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RESEARCH ARTICLE | *Signaling and Stress Response*

Effect of melatonin on EGF- and VEGF-induced monolayer permeability of HUVECs

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Yang L, Zhang Y, Ma Y, Du J, Gu L, Zheng L, Zhang X. Effect of melatonin on EGF- and VEGF-induced monolayer permeability of HUVECs. *Am J Physiol Heart Circ Physiol* 316: H1178–H1191, 2019. First published December 21, 2018; doi:10.1152/ajpheart.00542.2018.—Melatonin is a natural hormone involved in the regulation of circadian rhythm, immunity, and cardiovascular function. In the present study, we focused on the mechanism of melatonin in the regulation of vascular permeability. We found that melatonin could inhibit both VEGF- and EGF-induced monolayer permeability of human umbilical vein endothelial cells (HUVECs) and change the tyrosine phosphorylation of vascular-endothelial (VE-)cadherin, which was related to endothelial barrier function. In addition, phospho-AKT (Ser^{473}) and phospho-ERK(1/2) played significant roles in the regulation of VE-cadherin phosphorylation. Both the phosphatidylinositol 3-kinase/AKT inhibitor LY49002 and MEK/ERK inhibitor U0126 could inhibit the permeability of HUVECs, but with different effects on tyrosine phosphorylation of VE-cadherin. Melatonin can influence the two growth factor-induced phosphorylation of AKT (Ser^{473}) but not ERK(1/2). Our results show that melatonin can inhibit growth factor-induced monolayer permeability of HUVECs by influencing the phosphorylation of AKT and VE-cadherin. Melatonin can be a potential treatment for diseases associated with abnormal vascular permeability.

NEW & NOTEWORTHY We found that melatonin could inhibit both EGF- and VEGF-induced monolayer permeability of human umbilical vein endothelial cells, which is related to phosphorylation of vascular-endothelial cadherin. Blockade of phosphatidylinositol 3-kinase/AKT and MEK/ERK pathways could inhibit the permeability of human umbilical vein endothelial cells, and phosphorylation of AKT (Ser^{473}) might be a critical event in the changing of monolayer permeability and likely has cross-talk with the MEK/ERK pathway.

AKT; epidermal growth factor; growth factors; human umbilical vein endothelial cells; melatonin; permeability; vascular-endothelial cadherin; vascular endothelial growth factor

INTRODUCTION

Permeability is an important determinant of vascular function, which is determined by transendothelial channels and tight junctions of vascular endothelial cells (ECs) (15). As the familiar structure between ECs, the tight junction is regulated by extracellular stimuli such as hypoxia, cytokines, nutrients, and immune cells (11, 30, 40, 44). Increasing tight junction permeability can weaken vascular endothelial barrier function, which contributes to many types of vascular disease (38, 58, 64).

Vascular-endothelial (VE-)cadherin is the major tight junction protein in vascular ECs and is required to maintain the vascular endothelial barrier (48, 59). The regulation of VE-cadherin expression typically involves transcription and posttranslational modification. Posttranslational modification usually comprises ubiquitination and phosphorylation (42, 53, 57). The phosphorylation of VE-cadherin has been investigated extensively and is thought to affect endothelial permeability (16). When vascular ECs are stimulated by particular cytokines and inflammatory factors, VE-cadherin is phosphorylated and will lead to a change in permeability. Therefore, VEGF could induce VE-cadherin tyrosine phosphorylation, which, in turn, mediates increased vascular permeability (16, 19). In addition, EGF has been indicated to contribute to the permeability of ECs and vascular tube formation, in particular, inducing endothelial cadherin tyrosine phosphorylation (6, 13, 60, 68). Furthermore, both EGF and VEGF belong to the class of plasma growth factors; the expression of EGF and VEGF or their receptors is closely associated with atherosclerotic or other vascular diseases (2, 47, 72). It has also been reported that VEGF could upregulate the expression of EGF receptors (EGFRs); many inhibitors could block both EGFRs and VEGF receptors (VEGFRs), and there appears to be cross-talk between EGFR and VEGFR pathways (24, 31, 36, 41, 51, 65). Therefore, we used both VEGF and EGF to stimulate human umbilical vein ECs (HUVECs) to investigate their effect on VE-cadherin phosphorylation to explain the potential mechanism of increased permeability of vascular diseases.

Recently, there has been an increase in research interest in melatonin maintaining homeostasis in the human body. Lack of melatonin might lead to many diseases such as circadian rhythm sleep disorders as well as neurodegenera-

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tive and cardiovascular diseases (17, 49, 55, 75). Our research focused on the effect of melatonin on cardiovascular disease. It has been previously reported that melatonin takes part in the regulation of epithelial cell permeability, such as the blood-brain barrier, intestinal epithelium, and aortic epithelium. However, the molecular mechanism of this regulation of vascular endothelial permeability by melatonin is still unclear (34, 56, 61, 70). In the present investigation, we established a relationship among melatonin, EGF, and VEGF, as they both regulated phosphorylation of the same target protein (VE-cadherin), which contributes to vascular endothelial permeability.

METHODS

Cell and plasmids. HUVECs were obtained from the American Type Culture Collection (Rockville, MD) and cultured in DMEM (high glucose, Hyclone, ThermoFisher Scientific, Waltham, MA) supplemented with 10% (vol/vol) FBS (Hyclone) and antibiotics (100 U/ml streptomycin and 100 μg/ml penicillin, Invitrogen) in a humidified incubator at 37°C with 5% CO_2. The ERK2(WT) plasmid and ERK2(TAYF) plasmid

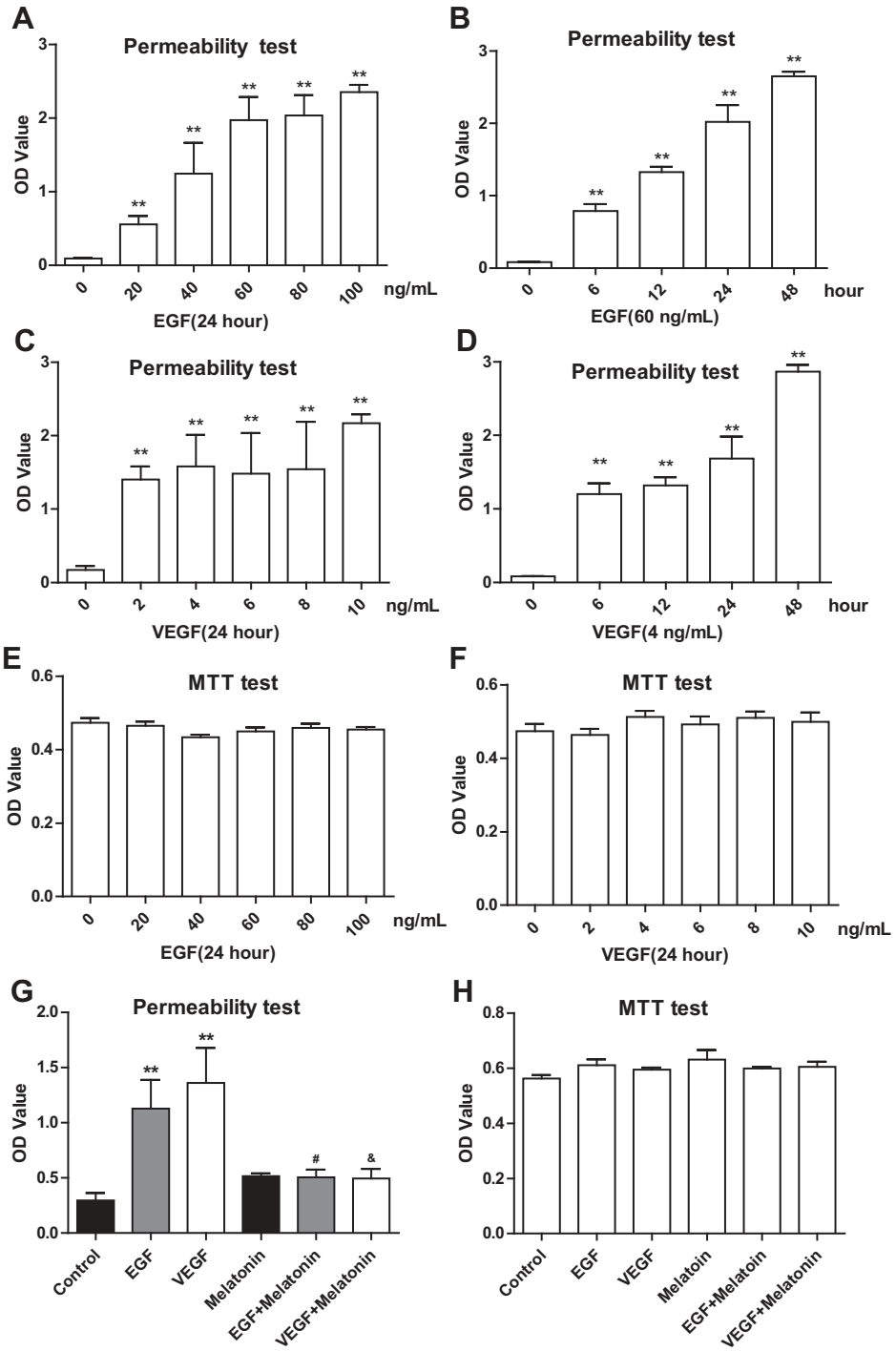


Fig. 1. Melatonin inhibits EGF- and VEGF-induced monolayer permeability of human umbilical vein endothelial cells (HUVECs). *A* and *C*: permeability tests were performed after HUVECs were incubated with various concentrations of EGF and VEGF for 24 h. *B* and *D*: HUVECs were incubated with 60 ng/ml EGF or 4 ng/ml VEGF, and permeability tests were performed after the indicted times. *E* and *F*: HUVECs were cultured in EGF or VEGF at different concentrations for 24 h, and cell proliferation was analyzed by the MTT assay. *G* and *H*: effect of melatonin on growth factor-induced monolayer permeability and cell proliferation of HUVECs. After a 24-h stimulation of melatonin and growth factors, permeability tests and MTT assays were performed. Repeated-measures ANOVA and Student's *t*-test were used to analyze the effect of EGF, VEGF, and melatonin on the permeability of HUVECs: $P = 0.012$ (*A*), $P = 0.002$ (*B*), $P = 0.011$ (*C*), $P = 0.005$ (*D*), $P = 0.013$ (*G*); $^{*}P < 0.05$; $^{**}P < 0.01$ compared with control; $^{\#}P < 0.05$ compared with EGF; $^{\&}P < 0.05$ compared with VEGF. $n \geq 3$. p, Phosphorylated.

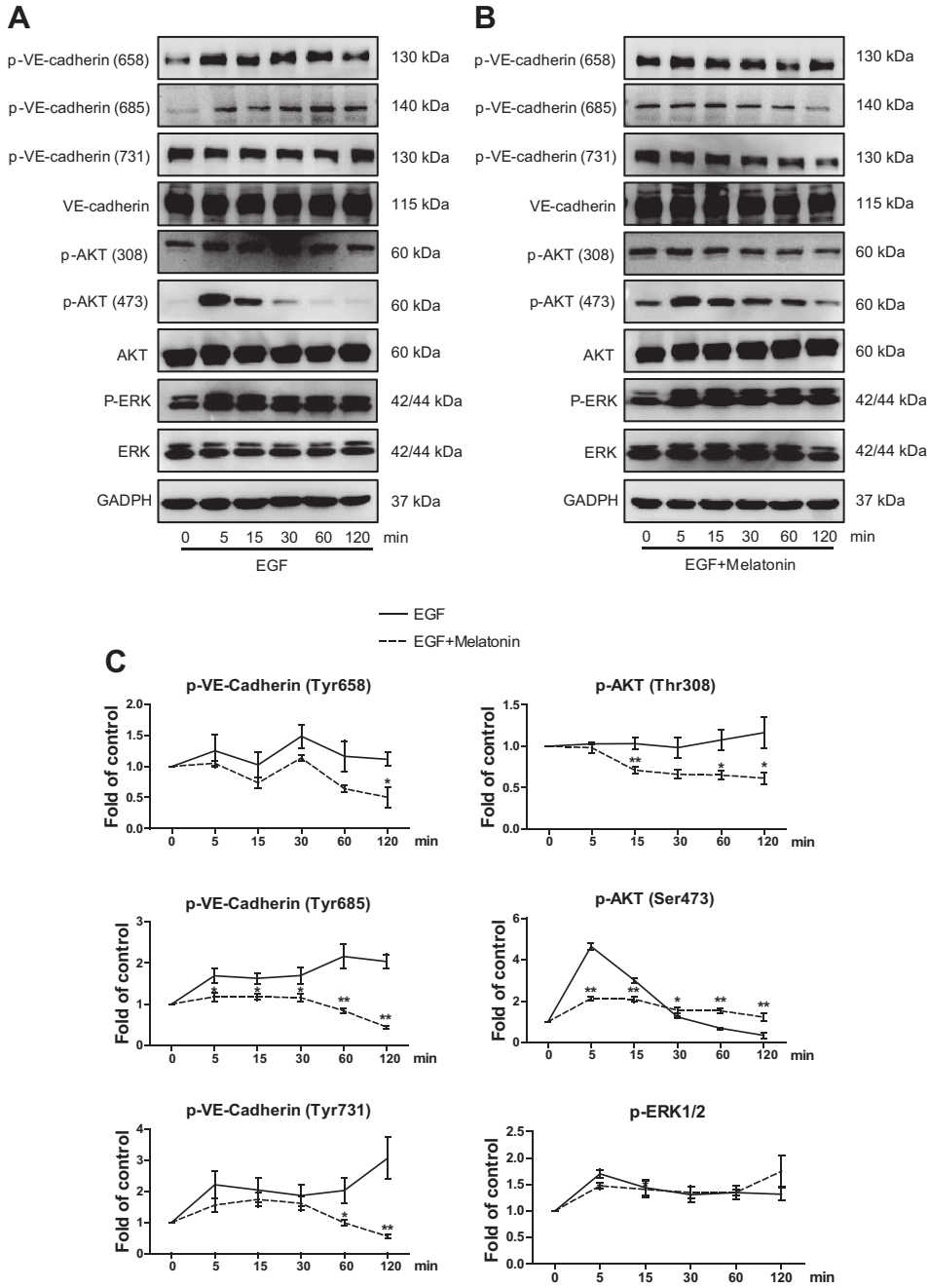


Fig. 2. Effect of melatonin on EGF-induced AKT, ERK, and vascular-endothelial (VE-)cadherin phosphorylation. *A*, *B*, *D*, and *E*: Western blot analysis of VE-cadherin (Tyr^{658}, Tyr^{685}, and Tyr^{731}), AKT (Thr^{308} and Ser^{473}), and ERK(1/2) phosphorylation in human umbilical vein endothelial cells treated with EGF and melatonin within 120 min or 48 h. *C* and *F*: ImageJ software was used to perform densitometry analyses of Western blot results. Repeated-measures or standard two-way ANOVA was used to analyze the effect of melatonin on the phosphorylation of VE-cadherin (Tyr^{658}, Tyr^{685}, and Tyr^{731}), AKT (Thr^{308} and Ser^{473}), and ERK(1/2) compared with EGF: $P = 0.091$, $P = 0.001$, $P = 0.077$, $P = 0.020$, $P = 0.038$, and $P = 0.749$ (*A* and *B*); $P = 0.000$, $P = 0.000$, $P = 0.015$, $P = 0.018$, $P = 0.015$, and $P = 0.026$ (*C* and *D*); $^{*}P < 0.05$; $^{**}P < 0.01$. $n = 4$. p, Phosphorylated.

were constructed as previously described (62), and the empty pEGFP-N1 vector was obtained from Clontech Laboratories (Palo Alto, CA). Cells were seeded in six-well plates, cultured to 80–90% confluence, and then transiently transfected with those plasmids using FuGENE HD Transfection Reagent (Promega, Madison, WI) in serum-free OPTI-MEM according to the manufacturer's instructions.

MTT assay. HUVECs were seeded at a density of 8×10^3 cells/well into a 96-well plate and made quiescent by serum starvation for 12 h. Cells were then treated with EGF (R&D Systems, Minneapolis, MN), VEGF (R&D Systems), and melatonin (Sigma-Aldrich, St. Louis, MO) for the indicated times, and cell proliferation was measured by the MTT assay as previously described (73).

Permeability assay. HUVECs (2×10^5 cells) were seeded in 150 μl medium on the membrane of each 3-μm Millicell Hanging Cell Culture insert (Millipore, Bedford, MA), and the 24-well plates were filled with 600 μl medium. After HUVECs were fully confluent (to ensure the formation of cell-cell junctions and a good endothelial barrier), EGF, VEGF, melatonin, 10 μM LY49002 (Sigma), or 10 μM U0126 (Alexis, Lausen, Switzerland) were added to the medium according to the following different combinations of treatment: control, EGF, VEGF, control + melatonin, EGF + melatonin, VEGF + melatonin, control + LY49002, EGF + LY49002, VEGF + LY49002, control + U0126, EGF + U0126, and VEGF + U0126. Horseradish peroxidase was added to the medium of the top chambers at the indicated times, and 20 μl of media from the lower chamber were transferred to a new 96-well plate after incubation for 30 min. The media reacted with TMB substrate (CWBio, Beijing, China), and the optical density (OD) value of 450 nm was measured by a microplate reader according to the manufacturer's instructions. After aspirating the medium of the

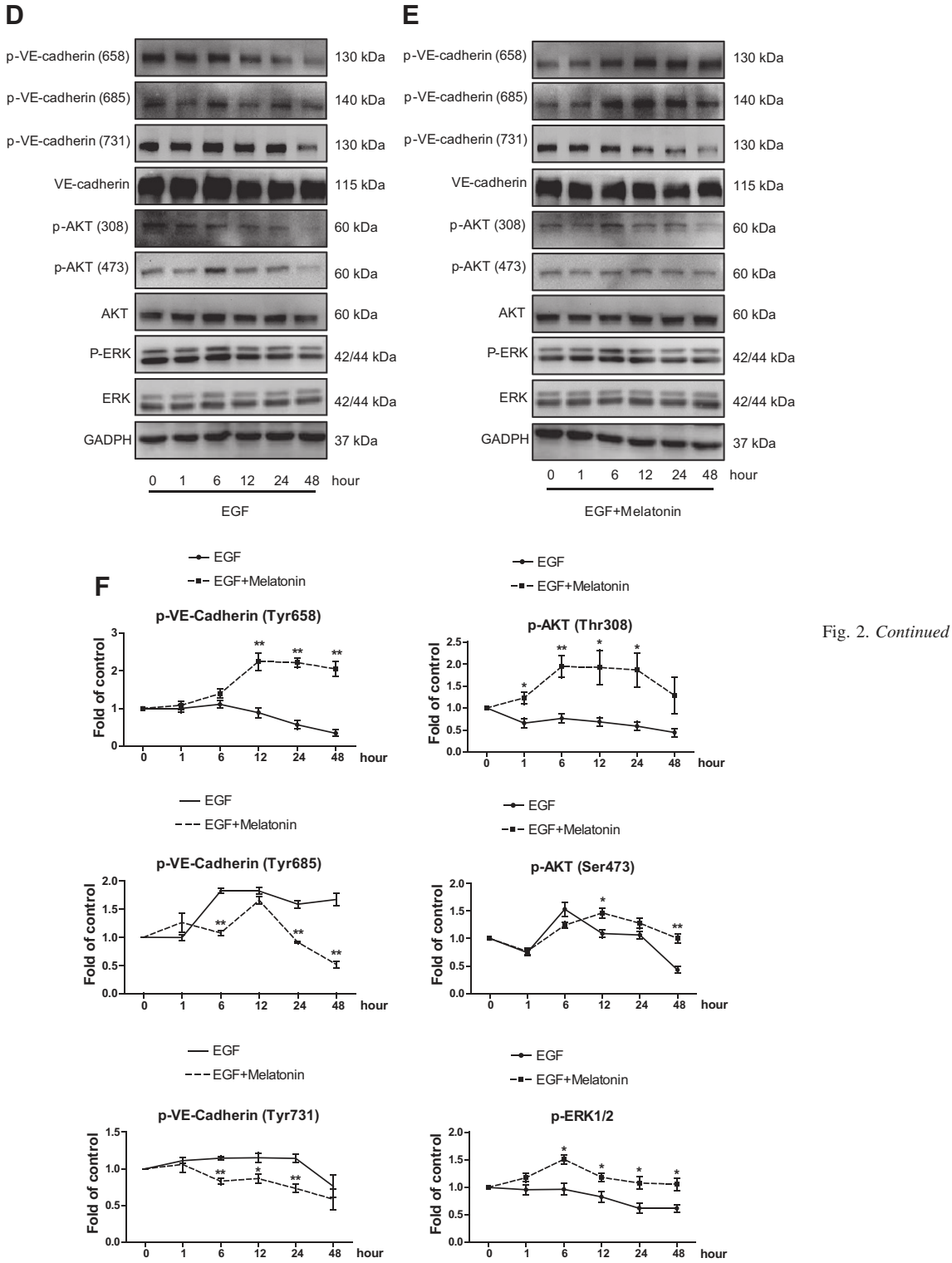


Fig. 2. *Continued*

inserts, the cell monolayers were fixed with 4% polyoxymethylene and stained with 0.1% crystal violet. A phase-contrast microscope was used to take images, and ImageJ software was used to measure the intercellular space volume. The data of the OD value and intercellular space volume were analyzed to reflect the change in cell permeability.

Western blot analysis. Sample protein extraction and concentration determination of whole cells were performed as previously described (18). Briefly, equal amounts of protein were run on SDS-polyacrylamide gels and transferred to a nitrocellulose membrane. The resulting blots were blocked with 5% nonfat dry milk and probed with antibodies. The following antibodies were used: GAPDH, ERK, phospho-ERK, EGFR (Cell Signaling Technology, Danvers, MA), VE-cadherin, phospho-VE-cadherin (Tyr^{685}, Abcam, Cambridge, MA), phospho-VE-cadherin (Tyr^{731}), phospho-VE-cadherin (Tyr^{658}, ThermoFisher Scientific), and VEGFR2 (Santa Cruz Biotechnology). Protein bands were detected by

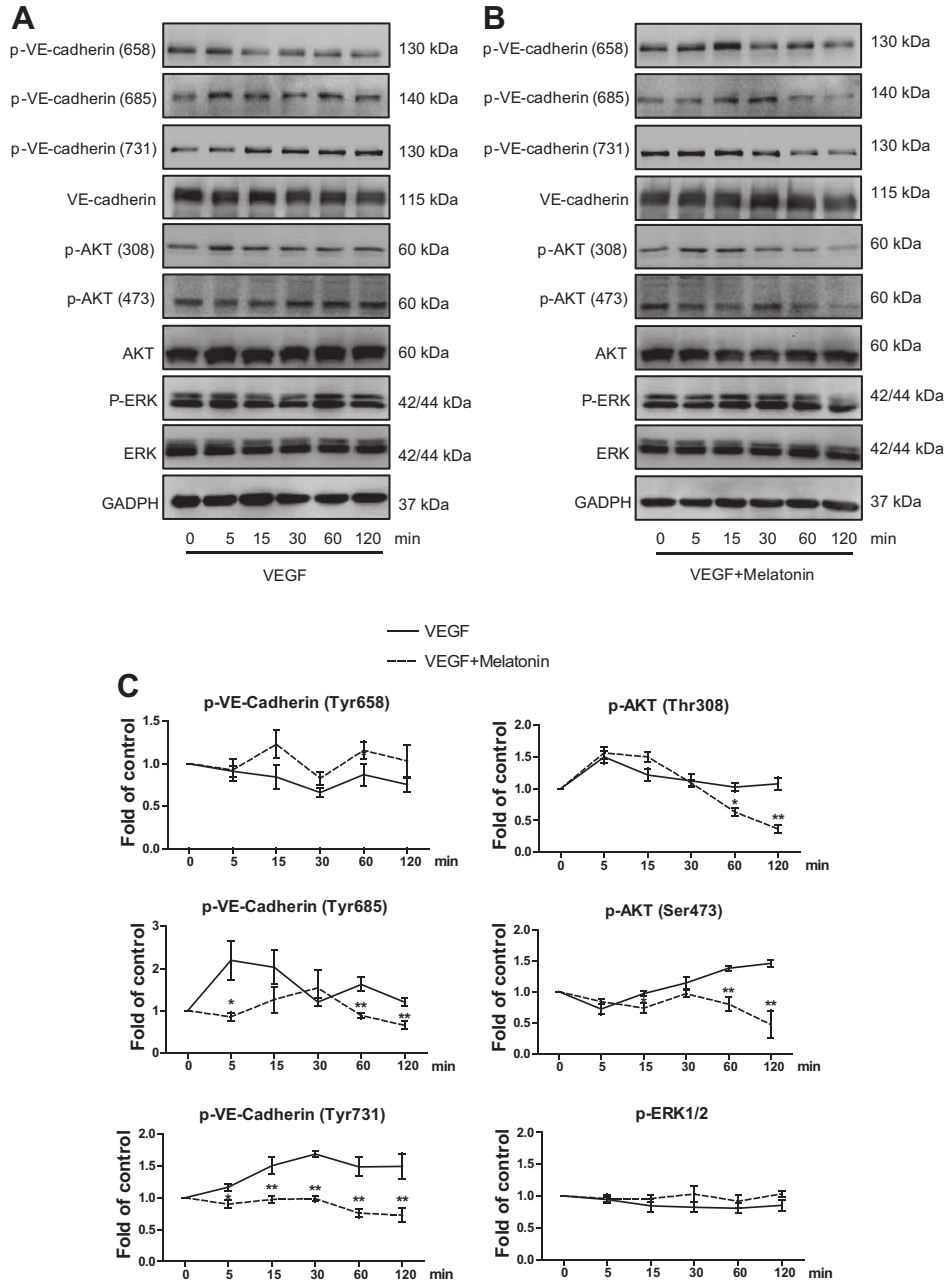


Fig. 3. Effect of melatonin on VEGF-induced AKT, ERK, and vascular-endothelial (VE-)cadherin phosphorylation. *A*, *B*, *D*, and *E*: Western blot analysis of VE-cadherin (Tyr^{658}, Tyr^{685}, and Tyr^{731}), AKT (Thr^{308} and Ser^{473}), and ERK(1/2) phosphorylation in human umbilical vein endothelial cells treated with VEGF and melatonin within 120 min or 48 h. *C* and *F*: ImageJ software was used to perform densitometry analysis of Western blot results. Repeated-measures or standard two-way ANOVA was used to analyze the effect of melatonin on the phosphorylation of VE-cadherin (Tyr^{658}, Tyr^{685}, and Tyr^{731}), AKT (Thr^{308} and Ser^{473}), and ERK(1/2) compared with VEGF: $P = 0.016$, $P = 0.065$, $P = 0.000$, $P = 0.172$, $P = 0.012$, and $P = 0.304$ (*A* and *B*); $P = 0.293$, $P = 0.000$, $P = 0.000$, $P = 0.672$, $P = 0.063$, and $P = 0.100$ (*C* and *D*); $^{*}P < 0.05$; $^{**}P < 0.01$. $n = 3$. p, Phosphorylated.

incubation with horseradish peroxidase-conjugated antibodies (Jackson ImmunoResearch, West Grove, PA) and visualized with ECL reagent (Millipore, Billerica, MA).

Quantitative RT-PCR. Total RNA was isolated with TRIzol reagent (ThermoFisher Scientific). Equal amounts of RNA (1 μg) from each sample were used for cDNA synthesis using HiScriptQ RT SuperMix for quantitative PCR (Vazyme, Nanjing, China). Quantitative RT-PCR was performed on the ABI StepOne Real-Time PCR System (Applied Biosystems, Foster City, CA) using HiScript Q RT SuperMix for quantitative PCR (Vazymea) and analyzed using StepOne software (v2.1, Applied Biosystems). The $2^{-\Delta C_T}$ method (where C_T is threshold cycle) was used to calculate gene expression levels. For the sample loading control, GAPDH was tested. Primers sequences were as follows: VEGFR2, forward 5′-GGCCCAATAATCAGAGTGGCA-3′ and reverse 5′-CCAGTGTCATTTCCGATCACTTT-3′; EGFR, forward 5′-AGGCACGAGTAACAAGCTCAC-3′ and reverse 5′-ATGAGGACATAACCAGCCACC-3′; and GAPDH, forward 5′-CATCAGCAATGCCTCCTGCAC-3′ and reverse 5′-TGAGTCCTTCCACGATACCAAAGTT-3′.

Statistical analysis. SPSS statistical software (version 19.0, SPSS, Chicago, IL) was used to perform all statistical analyses. Data were analyzed by repeated-measures or standard two-way ANOVA and a Student's *t*-test (32). *P* values of <0.05 were considered significant (two tailed). Data are presented as means ± SE.

RESULTS

Melatonin inhibits EGF- and VEGF-induced monolayer permeability of HUVECs. We found that the monolayer permeability of HUVECs was increased significantly with increasing doses of EGF or VEGF (Fig. 1, *A* and *C*), reaching peak permeability at 24−48 h (Fig. 1, *B* and *D*) with 60 ng/ml EGF or 4 ng/ml VEGF. To preclude the possibility that HUVEC

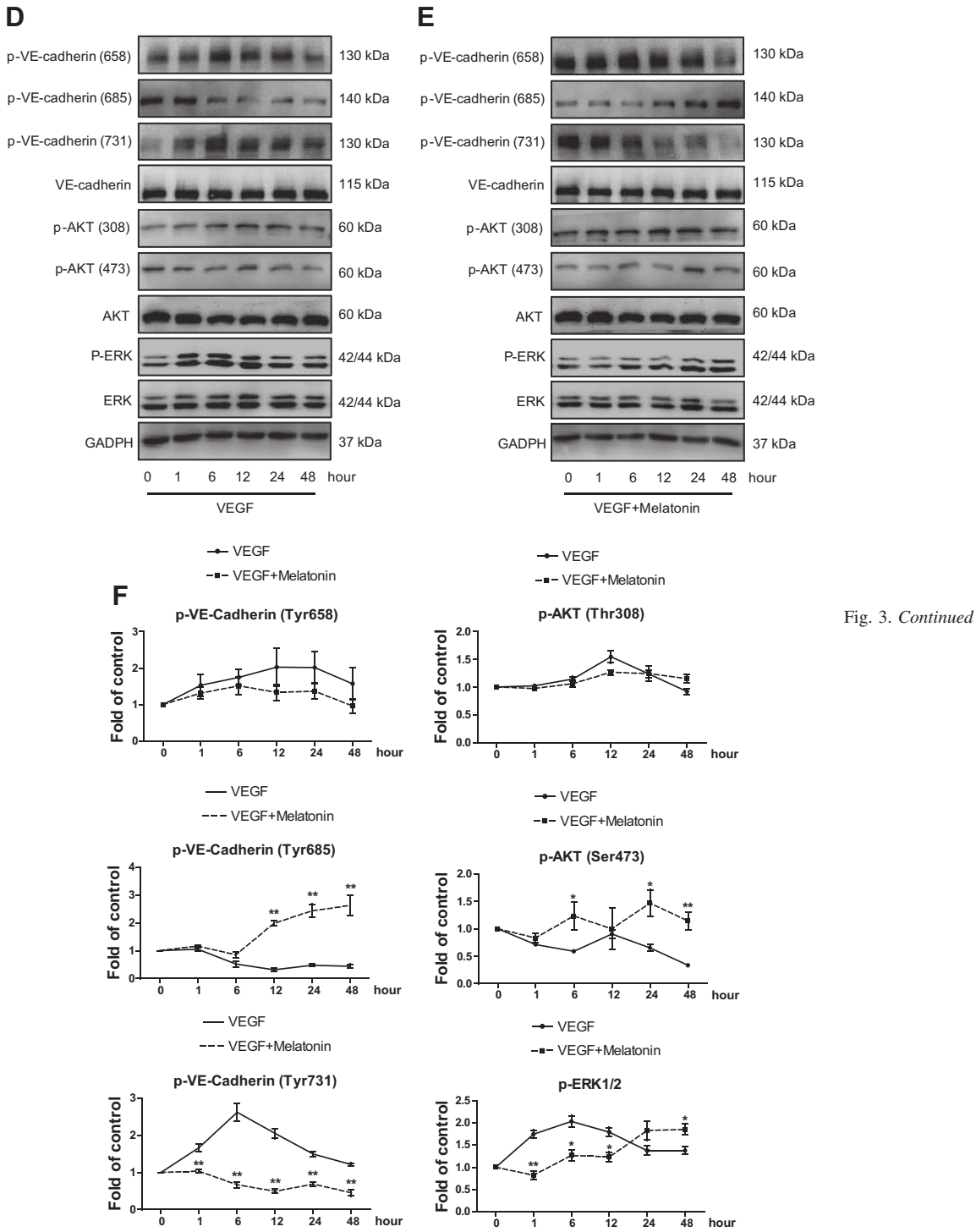


Fig. 3. *Continued*

proliferation would be associated with permeability, MTT tests were performed after cells were treated for 24 h. The results showed that stimulation of the two growth factors did not affect the proliferation of HUVECs within 24 h (Fig. 1, *E* and *F*). We then examined whether melatonin could affect the monolayer permeability of HUVECs induced by EGF and VEGF by stimulating HUVECs with 100 μM melatonin combined with EGF or VEGF. The permeability test results indicated that melatonin inhibited EGF- and VEGF-induced monolayer permeability of HUVECs (Fig. 1*G*), and proliferation of HUVECs was not affected by melatonin within 24 h (Fig. 1*H*). Taken together, these data suggest that melatonin could suppress the increasing monolayer permeability of HUVECs induced by EGF and VEGF.

Effect of EGF, VEGF, and melatonin on signal transduction of HUVECs. To investigate the mechanism of the two growth factors and melatonin affecting the permeability of HUVECs, we focused on the phosphatidylinositol 3-kinase (PI3K)/AKT

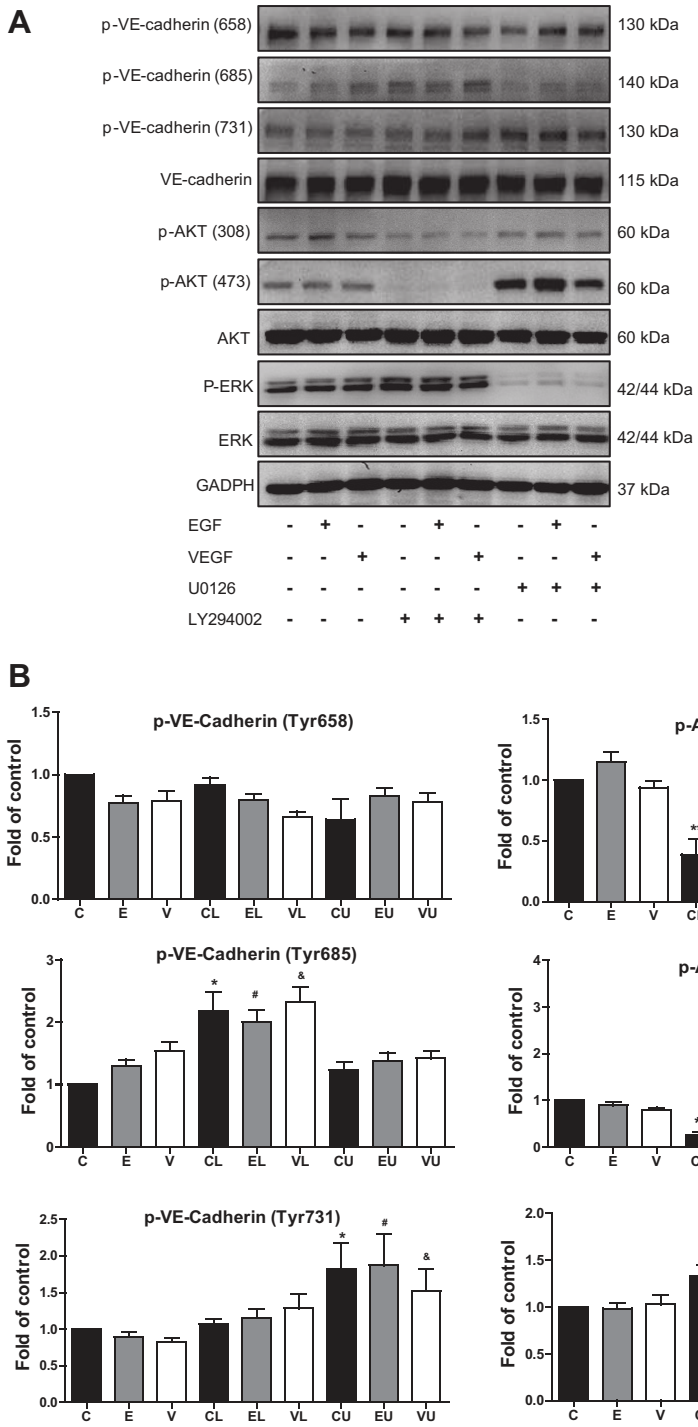
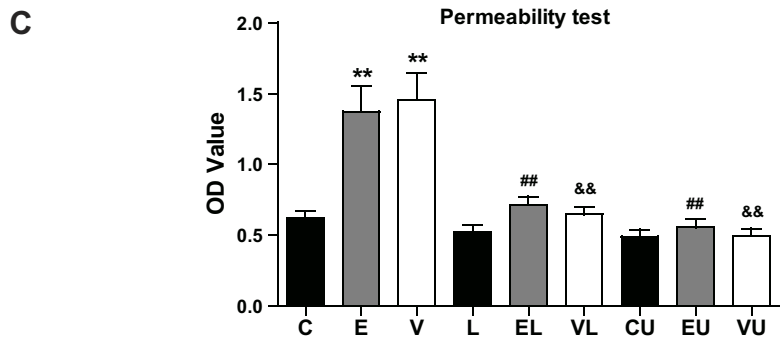


Fig. 4. Effect of the phosphatidylinositol 3-kinase/AKT inhibitor LY49002 and MEK/ERK inhibitor U0126 on growth factor-induced AKT, ERK, and vascular-endothelial (VE-)cadherin phosphorylation. *A*: VE-cadherin, AKT, and ERK phosphorylation was detected by Western blot analysis after human umbilical vein endothelial cells (HUVECs) were treated with EGF, VEGF, LY49002, and U0126 for 24 h. *B*: densitometry analyses of Western blot results. *C*: permeability tests were performed after HUVECs were incubated with growth factors and inhibitors for 24 h. *D*: a phase-contrast microscope was used to take HUVEC images, and ImageJ software was used to measure the areas of the gaps between them (red area, bar = 100 μM). C, control; E, EGF; V, VEGF; L, LY49002; U, U0126; CL, control + LY49002; EL, EGF + LY49002; VL, VEGF + LY49002; CU: control + U0126; EU, EGF + U0126; VU, VEGF + U0126. Repeated-measures or standard two-way ANOVA was used to analyze the effect of LY49002 and U0126 on the phosphorylation of VE-cadherin (Tyr^{658}, Tyr^{685}, and Tyr^{731}), AKT (Thr^{308} and Ser^{473}), and ERK(1/2): $P = 0.351$, $P = 0.006$, $P = 0.077$, $P = 0.000$, $P = 0.000$, and $P = 0.006$ (LY49002); $P = 0.287$, $P = 0.651$, $P = 0.043$, $P = 0.381$, $P = 0.001$, and $P = 0.000$ (U0126); $^{*}P < 0.05$; $^{**}P < 0.01$ compared with control; $^{\#}P < 0.05$; $^{\#\#}P < 0.01$ compared with EGF; $^{\&}P < 0.05$; $^{\&\&}P < 0.01$ compared with VEGF. $n = 5$. p, Phosphorylated.

and MEK/ERK pathways, which could be activated by growth factors, as well as phosphorylation of VE-cadherin related to the permeability detected by Western blot analysis. The results showed that EGF simultaneously activated both PI3K/AKT and MEK/ERK pathways and that phosphorylation of AKT (Ser^{473}) and ERK(1/2) rapidly increased and reached a peak at 5 min; VE-cadherin phosphorylation was also induced by EGF within 120 min, including Tyr^{658}, Tyr^{685}, and Tyr^{731} (Fig. 2*A*). We then tested the effect of melatonin on EGF-induced protein phosphorylation, and the results suggested that melatonin suppressed VE-cadherin (Tyr^{658}, Tyr^{685}, and Tyr^{731}) and AKT (Thr^{308}) phosphorylation compared with EGF but did not influence ERK phosphorylation. Interestingly, the duration of AKT (Ser^{473}) phosphorylation was extended but weakened compared with EGF (Fig. 2, *B* and *C*). To further determine whether melatonin inhibited EGF-induced protein phosphorylation in a time-dependent manner, we treated HUVECs with EGF and melatonin for a longer time (48 h). Melatonin increased phosphorylation of VE-cadherin (Tyr^{658}), AKT (Thr^{308} and Ser^{473}), and ERK(1/2) compared with EGF, and the inhi-



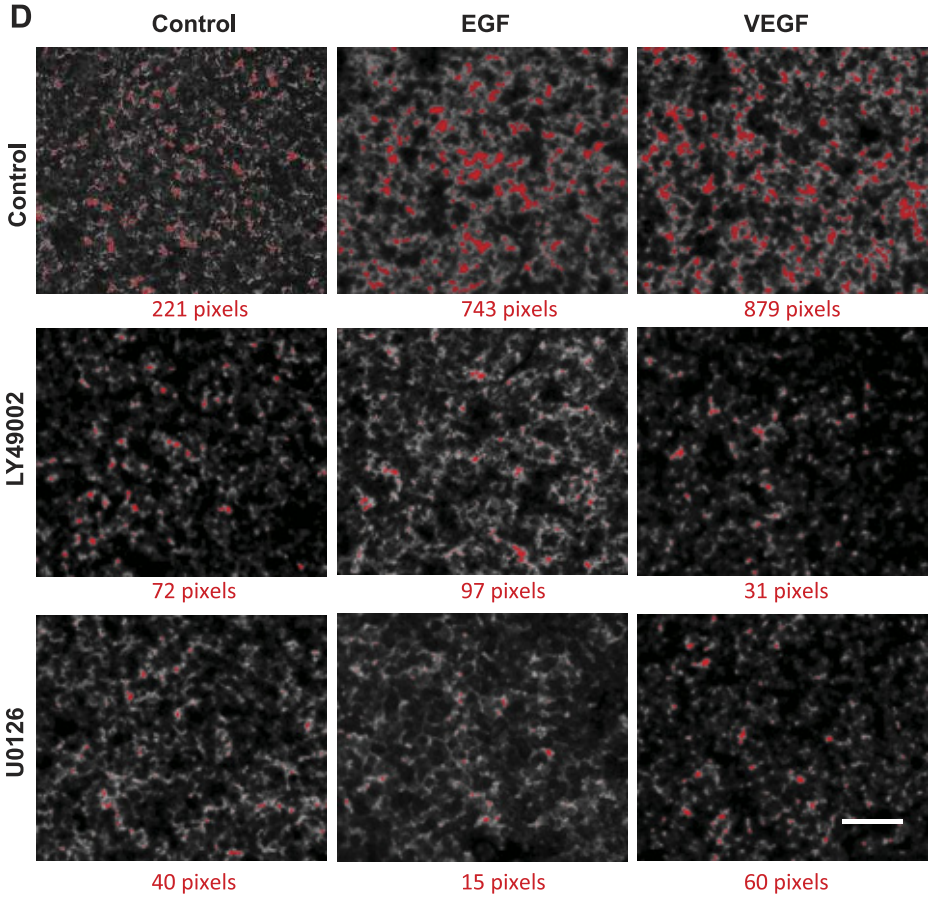


Fig. 4. *Continued*

bition of phosphorylation of VE-cadherin (Tyr^{685} and Tyr^{731}) continued for 48 h (Fig. 2, *D* and *E*). Taken together, these results demonstrate that melatonin disturbs EGF signal transduction, resulting in inhibition of VE-cadherin (Tyr^{685} and Tyr^{731}) phosphorylation, prolongation of AKT and ERK phosphorylation, and reversal of VE-cadherin (Tyr^{658}) phosphorylation.

We also investigated how melatonin influenced VEGF signal transduction. As shown in Fig. 3, *A–C*, melatonin inhibited VEGF-induced VE-cadherin (Tyr^{685} and Tyr^{731}) and AKT (Thr^{308} and Ser^{473}) phosphorylation but did not affect phospho-VE-cadherin (Tyr^{658}) or phospho-ERK within 120 min. Similarly, we treated HUVECs with VEGF and melatonin for 48 h, and the Western blot analysis showed differences with the EGF-treated group. Although melatonin suppressed VEGF-induced VE-cadherin (Tyr^{731}) phosphorylation equally to the EGF-treated group but enhanced phosphorylation of VE-cadherin (Tyr^{685}), melatonin also delayed the time to peak phosphorylation of phospho-AKT (Ser^{473}) and phospho-ERK(1/2) but had no correlation with VEGF-induced phospho-VE-cadherin (Tyr^{658}) or phospho-AKT (Thr^{308}; Fig. 3, *D* and *E*).

Melatonin could alter EGF and VEGF signaling by modulating phosphorylation of AKT, ERK, and VE-cadherin, which would then contribute to the monolayer permeability of HUVECs.

Both inhibition of PI3K/AKT and MEK/ERK signal pathways suppressed monolayer permeability of HUVECs. To evaluate the role of PI3K/AKT and MEK/ERK signal pathways in the regulation of HUVEC permeability, two inhibitors were applied under EGF or VEGF conditions (alone or in

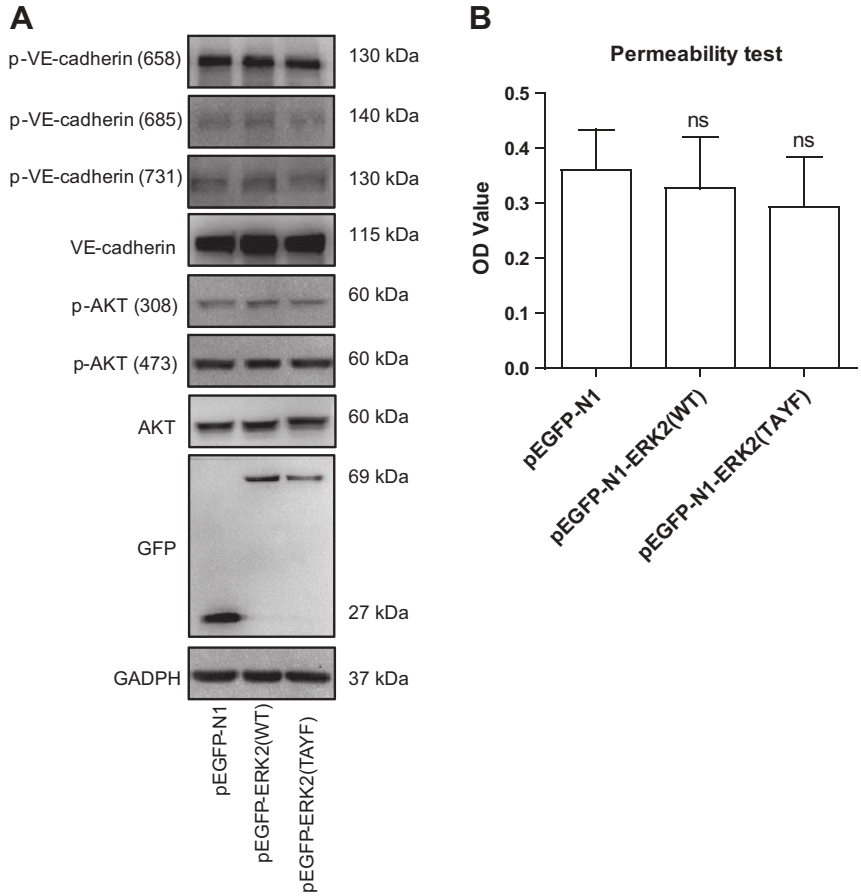


Fig. 5. Effect of wild-type and mutant ERK2 on AKT, ERK, vascular-endothelial (VE-)cadherin phosphorylation, and human umbilical vein endothelial cell (HUVEC) permeability. *A*: Western blot analysis of VE-cadherin (Tyr^{658}, Tyr^{685}, and Tyr^{731}), AKT (Thr^{308} and Ser^{473}), and ERK(1/2) phosphorylation in HUVECs transferred with ERK2 plasmid. *B*: permeability tests were performed after HUVECs were transferred with ERK2 plasmid. WT, wild-type sequence containing the nuclear location sequence; TAYF, phosphorylation site mutant containing the nuclear location sequence; ns, not significant; OD, optical density.

combination with other agents). After 24 h of treatment, the PI3K/AKT inhibitor LY49002 significantly reduced the activation of AKT (Thr^{308} and Ser^{473}) but increased the phosphorylation of VE-cadherin (Tyr^{685}); in addition, the MEK/ERK inhibitor U0126 obviously suppressed the activation of ERK(1/2) but enhanced VE-cadherin (Tyr^{731}) phosphorylation. It was noteworthy that LY49002 could increase ERK(1/2) phosphorylation and U0126 could enhance the activation of AKT (Ser^{473}; Fig. 4, *A* and *B*). There is possibly an inverse relationship between phospho-ERK and phospho-AKT (Ser^{473}), and VE-cadherin phosphorylation was regulated by both PI3K/AKT and MEK/ERK signal pathways. Further permeability tests indicated that both LY49002 and U0126 could inhibit the monolayer permeability of HUVECs induced by EGF and VEGF (Fig. 4, *C* and *D*). Together, PI3K/AKT and MEK/ERK signal pathways play an important roles in the regulation of HUVEC permeability, and there is potential cross-talk between these two pathways.

Effect of ERK2 and melatonin on AKT, ERK, VE-cadherin phosphorylation, and HUVEC permeability. Activated ERK2, a transcription factor that is one of the two isoforms of ERK, could regulate gene expression, which can affect many aspects of cellular function. We transferred ERK2 and control plasmids into the cells to evaluate its effect on HUVEC permeability. As shown in Fig. 5, *A* and *B*, both wild-type and mutant ERK2 did not affect the monolayer permeability of HUVECs or phosphorylation of AKT, ERK, and VE-cadherin compared with control plasmid. The ERK2 plasmids all contained a nuclear location sequence; therefore, the phosphorylation of ERK might be more effective on HUVEC permeability rather than location.

To exclude the influence of FBS, two groups of HUVECs were incubated in the medium with and without FBS, respectively. As shown in Fig. 6, *A–D*, treatment of the two groups of cells with melatonin had a weaker effect on phosphorylation of both those proteins compared with any combination with EGF or VEGF.

Effect of melatonin on EGFR and VEGFR expression. As melatonin could regulate the expression of genes (4), we investigated whether melatonin could influence the expression of EGFR and VEGFR2. The quantitative PCR and Western blot results showed that melatonin could inhibit the expression of both EGFR and VEGFR2, which could partly influence EGF and VEGF signaling transduction (Fig. 7, *A–H*).

In summary, melatonin inhibits EGF- and VEGF-induced HUVEC permeability by influencing the phosphorylation of AKT, ERK, and VE-cadherin in a time-dependent manner. Gene regulation by ERK2 and FBS in medium did not play an apparent role in the process, and expression of EGFR and VEGFR2 is involved in the regulation of signaling transduction.

DISCUSSION

Vascular endothelial permeability represents the endothelial barrier function of blood vessels, which has a clear relationship

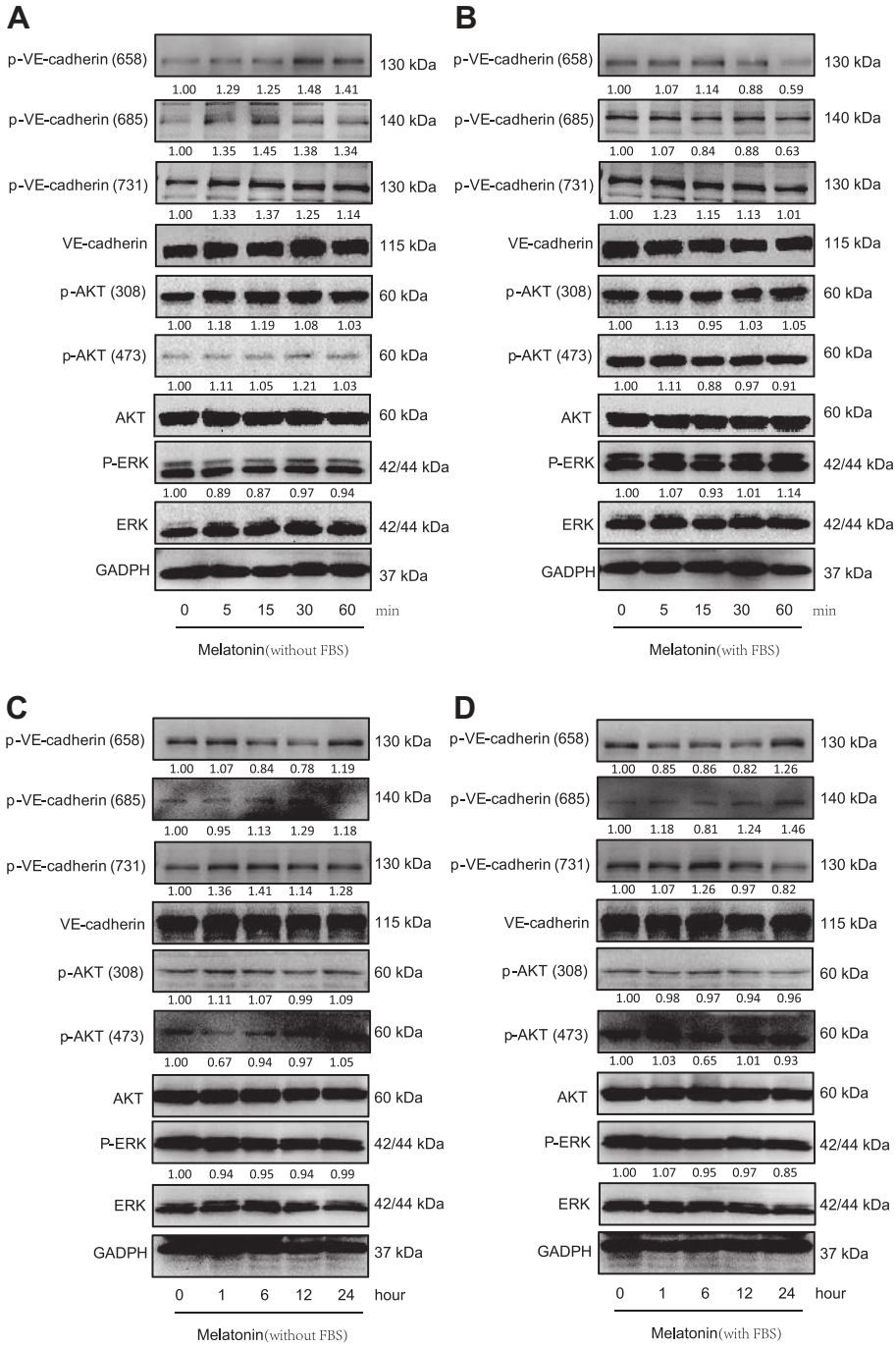


Fig. 6. Effect of melatonin on AKT, ERK, and vascular-endothelial (VE-)cadherin phosphorylation with or without FBS. *A* and *B*: Western blot analysis of VE-cadherin (Tyr^{658}, Tyr^{685}, and Tyr^{731}), AKT (Thr^{308} and Ser^{473}), and ERK(1/2) phosphorylation in human umbilical vein endothelial cells (HUVECs) treated with melatonin (with or without FBS) within 60 min. *C* and *D*: Western blot analysis of VE-cadherin (Tyr^{658}, Tyr^{685}, and Tyr^{731}), AKT (Thr^{308} and Ser^{473}), and ERK(1/2) phosphorylation in HUVECs treated with melatonin (with or without FBS) within 24 h. p, Phosphorylated.

with many cardiovascular diseases, including atherosclerosis (10, 15, 58). As the cardiovascular disease is often accompanied by loss of tight junctions between the vascular ECs and changes of cytokines in the serum, we aimed to determine the effect of VEGF and EGF on vascular endothelial permeability in the present study (11, 38, 40). The data shown in Fig. 1, *A–D*, suggest that both EGF and VEGF could induce the permeability of HUVECs, consistent with previous reports (6, 29, 37).

One of the most important junction proteins of vessel ECs, VE-cadherin, was detected in our study (8). As shown in Figs. 1–6, the permeability of HUVECs was induced by EGF and VEGF, but VE-cadherin expression did not change significantly. We then considered the role of VE-cadheirn phosphorylation in the regulation of HUVEC permeability, which could affect the function of VE-cadherin but not the expression at the indicated time (67). Previous studies have demonstrated that phosphorylation of VE-cadherin contributes to the regulation of vascular EC permeability (33, 39, 42, 54). Tyrosine phosphorylation of VE-cadherin was associated with weak junctions and impaired barrier function, and Tyr^{658}, Tyr^{685}, and Tyr^{731} phosphorylation of VE-cadherin was shown to be related to the permeability of vessel ECs (16, 66). In our study, melatonin inhibited both EGF and VEGF-induced Tyr^{685} and

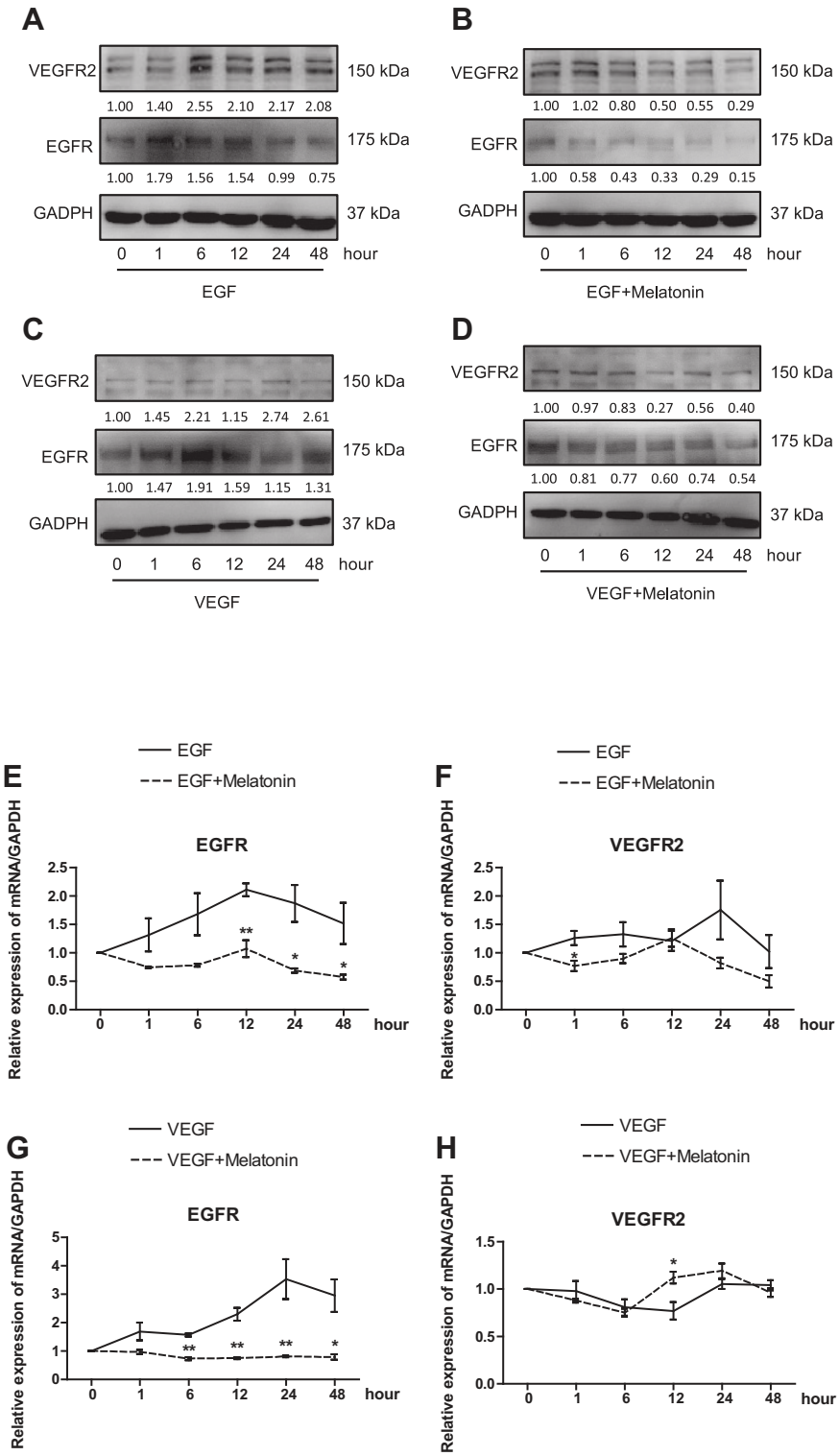


Fig. 7. Effect of melatonin on expression of EGF receptor (EGFR) and VEGF receptor 2 (VEGFR2). *A*−*D*: Western blot analysis of EGFR and VEGFR2 in human umbilical vein endothelial cells (HUVECs) treated with EGF/VEGF and melatonin within 48 h. *E*−*H*: quantitative RT-PCR analyses of EGFR and VEGFR2 mRNA expression in HUVECs that were incubated in EGF/VEGF and melatonin within 48 h. Repeated-measures or standard two-way ANOVA was used to analyze the effect of melatonin on the expression of EGFR and VEGFR2 compared with EGF/VEGF: $P = 0.000$ and $P = 0.034$ (*A* and *B*); $P = 0.000$ and $P = 0.021$ (*C* and *D*); $P = 0.009$ and $P = 0.031$ (*E* and *F*); $P = 0.000$ and $P = 0.456$ (*G* and *H*); $^{*}P < 0.05$; $^{**}P < 0.01$ compared with EGF/VEGF. $n = 3$.

Tyr^{731} phosphorylation of VE-cadherin in 2 h, and Tyr^{731} phosphorylation of VE-cadherin was also suppressed in 48 h (Figs. 2 and 3), consistent with previous studies (34, 61). The puzzling aspect of this phenomenon is the opposite trend of Tyr^{658} and Tyr^{685} phosphorylation of VE-cadherin was observed in 48 h, and melatonin showed different effects on phosphorylation of these two tyrosines (Fig. 2, *D–F*, and Fig. 3, *D–F*). Possible reasons for this discrepancy are: Tyr^{731} phosphorylation of VE-cadherin played an important role in the regulation of HUVEC permeability from the beginning to 48 h; Tyr^{658} and Tyr^{685} phosphorylation of VE-cadherin contributed to the internalization and ubiquitination of VE-cadherin (42), which often needs a longer time of action; melatonin stabilized the existence of VE-cadherin by balancing phos-

phorylation of Tyr^{658} and Tyr^{685}; and the tyrosine site was determined by different stimulation or signal pathways. These possibilities warrant exploration in future studies. Additionally, the role of phosphorylation of Tyr^{645}, Tyr^{733}, and Ser^{665} of VE-cadherin should also be discussed, which have been mentioned in previous studies and could be related to leukocyte adhesion and permeability (3, 16).

It has been reported that melatonin could influence G protein-related, NF-κB and hypoxia signaling pathways, which could be related to blood-brain barrier damage and retinal disease (23, 28). The MAPK/ERK and PI3K/AKT pathways were also influenced by melatonin, which are involved in many important physiological processes (35, 45). To explain the mechanism of melatonin inhibiting EGF- and VEGF-induced HUVEC permeability further, we detected the phosphorylation of AKT and ERK, common downstream proteins of EGF and VEGF, which are related to the permeability of vascular ECs (9, 26, 27). As shown in Figs. 2 and 3, melatonin inhibited the phosphorylation of AKT (Ser^{473}) induced by both EGF and VEGF at 2 h but prolonged the phosphorylation of AKT (Ser^{473}) and ERK(1/2) or delayed signaling transduction at 48 h. As the phosphorylation cascade determines signaling transduction, melatonin might suppress the EGF- and VEGF-induced permeability of HUVECs by disturbing AKT signaling transduction. When the PI3K/AKT inhibitor LY49002 and MEK/ERK inhibitor U0126 were used to block the phosphorylation of AKT and ERK, the permeability of HUVECs induced by EGF and VEGF was also inhibited, confirming that phospho-AKT and phospho-ERK contribute to EGF- and VEGF-induced permeability of HUVECs (Fig. 4, *C* and *D*). Our results are similar to a previous report (14) that described that blockade of MAPK activation could prevent endothelial hyperpermeability, although the method of inhibition was different. Figure 4, *A* and *B*, shows that Tyr^{685} and Tyr^{731} phosphorylation of VE-cadherin was increased by LY49002 and U0126, respectively. Of note, LY49002 blocked phospho-AKT but increased phospho-ERK, and U0126 blocked phospho-ERK but remarkably increased phospho-AKT (Ser^{473}). This suggested that there was feedback between phospho-AKT and phospho-ERK or that there was a phosphorylation balance between the two proteins. This balance has also been reported in many other proteins (7, 20, 25, 50). In summary, phospho-AKT (Ser^{473}) might contribute to the phosphorylation of VE-cadherin (Tyr^{731}), and phospho-ERK was related to the phosphorylation of VE-cadherin (Tyr^{685}) in a longer time period (24–48 h). In addition, there was cross-talk between PI3K/AKT and MEK/ERK pathways in HUVECs.

We also detected an effect of melatonin on the expression of EGFR and VEGFR2 related to the permeability of the blood-brain barrier and other vessels (12, 74). In our results, melatonin obviously inhibited the expression of the two receptors, which would thus delay signaling transduction or phosphortransfer.

As ERK2 could play a role as a transcription factor in many cell biological processes, and FBS might influence cell state (22, 26, 73), we determined the effect of ERK2 and FBS on the regulation of VE-cadherin phosphorylation (Figs. 5 and 6). Consequently, ERK2 and FBS did not significantly influence the phosphorylation of VE-cadherin.

This study had some limitations, as we did not study the role of Src, Rho GTPase, or other proteins related to VE-cadherin phosphorylation. However, previous work has already described the mechanism clearly (1, 16, 21, 71). Calmodulin has been previously reported to have an interaction with melatonin, and it influences cGMP production and regulates cell membrane permeability and endothelial barrier function (43, 46, 52, 63, 69); however, its structure was flexible and can bind to lots of target proteins (5). Thus, new research should confirm its role in a long-term experiment.

In conclusion, we found that melatonin could inhibit EGF- and VEGF-induced permeability of HUVECs by influencing the phosphorylation of VE-cadherin; phospho-AKT (Ser^{473}) might be the key protein regulating Tyr^{731} phosphorylation of VE-cadherin. Furthermore, the phosphorylation cascade of AKT (Ser^{473}) might be a time switch in the signaling transduction of HUVECs, which encompasses the phosphorylation balance with phospho-ERK. Melatonin plays an important role in the regulation of the phosphorylation cascade. Future research is needed to study the role of melatonin in vascular diseases in vivo.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

L.Y., Y.Z., Y.M., J.D., L.G., L.Z., and X.Z. performed experiments; L.Y., Y.Z., Y.M., and L.G. analyzed data; L.Y., Y.Z., Y.M., and L.G. interpreted results of experiments; L.Y. and Y.Z. prepared figures; L.Y. and Y.Z. drafted manuscript; L.Y. and Y.Z. edited and revised manuscript; L.Y. and Y.Z. approved final version of manuscript.

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