

The role of DRAM1 in mitophagy contributes to preeclampsia regulation in mice

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Abstract

Background: Preeclampsia (PE) is a pregnancy complication that is diagnosed by the new onset of hypertension and proteinuria. Although the pathogenesis of PE is still not fully understood, growing evidence indicates that oxidative stress and mitochondrial dysfunction may contribute to the progression of PE. Therefore, we aimed to determine the role of mitophagy in mitochondrial dysfunction and oxidative stress in PE. Moreover, we aimed to evaluate the role of DNA damage-regulated autophagy modulator 1 (DRAM1) in the development of PE.

Results: In this study, we first constructed a mouse model of PE induced by Hif-1 α and found a high level of oxidative stress, apoptosis and mitochondrial dysfunction in the placentas of PE mice. Additionally, the activity of mitophagy was decreased, and the level of DRAM1 was significantly decreased in the placentas of PE mice. To further explore the role of DRAM1 in mitophagy, DRAM1 was overexpressed in the placental tissues of PE mice. It was found that the overexpression of DRAM1 effectively improved the symptoms of PE mice and that blood lipid and urine protein levels were significantly reduced. Furthermore, DRAM1 overexpression also improved mitochondrial function and reduced oxidative stress in the placentas of PE mice. In addition, it improved mitochondrial fusion and fission and enhanced mitophagy.

Conclusions: our results indicate a key role of DRAM1 in mitophagy in contributing to the regulation of PE. To our knowledge, this is the first study to confirm the role of DRAM1 in PE, and the study provides a new understanding of the pathophysiological mechanisms of PE.

Background

Preeclampsia (PE) is characterized by hypertension with or without proteinuria after the 20th week of pregnancy and is associated with endothelial dysfunction, oxidative stress, systemic vasoconstriction, inflammation, intrauterine growth restriction, and multiorgan dysfunction [1, 2]. The World Health Organization reports that more than 60,000 pregnant women worldwide die of eclampsia each year [3]. Women with PE and their offspring have an increased risk of developing cardiovascular disease later in life [4].

Although the pathogenesis of PE is still not fully understood, increased oxidative stress along with antioxidative defense mechanisms may contribute to the progression of PE. Pregnancy itself increases susceptibility to oxidative stress, possibly resulting in tissue damage [5]. However, the production of pro-oxidants and reactive oxygen species (ROS) towards the end of pregnancy is increased in normal uncomplicated pregnancies and maintained through the accumulation of antioxidants, such as superoxide dismutase (SOD), glutathione (GSH), tocopherols, and carotenoids, as well as ascorbic acid [6]. Nevertheless, the pro-oxidant (malonaldehyde (MDA)) and antioxidant (GSH and SOD) balance is disturbed in pregnancies with PE [7], causing cellular dysfunction and death. Moreover, studies have reported that abnormal placentation and shallow trophoblast invasion stimulate placental oxidative

stress [8], which leads to an intravascular inflammatory response and endothelial dysfunction. Although multiple sources of ROS have been identified in the placenta, few studies have examined the role of mitochondrial dysfunction and ROS in the pathology of PE. Mitochondrial dysfunction causes a reduction in oxygen consumption and an increase in superoxide production [9].

Mitochondria play an important role in ATP synthesis during aerobic respiration. In this process, reactive ROS, such as the superoxide anion (O_2^-), hydroxyl radicals (OH^-), hydroperoxyl (HO_2^-), and hydrogen peroxide (H_2O_2), are formed as metabolites of mitochondrial oxidative phosphorylation [10]. Under conditions of oxidative stress, mitochondria can synthesize ROS at a level that extensively disrupts mitochondrial homeostasis, altering the composition of lipids, proteins, and nucleic acids. The resulting disruption in the composition of the mitochondrial membrane impairs the electrochemical potential of the membrane, leading to mitochondrial dysfunction and apoptosis [11].

Because mitochondrial damage can lead to apoptosis, the timely elimination of excess ROS is important for cell survival. Elevated levels of ROS trigger mitophagy, a cellular process in which lysosomes selectively scavenge for and eliminate damaged mitochondria [12]. Under conditions of oxidative stress, mitophagy is upregulated to prevent the accumulation of dysfunctional mitochondria. Thus, we suspect that mitochondrial dysfunction regulated by mitophagy plays an important role in PE [13].

DNA-damage regulated autophagy modulator 1 (DRAM1) is an evolutionarily conserved transmembrane protein [14]. DRAM1 was identified as the first direct molecular link between the tumor suppressor TP53/p53 and autophagy [15]. Since then, various roles for DRAM1 have been described in several processes, including autophagy, cell death, immunity and cellular differentiation. Elevated oxidative stress caused by mitochondrial dysfunction leads to apoptosis, and mitophagy plays an important role in maintaining mitochondrial function. However, neither mitochondrial dysfunction nor mitophagy has been examined in PE. Additionally, we do not know the contributions of mitochondrial dysfunction and mitophagy to the development of PE. Moreover, the cause of mitochondrial dysfunction and mitophagy are unknown in PE. Therefore, we wanted to evaluate the role of mitochondrial dysfunction and ROS in the development of PE. Moreover, we sought to determine the role of mitophagy in mitochondrial dysfunction in PE. We therefore aimed to investigate whether DRAM1 plays a role in mitophagy and mitochondrial dysfunction in PE.

In this study, we provide evidence that oxidative stress is induced by mitochondrial dysfunction and that mitochondrial dysfunction is caused by a decrease in DRAM1 and mitophagy in the placenta in PE. Additionally, we demonstrate that the dysfunction of mitophagy in PE may result from disturbances in mitochondrial fusion and fission and a lack of DRAM1. Altogether, our results indicate a key role for DRAM1 in mitophagy in contributing to the regulation of PE. To our knowledge, this is the first study to find that DRAM1 plays a role in PE, and it provides a new target for the treatment of PE and new insights into the pathophysiological mechanisms of PE.

Results

Hif-1 α induces preeclampsia in mice

To effectively evaluate whether DRAM1 plays an important role in PE, we first constructed a PE mouse model induced by hypoxia-inducible factor 1 α (Hif-1 α) [16]. To effectively evaluate the symptoms of PE mice, we examined the pathological and physiological indicators associated with PE.

Hypertension is a defining feature of PE [17]. We examined the dynamic changes in blood pressure in PE mice as pregnancy progressed. The first measurement was taken at E0.5, and blood pressure began to rise after the mice became pregnant. On E16.5, the blood pressure of the Hif-1 α group was significantly higher than that of the Veh-GFP and WT groups (Fig. 1A, * $p < 0.05$, # $p < 0.05$, $n = 8$), while there was no significant difference between the Veh-GFP group and the WT group (Fig. 1A, $n = 8$). On E19.5, the blood pressure of the Hif-1 α group was significantly higher than that of the Veh-GFP and WT groups (Fig. 1A, ** $p < 0.01$, ### $p < 0.01$, $n = 8$), and there was no significant difference between the Veh-GFP and WT groups (Fig. 1A, $n = 8$). The blood pressure of the mice was measured after delivery; blood pressure began to drop, and there was no significant difference between the three groups (Fig. 1A, $n = 8$).

Dyslipidemia is closely related to the occurrence and development of PE during pregnancy [1]. We measured triglyceride (TG), total cholesterol (TC), low density lipoprotein (LDL), and high density lipoprotein (HDL) levels on E19.5. The results showed that the levels of TG and TC in the Hif-1 α group were significantly increased compared with those in the WT group and the Veh-GFP group (Fig. 1B, * $p < 0.05$, ** $p < 0.01$, $n = 4$) and that there was no significant difference between the WT group and the Veh-GFP group. There was no significant change in the LDL content of the Hif-1 α group relative to that of both the WT and Veh-GFP groups (Fig. 1B, $n = 4$). In addition, the HDL content of the Hif-1 α group was significantly decreased compared with that of the WT group and the Veh-GFP group (Fig. 1B, ** $p < 0.01$, $n = 4$), and there was no significant difference between the WT group and the Veh-GFP group.

Next, we measured the presence of proteinuria, another defining feature of PE, on E16.5 and E19.5. The results showed that the urine protein content of the Hif-1 α group was significantly increased compared with that of the WT and Veh-GFP groups on both E16.5 and E19.5 (Fig. 1C, ** $p < 0.01$, $n = 8$). There were no significant differences between the WT group and the Veh-GFP group on either E16.5 or E19.5 (Fig. 1C).

Finally, we tested the kidney function of mice on E19.5 and found that the WT and Veh-GFP mice showed no glomerular changes. Glomerular shrinkage was observed in the Hif-1 α mice relative to the WT and Veh-GFP mice (Fig. 1D). In addition, the Hif-1 α mice developed moderate swelling of glomerular endothelial cells and mesangial cells with limited occlusion of the glomerular capillary lumen (Fig. 1D).

The hypertension, hyperlipidemia, high urine protein and renal dysfunction observed in the Hif-1 α mice were consistent with the pathological findings of PE. These results indicate that we successfully constructed a PE mouse model that could be used in subsequent experiments.

Excessive oxidative stress in the placentas of preeclampsia mice

The placenta is an important organ for the exchange of substances between the fetus and the mother. To test whether the placental function of mice with PE is normal, we evaluated the morphology and oxidative stress levels of these mice. As gestation proceeds, the relative area of the junctional zone (JZ) decreases as the labyrinth expands in size [18]. We first examined the morphological changes in the placentas of the mice and found that the JZ region was larger than that of the WT and Veh-GFP mice on E19.5 (Fig. 2A). To determine the level of oxidative stress in the placenta, we first tested the enzymatic activity of SOD, which is an important antioxidant enzyme. The results showed that the SOD activity in the Hif-1 α group was significantly decreased compared with that in the WT and Veh-GFP groups, while there was no significant difference between the WT and the Veh-GFP groups (Fig. 2B, ** $p < 0.01$, $n = 4$). We also examined the amount of MDA, which is a natural product of lipid oxidation in organisms and represents the level of oxidative stress. We found that the MDA content in the Hif-1 α group was significantly higher than that in the WT and Veh-GFP groups and that there was no significant difference between the WT and Veh-GFP groups (Fig. 2C, ** $p < 0.01$, $n = 4$). In addition, we measured the content of H₂O₂, the most common active oxygen molecule in organisms. The results showed that H₂O₂ levels were increased significantly in the Hif-1 α group compared to the WT and Veh-GFP groups and that there was no significant difference between the WT and Veh-GFP groups (Fig. 2C, ** $p < 0.01$, $n = 4$). These findings suggest excessive oxidative stress in the placentas of PE mice.

Mitochondrial dysfunction in the placenta of preeclampsia mice leads to apoptosis

High levels of oxidative stress usually trigger inflammation and apoptosis. There is a close relationship between oxidative stress and mitochondrial dysfunction, which can lead to the aggravation of oxidative stress, so we examined the level of apoptosis and mitochondrial function.

To investigate the growth of placental cells, we first examined the level of proteins associated with apoptosis. Bax is one of the earliest pro-apoptotic factors and mainly exists in the cytoplasm and on the surface of mitochondria. Bcl-2 is a classical genes that inhibits apoptosis [19]. It blocks apoptosis by inhibiting oxidative stress and is mainly distributed in the mitochondrial membrane. Therefore, the levels of Bax and Bcl-2, and the ratio of Bax/Bcl-2 can be detected to represent the level of apoptosis.

We used Western blotting (WB) to assess the abovementioned protein levels associated with apoptosis. Compared with that in the WT group and the Veh-GFP group, the Bax protein content in the Hif-1 α group was significantly increased, and there was no significant difference between the WT and the Veh-GFP groups (Fig. 2E, F, * $p < 0.05$, ** $p < 0.01$, $n = 6$). There was no significant difference in the protein level of Bcl-2 between the WT groups and the Veh-GFP or Hif-1 α group (Fig. 2E, F, $n = 6$). We calculated the Bax/Bcl-2 ratio and found that it was significantly increased in the Hif-1 α group compared to the WT and

Veh-GFP groups and that there was no significant difference between the WT and Veh-GFP groups (Fig. 2E, F, * $p < 0.05$, ** $p < 0.01$, $n = 6$).

Mitochondria are the core of energy metabolism, and the growth of the fetus requires a large amount of ATP from the placenta [20]. Therefore, mitochondrial dysfunction is an important factor that affects PE. The maintenance of energy metabolism, the regulation of mitochondrial function changes or adaptation to environmental stimulation, the generation of new mitochondria and the clearance of defective mitochondria are critical for eukaryotic cells to maintain mitochondrial health. In mitochondria, energy is mainly synthesized by the electron transport chain (ETC). Cytochrome c oxidase IV (COX IV) plays an important role in the ETC [21]. Therefore, we measured the protein expression level of COX IV in placental tissue by WB. The results showed that the level of COX IV in the Hif-1 α group was significantly lower than that in the WT and Veh-GFP groups (Fig. 2E, F, * $p < 0.05$, $n = 6$) and that there was no significant difference between the WT and Veh-GFP groups. These results suggest that apoptosis induced by oxidative stress in the placentas of PE mice may be caused by mitochondrial dysfunction.

Decreased DRAM1 in the placentas of preeclampsia mice leads to mitophagy dysfunction

Mitophagy is the most important pathway for clearing damaged mitochondria. The current focus of mitophagy is the phosphatase and tensin homolog-induced putative kinase 1 (PINK1)/E3 ubiquitin ligase (Parkin) regulatory pathway [12]. Protein aggregates and damaged organelles are ubiquitinated to initiate selective autophagy. To initiate mitophagy, phosphorylated PINK1 is ubiquitinated to activate Parkin, which establishes a ubiquitin chain on outer mitochondrial membrane proteins, recruits autophagy receptors, and triggers mitophagy.

Therefore, we used WB to detect the protein content of PINK and Parkin, and the results showed that the level of PINK in the Hif-1 α group was significantly lower than that in the WT and Veh-GFP groups and that there was no significant difference between the WT and Veh-GFP groups (Fig. 2G, H, * $p < 0.05$, $n = 6$). Compared with that in the Veh-GFP group, the Parkin protein level in the Hif-1 α group was significantly decreased, and there was no significant difference between the WT group and the Veh-GFP group (Fig. 2G, H, ** $p < 0.01$, $n = 6$).

To further investigate the causes of mitophagy, we examined the protein DRAM1 which plays an important role in autophagy. It was found that the level of DRAM1 in the Hif-1 α group was significantly decreased compared with that in the WT and Veh-GFP groups, while there was no significant difference between the WT and Veh-GFP groups (Fig. 2G, H, * $p < 0.05$, ** $p < 0.01$, $n = 6$).

These findings suggest that mitochondrial dysfunction is due to the inability of damaged mitochondria to be cleared in time and that the loss of DRAM1 leads to mitophagy dysfunction.

Overexpression of DRAM1 significantly improves the symptoms of preeclampsia mice

To further investigate whether PE is affected by DRAM1, we transferred a DRAM1 overexpression plasmid into the trophoblasts of the placentas of preeclampsia mice on E14.5 by embryonic electroporation (Fig. 3A). To verify the normal expression of DRAM1, the protein levels of DRAM1 were tested by WB, and the results showed that the level of DRAM1 in the DRAM group was significantly increased compared to that the control group (Fig. 3C, D, * $p < 0.05$, $n = 6$). This result indicates that we successfully constructed a DRAM1 overexpression model.

To evaluate the effect of DRAM1 overexpression on PE, we measured the total amount of urine protein in the mice, and the results showed that there was no significant difference between the DRAM and control groups on E16.5 but that the level of urine protein in the DRAM group decreased significantly on E19.5 (Fig. 3B, * $p < 0.05$, $n = 6$).

Meanwhile, the serum lipid levels of E19.5 mice were detected. The results showed that there was no significant difference in TG or LDL levels between the DRAM group and the control group (Fig. 3H). The TC content decreased significantly after DRAM1 overexpression (Fig. 3H, ** $p < 0.01$, $n = 6$). In addition, the HDL content of the DRAM group was significantly higher than that of the control group (Fig. 3H, * $p < 0.05$, $n = 6$). The above results show that the overexpression of DRAM1 effectively improves the symptoms of hyperlipidemia and high urine protein in PE mice.

Overexpression of DRAM1 effectively reduces apoptosis of placental cells in preeclampsia mice

In addition, we measured the level of oxidative stress in placental tissues on E19.5, and the results showed that the level of SOD in the DRAM group increased significantly compared with that in the control group (Fig. 3E, * $p < 0.05$, $n = 6$). The level of MDA content decreased significantly after DRAM1 overexpression (Fig. 3F, * $p < 0.05$, $n = 6$), and the level of H_2O_2 did not change significantly (Fig. 3G).

Next, we measured the apoptosis level of placental cells on E19.5. The results showed that there was no significant change in the level of Bax in the DRAM group and that the content of Bcl-2 was significantly increased compared with that in the control group (Fig. 3I, J, ** $p < 0.01$, $n = 3$). In addition, we calculated the ratio of Bax to Bcl-2, and the results showed that the Bax/Bcl-2 ratio decreased significantly after DRAM1 was overexpressed (Fig. 3J, ** $p < 0.01$, $n = 6$). The above results show that the overexpression of DRAM1 effectively reduces apoptosis induced by oxidative stress.

Overexpression of DRAM1 promotes mitochondrial fusion, fission and mitophagy

How does DRAM1 improve the levels of oxidative stress and apoptosis in the placentas of preeclampsia mice? We investigated the levels of mitophagy and energy synthesis in the placenta after DRAM1 overexpression. The results showed that the levels of PINK and Parkin protein in the DRAM group

increased significantly compared with those in the control group (Fig. 4A, B, $**p < 0.01$. $n = 6$). These results indicated that the overexpression of DRAM1 effectively promoted the occurrence of mitophagy. Meanwhile, we detected the protein level of COX IV in the placenta by Western blotting and found that the overexpression of DRAM1 significantly promoted the expression of COX IV (Fig. 4A, B, $*p < 0.05$. $n = 6$). The above results indicate that the overexpression of DRAM1 can promote mitophagy, clear damaged mitochondria, and improve mitochondrial function.

Mitochondrial kinetic balance is critical for regulating mitochondrial function and maintains energy metabolism changes and normal function through continuous fission and fusion [22]. Therefore, we examined major protein changes associated with mitochondrial fusion and mitochondria. Mitochondria have a double-layer membrane structure, and the fusion of mitochondria in vivo and in the outer membrane is independently carried out by OPA1 and mfn1/2, respectively [23]. The fusion of the outer mitochondrial membrane is mediated by MFN1/2, and OPA1 mediates the inner membrane. We first examined the protein level of Mfn1/2 in the placenta by WB. The results showed that compared with that in the control group, the Mfn1 protein level in the DRAM group was significantly increased (Fig. 4C, D, $*p < 0.05$, $n = 6$) and that Mfn2 was not significantly changed (Fig. 4C, D). OPA1 forms precursor proteins in the mitochondrial cytoplasm, which are transported to the outer membrane of the mitochondria during mitochondrial division[24]. We found that the protein level of OPA1 in the mouse placenta was significantly increased after DRAM1 overexpression (Fig. 4E, F, $*p < 0.05$, $n = 6$). These results indicate that DRAM1 can effectively promote the fusion and fission of mitochondria.

Discussion

Mitochondrial dysfunction and oxidative stress are associated with PE but have not been measured in a mouse model of PE; however, such an investigation is important to better understand the contribution of mitochondrial dysfunction and oxidative stress to the function of placental in PE. The present study reveals several novel findings. (1) The blood pressure of PE mice increases gradually with increasing gestation and recovers gradually after the end of gestation. (2) PE mice have high levels of oxidative stress in the placenta, which leads to apoptosis. (3) Mitochondrial dysfunction can be observed in the placentas of PE mice, mitophagy is weakened, and the level of DRAM1 is low. (4) DRAM1 overexpression can reduce urine protein and serum lipid levels effectively in the placentas of PE mice. (5) DRAM1 overexpression significantly reduces the level of oxidative stress, improves mitochondrial function and enhances the level of mitophagy in the placentas of PE mice. (6) DRAM1 improves mitophagy by promoting mitochondrial fusion and fission, thereby improving mitochondrial function. On the basis of these results, it is suggested that increased DRAM1 in the placenta contributes to improved mitochondrial dysfunction and decreased levels of oxidative stress in PE.

Although ROS production is known to be induced by ischemic placenta in PE, ROS are also produced by systemic blood vessels during the second phase of PE onset. In some studies, placental homogenates derived from patients with PE show 39% higher hydrogen peroxide production than those derived from normal pregnant women [3, 25]. We also found a significant increase in the level of H_2O_2 in a mouse

model of PE induced by Hif-1 α . However, the content of H₂O₂ decreased in the placenta after DRAM1 overexpression in PE mice. Additionally, SOD is an enzyme that catalyzes the scavenging of superoxide radicals, which are constitutively expressed in the mitochondria and cytoplasm. SOD is increased during normal pregnancy, but the activity and mRNA expression of SOD in placentas derived from patients with PE is decreased, which may result in increased oxidative stress in the placentas of patients with PE. It has been reported that SOD is decreased in erythrocytes derived from patients with PE. We also found a significant decrease in the activity of SOD in the placentas of PE mice. However, the activity of SOD increased significantly when DRAM1 was overexpressed.

Moreover, increased ROS concentrations in patients with PE have been confirmed by increased levels of MDA, an index of lipid peroxidation [26]. At the same time, we also found that the expression of MDA in the placentas of PE mice was significantly increased. However, the MDA content decreased significantly after DRAM1 overexpression. In addition, lipid peroxidation is closely related to heat-shock protein 70 (HSP70) [27]. The HSP70 level in the peripheral blood is significantly higher in both the fetal and maternal circulation in PE [28]. We also found that the levels of TC and TG in the serum were increased significantly and that their content gradually decreased after DRAM1 was overexpressed in the placentas of PE mice.

Oxidative stress is the result of many ROS exceeding antioxidant activity and one of the risk factors for the development of PE through vascular dysfunction [29]. The poor invasion of cytotrophoblasts into the uterine myometrium and disturbed spiral artery remodeling play important roles in the pathophysiology of PE, which results in placental hypoxia [30, 31]. At this time, the termination of pregnancy is the only way to avoid maternal eclampsia and fetal distress in PE [32]. Supplementation with many antioxidants may prevent or repair the occurrence of PE [33]. In addition, our results indicate that overexpression of DRAM1 can effectively reduce the oxidative stress level in the placentas of PE mice. This is likely to be useful in the treatment of PE.

Over the past 20 years, more than 800 peer-reviewed publications have confirmed the hypothesis that oxidative damage is involved in the pathophysiology of PE, but current antioxidant interventions are not clinically effective [34]. One possible explanation is that these antioxidant regimens fail to reach the mitochondria in the cell. Therefore, they fail to improve the pathology of oxidative damage. Great advances have recently been made in mitochondrial pharmacology due to the development of many different pharmacological strategies for addressing mitochondrial dysfunction [35]. Mitochondrial dysfunction is a pathogenic mediator of oxidative stress in PE, as increased mitochondrial lipid peroxidation and enhanced susceptibility to oxidation are evident in mitochondria in the placentas of women with pregnancies complicated by PE [36, 37]. We also found that COX IV levels decreased significantly in the placentas of PE mice.

There are a number of different instigators that disrupt mitochondrial function, including altered oxygen consumption, decreased ATP production, increased ROS production and mtDNA damage [38]. Mitochondrial damage is one of the most important factors that lead to mitochondrial dysfunction[8].

Therefore, preventing mitochondrial damage is very important. To prevent mitochondrial damage by preserving a population of healthy mitochondria, several quality control mechanisms have evolved. Selective autophagy of mitochondria, known as mitophagy, is an important mitochondrial quality control mechanism that eliminates damaged mitochondria [8]. The elimination of damaged mitochondria in mammals is mediated by a pathway comprised of PTEN-induced PINK1 and Parkin [13]. PINK1 and Parkin accumulate on damaged mitochondria, promote their segregation from the mitochondrial network, and target these organelles for autophagic degradation in a process that requires the Parkin-dependent ubiquitination of mitochondrial proteins [39, 40]. We found a significant decrease in the level of PINK1 and Parkin in the placental tissues of PE mice, which suggests that the mitochondrial dysfunction in PE is probably due to a decreased level of mitophagy. After DRAM1 overexpression, the levels of PINK1 and Parkin in the placentas of PE mice were significantly increased, indicating that mitophagy was activated effectively. At the same time, the COX IV level was also significantly elevated, indicating an effective improvement in mitochondrial function.

How can DRAM1 improve mitophagy and thus promote mitochondrial functional recovery? The regulation of mitochondrial dynamics occurs through mitochondrial biogenesis and continuous cycles of fission and fusion [22]. These processes are thought to target damaged/depolarized mitochondria for autophagy. Mitochondrial fission and fusion are regulated by members of a family of conserved large GTPases initially identified in yeast [23]. The dynamin-like GTPase dynamin-related protein 1 (Drp1) mediates fission by forming a multimeric complex that wraps around the outer membrane of mitochondrial tubules and exerts a mechanical force to produce membrane scission [12]. In contrast to fission, in which mitochondria are divided using only an outer membrane apparatus, two distinct machineries, Mitofusin 1 and 2 (Mfn1/2) and optic atrophy 1 (OPA1), are required for the fusion of the outer and inner membranes [23]. We first measured the levels of DRP1 and Mfn1/2 and found that mitochondrial fusion was significantly increased after DRAM1 overexpression. These findings are consistent with the findings of Yu et al., who reported that Mfn2 is downregulated in PE placentas [41]. In addition, Yu et al. demonstrated that ATP is significantly reduced after knockout of the Mfn2 gene in trophoblast cells [41]. Therefore, our findings suggest that DRAM1 can improve mitochondrial dysfunction by promoting mitochondrial fusion in PE. Alternatively, during fission, unhealthy, nonfunctional mitochondrial fragments that lack transmembrane potentials are discarded and targeted for degradation via a selective autophagic process termed mitophagy [42]. W. J. et al [41]. demonstrated that DRP1^{-/-} mice exhibit embryonic lethality due to a deficiency in the formation of trophoblast giant cells and consequent placental dysfunction, underscoring the requirement of mitochondrial fission for proper placental and embryonic development. Hence, DRAM1 was shown to promote mitochondrial fission to accelerate mitophagy to clear damaged mitochondria, which is significant for maintaining mitochondrial function.

Conclusion

Our present study is the first to demonstrate that DRAM1 reduces apoptosis induced by oxidative stress by improving mitochondrial dysfunction, enhancing mitophagy clearance through mitochondrial fission and fusion, and rescuing urinary protein and serum lipids levels in the placentas of PE mice. These data shed light on a novel mechanism behind the therapeutic effect of DRAM1 in PE.

Methods

Animals

C57BL/6J wild-type mice were purchased from Guangdong Medical Laboratory Animal Center (Guangdong, China), and all experiments have been approved by the Ethics Committee of Shenzhen Hospital of Peking University (Permit Number: SYXK-2015-0106, 2019-078), and were in accordance with the ARRIVE guidelines on the Care and Use of Experimental Animals. Male and female animals were fed separately and housed in groups of 4–5. All mice were maintained under standard laboratory conditions at 22 ± 2 °C, with $50 \pm 10\%$ relative humidity and on a 12 h-light/dark cycle, with food and water made available ad libitum. Their fertility cycle was controlled, and they were allowed to mate overnight when females were in a pro-estrus state. Vaginal smears on glass slides were examined on the following morning at 8:00 am; and if spermatozoa were found, it was designated as first day of pregnancy (E0.5). Pregnant mice at day 8 of gestation were randomly divided into three groups and injected systemically via tail vein with either 100 μ l of Ad-CMV-HIF (8×10^{10} viral particles) (n = 8), Ad-CMV-GFP (8×10^{10} viral particles) (n = 8), or saline solution (n = 8) (Biotechnology of Heyuan, China). At the end of the experiment, the mice were euthanized by CO₂ (70%).

Blood Pressure Measurement

Systolic blood pressure was measured in conscious mice on day 0.5, 8.5, 12.5, 16.5, 19.5 of gestation mice using the tail cuff technique (Yuyan Instruments, Shanghai, China). Mice were prewarmed at 37 °C for 30 minutes before measurements were taken. The mean of five consecutive readings was recorded as systolic blood pressure.

Urinary Albumin and Serum lipid Measurement

Urine was collected on day 16.5 and 19.5 of gestation for albumin measurements. Urinary albumin was quantified by bicinchoninic acid (BCA) protein assay of Easy II Protein Quantitative Kit (Cat. No. DQ111-01, TRAN, Beijing, China) according to the manufacturer's protocol. In brief, urine was collected for each mouse, Protein concentration was determined using BCA Protein Assay. Absorbance was measured at 595 nm with Microplate Reader and BSA was used as standard.

Blood was collected on day 19.5 of gestation for serum lipid measurements. Serum lipid was quantified by ultraviolet-visible spectrophotometry (UV-VIS) of Biochemical detection kit for blood lipid. These Kits were as follows: TC (Cat. No. BH016Z, TEGEN, China), TG (Cat. No. BH017Z, TEGEN, China), LDL (Cat.

No. BH019Z, TEGEN, China), HDL (Cat. No. BH018Z, TEGEN, China). All Kits according to the manufacturer's protocol.

Tissue processing

For histology and biochemical analyses, mice were anesthetized with sodium pentobarbital (1%) and the placentas and kidneys were rapidly removed. Some placentas were kept at 80 °C for biochemical analysis. The remaining placentas and kidneys were fixed with 4% (vol/vol) paraformaldehyde (PFA) overnight, and dehydrated with gradient sucrose (20% and 30%) for 24 h, after that they were embedded in OCT (optimal cutting temperature compound). At last, the embedded tissue was sagittal cut into 30-mm-thick sections using a freezing-sliding Microtome (Leica, Germany).

Histology

The obtained placental kidney tissue sections were washed by PBS and used for Hematoxylin and Eosin (H&E) staining. H&E staining were performed following the protocol of the Kit (Cat. No. G1120, Solarbio, China).

Oxidative stress Measurement

To test the level of oxidative stress in placental, different kits were used. Superoxide Dismutase (SOD) activity in placental tissue was detected by nitro-blue tetrazolium (NBT) (Cat. No. S0107, Biyuntian, China). The levels of malonic dialdehyde (MDA) were tested by thiobarbituric acid (TBA) (Cat. No. BC0025, Solarbio, China). The levels of H₂O₂ were tested by Titanium sulfate (TS) (Cat. No. BC3595, Solarbio, China). All Kits according to the manufacturer's protocol.

Western blot

Placental samples were homogenized in Tris-buffered saline (TBS) with a protease inhibitor cocktail and phosphatase inhibitors (Roche, Basle Switzerland). The samples were centrifuged at 13,000 g for 1.5 h at 4°C and the supernatants were collected. The protein concentration was measured with BCA kit (Cat. No. DQ111-01, TRAN, Beijing, China). The protein samples (20 µg) were loaded onto a 10% SDS-PAGE gel and then transferred onto 0.45 mm polyvinylidene difluoride membranes (Millipore, Massachusetts, USA) at 90 mA for 1–2 h. After blocked with 5% non-fat milk for 1 h at 37 °C, the membrane was incubated with primary antibody (Table 1) overnight at 4 °C and horseradish peroxidase-conjugated secondary antibodies (anti-mouse and anti-rabbit; NeoBioscience) for 1 h at 37 °C. The immunoreactive bands were visualized with an electroluminescence kit and scanned for densitometric analysis using an imaging system (Image Station 4000 M; Kodak) and Quantity One software (Bio-Rad). GAPDH or β-Actin were used as the loading control.

In utero electroporation was as previously described [43]. Briefly, pregnant female mice were anesthetized by diluting ketamine (100 mg/kg) and xylazine (10 mg/kg) with 0.9% saline. The uterine horns were carefully taken out following cutting the abdomen. Approximately 3 µg of plasmid DNA (V5-DRAM1-pCAGEN-EGFP or V5 -pCAGEN-EGFP) was mixed with 1 µg of RFP-pCAGGS and 0.025% of Fast Green

was pressure injected into the junctional zone of placenta by pulling the glass capillaries. Five pulses of current (40 mV for 40 ms) were sent to the placenta using an electroporator (BTX, T830). The uterus was moved into the peritoneal cavity, and the abdominal wall and skin were sutured.

Statistical Analysis

The data were analyzed using GraphPad Prism software. All data were expressed as the mean \pm SEM and considered statistically significant at a level of * $p < 0.05$, ** $p < 0.01$. A two-way t-test was used to analyze the data of immunoblot analysis.

Declarations

Ethics Approval and Consent to Participate: All animal experiments and procedures were approved by the Ethics Committee of Shenzhen Hospital of Peking University (Permit Number: SYXK-2015-0106, 2019-078).

Consent for Publication: The authors showed their consent for publication of the research results.

Availability of data and materials: All data generated or analyzed during this study are included in this published article.

Competing Interests: The authors have no conflict of interest to declare.

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Author Contributions: All authors have read and approved the manuscript. GQ. C contributed to data acquisition, interpretation, and performed the experiments, statistical analysis, and participated in the drafting of the manuscript. YL1, LC and FZ analyzed and interpreted data, LZ, YH and PPH performed statistical analysis. LL. L participated in the drafting of the manuscript. YL2. Y designed the study, performed the experiments, analyzed and interpreted data, drafted the manuscript, procured funding, and supervised the study.

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Figures

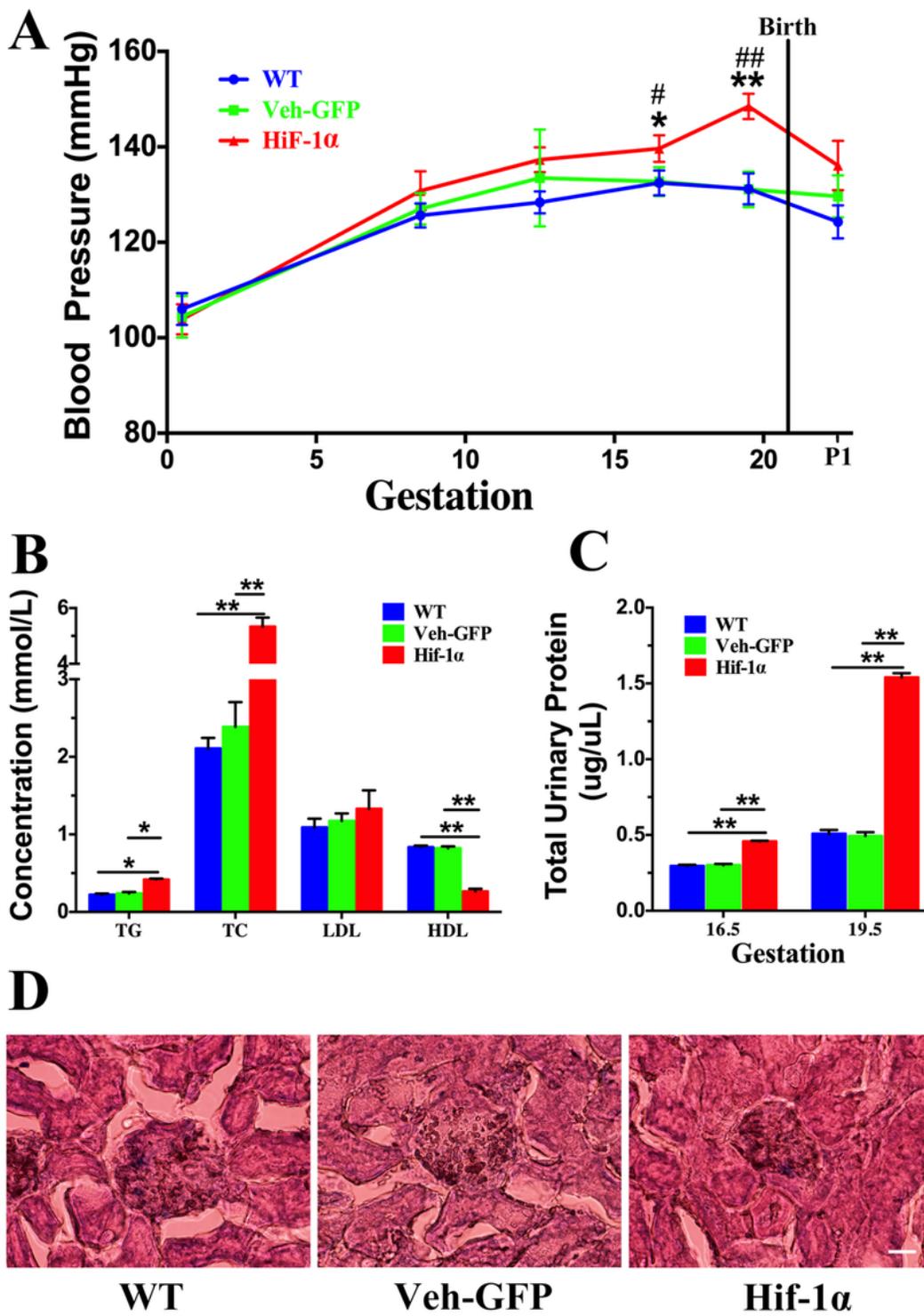
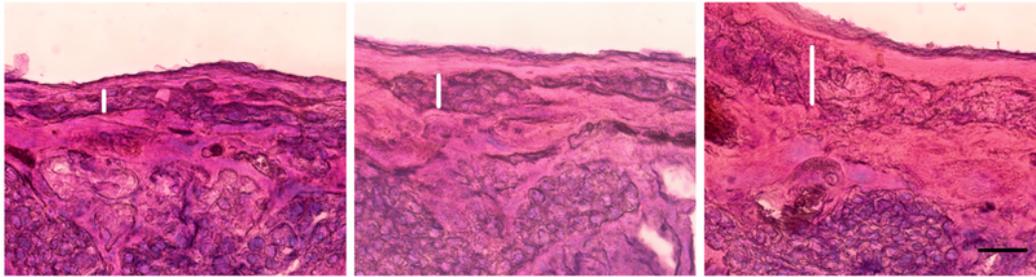


Figure 2

Construction of a pre-eclampsia mouse model by Hif-1α virus. Preeclampsia mice have elevated blood pressure, and the levels of urinary protein increased, and the function of renal impaired. A. Blood pressure was tested sequentially throughout gestation at the time points indicated in WT (Blue, n=8), Veh-GFP (Green, n=8), Hif-1α (Red, n=8) mice. Data are mean (±SEM) of several mice used for each experiment. *p<0.05 as compared with Veh-GFP group, #p<0.05 as compared with WT group. B. To measure serum

lipid levels at E19.5 in mice, Kits were used to detect triglyceride (TG), total cholesterol (TC), low density lipoprotein (LDL), high density lipoprotein (HDL) in WT, Veh-GFP and Hif-1 α mice (n=4). Data are mean (\pm SEM) of several mice used for each experiment. *p<0.05, **p<0.01. C. To determine total urinary protein, the bicinchoninic acid (BCA) protein assay was used in WT, Veh-GFP, Hif-1 α mice at E16.5 and E19.5. n=8, **p<0.01. D. Renal histology in WT, Veh-GFP and Hif-1 α mice by H&E hematoxylin and eosin stain in E19.5. Scale bar =50 μ m

A

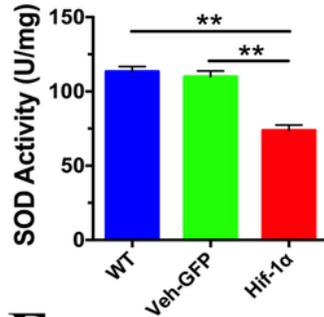


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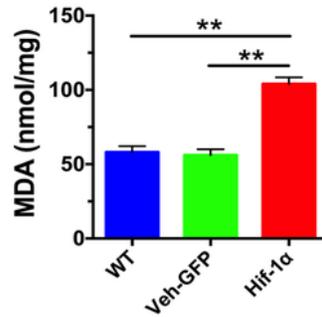
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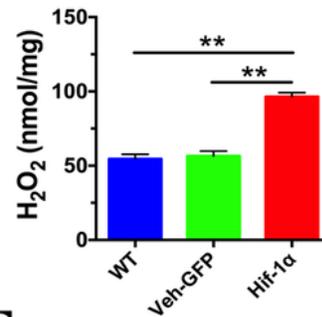
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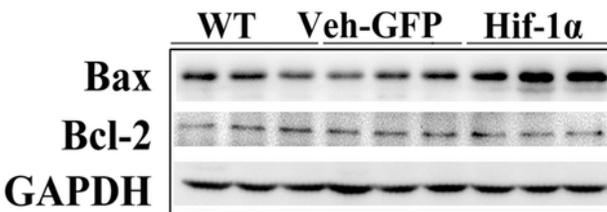
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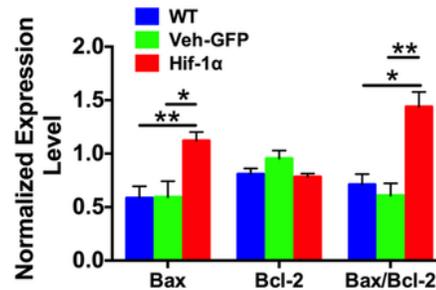
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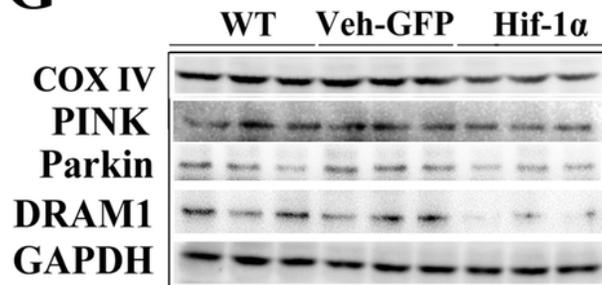
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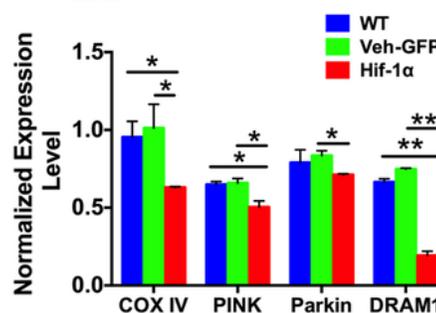


Figure 4

The levels of oxidative stress, apoptosis and mitochondrial autophagy were increased in the placenta of PE mice. A. Morphological examination of mouse placenta by H&E hematoxylin and eosin stain in E19.5, which revealed that the junctional zone (JZ) (white) region of Hif-1 α mice was larger than that of WT and Veh-GFP mice. Scale bar =100 μ m. B-D. The level of placental oxidative stress in Hif-1 α mice was significantly increased. Superoxide Dismutase (SOD) activity in placental tissue was detected by nitro-blue tetrazolium (NBT) (B); The levels of malonic dialdehyde (MDA) were tested by thiobarbituric acid (TBA) (C); The levels of H₂O₂ were tested by Titanium sulfate (TS) (D). **p<0.05, n=4. E-F. Representative Western blot images and quantification of Bax, Bcl-2 in the placenta of WT, Veh-GFP, and Hif-1 α mice. Densitometry results were normalized against the levels of GAPDH. G-H. Representative Western blot images and quantification of COX IV, PINK, Parkin and DRAM-1 in the placenta of WT, Veh-GFP, and Hif-1 α mice. Densitometry results were normalized against the levels of GAPDH. Values are expressed as mean \pm SEM (error bars). *p < 0.05, **p<0.01. n=6.

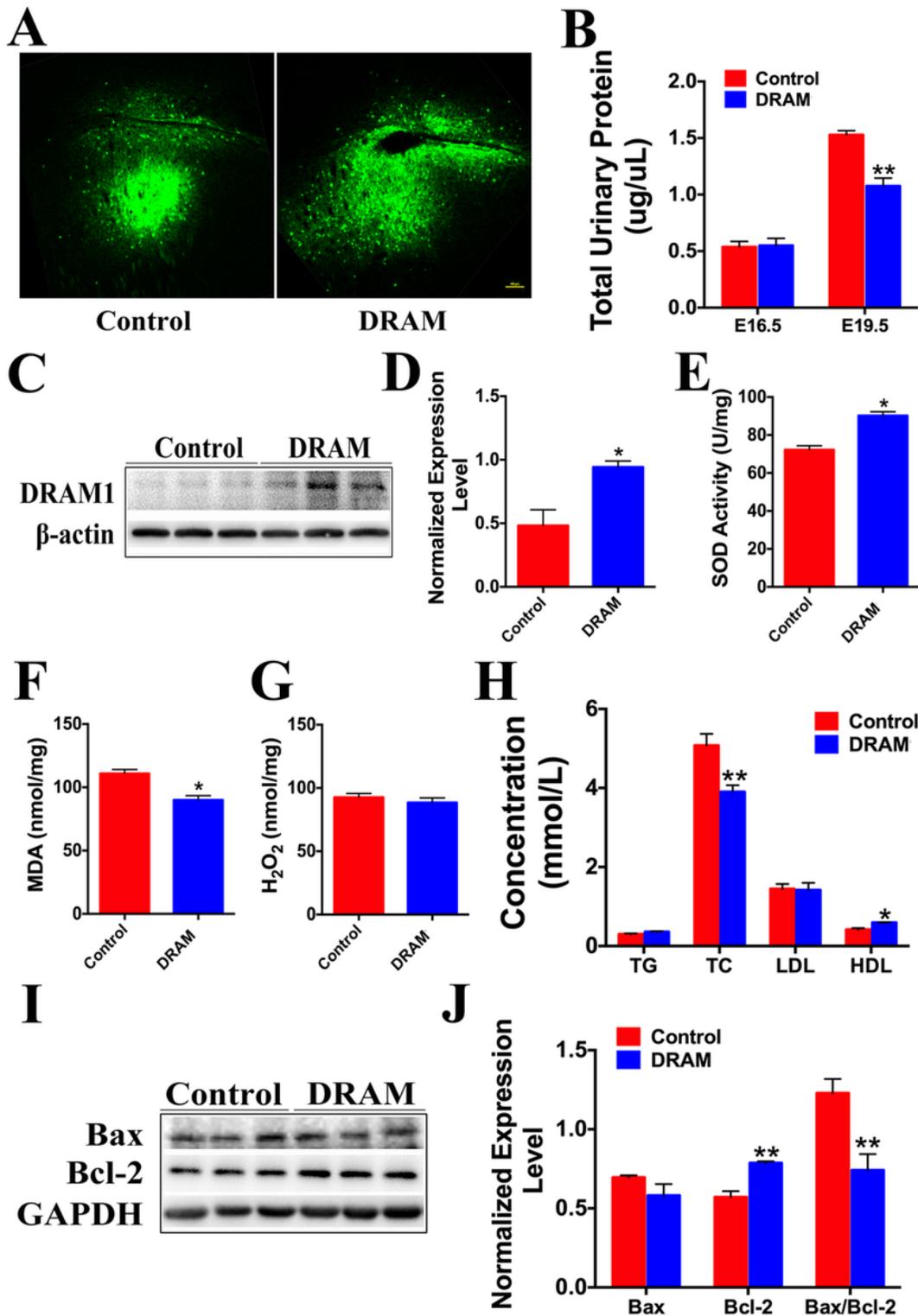


Figure 6

Overexpression of DRAM1 can effectively reduce apoptosis induced by oxidative stress and significantly reduce urine protein and blood lipid levels in preeclampsia. A. Sample images showing overexpression of DRAM or control in the placenta at E19.5 mice. Scale bars = 100 μ m. B. To determine total urinary protein, the bicinchoninic acid (BCA) protein assay was used in Control and DRAM mice at E16.5 and E19.5. n=6. C-D. Sample images of WB analysis of DRAM1 levels in the placenta from E19.5 mice with either DRAM1

overexpression or control. Representative Western blot images and quantification of DRAM1 in the placenta of Control and DRAM mice. Densitometry results were normalized against the levels of GAPDH. n=6. E-G. The level of placental oxidative stress in DRAM mice was significantly decreased. Superoxide Dismutase (SOD) activity in placental tissue was detected by (NBT) (E); The levels of malonic dialdehyde (MDA) were tested by thiobarbituric acid (TBA) (F); The levels of H₂O₂ were tested by Titanium sulfate (TS) (G). n=6. H. To measure serum lipid levels at E19.5 in mice, Kits were used to detect triglyceride (TG), total cholesterol (TC), low density lipoprotein (LDL), high density lipoprotein (HDL) in Control and DRAM mice. n=6. I-J. Representative Western blot images and quantification of Bax, Bcl-2 in the placenta of Control and DRAM mice. Densitometry results were normalized against the levels of GAPDH. n=3. Data are mean (\pm SEM) of several mice used for each experiment. *p<0.05, **p<0.01.

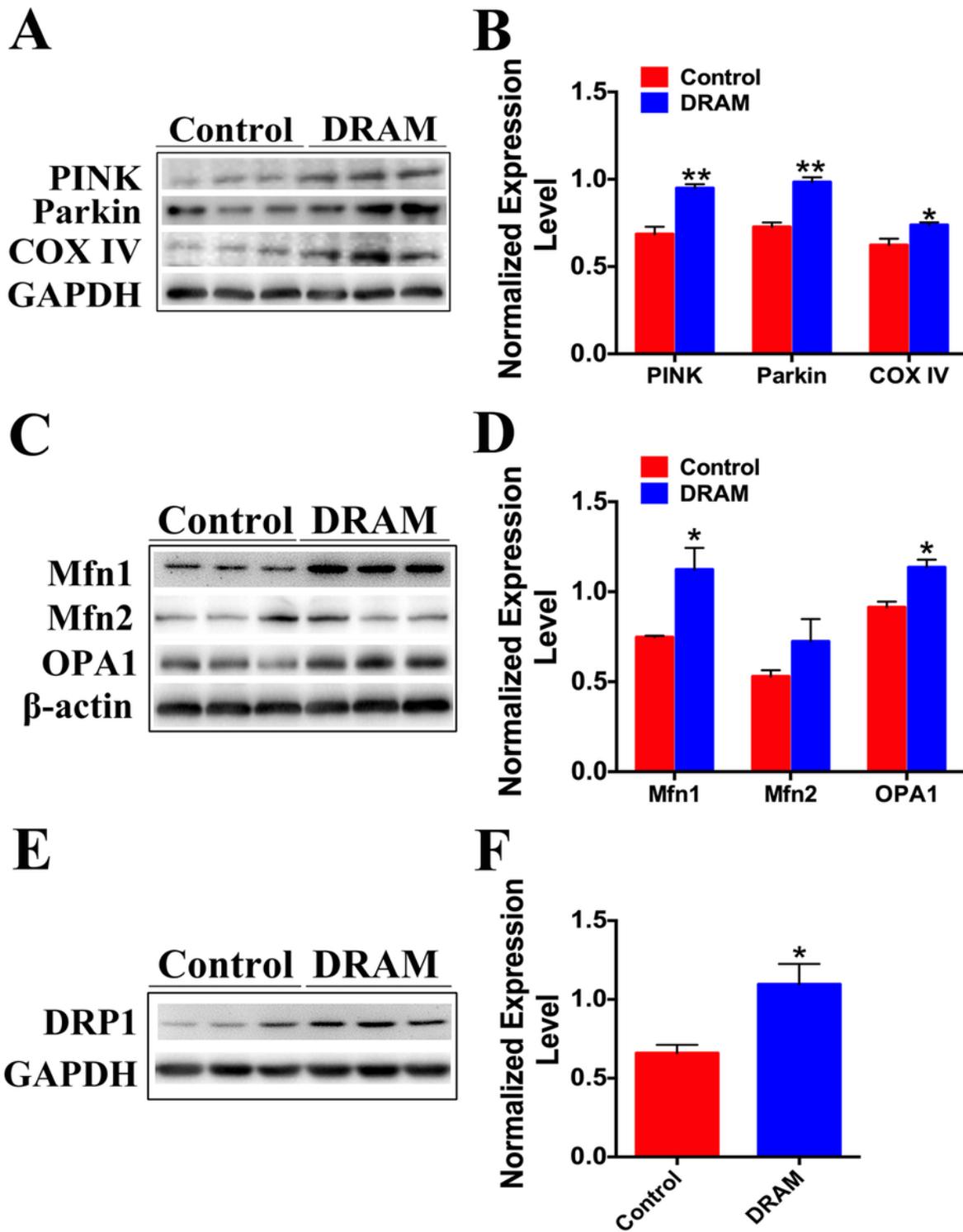


Figure 8

Overexpression of DRAM1 promotes mitochondrial fusion and fission, which promotes mitophagy. A-B, Overexpression of DRAM1 promotes mitophagy. Representative Western blot images and quantification of PINK, Parkin and COX IV in the placenta of Control and DRAM mice. C-D, Overexpression of DRAM1 promotes mitochondrial fusion. Representative Western blot images and quantification of Mfn1, Mfn2 and OPA1 in the placenta of Control and DRAM mice. E-F, Overexpression of DRAM1 promotes

mitochondrial fission. Representative Western blot images and quantification of DRP1 in the placenta of Control and DRAM mice. Densitometry results were normalized against the levels of GAPDH or β -actin. Values are expressed as mean \pm SEM (error bars). COX IV, cytochrome c oxidase IV. Mfn1, mitofusin 1. Mfn2, mitofusin 2. OPA1, optic atrophy 1. DRP1, dynamin-related protein 1. *p < 0.05, **p<0.01. n=6.

Table 1
Antibody information

Antibody	Host	Cat. No.	Source
Bax	Rabbit	ab32503	Abcam
Bcl-2	Rabbit	ab185002	Abcam
COX IV	Rabbit	4850	Cell Signaling
PINK	Rabbit	ab216144	Abcam
Parkin	Mouse	4211	Cell Signal
DRAM1	rabbit	PA5-69473	Invitrogen
Mfn1	Rabbit	14739	Cell Signaling
Mfn2	Rabbit	11925	Cell Signaling
OPA1	Rabbit	ab157457	Abcam
DRP1	Rabbit	ab184247	Abcam
GAPDH	Rabbit	ab181602	Abcam
β -Actin	Mouse	3700	Cell Signaling