H$_2$O$_2$ induces caveolin-1 degradation and impaired mitochondrial function in E11 podocytes

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Abstract. Increased intercellular reactive oxygen species (ROS) levels are the major cause of podocyte injury with proteinuria. Caveolin-1 (CAV-1) is an essential protein component of caveolae. CAV-1 participates in signal transduction and endocytic trafficking. Recent research has indicated that CAV-1 regulates oxidative stress-induced pathways. The present study used hydrogen peroxide (H$_2$O$_2$) at nontoxic concentrations to elevate the level of ROS in E11 podocytes. Treatment with 500 and 1,000 µM H$_2$O$_2$ for 1 h significantly reduced CAV-1 expression levels. Simultaneously, the treatment significantly reduced the expression of the antioxidant enzymes glutamine-cysteine ligase catalytic subunit, superoxide dismutase 2 and catalase. To determine the role of CAV-1 in mediating oxidative stress, E11 podocytes were administered antenapedia-CAV-1 (AP-CAV-1) peptide for 48 h. The AP-CAV-1 treatment enhanced CAV-1 expression and inhibited cyclophilin A expression, thus reducing ROS-induced inflammation. Moreover, CAV-1 protected against H$_2$O$_2$-induced oxidative stress responses by enhancing the expression of antioxidant enzymes. Furthermore, CAV-1 attenuated H$_2$O$_2$-induced changes oxidative phosphorylation, and the expression of optic atrophy 1 and translocase of the inner membrane 23, as well as preserving mitochondrial function. CAV-1 treatment significantly suppressed apoptosis, as indicated by a higher B-cell lymphoma 2/BCL2-associated X protein ratio. Therefore, enhancing the expression of CAV-1 may be an important therapeutic consideration in treating podocyte injury.

Introduction

Glomerular podocytes are highly specialized cells with a complex cellular organization that assist the kidneys in blood filtration. Podocytes also serve a crucial role in the synthesis of glomerular basement membrane components, the formation of the slit membrane and interactions that ensure endothelial cell viability (1,2). Several studies have revealed that podocyte injury results in the effacement of foot processes and proteinuria, and ultimately leads to consequences of acquired glomerular diseases (1,3). In 2009, Ronconi et al (4) indicated that the podocyte damage that occurs in the pathogenesis of glomerulosclerosis could potentially be repaired through stem cell regeneration in the kidney. Furthermore, the effacement of podocytes and the decrease in their density appear to be central to the pathogenesis of diabetic nephropathy (DN). Injuries sustained because of increased oxidative stress constitute the most crucial mechanism (5,6). Recently, Mallipattu and He (7) reported that podocytes are terminally differentiated and have a minimal capacity to self-replicate; therefore, they are extremely sensitive to cellular injury. When podocyte injury occurs, it directly causes the onset and progression of glomerular diseases such as focal segmental glomerular sclerosis, minimal change disease, DN and human immunodeficiency virus-associated nephropathy. Therefore, understanding the biological mechanisms involved in podocyte injury may provide novel therapeutic targets for preventing or mitigating progression to end-stage renal failure.

Caveolin (CAV)-1 is an essential protein component of caveolae, which are omega-shaped plasma membrane invaginations rich in sphingolipids and cholesterol. In addition to maintaining cholesterol homeostasis, CAV-1 is involved in regulating vesicular transport, signal transduction and tumor progression (8,9). Cellular organelles such as mitochondria, nuclei and endoplasmic reticuli are rich in CAVs, and CAV-1 is highly expressed in vascular endothelial cells, adipocytes, smooth muscle cells and fibroblasts (10). In CAV-1-deficient fibroblasts, >40 upregulated protein biomarkers have been identified. Most of these biomarkers are associated with myofibroblast differentiation or oxidative stress hypoxia (11). The absence of CAV-1 causes cholesterol-dependent...
mitochondrial dysfunction and apoptotic susceptibility (12). By contrast, previous studies have demonstrated that CAV-1 is highly expressed in podocytes and interacts with the podocyte slit diaphragm protein nephrin and CD2AP (13,14). Previous studies demonstrated that angiotensin II induces nephrin dephosphorylation and podocyte injury through a CAV-1-dependent mechanism; therefore, CAV-1 is potentially a novel therapeutic target in nephrotic syndrome and podocyte injury (15-17). Hence, demonstrating the cell-specific role of CAV-1 in the pathogenesis of renal-associated disease may be crucial.

The present study used antennapedia-conjugated CAV-1 peptide, which is a Drosophila transcription factor facilitating CAV-1 translocation across the cell membrane (18,19), in a H2O2-induced podocyte dysfunction model. To evaluate CAV-1-induced changes in the H2O2-dependent mechanism in injured podocyte cells, the present study examined the mRNA expression levels of CAV-1, cyclophilin A (CypA) and ATP-binding cassette transporter A1 (ABCA1), as well as the mitochondrial function, oxidative and anti-oxidative homeostasis, and apoptosis of E11 podocytes.

Materials and methods

Materials. Antennapedia-CAV-1 (AP-CAV-1) peptide [RQPKIWEFPNRRKPKWKK-DGIWKA SFTTFVTKYWFYR-(OH)] was obtained from AllBio Science, Inc., (Taiwan). Hydrogen peroxide solution (H2O2) was purchased from the Sigma-Aldrich; Merck KGaA (Darmstadt, Germany). Antibodies against monoclonal anti-CAV-1 (cat no. 1249-1; 1:1000; Epitomics; Abcam, Cambridge, MA, USA), monoclonal anti-CypA (cat no. GTX113520; 1:1000; GeneTex, Inc., Irvine, CA, USA), polyclonal anti-superoxide dismutase 2 (SOD2; cat no. NB100-1992; 1:1000; Novus Biologicals, LLC, Littleton, CO, USA), polyclonal anti-catalase (cat no. ab16731; 1:1000; Abcam), polyclonal anti-glutamate-cysteine ligase catalytic subunit (GCLC; cat no. GTX113197; 1:800; GeneTex, Inc.), mouse anti-optic atrophy 1 (OPA1; cat no. 612607; 1:1000; Abcam), mouse anti-CAV-1 (cat no. GTX113520; 1:1000; GeneTex, Inc.), mouse anti-CypA (cat no. ab110413; 1:800; MitoSciences; Abcam), and mouse monoclonal anti-CyP A (cat no. GTX113520; 1:1000; GeneTex, Inc.) according to the manufacturer's protocol. qPCR was performed using AllScript First-Strand cDNA Synthesis SuperMix and AllScript Green qPCR SuperMix UDG (AllBio Science, Inc.) according to the manufacturer's protocol. qPCR analysis was used to determine the relative levels of CAV-1, CypA, ATP-binding cassette transporter A1 (ABCA1), B-cell lymphoma 2 (Bcl2), and BCL2-associated X protein (Bax) mRNA. β-actin was performed in the same reaction on all samples tested as an internal control for variations in RNA amounts. Relative gene expression was quantified according to the comparative Cq method and normalized to β-actin mRNA levels (20). The gene-specific primers are listed in Table I. The thermocycling conditions for qPCR included an initial phase of 3 min at 50°C, followed by 10 sec at 94°C and 40 cycles of 5 sec at 94°C, 15 sec at 60°C and 15 sec at 72°C. Each sample was assayed in duplicate, and fluorescence spectra were continuously monitored using the LightCycler 480 Detection system (Roche, Basel, Switzerland).

Cell culture. The E11 murine kidney podocyte cell line was obtained from CLS Cell Lines Service GmbH (Germany) and was maintained in RPMI 1640 medium (Amimed, BioConcept Ltd., Switzerland) supplemented with 10% FBS (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), 100 U/ml penicillin and 100 g/ml streptomycin (TOKU-E, Bellingham, WA, USA), 20 U/ml human recombinant interferon gamma (IFN-γ; ProSpec-Tany TechnoGene Ltd., East Brunswick, NJ, USA) at 33°C in a humidified 5% CO2 incubator.

Western blot analysis and quantification. Cells were pretreated with the indicated concentration of H2O2 for 1 h, followed by treatment with an indicated concentration of AP-CAV-1 peptide for an addition 48 h. Cells were washed with ice-cold PBS and lysed in radioimmunoprecipitation assay buffer, and centrifuged at 20,000 × g for 20 min at 4°C. The protein concentration was detected using a Bicinchoninic Acid protein assay kit (Thermo Fisher Scientific, Inc.). Proteins (20 μg) were separated by 12% SDS-PAGE and then transferred to polyvinylidene difluoride membranes. The membrane was probed with the indicated primary antibodies at 4°C overnight, and then with horseradish peroxidase-conjugated goat anti-mouse (cat no. 115-035-003; 1:50,000; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) and goat anti-rabbit (cat no. 31460; 1:100,000; Thermo Fisher Scientific, Inc.) secondary antibodies at room temperature for 1 h, and signals were obtained using an enhanced chemiluminescence kit (EMD Millipore, Billerica, MA, USA). Blots were semi-quantified by densitometry using Fusion-Capt Advance FX7 software version 16.08a on a Fusion FX7 imaging system (Labtech International, Inc., Vilber Lourmat, France).

RNA isolation and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was prepared using an AllPure Total RNA Isolation kit (AllBio Science Inc., Taichung, Taiwan) according to the manufacturer’s protocol. Reverse transcription and qPCR were performed using AllScript First-Strand cDNA Synthesis SuperMix and AllScript Green qPCR SuperMix UDG (AllBio Science, Inc.) according to the manufacturer's protocol. qPCR analysis was used to determine the relative levels of CAV-1, CypA, ATP-binding cassette transporter A1 (ABCA1), B-cell lymphoma 2 (Bcl2), and BCL2-associated X protein (Bax) mRNA. β-actin was performed in the same reaction on all samples tested as an internal control for variations in RNA amounts. Relative gene expression was quantified according to the comparative Cq method and normalized to β-actin mRNA levels (20). The gene-specific primers are listed in Table I. The thermocycling conditions for qPCR included an initial phase of 3 min at 50°C, followed by 10 sec at 94°C and 40 cycles of 5 sec at 94°C, 15 sec at 60°C and 15 sec at 72°C. Each sample was assayed in duplicate, and fluorescence spectra were continuously monitored using the LightCycler 480 Detection system (Roche, Basel, Switzerland).

Statistical analysis. Statistical analyses were performed using one-way analysis of variance followed by Bonferroni’s post hoc test in SPSS software version 22.0 (IBM Corp., Armonk, NY, USA). Data are presented as mean ± standard deviation. P<0.05 was considered to indicate a statistically significant difference.

Results

Effects of H2O2 on expression levels of antioxidant-associated proteins, CAV-1 and CypA in E11 podocytes. To determine the effects of H2O2 on the antioxidant-associated proteins of podocytes, E11 cells were treated with various concentrations of H2O2 for 1 h. The expression of antioxidant-associated proteins was measured through western blot analysis. A significant and dose-dependent decrease was observed in the expression of the antioxidant enzymes GCLC, catalase and SOD2 in the H2O2-treated groups compared with the vehicle
control (Fig. 1A). Similarly, the H₂O₂ treatment markedly reduced the expression of CAV-1; whereas the expression of CypA, which is an inflammatory marker, was significantly upregulated. The quantification of the results is presented in Fig. 1B (P<0.001). These results suggested that H₂O₂ significantly affects the antioxidant capacities of podocytes, thus promoting intercellular inflammation and altering mitochondrial antioxidant capacity.

Table I. Primers used in reverse transcription-quantitative polymerase chain reaction analysis.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward (5'-3')</th>
<th>Reverse (5'-3')</th>
</tr>
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<tbody>
<tr>
<td>Caveolin-1</td>
<td>AGCCCAACAAACAAGGCCAT</td>
<td>GCAATCACATTTCAAAGTCAATCTT</td>
</tr>
<tr>
<td>Cyclophilin A</td>
<td>TGGCTGGACCAAAACACAAAAGCG</td>
<td>GCTCTTTCCTACCTTCCCAAATA</td>
</tr>
<tr>
<td>ABCA1</td>
<td>AACAGTTTGCTGCCCCCTTTTG</td>
<td>AGTTCCAGGCTGGGTACTTT</td>
</tr>
<tr>
<td>Bcl2</td>
<td>CTGAGTACCTGAACCGGCATC</td>
<td>GAGCAGTGCTTCAGAGACAG</td>
</tr>
<tr>
<td>Bax</td>
<td>GTTTACTCGAGGTAGCACAGG</td>
<td>AGCTGAGCGAGTGTCCTCGGCGG</td>
</tr>
<tr>
<td>β-actin</td>
<td>TGGAAATCCTGCGCATCAGTAAAC</td>
<td>TAAACGACGTCTGTAACAGTCGG</td>
</tr>
</tbody>
</table>

ABCA1, ATP-binding cassette transporter A1; Bcl2, B-cell lymphoma 2; Bax, BCL2-associated X protein.

Figure 1. Effects of H₂O₂ on antioxidant-associated proteins, CAV-1 and CypA expression levels. E11 cells, except for the CON group, were treated with the indicated concentration of H₂O₂ for 1 h. (A) Representative western blot images and (B) quantification of GCLC, catalase, SOD2, CAV-1 and CypA protein expression levels. GAPDH served as an internal control. Data are presented as the mean ± standard deviation of at least three independent experiments. a: CON group, b: 250 µM H₂O₂ group, c: 500 µM H₂O₂ group, d: 1 mM H₂O₂ group. CON, control; CAV-1, caveolin-1; CypA, cyclophilin A; SOD2, superoxide dismutase 2; GCLC, glutamine-cysteine ligase catalytic subunit.

Figure 2. Effects of AP-CAV-1 on H₂O₂-mediated CAV-1 mRNA and CypA mRNA expression levels. E11 cells were pretreated with 500 µM H₂O₂ for 1 h, followed by treatment with the indicated concentration of AP-CAV-1 peptide for an additional 48 h. mRNA expression levels of (A) CAV-1, (B) CypA and (C) ABCA1. β-actin served as an internal control. Data are presented as the mean ± standard deviation of at least three independent experiments. CAV-1, caveolin-1; CypA, cyclophilin A; AP-CAV-1, antenapedia-caveolin-1; ABCA1, ATP-binding cassette transporter A1.

Effects of CAV-1 on the mRNA expression levels of the CypA and ABCA1 genes. The present study examined whether AP-CAV-1 treatment exerted additional effects on the expression levels of CAV-1 and CypA in H₂O₂-treated E11 cells. RT-qPCR assay results revealed that the mRNA expression levels of CAV-1 and CypA in the AP-CAV-1-treated group were significantly elevated and diminished, respectively, compared with those of the H₂O₂-treated group (Figs. 2A and B, respectively).
quantification of the results is presented in Table II (CAV-1, 
P=0.018; CypA, P<0.001). Furthermore, the ABCA1 mRNA levels in the E11 podocytes were significantly higher in the H2O2-treated group compared with the control group. In the AP-CAV-1-treated E11 cells, CAV-1 provided protection from H2O2-associated damage and the change in the ABCA1 mRNA expression level was significantly reduced (Fig. 2C). However, no significant difference was observed for ABCA1 mRNA levels between AP-CAV-1-treated groups. Overall, these results indicated that CAV-1 diminished H2O2-induced E11 podocytes injuries and prevented ABCA1 compensatory action from becoming excessively active in the H2O2-treated E11 cells.

Effects of CAV-1 on apoptosis-associated gene mRNA expression levels. To investigate whether CAV-1 activity affects cell survival, the present study examined the expression of apoptosis-associated gene mRNA expression levels in E11 cells by RT-qPCR. A higher compensatory mRNA level of Bcl2 was observed in the H2O2-treated E11 cells. CAV-1 treatment significantly suppressed Bcl2 mRNA expression in a dose-dependent manner (Fig. 3A). The Bax mRNA expression levels were more markedly diminished in the CAV-1-treated groups than in the H2O2-treated group (Fig. 3B). The quantification of the results is presented in Table III. The Bcl2/Bax ratios were also higher in the CAV-1 groups compared with the H2O2-treated group. CAV-1 provided podocytes with resistance to apoptotic stimuli (Fig. 3C). These results suggested that CAV-1 may prevent apoptotic cell death in E11 podocytes.

Effects of CAV-1 on H2O2-induced changes. To determine whether AP-CAV-1 treatment was associated with a local decrease in oxidative stress, the present study measured the expression levels of the antioxidant enzymes GCLC, catalase and SOD2 in E11 podocytes. A significant elevation of three markers was observed in the AP-CAV-1-treated E11 cells (Fig. 4; P<0.001). The expressions of the pro-inflammatory markers CypA and CAV-1 were significantly lower and higher, respectively, in the AP-CAV-1-treated groups compared with the H2O2-treated groups (Fig. 4; P<0.001). Thus, AP-CAV-1 may have increased antioxidant enzyme activity and attenuated local oxidative damage in the CAV-1-treated E11 cells.

CAV-1 preserves mitochondrial respiratory function by upregulating OXPHOS expression. The present study used western blot analysis to examine mitochondrial OXPHOS complexes because they directly affect mitochondrial function and antioxidative capacity. The expression levels of the ATP synthase α-subunit (complex V), cytochrome c oxidase subunit 1 (MTCO1; complex IV), core 2 protein (complex III), Succinate dehydrogenase [ubiquinone] iron-sulfur subunit, mitochondrial (SDHB; complex II) and NADH dehydrogenase [ubiquinone] 1 β subcomplex subunit 8 (NDUFB8; complex I) were significantly enhanced in a dose-dependent manner in the AP-CAV-1-treated

Table II. mRNA expression levels of CAV-1, CypA and ABCA1 in the control and AP-CAV-1-H2O2-treated E11 cells.

<table>
<thead>
<tr>
<th>Gene</th>
<th>CON*</th>
<th>500 μM H2O2b</th>
<th>0.1 μM AP-CAV-1c</th>
<th>1.0 μM AP-CAV-1d</th>
<th>Post Hoc Tests</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAV-1</td>
<td>0.0047 ± 0.0014</td>
<td>0.0028 ± 0.0005</td>
<td>0.0035 ± 0.0008</td>
<td>0.0039 ± 0.0007</td>
<td>0.018 &gt;b</td>
</tr>
<tr>
<td>CypA</td>
<td>0.1296 ± 0.0031</td>
<td>0.1519 ± 0.0215</td>
<td>0.1288 ± 0.0170</td>
<td>0.0920 ± 0.0163</td>
<td>&lt;0.001 a,b,c&gt;d</td>
</tr>
<tr>
<td>ABCA1</td>
<td>0.0007 ± 0.0005</td>
<td>0.0059 ± 0.0092</td>
<td>0.0008 ± 0.0003</td>
<td>0.0016 ± 0.0021</td>
<td>0.515</td>
</tr>
</tbody>
</table>

*CON group, **500 μM H2O2 group, ***500 μM H2O2 + 0.1 μM AP-CAV-1 group, ****500 μM H2O2 + 1.0 μM AP-CAV-1 group. SD, standard deviation; CAV-1, caveolin-1; CypA, cyclophilin A; AP-CAV-1, antenapedia-caveolin-1; ABCA1, ATP-binding cassette transporter A1.
groups compared with the H$_2$O$_2$-treated group (P<0.001 vs. P=0.002). AP-CAV-1 treatment resulted in a significantly increased expression of electron transport chain complex I-V protein, suggesting that CAV-1 treatment preserves mitochondrial respiratory function in H$_2$O$_2$-treated E11 podocytes (Fig. 5).
CAV-1 prevents OPA1 and Tim23 degradation. The present study determined whether OPA1 and Tim23 expression levels that are upregulated by CAV-1 treatment prevent H$_2$O$_2$-induced apoptosis in E11 podocytes. OPA1 is a protein required for inner mitochondrial membrane fusion. H$_2$O$_2$ exposure decreased the intensity levels of both bands, whereas OPA1 levels markedly increased after CAV-1 treatment. Similarly, CAV-1 prevented a substantial decrease in the expression of the inner-membrane protein Tim23 from being induced by the H$_2$O$_2$ insult (Fig. 6, P=0.039 and P<0.001, respectively). These data suggested that CAV-1 prevents the mitochondrial fusion mechanism from undergoing OPA1 deregulation, and preserves the integrity of Tim23 content, mediating the translocation of proteins into the mitochondrial matrix in response to in vitro H$_2$O$_2$-induced toxicity.

Discussion

The present study demonstrated that H$_2$O$_2$ induces reactive oxygen species (ROS) production, oxidative stress, inflammation, cell apoptosis and mitochondrial dysfunction in podocytes, and that treatment with CAV-1 prevents H$_2$O$_2$-induced ROS production, oxidative stress, inflammation, cell apoptosis and mitochondrial dysfunction, indicating that CAV-1 functions as a positive regulator in podocyte injury.

ROS overexpression has been observed in glomerular endothelial and epithelial cells and has been demonstrated to disrupt normal glomerular permeability, thus leading to proteinuria (21,22). The inhibition of ROS generation through the use of NADPH oxidase inhibitors, renin-angiotensin-aldosterone system inhibitors, statins, anti-diabetic drugs and antioxidant vitamins may ameliorate the renal damage caused by diabetic nephropathy (5,6). CAV-1 has been associated with oxidative regulating pathways. Chen et al (23) demonstrated that CAV-1 is a negative regulator of NADPH oxidase-induced ROS in endothelial cells, and Sun et al (24) demonstrated that CAV-1 significantly reduces ROS production and apoptosis in podocytes. Similarly, the results of the present study revealed that increased CAV-1 expression not only promotes GCLC, SOD2 and catalase expression to increase antioxidant defensive capacity, but also downregulates the Bcl2/Bax expression ratio, thus preventing podocyte cell death. However, Volonte et al (25) revealed that the CAV-1 interaction with nuclear factor erythroid 2-related factor 2-GCLC proteins negatively regulated antioxidant defenses in fibroblasts. Hence, the differences observed in the roles of CAV-1 in oxidative regulating pathways are cell-type specific.

Crucially, the CAV-1 expression level was markedly affected by the addition of H$_2$O$_2$ to the culture medium; the CAV-1 level decreased by nearly 50-70%. Similarly, previous studies demonstrated that the expression of CAV-1 is significantly decreased after H$_2$O$_2$ treatment in cardiomyocytes and skeletal muscle cells (26,27). Several studies have suggested that the rapid degradation of CAV-1 protein could be caused by the ubiquitin-proteasome pathway, particularly after oxidative injury (28,29). The present study revealed that H$_2$O$_2$ had a reversible effect on CAV-1 expression when podocyte cells were incubated with an AP-CAV-1 peptide, and that CAV-1 protein degradation could then trigger cellular mitochondrial function and antioxidant defense.

In conclusion, the results of the present study demonstrated that CAV-1 provides protection against the H$_2$O$_2$-induced oxidative stress response, as demonstrated by an increase in the activity of the antioxidant enzymes GCLC, SOD2 and catalase. CAV-1 also attenuated the expression of the proinflammatory marker CypA, altered Bcl2/Bax mRNA expression levels, suppressed apoptotic cell death and preserved mitochondrial functions such as upregulated OXPHOS, OPA1 and Tim23 protein expression levels. Therefore, targeting enhanced CAV-1 expression levels in podocyte injury may have potential as a therapeutic strategy for the treatment of glomerular injury.

Acknowledgements

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References