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Reduction of DUSP4 contributes to podocytes oxidative stress, insulin resistance and diabetic nephropathy

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ABSTRACT

Podocytes are insulin-sensitive cells, and their loss is critical in diabetic nephropathy (DN) progression that could lead to end-stage kidney disease. We have previously shown that decreased DUSP4 expression caused elevated JNK phosphorylation in the diabetic kidney and worsened DN characteristics. Yet, the role of DUSP4 in diabetic podocyte insulin resistance and the progression of DN remains unclear. Here, we report that HG-exposed podocytes exhibited reduced DUSP4 expression, increased phosphorylation of JNK and serine 307 of IRS1 as well as Nox4 expression, while decreasing insulin signaling actions. DUSP4 overexpression, JNK and Nox1/4 inhibition prevented HG-induced serine 307 phosphorylation of IRS1 and restored insulin actions. Diabetic mice showed renal dysfunction and insulin resistance, characteristics that were exacerbated in diabetic DUSP4 deficient mice due to Nox1/4 upregulation. Thus, our results demonstrated that diabetes-induced reduction of DUSP4 leads to JNK activation and elevated Nox4 expression, which contributes to podocyte dysfunction, insulin resistance and progression of DN.

Diabetic nephropathy (DN) is a microvascular complication of diabetes which ultimately leads to end-stage kidney disease [1]. DN is characterized by morphological changes to the glomeruli and tubules triggering the onset of albuminuria and proteinuria. Podocytes are insulin-sensitive epithelial cells that create slit diaphragms within the glomeruli to ensure proper filtration. Evidence has shown that podocyte loss is an early event in the development of DN and the onset of albuminuria [2]. Furthermore, clinical studies have reported a negative correlation between podocyte density and the progression of DN, highlighting podocyte loss as an indicator of DN progression [3]. We have shown that hyperglycemia can lead to podocyte cell death [4] and insulin unresponsiveness. Nonetheless, mechanisms of podocyte loss by high glucose level conditions need to be further investigated.

Insulin signaling is important for podocyte function, proliferation, and survival. We, and other groups, have previously shown that hyperglycemia leads to insulin resistance in podocytes [5,6]. The loss of insulin signaling favors podocyte cell death by decreasing critical Akt actions, an effector protein downstream of the insulin receptor, such as GLUT4 translocation to the membrane and consequent glucose uptake in the cell [6]. Furthermore, insulin resistance has been shown to be associated with serine phosphorylation of insulin receptor substrate (IRS)-1 [7]. However, the precise mechanisms leading to insulin resistance in podocytes remains unclear.

Mitogen-activated protein kinase (MAPK) activation and oxidative stress are known mechanisms of podocyte dysfunction [8]. We have previously shown that HG increases the activation of pro-apoptotic c-Jun terminal kinase (JNK) and p38 MAPK in cultured mouse podocytes exposed to HG and renal glomeruli of diabetic mice [9]. Interestingly, JNK activation has been shown to contribute to insulin resistance in liver tissue of obese *ob*/ob mice [10]. Taken together, these data underline a potential role of JNK activity in podocyte insulin resistance. However, targeting the JNK pathway may not be suitable to prevent the progression of early diabetic renal injury [11].

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We have recently shown that HG-induced p38 and JNK MAPK activation is mediated by decreased DUSP4 expression, a dual Specificity Phosphatase (DUSP) known to endogenously modulate MAPK activation. More specifically, we have observed that DUSP4 overexpression by adenoviral transfection prevents both p38 and JNK phosphorylation in podocytes exposed to HG conditions [9]. Considering that insulin signaling is critical for podocyte viability and normal kidney function, uncovering mechanisms leading to insulin resistance in podocytes is fundamental. Because DUSP4 regulates JNK signaling pathways, we hypothesize that JNK activation, as a consequence of DUSP4 expression loss, contributes to insulin resistance, podocyte dysfunction and DN progression.

1. Research design and methods

Reagents and antibodies – The antibodies and reagents used in this study are found in supplemental methods.

Animals and experimental design - C57BL/6J (*Ins2*$^{+/+}$), diabetic heterozygous male *Ins2*$^{+/C96Y}$ (Akita) and DUSP4 null mice (*Dusp4*$^{-/-}$) were purchased from The Jackson Laboratory. Akita mice develop type 1 diabetes at ~3–4 weeks of age and were crossed with DUSP4 null mice to generate a diabetic DUSP4 null mouse (*Ins2*$^{+/C96Y}$- *Dusp4*$^{-/-}$). After 4 months of diabetes, the right kidney was clamped and harvested, followed by a 5 mU/g insulin injection for 15 min before the sacrifice. Throughout the study, the animals had free access to water and standard rodent chow (Envigo). All experiments performed were conducted in accordance with the Canadian Council of Animal Care and Institutional Guidelines as approved by the University of Sherbrooke Animal Care and Use Committees according to the guidelines of the National Institutes of Health.

Blood glucose, renal function and pathology – Blood glucose was measured by a Glucometer (Contour; Bayer, Pointe-Claire, QC, Canada). Urinary albumin concentrations, glomerular filtration [4], foot process effacement, glomerulosclerosis, glomerular hypertrophy, mesangial expansion, transmission electron microscopy, immunohistochemistry and WT-1 immunofluorescence were measured as previously described [9].

Cell culture and adenoviral vector transfection - A mouse podocyte cell line was cultured as previously described (8). After differentiation, podocytes were treated with RPMI +0.1% FBS containing either normal glucose (NG; 5.6 mmol/L + 19.5 mmol/L mannitol to adjust osmotic pressure) or high glucose (HG; 25 mmol/L) for 72 h. JNK inhibitor SP600125 (5 μmol/L) and antioxidant N-acetyl cysteine (NAC; 50 μmol/L) were added for the last 24 h of NG or HG treatment and Nox1/4 inhibitor (500 nmol/L) added 30 min prior to NG or HG treatment and maintained throughout the experience. Adenoviral vector infections were performed as previously reported [9].

Reactive oxygen species (ROS) detection by dihydroxyethidium (DHE) and HPLC - Cell cultures exposed to various conditions were incubated with HBSS/DTPA containing 50 μmol/L of DHE at 37 °C for 30 min. Samples were washed and lysed with acetonitrile. The supernatant was collected following centrifugation and samples were vacuum dried without heat. Samples were resuspended then separated on a methanol gradient by HPLC to determine the relative quantity of ethidium (catalyzed by ROS, for example H2O2) and 2-hydroxyethidium (catalyzed by O2-) produced by DHE degradation as described by Burger et al. [12].

Real-time PCR and immunoblot analyses - Real-time PCR and immunoblots were performed as previously described [4]. PCR primers are listed in the Supplementary Table 1. GAPDH and B2M expression were used to normalize data.

Statistical analyses - In vitro and *in vivo* data are presented as the mean ± SD for each group. Statistical analysis was performed by unpaired *t*-test, by non-paired one-way ANOVA, or paired one-way ANOVA (Fig. 3E) followed by the Tukey test corrections for multiple comparisons. Data in each group were checked for normal distribution using the D'Agostino and Pearson normality test based on $\alpha = 0.05$. Results were considered statistically significant at $P < 0.05$.

2. Results

2.1. Diabetic DUSP4 deficient mice showed characteristics of worsened renal function and glomerular pathology

Blood glucose, renal function and early-stage kidney pathology were evaluated in diabetic Akita mice (*Ins2*$^{+/C96Y}$) with or without a systemic deletion of the DUSP4 gene (*Dusp4*$^{-/-}$). Deletion of DUSP4 had no effect on body weight and blood glucose levels in diabetic mice (Supplementary Table 2). *Ins2*$^{+/C96Y}$ mice exhibited albuminuria as compared to nondiabetic (*Ins2*$^{+/+}$) littermates (2.2-fold increase), which was similar in *Ins2*$^{+/C96Y}$-*Dusp4*$^{-/-}$ mice (Fig. 1A). GFR levels were increased by 1.47-fold in *Ins2*$^{+/C96Y}$ mice and worsened to 2.61-fold in *Ins2*$^{+/C96Y}$-*Dusp4*$^{-/-}$ mice as compared to nondiabetic controls (Fig. 1B). Moreover, diabetic mice exhibited an increase in mesangial expansion (Fig. 1C and D; 1.42-fold), glomerular hypertrophy (Supplementary Fig. 1A; 1.21-fold), glomerulosclerosis (Fig. 1E and F; 1.72-fold), and podocyte foot process effacement and loss (Supplementary Figs. 1B, 1C, 1D and 1E), as compared to nondiabetic controls. Interestingly, *Ins2*$^{+/C96Y}$-*Dusp4*$^{-/-}$ mice had exacerbated levels of mesangial expansion and glomerulosclerosis (Fig. 1C, D, 1E and 1F) as compared to *Ins2*$^{+/C96Y}$ mice. Taken together, this data suggests DUSP4 loss accelerates select markers of DN progression.

2.2. Absence of DUSP4 deteriorated insulin signaling actions in diabetic mice

To evaluate the effect of DUSP4 loss on insulin resistance in the kidney cortex of our mice, insulin was injected for 15 min prior to sacrifice. Insulin stimulation increased Akt and ERK phosphorylation (4.09-fold and 4.36-fold respectively) in nondiabetic controls as compared to non-stimulated controls (Fig. 1G and H and Supplementary Fig. 2, respectively). Compared to nondiabetic controls, diabetes caused decreased Akt phosphorylation by 33%, an effect associated with increased JNK and serine 307 phosphorylation of IRS1. Interestingly, Akt activation was worsened by 42% in diabetic mice lacking DUSP4 compared to diabetic mice without exacerbating JNK and serine 307 phosphorylation of IRS1 (Fig. 1G). These observations suggest that deletion of DUSP4 may trigger additional pathways leading to insulin resistance.

2.3. JNK inhibition and DUSP4 overexpression restored insulin action in HG-exposed podocytes

Since podocytes are the highest insulin-sensitive cells in the kidney, we evaluated HG-induced insulin unresponsiveness by immunoblot of the serine (inhibitory) and tyrosine (activation) phosphorylation of IRS1 as well as Akt phosphorylation. In NG conditions, insulin-stimulated podocytes showed increased phosphorylation of Akt (Fig. 2A and C). HG exposure caused a 1.4-fold increase in phospho-serine 307 of IRS1, 36% decrease in phospho-tyrosine 608 of IRS1 and 41% decrease in phospho-Akt (Fig. 2A) which confirmed insulin resistance. To verify if JNK

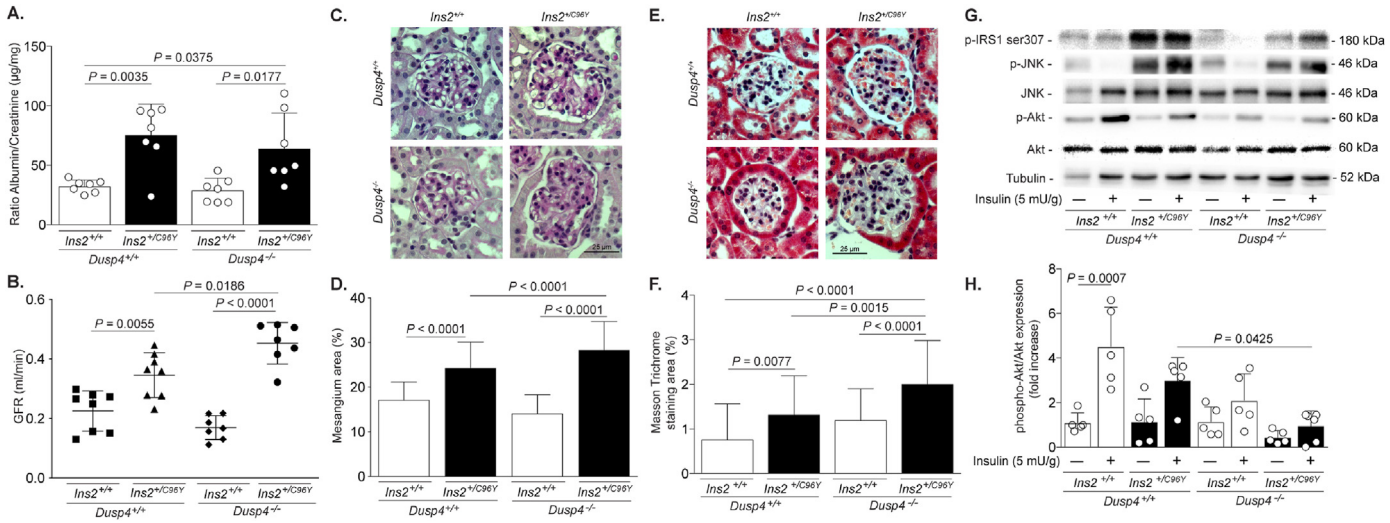

Fig. 1. Diabetes-induced renal function decline and insulin resistance. (A) Albumin levels and (B) glomerular filtration rate were measured in nondiabetic ($Ins2^{+/+}$) and diabetic ($Ins2^{+/C96Y}$) with or without systemic deletion of DUSP4 ($Dusp4^{-/-}$). Kidney cross sections were stained with (C) Periodic Acid Schiff to quantify (D) mesangial expansion and (E) Masson Trichrome staining to quantify (F) glomerular fibrosis. (G and H) Each group of mice had one kidney stimulated with 5 mU/g of insulin for 15 min prior to sacrifice. Akt, serine 307 of IRS1 and JNK phosphorylation were observed by immunoblot and densitometry for quantification. Results are shown as mean ± SD of 7 (A), 7–8 (B), 5 (G and H) and 5–6 mice per group with 10 glomerular measurements chosen at random per mouse (C, D, E, and F).

phosphorylation mediates insulin resistance in podocytes, JNK inhibitor (SP600125) was used. JNK inhibition was validated by a decrease in the phosphorylation and expression of its substrate c-Jun (Fig. 2A). JNK inhibition prevented the phosphorylation of serine 307 of IRS1 and restored tyrosine 608 phosphorylation of IRS1 and Akt activation (Fig. 2A), suggesting that JNK phosphorylation contributes to HG-induced insulin resistance in podocytes. Since JNK inhibition is not favored to prevent early diabetic kidney injury [11,13], DUSP4 implication in insulin resistance was evaluated using an adenoviral vector overexpressing mouse DUSP4. DUSP4 overexpression not only suppressed JNK phosphorylation in cells exposed to HG levels (Fig. 2B), but it also prevented HG-induced insulin resistance by reducing serine 307 phosphorylation of IRS1 as well as reestablishing tyrosine 608 phosphorylation of IRS1 and Akt activation (Fig. 2C).

2.4. JNK and DUSP4 overexpression prevented HG-mediated Nox4 expression but not total ROS production

DUSP4 was shown to modulate Nox4 expression in a model of hypoxia/reperfusion [14]. Interestingly, in endothelial cells, oxidative stress producer Nox2 seemed to induce insulin resistance [15]. The literature suggests that DUSP4 could be implicated in ROS production modulation and consequent insulin resistance, thus, we first treated podocytes exposed to HG with a strong antioxidant N-acetyl cysteine (NAC). HG exposure decreased DUSP4 mRNA expression by 31% which was prevented with NAC treatment (Fig. 3A), suggesting a link between HG-reduced DUSP4 expression loss and ROS. Since Nox4 is the most important ROS producer in the kidney [16] and DUSP4 deletion in diabetic mice exacerbates Nox4 expression [9], we further investigated the implication of DUSP4 in ROS production. HG exposure increased Nox4 mRNA expression in podocytes compared to NG conditions (Fig. 3B and C) which was prevented with DUSP4 overexpression, JNK inhibition and antioxidant NAC (Fig. 3B and C and Supplementary Fig. 3, respectively). In contrast, total HG-induced ROS production was not prevented by DUSP4 overexpression (Fig. 3D) suggesting that DUSP4 overexpression is not sufficient to decrease total HG-induced ROS production.

2.5. Nox1/4 inhibition restored HG-induced insulin resistance in podocytes

To further evaluate the implication of Nox4 in insulin resistance, we treated cells with a potent Nox1/4 inhibitor (GKT136901). We observed that Nox1/4 inhibition significantly restored insulin stimulated Akt phosphorylation and prevented serine 307 phosphorylation of IRS1 (Fig. 3E) in HG exposed podocytes. However, Nox1/4 inhibition did not prevent HG-induced DUSP4 expression loss and increased JNK phosphorylation (Fig. 3F). These data support the notion that Nox1/4 inhibitor acts downstream of DUSP4 and JNK to prevent HG-induced podocyte insulin resistance.

2.6. $Ins2^{+/C96Y}$-$Dusp4^{-/-}$ mice show Nox expression deregulation

Since our podocyte data suggests that DUSP4 regulates Nox1/4 expression and insulin resistance, we have measured Nox expression in renal cortex of all groups of mice. Interestingly, $Ins2^{+/C96Y}$-$Dusp4^{-/-}$ mice presented a significant deregulation of Nox1 expression (6.27-fold increase; Fig. 4A) and Nox4 (1.32-fold increase; Fig. 4C), but not Nox2 expression (Fig. 4B) compared to $Ins2^{+/C96Y}$ mice.

3. Discussion

Podocyte injury is a critical factor in the progression of DN [2]. Unfortunately, these cells are known to have a limited capacity of renewal [17], strengthening the need to understand the molecular mechanisms leading to their dysfunction and loss. Both nondiabetic podocyte-specific insulin receptor null or Akt2 null mice develop extensive albuminuria and renal impairment similar to diabetic mice [18,19], suggesting the necessity of insulin signaling in podocyte survival. Furthermore, we and other groups have shown that insulin resistance is a major predictor of podocyte dysfunction in diabetic mice [5,6]. In this study, we were able to characterize that DUSP4 reduction by HG conditions contributed to podocyte insulin resistance through phosphorylation on serine 307 of IRS1. Similarly, diabetes increased insulin resistance and was associated with increased serine 307 phosphorylation of IRS1 suggesting that

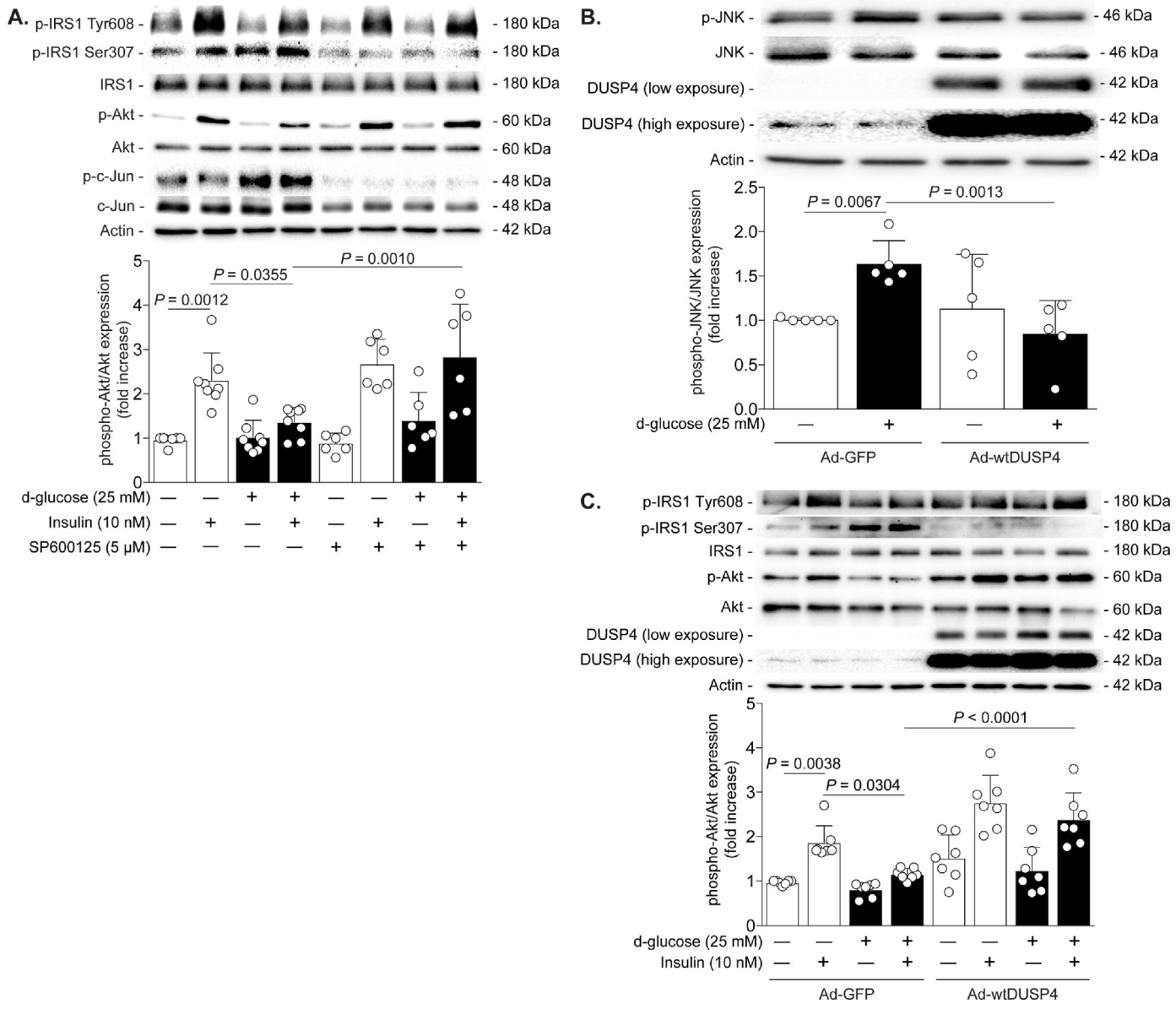

Fig. 2. HG-induced insulin resistance is prevented with JNK inhibition and DUSP4 overexpression. Cultured podocytes were exposed to NG (5.6 mmol/L D-glucose) or HG (25 mmol/L D-glucose) levels for 72 h and either treated with (A) JNK inhibitor SP600125 for the last 24 h or (B and C) infected with GFP (Ad-GFP) and native form of DUSP4 (Ad-wtDUSP4) 24 h prior to the NG or HG exposure time. Expression of (A) phospho-*c*-Jun, (A and C) phospho-Akt, serine 307 and tyrosine 608 phosphorylation of IRS1, (B) phospho-JNK, and (B and C) DUSP4 were observed by immunoblot and densitometry for quantification. Results are shown as mean ± SD of 6–8 (A), 5 (B), 7 (C) independent experiments.

serine 307 of IRS1 is a primary target for inhibitory phosphorylation.

Over the years, many groups have demonstrated the importance of MAPK activation in health and disease. Protein kinases p38 and JNK are known for their duality in exhibiting pro-apoptotic and anti-apoptotic features. Sustained p38 activation has been shown to exacerbate glomerular pathology [9,20] whereas JNK activation is known to trigger insulin resistance through phospho-serine 307 of IRS1 in several cell types [10,21]. Thus, we demonstrated that JNK phosphorylation was increased in podocytes exposed to HG conditions. Furthermore, JNK inhibition was able to prevent insulin resistance in podocytes exposed to HG conditions by halting serine 307 phosphorylation of IRS1 suggesting an importance of JNK modulation on podocyte insulin resistance.

The DUSP family of phosphatases, more specifically the mitogen-activated protein kinase phosphatases, has been shown to have important roles in the progression or slowdown of pathologies. Notably, DUSP6 overexpression has been shown to exhibit antitumor effects [22] and DUSP1 loss is known to lead to sustained ERK activation in tumorigenesis [23]. On the other hand, conditional activation of DUSP4 decreased genotoxic stress-induced apoptosis in human embryonic kidney cells 293 via suppression of JNK phosphorylation but not ERK *in vivo* [24]. Starting at the cellular level, we showed that overexpression of DUSP4 in mouse podocytes exposed to HG conditions prevented not only JNK phosphorylation but also insulin resistance. Furthermore, we were able to identify that mesangial expansion, glomerulosclerosis, hyperfiltration, oxidative stress and insulin resistance were early DN characteristics associated with the loss of DUSP4. In a previous study, we demonstrated that chronic exposure to hyperglycemia (6 months of diabetes) in *Ins2*$^{+/C96Y}$-*Dusp4*$^{-/-}$ worsened all DN characteristics (including podocyte loss), oxidative stress, and

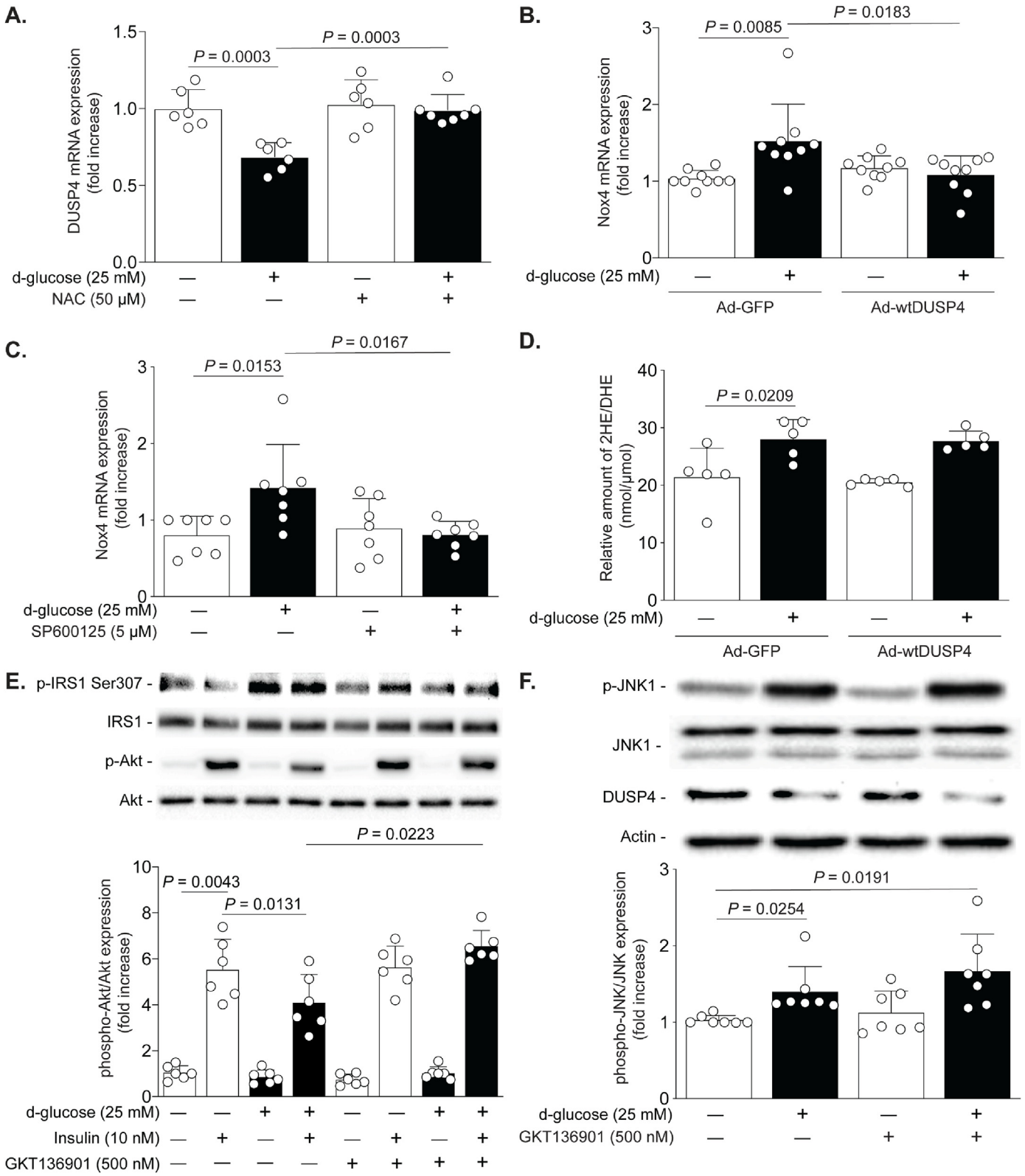

Fig. 3. Nox4 related oxidative stress implication in high glucose mediated insulin resistance. Podocytes were exposed to NG (5.6 mmol/L D-glucose; white bars) or HG (25 mmol/L D-glucose; black bars) levels for 72 h and treated with (A) NAC, (C) SP600125 for the last 24 h, (E and F) Nox1/4 inhibitor for 30 min prior to NG or HG treatment and maintained throughout the 72 h experiment, or (B and D) infected with GFP (Ad-GFP) and native form of DUSP4 (Ad-wtDUSP4) 24 h prior to the NG or HG exposure time. (A) DUSP4 and (B and C) Nox4 mRNA expressions were measured by quantitative PCR. (D) Relative amount of ROS production (ratio 2DHE/DHE) was measured by fluorescent HPLC. (E) Akt and serine 307 phosphorylation of IRS1, (F) JNK phosphorylation and DUSP4 expression were observed by immunoblot and densitometry for quantification. Results are shown as mean ± SD of 5 (D), 6 (A and E), 7 (C, F) and 9 (B) independent experiments.

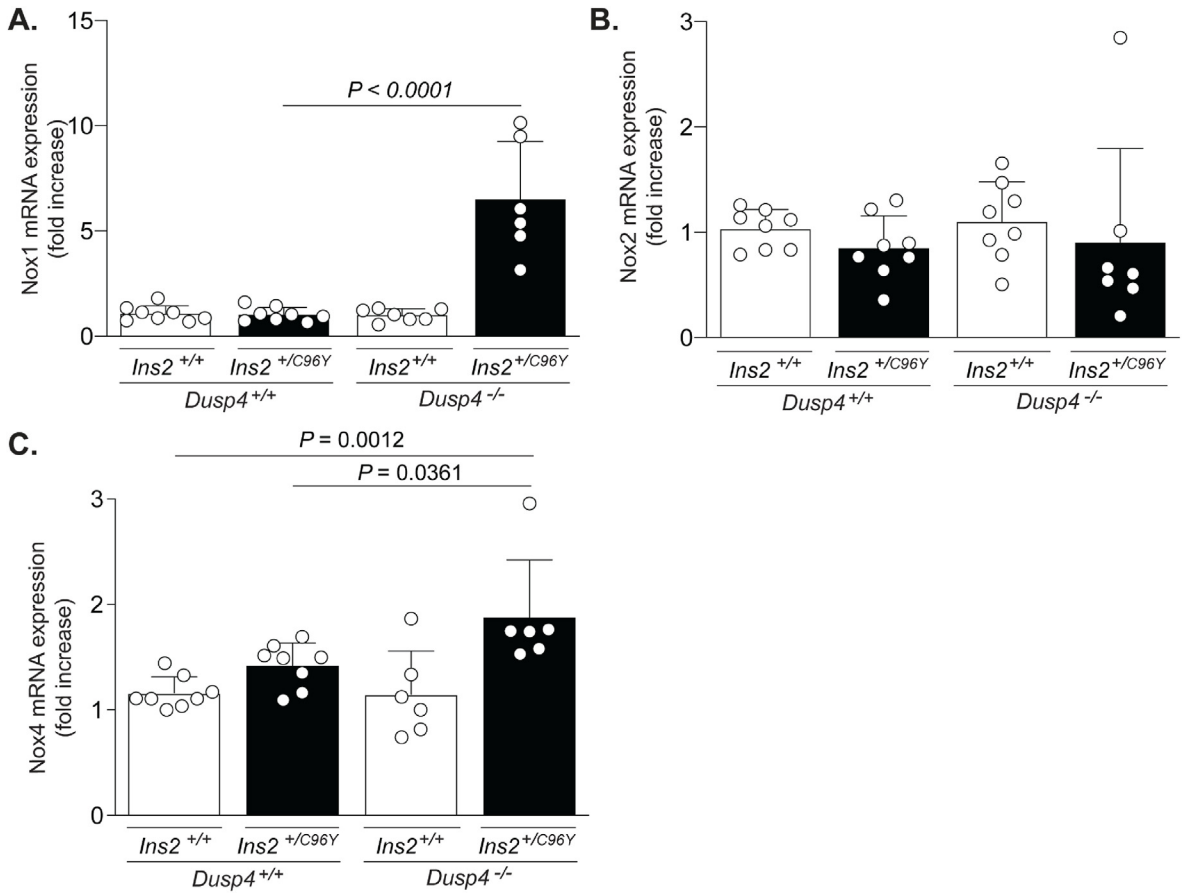

Fig. 4. Nox expression in the kidney cortex of diabetic and *Ins2*$^{+/C96Y}$-*Dusp4*$^{-/-}$ mice. (A) Nox1, (B) Nox2 and (C) Nox4 mRNA expressions were determined by quantitative PCR in nondiabetic (*Ins2*$^{+/+}$; white bars) and diabetic (*Ins2*$^{+/C96Y}$; black bars) mice with or without deletion of DUSP4 (*Dusp4*$^{-/-}$). Results are presented as mean ± SD of 6–8 mice per group.

phosphorylation of p38 and JNK [9]. These results suggest that insulin resistant *Ins2*$^{+/C96Y}$-*Dusp4*$^{-/-}$ mice seem to have more rapid progression of DN characteristics. Unfortunately, insulin resistance in *Ins2*$^{+/C96Y}$-*Dusp4*$^{-/-}$ mice cannot be completely explained by increased JNK or serine phosphorylation of IRS1. Thus, Nox and subsequent ROS production could be a potential avenue to investigate.

Nox4 has been shown to be the most important ROS producer in the kidney [16]. However, other Nox isoforms are equally important since Nox2 has been shown to be the driving force in ROS production in obese Zucker rats [25] and Nox5 has been shown to play a role in the progression of DN in humans [26]. However, whole body or podocyte-specific Nox4 but not Nox2 deletion has been shown to prevent glomerular changes and kidney injuries in DN [8,27,28]. DUSP4 deletion has been shown to exacerbate Nox4 mRNA expression in cardiomyocytes [9,29] and other Nox isoforms have been associated with insulin resistance [15]. Our results suggest that HG-increased Nox4 mRNA expression is mediated by JNK phosphorylation since overexpression of DUSP4 and JNK inhibition prevented elevated Nox4 expression. Astonishingly, DUSP4 overexpression did not reduce total ROS production suggesting other pathways leading to total ROS production in podocytes. However, increased Nox1/4 expression seems to play an indirect role in insulin resistance since podocytes treated with Nox1/4 inhibitor were capable of reestablishing insulin signaling without preventing upstream HG-induced reduction in DUSP4 expression and JNK phosphorylation. For the first time, we identified a link between DUSP4 expression loss and increased oxidative stress leading to podocyte insulin resistance. *In vivo*, our *Ins2*$^{+/C96Y}$-*Dusp4*$^{-/-}$ mice show significant Nox1 and Nox4 deregulations. It is possible that Nox1/4 upregulation in *Ins2*$^{+/C96Y}$-*Dusp4*$^{-/-}$ mice contributes to the observed insulin unresponsiveness; however, further studies using the Nox1/4 inhibitor will be needed to investigate the exact mechanisms.

In conclusion, we have demonstrated that hyperglycemia leads to reduced DUSP4 expression and consequent increased JNK and Nox4 activation is podocytes. Conjointly, these two pathways contribute to podocyte insulin resistance. Yet, *in vivo*, Nox1/4 upregulation in diabetic DUSP4 null mice seems to have greater importance on early insulin resistance. Nonetheless, taken together, these two mechanisms contribute to podocyte insulin resistance, dysfunction, and ultimately the progression of DN.

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Disclosures

All authors declare no conflicts of interest relevant to this article.

Author contributions

M.R., C·S., B.D. and F.L. performed experiments and researched data. A.G. performed animal care. D.B. performed the ROS detection. M.R., F.L. and P.G. analyzed the data and wrote the manuscript. A-M.C. revised the manuscript and provided clinical significance.

Declaration of competing interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrc.2022.07.067.

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