Resveratrol Protects against Oxidized LDL-Induced Breakage of the Blood-Brain Barrier by Lessening Disruption of Tight Junctions and Apoptotic Insults to Mouse Cerebrovascular Endothelial Cells\textsuperscript{1,2}

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Abstract

Cerebrovascular endothelial cells (CEC) comprise the blood-brain barrier (BBB). In a previous study, we showed that oxidized LDL (oxLDL) can induce apoptosis of mouse CEC. Resveratrol possesses chemopreventive potential. This study aimed to evaluate the effects of resveratrol on oxLDL-induced insults to mouse CEC and its possible mechanisms. Exposure of mouse CEC to 200 \( \mu \)mol/L oxLDL for 1 h did not cause cell death but significantly altered the permeability and transendothelial electrical resistance of the cell monolayer. However, resveratrol completely normalized such injury. As for the mechanisms, resveratrol completely protected oxLDL-induced disruption of F-actin and microtubule cytoskeletons as well as occludin and zona occludens-1 (ZO-1) tight junctions. The oxLDL-induced decreases in the mitochondrial membrane potential and intracellular ATP levels were normalized by resveratrol. Exposure of mouse CEC to 200 \( \mu \)mol/L oxLDL for 24 h elevated oxidative stress and simultaneously induced cell apoptosis. However, resveratrol partially protected against oxLDL-induced CEC apoptosis. The oxLDL-induced alterations in levels of Bcl-2, Bax, and cytochrome c were completely normalized by resveratrol. Consequently, resveratrol partially decreased oxLDL-induced activation of caspases-9 and -3. Therefore, in this study, we show that resveratrol can protect against oxLDL-induced damage of the BBB through protecting disruption of the tight junction structure and apoptotic insults to CEC. J. Nutr. 140: 2187–2192, 2010.

Introduction

Cerebrovascular endothelial cells (CEC)\textsuperscript{8} make up the blood-brain barrier (BBB) by forming complex tight junctions (1,2). Structurally, the BBB is a unique membranous barrier in brain capillaries that functionally maintains homeostasis of the cerebral microenvironment (3). Occludin and ZO-1 participate in CEC-involved construction of the BBB (4,5). When CEC are damaged, the BBB can be broken down, leading to irreversible injuries to brain neurons (6). A variety of harmful substances, including peripheral immune cells, oxidized lipid derivatives, and toxins, were shown to induce damage to CEC (7,8). Reactive oxygen species (ROS) originating from inflammatory reactions is another crucial factor that can cause CEC dysfunction (9). Thus, diminishing the insults to the BBB can support the prevention or treatment of brain-related diseases such as ischemic stroke.

LDL are frequently transformed into oxidized (ox)LDL by divalent cations or endothelial cells at the moieties of apolipoprotein B or lipids (10). Universally, oxidation of LDL is thought to be one of the earliest events in atherosclerosis (11,12). However, oxLDL at high levels can also cause pathophysiological conditions in various tissues and cells. For example, oxLDL suppress nuclear translocation of cell cycle proteins in vascular smooth muscle cells, ultimately retarding cell growth (13). In addition, oxLDL can decrease neuronal viability (14). Previous studies further showed that oxLDL elevate oxidative stress and induce apoptotic insults to mouse CEC (15,16). Therefore, ROS are critical factors that mediate oxLDL-induced injuries of tissues/cells (15,16).
Resveratrol, a polyphenolic natural product that is synthesized by various plant species, has attracted considerable attention because of its therapeutic potential (17,18). Basically, resveratrol has a plethora of health benefits that control atherosclerosis, heart disease, arthritis, autoimmune disorders, and cancers (19,20). Furthermore, resveratrol can also be a powerful antioxidant to prevent doxorubicin cardiotoxicity through reducing oxidative stress-induced changes in mitochondrial function (21). Our previous study showed that oxLDL increased levels of intracellular ROS and induced apoptotic damage to mouse CEC (15). Loss of BBB integrity is recognized as a cause of profound brain alterations (22). Nevertheless, reducing damage to CEC can prevent BBB injuries and protect against brains insults. Therefore, this study aimed to evaluate the effects of resveratrol on oxLDL-induced injuries of mouse CEC from the viewpoints of tight junction construction and antiapoptosis.

Materials and Methods

Isolation of mouse CEC and drug treatment. Mouse CEC were prepared from cerebral capillaries as described previously (23). Ten male ICR mice (30 g) were purchased from the Animal Center, National Taiwan University, Taipei, Taiwan. This investigation conformed to the Guide for the Care and Use of Laboratory Animals published by the US NIH (NIH publication no. 85-23, revised 1996) and all procedures werepreapproved by the Institutional Animal Care and Use Committee of Taipei Medical University, Taipei, Taiwan. Mouse CEC were immunocytochemically verified and seeded as described previously (15). The purity of resveratrol (trans-3,4'-5-trihydroxystilbene), purchased from Sigma, was >99%. Resveratrol was freshly dissolved in dimethyl sulfoxide (DMSO). Mouse CEC were cotreated with resveratrol and oxLDL. Control cells received only DMSO.

Preparation of LDL and oxLDL. LDL and oxLDL were prepared using a previously published method (24). The blood of the normal healthy patients (6 males and 12 females, 20-65 y old) was isolated for LDL preparation. The protocol of this study was approved by the Institutional Review Board of Taipei Medical University-Wan Fang Hospital (approval no. 96039) and all participants provided written informed consent. The oxLDL were prepared by reacting purified LDL with phorbol-12-myristate-13-acetate (PMA) (Sigma, St. Louis, Missouri, lot no. 96039) and all participants provided written informed consent. The protocol of this study was approved by the Institutional Animal Care and Use Committee of Taipei Medical University, Taipei, Taiwan. Mouse CEC were immuno-construct and antiapoptosis.

Cytotoxicity assay. Cell viability was assayed following a colorimetric method (26). After drug treatment, mouse CEC were cultured with 1, 5, and 10 μM of resveratrol toward mouse CEC. Exposure of mouse CEC to 1, 5, and 10 μM/L resveratrol for 24, 48, and 72 h did not affect cell viability (data not shown).

Confocal microscopic studies of F-actin, microtubules, ZO-1, and occludin. For imaging analysis of F-actin filaments and the microtubule cytoskeleton, cells were stained with phalloidin-FITC (Molecular Probes) or immunodetected using a mouse monoclonal antibody labeled with FITC against mouse α-tubulin (Molecular Probes). For imaging analyses of ZO-1 and occludin tight junctions, these 2 proteins were immunodetected using polyclonal antibodies against rabbit anti-ZO-1 and occludin (Zymed). A confocal laser scanning microscope (Model FV500, Olympus) was utilized to analyze the samples (28).

Measurement of the mitochondrial membrane potential. The mitochondrial membrane potential (MMP) of mouse CEC was determined according to a previous method (29). After drug administration, cells were harvested and incubated with 3,3-dihexyloxacarbocyanine iodide (Molecular Probes) at 37°C for 30 min. The fluorescent intensities were analyzed by flow cytometry (Becton Dickinson).

Measurement of cellular ATP levels. Amounts of cellular ATP in mouse CEC were determined as previously described (30). The luminescence (560 nm) emitted by the luciferase-mediated reaction of ATP and luciferin was detected using a multilabel counter (Walach Allyn).

Quantification of intracellular ROS. Levels of intracellular ROS were quantified according a previously described method (31). The cells were cotreated with drugs and 2,7’-dichlorofluorescin diacetate (Molecular Probes), an ROS-sensitive dye. Relative fluorescence intensities of cells were quantified using a flow cytometer (Becton Dickinson).

Analysis of apoptotic cells. Apoptotic cells were determined according to a previous method (32). Following a process of centrifugation and washing, fixed cells were stained with propidium iodide (Sigma) and analyzed using a flow cytometer (Becton Dickinson).

Immunoblot analyses for Bcl-2, Bax, cytochrome c, and β-actin. Protein analyses were carried out according to a previous method (33). Bcl-2 and Bax were immunodetected using monoclonal antibodies against human Bcl-2 and rat Bax (Santa Cruz Biotechnology), respectively. Amounts of cytochrome (Cyt) c were detected by a mouse monoclonal antibody against the human Cyt c (Transduction Laboratories). Cellular β-actin protein was immunodetected as the internal standard. These protein bands were quantified using a digital imaging system (Uvtec).

Fluorogenic substrate assay for caspase activities. Activities of caspases-3 and -9 in mouse CEC were determined as previously described (34). The peptide substrates for the assays of caspase-3 and -9 activities were Asp-Glu-Val-Asp and Leu-Glu-His-Asp, respectively. Intensities of the fluorescent products were measured using a spectrometer (PerkinElmer Instruments).

Statistical analysis. Two-way ANOVA was used to compare the permeability, MMP, ATP, ROS, cell viability, and apoptosis in response to different treatments of resveratrol or oxLDL. Values in the text are means ± SEM. Differences were considered significant at P < 0.05.

Results

Toxicity of resveratrol toward mouse CEC. Exposure of mouse CEC to 1, 5, and 10 μM/L resveratrol for 24, 48, and 72 h did not affect cell viability (data not shown).

Resveratrol normalizes the oxLDL-induced decrease in the permeability of the CEC monolayer. Resveratrol alone did not affect the permeability of the CEC monolayer (Table 1). Exposure of mouse CEC to oxLDL for 1 h increased the permeability by 23%, which was completely normalized by resveratrol. Exposure to oxLDL induced a significant 21% reduction in TEER (Table 1). Resveratrol did not alter the...
resistance but completely protected the oxLDL-induced decrease in TEER values.

oxLDL-induced disruption of F-actin and microtubule cytoskeletons is normalized by resveratrol. In untreated mouse CEC, long-form and regular F-actin filaments were observed (Fig. 1A). Administration of resveratrol did not affect the F-actin cytoskeleton. After exposure to oxLDL, F-actin filaments in mouse CEC were disrupted. Resveratrol reduced oxLDL-induced disruption of F-actin polymerization. In control mouse CEC, the microtubule cytoskeleton was uniformly distributed (Fig. 1B). Treatment of mouse CEC with resveratrol did not affect the microtubular cytoskeleton. oxLDL could interfere with polymerization of the microtubular cytoskeleton in mouse CEC. However, resveratrol completely normalized oxLDL-induced alterations in remodeling of the microtubular cytoskeleton. oxLDL decreased the fluorescent intensities of F-actin and microtubule cytoskeletons by 40 and 34%, respectively, and these effects were completely protected by resveratrol (Table 1).

Resveratrol defends against oxLDL-induced damage to occludin and ZO-1 tight junctions. In control CEC, continuous occludin polymers were observed (Fig. 1C). However, after oxLDL administration, the occludin-formed polymers were disrupted into discontinuous spots. Resveratrol did not affect occludin polymerization but lessened oxLDL-induced disruption of the tight junction structure. Similarly, continuous and uniform ZO-1 polymers were observed in control mouse brains (Fig. 1D). After exposure to oxLDL, the structure of ZO-1 polymerization was disrupted. Resveratrol did not change ZO-1 tight junctions but alleviated the oxLDL-induced disruption of ZO-1 polymerization.

TABLE 1 Effects of resveratrol on oxLDL-induced alterations in cell permeability, TEER, F-actin, and microtubule cytoskeletons, MMP, ATP, intracellular ROS, cell viability, apoptotic cells, and activities of caspase-9 and 3 in mouse CEC

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Control</th>
<th>Res</th>
<th>oxLDL</th>
<th>Res+oxLDL</th>
<th>Res</th>
<th>oxLDL</th>
<th>Res+oxLDL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell permeability²</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fluorescence intensity</td>
<td>350 ± 20</td>
<td>346 ± 11</td>
<td>430 ± 19</td>
<td>355 ± 21</td>
<td>0.856</td>
<td>0.011</td>
<td>0.011</td>
</tr>
<tr>
<td>TEER, Ω/cm²</td>
<td>318 ± 6</td>
<td>310 ± 11</td>
<td>250 ± 12</td>
<td>290 ± 11</td>
<td>0.445</td>
<td>&lt;0.001</td>
<td>0.009</td>
</tr>
<tr>
<td>F-actin cytoskeleton, Fluorescence intensity</td>
<td>87 ± 10</td>
<td>84 ± 11</td>
<td>52 ± 9</td>
<td>82 ± 14</td>
<td>0.778</td>
<td>0.003</td>
<td>0.007</td>
</tr>
<tr>
<td>Microtubule cytoskeleton, Fluorescence intensity</td>
<td>73 ± 8</td>
<td>76 ± 10</td>
<td>48 ± 12</td>
<td>68 ± 13</td>
<td>0.692</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>MMP, FL</td>
<td>33.0 ± 3.1</td>
<td>32.5 ± 0.6</td>
<td>26.8 ± 0.6</td>
<td>31.3 ± 0.5</td>
<td>0.588</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>ATP, % of control</td>
<td>100 ± 4</td>
<td>104 ± 4</td>
<td>80 ± 6</td>
<td>104 ± 6</td>
<td>0.242</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
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<tr>
<td>Intracellular ROS, Fluorescence intensity</td>
<td>139 ± 10</td>
<td>139 ± 37</td>
<td>381 ± 16</td>
<td>207 ± 12</td>
<td>0.992</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Cell viability, OD₅₅₀</td>
<td>1.20 ± 0.05</td>
<td>1.25 ± 0.07</td>
<td>0.70 ± 0.04</td>
<td>1.03 ± 0.05</td>
<td>0.501</td>
<td>&lt;0.001</td>
<td>0.003</td>
</tr>
<tr>
<td>Apoptotic cells, %</td>
<td>6 ± 1³</td>
<td>5 ± 1²</td>
<td>58 ± 5</td>
<td>16 ± 2²</td>
<td>0.226</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Caspase-9 activities, FL</td>
<td>26 ± 4³</td>
<td>20 ± 2²</td>
<td>74 ± 8³</td>
<td>46 ± 3²</td>
<td>0.252</td>
<td>&lt;0.001</td>
<td>0.007</td>
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<tr>
<td>Caspase-3 activities, FL</td>
<td>18 ± 3³</td>
<td>19 ± 2²</td>
<td>63 ± 7³</td>
<td>36 ± 4³</td>
<td>0.678</td>
<td>&lt;0.001</td>
<td>0.003</td>
</tr>
</tbody>
</table>

² F.I., Fluorescence intensity.

1 Data are presented as mean ± SEM, n = 6. Means without a common letter differ, P < 0.05.

FIGURE 1 Effects of resveratrol (Res) on oxLDL-induced disruption of F-actin and microtubule cytoskeletons and occludin and ZO-1 tight junctions of mouse CEC. F-actin (A) and microtubules (B) cytoskeletons and occludin (C) and ZO-1 (D) tight junctions were observed and analyzed using confocal microscopy. The number of replicates is 6.
OxLDL reduces the MMP and ATP levels, but resveratrol protects these alterations. Treatment of mouse CEC with resveratrol did not affect the MMP or ATP levels, but oxLDL caused significant 19 and 20% decreases, respectively (Table 1). Resveratrol normalized the oxLDL-induced changes in the membrane potential and ATP synthesis (Table 1).

Resveratrol partially decreases oxLDL-induced apoptotic insults to mouse CEC. Exposure of mouse CEC to oxLDL for 24 h increased levels of intracellular ROS by 1.7-fold (Table 1). Resveratrol did not influence basal levels of intracellular ROS but significantly decreased oxLDL-enhanced oxidative stress by 46%. In parallel, treatment with oxLDL for 24 h induced a significant 42% decrease in cell viability (Table 1). Resveratrol did not cause cell death but partially decreased oxLDL-induced death of mouse CEC by 47%. Exposure to oxLDL for 24 h induced CEC apoptosis by 8.7-fold (Table 1). Resveratrol induced a significant 72% reduction in oxLDL-induced cell apoptosis.

Protection of resveratrol against oxLDL-induced apoptosis involves a mitochondrion-dependent pathway. Exposure of mouse CEC to oxLDL for 24 h decreased the levels of Bcl-2 but increased the amounts of Bax and Cyt c (Fig. 2A). Resveratrol did not affect the levels of Bcl-2, Bax, or Cyt c in mouse CEC but lowered oxLDL-induced changes in these apoptosis-related proteins. oxLDL decreased levels of Bcl-2 by 68% but augmented the amounts of Bax and Cyt c by 1.5-fold and 80%, respectively (Fig. 2B). Resveratrol normalized oxLDL-induced alterations in Bcl-2, Bax, and Cyt c levels. Exposure of mouse CEC to oxLDL induced significant 1.8- and 2.5-fold increases in caspase-9 and -3 activities, respectively (Table 1). Resveratrol did not affect the activities of caspases-9 and -3 but reduced oxLDL-induced activation of caspases-9 and -3 by 38 and 43%. However, resveratrol did not completely normalize oxLDL-induced activation of caspase-9 and -3 (Table 1).

Discussion

In this study, we show that treatment of mouse CEC with resveratrol protected against oxLDL-induced damage of the cell monolayer and cell apoptosis without affecting cell viability. Loss of BBB integrity is recognized as a cause of profound brain damage (22). CEC are vital components of the BBB; they form complex tight junctions to force most molecular traffic to take a transcellular route across the barrier (1,2). Being a lipophilic compound, resveratrol can cross the plasma membrane and has its diverse targeting of intracellular signaling molecules, biogenesis enzymes, oxidative systems, DNA-repair mechanisms, and transcription factors (35). Therefore, resveratrol may accumulate in CEC and trigger intracellular responses. This provides in vitro data to show that resveratrol can protect oxLDL-induced damage of the BBB by suppressing damage of the tight junction structure and apoptotic insults.

Cytoskeletons participate in the construction of tight junctions. F-actin can modulate BBB permeability (5). Microtubule integrity is essential for apical polarization and epithelial tight junctions in the thyroid (36). This study showed that resveratrol completely normalized oxLDL-induced disruption of F-actin and microtubule cytoskeletons and concurrently shielded against damage of CEC tight junctions. As a result, one of the possible mechanisms to explain how resveratrol can protect against oxLDL-induced injury of tight junctions can be attributed to the fortification of cytoskeletons. Furthermore, occludin and ZO-1 contribute to the configuration of tight junctions (4). The oxLDL-induced damage of occludin and ZO-1 tight junctions was significantly alleviated following resveratrol treatment. Taken together, resveratrol can improve oxLDL-induced disruption of cytoskeletons and tight junctions and consequently lessens damage to the permeability of the CEC monolayer and BBB.

Assembly of cytoskeletons and tight junctions requires energy (37,38). Preservation of the MMP is a key step in ATP synthesis (27). The present results reveal that resveratrol completely normalized oxLDL-involved reduction in the membrane potential of CEC mitochondria. In parallel, the oxLDL-induced decrease in the levels of ATP was completely protected by resveratrol. Thus, resveratrol can normalize oxLDL-induced suppression of ATP synthesis via recovering the MMP. Intracellular ATP reduction was reported to perturb the tight junction structure and cell-cell adhesion, eventually leading to alveolar flooding associated with high tidal volumes (38). Therefore, recovery of the MMP and ATP synthesis may explain how resveratrol protects against oxLDL-provoked disturbances of CEC tight junction.

Resveratrol is thought to be an antioxidant for scavenging ROS and diminishing oxidative stress-induced cell damage (17,21). One possible mechanism to explain the defense of...
resveratrol against oxLDL-triggered insults to mouse CEC may be attributed to its antioxidant properties. Resveratrol completely normalized oxLDL-induced alterations in the levels of Bcl-2 and Bax. Bcl-2 and Bax have opposite functions in driving cells toward survival or apoptosis (39,40). After Bax translocation from the cytoplasm to mitochondrial membranes, Cyt c, an apoptotic factor, is released into the cytoplasm from mitochondria, consequently triggering activation of caspases-9 and -3 (41). Eventually, consecutive activation of caspases-9 and -3 leads to DNA fragmentation and cell apoptosis (42). Bcl-2 can stop Bax-stimulated apoptotic events. Resveratrol could sequentially decrease oxLDL-induced alterations in Cyt c release and caspase-9 and -3 activations. Therefore, resveratrol’s protection against oxLDL-induced apoptosis of mouse CEC occurs via an intrinsic Bax/Bcl-2-mitochondrion-Cyt c-caspase protease pathway.

In conclusion, this study shows that resveratrol can protect against oxLDL-induced breakage of the BBB by alleviating damage of tight junction structures and consequent apoptotic insults. However, there are certain study limitations to this study, including: 1) because in vitro CEC were used as our present model, the physiological relevance of resveratrol needs to be further verified; 2) free resveratrol was used instead of the glucuronide- or sulfate-conjugated forms; and 3) the clinical concentrations of resveratrol used here may be higher. Therefore, we are investigating the effects of oral resveratrol on high-fat diet-induced insults to the BBB.

Acknowledgments
Y.-L.L., H.-C.C., and R.-M.C. designed the research. Y.-L.L., H.-C.C., and J.-H.C conducted the research. T.-L.C., J.-W.L., and R.-M.C. analyzed data and performed statistical analysis. H.-C.C., W.-T.C., and R.-M.C. wrote the paper. All authors read and approved the final manuscript.

Literature Cited
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