Diabetic Complications Consortium

Application Title: S-nitrosylation of extracellular matrix proteins in diabetic nephropathy

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1. Project accomplishments:

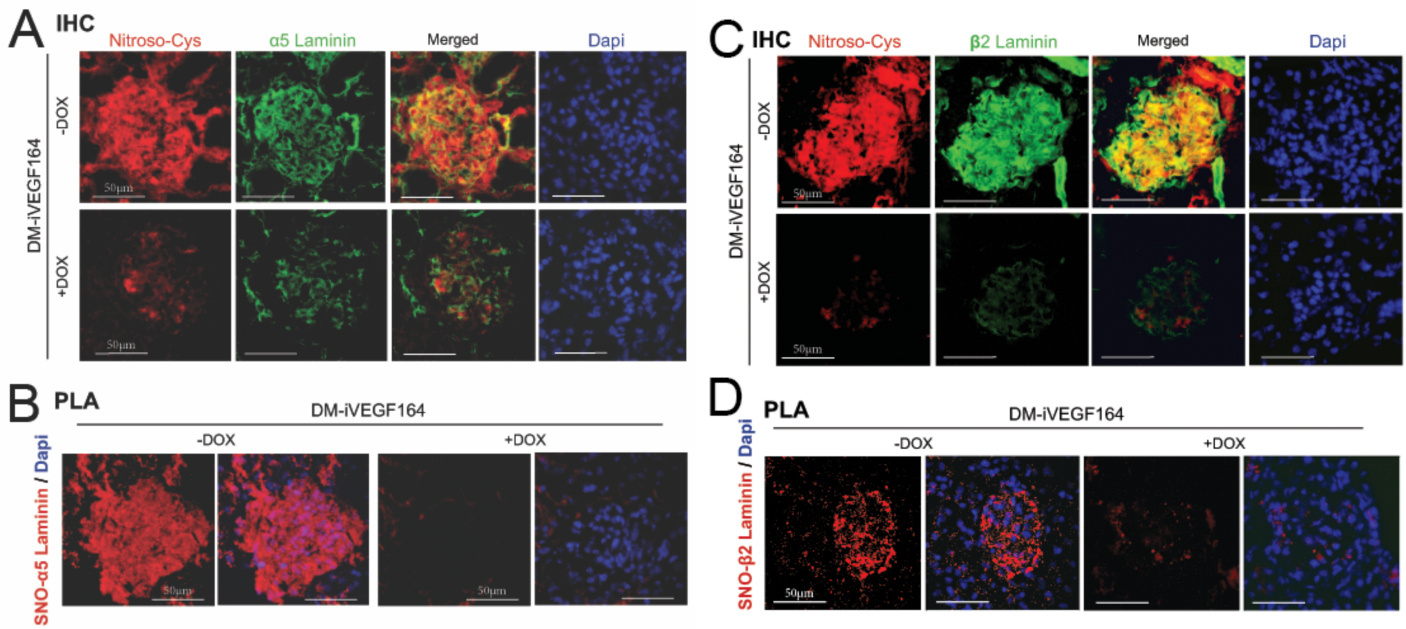
We established that α5-laminin and β2-laminin, two components of the laminin 521 trimer, a key glomerular basement membrane protein complex that contributes significantly to extracellular matrix accumulation and nodular glomerulosclerosis in diabetic nephropathy, are S-nitrosylated in normal mice and podocytes. We determined that α5-laminin and β2-laminin denitrosylation occurs in the setting of advanced diabetes or high glucose, leading to increased α5-laminin and β2-laminin secretion and cell migration disruption.

2. Specific Aims:

.Aim 1: Evaluate laminin 521 S-nitrosylation in diabetic nephropathy.

We have shown that laminin 521, a main component of the normal mature glomerular basement membrane, is nitrosylated in normal kidney glomeruli (1). S-nitrosylation of laminin, a reversible post-translational modification, is controlled by nitric oxide availability and by VEGF-A in mouse kidneys and podocytes (1). Laminin 521 is increased in the glomerular nodules observed in several experimental models of diabetic nephropathy (DN) and is thought to be involved in the pathogenesis of nodular glomerulosclerosis.

We performed a series of experiments to determine which laminin chain is S-nitrosylated using dual immunohistochemistry (anti-SNO-Cys + anti-chain specific laminin antibodies), biotin switch assay (BST) and proximity link assay (PLA) in kidney tissue and cultured cells. First, we determined that immunoreactive α5-laminin and β2-laminin co-localize with SNO-Cys residues in glomeruli from mice with mild diabetic nephropathy (Fig. 1A/C, *top panels*) and normal mice (not shown). Second, we determined that α5-laminin and β2-laminin are S-nitrosylated in kidneys from mice with mild DN (Fig.1 B/D, - dox, *left panels*) and from non-diabetic mice (not shown), whereas both SNO-α5-laminin (Figure 1A/B) and SNO-β2-laminin (Figure 1C/D) decrease dramatically in mice with advanced DN (+ dox) (2).



Next, we established that α5-laminin is S-nitrosylated in immortalized human podocytes by detecting co-localization of α5-laminin and SNO-Cys residues (Figure 2A), SNO-α5-laminin by BST (Figure 2B) and PLA (Figure 2C). Notably, α5-laminin nitrosylation status is regulated by glucose (and not by mannitol, suggesting this not due to increased osmolarity), such that high glucose significantly decreases SNO-α5-laminin, as measured by three independent methods (Figure 2A-D).

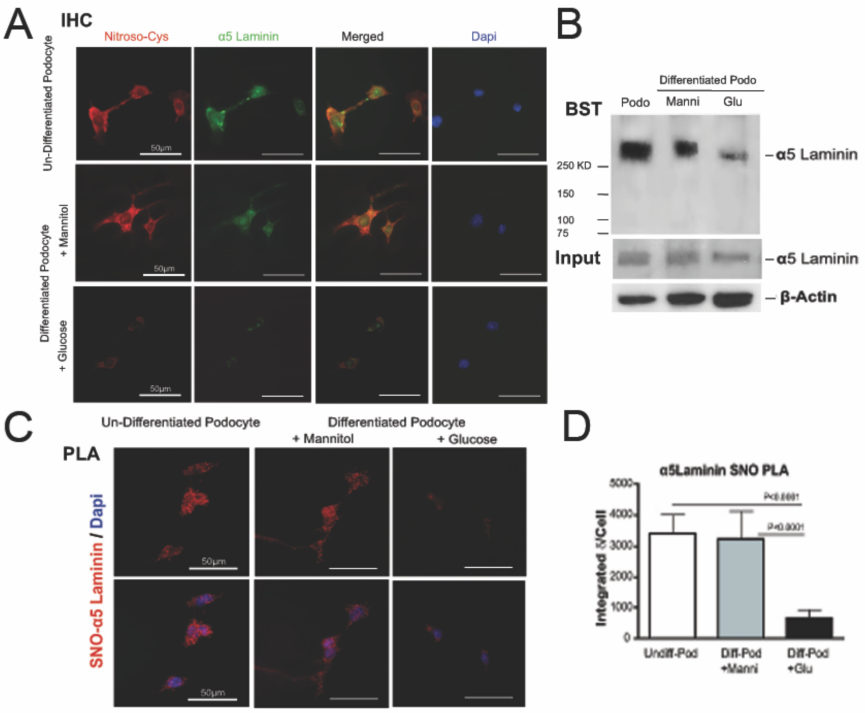
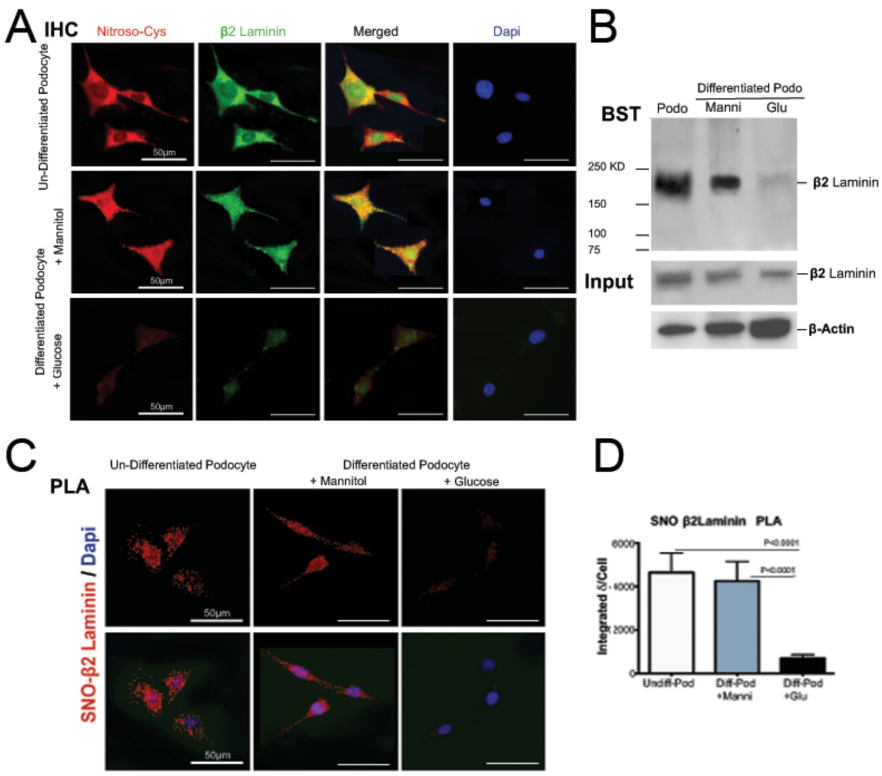


Figure 2: A) IHC co-localization of α5-laminin and SNO-Cys decreases in high glucose; B) BST: high glucose decreases SNO α5-laminin, input: WB total α5-laminin; C) PLA: localization of SNO-α5-laminin; D) PLA signal quantitation (integrated δ/ cell).



We evaluated nitrosylation of β2-laminin by identical methods, and detected SNO-β2-laminin in control podocytes, which was intensely downregulated by high glucose, while no effect was noted with the same hyperosmolarity (Figure 3A-D). Results from dual IF, BST and PLA experiments were congruent and consistent with our in vivo findings in diabetic mice, leading to the conclusion that high glucose and advanced diabetes induce denitrosylation of α5 and β2-laminin.

Figure 3: A) IHC co-localization of β2-laminin and SNO-Cys decreases in high glucose; B) BST: high glucose decreases SNO β2-laminin, input: WB total β2-laminin; C) PLA: localization of SNO-β2-laminin; D) PLA signal quantitation (integrated δ/ cell).

To understand whether disregulation of laminin 521 S-nitrosylation alters laminin secretion leading to glomerular nodule development in diabetic nephropathy we first compared S-nitrosylation (SNO) of α5-laminin and β2-laminin in kidneys from normoglycemic and T1D mice with mild DN and advanced DN (2). Clearly, α5-laminin and β2-laminin de-nitrosylation correlates with nodular glomerulosclerosis in T1D mice (Figure 3A). Consistent with this, podocytes cultured in normal glucose or mannitol containing medium express abundant SNO-α5-laminin and SNO-β2-laminin,

while podocytes exposed to high glucose showed minimal SNO-α5-laminin and SNO-β2-laminin, as assessed by three independent methods (Figure 2).

Next, we evaluated the secretion of each laminin chain by immortalized human podocytes exposed to medium with normal glucose [5mM], high glucose [25mM], or mannitol [25mM]. The decrease in SNO-α5-laminin and SNO-β2-laminin upon exposure to high glucose was associated with several fold increase in α5-laminin and β2-laminin secretion to the cultured medium (Figure 3B, *first and second panels*). Notably, TGFβ1 was detected in podocyte cell lysates but not secreted to the medium (Figure 3B, *third panel*).

Ongoing experiments are testing whether nitric oxide donors can reverse the increased α5-laminin and β2-laminin secretion in the setting of high glucose, and will identify specific Cys residues undergoing S-nitrosylation.

Figure 3

A

χ^2 = 11, p<0.01	SNO-α5 Laminin	α5 Laminin
NO nodules	6	0
Nodules	0	5

χ^2=13.4,p<0.01	SNO-β2 Laminin	β2 Laminin
NO nodules	8	0
Nodules	1	8

B

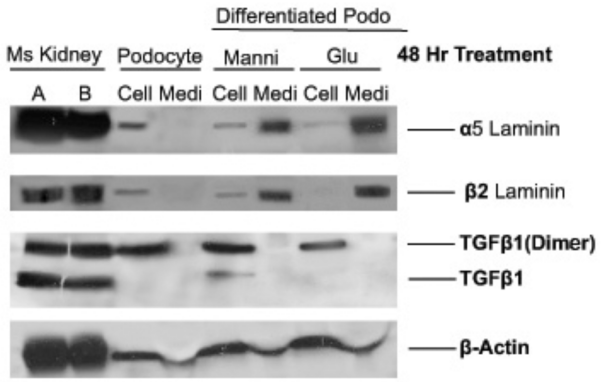


Figure 3: A) X^2 test demonstrates high association of diabetic nodular glomerulosclerosis and laminin α5 and β2 de-nitrosylation, n=6 (control) and n=5 (advanced DN) mice; B) WB: high glucose induces α5 and β2 laminin secretion, but not TGFβ1 secretion.

Wound assays showed that podocyte migration is significantly decreased in the setting of denitrosylated α5-laminin and β2-laminin (high glucose) (Figure 4). Ongoing experiments are testing whether NO donors can rescue this migration defect.

Figure 4

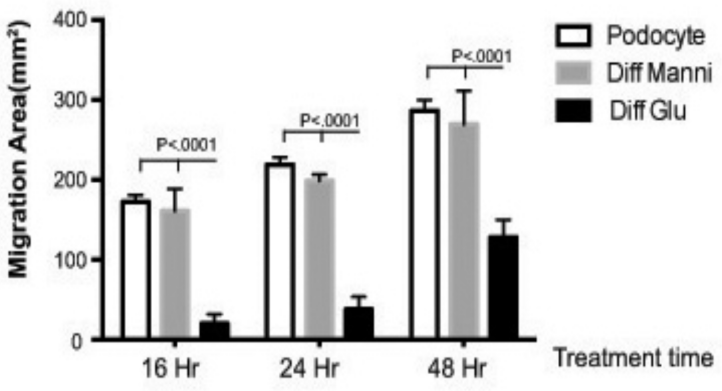


Figure 4: High glucose impairs podocyte migration in the setting of α5 and β2 laminin de-nitrosylation. High osmolarity does not regulate migration or SNO-laminin.

Conclusions:

De-nitrosylation of α5-laminin and β2-laminin leads to increased laminin secretion by podocytes and contributes to development of glomerular nodules in advanced diabetic nephropathy. The inherent reversibility of S-nitrosylation implies that this pathogenic mechanism may be a target for therapeutic intervention in DN.

Aim 2: Examine TFGβ1 and CTGF S-nitrosylation in diabetic nephropathy.

These experiments are ongoing and analysis of the data is in progress.

3. Publications:

Manuscript describing Aim 1 accomplishments is in preparation.

References:

1. Veron D, Aggarwal PK, Velazquez H, Kashgarian M, Moeckel G, Tufro A. Podocyte-specific VEGF-a gain of function induces nodular glomerulosclerosis in eNOS null mice. J Am Soc Nephrol. 2014 Aug;25(8):1814-24. PubMed PMID: 24578128; PubMed Central PMCID: PMC4116059.
2. Veron D, Bertuccio CA, Marlier A, Reidy K, Garcia AM, Jimenez J, Velazquez H, Kashgarian M, Moeckel GW, Tufro A. Podocyte vascular endothelial growth factor ($Vegf_{164}$) overexpression causes severe nodular glomerulosclerosis in a mouse model of type 1 diabetes. Diabetologia. 2011 May;54(5):1227-41. PubMed PMID: 21318407; PubMed Central PMCID: PMC3397150.