Reduction of Mitochondria–Endoplasmic Reticulum Interactions by Acetylcholine Protects Human Umbilical Vein Endothelial Cells From Hypoxia/Reoxygenation Injury

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Objective—We explored the role of endoplasmic reticulum (ER)–mitochondria Ca\(^{2+}\) cross talk involving voltage-dependent anion channel-1 (VDAC1)/glucose-regulated protein 75/inositol 1,4,5-trisphosphate receptor 1 complex and mitofusin 2 in endothelial cells during hypoxia/reoxygenation (H/R), and investigated the protective effects of acetylcholine.

Approach and Results—Acetylcholine treatment during reoxygenation prevented intracellular and mitochondrial Ca\(^{2+}\) increases and alleviated ER Ca\(^{2+}\) depletion during H/R in human umbilical vein endothelial cells. Consequently, acetylcholine enhanced mitochondrial membrane potential and inhibited proapoptotic cascades, thereby reducing cell death and preserving endothelial ultrastructure. This effect was likely mediated by the type-3 muscarinic acetylcholine receptor and the phosphatidylinositol 3-kinase/Akt pathway. In addition, interactions among members of the VDAC1/glucose-regulated protein 75/inositol 1,4,5-trisphosphate receptor 1 complex were increased after H/R and were associated with mitochondrial Ca\(^{2+}\) overload and cell death. Inhibition of the partner of the Ca\(^{2+}\) channeling complex (VDAC1 siRNA) or a reduction in ER–mitochondria tethering (mitofusin 2 siRNA) prevented the increased protein interaction within the complex and reduced mitochondrial Ca\(^{2+}\) accumulation and subsequent endothelial cell death after H/R. Intriguingly, acetylcholine could modulate ER–mitochondria Ca\(^{2+}\) cross talk by inhibiting the VDAC1/glucose-regulated protein 75/inositol 1,4,5-trisphosphate receptor 1 complex and mitofusin 2 expression. Phosphatidylinositol 3-kinase siRNA diminished acetylcholine-mediated inhibition of mitochondrial Ca\(^{2+}\) overload and VDAC1/glucose-regulated protein 75/inositol 1,4,5-trisphosphate receptor 1 complex formation induced by H/R.

Conclusions—Our data suggest that ER–mitochondria interplay plays an important role in reperfusion injury in the endothelium and may be a novel molecular target for endothelial protection. Acetylcholine attenuates both intracellular and mitochondrial Ca\(^{2+}\) overload and protects endothelial cells from H/R injury, presumably by disrupting the ER–mitochondria interaction. (Arterioscler Thromb Vasc Biol. 2015;35:1623-1634. DOI: 10.1161/ATVBAHA.115.305469.)

Key Words: acetylcholine ■ calcium ■ endothelial cells ■ mitochondria ■ reperfusion injury

Lining the inner surface of the circulatory system, the vascular endothelium plays an important role in maintaining cardiovascular homeostasis. Ischemia followed by reperfusion leads to tissue injury under various pathophysiological conditions, and the vascular endothelium is implicated in the pathogenesis of ischemia/reperfusion (I/R) injury.\(^1\)\(^2\) Given the critical role of endothelium in the regulation of vascular tone, as well as platelet and leukocyte function, protection of vascular endothelial cells is an important therapeutic target.\(^3\)\(^4\) The mechanisms of I/R injury are complex and multifactorial, including excessive generation of reactive oxygen species (ROS), inflammation, mitochondrial dysfunction, and calcium overload.\(^5\)\(^6\) The development of novel pharmacological therapeutics affecting multiple targets such as calcium overload and mitochondria may afford better protection against I/R injury.

Recent clinical studies have evaluated vagal nerve stimulation as a new and promising therapeutic approach for chronic heart failure.\(^7\)\(^8\) Vagal nerve stimulation also has protective effects at a much earlier stage when the initial ischemic stress and reperfusion insult occurs.\(^9\)\(^10\) Previous studies have shown that acetylcholine, the principal neurotransmitter of the vagus nerve, protects cardiomyocytes from ischemia or I/R injury by inhibiting ROS formation, improving mitochondrial biogenesis and function, and decreasing inflammatory markers.\(^11\)\(^12\) In addition, acetylcholine has protective effects on rat

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cardiomyocytes subjected to hypoxia by activating the phosphatidylinositol 3-kinase (PI3K)/Akt pathway. Although these antioxidative and anti-inflammatory effects of acetylcholine have been well documented in cardiomyocytes, the effects on calcium overload, a major determinant of I/R injury, have not been characterized, especially in endothelial cells.

During I/R, cytosolic accumulation of Ca\(^{2+}\) leads to mitochondrial Ca\(^{2+}\) overload, which triggers opening of the mitochondrial permeability transition pore and results in cell death. There is a close anatomic and functional relationship between endoplasmic reticulum (ER) and mitochondria in the myocardium; together, ER and mitochondria establish junctions to cooperate in the propagation of Ca\(^{2+}\) signals. This occurs via a macromolecular complex composed of voltage-dependent anion channel-1 (VDAC1), glucose-regulated protein 75 (Grp75), and inositol 1,4,5-trisphosphate receptor 1 (IP3R1) that regulates direct Ca\(^{2+}\) transfer from ER to mitochondria. Nevertheless, the role of ER–mitochondria interplay in reperfusion-induced vascular endothelial cell death has not been previously reported. Therefore, we explored whether Ca\(^{2+}\) flux from ER to mitochondria plays a role in endothelial cell death after hypoxia/reoxygenation (H/R). We further examined the protective effects of acetylcholine in endothelial cells against H/R-induced calcium overload and ER–mitochondria–dependent apoptotic signals, with a focus on ER–mitochondria cross talk.

**Materials and Methods**

Materials and Methods are available in the online-only Data Supplement.

**Results**

### Muscarinic Acetylcholine Receptor Mediated the Inhibition of ER Ca\(^{2+}\) Depletion and Mitochondrial Ca\(^{2+}\) Overload by Acetylcholine During H/R

Recent studies have shown that I/R is related to phospholipase C–induced IP3 production and abnormal ER Ca\(^{2+}\) release, inducing large increases in cytoplasmic Ca\(^{2+}\) and mitochondrial Ca\(^{2+}\) loading. Thus, we examined ER Ca\(^{2+}\) using MagFura2 staining. We found that the Ca\(^{2+}\) fluorescence intensity of the ER in the H/R group was significantly lower than that in the control group (Figure 2A). Acetylcholine significantly increased Ca\(^{2+}\) fluorescence intensity of the ER compared with the H/R group, and this effect of acetylcholine was abrogated by atropine but not affected by hexamethonium. These results suggested that acetylcholine inhibited the release of calcium from the ER during H/R.

As a part of the mitochondrial network is in close spatial proximity to ER Ca\(^{2+}\) release sites, Ca\(^{2+}\) cross talk between the ER and the mitochondria has been implicated in myocardial reperfusion injury. Here, we explored the effect of acetylcholine on mitochondrial Ca\(^{2+}\) content. Mitochondrial Ca\(^{2+}\) accumulation measured by Rhod-2 staining was significantly greater than that in the control group, as shown in Figure 2B. In sharp contrast, acetylcholine attenuated the increase in mitochondrial Ca\(^{2+}\) relative to the H/R group. However, this protective effect of acetylcholine was blocked by atropine, whereas hexamethonium had no significant effect.

**Acetylcholine Mediated the Inhibition of ER Ca\(^{2+}\) Overload via Acetylcholine**

We first determined the time course of changes in Ca\(^{2+}\) after reoxygenation. Human umbilical vein endothelial cells (HUVECs) were subjected to 8 hours of hypoxia followed by 1, 2, 4, 8, 16, or 24 hours of reoxygenation. As shown in Figure 1A and 1B, the intracellular Ca\(^{2+}\) level measured by Cal-520 staining increased rapidly and peaked at 2 hours after reoxygenation.

To examine whether acetylcholine could decrease H/R-induced Ca\(^{2+}\) overload, HUVECs were treated with acetylcholine at 10\(^{-8}\) to 10\(^{-4}\) mol/L at the beginning of reoxygenation. Treatment with acetylcholine attenuated the H/R-induced Ca\(^{2+}\) increase in a concentration-dependent manner (Figure 1C). Therefore, in subsequent experiments, a reoxygenation time of 2 hours was chosen, and acetylcholine was used at a concentration of 10\(^{-5}\) mol/L, which was consistent with previous reports.

To determine which type of acetylcholine receptor (ACHR) plays the predominant role in acetylcholine-mediated suppression of Ca\(^{2+}\) overload, HUVECs were exposed to acetylcholine (10\(^{-5}\) mol/L) and cotreated with atropine (Atro; muscarinic AChR inhibitor, 10\(^{-5}\) mol/L) or hexamethonium (Hex; nicotinic AChR inhibitor, 10\(^{-5}\) mol/L). Acetylcholine markedly reduced H/R-induced Ca\(^{2+}\) overload (Figure 1D), and this protective effect of acetylcholine was abolished by atropine. The protective effect was not affected by nicotinic AChR inhibitor hexamethonium, indicating that muscarinic AChR plays an important role in acetylcholine-mediated suppression of Ca\(^{2+}\) overload. Acetylcholine treatment had no significant effect on the intracellular Ca\(^{2+}\) level of control cells.
apoptotic signals. Loss of mitochondrial membrane potential seems to be an early event in apoptosis. We detected the mitochondrial membrane potential using the dye 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazole-carbocyanine iodide (JC-1). If a loss or collapse of mitochondrial membrane potential occurred, the ratio of red:green fluorescence was reduced. After H/R, the dye remained mostly in the cytoplasm and showed green fluorescence (Figure 3A). In contrast, the dye in acetylcholine-treated HUVECs seemed to accumulate within mitochondria and showed red fluorescence. Treating the cells with atropine inhibited the protective effects of acetylcholine, confirming that acetylcholine acts through muscarinic receptors in HUVECs.

As shown in Figure 3B, acetylcholine increased antiapoptotic factor Bcl-2 expression and upregulated the Bcl-2/Bax ratio during the reoxygenation period. We also determined the release of cytochrome C from mitochondria. In the control group, relatively low levels of cytochrome C were released from the mitochondria to cytosol. After H/R procedures, the cytochrome C levels were significantly increased in the cytosol and decreased in the mitochondria. Administration of acetylcholine inhibited the release of cytochrome C to the cytosol. Depletion of the Ca2+ pool in the ER can trigger cellular apoptosis through activation of caspase 12, which resides on the outside the ER membrane. Acetylcholine inhibited activation of caspase 12 during reoxygenation. These effects of acetylcholine were reversed by treatment with atropine or a selective M3AChR antagonist 4-diphenylacetoxy-N-methylpiperidine methiodide (4-DAMP, 10−6 mol/L). Acetylcholine treatment alone had no significant effect on the apoptotic signal cascade in control cells (Figure I in the online-only Data Supplement).

Acetylcholine Protected Against H/R Injury via the PI3K/Akt/Endothelial Nitric Oxide Synthase Signaling Pathway

We further explored the involvement of the PI3K/Akt/endothelial nitric oxide synthase (eNOS) signaling pathway in
Acetylcholine-mediated endothelial protection. PI3K inhibitor LY294002 abolished acetylcholine-induced preservation of Akt and eNOS phosphorylation and eNOS expression, as well as NO production. N\textsuperscript{G}-nitro-l-arginine methyl ester (L-NAME) suppressed eNOS phosphorylation at Ser1177 and expression and NO production (Figure 4A). Acetylcholine increased sarcoplasmic reticulum Ca\textsuperscript{2+}-ATPase 2 expression and the Bcl-2/Bax ratio and suppressed the release of mitochondrial cytochrome C to the cytosol and activation of caspase 12 during reoxygenation (Figure 4A and 4B). The endothelial protective effects of acetylcholine were lost in HUVECs treated with the PI3K inhibitor LY294002 or the NOS inhibitor L-NAME.

In addition, lactate dehydrogenase (LDH), an indicator of cell injury, was measured in the supernatant at the end of reoxygenation. H/R injury caused a significant increase in LDH release, and acetylcholine administration markedly decreased LDH release (Figure II in the online-only Data Supplement). The effects of acetylcholine were reversed by treatment with atropine, 4-DAMP, LY294002, or L-NAME.

We also examined the effects of acetylcholine on ultrastructural changes in HUVECs using transmission electron microscopy. As shown in Figure 4C, HUVECs subjected to H/R showed dilated ER and swollen or ruptured mitochondria compared with control cells. In acetylcholine-treated endothelial cells, the cellular ultrastructure appeared similar to that of the control endothelial cells. In the atropine, 4-DAMP–treated, LY294002-treated, or L-NAME–treated HUVECs, more severe ultrastructural changes were observed. The cristae in the mitochondria appeared distorted and in some cases were completely lysed. Furthermore, the ER appeared dilated and swollen. The transmission electron microscopic observations indicated that acetylcholine could preserve cellular ultrastructural changes induced by H/R.

**Downregulation of VDAC1 or Mitofusin 2 Protected HUVECs Against H/R Injury**

Recent studies have identified new proteins enriched at the ER–mitochondria junctions, including the VDAC1/Grp75/IP3R1 complex, which directly controls Ca\textsuperscript{2+} transfer from

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**Figure 2.** Acetylcholine (ACh) inhibited endoplasmic reticulum (ER) Ca\textsuperscript{2+} depletion and mitochondrial Ca\textsuperscript{2+} overload during hypoxia/reoxygenation (H/R) via muscarinic acetylcholine receptor. A, Changes in fluorescence intensity of Ca\textsuperscript{2+} in the ER were measured using Mag-Fura2. B, Measurement of mitochondrial Ca\textsuperscript{2+} by Rhod-2 loading after 2 hours of reoxygenation. Open bar, normoxia; filled bar, H/R; Data represent the mean±SEM (n=4). *P<0.001 vs Con; #P<0.001 vs H/R; &P<0.001 vs ACh-treated H/R group. Scale bar, 50 μm. Atro indicates atropine group; and Hex, hexamethonium group.
the ER to mitochondria. To explore the involvement of the VDAC1/Grp75/IP3R1 complex during H/R injury, we downregulated 1 partner of this complex, VDAC1 (Figure IIIA in the online-only Data Supplement). The association among VDAC1, Grp75, and IP3R1 was confirmed by cross coimmunoprecipitation with anti-IP3R1 and anti-Grp75 antibodies. H/R of HUVECs led to an increase in interactions among VDAC1, Grp75, and IP3R1 (Figure 5A and 5B). These increased protein–protein interactions were associated with mitochondrial Ca$^{2+}$ overload (Figure 5C) and a marked increase in cell death (Figure IV in the online-only Data Supplement) compared with the control group. After H/R, the downregulation of VDAC1 reduced the VDAC1/Grp75/IP3R1 interactions, mitochondrial Ca$^{2+}$ content (Figure 5A
Figure 4. Acetylcholine (ACh) protected against hypoxia/reoxygenation (H/R) injury via the phosphatidylinositol 3-kinase/Akt/endothelial nitric oxide synthase (eNOS) signaling pathway. A and B, ACh treatment prevented hypoxia/reoxygenation (H/R)–induced decreases in the phosphorylated Akt, eNOS phosphorylation and expression, and NO release. ACh also upregulated sarcoplasmic reticulum Ca\(^{2+}\)-ATPase 2 (SERCA2) expression, increased the Bcl-2/Bax ratio, suppressed cytochrome C release and caspase 12 activation, and prevented an increase in mitofusin 2 (Mfn2) expression after H/R. These effects of ACh were abrogated by treatment with LY294002 (LY, 10\(^{-5}\) mol/L) or \(N^\prime\)-nitro-\(L\)-arginine methyl ester (L-NAME, 10\(^{-4}\) mol/L). Open bar, normoxia; filled bar, H/R. Data represent the mean±SEM (n = 5). **\(P<0.01\), ***\(P<0.001\) vs Con; ##\(P<0.01\), ###\(P<0.001\) vs H/R; &\(P<0.05\), &&\(P<0.01\), &&&\(P<0.001\) vs ACh-treated H/R group. C, Ultrastructural changes in HUVECs. Scale bar, 1 \(\mu\)m.
Figure 5. Decreasing endoplasmic reticulum (ER)–mitochondria interactions by genetic downregulation of voltage-dependent anion channel-1 (VDAC1) or mitofusin 2 (Mfn2) protects human umbilical vein endothelial cell (HUVECs) from hypoxia/reoxygenation (H/R) injury. A and B, Protein lysates from HUVECs were immunoprecipitated with anti–inositol 1,4,5-trisphosphate receptor 1 (IP3R1) or anti–glucose-regulated protein 75 (Grp75) antibodies, and IP3R1, Grp75, and VDAC1 levels in input samples. Data represent the mean±SEM (n=3). C, Measurement of mitochondrial Ca\(^{2+}\) by Rhod-2 loading after 2 hours of reoxygenation. Scale bar, 50 \(\mu\)m. Data represent the mean±SEM (n=4). *\(P<0.05\), **\(P<0.01\), ***\(P<0.001\) vs NC siRNA-treated control group; ##\(P<0.01\), ###\(P<0.001\) vs NC siRNA-treated H/R group.

and 5C) and LDH release (Figure IV in the online-only Data Supplement). These data support the conclusion that inhibiting partners of the VDAC1/Grp75/IP3R1 complex prevent \(\text{Ca}^{2+}\) transfer from the ER to mitochondria during H/R and attenuates mitochondrial \(\text{Ca}^{2+}\) overload and subsequent endothelial cell death.
Mitofusin 2 is involved in tethering ER to mitochondria and regulating ER–mitochondria cross talk. Mitofusin 2 protein expression was significantly increased after H/R compared with the control group, and the increase in mitofusin 2 expression was inhibited by acetylcholine treatment (Figure 3B). This effect of acetylcholine was reversed by the treatment with atropine, 4-DAMP, LY294002, or L-NAME (Figures 3B and 4B). Furthermore, mitofusin 2 protein expression was downregulated by the corresponding siRNA (Figure IIIIB in the online-only Data Supplement). To investigate the ER–mitochondria interaction, we analyzed important parameters for the functional interaction between ER and mitochondria, the contact length of mitochondria with junctional ER/mitochondrial perimeter, and the distance between ER and the outer mitochondrial membrane, in the transmission electron micrographs, as described previously. Our results showed that the length of interface/mitochondrial perimeter was increased and the mean distance between ER and the outer mitochondrial membrane was decreased in the H/R group. The length of interface/mitochondrial perimeter was decreased, and the ER–mitochondria distance at these sites was increased in the mitofusin 2 siRNA-treated H/R group (Figure V in the online-only Data Supplement). Our results suggested that mitofusin 2 siRNA significantly disrupted ER–mitochondria tethering in H/R-injured HUVECs. HUVECs in which mitofusin 2 was silenced showed decreased interaction among VDAC1, Grp75, and IP3R1 during H/R (Figure 5B). Furthermore, the increase in mitochondrial Ca^{2+} content and cell death observed after H/R was significantly attenuated in the mitofusin 2 siRNA group (Figure 5C; Figure IV in the online-only Data Supplement). These results indicate that disruption of the ER–mitochondria interaction protects endothelial cells against H/R injury by reducing mitochondrial Ca^{2+} overload via the VDAC1/Grp75/IP3R1 complex.

**Acetylcholine Inhibited the VDAC1/Grp75/IP3R1 Complex via the PI3K/Akt Pathway**

As shown in Figure 6A, H/R caused an increase in the association among VDAC1, Grp75, and IP3R1, as revealed by coimmunoprecipitation. Interestingly, treatment with acetylcholine during reoxygenation reduced these increased protein–protein interactions within the complex. Furthermore, we examined the role of the PI3K/Akt pathway and found that endothelial cells transfected with PI3K siRNA showed higher LDH release and mitochondrial Ca^{2+} levels, as well as increased protein interactions within the VDAC1/Grp75/IP3R1 complex, compared with the NC siRNA group in the presence of acetylcholine (Figure 6B–6D), suggesting that the PI3K/Akt pathway plays a role in acetylcholine-mediated inhibition of the VDAC1/Grp75/IP3R1 complex, mitochondrial Ca^{2+} overload, and subsequent cell death observed during H/R.

**ROS Mediated the VDAC1/Grp75/IP3R1 Complex**

We used MitoSOX Red to detect changes in mitochondrial ROS levels. We found that mitochondrial ROS levels were significantly elevated after H/R, and observed that acetylcholine administration decreased H/R-induced mitochondrial ROS levels (Figure VI in the online-only Data Supplement). These results indicated that intracellular oxidative stress occurred in endothelial cells subjected to H/R and that mitochondria play an important role in this process. Interestingly, mitofusin 2 siRNA also decreased H/R-induced mitochondrial ROS levels (Figure VII in the online-only Data Supplement). The antioxidant N-Acetyl-cysteine diminished VDAC1/Grp75/IP3R1 complex formation, suggesting that ROS plays a role in mediating this Ca^{2+} channeling complex (Figure VIII in the online-only Data Supplement).

**Discussion**

The present study demonstrates that the enhanced interplay between ER and mitochondria in endothelial cells and the subsequent increased Ca^{2+} flux from ER to mitochondria through the VDAC1/Grp75/IP3R1 complex may play important roles in H/R injury. Acetylcholine could reduce both intracellular and mitochondrial Ca^{2+} levels and sustain ER Ca^{2+} content, thus protecting vascular endothelial cells based on the restoration of mitochondrial membrane potential, preservation of endothelial ultrastructure, inhibition of proapoptotic signals, and reduction in LDH release. These effects were likely mediated by M3AChR and the PI3K/Akt signaling pathway. Disruption of ER–mitochondria interaction via the down-regulation of VDAC1 or mitofusin 2 reduced the increased interaction within VDAC1/Grp75/IP3R1 complex and protected against H/R injury. Importantly, acetylcholine could modulate ER–mitochondria Ca^{2+} cross talk by suppressing the VDAC1/Grp75/IP3R1 complex and mitofusin 2 expression. PI3K siRNA diminished acetylcholine-mediated inhibition of mitochondrial Ca^{2+} overload, as well as VDAC1/Grp75/IP3R1 complex formation induced by H/R, indicating the involvement of PI3K/Akt pathway in the benefits of acetylcholine. These results suggest that the ER–mitochondria interaction may be a novel molecular target for endothelial protection and provide insights into the cellular protective mechanisms of acetylcholine.

Cumulative evidence suggests that Ca^{2+} overload and altered Ca^{2+} handling play important roles in the induction of cell death during reperfusion or reoxygenation. Our results demonstrated that Ca^{2+} overload (both cytosolic and mitochondrial) and depletion of the Ca^{2+} pool in the ER occurred during H/R in endothelial cells, consistent with previous observations in cardiomyocytes. Thus, understanding endothelial Ca^{2+}-handling changes during H/R processes is important. This impairment in cellular Ca^{2+} signaling could affect endothelial function, and in the worst case, excess mitochondrial Ca^{2+} and ER stress could both trigger apoptotic pathways. Our findings suggest that, in response to H/R stimuli, mitochondrial Ca^{2+} overload can induce mitochondrial membrane depolarization and coordinated cytochrome C release from mitochondria, culminating in cell death. In addition, low levels of Ca^{2+} in the ER result in ER stress, triggering cellular apoptosis through activation of caspase 12.

Another important factor is the interplay between the ER and mitochondria. Ca^{2+} exchange between the 2 organelles has been implicated in myocardial I/R injury, and ER–mitochondria communication may amplify reperfusion injury. Our study demonstrated that during H/R in endothelial cells, the interactions among partners of the VDAC1/Grp75/IP3R1 complex were significantly increased.
concomitantly with mitochondrial Ca\textsuperscript{2+} overload and cell death, which is consistent with a previous report in cardiomyocytes.\textsuperscript{24} siRNA removal of VDAC1 decreased protein interactions within the complex and attenuated mitochondrial Ca\textsuperscript{2+} overload and endothelial cell death. Furthermore, mitofusin 2 has been proposed as a key protein that bridges the ER and mitochondria and provides the physical basis of their intercommunication during Ca\textsuperscript{2+} signaling.\textsuperscript{20,25} Recent studies...
showed that the association of sarcoplasmic reticulum and mitochondria was decreased in mitofusin 2-knockout cardiomyocytes based on the reduced cardiomyocyte sarcoplasmic reticulum–mitochondrial contact length. Our results demonstrated that mitofusin 2 siRNA disrupted ER–mitochondria tethering in H/R-injured HUVECs. Downregulation of mitofusin 2 similarly decreased the Ca$^{2+}$ channeling complex and blunted reoxygenation injury. These results suggest that Ca$^{2+}$ cross talk between the ER and mitochondria in endothelial cells plays an important role in H/R injury.

Autonomic imbalance, characterized by suppressed vagal activity and increased sympathetic activity, is correlated with various pathological conditions including I/R injury. Conventionally, pharmacological interventions have primarily suppressed sympathetic overactivation, whereas vagal modulation has always been neglected. More recently, the importance of elevating vagal activity has been discussed.\textsuperscript{7–9,26} A growing body of evidence indicates that the vagal nerve and its neurotransmitter acetylcholine play defensive roles in the pathogenesis of cardiovascular diseases\textsuperscript{27}; however, few studies have explored the effects of acetylcholine treatment on Ca$^{2+}$ regulation in endothelial cells in the context of I/R. Importantly, this study demonstrated that acetylcholine reduced both mitochondrial and cytosolic Ca$^{2+}$ overload that inhibiting the Ca$^{2+}$-related apoptotic pathway, leading to attenuation of cell death and preservation of endothelial structure. Acetylcholine acts on either muscarinic or nicotinic AChR to exert its effect. Addition of atropine (but not hexamethonium) significantly reversed the effect of acetylcholine on Ca$^{2+}$ homeostasis, indicating that the effect of acetylcholine on Ca$^{2+}$ modulation is mediated primarily by muscarinic AChR. Our data conflict with reports documenting the functional importance of nicotinic AChR in I/R injury.\textsuperscript{10,28} It is likely that nicotinic AChR, especially the $\alpha_7$ receptor subunit, mediates the anti-inflammatory effects and contributes to the cardiovascular protective properties of the nicotinic component. This may be responsible for the absence of a nicotinic AChR protective role in Ca$^{2+}$ modulation observed here. In our study, the M3AChR-specific antagonist 4-DAMP prevented the antiapoptotic effects of acetylcholine, suggesting an important role of M3AChR in acetylcholine-induced endothelial protection from H/R injury.\textsuperscript{29} Moreover, previous studies have demonstrated that choline, an agonist of M3AChR, reduces intracellular Ca$^{2+}$ overload in cardiomyocytes.\textsuperscript{30–32} However, additional studies are required to identify the link between M3AChR and attenuation of Ca$^{2+}$ overload.

We further explored the signaling pathway mediated by acetylcholine in endothelial cells. Previous studies have demonstrated that acetylcholine and vagal nerve stimulation prevent cardiomyocyte apoptosis induced by multiple pathological insults, including ischemia or burn injury, likely through the PI3K/Akt pathway.\textsuperscript{43,33} Consistent with those results, we found that the antiapoptotic effects of acetylcholine were reversed by the PI3K inhibitor LY294002. In addition, we found that acetylcholine treatment balanced the reduction of eNOS expression and phosphorylation after H/R and L-NAME abrogated the effects of acetylcholine, suggesting that acetylcholine likely acts, at least in part, by increasing eNOS levels. These data are in agreement with the hypothesis that PI3K and Akt are important upstream regulators of eNOS activation\textsuperscript{34} and the fact that acetylcholine increases the production of NO from cardiomyocytes.\textsuperscript{35} Acetylcholine stimulates the release of soluble mediators in vascular endothelium; the main relaxing factors include NO, arachidonic acid metabolites, and endothelium-derived hyperpolarizing factors.\textsuperscript{36} In the present study, we explored the potential role of NO in mediating the protective effects of acetylcholine on endothelial cells. However, it remains possible that other factors, such as arachidonic acid metabolites and endothelium-derived hyperpolarizing factors, contribute to acetylcholine-mediated endothelial protection. Previous studies have demonstrated that cytochrome P-450 metabolites of arachidonic acid protect human endothelial cells by inhibiting apoptotic pathways via the PI3K/Akt survival signaling cascade.\textsuperscript{37} Thus, it is of interest to further investigate the potential involvement of arachidonic acid metabolites and endothelium-derived hyperpolarizing factors in protection elicited by acetylcholine in the future.

Notably, endothelial NO, on stimulation by acetylcholine, may be partially involved in acetylcholine-mediated Ca$^{2+}$ handling. Previous studies have demonstrated that in vascular endothelial cells, NO donor enhances ER Ca$^{2+}$ uptake and inhibits mitochondrial Ca$^{2+}$ overload.\textsuperscript{38,39} Importantly, acetylcholine prevented the increased interaction within the VDAC1/Grp75/IP3R1 complex and mitofusin 2 expression, which explains, at least in part, its protective effect against endothelial injury induced by H/R. It is noteworthy that the decrease in mitochondrial Ca$^{2+}$ content paralleled a similar decrease in mitochondrial ROS levels by acetylcholine, suggestive of a possible correlation between mitochondrial Ca$^{2+}$ levels and ROS generation. N-Acetyl-L-cysteine suppressed VDAC1/Grp75/IP3R1 complex formation, suggesting a role for ROS in mediating this Ca$^{2+}$ channeling complex. Thus, it is plausible to speculate that the inhibition of ROS, at least in part, is responsible for acetylcholine-mediated protection.

Acetylcholine shows a remarkable ability to modulate Ca$^{2+}$ handling and organ homeostasis. A major difference between a simple pharmacological inhibition of calcium channel and improved vagal activity is that the latter represents an already existing physiological protective mechanism that requires reinforcement. The possibility of fine tuning the complex pathophysiologic process involving Ca$^{2+}$ modulation and ER/mitochondria homeostasis is especially attractive. The present in vitro study in HUVECs yielded promising results, revealing a novel mechanism underlying the endothelial protection afforded by acetylcholine against H/R. Additional studies on the effects of acetylcholine or improved vagal activity on Ca$^{2+}$ modulation in vivo in experimental animals are required. In addition, the acetylcholine concentration used in this study seems higher than acetylcholine levels in vivo (presumably in response to vagal stimulation), and this may be a limitation of the present study. In general, the acetylcholine concentration in whole blood is low under physiological conditions.\textsuperscript{40} Using a microdialysis technique, Kawada et al demonstrated that acetylcholine level in the rabbit left ventricle induced by vagal stimulation is at the nanomolar level.\textsuperscript{41} In the synaptic space after quantal release, the amount of acetylcholine can reach the millimolar range.\textsuperscript{42,43} Moreover, the concentration of
acetylcholine for therapy in the pathological conditions is usually higher than the physiological level. In the present study, treatment with acetylcholine (10^{-4}–10^{-2} mol/L) attenuated H/R-induced Ca^{2+} elevation in a concentration-dependent manner, in which acetylcholine (10^{-1} mol/L) more effectively protected against calcium overload and showed dominant endothelial protection. Therefore, we chose 10^{-1} mol/L acetylcholine for our experiments, which is consistent with previous studies. Overall, our data suggest that acetylcholine prevented H/R-induced intracellular and mitochondrial Ca^{2+} overload and inhibited the apoptotic pathway in endothelial cells, presumably through the M3AChR and PI3K/Akt pathway. Notably, acetylcholine depressed ER–mitochondria interaction at reperfusion by inhibiting VDAC1/Grp75/IP3R1 complex and mitofusin 2 expression, thereby preventing mitochondrial Ca^{2+} overload and subsequent cell death (Figure IX in the online-only Data Supplement). The present findings provide novel insights into the mechanism of endothelial I/R injury and reveal novel therapeutic perspectives. The ER–mitochondria interaction may be a new molecular target for vascular endothelial protection. These results also increase our understanding of acetylcholine-mediated favorable effects, contributing to the development of future therapeutic strategies and pharmacological manipulations aimed at exploiting this endogenous protective mechanism to prevent or alleviate I/R injury.

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Disclosures
None.

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2. He X, Zhao M, Bi XY, Yu XJ, Zang WJ. Delayed preconditioning prevents mitochondrial Ca^{2+} overload and subsequent cell death (Figure IX in the online-only Data Supplement). The present findings provide novel insights into the mechanism of endothelial I/R injury and reveal novel therapeutic perspectives. The ER–mitochondria interaction may be a new molecular target for vascular endothelial protection. These results also increase our understanding of acetylcholine-mediated favorable effects, contributing to the development of future therapeutic strategies and pharmacological manipulations aimed at exploiting this endogenous protective mechanism to prevent or alleviate I/R injury.

ER–Mitochondria Interplay in Endothelial H/R


**Significance**

Interorganelle cross talk is generally underestimated; however, the functional relationship between the endoplasmic reticulum and mitochondria and the pathophysiological relevance of this interaction has been increasingly recognized. We show that enhanced interplay between the endoplasmic reticulum and mitochondria plays important roles in endothelial cells during hypoxia/reoxygenation through Ca2+ flux from endoplasmic reticulum to mitochondria via the VDAC1/Grp75/P38R1 complex, suggesting novel molecular targets for endothelial protection. Vagal nerve activation is currently being explored for the treatment of cardiovascular diseases. We show that ACH prevents hypoxia/reoxygenation-induced intracellular and mitochondrial Ca2+ overload and inhibits the apoptotic pathway in endothelial cells, presumably through the M3A-Chr and P38K/Akt pathway. Notably, ACh depresses the endoplasmic reticulum–mitochondria interaction by inhibiting voltage-dependent anion channel-1 (VDAC1)/glucose-regulated protein 75/insolit 1,4,5-trisphosphate receptor 1 complex and mitofusin 2 expression, thereby preventing mitochondrial Ca2+ overload and subsequent cell death. These results increase our understanding of acetylcholine-mediated favorable effects, indicating that vagal modulation is a promising strategy to alleviate reperfusion injury.
Reduction of Mitochondria–Endoplasmic Reticulum Interactions by Acetylcholine Protects Human Umbilical Vein Endothelial Cells From Hypoxia/Reoxygenation Injury

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Materials and Methods

Cell culture. The HUVEC line was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA) (ATCC code: CRL-1730) and was cultured at 37°C under a humidified 5% CO₂ atmosphere in Ham’s F12K medium (Macgene Biotech Co., Ltd, Beijing, China), supplemented with 10% fetal bovine serum (HyClone, Logan, UT, USA), 0.03 mg/mL endothelial cell growth supplement (Macgene Biotech Co., Ltd), 100 U/mL penicillin (Sigma, St. Louis, MO, USA), and 100 μg/mL streptomycin (Sigma). The 4th to 8th passages of HUVECs were used for this study.

Treatment protocol. Before experimental intervention, confluent cultured cells were serum starved (fetal bovine serum was eliminated in all of the following experiments) for 12 h. A hypoxic condition was produced in modified ischemia-mimetic solution (in mmol/L: NaCl, 135; KCl, 8; NaH₂PO₄, 0.33; MgCl₂, 0.5; Hepes, 5.0; CaCl₂, 1.8; and lactate, 20, pH 6.80) and hypoxia (1% O₂, 5% CO₂, and 94% N₂) for 8 h, as described previously. After hypoxia, the culture medium was rapidly replaced with fresh F12K, and the plates were transferred to a normoxic incubator (95% air and 5% CO₂) for 2 h of reoxygenation. The following experimental groups were included: (1) Con: normoxia and cultured with F12K; (2) ACh: administration of ACh (10⁻⁵ mol/L) alone; (3) H/R group: hypoxia for 8 h followed by reoxygenation for 2 h; (4) H/R + ACh group: administration of ACh (10⁻⁵ mol/L) at the onset of reoxygenation; (5) Atro group (H/R + ACh + atropine): atropine (non-specific inhibitor of MACHR, 10⁻⁵ mol/L) was co-applied with ACh (10⁻⁵ mol/L); (6) Hex group (H/R + ACh + hexamethonium): nicotinic ACh receptor (NACHR) non-specific blocker hexamethonium (10⁻⁵ mol/L) was used in combination with ACh (10⁻⁵ mol/L).

To further clarify the potential mechanism of the ACh-mediated protective effect, 4-DAMP (a selective M3AChR antagonist, 10⁻⁶ mol/L), LY294002 (the PI3K inhibitor, 10⁻⁵ mol/L), and L-NAME (the NOS inhibitor, 10⁻⁴ mol/L) were used in combination with ACh (10⁻⁵ mol/L). The following experimental groups were included: Con, H/R, H/R + ACh, 4-DAMP (4-DAMP 10⁻⁶ mol/L + ACh + H/R), LY (LY294002 10⁻⁵ mol/L + ACh + H/R), and L-NAME (L-NAME 10⁻⁴ mol/L + ACh + H/R).

Measurement of intracellular, ER, and mitochondrial Ca²⁺ levels. The intracellular Ca²⁺ level was determined using the fluorescent Ca²⁺ indicator Cal-520 acetoxymethyl ester (Cal-520 AM) (AAT Bioquest, Sunnyvale, CA, USA) according to the manufacturer’s instructions. Briefly, after different treatments, HUVECs (4 × 10⁴ cells/well) were cultured on a 24-well plate and then loaded with 5 μmol/L Cal-520 AM and 0.02% Pluronic F-127 (AAT Bioquest) for 2 h at 37°C in the dark in modified Tyrode buffer with the following composition (in mmol/L: NaCl, 135; KCl, 5.4; CaCl₂, 1.8; MgSO₄, 1.0; glucose, 10.0; and HEPES, 5.0 at pH 7.4). After incubation, the dye loading medium was replaced with pre-warmed modified Tyrode buffer and imaged with a fluorescence microscope (TE-2000U, Nikon, Tokyo, Japan) at 492 nm excitation and 514 nm emission wavelengths. The obtained images were quantitatively analyzed for changes in fluorescence intensities using Image-Pro Plus 5.0 (Media Cybernetics, Silver Spring, MD, USA). Relative changes in intracellular Ca²⁺ levels were calculated by normalizing the fluorescence intensity to the control group.

ER Ca²⁺ content in HUVECs was measured as described previously, with minor modifications. HUVECs were loaded with Mag-Fura2 AM (5 μmol/L; Biotium, Hayward, CA, USA) and 0.02% Pluronic F-127 for 20 min in the dark at room temperature, and superfused with modified Tyrode buffer for 90 min at 37°C to wash away cytosolic dye. Mag-Fura2 fluorescence (380 nm excitation; 510 nm emission)
were imaged using a fluorescence microscope.

To measure mitochondrial Ca\(^{2+}\) levels, HUVECs were loaded with 10 μmol/L Rhod-2 AM (Biotium) and 0.02% Pluronic F-127 for 120 min at 4°C, followed by incubation for 30 min at 4°C, as described previously.\(^3\) This two-step cold loading/warm incubation protocol facilitates the selective accumulation of Rhod-2 in mitochondria. Fluorescence intensities of Rhod-2 AM-loaded cells were detected using the fluorescence microscope at 556 nm excitation and 576 nm emission wavelengths.

**Measurement of mitochondrial membrane potential.** Mitochondrial membrane potential was assessed using the JC-1 assay kit (Beyotime Biotech, Haimen, China) according to the manufacturer’s instructions. After the treatments, HUVECs cultured in 24-well plates were stained with JC-1 staining solution for 20 min at 37°C in the dark and rinsed twice with the buffer provided as part of the kit, which was pre-cooled at 4°C. JC-1 fluorescence was measured using a fluorescent microscope. Red emission of the dye represented a potential-dependent aggregation in the mitochondria. Green fluorescence represented the monomeric form of JC-1, appearing in the cytosol after mitochondrial membrane depolarization. The ratio of JC-1 aggregate to monomer intensity reflects changes in the mitochondrial membrane potential of HUVECs.

**Measurement of mitochondrial ROS levels**

Changes in mitochondrial ROS levels were detected using a fluorescent probe specific to anion superoxide produced in the inner mitochondrial compartment, MitoSOX Red. Briefly, HUVECs were loaded with MitoSOX Red (5 μmol/L; Invitrogen, Carlsbad, CA, USA) for 30 min at 37°C, washed twice with phosphate-buffered saline (PBS), fixed with 4% paraformaldehyde in PBS for 30 min at room temperature, washed twice with PBS, incubated for 30 min at 37°C with MitoTracker Green (200 nmol/L, Beyotime) to label mitochondria, and washed again before mounting. The fluorescent images were captured using a laser confocal microscope (Nikon C2, Nikon, Tokyo, Japan). MitoSOX Red fluorescence (excitation at 510 nm) was detected at a wavelength of 580 nm. MitoTracker Green fluorescence was observed by excitation with 490 nm and examination of emission at 516 nm.

**Isolation of cytosolic and mitochondrial fractions.** Cytosolic and mitochondrial fractions were isolated as described previously.\(^4\) Briefly, cultured HUVECs were harvested by scraping and then pelleted by centrifugation at 1000x g for 10 min at 4°C in PBS. The resulting pellet was then resuspended in 1 mL of mitochondrial isolation buffer (in mmol/L: NaCl, 10; MgCl\(_2\), 2.5; Tris, 10; pH 7.5 adjusted by HCl). After incubation in an ice bath for 10 min, the cell suspension was transferred to a Dounce glass homogenizer, and 0.7 mL of 2.5× mitochondrial suspension was added (in mmol/L: mannitol, 525; sucrose, 175; Tris, 12.5; EDTA-Na\(_2\), 1; pH 7.5 adjusted by HCl). The supernatant was purified by centrifugation at 13000 x g for 5 min at 4°C twice. The supernatant was retrieved again and centrifuged at 17000 x g for 15 min at 4°C to pellet the mitochondria. The resultant supernatant was designated as the cytosol. The pellet was resuspended in 1× mitochondrial suspension and stored at -80°C. Protein concentrations were determined using the Bicinchoninic acid protein assay kit (Beyotime Biotech).

**Protein preparation and Western blotting.** HUVECs\(^3\) were lysed in RIPA buffer (Beyotime Biotech) containing 1 mmol/L phenylmethylsulfonyl fluoride. Cell lysate was centrifuged at 13000 rpm for 10 min at 4°C, and the supernatants were used as sample protein. Samples (30 μg) were separated on SDS-polyacrylamide gels and transferred to nitrocellulose membranes (Millipore, Billerica, MA, USA). After blocking
with 5% nonfat milk in Tris-buffered saline supplemented with 0.1% Tween 20 for 1 h at room temperature, the membranes were incubated at 4°C overnight with primary antibody against Akt (diluted 1:500; Signalway Antibody, Pearland, TX, USA), phospho-Akt (Ser473; diluted 1:1000; Cell Signaling Technology Inc., Beverly, MA, USA), eNOS (diluted 1:500; Signalway Antibody), phospho-eNOS (Ser1177; diluted 1:500; Cell Signaling Technology), SERCA2 (diluted 1:500, Fantibody; New York, USA), Bcl-2 (diluted 1:1000; Cell Signaling Technology), caspase 12 (diluted 1:1000; Abnova, Walnut, CA, USA), Mfn2 (diluted 1:1000, Millipore), PI3K (diluted 1:1000; Millipore), VDAC1 (diluted 1:1000; Cell Signaling Technology, Santa Cruz, CA, USA) or IP3R1 (diluted 1:1000; Cell Signaling Technology). The membranes were then incubated with the appropriate horseradish peroxidase-conjugated secondary antibodies (Signalway Antibody) at 1:5000 dilution for 30 min at room temperature. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH; diluted 1:5000, Sinopept; Beijing, China) was also determined and used as an internal loading control. The bands were visualized with ECL-Plus reagent (Millipore), and graphs were analyzed using the Gel-Pro Analyzer (Media Cybernetics, Bethesda, MD, USA). Cytochrome C was detected using the antibody against cytochrome C (diluted 1:500, Signalway Antibody) from the cytosolic and mitochondrial fractions as described previously.\(^5\)

**Measurement of lactate dehydrogenase.** Cell death was determined by measuring LDH. HUVECs were cultured in 6-well plates at 5 × 10^5 cells/well. After the different treatments, the supernatant was collected, and the amount of LDH released from cells was determined using an AU2700 automatic biochemical analyzer (Olympus, Tokyo, Japan). The data for each treatment group are expressed as a percentage of the control.

**Transmission electron microscopy.** HUVECs were harvested and fixed with 2.5% glutaraldehyde in 0.1 mol/L phosphate buffer (pH 7.2–7.4) for 2 h at 4°C and then post-fixed with 1% osmium tetroxide in 0.1 mol/L phosphate buffer for 2 h. The samples were dehydrated in a graded ethanol series, infiltrated with propylene oxide, and embedded in Epon 812. Ultrathin sections were sliced with glass knives on a LKB V ultramicrotome, stained with uranyl acetate and lead citrate, and examined using a transmission electron microscope (H-7650; Hitachi, Tokyo, Japan). ER–mitochondrial associations were quantified using NIH ImageJ software (NIH, Bethesda, MD, USA) as described previously.\(^6,7\)

**Small interfering RNA.** HUVECs were seeded into six-well plates. When the cells reached 70-80% confluence, they were transiently transfected with VDAC1 siRNA (100 nmol/L), Mfn2 siRNA, PI3K siRNA, or negative control (NC) siRNA (GenePharm, Shanghai, China) using Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. Gene silencing was monitored by Western blot of cell extracts isolated 48 h post-transfection.

**Coimmunoprecipitation.** Extracts from HUVECs (500 µg) were incubated overnight with 2 µg of anti-IP3R1 antibody (Cell Signaling Technology) or anti-Grp75 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA). On the next day, the mixture was incubated for 3 h at 4°C with Protein A Agarose (Beyotime Biotech). The pellets were collected by centrifugation, washed 3 times with RIPA buffer, and resuspended in electrophoresis sample buffer.

**Measurement of NO release.** NO levels in cell-culture supernatants were determined by measuring the concentration of nitrite (NO₂), the major metabolite of NO, with a commercially available kit (Jiancheng Bioengineering Institute, Nanjing,
China) according to the manufacturer’s instructions. Absorbance was detected at 550 nm.

**Statistical analysis.** All values are expressed as the mean ± SEM. Statistical analysis was performed using the Student’s t-test or one-way analysis of variance followed by Tukey’s multiple-comparison post hoc test. $P < 0.05$ was considered statistically significant.

**Supplemental References**


Supplementary Figure I. ACh treatment did not affect apoptotic signal cascades in control cells. Open bar, normoxia. Data represent the mean ± SEM (n = 5).
Supplementary Figure II. ACh reduced LDH release via the M3AChR and PI3K/Akt/eNOS signaling pathway. The supernatant was collected for measuring the LDH level with a biochemical detecting system after 2 h of reoxygenation. ACh treatment reduced cell injury indicated by LDH release. These effects of ACh were abrogated by treatment with atropine (Atro, 10⁻⁵ mol/L), M3AChR antagonist 4-DAMP (10⁻⁶ mol/L), LY294002 (LY, 10⁻⁵ mol/L), or L-NAME (10⁻⁴ mol/L). Open bar, normoxia; filled bar, H/R. Data represent the mean ± SEM (n = 6). ***P < 0.001 vs Con; ##P < 0.01 vs H/R; &P < 0.05, &&P < 0.01 vs ACh-treated H/R group.
Supplementary Figure III. The silencing efficiency of VDAC1 siRNA (A), Mfn2 siRNA (B), and PI3K siRNA (C) was detected by Western blot. Data represent the mean ± SEM (n = 4). **P < 0.01 vs NC siRNA group.
Supplementary Figure IV. Downregulation of VDAC1 or Mfn2 decreased LDH release and protected HUVECs from H/R injury. Data represent the mean ± SEM. (n = 6). ***P < 0.001 vs NC siRNA-treated control group; #P < 0.05, ##P < 0.01 vs NC siRNA-treated H/R group.
Supplementary Figure V. Mfn2 siRNA disrupts ER–mitochondria tethering in H/R-injured HUVECs. Transmission electron micrographs images (A) (red arrowheads depicting the ER–mitochondria contacts) and measurements of the ER–mitochondria interactions (B). Scale bar = 200 nm. Data are the mean ± SEM. A total of 108–116 ER–mitochondria contacts were analyzed from each of the groups. **P < 0.01, ***P < 0.001 vs NC siRNA-treated control group; ###P < 0.001 vs NC siRNA-treated H/R group.
**Supplementary Figure VI.** ACh administration decreased H/R-induced mitochondrial ROS levels. For the mitochondrial ROS assay, the MitoSOX Red fluorescence was colocalized with MitoTracker Green. Open bar, normoxia; filled bar, H/R. Data represent the mean ± SEM (n = 4). *P < 0.001 vs Con; #P < 0.001 vs H/R. Scale bar = 50 μm.
Supplementary Figure VII. Mfn2 siRNA decreased H/R-induced mitochondrial ROS levels. For the mitochondrial ROS assay, the MitoSOX Red fluorescence was colocalized with MitoTracker Green. Data represent the mean ± SEM (n = 4). *P < 0.001 vs NC siRNA-treated control group; #P < 0.001 vs NC siRNA-treated H/R group. Scale bar = 50 μm.
Supplementary Figure VIII. ROS mediates the VDAC1/GRP75/IP3R1 complex. Treatment with NAC reduced H/R-induced increased VDAC1/Grp75/IP3R1 interactions. Data are the mean ± SEM (n = 3). **P < 0.05, ***P < 0.001 vs Con; #P < 0.05, ##P < 0.01, ###P < 0.001 vs H/R.

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![Bar graph showing protein interaction](image-url)
Supplementary Figure IX. Proposed schematic illustration of the mechanism by which ACh exerts its endothelial protective effects against H/R injury in the present study. ACh prevents both intracellular and mitochondrial Ca\(^{2+}\) elevation and alleviates ER Ca\(^{2+}\) depletion during H/R. Consequently, ACh enhances mitochondrial membrane potential (\(\Delta \psi_m\)) and inhibits proapoptotic cascades, thereby reducing cell death and preserving endothelial ultrastructure, presumably through the M3AChR and PI3K/Akt/eNOS signaling pathway. Notably, ACh depresses the ER–mitochondria interaction by inhibiting VDAC1/Grp75/IP3R1 complex and Mfn2 expression, thereby preventing mitochondrial Ca\(^{2+}\) overload and subsequent cell death. Red circle represents Ca\(^{2+}\).