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Blood-brain barrier disruption in diabetic mice is linked to Nrf2 signaling deficits: Role of ABCB10?

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Abstract

Blood-brain barrier (BBB) damage is a critical neurovascular complication of diabetes mellitus that adversely affects the CNS health and function. Previously, we showed the protective role of NF-E2 related factor-2 (Nrf2), a redox sensitive transcription factor, in regulation of BBB integrity. Given the pathogenic role of mitochondrial oxidative stress in diabetes-related microvascular complications, we focused on assessing: 1) the impact of diabetes on brain Nrf2 in correlation with BBB permeability and 2) Nrf2-dependent regulation of the mitochondrial transporter ABCB10, an essential player in mitochondrial function and redox balance at BBB endothelium. Using live animal fluorescence imaging, we demonstrated a strong increase in BBB permeability to 70kDa dextran in db/db diabetic mice that correlated with significant down-regulation of brain Nrf2 protein. Further, Nrf2 gene silencing in human BBB endothelial cells markedly suppressed ABCB10 protein, while Nrf2 activation by sulforaphane up-regulated ABCB10 expression. Interestingly, ABCB10 knockdown resulted in a strong-induction of Nrf2 driven anti-oxidant responses as evidenced by increased expression of Nrf2 and its downstream targets. Nrf2 or ABCB10 silencing elevated endothelial-monocyte adhesion suggesting an activated inflammatory cascade. Thus, our results demonstrate a novel mechanism of ABCB10 regulation driven by Nrf2. In summary, Nrf2 dysregulation and ABCB10 suppression could likely mediate endothelial oxidative/inflammatory stress and BBB disruption in diabetic subjects.

Keywords

ABCB10; db/db mice; *in vivo* imaging; Leukocyte; Mitochondria; Oxidative stress

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5. Conflict of Interest

The authors disclose that no conflicts of interest are perceived in publishing this study.

7. Authors' contributions

Conceived and designed the experiments: RKS; Performed the experiments: RKS, SP, ST; Data analysis and interpretation: RKS, SP, ST; Contributed reagents/materials/analysis tools: LC; Manuscript preparation: RKS, MAK, LC; Manuscript revision: SP, MAK, ST. All authors approved the final version of this manuscript.

1. Introduction

Diabetes mellitus adversely affects the central nervous system leading to a host of neurobehavioral and degenerative disorders [22]. Emerging preclinical and clinical studies indict blood-brain barrier (BBB) pathophysiology as a critical neurovascular ramification linked to various CNS morbidities of diabetic milieu [9, 12, 22, 26]. Endothelial oxidative stress (and subsequent mitochondrial damage) through abnormal production of reactive oxygen species (ROS), has been established as a central pathological mechanism for BBB dysfunction in diabetes [22, 25].

NF-E2 related factor-2 (Nrf2) is a redox-sensitive master regulator of transcriptional expression of an elaborate network of cytoprotective and anti-oxidant genes involved in cellular anti-oxidant defense including mitochondrial function [7, 15]. In fact, independent studies have established the protective role of Nrf2 against BBB damage in various CNS pathologies [4, 21, 23, 36]. Additionally, Nrf2 activation by sulforaphane (SFN) elevated the expression/activity of major BBB efflux transporters of ABC superfamily that further strengthens the BBB integrity [30]. However, the impact of Nrf2 on other ABC transporters at BBB endothelium is unknown.

Mitochondrial ABCB10 has been well studied for its established role in heme biosynthesis and erythroid maturation [19]. Recent studies have also demonstrated the non-erythroid functions of ABCB10 in redox biology and mitochondria protection against oxidative insults [18, 35]. While ABCB10 gene was also found to be enriched at human and mouse brain endothelial cells [31], its functional relevance at BBB remains elusive.

Therefore, our objectives were to assess the impact of diabetes on cerebral Nrf2 response *in vivo* and to study Nrf2-dependent regulation of ABCB10. Additionally, we delineated the role of mitochondrial ABCB10 in BBB endothelial redox biology.

2. Materials and methods

2.1. Animals

Adult male db/db mice (BKS.Cg-Dock7^{m} +/+ Lepr^{db}/J; Genotype: Lepr^{db}/Lepr^{db}; Stock # 000642), a widely used model of diabetes [16, 37] and non-diabetic db/+ control mice (C57BLKS/J) were obtained from Jackson Laboratory (Bar Harbor, ME). Animals received *ad libitum* access to diet and water under controlled conditions of temperature and humidity with a 12h/12h light/dark cycle. Animal handling and experimental protocols complied with NIH guidelines and were approved by the Institutional Animal Care and Use Committee at Texas Tech University.

2.2. In vivo fluorescence imaging

Mice (n=5/group and 9 weeks old) were administered 10mg/ml Rhodamine-B isothiocyanate conjugated dextran (RITC, 70kDa; Sigma-Aldrich, Saint Louis, MO) by tail vein. This size of the animal group was adequate to produce a significant mean difference of ≥ 50% between the groups with type 1 error rate of 0.05 for qualitative and quantitative

results reported in this study. Live animal fluorescence imaging was performed on anesthetized mice at 10 and 30 min after injection with optimized settings (Em/Ex=530/620nm) in IVIS Lumina XR (Caliper Life Sciences). Fluorescence intensity was measured across an identical region of interest in each animal by Living Image software (v 4.4).

2.3. Cell culture

Human cerebral microvascular endothelial cell line (hCMEC/D3), a well-established *in vitro* model of human BBB [6, 23, 32], was used in this study. Cells were seeded on Matrigel coated sterile plates or chamber slides and cultured in HEPES-buffered EBM2 medium supplemented with Lonza EGM2 MV BulletKit™. Cellular ROS levels were measured by H_2- DCFDA (10 μM) assay.

2.4. Gene silencing

After reaching 50–60% confluence, hCMEC/D3 cells were transfected with *Silencer*® Select pre-designed and validated siRNAs (Nrf2: s9491; ABCB10: s223600; negative control: 40424303; Life Technologies, Carlsbad, CA), as described earlier [23]. Following 72h transfection, cells were harvested for protein collection or fluorescence imaging.

2.5. Western blotting

Equal loads of denatured protein samples obtained from total cell lysates were subjected to SDS-PAGE [21, 23]. After electrotransfer and blocking with 5% non-fat dry milk, membranes were incubated with primary antibodies, washed and subsequently incubated with secondary antibodies (1:8000). Band densities were measured and normalized to loading control. Antibodies against Nrf2 (sc-722 and detects ~61kDa), NQO-1 (sc-271116) were from Santa Cruz, ABCB10 antibody (SAB2701235) was from Sigma Aldrich, while AKT/pAKT (9916S) were obtained from Cell Signaling Technology.

2.6. Immunofluorescence

A similar procedure [21, 23] was followed for immunofluorescence analysis of protein expression/localization. Briefly, cells were fixed, permeabilized and blocked with 10% goat serum in 1x PBS for 30 min. Following incubation with primary antibodies in blocking buffer (1:200), cells were rinsed and added Alexa Fluor® 555 conjugated goat anti-rabbit antibody (1:1000). After multiple rinses, cells were mounted and imaged with EVOS digital inverted fluorescence microscope.

2.7. Endothelial-monocyte adhesion assay

Human peripheral blood monocytes (THP-1 cell line) obtained from American Type Culture Collection (TIB-202™) was maintained as suspension culture in the recommended RPMI-1640 medium. Cells (2×10^5/ml) were incubated with 10μM calcein-AM for 20 min, centrifuged and suspended (x2) in fresh medium. Labeled monocytes were added to hCMEC/D3 cell monolayers for 15 min on a shaker at constant speed. Slides were gently rinsed with ice cold PBS and fixed with cold 4% buffered formalin for 10 min. Cells were

washed, cover slipped and imaged at 40X magnitude using EVOS digital inverted fluorescence microscope.

2.8. Statistical analyses

Data were expressed as mean ± S.E.M and were analyzed by unpaired student's *t*-test using GraphPad Prism 7 (La Jolla, CA). P value <0.05 was used for statistical significance.

3. Results

3.1. BBB permeability in diabetic mouse brain correlates with Nrf2 down-regulation

The mean weight and non-fasting blood glucose levels in db/db mice (41g and 450mg/dl) were significantly higher compared to db/+ mice (26g and 160mg/dl) at 9 weeks. As shown in Fig. 1A & 1B, live animal imaging demonstrated a significant increase in brain fluorescence intensity of RITC-dextran in db/db mice at 10 min ($p<0.01$) and 30 min ($p<0.05$ vs. control mice), indicating an increased BBB permeability. Moreover, the fluorescence intensity decreased by 10-fold as the imaging time advanced from 10 to 30 min that indicates a rapid clearance of the dextran from circulation. Also, we did not observe fluorescence in control db/db mouse that did not receive RITC-dextran injection, thus ruling out auto-fluorescence. Importantly, there was a marked decline in the steady state Nrf2 protein content in brain homogenates of diabetic mice, compared to db/+ mice (Fig. 1C). Thus, BBB disruption in diabetic subjects strongly correlated with Nrf2 down-regulation.

3.2. Nrf2 regulates mitochondrial ABCB10 expression in human BBB endothelium

Given the important role of ABCB10 transporter in mitochondria function, we next determined if Nrf2 regulates ABCB10 expression at human BBB endothelium. As shown in Fig. 2A, Nrf2 gene silencing by siRNA resulted in a significant down-regulation of mitochondrial ABCB10 including NAD(P)H Quinone Dehydrogenase 1 (NQO1), a downstream effector of Nrf2 in hCMEC/D3 cell line. In contrast, pharmacological activation of Nrf2 by SFN (5μM; [30]) potentiated ABCB10 expression (Fig. 2B). Additionally, we demonstrated that SFN treatment (24h) significantly increased AKT phosphorylation (at Ser473; Fig. 2B). Thus, these results converge to the novel finding that mitochondrial ABCB10 expression is an extension to regulatory arm of Nrf2 signaling axis at BBB endothelium.

3.3. ABCB10 regulates BBB endothelial redox homeostasis

We next assessed the role of ABCB10 in BBB endothelial redox biology and inflammation. Mitochondrial ABCB10 knockdown by siRNA (as determined by western blotting) generated a strong activation of Nrf2 regulated anti-oxidant gene induction responses in hCMEC/D3 cells, as explained by an increased expression of Nrf2 ($p<0.05$) and its downstream targets, HO1 ($p<0.01$) and NQO1 ($p<0.05$) vs. negative siRNA control (Fig. 3A). We further examined the effects of hypo- (2.2mM D-glucose) and hyperglycemia (25mM D-glucose) on ABCB10 expression. The effects of hypoglycemia were tested to validate the Nrf2-depenent regulation of ABCB10, as hypoglycemia exposure (12h) significantly diminished Nrf2 expression in hCMEC/D3 cells [23]. As demonstrated in Fig. 3B1, hypoglycemia (12h) or hyperglycemia (48h) markedly reduced BBB endothelial

ABCB10 expression, with a corresponding up-regulation of endothelial ROS generation (oxidative stress) following exposure to hyperglycemic conditions in glucose concentration-dependent manner (Fig. 3B2).

Importantly, we observed a significant increase in endothelial adhesion of human monocytes (THP-1 cells), following Nrf2 or ABCB10 gene silencing in hCMEC/D3 cells (Fig. 3C). These data likely suggest the potential role of Nrf2 and its downstream regulation of ABCB10 in BBB oxidative stress and neuroinflammation.

4. Discussion

Previously, diabetes-related hyperglycemia was shown to progressively compromise BBB integrity by destabilization of selective tight junction proteins *in vitro* [24] and *in vivo*[10, 25]. For example, a significant extravasation of plasma proteins (e.g. 60kDa albumin) was reported in diabetic rodents [8, 13] and humans [14]. In line with these findings, here we demonstrate an increased BBB permeability to 70kDa dextran in transgenic type 2 diabetic model (db/db mice) by a non-invasive live animal fluorescence imaging approach (Fig. 1). Our data corroborate with magnetic resonance imaging studies demonstrating a substantial loss of BBB integrity in type 2 diabetic patients [28] and rhesus monkeys [33]. The increased fluorescence intensity in db/db mouse brain also suggests an edematous brain tissue that is linked to BBB damage [2]. However, there was a remarkable decrease in dextran flux from 10–30min which can be explained by the rapid systemic clearance of the dextran. One limitation of this experimental approach is its inability to capture the BBB leak at higher resolution for rigorous assessment of BBB permeability changes across various regions. Nevertheless, fluorescence imaging method is a simple and non-invasive technique that can be multiplexed for simultaneous imaging of two or more molecular events to assess the size-selective paracellular flux of dextrans of various molecular sizes depending on the instrument’s detection limits.

The BBB damage in diabetic mouse brain could be a clinical manifestation of a parallel down-regulation in cerebral Nrf2 levels (Fig. 1C), as supported by recent findings [3, 21, 23, 36]. For example, Nrf2 silencing adversely affected tight junction protein composition and integrity in hCMEC/D3 cell line [23], while its activation by SFN pretreatment protected against BBB disruption and neurological deficits following ischemic stroke in rats [3]. Importantly, earlier studies also reported a similar deficit in brain Nrf2 signaling cascade in diabetic subjects leading to potential neurocognitive complications [1, 5, 20]. While the precise changes in Nrf2 content at the BBB endothelium in diabetes subjects have yet to be established, it is very likely that diminished Nrf2-driven responses account for increased endothelial oxidative stress and mitochondrial dysfunction resulting in BBB impairment [17, 21, 22, 37].

We also provide novel mechanistic evidence demonstrating Nrf2-dependent regulation of mitochondrial ABCB10 at human BBB endothelium (Fig. 2). Further, our results suggest that Nrf2-driven ABCB10 expression is a downstream molecular event to AKT (Ser473) phosphorylation [20]. However, additional studies are required to further probe the presence of Nrf2 binding site (ARE sequence) in promoter region for transcriptional regulation of

ABCB10 in BBB endothelium. Data from our study also reveal that ABCB10 critically regulates redox homeostasis in BBB endothelium (Fig. 3). These results further corroborate with emerging studies implying the potential role of ABCB10 in protection against oxidative stress in various cell types [18, 34, 35]. For instance, a significant elevation of mitochondrial ROS following the depletion of ABCB10 in hepatocytes [35], while ABCB10$^{+/-}$ mice exhibited increased susceptibility to oxidative damage and poor outcome following cardiac ischemia/reperfusion injury [18]. Moreover, ABCB10 knockdown markedly up-regulated various redox-related proteins and detoxifying enzymes [35] that are known to be transcriptionally regulated by Nrf2 signaling [7]. Importantly, we show that prolonged hyperglycemic exposure (48h) significantly reduced ABCB10 expression in human BBB endothelium (Fig. 3B1) with a concomitant rise in the production of ROS (Fig. 3B2). Thus, these results provide a rationale evidence suggesting a likely association between ABCB10 down-regulation and oxidative stress response provoked by hyperglycemia. Thus, our study warrants for additional investigations to determine the functional relevance of ABCB10 in diabetes-induced BBB complication.

Importantly, silencing either BBB Nrf2 or ABCB10 increased THP-1 monocyte adhesion to endothelial cells (Fig. 3), indicating a profound inflammatory response, possibly through increased expression of endothelial adhesion molecules [6, 11, 21]. Thus, we provide an additional evidence for the role of Nrf2 and importantly, ABCB10, in protection of BBB endothelium against inflammation. Recent elucidations indicate a significant level of neuroinflammation in diabetic mice as evidenced by increased BBB permeability, macrophage infiltration and induction of pro-inflammatory genes [26, 29]. Therefore, we speculate that down-regulation of Nrf2 functional responses could trigger BBB endothelial dysfunction and neuroinflammatory cascade in diabetic subjects [27].

In summary, this study suggests that targeting Nrf2 pathway could have therapeutic utility to preserve BBB integrity and CNS function in type 2 diabetes mellitus, while demonstrating the putative role of ABCB10 in diabetes-related BBB pathophysiology

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Highlights

- *In vivo* fluorescence imaging revealed BBB hyper-permeability in diabetic mice
- Nrf2 is significantly down-regulated in diabetic brain
- Nrf2 regulates mitochondrial ABCB10 expression in human BBB endothelium
- Hyperglycemia markedly reduces ABCB10 expression in BBB endothelial cells
- Nrf2 or ABCB10 gene silencing increases BBB endothelial-monocyte adhesion

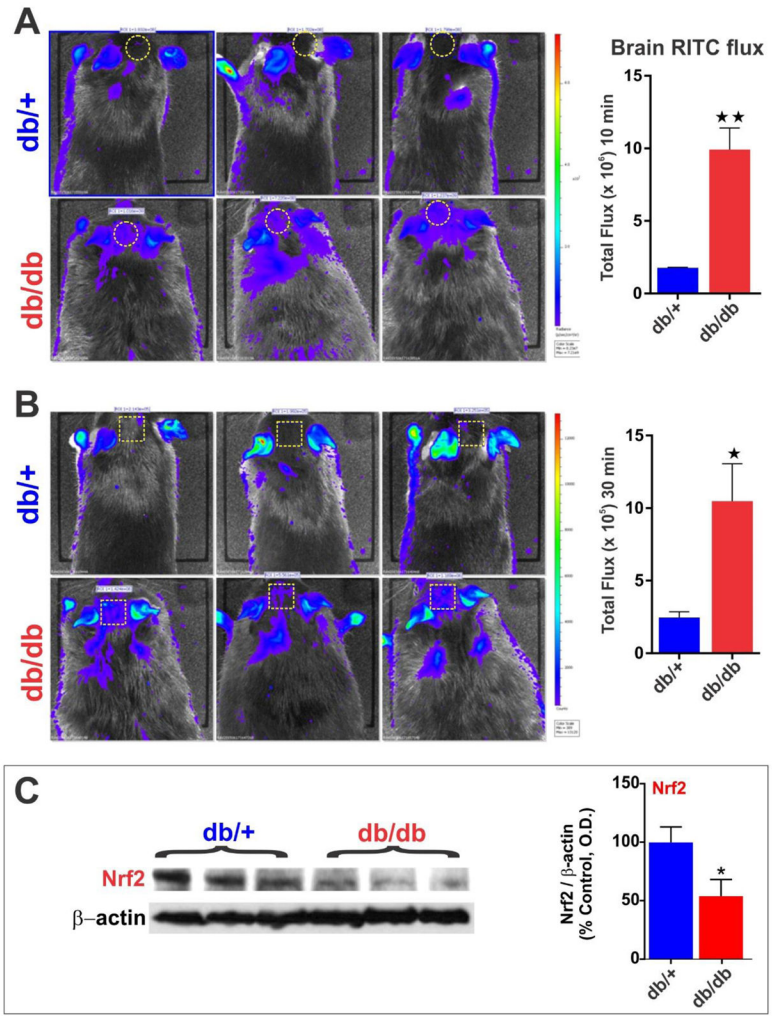

Fig. 1. Increased BBB permeability in diabetic mouse brain correlates with Nrf2 down-regulation

Live animal florescent imaging of diabetic (db/db) and control (db/+) mouse brains at 10 (**A**) and 30min (**B**) following tail vein injection of RITC dextran (n=5 mice/group). Fluorescence intensity was measured across identical regions of interest and expressed in terms of total radiance. (**C**) Nrf2 expression were analyzed by western blotting in brain homogenates extracted from diabetic and control mice (n=5 mice/group).

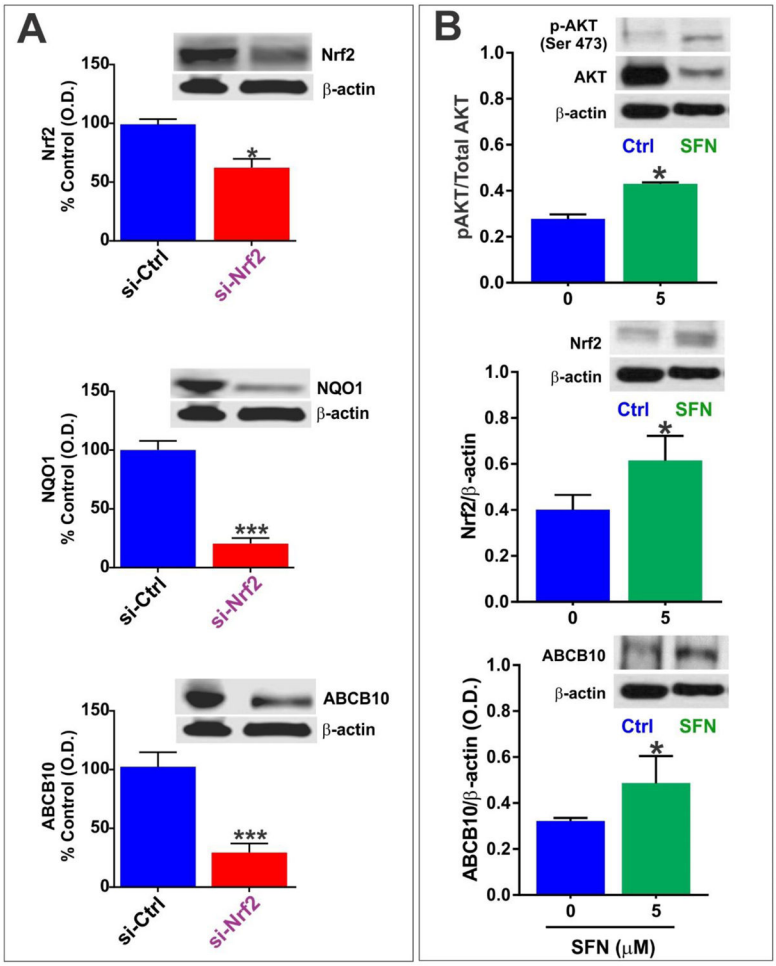

Fig. 2. Nrf2 regulates mitochondrial ABCB10 expression in BBB endothelial cells

(**A**) Nrf2 knockdown by siRNA in hCMEC/D3 cells leads to subsequent down-regulation of NQO1 and ABCB10, as determined by western blots. (**B**) Treatment with SFN (5μM) for 24h significantly enhanced Nrf2 and ABCB10 protein expression in hCMEC/D3 cells, possibly through increased phosphorylation of AKT (Ser 473). Representative western blots were shown. N=3 biological replicates and two independent experiments. *** $p < 0.001$ and * $p < 0.05$, vs. control.

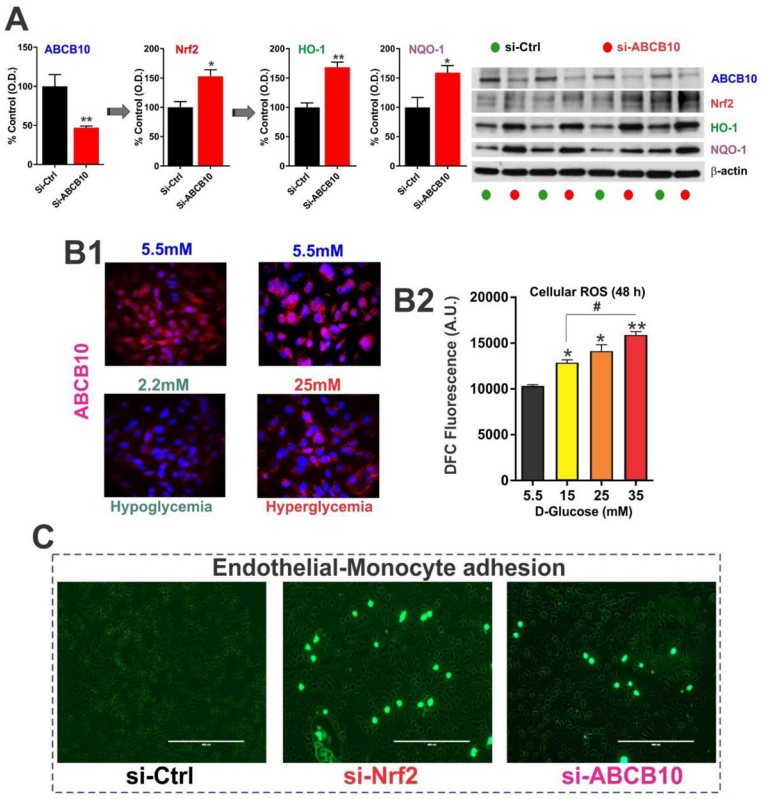

Fig. 3. ABCB10 gene silencing induces oxidative stress response in BBB endothelial cells

(**A**) Knockdown efficiency of ABCB10 and its effects on the protein expression of Nrf2 and its downstream targets heme oxygenase 1 (HO-1) and (NQO1) in hCMEC/D3 cells were analyzed by western blotting. (**B1**) Immunofluorescence analyses ABCB10 expression in hCMEC/D3 cells following exposure to low and high glucose conditions and (B2) total cellular levels of ROS in hCMEC/D3 cells. (**C**) Nrf2 or ABCB10 gene silencing in hCMEC/D3 cells by siRNA significantly enhance THP-1 monocyte adhesion to endothelial cells.. Immunofluorescence images were obtained at 40X magnitude and scale size was 100μm. *** $p < 0.001$, ** $p<0.01$ and * $p < 0.05$, vs. control; # $p<0.05$ vs 15 mM D-Glucose