

Endothelial protective effect of rapamycin against simulated ischemia injury through up-regulation of autophagy and inhibition of endoplasmic reticulum stress

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Submitted: 21 October 2019

Accepted: 16 February 2020

Arch Med Sci Civil Dis 2020; 5: e14–e21
DOI: <https://doi.org/10.5114/amscd.2020.94101>
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Abstract

Introduction: Rapamycin has been shown to have cytoprotective properties in some experimental models of ischemia. However, the precise molecular mechanisms underlying the positive effect of rapamycin on endothelial cells in ischemic injury remain unknown. It is very important because endothelial cells are firstly exposed to ischemia and play an important role in ischemic organ damage. Autophagy and endoplasmic reticulum stress are suggested to be implicated in hypoxic/ischemic injury of endothelial cells. This study aims to explore whether the endothelial protective effect of rapamycin is associated with exacerbation of autophagy and attenuation of endoplasmic reticulum stress.

Material and methods: The protective effects of rapamycin against oxygen and glucose deprivation (OGD)-induced cell injury were explored in human vascular endothelial cells (HUVECs). Cell viability was measured by MTT assay. The protein levels of Beclin 1, p62, p-mTOR, p-S6K, p-4EBP, GRP78, p-PERK and p-IRE1 were analyzed using immunoblotting.

Results: Rapamycin in the simulated ischemia model increased the cell viability, indicating its cytoprotective effect ($p < 0.05$). Experiments with 3-methyladenine as an inhibitor of autophagy and thapsigargin as an inducer of endoplasmic reticulum stress support that rapamycin exerts endothelial protective effects against OGD-induced damage via autophagy – endoplasmic reticulum stress pathway.

Conclusions: This study demonstrated that rapamycin protects ischemic HUVECs via down-regulation of the mTOR pathway, enhancement of autophagy and inhibition of endoplasmic reticulum stress.

Key words: rapamycin, autophagy, ER stress, endothelial cells, ischemia.

Introduction

Autophagy is a catabolic process that is responsible for the elimination of aggregated proteins and damaged organelles [1]. In the initial step of autophagy the cytoplasmic components are sequestered in double-membrane vesicles, referred to as autophagosomes. Autophagosomes fuse with acidified endosomal and/or lysosomal vesicles to generate autolysosomes, where the luminal content is degraded and recycled [2]. The essential role of autophagy in most cells is the basal turnover of cellular

components and maintenance of the function [1]. Autophagy principally is an important adaptive mechanism under different stress conditions such as exposure to hypoxia, ischemia or starvation [3]. Under ischemic/hypoxic stress induction of autophagy is considered as a process that promotes cell survival and counteracts apoptosis [4]. However, excessive autophagy may destroy essential cellular compounds and lead to cell death [2].

Beclin 1 and p62/SQSTM1 (p62) are the main autophagy markers. Beclin 1, a BH3-only domain protein, forms a complex with class III phosphoinositide 3-kinase (PI3K) responsible for autophagic vesicle nucleation and autophagy induction [5]. The p62 binds directly to microtubule-associated protein 1 light chain 3 (LC3) protein, is incorporated into the autophagosomes and degraded in autolysosomes. A measure of p62 expression is commonly used as a marker to study autophagic flux [6].

Accumulating evidence suggests that ischemic organ damage is linked to autophagy. In vivo studies performed in experimental models of ischemia show the increased expression of Beclin 1 and LC3-II [7–9]. However, it is still controversial whether autophagy is beneficial or fatal to ischemic tissue. Furthermore, the impact of autophagy induced by ischemia on endothelial cells (ECs) is unknown. ECs play a key role in regulation of blood flow, suppression of vascular inflammation and thrombosis as well as in release of trophic factors. Injury of ECs leads to platelet activation, increased reactive oxygen species production, inflammation in blood vessels and progression of ischemic tissue damage [10]. For this reason, the identification of the molecular mechanism of autophagy in ECs constitutes a potential target for the development of possible strategies to minimize ischemic injury.

Ischemia also contributes to the aggregation of misfolded or unfolded proteins within the endoplasmic reticulum (ER), which results in ER stress and unfolded protein response (UPR) [11]. There are three major ER-resident proteins involved in UPR, namely protein-kinase-RNA like ER kinase (PERK), inositol-requiring enzyme 1 (IRE1) and activating transcription factor (ATF6) [12]. Physiologically, their activity is suppressed by binding of the intraluminal chaperone glucose regulated protein 78 (GRP78). In response to ER stress, GRP78 is released and binds to the unfolded proteins, which leads to the activation of PERK, IRE1 and ATF6 [12]. Furthermore, there is evidence that ER stress is linked to autophagy [13, 14].

Numerous studies have demonstrated that PI3K/Akt/mTOR axis is involved in ischemic injury in various pathophysiological conditions [7, 15, 16]. mTOR has been reported to play a sig-

nificant role in regulation of metabolism, gene transcription, protein synthesis and degradation, autophagy, apoptosis and necroptosis [17]. After activation, mTOR phosphorylates its downstream target proteins: ribosomal S6 kinase (S6K) and eukaryote initiation factor 4E binding protein 1 (4EBP1) [17]. It was shown that mTOR inhibition is associated with the up-regulation of autophagy and protective effects in experimental ischemia and reperfusion [7]. Rapamycin, an immunosuppressive agent and mTOR inhibitor, exerts cytoprotective effects in both cell and animal models of ischemia [18, 19]. The available data suggest that rapamycin activates protective autophagy and inhibits ER stress in ischemic neurons, hepatocytes and nephrons [20–22]. However, whether up-regulation of autophagy and attenuation of ER stress by rapamycin has a protective effect on ischemic ECs remains to be elucidated.

In this paper the ability of rapamycin to induce autophagy and attenuate ER stress in human umbilical vein endothelial cells (HUVECs) following in vitro simulated ischemia injury was determined.

Material and methods

Cell culture and treatment

HUVECs (Lonza, USA) from passages 3 through 5 were cultured in EGM-2 medium (Lonza) supplemented with EGM-2 Bullet Kit (Lonza) under standard conditions. For simulated ischemia (3% O₂, no glucose), confluent cells in DMEM without glucose were transferred to a hypoxic chamber (Eppendorf Inc., USA) which was flushed with 92% N₂, 5% CO₂ and 3% O₂ at 37°C. Cells were maintained under oxygen-glucose deprivation (OGD) for 6 h and treated with 100 nM rapamycin (Rapa, Sigma-Aldrich, USA) without/with 3-methyladenine (3-MA, Sigma-Aldrich, 5 mM) or thapsigargin (TG, Sigma-Aldrich, 1 μM). The control groups (Normoxia) were placed in EGM-2 with EGM-2 Bullet Kit in the incubator with normal O₂ content.

Cell viability

The cell viability was assessed by [3(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT, Sigma-Aldrich) assay using a final concentration of MTT of 0.25 mg/ml and 3 h incubation. The cells were lysed in 100 μl DMSO and optic density (OD) was measured on the microplate reader at 570 nm.

Western blot

Cells were homogenized in RIPA lysis buffer. Twenty μg lysates per sample were separated on 10% SDS-PAGE gels, which were then transferred to PVDF membranes. After blocking with 5% dry

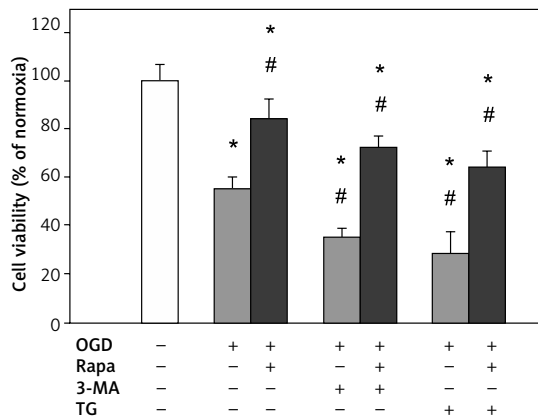


Figure 1. Rapamycin (Rapa) attenuates and 3-methyladenine (3-MA) and thapsigargin (TG) potentiate oxygen and glucose deprivation (OGD)-induced injury of HUVECs. Cell survival was measured by MTT assay

Results are mean \pm SD ($n = 8$), * $p < 0.05$ vs. Normoxia # $p < 0.05$ vs. OGD.

milk in TBST, the implied primary and secondary antibodies were added. Afterwards, the blots were developed using ECL reagents and OD was quantified with ImageJ software. The following primary antibodies purchased from Santa Cruz Biotechnology (Santa Cruz, USA) and dilutions were used: Beclin 1 (1/500), p62 (1/2000), p-mTOR (1/1000), mTOR (1/1000), p-S6K (1/500), S6K (1/500), p-4EBP1 (1/500), 4EBP1 (1/500), GRP78 (1/1000), p-PERK (1/1000), PERK (1/1000), p-IRE1 (1/1000), IRE1 (1/1000) and β -actin (1/1000).

Statistical analysis

Statistical significance was determined by one-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparison test. A value of $p < 0.05$ was accepted as statistical significance.

Results

Rapamycin increases and 3-MA and TG decrease cell viability

To determine the ability of rapamycin to protect OGD-injured HUVECs through autophagy activation and ER stress attenuation, the cells were treated with 3-MA and TG. 3-MA is a widely used inhibitor of autophagy acting by PI3K inhibition, and thus it suppresses autophagosome formation [23]. TG, a specific inhibitor of ER Ca^{2+} -dependent ATPase, is ER stress inducer. Furthermore, it was shown that TG blocks autophagosome-lysosome fusion and inhibits autophagic flux [24]. The concentrations of 3-MA and TG (5 mM and 1 μM , respectively) were selected on the basis of studies carried out on HUVECs [25, 26].

The viability of HUVECs was significantly reduced by 6 h OGD treatment to 55% ($p < 0.05$,

Figure 1). Compared with both Normoxia and OGD groups, 3-MA and TG substantially reduced HUVECs viability to 35% and 29%, respectively ($p < 0.05$, Figure 1). In preliminary dose-response experiments, rapamycin (10–1000 nM) did not have any influence on cell survival for 6 h in normoxia (data not shown). In the present study, the cells were incubated with 100 nM rapamycin. At this concentration, the autophagy induction and mTOR inhibition in ischemic HUVECs was observed (Figures 2 and 3). Moreover, the cell viability was significantly increased after simultaneous treatment with rapamycin with 3-MA or TG under OGD to 72% and 65% respectively, as compared with the OGD alone ($p < 0.05$).

Rapamycin counters the inhibitory effects of 3-MA and TG on autophagy

The expressions of Beclin 1 and p62 as important autophagy biomarkers were selected to assess the activation autophagy flux in HUVECs. As shown in Figure 2, a significant increase in Beclin 1 expression was observed following OGD ($p < 0.05$). Furthermore, 3-MA and TG treatment attenuated Beclin 1 expression, which was coincident with significant p62 up-regulation and reduced degradation ($p < 0.05$). Rapamycin significantly increased both the Beclin 1 level and p62 degradation (Figure 2, $p < 0.05$). Furthermore, the drug was able to effectively counteract the blockade of autophagy flux by both 3-MA and TG treatment during OGD.

OGD and rapamycin inhibit mTOR signaling pathway

The mTOR pathway plays an essential role in pathophysiology of ischemic injury [27]. mTOR is a negative regulator of autophagy, e.g. activation of mTOR resulted in decreased autophagic flux [28]. The expressions of phosphorylated forms of S6K and 4EBP1 are indicators of mTOR activity [27].

As shown in Figure 3, the expressions of p-mTOR, p-S6K and p-4EBP1 were significantly decreased after OGD and rapamycin in comparison to normoxic cells ($p < 0.05$). In addition, total mTOR, S6K and 4-EBP1 levels were unchanged in all experimental groups. The results suggest that the mTOR pathway was effectively suppressed by rapamycin in this experimental model.

Rapamycin attenuates ER stress in OGD-injured HUVECs

To determine whether attenuation of ER stress is involved in the endothelial protective effect of rapamycin against OGD-induced injury, the expressions of ER stress-related proteins were determined. The analysis of Western blot data showed that the ex-

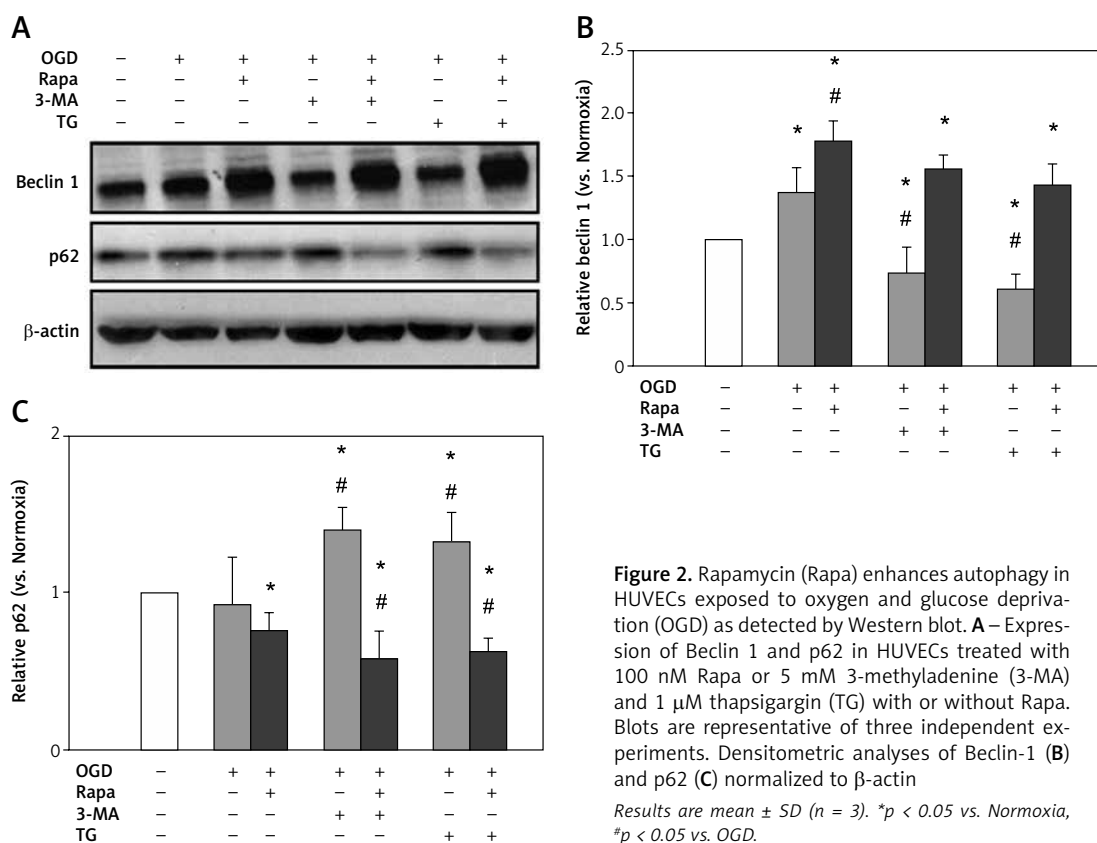


Figure 2. Rapamycin (Rapa) enhances autophagy in HUVECs exposed to oxygen and glucose deprivation (OGD) as detected by Western blot. **A** – Expression of Beclin 1 and p62 in HUVECs treated with 100 nM Rapa or 5 mM 3-methyladenine (3-MA) and 1 μM thapsigargin (TG) with or without Rapa. Blots are representative of three independent experiments. Densitometric analyses of Beclin-1 (**B**) and p62 (**C**) normalized to β-actin

Results are mean ± SD (n = 3). *p < 0.05 vs. Normoxia, #p < 0.05 vs. OGD.

pression levels of GRP78, p-PERK and p-IRE1 were significantly increased in ischemic HUVECs revealing OGD-induced ER stress ($p < 0.05$, Figure 4). In the rapamycin-treated group, the expression levels of ER-stress related proteins were significantly decreased compared with OGD (Figure 4, $p < 0.05$). It should be noted that the expressions of total PERK and IRE1 were not significantly changed. This demonstrated that rapamycin inhibited ER stress through down-regulating the phosphorylation of signaling proteins, e.g. p-PERK and p-IRE1.

Discussion

Autophagy is a catabolic process of degradation of cellular constituents [1]. Numerous reports have demonstrated that autophagy is impaired in ischemia/hypoxia and autophagosomes are accumulated within cells [29, 30]. Moreover, it has been proposed that autophagy dysfunction in endothelium following ischemia might be involved in a breakdown of the endothelial barrier resulting in vascular hyperpermeability and induction of autophagy in ECs has a protective effect [31, 32]. However, the involvement of ER stress-mediated autophagy in endothelial dysfunction in response to ischemic injury remains unclear. On one hand, there is evidence that ER stress might contribute to activation of autophagy [9]. But on the other, it was shown that TG, an ER stress

inductor, is able to inhibit autophagy. TG-inhibited autophagy might result from block fusion of autophagosomes with lysosomes and failure of autophagic flux [24]. The presented results from MTT assay clearly indicate that both inhibition of autophagy by 3-MA and activation of ER stress by TG decreased viability of HUVECs under OGD (Figure 1). This is consistent with the observations by Xie *et al.* [33], who described the increased advanced glycation end product (AGE)-induced injury of HUVECs after 3-MA treatment. The authors suggested that early autophagy activation in ECs is involved in the protective mechanism and promotes cell survival [33].

It should be also noted that during normal growth conditions HUVECs expressed a relatively high basal level of autophagy as shown by marked Beclin 1 expression (Figure 2 A). The constitutive active basal autophagy in ECs might contribute to their resistance to ischemia by maintaining the sufficient level of intracellular ATP [8]. Instead, failure of basal autophagy causes the increase in ROS production that activates inflammatory processes and apoptosis [34, 35].

This study has shown that rapamycin increased cell viability following autophagy inhibition and ER stress induction in ischemic HUVECs (Figure 1). Moreover, inhibition of mTOR by rapamycin clearly raised Beclin 1 level and p62 degradation in

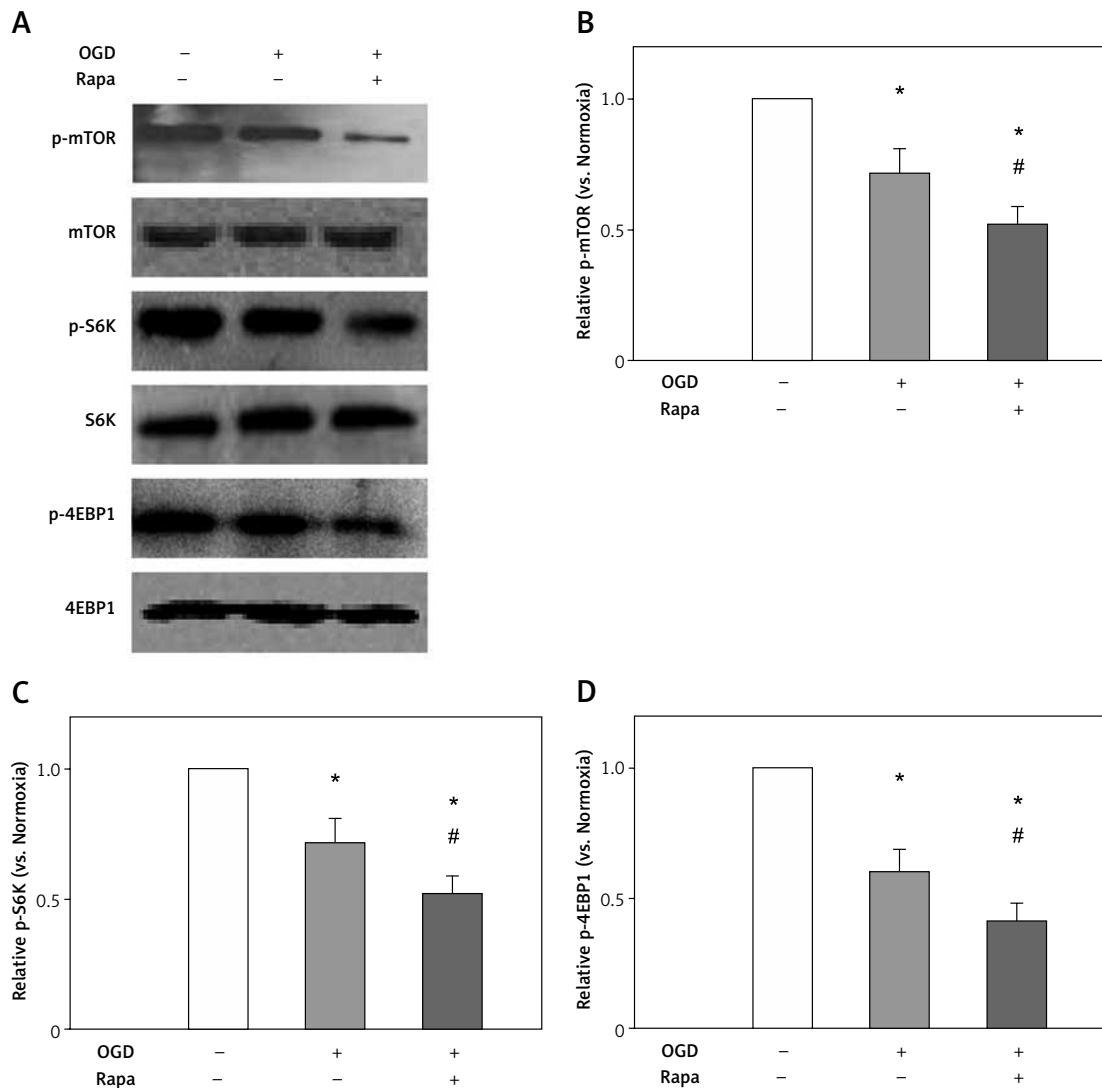


Figure 3. Rapamycin (Rapa) inhibits the mTOR pathway in HUVECs exposed to oxygen and glucose deprivation (OGD) as detected by Western blot. **A** – Expression of p-mTOR, mTOR, p-S6K, S6K, p-4EBP1 and 4EBP1 in HUVECs treated with 100 nM Rapa. Blots are representative of three independent experiments. Densitometric analyses of p-mTOR (**B**), p-S6K (**C**) and p-4EBP1 (**D**) normalized to mTor, S6K and 4EBP1, respectively

Results are mean \pm SD ($n = 3$). * $p < 0.05$ vs. Normoxia, # $p < 0.05$ vs. OGD.

HUVECs treated with/without 3-MA or TG in OGD (Figure 2). The role of the mTOR pathway in ECs in various vascular disorders remains controversial. The inhibition of mTOR pathway using curcumin protects HUVECs from oxidative stress and activates of autophagy [30]. However, Xie *et al.* [36] showed that α -lipoic acid through mTOR activation reduces OGD-induced endothelial injuries, thereby suggesting that down-regulation of the mTOR pathway may be unfavorable to ischemic ECs. It seems that these differences may result from distinct cell types and experimental ischemic models. In this study, rapamycin used as a mTOR inhibitor and autophagy agonist effectively protected ECs against ischemic injury.

mTOR is known as an universal regulator of autophagy. In experimental models of the cerebral

stroke, it was shown that inhibition of mTOR by rapamycin activated autophagy in penumbra and had a neuroprotective effect [7, 18]. This study also demonstrated that mTOR is a negative regulator of protective autophagy in OGD-injured HUVECs. Furthermore, the activation of autophagy by rapamycin in ischemic ECs was connected to the decreased phosphorylation levels of mTOR, S6K and 4EBP1 (Figure 3).

The molecular mechanism leading to the autophagy activation by rapamycin remains unknown. The available data indicate that rapamycin inactivates mTORC1, thereby enhancing autophagosome formation through ulc-51-like autophagy activating kinase 1 (ULK1) [37]. In addition, the autophagy induced by rapamycin in Beclin 1-dependent mechanism protects against neonatal ischemia [38, 39].

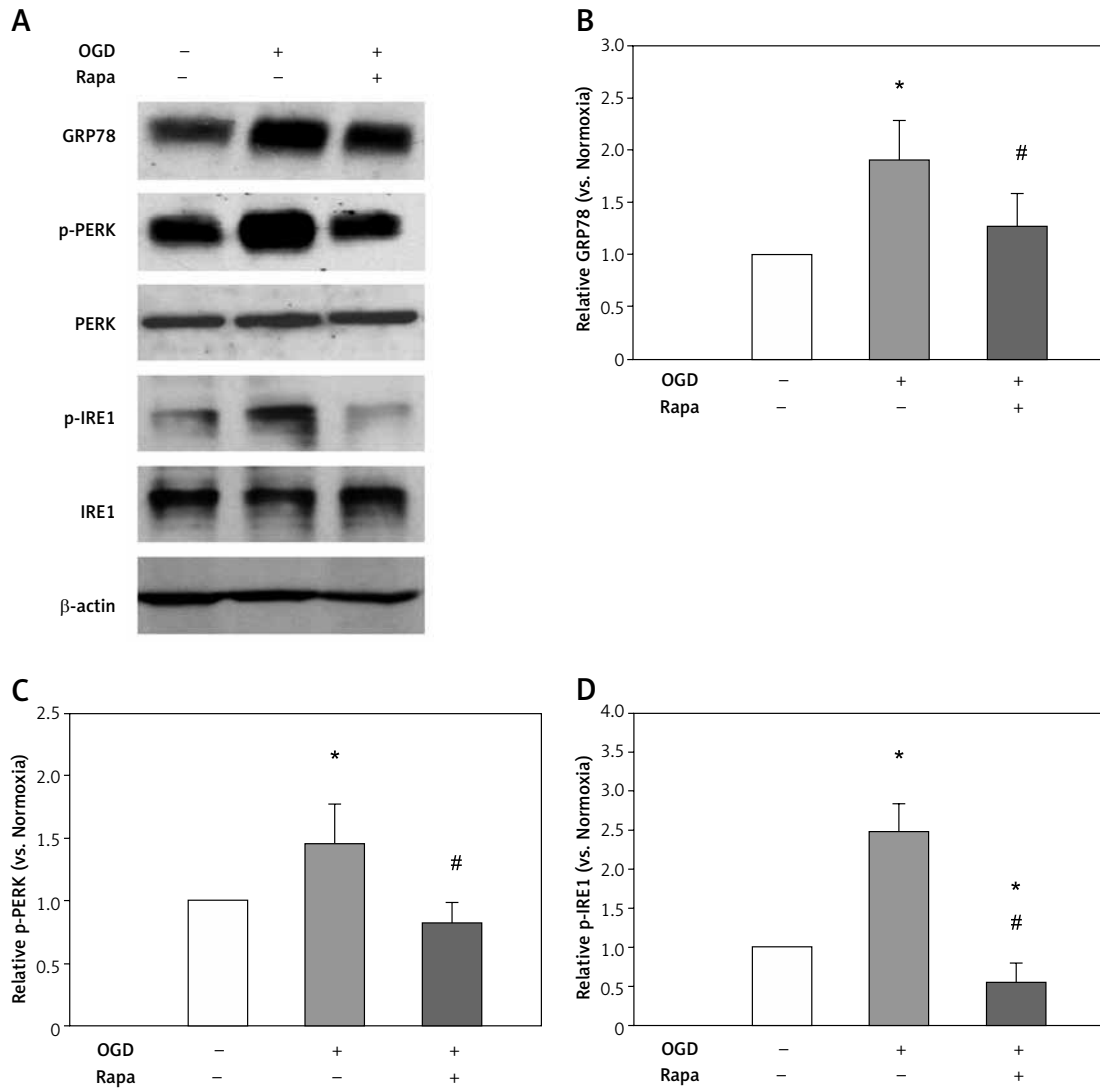


Figure 4. Rapamycin (Rapa) attenuates ER stress in HUVECs exposed to oxygen and glucose deprivation (OGD) as detected by Western blot. **A** – Expression of GRP78, p-PERK, PERK, p-IRE and IRE in HUVECs treated with 100 nM Rapa. Blots are representative of three independent experiments. Densitometric analyses of GRP78 (**B**), p-PERK (**C**) and p-IRE1 (**D**) normalized to β -actin, PERK and IRE1, respectively
 Results are mean \pm SD (n = 3). *p < 0.05 vs. Normoxia, #p < 0.05 vs. OGD.

However, as mTOR modulates a number of cellular functions, the prevalence of events other than autophagy induction could be responsible for the endothelial protective effect of rapamycin.

It has been proposed that mild ER stress is an activator of protective autophagy in ischemic stroke. However, prolonged and severe ER stress leads to apoptosis and autophagy disturbances [40]. There is some evidence suggesting the cross-talk between ER stress and the mTOR signaling pathway [41]. In addition, the link between autophagy and ER stress at the level of molecules as Beclin 1 and ER-stress related proteins was suggested [9]. Yet, little is known about the molecular mechanisms underlying the interactions between autophagy and ER stress after rapamycin treatment. It was shown that rapamycin enhances

cell survival under ER stress and protects against apoptosis [42–44]. Furthermore, significant suppression of ER-stress induced IRE1 pathway by rapamycin was described [42]. In an *in vitro* experimental model it was shown that inactivation of mTOR by rapamycin leads to an increase in cell survival under ER stress and activation of autophagy [45]. This study demonstrated that rapamycin inhibits the expression of ER-stress related proteins in OGD-injured HUVECs (Figure 4). At the same time, rapamycin induces protective autophagy and inhibits mTOR pathway (Figures 2 and 3).

In the present study, endothelial cell cultures were treated with rapamycin at a concentration of 100 nM which does not have toxic effects and is commonly used in scientific works on cytoprotection. The concentration range of 10–100 nM is

used in most *in vitro* studies on rapamycin in order to suppress mTORC1 and induce autophagy. The higher doses of rapamycin (0.2–20 μ M) targeting mTORC2 and having non-specific effects are far beyond that tolerated in humans [46].

In conclusion, the present study shows that OGD induces endothelial cell injury, activates autophagy, mTOR pathway and ER stress. Rapamycin has an apparent endothelial protective effect against ischemic damage through enhancement of autophagic flux together with inhibition of the mTOR pathway and ER stress-related proteins. The author is aware of the limitations of this study; the most important is that all experiments were performed only in *in vitro* conditions. Therefore, additional studies are needed to confirm these protective mechanisms of rapamycin towards ECs in experimental models of ischemia.

Acknowledgments

The work was partially supported by grant No. N N401 072139 from the Ministry of Sciences and Higher Education, Warsaw, Poland.

Conflict of interest

The authors declare no conflict of interest.

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