

Original Article

# Carbachol inhibits TNF- $\alpha$ -induced endothelial barrier dysfunction through alpha 7 nicotinic receptors

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**Aim:** To test whether carbachol can influence endothelial barrier dysfunction induced by tumor necrosis factor (TNF)- $\alpha$  and whether the alpha 7 nicotinic receptor can mediate this process.

**Methods:** Rat cardiac microvascular endothelial cells were exposed to carbachol followed by TNF- $\alpha$  treatment in the presence or the absence of  $\alpha$ -bungarotoxin (an antagonist of the alpha 7 nicotinic receptor). Permeability of endothelial cells cultured on Transwell filters was assayed using FITC-albumin. F-actin was stained with FITC-phalloidin. Expression of vascular endothelial cadherin, intercellular adhesion molecule 1 (ICAM-1), phosphor-ERK1/2 and phosphor-JNK was detected using Western blot.

**Results:** Carbachol (2  $\mu$ mol/L–2 mmol/L) prevented increase in endothelial cell permeability induced by TNF- $\alpha$  (500 ng/mL) in a dose-dependent manner. Further, it attenuated the down-regulation of vascular endothelial cadherin and the up-regulation of ICAM-1 induced by TNF- $\alpha$ . In addition, treatment of endothelial cells with carbachol decreased phosphor-ERK1/2 and phosphor-JNK. These effects of carbachol were blocked by  $\alpha$ -bungarotoxin 3  $\mu$ g/mL.

**Conclusion:** These data suggest that the inhibitory effect of carbachol on TNF- $\alpha$ -induced endothelial barrier dysfunction mediated by the alpha 7 nicotinic receptor.

**Keywords:** carbachol; alpha 7 nicotinic receptors; endothelium; barrier function; tumor necrosis factor  $\alpha$ ; intercellular adhesion molecule 1; ERK1/2

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## Introduction

Systemic inflammatory response and vascular hyperpermeability are important for the pathophysiological basis of acute tissue injury induced by trauma, infection and shock. Proinflammatory molecules produced during trauma, infection and shock activate endothelial cells. Upon activation, endothelial cells interact with inflammatory cells. This interaction disrupts the integrity of vascular barrier function, resulting in an increase of vascular permeability<sup>[1]</sup>. Therefore, inhibition of the systemic inflammatory response and a reduction of vascular hyperpermeability are important for the prevention and treatment of acute tissue injury.

It has been documented that the cholinergic anti-inflammatory pathway is a physiological mechanism that modulates inflammatory responses. For example, nicotinic acetylcholine receptor (nAChR) agonists block tumor necrosis factor (TNF) $\alpha$  production by lipopolysaccharide (LPS)-stimulated mac-

rophages via alpha 7 nAChR<sup>[2]</sup>. Stimulation of the vagus nerve releases ACh, leading to suppression of TNF- $\alpha$  production *in vivo* via alpha 7 nAChR<sup>[3]</sup>. Unfortunately, the use of acetylcholine and nicotine as therapeutic agents is limited by their easy hydrolysis and toxicity, respectively<sup>[4, 5]</sup>.

Carbachol is an artificially synthesized cholinomimetic agonist<sup>[6]</sup>. Among its important features are stability to hydrolysis and low toxicity<sup>[6, 7]</sup>. We and others have shown that carbachol inhibits the release of TNF- $\alpha$ <sup>[3]</sup> and reduces the levels of TNF- $\alpha$  and interleukin-6 (IL-6) released from rat peritoneal macrophages stimulated by LPS<sup>[8]</sup>. These effects of carbachol are mediated by alpha 7 nAChR. The data indicate that carbachol has the ability to inhibit the inflammatory response. However, it is not yet clear whether carbachol is able to inhibit vascular hyperpermeability.

The endothelium is the first barrier influencing vascular permeability<sup>[9]</sup>. Barrier function of the endothelium is associated with endothelial cell permeability, cytoskeletal reorganization and expression of adherent molecules<sup>[10]</sup>. In the present study, employing an *in vitro* endothelial cell model, we observed the effect of carbachol on endothelial permeability, the rearrange-

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ment of F-actin and the expression of vascular endothelial cadherin (VE-cadherin) and intercellular adhesion molecule 1 (ICAM-1). Also, we tested whether alpha 7 nAChR mediated these effects of carbachol.

## Materials and methods

### Animals and chemicals

Male Sprague-Dawley rats weighing 80–100 g (1 month old) were used for EC culture. All animals were from the Experimental Animal Center, PLA General Hospital, Beijing, China. The investigation conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication No 85-23, revised 1996) and approved by the local animal care and use committee. Carbachol, TNF- $\alpha$ ,  $\alpha$ -bungarotoxin, collagenase I, endothelial cell growth supplement (ECGS),  $\beta$ -glycerophosphate, sodium orthovanadate, leupeptin, DTT, FITC-phalloidin, FITC-albumin, EDTA and HEPES were purchased from Sigma (St Louis, MO, USA). Trypsin and M199 medium were from Difco (USA). Anti-VE-cadherin, anti-ICAM-1, anti-phospho-ERK1/2, and anti-phospho-JNK antibodies were from Santa Cruz Biotechnology (USA). Newborn calf serum was from Hong Zhou Biological Research Institute (China). Other chemicals were purchased from Sigma.

### Isolation and culture of rat cardiac microvascular endothelial cells and experimental protocols

Rats were anesthetized with 20% urethane by abdominal injection (10 mL/kg). The left ventricles were fully minced and digested with 0.1% collagenase I for 6 min at 37 °C in a shaking water bath. Then, 0.1% trypsin was added and incubated for 4 min at 37 °C. The digested solution was filtered through 100- $\mu$ m mesh filter, and the filtrates were collected and suspended in standard M199 medium containing 2 mmol/L *L*-glutamine, 100 U/mL penicillin, 100  $\mu$ g/mL streptomycin, 25% newborn calf serum, 40 U/mL heparin and 100  $\mu$ g/mL ECGS. Then the suspension was cultured in a humidified chamber with 5% CO<sub>2</sub> at 37 °C, and the medium was changed every 3 d. The cells were characterized based on their typical cobblestone morphology and on the presence of CD31, a surface marker of microvascular endothelial cells.

All studies were performed on cells between passages three and five. Endothelial cells were exposed to concentration gradients of carbachol (2 mmol/L, 0.2 mmol/L, 0.02 mmol/L, 2  $\mu$ mol/L, 0.2  $\mu$ mol/L) for 15 min. Then they were treated with TNF- $\alpha$  at a final concentration of 500 ng/mL for 24 h. Our preliminary results showed that 500 ng/mL TNF- $\alpha$  consistently induces a significant increase in the permeability of endothelial cells. For experiments using the specific antagonist of the alpha 7 nicotinic acetylcholine receptor,  $\alpha$ -bungarotoxin (3  $\mu$ g/mL) was added to the cultures 15 min before carbachol administration.

### Characterization of the cultured cells by cDNA microarray analysis

Total RNA was prepared from the cultured cells using TRI-

zol (Invitrogen Life Technologies). cDNA probe synthesis and hybridization were made according to manufacturer's instructions (GEArray™ Q serial endothelial cell biology gene array bulk kit, Bethesda, MD). The hybridization signals on the X-ray film were scanned, and the image was converted to grayscale to obtain digital number for its density. Each GEArray™ Q Series membrane was spotted with negative controls (pUC18 DNA and blanks) and housekeeping genes, including  $\beta$ -actin, GAPDH, cyclophilin A and ribosomal protein L13a. All raw signal intensities were corrected for background by subtracting the minimum value to avoid the appearance of negative numbers. All signal intensities were normalized to that of a housekeeping gene. The corrected and normalized signals were then used to estimate the relative abundance of particular transcripts.

### Permeability study of endothelial cells

Endothelial cells were seeded on Transwell filters (Corning Costar). After reaching confluence, cells were treated according to experimental protocols. Then, 100  $\mu$ L FITC-albumin (1 mg/mL) were added gently to the upper chamber and incubated for 45 min in a humidified atmosphere with 5% CO<sub>2</sub> at 37 °C. The medium in the lower chamber was removed, and the fluorescence intensity was measured with a fluorescence spectrophotometer (excitation: 490 nm; emission: 525 nm).

### F-actin staining

Endothelial cells were grown to confluence on gelatin-coated glass cover slips. After exposure to experimental conditions, endothelial cells were washed five times with PBS and fixed with 2.5% glutaraldehyde for 30 min at room temperature. Samples were washed three times with PBS and permeabilized with 1% Triton X for 10 min at room temperature. Then, the cells were incubated for 1 h at room temperature with 0.33  $\mu$ mol/L FITC-phalloidin. Cover slips were routinely screened by fluorescence microscopy.

### Western blot

Cells were lysed at 4 °C in a lysis buffer containing (mmol/L) HEPES 20 (pH 7.7), MgCl<sub>2</sub> 2.5, EDTA 0.1,  $\beta$ -glycerophosphate 20, DTT 0.5, sodium orthovanadate 0.1, NaCl 75, leupeptin 4  $\mu$ g/mL, PMSF 20  $\mu$ g/mL and Triton X-100 0.05% (*v/v*). Samples were subjected to 8% SDS-PAGE and transferred to nitrocellulose membranes (Millipore Corporation, Bedford). The blotted membranes were incubated with primary antibodies, followed by a peroxidase-conjugated secondary antibody. Antigen-antibody complexes were visualized by enhanced chemiluminescence.

### Statistical analysis

The results are expressed as the mean $\pm$ SEM. For the comparison between two groups, the Student's *t*-test was employed. A one-way ANOVA was used for multiple comparisons. A value of *P*<0.05 was considered significant.

## Results

### Characterization of the cultured cells by microvascular endothelial markers

cDNA microarray was used to identify the expression of microvascular endothelial markers including CD31, cadherin 5 and von Willebrand factor. As shown in Table 1, CD31, cadherin 5 and von Willebrand factor were highly expressed in the cultured cells. These data indicate that the cultured cells were microvascular endothelial cells.

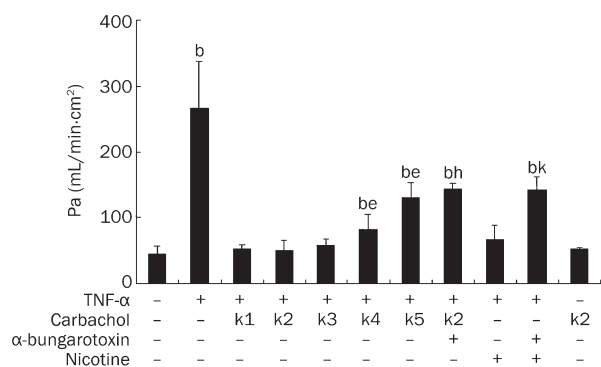
**Table 1.** Expression of microvascular endothelial markers in the cultured cells.

Markers	Full name	Accession number	Expression intensity
Cdh5	Cadherin5	NM009868	+++
CD31	Platelet/endothelial cell adhesion molecule	NM008816	+++
Vwf	Von Willebrand factor homolog	NM011708	+++

-: non-expressing genes; +: slightly expressing genes; ++: moderately expressing genes; +++: high expressing genes.

### Carbachol inhibits TNF- $\alpha$ -induced increase in permeability of endothelial cells by alpha 7 nAChR

We first examined whether carbachol had a protective effect on endothelial monolayer permeability. As shown in Figure 1, in the control group, the permeability index of endothelial cells was  $44.43 \pm 12.39$  (Pa $\times 10^5$ ). In TNF- $\alpha$ -challenged cells, the permeability index was significantly increased ( $P < 0.05$ , compared with the control group). However, when carbachol was

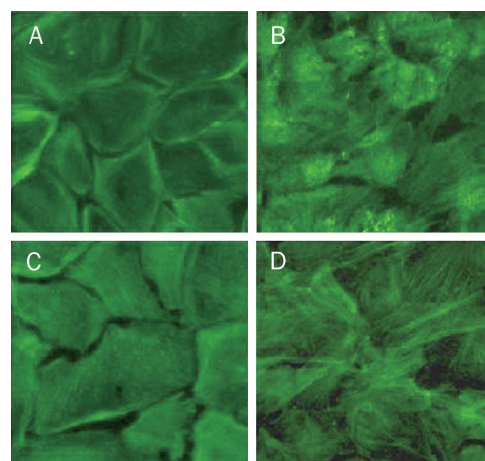


**Figure 1.** Carbachol inhibits TNF- $\alpha$ -induced hyperpermeability of endothelial cells by alpha 7 nicotinic receptors. Endothelial cells were exposed to concentration gradients of carbachol (K1: 2 mmol/L; K2: 0.2 mmol/L; K3: 0.02 mmol/L; K4: 2  $\mu$ mol/L; K5: 0.2  $\mu$ mol/L) for 15 min. Then they were treated with TNF- $\alpha$  at a final concentration of 500 ng/mL for 24 h. For experiments using the antagonist of alpha 7 nicotinic receptor,  $\alpha$ -bungarotoxin 3  $\mu$ g/mL was added to the cultures 15 min before carbachol treatment. Nicotine (10  $\mu$ mol/L) was used as a positive control. <sup>b</sup> $P < 0.05$  vs control group; <sup>a</sup> $P < 0.05$  vs TNF- $\alpha$  group; <sup>h</sup> $P < 0.05$  vs K2+TNF- $\alpha$  group; <sup>k</sup> $P < 0.05$  vs nicotine+TNF- $\alpha$  group.

added to the cells at concentrations of 2  $\mu$ mol/L (K4 group) and 0.2  $\mu$ mol/L (K5 group) followed by exposure to TNF- $\alpha$ , the endothelial cell-permeable indexes were lower than those of the TNF- $\alpha$ -challenged group ( $P < 0.05$ ), but still higher than those of the control group ( $P < 0.05$ ). When the cells were subjected to carbachol at concentrations of 2 mmol/L (K1 group), 0.2 mmol/L (K2 group) and 0.02 mmol/L (K3 group), respectively, before stimulation with TNF- $\alpha$ , there was no significant difference in permeability index between the carbachol-treated group and the control group ( $P > 0.05$ ). It is possible that  $\alpha$ -bungarotoxin 3  $\mu$ g/mL antagonizes the inhibitory effect of carbachol on TNF- $\alpha$ -challenged hyperpermeability of endothelial cells, indicating that alpha 7 nAChR may participate in mediating the protective effect of carbachol on endothelial monolayer permeability. In addition, we analyzed the effect of carbachol alone in the absence of TNF- $\alpha$  and found that carbachol alone had no effect on endothelial cell permeability ( $P > 0.05$ , compared with control group). These data indicate that carbachol inhibits the hyperpermeability of endothelial cells induced by TNF- $\alpha$  and that this effect is mediated by alpha 7 nAChR.

### Carbachol prevents F-actin rearrangement of endothelial cells exposed to TNF- $\alpha$ via alpha 7 nAChR

It has been well documented that the cytoskeleton exerts a critical role in the regulation of endothelial monolayer permeability. To better understand the effects of carbachol on the cytoskeleton and the contribution of alpha 7 nAChR activation to these effects, we next investigated the roles of carbachol and alpha 7 nAChR in F-actin rearrangement of endothelial cells by analyzing the spatial distribution of F-actin using immunofluorescent microscopy. Under the control conditions, endothelial cells maintained their polygonal shape, with a prominent dense peripheral actin-containing cortical band (Figure 2A). TNF- $\alpha$  induced a significant decrease in circumferential actin staining in association with a dramatic

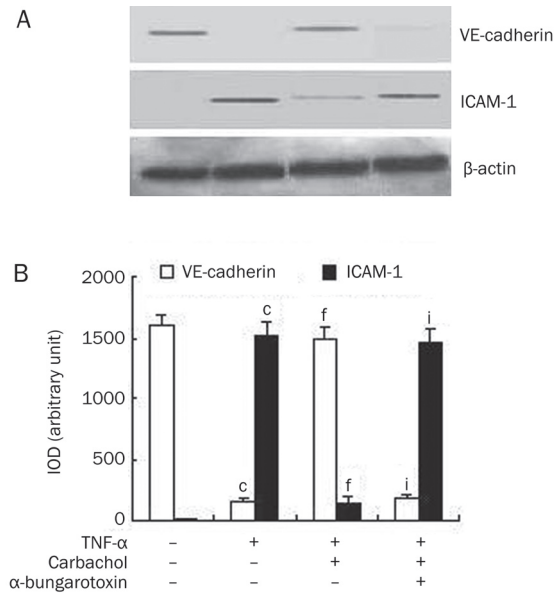


**Figure 2.** Carbachol prevents F-actin rearrangement of endothelial cells exposed to TNF- $\alpha$  via alpha 7 nicotinic receptors. Cells were treated as described in Figure 1. F-actin was stained with FITC-phalloidin. (A) Control; (B) TNF- $\alpha$ ; (C) carbachol+TNF- $\alpha$ ; (D)  $\alpha$ -bungarotoxin+carbachol+TNF- $\alpha$ .

increase in stress fiber formation, particularly in the central regions of the cells (Figure 2B). These changes were inhibited by carbachol (Figure 2C). However, blocking alpha 7 nAChR by  $\alpha$ -bungarotoxin, the protective role of carbachol in F-actin was abolished (Figure 2D). These data suggest that carbachol prevents F-actin rearrangement of endothelial cells exposed to TNF- $\alpha$  via alpha 7 nAChR.

### Carbachol regulates the expression of VE-cadherin and ICAM-1 in endothelial cells by activation of alpha 7 nAChR

The effects of carbachol on the changes of TNF- $\alpha$ -induced VE-cadherin and ICAM-1 expression by endothelial cells were studied using Western blot. As shown in Figure 3A, VE-cadherin, but not ICAM-1, was expressed in the control group of endothelial cells. When endothelial cells were exposed to TNF- $\alpha$ , the expression of VE-cadherin was down-regulated and the expression of ICAM-1 was up-regulated. Treatment of endothelial cells with carbachol attenuated the VE-cadherin down-regulation and ICAM-1 up-regulation induced by TNF- $\alpha$ . Furthermore, the effect of carbachol on the expression of VE-cadherin and ICAM-1 was decreased when endothelial cells were subjected to  $\alpha$ -bungarotoxin. To quantify the expression levels of VE-cadherin and ICAM-1, we used Image-Pro Plus software to analyze the integrated optical density (IOD) of VE-cadherin and ICAM-1 bands. As shown in Figure

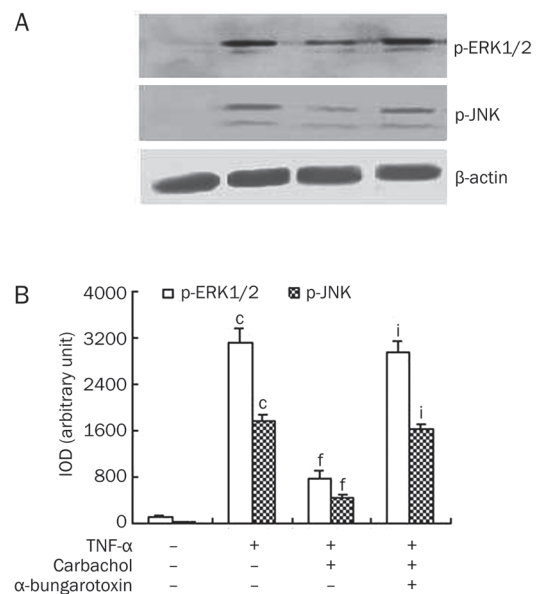


**Figure 3.** Carbachol 0.2 mmol/L regulates the expression of VE-cadherin and ICAM-1 in endothelial cells by activation of alpha 7 nicotinic receptors. (A) The expression of VE-cadherin and ICAM-1 was assessed by Western blot using protein lysates extracted from cells exposed to carbachol followed by TNF- $\alpha$  500 ng/mL in the presence or the absence of  $\alpha$ -bungarotoxin 3  $\mu$ g/mL.  $\beta$ -Actin was used as a normalization control. (B) Integrated optical density (IOD) of VE-cadherin and ICAM-1 expression bands was analyzed by Image-Pro Plus software. IOD=area $\times$ average density. <sup>c</sup> $P$ <0.01 vs control, <sup>f</sup> $P$ <0.01 vs TNF- $\alpha$  group, <sup>i</sup> $P$ <0.01 vs carbachol+TNF- $\alpha$  group.

3B, carbachol induced an increase in VE-cadherin expression ( $P$ <0.001 vs TNF- $\alpha$  group) and a reduction in ICAM-1 expression ( $P$ <0.001 vs TNF- $\alpha$  group) in endothelial cells. However,  $\alpha$ -bungarotoxin significantly blocked the increase of VE-cadherin expression ( $P$ <0.001 vs carbachol+TNF- $\alpha$  group) and the reduction of ICAM-1 expression ( $P$ <0.001 vs carbachol+TNF- $\alpha$  group) induced by carbachol. These data indicate that carbachol regulates the expression of VE-cadherin and ICAM-1 in endothelial cells induced by TNF- $\alpha$  through activation of alpha 7 nAChR.

### Alpha 7 nAChR mediates the inhibitory effects of carbachol on the phosphorylation of ERK1/2 and JNK in endothelial cells induced by TNF- $\alpha$

TNF- $\alpha$  can activate mitogen-activated protein kinases (MAPKs) in the signaling pathway leading to changes of VE-cadherin and ICAM-1 expression<sup>[7, 8]</sup>. To examine whether carbachol regulates MAPK activation induced by TNF- $\alpha$ , we employed Western blot to analyze the expression of phosphor-ERK1/2 and phosphor-JNK in endothelial cells. As shown in Figure 4, TNF- $\alpha$  stimulated the expression of phosphor-ERK1/2 and phosphor-JNK ( $P$ <0.001 vs control group). In contrast, treatment of endothelial cells with carbachol inhibited the levels of phosphor-ERK1/2 and phosphor-JNK induced by TNF- $\alpha$  ( $P$ <0.001 vs TNF- $\alpha$  group). The presence of  $\alpha$ -bungarotoxin blocked the inhibitory effect of carbachol on



**Figure 4.** Alpha 7 nicotinic receptors mediate the inhibitory effects of carbachol 0.2 mmol/L on the phosphorylation of ERK1/2 and JNK in endothelial cells induced by TNF- $\alpha$  500 ng/mL. (A) The levels of phosphor-ERK1/2 and JNK were assessed by Western blot using protein lysates extracted from cells exposed to carbachol followed by TNF- $\alpha$  in the presence or the absence of  $\alpha$ -bungarotoxin 3  $\mu$ g/mL.  $\beta$ -Actin was used as a normalization control. (B) Integrated optical density (IOD) of ERK1/2 and JNK levels was analyzed by Image-Pro Plus software. IOD=area $\times$ average density. <sup>c</sup> $P$ <0.01 vs control, <sup>f</sup> $P$ <0.01 vs TNF- $\alpha$  group, <sup>i</sup> $P$ <0.01 vs carbachol+TNF- $\alpha$  group.

the levels of phosphor-ERK1/2 and phosphor-JNK ( $P < 0.001$  vs carbachol+TNF- $\alpha$  group). These results suggest that alpha 7 nAChR mediates the inhibitory effects of carbachol on the phosphorylation of ERK1/2 and JNK in endothelial cells induced by TNF- $\alpha$ .

## Discussion

In the present study, we showed that carbachol can inhibit the hyperpermeability and F-actin rearrangement of endothelial cells induced by TNF- $\alpha$ . Also, carbachol can regulate the expression of VE-cadherin and ICAM. These effects of carbachol are mediated by alpha 7 nAChR.

The permeability of monolayer endothelial cells is controlled by the equilibrium between the endothelial cell contraction force and the intercellular contact force. This equilibrium is regulated by the cytoskeleton<sup>[11]</sup>. It has been reported that cytokines can destroy the endothelial cell cytoskeleton, cause gaps between the cells and increase transendothelial permeability<sup>[12]</sup>. Our results show that exposure of endothelial cells to TNF- $\alpha$  led to the increase of endothelial cell permeability accompanied by F-actin rearrangement and stress fiber formation in endothelial cells. Intriguingly, pre-treatment of endothelial cells with carbachol prevents hyperpermeability, cytoskeletal rearrangement and the formation of stress fibers in endothelial cells. Therefore, these data suggest that carbachol suppresses the increase of endothelial permeability by protecting the endothelial cell cytoskeleton.

It has been well documented that VE-cadherin and ICAM-1, as adhesion proteins, play a pivotal regulatory role in the permeability of endothelial cells. Inhibition of VE-cadherin induces a reorganization of the actin cytoskeleton, reduces cell-cell adhesion and increases the permeability of endothelial cells<sup>[13]</sup>. Also, overexpression of ICAM-1 causes alteration of the actin cytoskeleton-endothelial junction, thereby inducing the hyperpermeability of endothelial cells<sup>[14]</sup>. It has been reported that TNF- $\alpha$  induces an increase in the permeability of endothelial cells by decreasing VE-cadherin protein expression and stimulating ICAM-1 protein expression<sup>[13, 15]</sup>. In the present study, we found that TNF- $\alpha$  down-regulates VE-cadherin expression and up-regulates ICAM-1 expression. Our observation is consistent with previously reported results. Further, we showed that carbachol inhibits the effect of TNF- $\alpha$  on the expression of VE-cadherin and ICAM-1. It seems that carbachol inhibits the hyperpermeability of endothelial cells by regulating the expression of VE-cadherin and ICAM-1.

MAPKs are highly conserved serine/threonine kinases that are activated in response to a wide variety of stimuli. TNF- $\alpha$  inhibits VE-cadherin expression and stimulates ICAM-1 expression through the activation of ERK1/2 and JNK and, subsequently, increases the permeability of endothelial cells<sup>[13, 16]</sup>. In the present study, we showed that carbachol inhibits ERK1/2 and JNK phosphorylation induced by TNF- $\alpha$ . It is possible that carbachol exerts its function by ERK1/2 and JNK pathways. However, it is necessary to determine the kinase phosphorylation sites and whether the inhibitors of ERK1/2 and JNK block the effects of carbachol.

We have previously shown that alpha 7 nAChR mediates the anti-inflammatory effect of carbachol<sup>[8]</sup>. Also, others have reported that administration of acetylcholine inhibits LPS-induced TNF- $\alpha$  release by a reduction in phosphorylation of MAPKs. This inhibitory effect of acetylcholine is blocked by  $\alpha$ -bungarotoxin, a specific antagonist of alpha 7 nAChR<sup>[17]</sup>. Our present study indicates that  $\alpha$ -bungarotoxin blocks the effect of carbachol on ERK1/2 and JNK activation and the expression of VE-cadherin and ICAM-1 in endothelial cells. Additionally,  $\alpha$ -bungarotoxin blocks the inhibitory effect of carbachol on the permeability index of endothelial cells and F-actin rearrangement induced by TNF- $\alpha$ . Our findings suggest that, similar to anti-inflammatory effects, the inhibitory effect of carbachol on endothelial barrier dysfunction is also mediated by alpha 7 nAChR.

The present study provides evidence that carbachol improves endothelial barrier dysfunction induced by TNF- $\alpha$  and that alpha 7 nAChR mediates the process. Because endothelial barrier dysfunction is crucial for the pathophysiological basis of acute tissue injury, our study implies that carbachol may have therapeutic potential for acute tissue injury.

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## Author contribution

Xiu-hua LIU, Sen HU, and Zhi-yong SHENG designed research; Yu-zhen LI and Fei RONG performed research; Yu-zhen LI analyzed data and wrote the paper.

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