. For example, the exposure of microvascular endothelial cells to H_2O_2 promotes tube formation and angiogenesis [43]. We indicated that the apigenin increased apelin expression by H/R at HG. A report demonstrated that the potent antioxidant effects of apigenin protect the artery from oxidative stress [44]. In response to the signal for angiogenesis through the ROS induced by H/R, apigenin may have modified ROS stimuli through the antioxidant effects. On the other hand, apigenin may influence apelin protein and gene expression. Apigenin exerts an inhibitory effect on choroidal angiogenesis in laser-induced experimental choroidal neovascularization [45]. More recently, we showed that apigenin strongly inhibited the expression of vascular cell adhesion molecule-1, IkappaB kinase (IKK) alpha and IKK epsilon/IKKi and suppressed the adhesion of U937 cells and human endothelial cells [22]. Apigenin may subtly regulate this pathway via the phosphorylation of IKK as a means of controlling the gene expression of VEGFR. VEGF proteins use the Flk-1/Cbl/Akt pathway to achieve IKK activation in vascular endothelial cells [46]. In chronic lymphocytic leukemia cells, dietary polyphenol epigallocatechin-3-gallate suppressed VEGFR1 and VEGFR2 phosphorylation, albeit incompletely [47]. From these findings, we suggest that plant components are able to regulate the expression of genes related to vascular angiogenesis and maturity [43,48]. In this study, we found that apigenin induced the expression of apelin in human endothelial cells, and it might be able to influence the expression of other angiogenic proteins. Therefore, the dietary flavonoid apigenin might modulate the expression of angiogenic genes [49]. In addition, the antioxidative effects of apigenin subtly regulate gene expression and might be involved in the control of angiogenesis [12].

In summary, we examined the expression of apelin mRNA and protein in angiogenic conditions such as HG plus TNFα, and H/R, as well as the regulatory effects of the dietary flavonoid apigenin. In particular, stimulation with HG plus TNFα or H/R changed the expression of apelin compared with the control conditions for a short period. Furthermore, we demonstrated that apigenin reverses the reductions in the expression levels of apelin and FATP1 induced by H/R in human endothelial cells. These findings suggest that the early stages of TNFα and H/R subtly influence apelin angiogenesis and that HG stimulates vascular rearrangement. In addition, the dietary flavonoid apigenin might modulate the expression of apelin and fatty acid transport.

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