

Type 3 muscarinic acetylcholine receptor stimulation is a determinant of endothelial barrier function and adherens junctions integrity: role of protein-tyrosine phosphatase 1B

Zhou-Yang Jiao^{1,2}, Jing Wu³, Chao Liu², Bing Wen², Wen-Zeng Zhao² & Xin-Ling Du^{1,*}

¹Department of Cardiovascular Surgery, Xiehe Hospital, Huazhong University of Science and Technology, Wuhan 430022, ²Department of Cardiovascular Surgery, First Affiliated Hospital of Zhengzhou University, Zhengzhou 450052, ³Department of Pediatrics, First Affiliated Hospital of Zhengzhou University, Zhengzhou 450052, China

The main purpose of this study was to investigate whether type 3 muscarinic acetylcholine receptor (M3R) dysfunction induced vascular hyperpermeability. Transwell system analysis showed that M3R inhibition by selective antagonist 4-diphenylacetoxy-N-methylpiperidine methiodide (4-DAMP) and small interfering RNA both increased endothelial permeability. Using coimmunoprecipitation and Western blot assay, we found that M3R inhibition increased VE-cadherin and β -catenin tyrosine phosphorylation without affecting their expression. Using PTP1B siRNA, we found that PTP1B was required for maintaining VE-cadherin and β -catenin protein dephosphorylation. In addition, 4-DAMP suppressed PTP1B activity by reducing cyclic adenosine monophosphate (cAMP), but not protein kinase C α (PKC α). These data indicate that M3R preserves the endothelial barrier function through a mechanism potentially maintaining PTP1B activity, keeping the adherens junction proteins (AJPs) dephosphorylation. [BMB Reports 2014; 47(10): 552-557]

INTRODUCTION

Endothelial permeability plays a particularly important role in the initiation and progression of atherosclerosis. Endothelial permeability is regulated in part by the dynamic opening and closing of cell-cell adherens junctions (AJs). Endothelial cell-specific membrane protein vascular endothelial cadherin (VE-cadherin), the major adhesive protein of endothelial adherens junctions, is linked to the actin cytoskeleton via the armadillo family members β - and γ -catenin.

*Corresponding author. Tel: +86-13-213243710; Fax: +86-27-85726337; E-mail: realduxinling@163.com

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Acetylcholine (ACh) induces nitric oxide (NO) release and thereby causes endothelial vasodilation mainly through the type 3 muscarinic acetylcholine receptor (M3R) (1). Impaired ACh-mediated vasodilation is an important feature of atherosclerosis caused mainly by muscarinic receptor defect (2). Furthermore, M3R is involved in regulation of E-cadherin-mediated cell-cell adhesion in diseases of the skin and tumor metastasis (3, 4). Thus, these preliminary studies raise the possibility that M3R subtype is involved in regulation of VE-cadherin mediated AJs and endothelial permeability.

The adherens junction proteins (AJPs) tyrosine phosphorylation is associated with increased endothelial permeability. Previous study demonstrates that M3R antagonist increases the phosphorylation levels of E-cadherin, β -catenin, and γ -catenin in the stratified epithelial cell keratinocytes (3). However, in endothelium, whether M3R inhibition will induce AJPs tyrosine phosphorylation is unknown. The tyrosine phosphorylation of AJPs is regulated by a dynamic balance between competing protein tyrosine phosphatase (PTP) and protein tyrosine kinase (PTK). The ubiquitously expressed endoplasmic reticulum-associated protein tyrosine phosphatase 1B (PTP1B) is abundantly expressed, has broad tissue distribution, and has robust catalytic capabilities (5-7). Moreover, PTP1B binds to E-cadherin, VE-cadherin, N-cadherin, as well as β -catenin and appears to be required to maintain them in a dephosphorylated state, thereby stabilizing AJs (5-7). Muscarinic acetylcholine receptor (mAChR) stimulation can activate both PTP and PTK in many cells (8). However, the role of M3R signal inhibition on PTP1B activity is incompletely understood.

RESULTS

M3R inhibition increased the endothelial permeability

The permeation of FITC-dextran through the cell monolayer is used as a marker to demonstrate the endothelial permeability *in vitro* (9). To assess the role of M3R inhibition in the endothelial permeability, we performed FITC-dextran passage leakage assays using the Transwell system. As shown in Fig. 1A, the endothelium was treated with increasing concentrations of

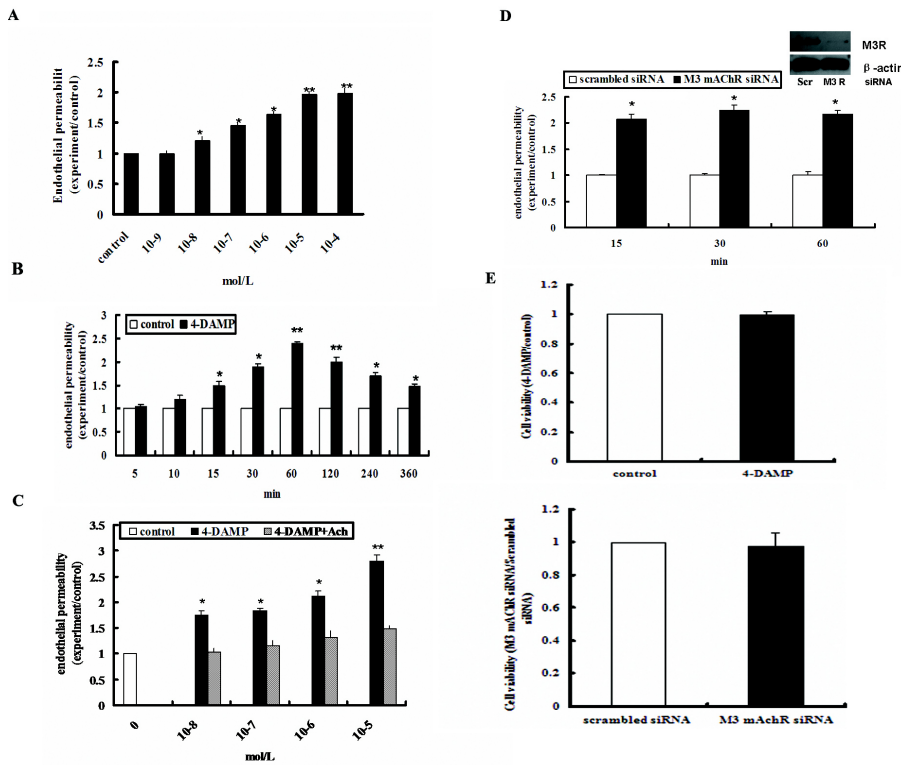


Fig. 1. M3R inhibition increases endothelial permeability. 4-DAMP increased endothelial permeability in concentration-dependent (A) and time-dependent (B) manner. Acetylcholine chloride partly prevents 4-DAMP-induced changes (C). They show changes in endothelial permeability (D) and MTT (E) in cells treated with siRNA. *P < 0.05, **P < 0.01. Data are shown as means ± S.E.M; n=3.

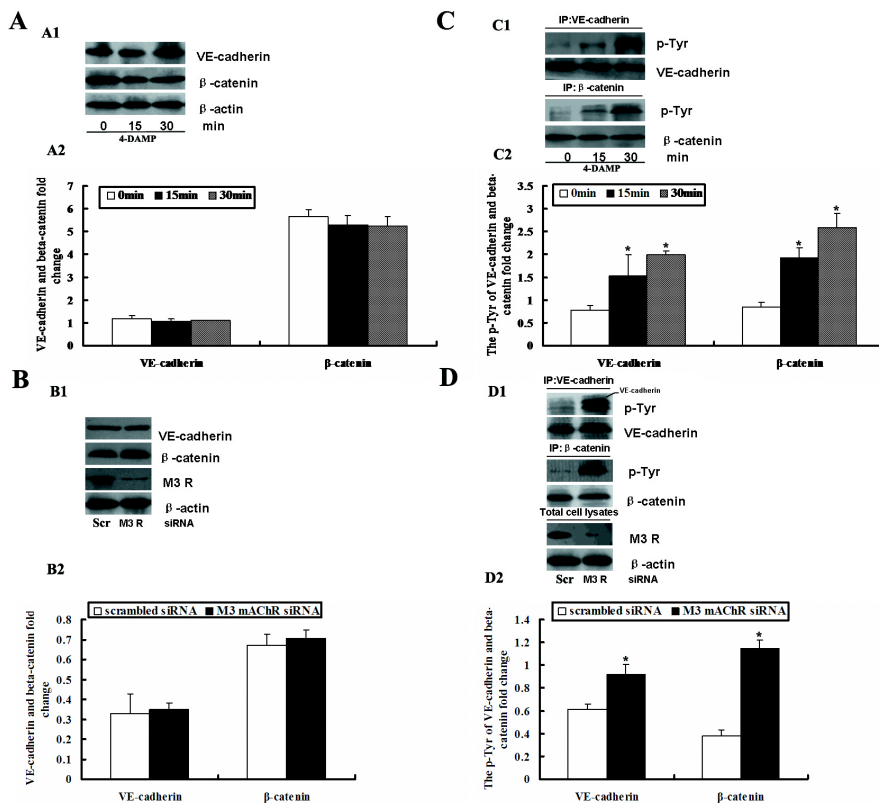


Fig. 2. M3R inhibition increased the tyrosine phosphorylation of VE-cadherin and β-catenin without affecting their expression. HUVEC cells were incubated with 4-DAMP (A, C) or siRNA (B, D) for incubated times. The total cell protein was subjected to Western blot analysis using antibodies to total VE-cadherin and β-catenin (A, B). The phosphorylation levels of VE-cadherin and β-catenin were immunoprecipitated from common lysates and assayed (C, D). *P < 0.05. The results represent the means ± S.E.M; n=3.

4-diphenylacetoxy-N-methylpiperidine methiodide (4-DAMP) (10^{-9} , 10^{-8} , 10^{-7} , 10^{-6} , 10^{-5} , and 10^{-4} mol/L). FITC-dextran flux appeared to gradually increase with increasing concentration of 4-DAMP compared with the media control for 3 h. The minimum and maximum concentration was 1×10^{-8} mol/L and 1×10^{-5} mol/L respectively, which suggested that 4-DAMP increased the endothelial permeability in a concentration-dependent manner. The effect of 4-DAMP on the endothelial permeability is also time-dependent (Fig. 1B). The fixed concentration of 4-DAMP (1×10^{-6} mol/L) increased FITC-dextran flux from 10 min to 6 h, reaching a maximum at 1 h. To further demonstrate the specificity of M3R's effect on endothelial permeability, acetylcholine chloride, an M3R agonist, was pretreated. Within the same system, acetylcholine chloride (1×10^{-8} mol/L) partially blocked 4-DAMP-induced endothelial permeability (Fig. 1C). In addition, using RNA interference, M3R siRNA resulted in a significant increase in the endothelial permeability compared with scrambled siRNA (Fig. 1D). Because cell injury also induces endothelial supermeability, we measured the cell viability with a MTT assay. HUVEC monolayers were exposed for 10h to 4-DAMP (1×10^{-4} mol/L), M3R siRNA, control siRNA, or media alone. There was no significant difference among them in cell viability (Fig. 1E). These results suggest that M3R inhibition increases endothelial permeability without causing cell injury.

M3R inhibition enhanced tyrosine phosphorylation of VE-cadherin and β -catenin

The expression of AJs molecules are closely related to endothelial integrity and permeability. To determine the role of M3R inhibition in the expression of AJs, we examined the level of VE-cadherin and β -catenin using Western blot assay. Interestingly, as shown in Fig. 2A and B, 4-DAMP and M3R siRNA did not affect VE-cadherin and β -catenin expression. The tyrosine phosphorylation of the AJs correlates with a rapid disruption of AJs, which is a critical mechanism involved in endothelial permeability. To investigate whether M3R inhibition induced AJs tyrosine phosphorylation, we measured the tyrosine phosphorylation of VE-cadherin and β -catenin using coimmunoprecipitation and Western blot assay. As shown in Fig. 2C and D, the tyrosine phosphorylation of VE-cadherin and β -catenin are markedly raised in the 4-DAMP and M3R siRNA groups compared with controls. These results suggest that M3R inhibition increases endothelial permeability associated with AJs tyrosine phosphorylation but without affecting their expression.

PTP1B negatively regulated M3R inhibition related AJs tyrosine phosphorylation

PTP1B maintains E-cadherin, VE-cadherin, N-cadherin, and β -catenin in a dephosphorylated state, thereby stabilizing cell-cell adhesions (5-7). To examine the role of PTP1B in M3R inhibition related tyrosine phosphorylation, HUVEC cells were transfected with PTP1B siRNA. As shown in Fig. 3, PTP1B siRNA, but not scrambled siRNA, knocked down PTP1B pro-

tein and decreased its activity without affecting β -actin protein expression. Moreover, PTP1B knockdown increased the tyrosine phosphorylation of VE-cadherin and β -catenin and augmented 4-DAMP-induced tyrosine phosphorylation. These results suggest that PTP1B is required for maintaining AJs dephosphorylation in unstimulated cells and it negatively regulates M3R inhibition related tyrosine phosphorylation.

M3R inhibition decreased PTP1B activity through cAMP but not PKC α

A possible link of muscarinic receptors and PTP has been previously demonstrated in skin disease, insulin resistance, and nervous synaptic plasticity (8, 10, 11). To further characterize the effect of PTP1B on M3R signal termination, we examined

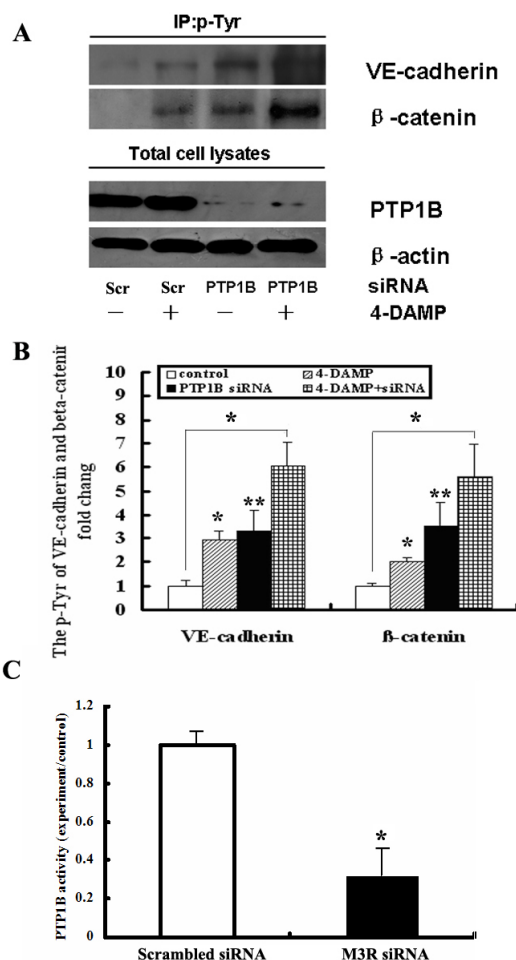


Fig. 3. PTP1B negatively regulates M3R inhibition related AJs tyrosine phosphorylation. HUVEC cells transfected with scrambled siRNA or PTP1B siRNA and then treated with 4-DAMP. (A, B) was immunoprecipitated from total cell lysates and assayed, (C) indicated PTP1B activity change. * $P < 0.05$, ** $P < 0.01$. Data are shown as means \pm S.E.M; $n=3$.

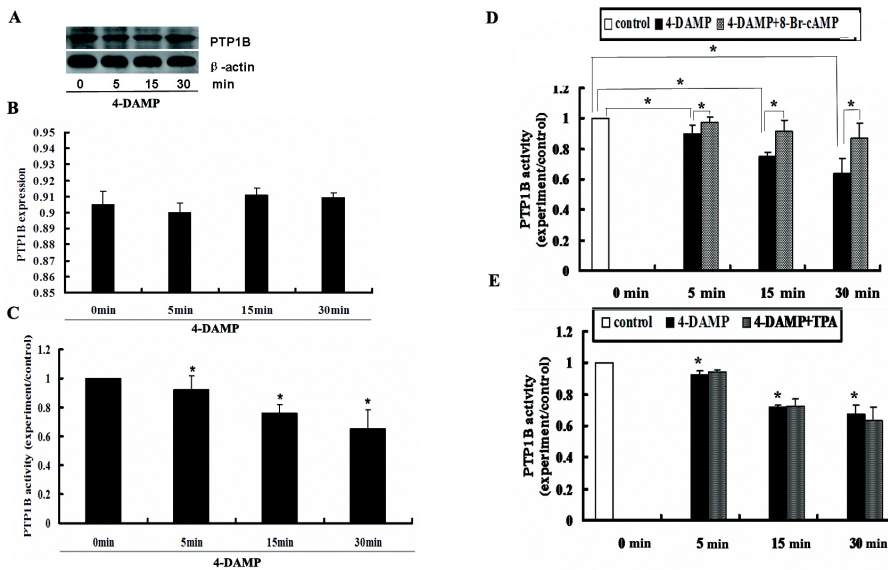


Fig. 4. M3R inhibition modulates PTP1B activity through cAMP but not PKC α in HUVEC cells. When treated with 4-DAMP at indicated times (A, B), total cell protein was subjected to Western blot analysis using antibodies to PTP1B. PTP1B activity change was indicated when treated with 4-DAMP (C), 4-DAMP+8-Br-cAMP (D) and 4-DAMP+ PMA (E). *P < 0.05. Data were shown as means \pm S.E.M; n=3.

PTP1B activity after 4-DAMP (1×10^{-6} mol/L 0, 5, 15 and 30 min) incubation. In HUVEC cells, we demonstrated that 4-DAMP decreased PTP1B activity but without affecting its expression (Fig. 4A-C). These data clearly indicate M3R inhibition decreases PTP1B activity.

Proper function of PTP1B activity is dependent on PKC α and/or cAMP (12, 13), which are both important downstream molecules of M3R signaling. To evaluate the possible mechanisms of M3R inhibition downregulated PTP1B activity, HUVEC cells were pretreated for 10 min with cAMP analogues, 8-Br-cAMP (1×10^{-5} mol/L) or PKC α activator, PMA (1×10^{-7} mol/L) prior to 4-DAMP. As shown in Fig. 4D, 8-Br-cAMP significantly restored PTP1B activity reduced by 4-DAMP. However, PMA had no effect on PTP1B activity suppression by 4-DAMP (Fig. 4E). These results suggest that cAMP not PKC α is required for M3R regulation on PTP1B activity.

DISCUSSION

Endothelial barrier function is important for the pathophysiological processes of atherosclerosis. Previous studies demonstrated that M3R signaling regulates E-cadherin mediated epithelial cell-cell adhesion (3, 4, 8). However, the precise role of M3R in VE-cadherin-mediated AJs and endothelial permeability is unknown. Using Transwell system analysis, we demonstrated that M3R inhibition increased endothelial permeability. Interestingly, 4-DAMP led to a decrease in endothelial permeability with long exposure, perhaps due to regulated, non-neuronal ACh synthesis and auto-/para-crine modulation in endothelial permeability (14). M3R is required for establishment of endothelial barrier function.

We observed that M3R inhibition increased the level of VE-cadherin and β -catenin tyrosine phosphorylation, which

potentially caused high permeability of the endothelium. The downregulation of AJs expression is another mechanism involved in cell adhesion disruption. For example, the dengue-2 virus increases endothelial permeability concomitant with the decrease in VE-cadherin expression (15). On the other hand, Aprotinin preserves cell junctions and reduces myocardial edema after cardioplegic arrest partially by preventing the reduction of VE-cadherin levels (16). However, in our study M3R inhibition had no impact on VE-cadherin and β -catenin expression. Consistent with our results, VEGF, thrombin, H₂O₂, and fluid shear stress increase endothelial permeability without changing AJs expression in the endothelial monolayers (5). This conflicting evidence may be attributable to differences in experimental conditions, stimulation, compensation, and feedback mechanisms (cell culture conditions or cell types or species, stimulation intensity or time).

Protein tyrosine phosphorylation is tightly regulated through the actions of both PTK and PTP. Disequilibrium between PTK and PTP will alter protein tyrosine phosphorylation. In actuality, PTK-driven tyrosine phosphorylation appears to prime and maintains endothelial cells in a tonic state with a lowered threshold for PTP inhibition (17). The general PTP inhibitors vanadate and phenylarsine oxide (PAO) considerably increase AJs tyrosine phosphorylation and endothelial permeability (18, 19). Using PTP1B siRNA, we found that PTP1B is required for maintaining VE-cadherin and β -catenin in a dephosphorylated state in quiescent endothelium PTP1B negatively regulated tyrosine phosphorylation triggered by M3R inhibition. Consistent with our result, Yoshimasa Nakamura et al reported that PTP1B stabilized cell-cell adhesions by reducing the tyrosine phosphorylation of VE-cadherin (5).

PTP has been implicated in a myriad of mAChR-dependent physiological and pathological processes. In our study, M3R

inhibition decreased PTP1B activity. Motility and proliferation of the endothelial cells play critical role in neointima formation closely related to the progression of atherosclerosis. One of the initial responses of quiescent endothelial cells to induce migration and proliferation is the loss of established cell-cell contacts (20). PTP1B maintained AJs low levels of tyrosine phosphorylation and PTP1B knockdown augments M3R inhibition-induced tyrosine phosphorylation. These data suggested that M3R dysfunction contributed to neointima and atherosclerotic plaque formation in atherosclerosis partially by suppressing PTP1B activity directly.

Although PTP1B could maintain VE-cadherin and β -catenin in a dephosphorylated state (5-7) and PTP1B activity was suppressed by M3R inhibition, little is known about the mechanisms whereby M3R inhibition influences this phosphatase in the endothelium. Proper function of PTP1B is dependent on Ser (378) phosphorylation by PKC α and/or Ser (352) phosphorylation by cAMP. In addition, M3R-mediated activation of adenylyl cyclases (AC) increases cAMP levels and its G $\alpha_{q/11}$ subunit stimulation generates the signaling diacylglycerol pathway that activates PKC α , we focused on cAMP and PKC α ; in this study as likely candidate intermedium. In cultured endothelial cells, M3R inhibition suppressed the effect of autocrine Ach synthesized by non-neuronal acetylcholine system (14). In our study, cAMP restored PTP1B activity down-regulated by 4-DAMP. Consistent with our results, Tao J et al reported that the Q205L G α_i2 -induced reduction in cAMP levels provided the decline in PTP1B activity and enhanced insulin signaling (21). In our study, M3R inhibition regulated PTP1B activity in a PKC α -independent pathway and cAMP contributed to the mechanism of M3R inhibition related PTP1B activity down-regulation, however other signaling molecules of M3R may also be involved, such as calmodulin (8).

In conclusion, we demonstrate constitutive M3R stimulation is a determinant of the endothelial barrier function and the AJs integrity. M3R inhibition decreases PTP1B activity through cAMP which partially induces AJs tyrosine phosphorylation. These findings may have implications for the prevention of disease and treatment of patients with a high risk of developing atherosclerosis.

MATERIALS AND METHODS

Materials and chemical, Cell culture, Immunoprecipitation and immunoblotting and PTP1B activity assay are described in the expanded Material and Methods section in the online data supplement, available at http://www.bmbreports.org/jbmb_by_volume.html?vol=47.

Suppression of M3R and PTP1B by siRNA

M3R siRNA and PTP1B siRNA oligonucleotides designed as described previously were purchased from Eurogentec (GenePharma Co,Ltd, Shanghai, China) (22). The sequences of specific siRNA against M3R is 5'-CAAUGACCUUGCACAA

UAATT-3'. The scrambled siRNA control is 5'-ACGUGACAC GUUCGGAGAATT-3'. The sequences of specific siRNA against PTP1B is 5'- CGGCCAUUUACCAGGAUAUTT-3'. The scrambled siRNA control is 5'-UUCUCCGAACGUGUCACGU TT-3'. Transient transfection of siRNA was performed using LipofectamineTM 2000 according to the manufacturer's instructions. In brief, the endothelial cells (1×10^5 cells/ml) were routinely grown on 24-well plates. After 24 hours at approximately 50% confluences, cells were transfected with M3R siRNA or PTP1B siRNA. The final siRNA concentration was 20 nmol/L. The cells were incubated with the transfection complexes at 37°C for 48 hours.

Permeability assay

Endothelial permeability was assessed as described previously (9). HUVEC cells (5×10^5 cells/ml) or HUVEC cells transfected with scrambled siRNA or with siRNA against M3R were seeded on collagen-coated transwell filters (0.4 μ m pore size, Costar, Cambridge, USA) in 24-well dishes and grown until they reached confluence. They were pretreated with (1) different concentrations of 4-DAMP (10^{-9} , 10^{-8} , 10^{-7} , 10^{-6} , 10^{-5} , and 10^{-4} mol/L) for 3 h, (2) fixed concentrations of 4-DAMP (1×10^{-6} mol/L) over a time period from 5 min to 6 h, (3) M3R agonist acetylcholine chloride (1×10^{-8} mol/L, 10 min) before 4-DAMP (10^{-8} , 10^{-7} , 10^{-6} and 10^{-5} mol/L, 30 min), or (4) scrambled siRNA or with specific siRNA against M3R. Afterward, FITC-dextran (Mr 40,000; Sigma, St. Louis, USA), at a final concentration of 1 mg/ml, was added to the upper chamber. At the indicated time points 50 μ l samples were taken from the lower compartment and replaced with the same volume of growth medium. The sample was diluted with 950 μ l of PBS, and the fluorescent intensity was measured with a spectrophotometer F-4500 an excitation wavelength of 485 nm and an emission wavelength of 530 nm.

Cell viability assay (MTT assay)

HUVEC cells (1×10^5 cells/mL) were incubated in 96 well cell culture microplates for 10 h with 4-DAMP (1×10^{-4} mol/L), M3R siRNA, or media alone for 10h. Then, cells were treated with MTT (2 mg/ml) for 4 h at 37°C, and were lysed with dimethyl sulfoxide (DMSO, 150 μ l/well). The metabolized MTT was determined photometrically at a wavelength of 570 nm in a spectrophotometer.

Statistical analysis

Results are presented as the percentage of control values (means \pm S.E.M). Differences at $P < 0.05$ were considered statistically significant. The data were analyzed using SPSS 17.0.

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