Apocynin ameliorates stroke-induced stress in the kidney

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Abstract

Stroke leads to the activation of multiple cell death pathways and affects various distal organs, including the kidney. Thus understanding systemic complications arising in non-neurological origin has implications for treating long-term consequences after stroke. Our recent study showed that apocynin curtails post-stroke brain damage by regulating the early onset of oxidative and endoplasmic reticulum (ER) stress. We collected kidney tissues from rats subjected to Middle Cerebral Artery Occlusion (MCAO) plus treated with apocynin to evaluate stroke-induced stress signaling in the kidney. We observed that MCAO induced protein ubiquitination, expression of ER stress markers, GRP78 (Glucose regulated protein 78), and peIF2α (Phosphorylated eukaryotic translation initiation factor 2 alpha subunit), and altered morphology in the glomerulus after 24h of brain ischemia/reperfusion. Apocynin treatment showed a systemic effect that reduced the expression of ER stress markers and improved glomerulus-altered morphology. Of note, apocynin sustained stroke-induced protein ubiquitination in the glomerulus. Thus our study suggests experimental stroke also induces non-neurological ER stress signaling in the kidney glomerulus, which has potential implications for treating stroke-induced acute and chronic consequences of kidney dysfunction. Further, we observed (in silico) up-regulated expression of ER stress-responsive/unfolded protein response pathway genes in clinical samples of chronic kidney disease and Diabetic Nephropathy. Thus our study has implications for developing novel therapeutic strategies for stroke-induced kidney dysfunction and suggests an emerging need to understand the brain-kidney pathological crosstalk underlying stroke damage.

Key words: Stroke; Organ stress; Kidney dysfunction; ER stress; Nephroseq

I. Introduction

Stroke and kidney dysfunction are pressing public health concerns, but the underlying molecular mechanisms that link these pathologies and their dire consequences are not clearly understood. Chronic Kidney Disease (CKD) patients are more vulnerable to experiencing recurrent strokes (Lu et al., 2015), while poor glomerular filtration rate is associated with an increased risk for stroke (Masson et al., 2015). After analyzing a large number of patient data, a recent study

concluded that prior stroke independently contributes to mortality, end-stage kidney disease, and cardiovascular complications, thus highlighting the pathological relationship between the brain and kidney (Tollitt et al., 2019). Acute injury to the kidney leads to brain-kidney crosstalk via cytokine signaling, leukocyte infiltration, oxidative stress, etc. (Lu et al., 2015). We reported that experimental stroke induces proteinuria via activating Hypoxia Inducible Factor 1 subunit alpha (HIF1α)/ Zinc finger E-box-binding homeobox 2 (ZEB2), indicating a pathological link between stroke and kidney (Nakuluri et al., 2019).

Oxidative stress in the post-ischemic brain seems like a master switch that regulates multiple neuronal cell death pathways, including ER stress (Nakka et al., 2016). We recently reported that apocynin (a potent anti-oxidant agent) administration during early reperfusion time points curtails ER stress/UPR and reduces brain damage (Nakka et al., 2022). Stroke activates ER stress and unfolded protein response (UPR) pathways such as PERK (Protein Kinase RNA-like ER kinase)-eIF2α (Eukaryotic translation Initiation factor 2 alpha subunit) and IRE1α (Inositol Requiring Enzyme 1 alpha subunit)-XBP1(X-Box binding Protein 1) after dissociating from ER-resident chaperone GRP78(Glucose Regulated Protein 78) to restore normal protein folding machinery in the stressed ER lumen, however, prolonged ER stress become detrimental to the survival of neurons in the post-stroke brain tissue (Nakka et al., 2010).

Consistent with the notion that stroke induces kidney dysfunction, we asked whether ER stress is also concomitant in the kidney tissues collected from stroke-subjected animals. If yes, the neuroprotective property of apocynin could regulate ER stress in the kidney. Further, our study highlights the clinical significance of ER stress markers underlying CKD and diabetic nephropathy (DN) obtained by data mining from the nephroseq database.

II. Material and Methods:

Compliance with ethical standards: We conducted all animal experiments with Institutional Animal Ethics Committee (IAEC) permission at Acharya Nagarjuna University. The IAEC approval number is ANUCPS/IAEC/AH/P/10/2018. We minimized the animal numbers wherever necessary and took proper post-operative care of animals that underwent surgery, such as pain management and providing food and water ad libitum.

Experimental stroke induction: We induced transient focal cerebral ischemia/stroke in adult male Sprague-Dawley rats by Middle cerebral Artery Occlusion (MCAO) using 3-0 nylon monofilament (Ethicon, Johnson & Johnson Ltd.) as described earlier (Nakka et al., 2022; Longa et al., 1989). In brief, we made a small nick in the external carotid artery, inserted the filament, and advanced it through the internal carotid artery to block MCA to induce cerebral ischemia. After occluding the MCA for about 60 minutes, the blood flow was re-established (called reperfusion), and rats have sacrificed after 6 hours and 24 hours after reperfusion. We did surgical incisions for the sham control group except for the filament insertion to nullify surgical stress.

Apocynin administration: We prepared and administered apocynin (Cat#W508454; Sigma-Aldrich) intraperitoneal route to the experimental groups of sham, MCAO plus vehicle, apocynin

plus MCAO by dissolving it in 10% dimethylsulfoxide (w/v) prepared using water for injection. We injected an effective dose of 1 mg/kg at 0 hours and 6 hours of reperfusion (called post-treatment), as described previously titrated by us (Nakka et al., 2022).

Histology: We used paraformaldehyde fixed and paraffin-embedded tissue sections to perform Periodic Acid Schiff (PAS) staining and Haematoxylin and Eosin (H&E) staining for altered kidney morphology and immunofluorescence for protein expression and localization. In brief, rats subjected to sham and MCAO underwent transcardial perfusion with buffered 4% paraformaldehyde for tissue fixation (Nakka et al., 2011). Later, we collected tissue sections of the brain and kidney (8-10µ thick) using a microtome for staining. We performed PAS staining in kidney paraffin-embedded sections (Babelova et al., 2013) with slight modifications to measure glomerular tuft, urinary space, and bowman capsule from kidney tissues collected from stroke animals.

The 2, 3, 5-Triphenyltetrazolium chloride (TTC) staining and golgi-cox staining were performed to assess brain infarction and neuronal morphology, respectively, as described previously (Nakka et al., 2022).

Immunoflourescence staining: Immunofluorescence staining for ER stress markers was performed as described previously (Nakka et al., 2010; Zaqout et al., 2020). In brief, we carried out procedures such as deparaffinization, antigen retrieval, cell permeabilization, and blocking with 1% BSA. Kidney sections were incubated with primary antibodies overnight at 4° C, and antibody details are as follows. Anti-Ubiquitin (Catalog # 13-1600; Thermo-Scientific), Anti-BiP/GRP78 and Phospho-eIF2α (Ser51) antibody (Cat#3177 and Catalog#9721, respectively, from Cell Signalling Technology, USA. We used corresponding secondary antibodies conjugated with Alexa Fluor™ plus 488 (Catalog # A32731; Thermo-Scientific) and counterstained with ProLong™ Gold Antifade Mountant (Catalog # P36941; Thermo-Scientific) and images were captured and analyzed using a fluorescence microscope (Olympus 1X81).

Data mining: We analyzed ER stress-responsive gene expression profile data from clinical samples of CKD (Nakagawa CKD Kidney) and diabetic nephropathy (Woroniecka Diabetes Glom) available on the Nephroseq platform (www.nephroseq.org) database.

III. Results

Apocynin ameliorates stroke-induced altered morphology in kidney glomerulus

We showed stroke-induced brain damage and neuronal morphology in rats subjected to MCAO and apocynin treatment as preliminary screening data to continue with further experiments (Figure 1A). H&E staining of glomeruli from MCAO rats suggest that there is significant change in the glomerular histology. The predominant structural changes include increase in the bowman's space, collapsed tuft, and focal segmental sclerosis of the glomerulus. Interestingly, in the apocynin treated rats significant improvement in the glomerular architecture in the time-dependent manner (Figure 1A). Further, PAS staining of glomeruli from MCAO rats suggest that glomerular injury is accompanied with mesangial hypercellularity that accentuate the lobules of

the glomeruli, obstruction in the capillary lumen, and increased bowman's space. Treatment with apocynin ameliorated the MACO induced glomerular manifestations in the kidney (Figure 1B).
Apocynin improved kidney function in the MCAO rats: Since we observed apocynin treatment ameliorated glomerular manifestations in MCAO rats, we assessed kidney function in these rats.

Figure 1A

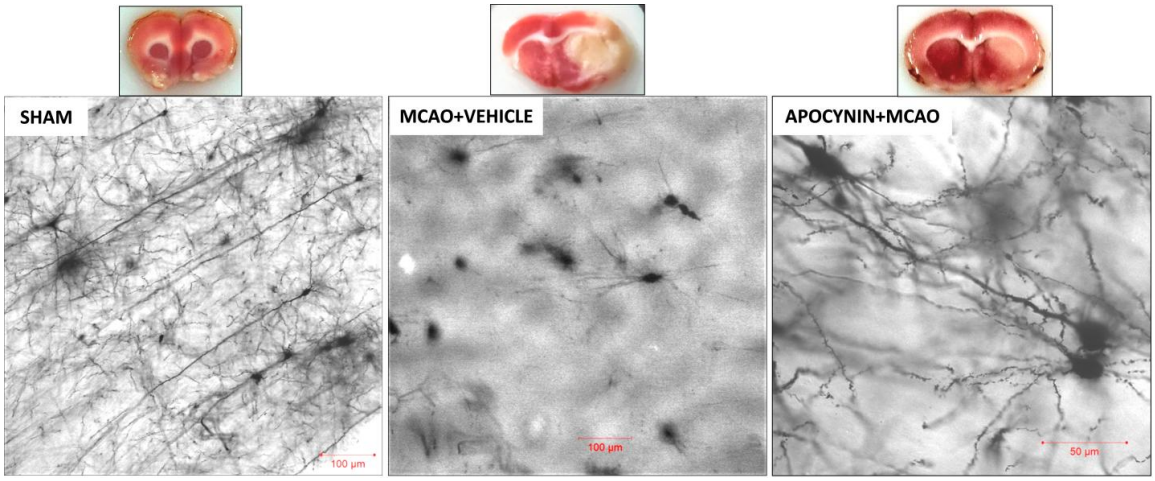


Figure 1A: Shows stroke/MCAO-induced brain damage in the striatum and cortex (TTC staining) and massive neuronal loss (Golgi-Cox staining) compared to sham control (scale bar 100µm). Apocynin treatment group showed reduced brain damage (TTC staining) and preserved neuronal morphology (Golgi-Cox staining) compared to MCAO group (n=4/group).

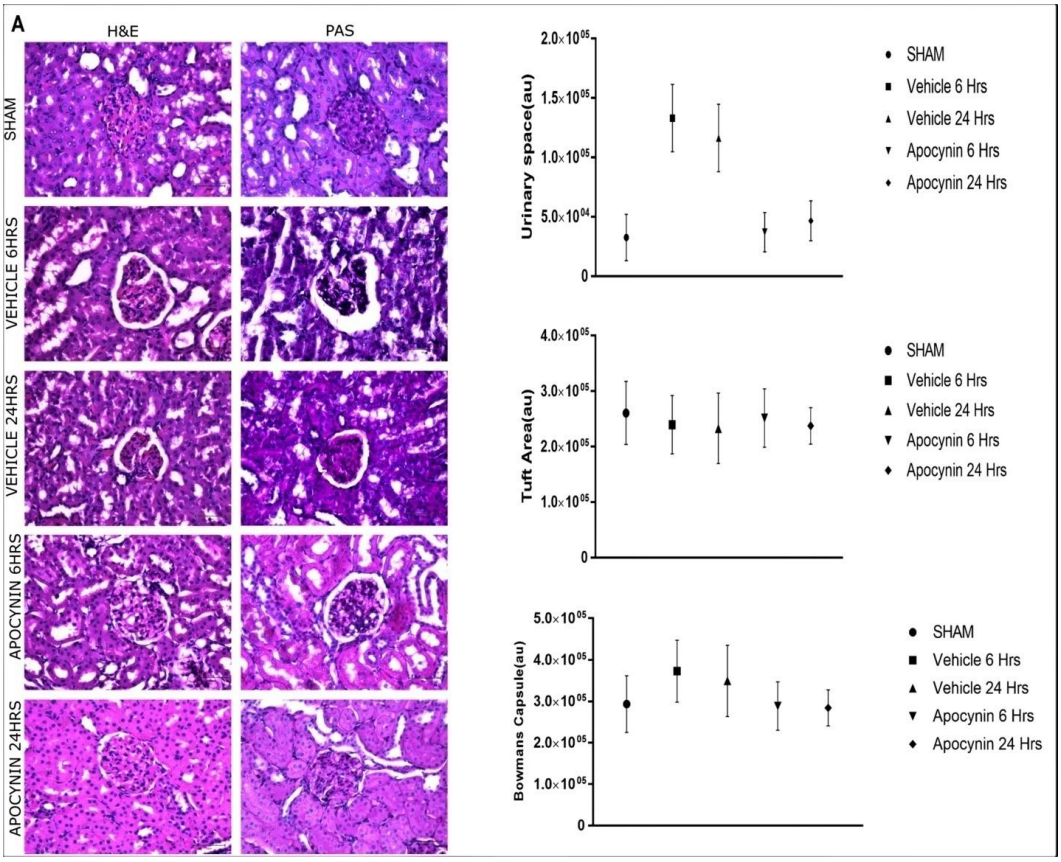


Figure 1B

Figure 1B: Shows stroke-induced morphological changes in the kidney tissue. H&E staining (left panel) shows morphological alterations such as an increase in the Bowman's capsule space associated with collapsed tuft and segmental sclerosis after 6 hours and 24 hours of reperfusion compared to sham controls. Apocynin treatment immediately after ischemia ameliorated stroke-induced altered morphology (n=4 per group). PAS staining (right panel) showed prominent morphological features of kidney injury in glomeruli, such as mesangial hypercellularity, capillary lumen obstruction, and increased Bowman's space after 6 and 24 hours of reperfusion in vehicle plus MCAO group compared to sham controls. Apocynin curtailed such abnormalities at 6 and 24 hours after reperfusion. Statistical significance as follows (n=4 per group): Bowman's capsule ($p<0.001$***), tuft area (not significant), and urinary space ($p<0.001$***). We applied One-way ANOVA followed by Tukey's multiple comparison post-hoc tests. Scale bar 2μm.

Apocynin reduces stroke-induced expression of GRP78 and eIF2α phosphorylation in the kidney glomerulus

Transient focal cerebral ischemia prominently induces the expression of ER stress markers such as GRP78 and eIF2α phosphorylation (peIF2α), contributing to brain damage (Nakka et al., 2010). Immunofluorescence staining of glomeruli from MCAO rats showed expression of GRP78 and peIF2α in a time-dependent manner post-cerebral reperfusion. Early 6 hours of reperfusion did not show a significant increase in the ER stress markers of the glomerulus. However, we noticed a pronounced expression of GRP78 and peIF2α in the glomerulus at 24 hours post-cerebral reperfusion. Interestingly, the apocynin treatment group curtailed such stress in the glomerulus, indicating that apocynin is also protective against stroke-induced stress in the kidney glomerulus showing a systemic effect (Figures 2A&B).

Figure 2

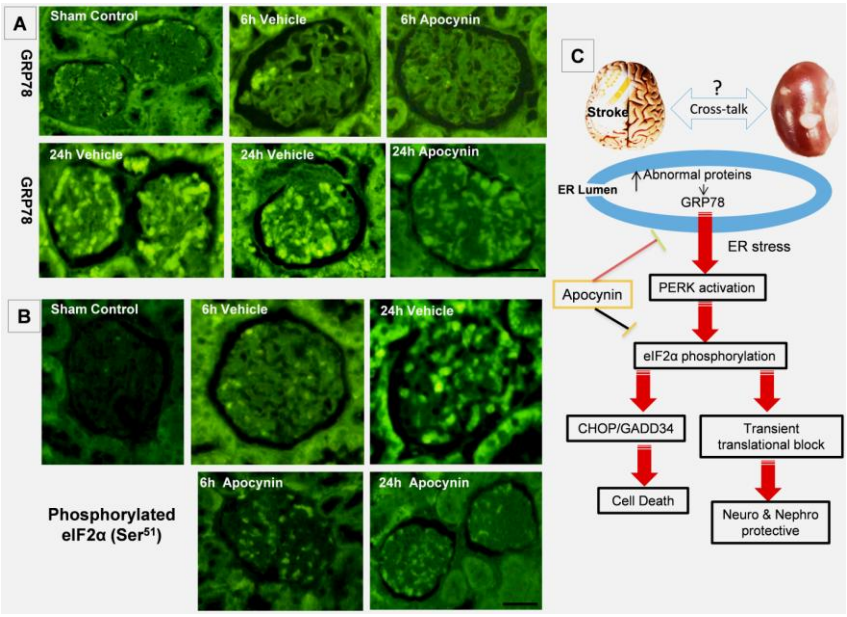


Figure 2: Shows stroke-induced activation of ER stress markers GRP78 and phosphorylated eIF2α in the glomerulus after 6 and 24 hours of cerebral reperfusion **(A&B)**. Both the markers showed increased expression after 24 hours, while apocynin treatment markedly reduced stroke-induced expression of GRP78 and eIF2α in the glomerulus (n=4 per group); scale bar 2µm. **C)** Exemplifies the PERK/ eIF2α axis of ER stress/UPR pathway of stroke-induced kidney injury. Under mild ER stress conditions, UPR serves as an adaptive stress response; however, sustained ER stress leads to cell death. Upon accumulation of abnormal protein load in the ER lumen BIP/GRP78 dissociate from the ER transmembrane kinase PERK leading to its activation. The PERK gets activated by auto-phosphorylation and activated PERK promotes eIF2α phosphorylation downstream. The peIF2α transiently attenuates global protein synthesis to reduce the protein load in the ER lumen; however, paradoxically allows the synthesis and accumulation of pro-apoptotic CHOP and GADD34, which contributes to cell death. Apocynin treatment showed a systemic effect of ER stress regulation underlying stroke-induced stress in the kidney.

Stroke induces ubiquitin accumulation in the kidney glomerulus

Stroke leads to the ubiquitination of abnormal proteins, which are subjected to degradation (Nakka et al., 2020). We observed the expression of ubiquitin after 24 hours post-cerebral reperfusion in the glomerulus of MCAO rats. However, apocynin treatment sustained the expression of ubiquitin even after 24 hours compared to sham and MCAO (Figure 3).

Figure 3

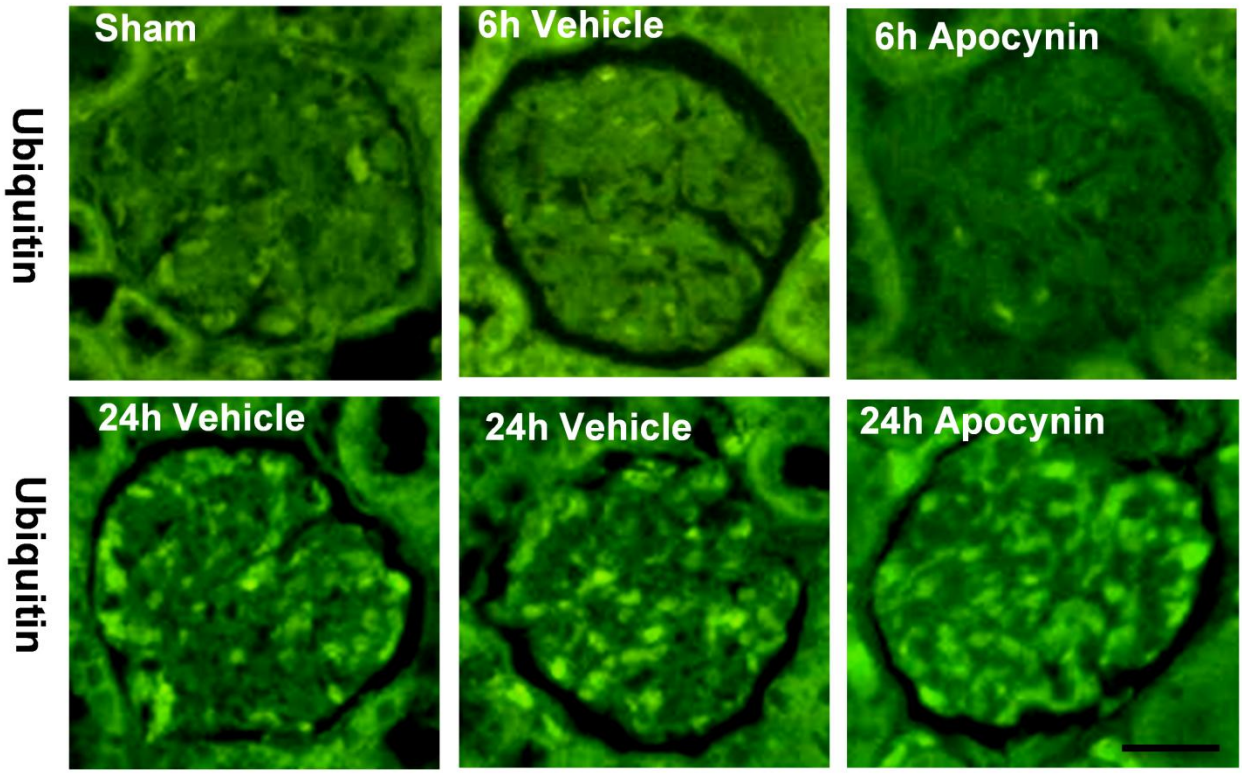
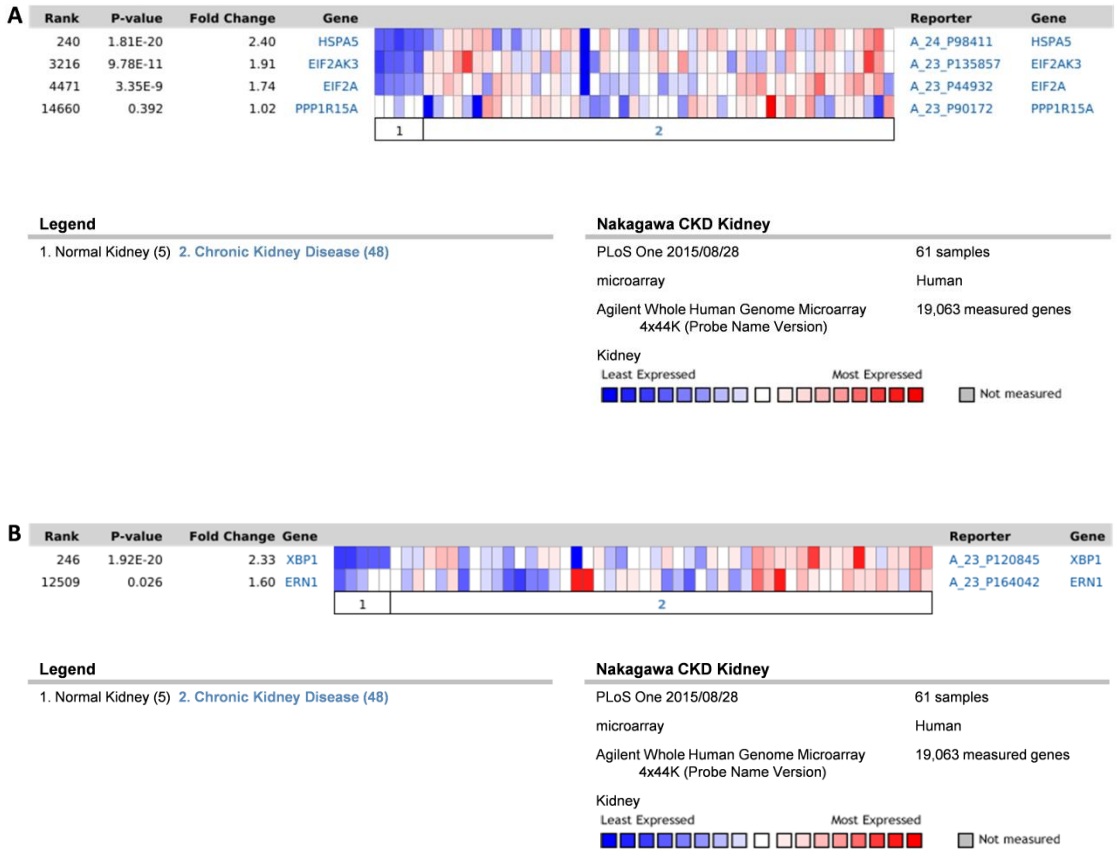


Figure 3: Shows stroke-induced expression of ubiquitin in the glomerulus of post-cerebral reperfusion. Ubiquitin expression is pronounced at 24 hours in the glomeruli of MCAO rats compared to sham control, while no significant change was observed after 6 hours of reperfusion in all experimental groups. Of note, apocynin did not affect the glomerular ubiquitin expression 24 hours post-cerebral reperfusion (n=4 per group; scale bar 2µm).

Data mining suggests increased expression of ER-stress responsive genes in CKD and DN

Data mining and visualization of nephroseq suggest increased renal expression of ERN1(ER to nucleus signalling 1 or IRE1α) and XBP1 in the Nakagawa CKD dataset. In addition, we observed increased expression of EIF2A/eIF2α, EIF2AK3/PERK, and HSPA5 (Heat Shock Protein Family A (HSP70) member 5 or BIP/GRP78, and PPP1R15A (Protein Phosphatase 1 Regulatory Subunit 15A) or GADD34 (Growth Arrest and DNA Damage inducible protein 34) in the Nakagawa CKD dataset (Figure 4A&B). Furthermore, glomeruli from DN patients presented elevated expression of ERN1 and XBP1, as revealed by the Woroniecka Diabetes Glomerulus data set (Figure 4C).

Figure 4



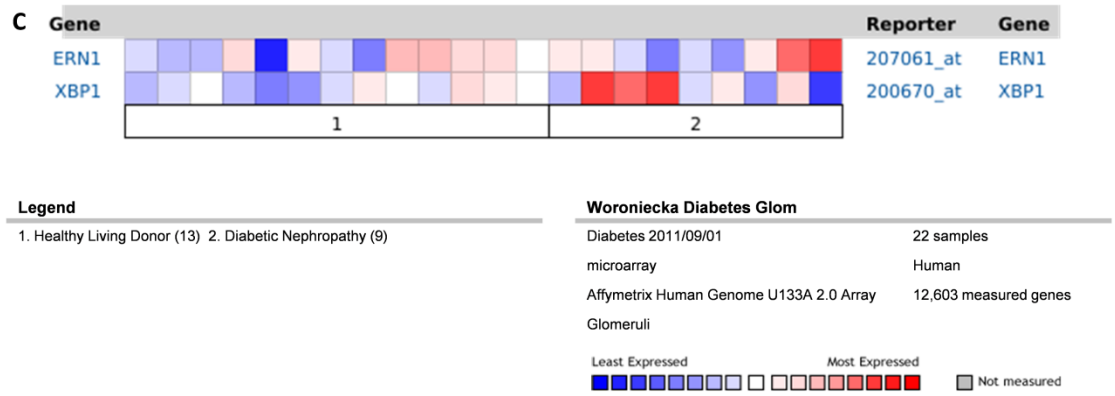


Figure 4: Represents microarray gene expression profile of ER stress/UPR pathways of normal kidney, CKD, and DN in clinical samples (Human) **A)** CKD samples showed increased renal expression of GPR78, PERK, eIF2α, and its downstream GADD34, thus indicating the activation of the UPR arm of the PERK- eIF2α axis in the diseased kidney**. B)** Increased renal expression of ERN1/IRE1α and its downstream XBP1 indicating the activation of the UPR arm of the IRE1α-XBP-1 axis in the diseased kidney. P-Values and rank scores included in the figures indicate the statistical significance of the data. **C)** Clinical samples of diabetic nephropathy showed an increased tendency of the IRE1α-XBP-1 axis. However, further analysis with more sample size is required to obtain statistical significance.

Source reference: We used Nephroseq (The Regents of The University of Michigan, Ann Arbor, MI) for analysis and visualization (https://www.nephroseq.org/resource/login.html)
Figure 4 A:
#a:1N10846;d:1N156636784;dso:geneOverex;dt:predefinedClass;ec:[1N2];epv:1N1.1N3;et:over;f:195329567;g:3309;p:1N200015275;pg:1;pvf:11016,11019[PERK],15038[GRP78],32066[GADD34],1N3;scr:datasets;ss:analysis;v:17).
Figure 4 B:
#a:1N10846;d:1N156636784;dso:geneOverex;dt:predefinedClass;ec:[1N2];epv:1N1.1N3;et:over;f:194968227;g:7494;p:1N200015275;pg:1;pvf:11310,39514,1N3;scr:datasets;ss:analysis;v:17
Figure4C:
#d:1N156636797;dso:geneOverex;dt:dataset;ec:[1N2];epv:1N1.1N3;et:over;f:2572641;g:7494;p:1N200015190;pg:1;pvf:11310[IRE1a],39514,1N3;scr:datasets;ss:all;v:17

IV. Discussion

The present study demonstrates apocynin treatment ameliorated MCAO-induced manifestation in kidney histology, in turn, kidney dysfunction, mimicking stroke in rats by MACO imposed stress in tissues of non-neurological origin, such as the kidney. We observed a significant change in morphological features and activation of ER stress/UPR markers in the glomeruli of stroke-

subjected animal groups. Further, analysis of clinical data of CKD and DN for ER stress/UPR-specific pathway markers suggests a clinical relevance of the study.

Stroke-induced dire consequences play a critical role in the dysfunction of multi-organs. Acute stroke is associated with non-neurological complications such as cardio-pulmonary, kidney injury, etc., indicating an emerging need to understand the effect of stroke on multi-organ dysfunction (Robba et al., 2020). We recently showed that transient focal cerebral ischemia induces proteinuria via HIF1α/ZEB2 pathway, indicating a pathological link between stroke-induced kidney injuries (Nakuluri et al., 2019). We and others reported the significance of ER stress underlying stroke damage and neurodegeneration (Nakka et al., 2010; Paschen and Mengesdorf, 2005). Multiple cell death pathways are activated in the post-stroke brain (Nakka et al.. 2008), and oxidative stress amongst all seems like a master switch that regulates cell death pathways, including ER stress (Nakka et al., 2016; Nakka et al., 2022).

We hypothesized that apocynin post-treatment (1mg/kg) that protects against brain damage curtailing oxidative stress and ER stress would have implications for stroke-induced kidney injury. Apocynin treatment significantly ameliorated stroke-induced altered morphology in the glomeruli. Further, apocynin curtailed stroke-induced ER stress marker GRP78 responsible for misfolded protein repair and UPR pathways. Phosphorylated eIF2α reduces the overload of abnormal proteins in the ER lumen via translational block (see Figure 2C for pathway description). The time-dependent profile of peIF2α appears early after acute ischemic insult in the brain(Nakka et al., 2010).

Increased expression of GRP78 and peIF2α in MCAO rats of glomerulus post-cerebral reperfusion indicate sustained stress in the glomerulus, which apocynin treatment curtailed efficiently. Ubiquitin removes stroke-induced misfolded protein aggregation via degradation machinery, while defects in ubiquitination machinery are associated with neurodegeneration (Nakka et al., 2020). Apocynin treatment appears to sustain the ubiquitination process in the glomerulus after post-24 hours of cerebral reperfusion, indicating post-translational modification and an ongoing process of abnormal protein degradation. Apocynin seems to regulate stroke-associated stress systemically.

Nephroseq analysis of data from CKD and DN subjects suggests the activation of ER stress and UPR branch pathways of PERK- eIF2α and IRE1α-XBP-1, emphasizing the clinical relevance. Overall, the present study suggests stroke-induced stress signaling in distal organs such as the kidney, thus having implications for understanding stroke-associated multi-organ dysfunction and establishing therapeutic strategies for stroke-induced kidney dysfunction. There is an emerging need to understand the brain-kidney pathological crosstalk underlying stroke damage.

Conflict of interest: None

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V. References

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