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ORIGINAL ARTICLE

Telmisartan-enhanced hypercholesterolaemic serum-induced vascular endothelial growth factor expression in immortalized human umbilical vascular endothelial cells

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Abstract

Objective. To clarify whether hypercholesterolaemia can increase vascular endothelial growth factor (VEGF) expression in human umbilical vascular endothelial cells (HUVECs) through the phosphatidylinositol 3-kinase (PI3K) pathway, and whether a special angiotensin II receptor blocker, telmisartan, can attenuate VEGF expression induced by hypercholesterolaemia. ***Methods***. Levels of VEGF expression, PI3K activity and angiogenesis *in vitro* were determined by various methods after HUVECs were incubated with hypercholesterolaemic serum or combined with telmisartan and/or wortmannin. ***Results***. We found that hypercholesterolaemic serum (cholesterol $\geqslant$0.08 mmol/L) can increase VEGF expression in HUVECs and that telmisartan cooperates with hypercholesterolaemic serum in promoting VEGF expression. The increased VEGF expression was associated with enhanced PI3K activity and could be significantly inhibited by wortmannin, a potent PI3K inhibitor. Likewise, hypercholesterolaemic serum significantly promoted angiogenesis *in vitro*, which could be inhibited when PI3K activity was suppressed. ***Conclusions***. Our study suggests that hypercholesterolaemic serum induces VEGF expression through PI3K in HUVECs and that telmisartan cooperates with hypercholesterolaemia in promoting VEGF expression.

Key Words: Endothelium, hypercholesterolaemia, PI3-kinase, telmisartan, VEGF

Introduction

Hypercholesterolaemia, a major risk factor for atherosclerosis [1], can cause vascular inflammation through increasing oxidative stress injury, activating platelets and leucocytes, and promoting cytokine expression. Recent studies suggest that vascular endothelial growth factor (VEGF) may be involved in hypercholesterolaemia-induced atherosclerosis [2]. In several *in vivo* studies it has been found that hypercholesterolaemia can significantly

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upregulate VEGF in serum and endothelial cells [1,3], but it is still unknown how hypercholesterolaemia upregulates the expression of VEGF. Phosphatidylinositol 3-kinase (PI3K) regulates VEGF expression and has an important role in angiogenesis [4]. Thus far, there has been no research on telmisartan, which antagonizes both the angiotensin II receptor blocker (ARB) and peroxisome proliferator-activated receptor-gamma (PPAR-gamma) in hypercholesterolaemia-induced dysfunction. Activation of PPAR-gamma has anti-inflammatory and anti-oxidative effects [5], and anti-atherogenic actions of AT1-receptor blockade, independently of antihypertensive or antilipidaemic effects, have also been reported [6].

Therefore, based on the conditionally immortalized human umbilical vascular endothelial cells (HUVECs), we investigated the effect of hypercholesterolaemic serum on VEGF expression in HUVECs, its signal pathway and the role of telmisartan in the process. We found that hypercholesterolaemic serum could induce VEGF expression in immortalized HUVECs via the PI3K pathway. Telmisartan did not reduce the hypercholesterolaemic serum-induced VEGF expression, but instead interacted with hypercholesterolaemic serum promoting VEGF expression.

Material and methods

Animal and reagents

New Zealand white male rabbits (~2 kg) were purchased from the Experimental Animal Center, Zhejiang Chinese Traditional Medical College. RPMI-1640 was obtained from Genom BioMed Technology Inc. (China); fetal bovine serum (FBS) from Hangzhou Sijiqing Biological Engineering Materials Co., Ltd. (China); wortmannin from Sigma-Aldrich Co. (USA); telmisartan from the HiSun Pharmaceutical Co. (China); and VEGF antibody from the Boster Bioengineering Co. (China). PI3K, β-actin antibodies and ECL (enhanced luminar) were purchased from Santa Cruz Biotechnology (USA). Biotinylated secondary antibodies and avidin/streptavidin-HRP conjugates were obtained from Maixin-Bio Co. (China); matrigel from BD Biosciences (USA) and the VEGF ELISA kit was bought from Jingmei Biotech Co. (China).

Hypercholesterolaemic serum preparation

Rabbits were fed a 1 % high cholesterol diet for 4 weeks or normal control. The peripheral blood of five rabbits was collected via the ear vein. Serum was isolated by centrifugation and mixed well for treating cultured cells. Total serum cholesterol was measured automatically using an enzymatic timed end-point method. Briefly, serum specimens were diluted 1:100 with reagents. The cholesterol esters were hydrolysed by the enzyme cholesterol esterase to yield free cholesterol and fatty acids. The free cholesterol was oxidized to cholesten-3-one and hydrogen pyroxide by cholesterol oxidase. Peroxidase then catalysed the reaction of hydrogen pyroxide with 4-aminoantipyrine and phenol to produce a coloured quinoneimine (Trinder's reaction). The change in absorbence at 520 nm is proportional to the cholesterol concentration in the specimen. The test was repeated three times.

VEGF ELISA assay

Serum from normal and hypercholesterolaemic rabbits was used for VEGF ELISA detection and the assay was done in accordance with the manufacturer's instructions. One

hundred microlitres of different concentrations of standards and samples was added to the appropriate wells and the mixture was incubated for 2 h at room temperature. The wells were washed four times with TBS-T buffer (pH 7.4, 0.02 mol/L and 0.1 % Tween-20). One hundred microlitres of biotinylated anti-VEGF (Biotin Conjugate) solution was added to each well and the mixture was incubated for 1 h at room temperature. The wells were washed four times with TBS-T buffer. One hundred microlitres of streptavidin-HRP working solution was added to each well and the mixture was incubated for 30 min at room temperature. The wells were washed four times with TBS-T buffer. One hundred microlitres of stabilized chromogen was added to each well and the mixture was incubated for 30 min at room temperature and in the dark. One hundred microlitres of stop solution was added to each well. The absorbance of each well was read at 450 nm. Detection of VEGF in culture supernants was performed as above, and the procedure was repeated three times for each sample.

HUVEC culture and treatment

HUVECs were prepared as previously reported [7]. Cells were cultured in RPMI-1640 medium containing 20 % FBS and passaged every 2–3 days until 60–70 % confluence was achieved. The cells were washed with PBS and replaced by serum-free medium before testing. Cultured HUVECs were divided as follows: serum-free medium control, normal rabbit serum, hypercholesterolaemic serum of various cholesterol concentrations and combinations with wortmannin and/or telmisartan.

Western blot analysis

Aliquots of cell lysates containing 50 μg of proteins were separated using 11 % SDS-polyacrylamide gel and transferred to nitrocellulose membrane filters. The filters were blocked with TBS-T buffer containing 20 % skimmed milk, then incubated with a rabbit polyclonal antibody to human VEGF (1:400) for 2 h at room temperature, followed by the addition of biotinylated secondary antibody (1:25), avidin/streptavidin-HRP conjugates (1:25) and ECL visualization of the bands. All tests analysed by Western blot were repeated three times. VEGF expression was described using the software Quantity-one.

PI3K activity assay

PI3K activity assay was performed as described previously [8]. Briefly, cells were lysed in 137 mM NaCl, 20 mM Tris, pH 7.5, 1 mM MgC l2,10 % glycerol, 1 % Triton X-100, 10 mM phenylmethylsulfonyl fluoride and 10 mM each of leupeptin, aprotinin and soybean trypsin inhibitor. Detergent lysates were immunoprecipitated with the p85-PI3K antibody for 3 h; the beads were then washed twice with lysis buffer and three times with Tris-HCl (pH 7.4). Subsequently, the precipitated immunocomplex was prepared by drying with nitrogen and resuspended in 10 mL of 30 mM HEPES. This was added to wash the beads and the tube was left on ice for 10 min. Forty microlitres of kinase buffer (30 mM HEPES, 30 mM MgCl2, 50 mM ATP, 200 mM adenosine and 10 mCi [g-32P]ATP) was then added to each tube and the reaction was allowed to proceed at room temperature for 15 min. The reaction was stopped with 0.1 N HCl and the lipids extracted with 200 mL of chloroform/methanol (1:1). The products were separated on potassium oxalate pretreated TLC plates by developing with chloroform, methanol, water and 30 % ammonium hydroxide (112:88:19:6, by vol). After drying, the plates were exposed to

autoradiography and the phosphorylated products quantified by excising the spot and scintillation counting (count per minute). Every sample was assayed three times.

Angiogenesis assay in vitro

Angiogenesis assay was performed as described by Lee [9]; 250 μL growth factor-reduced Matrigel (10 mg protein/mL) was pipetted into each well of a 24-multiwell culture plate and polymerized for 30 min at 37°C. HUVECs that were incubated in RPMI-1640 with 20 % FBS overnight were harvested after trypsin treatment, resuspended in RPMI-1640 containing 1 % FBS, loaded onto a layer of Matrigel at a density of 1×10^5 cells/mL, followed by the addition of various combinations of hypercholesterolaemic serum (cholesterol 0.32 mmol/L), telmisartan (1×10^{-5} mol/L) and wortmannin (100 nmol/L). Serum-free medium was used as control. Matrigel cultures were incubated at 37°C for up to 24 h, then photographed. Control or test reagent was assayed in duplicate.

Statistical analysis

The data were expressed as mean ± standard deviation. Results were analysed by one-way ANOVA. Student's *t*-test was used to compare cholesterol level of rabbit serum. $P \leqslant 0.05$ was considered to be statistically significant.

Results

Serum cholesterol level after a high-cholesterol diet

After 4 weeks of cholesterol diet containing 1 % cholesterol, there was a significant increase in serum cholesterol compared with controls (33.9 ± 9.4 versus 1.3 ± 0.6 mmol/L; $p < 0.01$) (Figure 1), indicating that the diet-induced hypercholesterolaemic model had been successfully established.

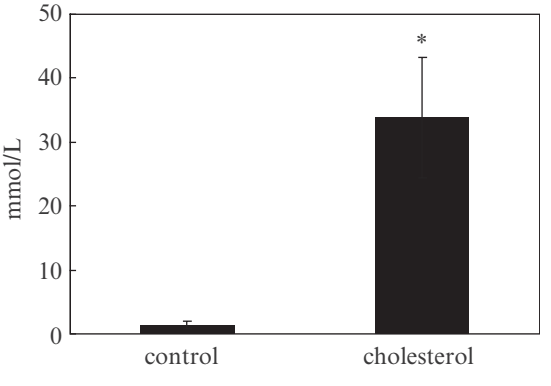

Figure 1. A 1 % cholesterol diet increased serum cholesterol level (33.9 ± 9.4 versus 1.3 ± 0.6 mmol/L; $p < 0.01$). *$p < 0.05$ versus control.

Hypercholesterolaemic serum-induced VEGF expression in HUVEC

In order to exclude the influence of VEGF in rabbit serum, we first determined the VEGF levels in normal and hypercholesterolaemic rabbit serum using ELISA. The content of VEGF was less than the lowest quantity of standard curve (31.25 pg/mL OD450: 0.214, 31.25–2500 pg/mL) and similar between hypercholesterolaemic and normal serum (OD450: 0.093 ± 0.010 versus 0.0100 ± 0.009; $p > 0.05$).

After incubation of HUVECs in serum-free medium control, normal rabbit serum and hypercholesterolaemic serum at different cholesterol concentrations (0.04, 0.08, 0.16 and 0.32 mmol/L) for 24 h, VEGF protein expression in the cell lysates was determined by Western blot analysis, as well as VEGF in culture medium by ELISA. We observed that VEGF expression in HUVECs treated with hypercholesterolaemic serum (cholesterol concentration $\geqslant 0.08$ mmol/L) was significantly higher than that treated with serum-free medium control, normal rabbit serum or hypercholesterolaemic serum with cholesterol concentration < 0.08 mmol/L (Figure 2). To explore the mechanisms by which hypercholesterolaemia led to increased VEGF expression in HUVECs, we investigated the role of PI3K and found that PI3K activity increased with hypercholesterolaemic serum (cholesterol 0.32 mmol/L). Treated with the PI3K specific inhibitor wortmannin (100 nmol/L) for 24 h, hypercholesterolaemic serum-induced VEGF expression was attenuated so that it was no longer significantly different from control (Figure 3). The PI3K activity assay revealed that PI3K activity was positively correlated with VEGF expression, and that wortmannin (100 nmol/L) could significantly inhibit PI3K activity induced by hypercholesterolaemic serum (Figure 4).

Telmisartan enhanced hypercholesterolaemic serum-induced VEGF expression

To determine the role of telmisartan on hypercholesterolaemic serum-induced VEGF expression, we exposed HUVECs to telmisartan (1×10^{-5}–1×10^{-8} mol/L) and found that it had no effect on VEGF expression. While HUVECs were incubated with telmisartan at different concentrations (1×10^{-5}–1×10^{-8} mol/L) in the presence of hypercholesterolaemic serum (cholesterol 0.32 mmol/L), VEGF expression was significantly increased compared with that caused by hypercholesterolaemic serum alone, especially when the concentration of telmisartan was $> 1 \times 10^{-7}$ mol/L (Figure 5). Simultaneously, PI3k activity in HUVECs incubated with both telmisartan and hypercholesterolaemic serum was significantly higher than that induced by hypercholesterolaemic serum alone (Figure 4); and VEGF expression induced by telmisartan combined with hypercholesterolaemic serum could be attenuated by wortmannin (Figure 6), which suggested that the synergistic effect of telmisartan on VEGF expression induced by hypercholesterolaemic serum was at least partly through the PI3K signalling pathway.

Telmisartan enhances hypercholesterolaemic serum-induced angiogenesis in vitro

Formation of tubular structures by endothelial cells is a critical step in angiogenesis. VEGF is a known potent angiogenic factor. Therefore, to determine whether VEGF induced by hypercholesterolaemic serum or combined with telmisartan possessed bioactivity, we investigated the formation of tube-like structures by HUVECs *in vitro*. When placed on matrigel matrix in the presence of hypercholesterolaemic serum (cholesterol 0.32 mmol/L) for 24 h, HUVECs formed incomplete and narrow tube-like structures (Figure 7). It could be observed that in the presence of hypercholesterolaemic serum plus telmisartan (1×10^{-5} mol/L), the formation of tube-like structures was more evident than that of

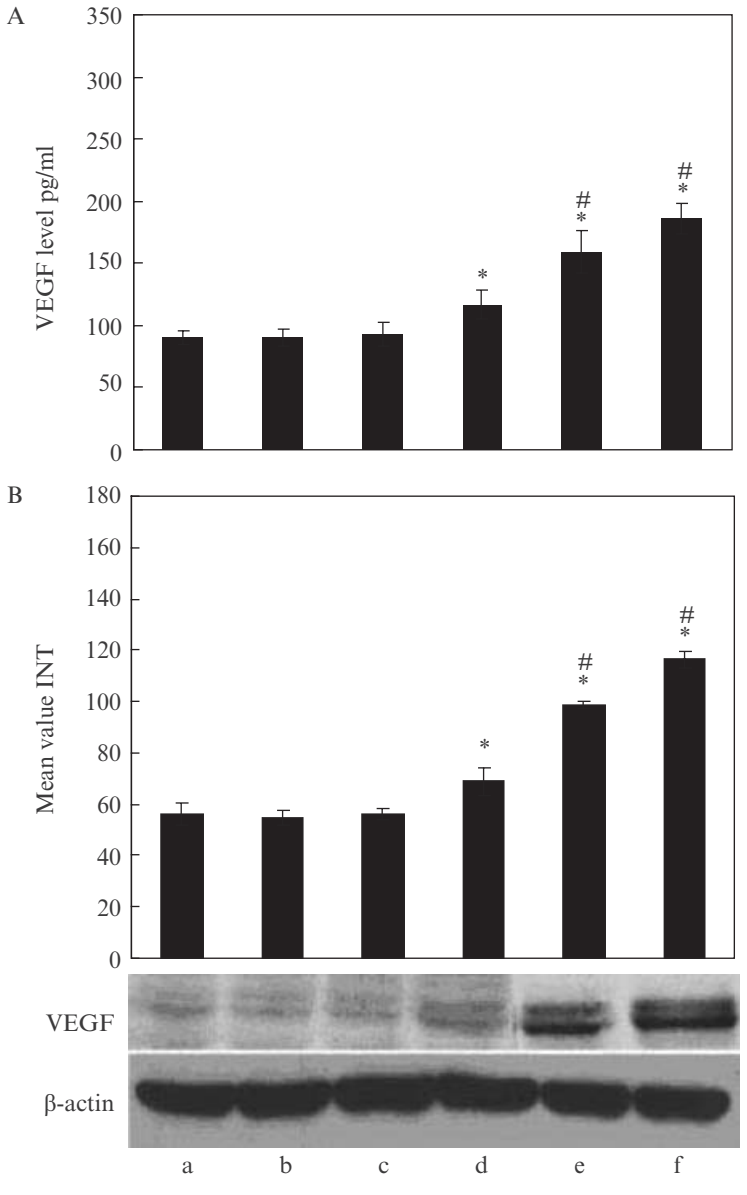

Figure 2. Hypercholesterolaemic serum induced VEGF expression. A. (VEGF in culture supernants, pg/mL): (a) serum-free medium control (90.0 ± 5.1), (b) normal rabbit serum (90.3 ± 7.3), (c–f) hypercholesterolaemic serum at different cholesterol concentrations (93.2 ± 9.2, 116.7 ± 12.1, 159.2.7 ± 16.6, 186.0 ± 12.4). B. (VEGF in cultured HUVECs cell lysates): (a) serum-free medium control (56.3 ± 4.2), (b) normal rabbit serum (54.7 ± 2.9), (c–f) hypercholesterolaemic serum at different cholesterol concentrations (56.0 ± 2.0, 69.0 ± 5.3, 98.7 ± 1.5, 116.3 ± 3.2). $^{*}p < 0.05$ versus serum-free medium control. $^{\#}p < 0.05$ versus previous group.

hypercholesterolaemic serum alone (Figure 7). The addition of telmisartan (1×10^{-5} mol/L) to HUVECs in the absence of hypercholesterolaemic serum, tube-like structures could not be seen (Figure 7). Wortmannin (100 nmol/L) significantly suppressed the formation of tube-like structures induced by hypercholesterolaemic serum or combined with telmisartan (Figure 7).

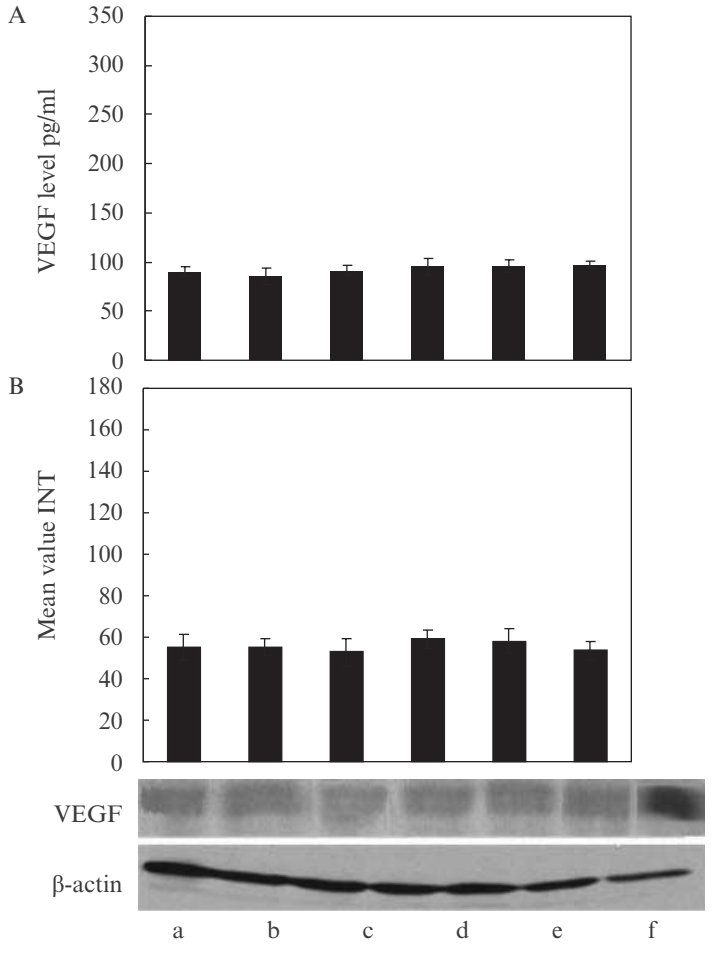

Figure 3. Wortmannin (100 nmol/L) attenuated the expression of VEGF induced by hypercholesterolaemia through PI3K inhibition. A. (VEGF in culture supernants, pg/mL): (a) serum-free medium control (90.0±5.1), (b) wortmannin (85.3±8.5), (c–f) hypercholesterolaemic serum at different concentrations combined with wortmannin (91.5±5.1, 95.1±8.3, 95.9±7.0, 96.7±3.6). B. (VEGF in cultured HUVEC cell lysates): (a) serum-free medium control (55.2±6.4), (b) wortmannin (55.3±4.1), (c–f) hypercholesterolaemic serum at different cholesterol concentrations combined with wortmannin (52.8±6.5, 59.0±4.4, 58.2±5.6, 53.5±4.7), (g) positive control.

Discussion

Hypercholesterolaemia is closely related with the function of vascular endothelial cells [10]. It can reduce endothelial nitric oxide synthase expression [11], increase oxidative stress of endothelial cells, stimulate the expression of adhesion molecules [12], increase vascular permeability and lead to atherosclerosis [13]. Researchers have suggested that VEGFs have an important role in atherosclerosis induced by hypercholesterolaemia [2]. VEGF is the major cytokine responsible for physiological or pathological angiogenesis through inducing chemotaxis and proliferation of vascular endothelial cells [14] and influences the permeability of blood vessels by influencing the expression or arrangement of VE-cadherin and occluding [15,16]. It is of great significance in the treatment of ischaemic diseases [17].

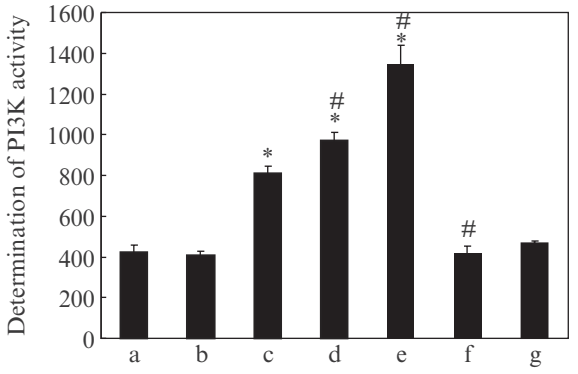

Figure 4. Wortmannin (100 nmol/L) can significantly inhibit PI3K activity induced by hypercholesterolaemia alone or in combination with telmisartan. (a) Serum-free medium control (423.2 ± 33.4), (b) wortmannin plus 1×10^{-5} mol/L telmisartan (406.2 ± 26.8), (c) 1×10^{-5} mol/L telmisartan (808.6 ± 37.5), (d) hypercholesterolaemic serum with 0.32 mmol/L cholesterol (970.9 ± 43.1), (e) hypercholesterolaemic serum combined with 1×10^{-5} mol/L telmisartan (1350.6 ± 88.5), (f) hypercholesterolaemic serum combined with wortmannin (420.2 ± 32.7), (g) hypercholesterolaemic serum combined with telmisartan and wortmannin (466.2 ± 14.7). $^{*}p < 0.05$ versus serum-free medium control. $^{\#}p < 0.05$ versus previous group.

At the same time, VEGF can promote the development of inflammation and atherosclerosis [18]. To elucidate whether hypercholesterolaemic serum can induce VEGF expression in HUVECs *in vitro*, hypercholesterolaemic serum was added to cultured HUVECs. It was found that hypercholesterolaemic serum significantly increases VEGF expression, which was consistent with previous studies [1]. However, the mechanism by which hypercholesterolaemia induces VEGF expression in vascular endothelial cells is still unclear. PI3K signalling pathway is important in pathological angiogenesis [19]. Recent studies have found that increased VEGF expression induced by various factors is associated with increased PI3K activity, and specific inhibitors to PI3K can reduce VEGF expression, both of which suggest that PI3K signalling is a major pathway in the regulation of VEGF expression [20]. In the present study, the upregulation of VEGF in HUVECs exposed to hypercholesterolaemic serum was associated with increased PI3K activity. It has been reported that VEGF can increase PI3K activity [21], although in our study there was no significant difference of VEGF between normal and hypercholesterolaemic rabbit serum. Only when incubated with hypercholesterolaemic serum was the expression of VEGF protein and activity of PI3K markedly elevated in HUVECs; moreover, the increase in VEGF protein expression by hypercholesterolaemic serum or combined with telmisartan was significantly inhibited after the addition of wortmannin, suggesting that the PI3K signalling pathway was involved in upregulation of VEGF caused by hypercholesterolaemic serum in HUVECs.

Telmisartan is a special ARB antagonizing both ARB and PPAR-gamma [22]. In addition to producing vasoconstriction, angiotensin II (Ang II) can induce the expression of inflammatory factors, cell growth and proliferation, and play an important part in the formation of atherosclerotic plaque and thrombosis [23,24]. ARB has an anti-inflammatory effect and can inhibit neovascularization through VEGF [25]. Tamarat et al. reported that ARB valsartan could completely prevent an Ang II angiogenic effect in Ang II-treated rats [26]. In the study by Murakami et al., circulating VEGF level, and its production by peripheral blood mononuclear cells, was elevated during the course of AMI, and early administration of ACEI and ARB candesartan did not affect the levels [27]. However, there

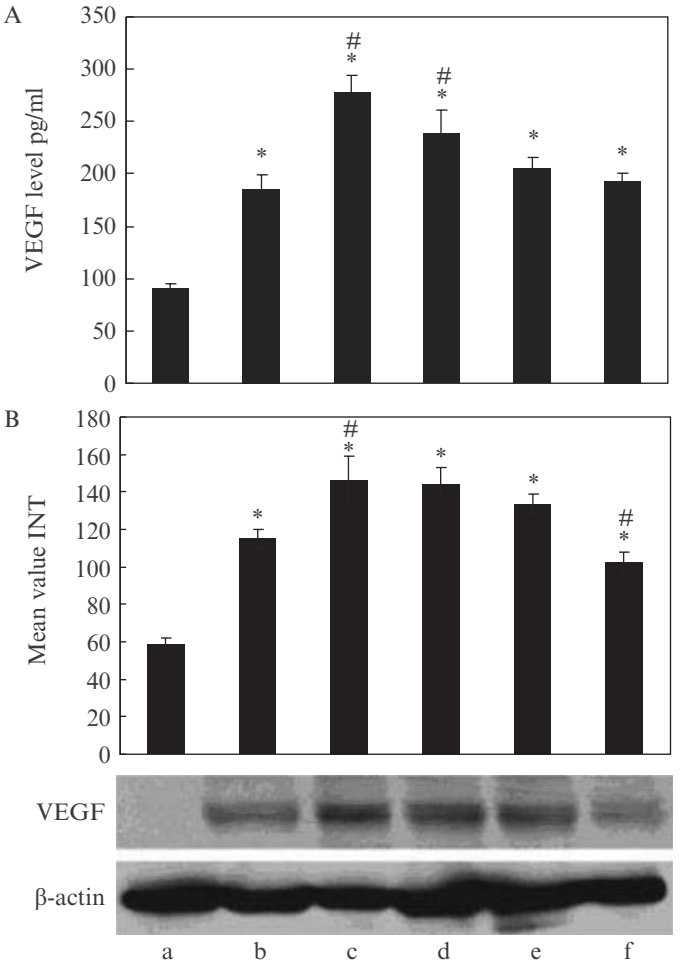

Figure 5. Telmisartan enhanced hypercholesterolaemia-induced VEGF expression. A. (VEGF in culture supernants, pg/mL): (a) serum-free medium control (90.0 ± 5.1), (b) hypercholesterolaemic serum$^{\Delta}$ (186.0 ± 12.4), (c–f) hypercholesterolaemic serum$^{\Delta}$ combined telmisartan at different concentrations (1×10^{-5}–1×10^{-8} mol/L) (278.2 ± 15.6, 238.6 ± 21.7, 205.2 ± 10.0, 192.8 ± 8.8). B. (VEGF in cultured HUVEC cell lysates): (a) serum-free medium control (58.7 ± 3.5), (b) hypercholesterolaemic serum$^{\Delta}$ (110.2 ± 5.0), (c–f) hypercholesterolaemic serum$^{\Delta}$ combined telmisartan at different concentrations (1×10^{-5}–1×10^{-8} mol/L) (146.0 ± 12.8, 144.3 ± 8.9, 133.3 ± 5.5, 102.5 ± 5.3). $^{\star}p < 0.05$ versus serum-free medium control. $^{\#}p < 0.05$ versus previous group. $^{\Delta}$Cholesterol level=0.32 mmol/L.

is no report of ARB increasing expression of VEGF, which makes us believe that increasing expression of VEGF in the present study may not be related to the Ang II-related pathway. In our study, we found that telmisartan could not reduce the hypercholesterolaemia-induced increase in VEGF expression in HUVECs. Instead, it further increased the hypercholesterolaemia-induced increase in VEGF expression. After wortmannin treatment, the telmisartan-induced and hypercholesterolaemia-induced increase in VEGF expression were significantly reduced. The above effect of telmisartan was confirmed by determining PI3K activity and angiogenesis levels, indicating that telmisartan acted with hypercholesterolaemia in promoting VEGF expression in HUVECs via the PI3K pathway. However, telmisartan alone had no effect on VEGF expression in HUVECs.

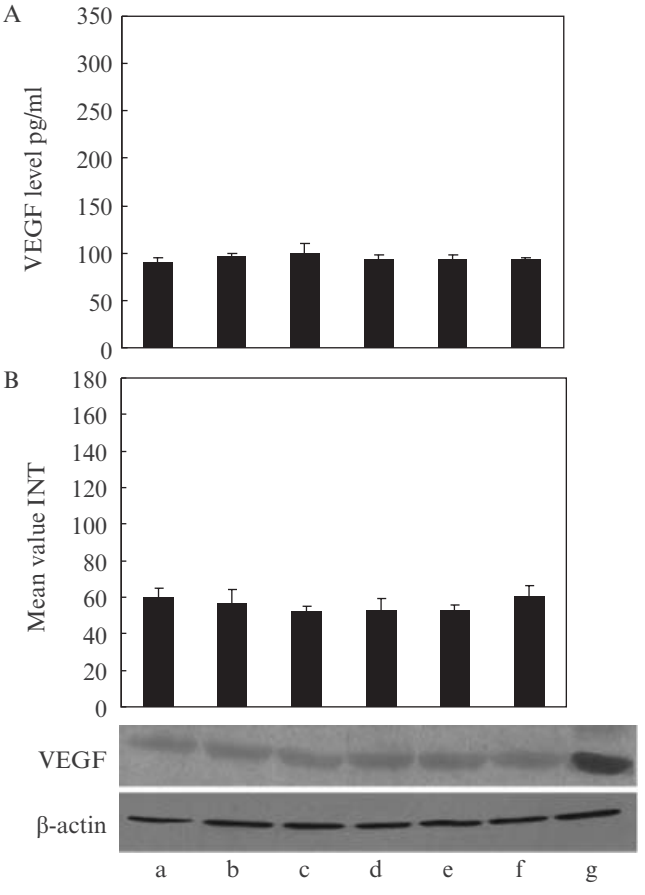

Figure 6. Wortmannin (100 nmol/L) attenuated VEGF expression induced by telmisartan combined with hypercholesterolaemia serum. A. (VEGF in culture supernants, pg/mL): (a) serum-free medium control (90.0±5.1), (b) hypercholesterolaemic serum$^{\Delta}$ combined with 100 nmol/L wortmannin (96.7±3.6), (c–f) hypercholesterolaemic serum$^{\Delta}$ combined with 100 nmol/L wortmannin and telmisartan at different concentrations (1×10^{-5}–1×10^{-8} mol/L) (100.1±9.8, 93.7±4.9, 92.9±5.7, 93.1±2.3). B. (VEGF in cultured HUVEC cell lysates): (a) Serum-free medium control (60.2±4.6), (b) hypercholesterolaemic serum$^{\Delta}$ combined with 100 nmol/L wortmannin (56.8±7.5), (c–f) hypercholesterolaemic serum$^{\Delta}$ combined with 100 nmol/L wortmannin and telmisartan at different concentrations (1×10^{-5}–1×10^{-8} mol/L) (53.3±3.0, 54.7±6.4, 53.9±3.1, 61.0±5.1), (g) positive control. $^{\Delta}$cholesterol level=0.32 mmol/L.

On the other hand, telmisartan can antagonize PPAR-gamma, which exerts its regulatory action through anti-inflammatory, anti-oxidative and anti-proliferative mechanisms [5] and reduces the expression of many pathogenic genes for atherosclerosis [28]. Mutations in PPAR-gamma results in severe metabolic disturbances [29], indicating that PPAR-gamma is important in the regulation of pathologic changes induced by hypercholesterolaemia. Up to now, the effects of PPAR-r on VEGF expression contradict. Kanata et al. reported that the PAAR-r antagonist GW9662 significantly suppressed VEGF mRNA expression by Ox-LDL [30]. Yang et al. reported that the PPAR-r agonist GI26257 increased VEGF basal secretion from adipocytes *in vitro* [31]. Nagai et al. demonstrated that the administration of GW9662 did not reverse the suppressive effect of telmisartan on VEGF production [32]. Therefore, the synergistic effect of telmisartan on the increase in expression of VEGF in HUVECS induced by hypercholesterolaemic serum might be the consequence of complex

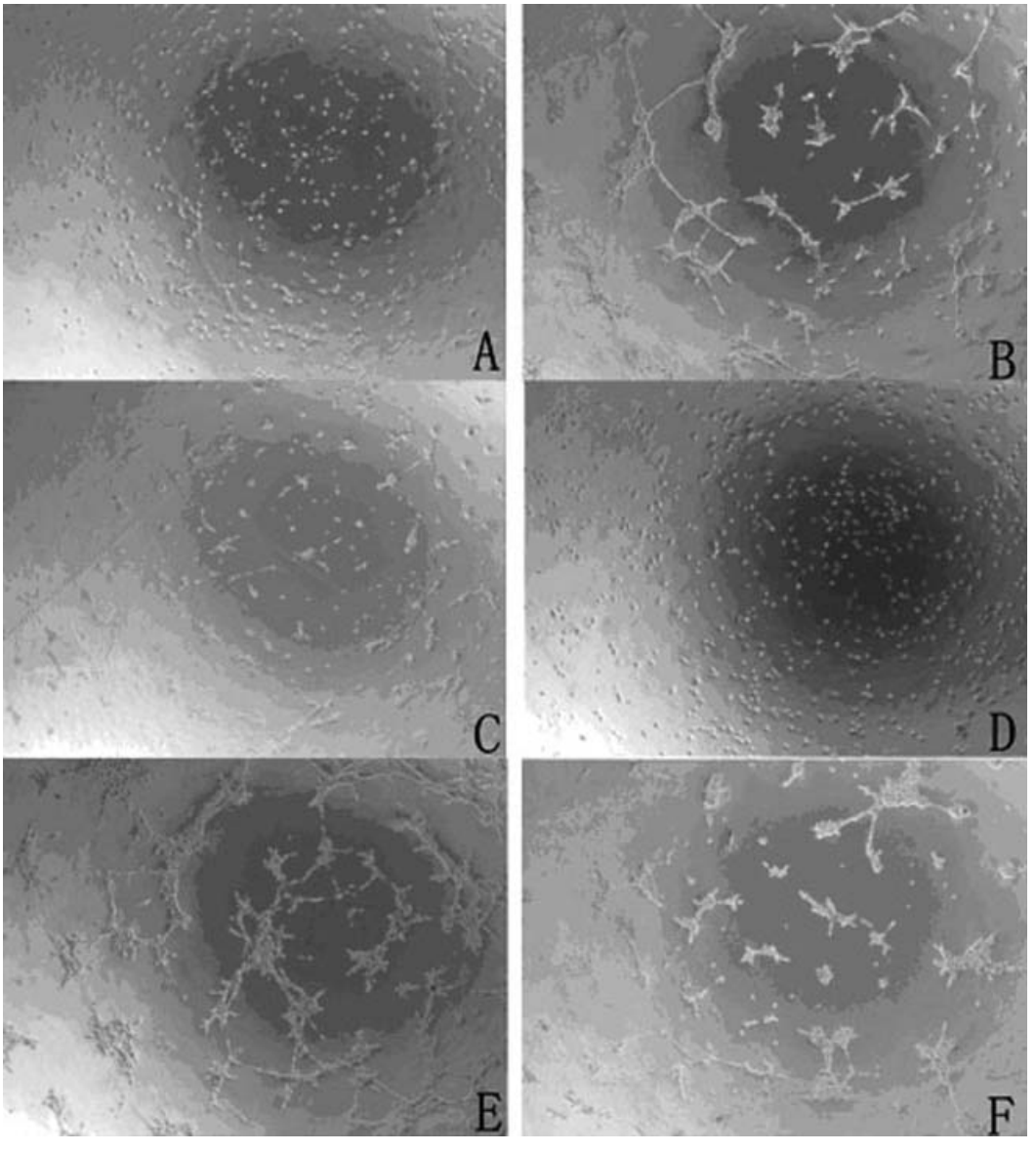

Figure 7. Telmisartan enhanced hypercholesterolaemia-induced angiogenesis *in vitro*. A. Serum-free medium control. B. Hypercholesterolaemic serum with 0.32 mmol/L cholesterol. C. 1×10^{-5} mol/L telmisartan. D. 100 nmol/L wortmannin. E. Hypercholesterolaemic serum combined telmisartan F. Hypercholesterolaemic serum combined with wortmannin and telmisartan.

molecular interplay relating to various cell types and pathophysiologic conditions. In our study, we could not clarify the mechanism concerning how telmisartan cooperates with hypercholesterolaemia in increasing VEGF expression in HUVECs. Further study is therefore required.

In summary, hypercholesterolaemia can induce VEGF expression in HUVECs via the PI3K pathway, and telmisartan in conjunction with hypercholesterolaemic serum can further increase VEGF expression.

Acknowledgements

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