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Reactive oxygen species deglycosilate glomerular α-dystroglycan

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In the kidney, dystroglycan (DG) has been shown to cover the basolateral and apical membranes of the podocyte. α-DG is heavily glycosilated, which is important for its binding to laminin and agrin in the glomerular basement membrane. Furthermore, α-DG is negatively charged, which maintains the filtration slit open. Reactive oxygen species (ROS) are known to degrade and depolymerize carbohydrates, and to play a role in several glomerular diseases. Therefore, we evaluated the effect of ROS on the glycosilation of glomerular α-DG. By using specific antibodies directed against the core protein or glyco-epitopes of α-DG, this was studied in a solid-phase assay, *in situ* on kidney sections, and *in vivo* in adriamycin nephropathy. A ligand overlay assay was used to study binding of α-DG to its ligands. Exposure to ROS leads to a loss of carbohydrate epitopes on α-DG both *in vitro* and on kidney sections. In the *in vitro* assays, a decreased binding of deglycosilated α-DG to laminin and agrin was found. In adriamycin nephropathy, where radicals play a role, we observed a loss of α-DG carbohydrate epitopes. We conclude that deglycosilation of glomerular α-DG by ROS leads to disruption of the agrin–DG complex, which *in vivo* may lead to the detachment of podocytes. Furthermore, loss of negative charge in the filtration slit may lead to foot process effacement of podocytes.

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Podocytes are highly specialized visceral epithelial cells, which cover the outside of the glomerular basement membrane (GBM). Together with the GBM and the fenestrated glomerular endothelial cells they form the capillary filter, which allows size- and charge-specific filtration of molecules from the circulation to the urinary space. Dystroglycan (DG) is, besides the $\alpha_3\beta_1$-integrin, a major connecting molecule between the podocyte and the GBM.[1] Furthermore, it has been shown that α-DG covers the apical cell membrane of the podocyte, where it plays a role in the maintenance of the filtration slit by its negative charge.[1,2] DG has originally been described as a component of the dystrophin–glycoprotein complex in skeletal muscle.[3] DG, encoded by a single gene, *DAG1*, is cleaved into two proteins by post-translational processing events: the extracellular α-DG and the transmembrane β-DG.[4] Within the podocyte β-DG is intracellularly linked to utrophin, which interacts with the actin cytoskeleton.[1] The extracellular domain of β-DG binds to α-DG, which on its part interacts with laminin G modules present in laminin, agrin, and perlecan in the GBM. DG has been shown to play an important role in basement membrane assembly. Mice with a targeted disruption of DG ($DAG1^{-/-}$) do not develop Reichert's membrane, at which stage further development of the embryo stops.[5] Blocking of the binding site of α-DG to laminin with antibody IIH6 in kidney organ culture pertubates development of epithelium.[6] α-DG is heavily glycosilated, especially by *O*-mannosyl glycosilation, which is one of its unique features.[7] The predicted molecular weight of the core protein of α-DG is about 74 kDa, whereas its apparent molecular mass, as detected by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), is about 156 kDa in skeletal muscle, ranging from 120 kDa in brain to 190 kDa in the Torpedo electric organ.[8–10] As no differences in primary protein structure are described, these observed differences in molecular mass point to different degrees of glycosilation.[11] The glycosilation of α-DG is necessary for the binding to laminin G. In inherited disorders of the glycosilation of α-DG, like muscle–eye–brain disease and Fukuyama-muscular dystrophy, a hypoglycosilated muscle α-DG is expressed, which shows a decreased binding to agrin and laminin.[12–14]

Reactive oxygen species (ROS) are involved in several forms of experimental nephropathies.[15–19] Besides infiltrating

neutrophils and macrophages, podocytes and other glomerular cells are able to produce ROS.[20–23] Agrin is the major heparan sulfate proteoglycan in the GBM.[24,25] Agrin is composed of a core protein with four attached negatively charged heparan sulfate polysaccharide side chains. We have previously described that hydroxyl radicals degrade and depolymerize these heparan sulfate side chains, whereas the core protein of agrin remains intact. In the adriamycin nephropathy in the rat, treatment of rats with a scavenger for hydroxyl radicals protected for heparan sulfate loss from the GBM and led to a decrease of albuminuria.[15]

In the present study, we investigate how the glycosilation of glomerular α-DG is affected by exposure to ROS, and whether this influences the binding of α-DG to laminin and agrin. This could reveal a novel mechanism of podocyte loss, which is associated with progressive decline of kidney function, proteinuria, and a poor prognosis in several glomerular diseases.[26–28]

RESULTS

Differences in accessibility of core protein epitopes of α-DG in muscle and kidney

As the antibodies against the α-DG core protein as described by Holt *et al.*[29] are the only ones to stain glomerular α-DG (data not shown), we raised antibodies against the same three peptides by the same procedure.[12,29–31] All three antibodies (i.e. affinity purified on the appropriate peptide) showed a high reactivity with their respective peptide in enzyme-linked immunosorbent assay (ELISA) (not shown). The specificity of the anti-α-DG amino acids (a.a.) 549–572 antibody (sheep anti-rabbit α-DG (ShαRbDG) 549–572), directed at the binding site for *β*-DG, was proven in Western blot analysis and immunoprecipitation (Figure 1, lanes 2 and 5). While the antibody specific for α-DG a.a. 517–526 (ShαRbDG 517–526) did not reveal a signal in Western blot analysis, it turned out specific for α-DG in immunoprecipitation (Figure 1, lane 1). Furthermore, these two antigen-purified polyclonal α-DG antibodies were used to stain rabbit muscle and kidney sections (Figure 2). The 517–526 epitope is only expressed in muscle and weakly in tubuli but not in glomeruli, whereas the 549–572 epitope is expressed in the glomerulus as well. To evaluate more precisely the localization of the epitope of ShαRbDG 549–572 in the glomerulus, we performed confocal laser microscopy analysis of double stainings of ShαRbDG 549–572 with antibodies specific for agrin (as a marker for the GBM), the carbohydrate epitopes of α-DG and podocalyxin-like protein (PCLP-1) as markers for podocytes, and perlecan as a marker for the mesangium. This suggested an intracellular localization in the podocyte of the epitope of ShαRbDG 549–572 (Figure 3).

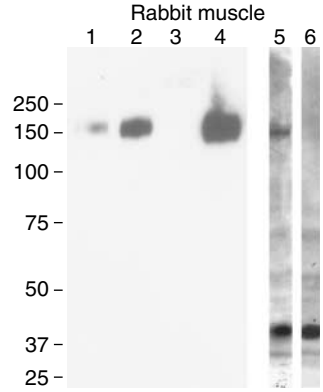

Figure 1 | Western blot analysis and immunoprecipitation from homogenized rabbit muscle by the polyclonal antibodies ShαRbDG 517–526 and 549–572 directed against the core protein of α-DG. Antigen affinity-purified sheep polyclonal antibodies against rabbit α-DG a.a. 517–526 (lane 1) and a.a. 549–572 (lane 2) bound to gammabind sepharose were able to precipitate rabbit muscular α-DG. Nonimmune sheep IgG served as a control (lane 3). The input extract is depicted in lane 4. These (precipitated) extracts were reduced and subsequently separated by 8% SDS-PAGE and blotted to nitrocellulose. The blot (lane 1–4) was probed with monoclonal antibody IIH6. Western blot analysis of unprecipitated rabbit muscle lysate with ShαRbDG 549–572 revealed a band at approximately 156 kDa (lane 5), which was not observed with nonimmune IgG (lane 6).

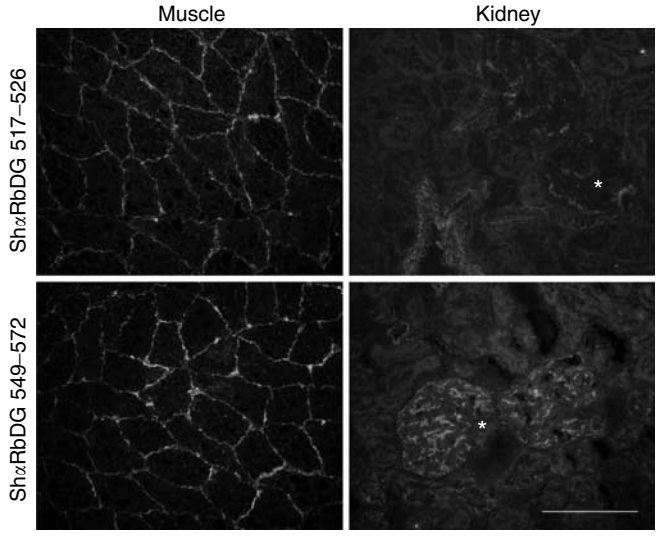

Figure 2 | Indirect immunofluorescence staining of rabbit muscle and kidney by the polyclonal antibodies ShαRbDG 517–526 and 549–572 against the core protein of α-DG. Antigen affinity-purified sheep polyclonal antibodies against rabbit α-DG a.a. 517–526 (upper panels) and a.a. 549–572 (lower panels) stain rabbit skeletal muscle and kidney sections differently. With ShαRbDG 517–526, a strong staining of muscle and a weak staining of some tubuli in the kidney were observed. However, a glomerular staining of α-DG with this antibody was not detected. With ShαRbDG 549–572 a similar staining of muscle and tubuli was observed, but strikingly also a glomerular staining of α-DG was seen (Bar = 100 μm, asterisk = glomerulus).

Effect of ROS on glomerular α-DG

To study the effect of ROS on native α-DG, rabbit kidney sections were exposed to ROS generated via the Haber–Weiss reaction (Table 1). Different concentrations of ROS were produced by varying the concentration of xanthine oxidase (XO). A concentration-dependent loss of the carbohydrate epitopes of α-DG was observed for IIH6 and VIA4.1, in which the epitope of IIH6 was more sensitive to ROS (Figure 4, first and third rows). However, the core epitope of

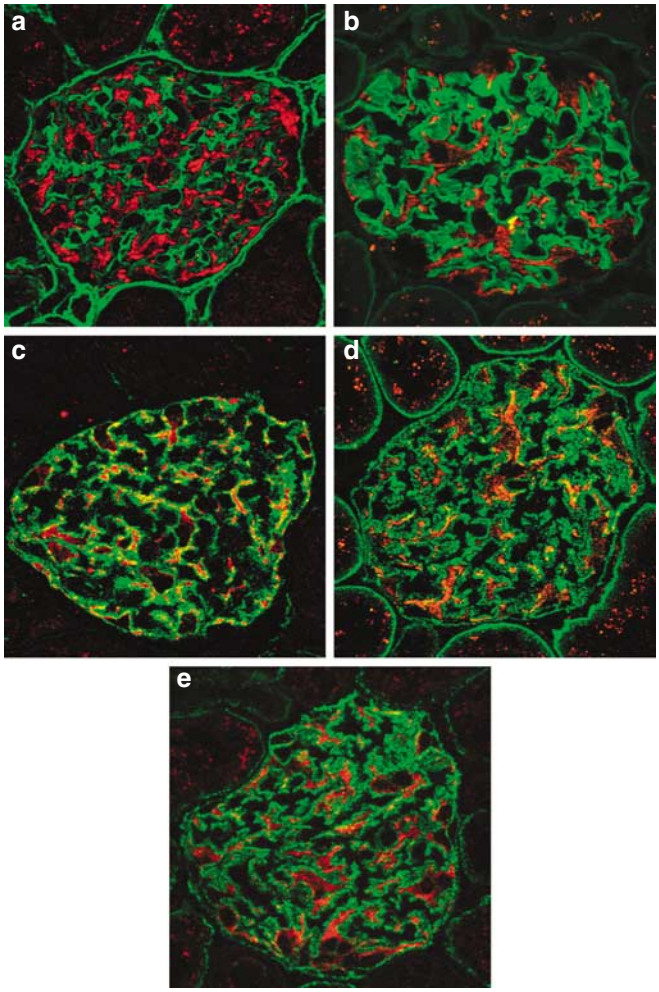

Figure 3 | The epitope of ShαRbDG 549–572 is expressed by podocytes. Confocal microscopy analysis of double stainings with ShαRbDG 549–572 (red), and (**a**) anti-perlecan, (**b**) anti-agrin, (**c**) anti-podocalyxin-like protein, or antibodies against the carbohydrate epitopes of α-DG ((**d**) VIA4.1 and (**e**) IIH6, all green). (**a**) No colocalization with the mesangium was observed. (**b**) All staining with ShαRbDG 549–572 was found outside the GBM. (**c**) The cells that were positive for the podocyte membrane marker PCLP-1 were also positive for ShαRbDG 549–572. The epitope of the core protein of α-DG was also localized intracellularly in the podocytes, since in the merge there is also red staining visible. (**d**, **e**) This intracellular localization is visualized by the double staining with the carbohydrate epitopes of α-DG, which are expressed on the podocyte membrane.

α-DG was conserved, as revealed by double staining with ShαRbDG 549–572 (Figure 4, second and fourth rows). This suggests a differential sensitivity of the respective carbohydrate epitopes of the monoclonal anti-α-DG antibodies IIH6 and VIA4.1 for ROS, whereas the peptide epitope of ShαRbDG 549–572 was not affected by these concentrations of ROS. Three scavengers for ROS: superoxide dismutase (SOD) for superoxide, deferoxamine (DFO) for ferrous ions, and dimethylthiourea (DMTU) for hydroxyl radicals, prevented to a certain extent the loss of the carbohydrate epitopes (Figure 4, columns 4–6).

Table 1 | Generation of reactive oxygen species

	Reaction	Scavenger
Haber–Weiss reaction	Xanthine oxidase ↺ Hypoxanthine → xanthine+uric acid+O_2^-	Superoxide dismutase (SOD)
	$2\ O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$	
Fenton reaction	$Fe^{3+} + O_2^- \rightarrow Fe^{2+} + O_2$	Deferoxamine (DFO)
	$Fe^{2+} + H_2O_2 \rightarrow {}^{\bullet}OH + OH^- + Fe^{3+}$	Dimethylthiourea (DMTU)

The effect of ROS on the molecular mass of bovine glomerular α-DG was analyzed by SDS-PAGE and Western blot. With increasing concentrations of XO the epitope of IIH6 disappeared (Figure 5, upper panel), and a band appeared at approximately 100 kDa, as revealed by ShαRbDG 549–572 (Figure 5, lower panel).

The effect of exposure of α-DG to ROS was quantified by incubating immobilized bovine glomerular α-DG with increasing concentrations of XO (Figure 6). Similar to the observations on kidney sections, the carbohydrate epitope of monoclonal antibody (MoAb) IIH6 was more sensitive to ROS than the carbohydrate epitope of MoAb VIA4.1 (Figure 6a and c). Importantly, the core protein was not affected by ROS, as evaluated by staining with ShαRbDG 549–572 (Figure 6e). As was observed on kidney sections, the loss of IIH6 and VIA4.1 epitopes could be prevented by either scavenging hydroxyl radicals or by preventing the formation of hydroxyl radicals by scavenging the ferrous ions (Figure 6b and d).

The effect of ROS treatment on the binding of glomerular α-DG to laminin and agrin was investigated first in a ligand overlay assay (Figure 7). Agrin and laminin could bind to α-DG not exposed to ROS, but the binding was lost after exposure to ROS.

In addition, a solid-phase assay was performed (Figure 8). At increasing concentrations of XO, a decrease in binding of Engelbreth–Holm–Swarm (EHS) laminin and bovine glomerular agrin to α-DG was observed (Figure 8a and c). Even at high concentrations of ROS, the α-DG core protein remained attached to these immunoplates, as measured by ShαRbDG 549–572 (Figure 8e). Scavenging hydroxyl radicals with DMTU and preventing the formation of hydroxyl radicals with DFO prevented the loss of binding of both laminin and agrin to α-DG (Figure 8b and d). In contrast to the binding studies with MoAb IIH6 and VIA4.1 in ELISA, SOD treatment protected the binding site of α-DG for both laminin and agrin. This latter finding implies that other carbohydrate epitopes, which are not recognized by IIH6 and VIA4.1, are involved in binding to laminin and agrin.

Adriamycin nephropathy

The observed deglycosilation of α-DG, both *in vitro* accompanied with a decrease in binding of α-DG to agrin and laminin, questioned the effect of ROS on α-DG *in vivo*.

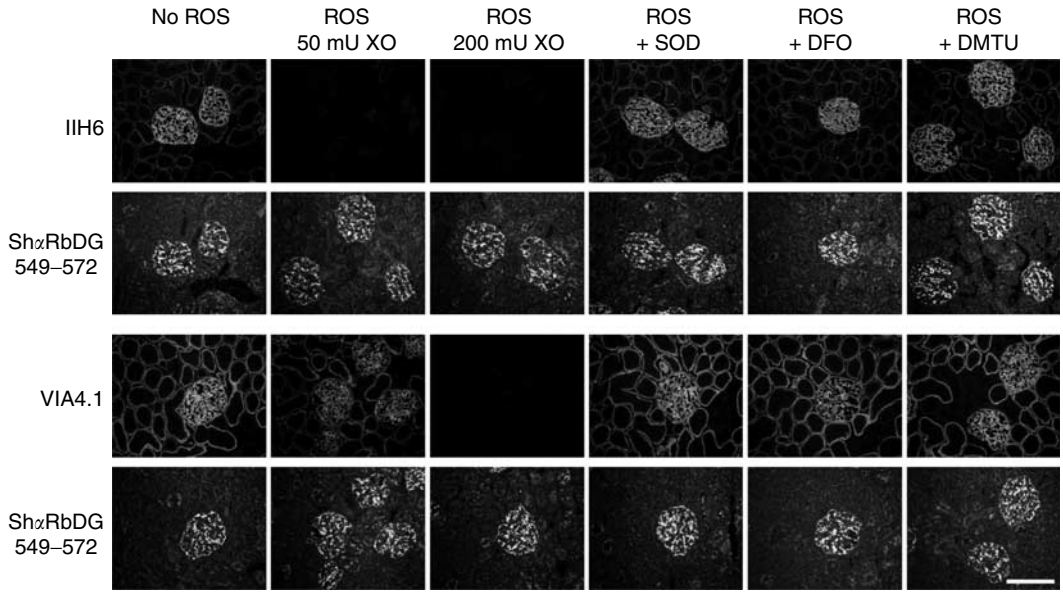

Figure 4 | Effect of *in situ* exposure of kidney sections to ROS on carbohydrate epitopes of α-DG. Indirect immunofluorescence of 2 μm rabbit kidney cryostat sections exposed to ROS. The epitope of antibody IIH6 was lost at the lowest concentration of XO (50 mU/ml), whereas both epitopes of MoAb IIH6 and VIA4.1 were lost at the highest concentration of XO (200 mU/ml). In all cases, the core epitope of α-DG remained intact. This ROS-dependent epitope loss at 200 mU/ml of XO was prevented by scavenging the superoxide with SOD (fourth column), as well as by scavenging hydroxyl radicals with DMTU (sixth column) or preventing the formation of hydroxyl radicals by chelation of ferrous ions with DFO (fifth column). After scoring of blinded sections, the glomerular staining on a scale from 1–10, the epitope loss compared to control sections was significantly different (VI4.1 50 mU XO, $P<0.05$; VIA4.1 200 mU XO, IIH6 50, and 200 mU XO, $P<0.01$). Furthermore, the protection by all three scavengers compared to ROS-treated sections was significantly different ($P<0.01$, Bar = 100 μm).

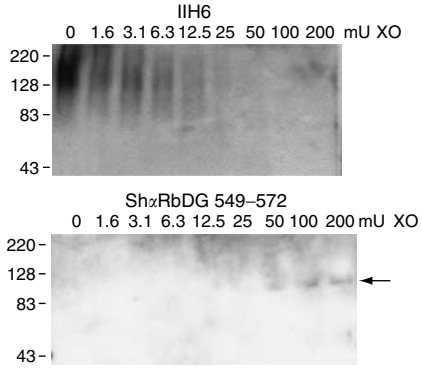

Figure 5 | Exposure of glomerular α-DG to ROS leads to hypoglycosilated α-DG. Purified bovine glomerular α-DG was exposed to increasing concentrations of ROS and subsequently separated by 4–15% SDS-PAGE. At lower concentrations of XO, the molecular mass of α-DG decreased, as revealed by probing with IIH6 (upper panel). At higher concentrations of XO the molecular mass decreased to approximately 100 kDa (arrow), as revealed by probing with ShαRbDG 549–572 (lower panel).

We observed a decrease in the expression of the specific α-DG carbohydrate epitopes recognized by IIH6 and VIA4.1 in the adriamycin-treated rats, which could not be prevented by scavenging hydroxyl radicals with DMTU (Figure 9). This seems to be in contrast with our earlier observations that DMTU prevented partially the depolymerization of heparan sulfate in adriamycin-injected rats. The expression of other components of the glomerular DG complex remained stable, as revealed by the glomerular staining for utrophin. However, ShαRbDG 549–572 failed to stain (ROS-treated) rat kidney sections.

The failure to demonstrate the *in vivo* protection of loss of α-DG carbohydrate epitope by DMTU in the adriamycin-treated rat made us compare the *in situ* susceptibility to ROS of rat glomerular heparan sulfate on agrin (assessed by JM403) and the oligosaccharides on α-DG (Figure 10). The staining of both IIH6 and VIA4.1 was lost at 200 mU XO, whereas still some staining of JM403 was visible. Furthermore, heparan sulfate loss could be prevented with lower concentrations of DMTU compared to loss of oligosaccharides on α-DG (Figure 10, columns 4 and 5). This indicates that oligosaccharides on α-DG are more susceptible to ROS than heparan sulfate. Therefore, prevention of ROS-mediated deglycosilation of α-DG requires higher concentrations of DMTU, which was not achieved *in vivo* in adriamycin nephropathy.

DISCUSSION

We have raised in sheep new antibodies specific for peptides a.a. 517–526 and 549–572 of α-DG, as described by others.[29,32] Their specificity was proven in ELISA, immunoprecipitation, Western blot analysis, and immunofluorescence. In immunofluorescence, we found an (intracellular) expression of the epitope of ShαRbDG 549–572 in podocytes. This localization was recently also found by Pavoni *et al.*,[32] with new antibodies raised against the a.a. 549–569 peptide of α-DG. It may reflect DG trafficking from the endoplasmatic reticulum to the Golgi, where it undergoes post-translational

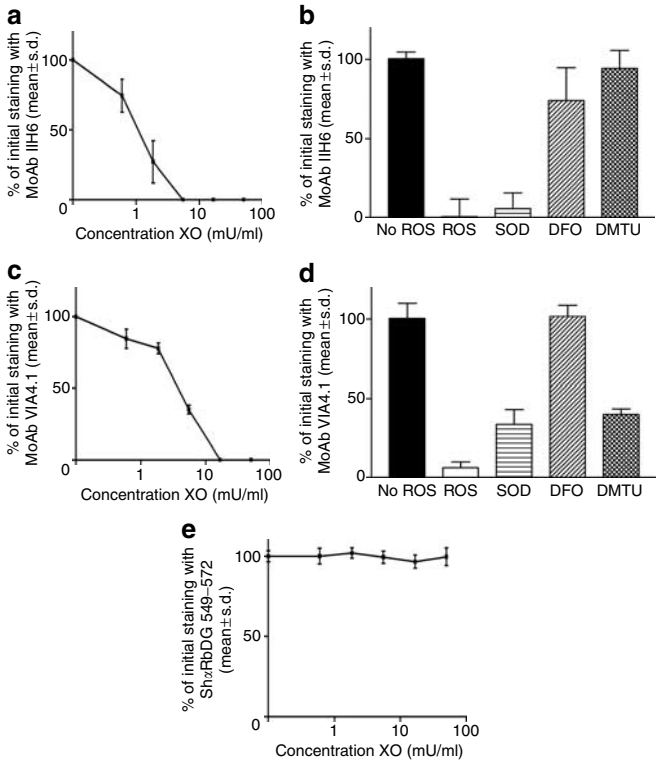

Figure 6 | Exposure of immobilized glomerular α-DG to ROS leads to loss of carbohydrate epitopes on α-DG. Purified bovine glomerular α-DG was coated in ELISA plates and exposed to different concentrations of XO. The carbohydrate epitope of (**a**) MoAb IIH6 is more sensitive to ROS than the epitope of (**c**) MoAb VIA4.1. At an XO concentration of 50 mU/ml, the concentration at which in ELISA all staining of α-DG with IIH6 and VIA4.1 was lost, SOD, DFO or DMTU was added to the reaction buffer. Preventing the formation of hydroxyl radicals with DFO as well as scavenging hydroxyl radicals prevented the loss of the epitope of (**b**) MoAb IIH6 and (**d**) VIA4.1. Scavenging the superoxide with SOD only partially prevented the ROS-mediated epitope loss (**b**, **d**). The binding of IIH6 and VIA4.1 to α-DG not exposed to ROS is set at 100%. (**e**) Note that the core-protein epitope a.a. 549–572 was conserved.

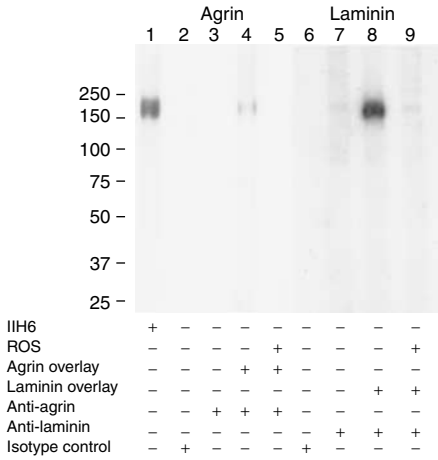

Figure 7 | Ligand overlay assay of purified bovine glomerular α-DG after exposure to ROS. Purified bovine glomerular α-DG was either exposed to ROS using an XO concentration of 200 mU/ml (lanes 5 and 9), or directly separated by 8% SDS-PAGE (lanes 1–4 and 5–8) and blotted to polyvinylidene difluoride membranes. These membranes were incubated with agrin (lanes 4 and 5) and laminin (lanes 8 and 9). α-DG was stained by MoAb IIH6 (lane 1), the overlay lanes were probed for agrin (lanes 3–5, isotype control lane 2) and laminin (lanes 7–9, isotype control lane 6) using specific antibodies. Lane 1 shows the presence of α-DG not exposed to ROS as probed by MoAb IIH6. This purified α-DG did not contain agrin (lane 3). Agrin could bind to α-DG not exposed to ROS (lane 4), but the binding was lost after exposure to ROS (lane 5). Purified α-DG still contained a faint amount of laminin (lane 7). This is probably contamination due to release of some laminin from the EHS laminin sepharose column used for purification of α-DG. However, laminin could clearly bind to α-DG (lane 8), while exposure of α-DG to ROS prevented binding of laminin (lane 9).

processing events. Extracellularly, the epitope is hardly recognized because of sterical hindrance by β-DG, as the epitope corresponds to the binding site to β-DG. Comparing our two antibodies to the core protein of α-DG, a difference in the accessibilities of these epitopes on the core protein of muscle and glomerular α-DG was found. The epitope of ShαRbDG 549–572 is accessible in muscle, some renal tubuli, and in the glomerulus, in contrast to ShαRbDG 517–526, which has a similar accessibility in muscle and renal tubuli, but not in the glomerulus. As there are no splice variants known for α-DG, the glomerular epitope of ShαRbDG 517–526 is either masked by a different tertiary structure in the glomerulus compared to muscle or, more likely, by a difference in glycosilation. This may also explain the higher molecular mass of glomerular α-DG compared to muscle α-DG observed by SDS-PAGE/Western blot. The peptide 517–526 contains one potential *O*-mannosyl glycosilation site at position 520. We suggest that in glomerular α-DG, in contrast to muscle α-DG, this site is glycosilated.

ROS play an important role in several forms of experimental nephropathies. We have previously shown that ROS can degrade and depolymerize the heparan sulfate side chains of agrin *in vitro* as well as *in vivo*.[15] Here, we show that glomerular α-DG is also sensitive to deglycosilation by ROS, as determined by the decrease in staining of kidney sections of two specific carbohydrate epitopes of α-DG recognized by MoAb IIH6 and VIA4.1, whereas the core epitope was not affected. Dependent on the concentration of XO, we observed a decrease in molecular mass of α-DG. First, the high molecular part of the 150–200 kDa smear disappeared, finally to a size of approximately 100 kDa. This indicates that some of the carbohydrate moieties of α-DG are resistant to ROS treatment, since the predicted molecular mass of completely unglycosilated α-DG is 74 kDa. The approximate molecular mass of hypoglycosilated α-DG in muscle–eye–brain disease is indeed about 74 kDa.[12,29] ShαRbDG 549–572 did not stain the broad smear of the fully glycosilated α-DG from bovine glomeruli. However, it did stain the more compact smear of rabbit muscle α-DG at approximately 156 kDa. Apparently, ShαRbDG 549–572 can only stain bovine glomerular α-DG in Western blot after exposure to ROS, which may be due to the more heterogeneous glycosilation of native bovine

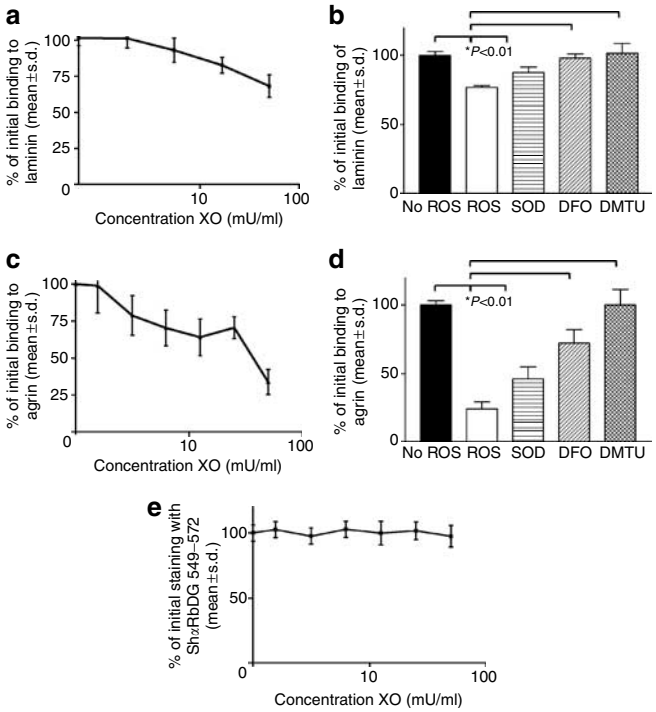

Figure 8 | Functional implications of deglycosilation of glomerular α-DG by ROS. (**a**) The binding of laminin to purified bovine glomerular α-DG exposed to ROS decreases in the solid-phase assay. (**b**) The decrease in laminin binding to ROS-treated α-DG was prevented partially by scavenging superoxide with SOD and almost completely by blocking the formation of hydroxyl radicals with DFO or scavenging hydroxyl radicals with DMTU (*$P < 0.01$). (**c**, **d**) Similar effects were observed with purified bovine glomerular agrin (*$P < 0.01$). (**e**) Importantly, the core protein as measured by ShαRbDG 549–572 was not affected by ROS. The binding of laminin or agrin to α-DG not exposed to ROS is set at 100%.

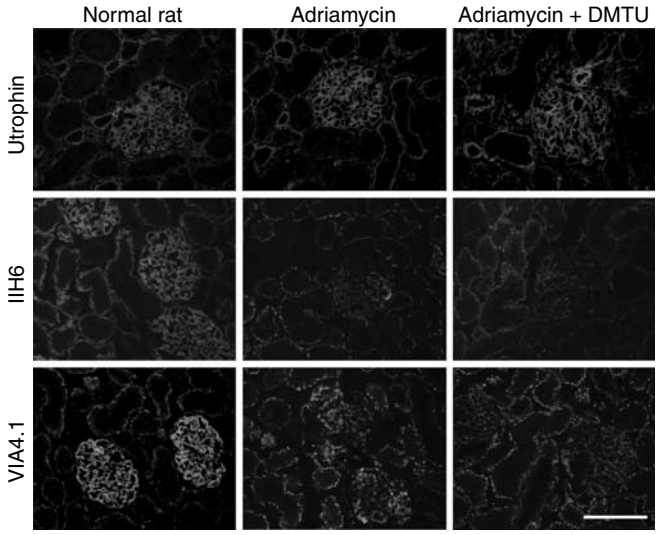

Figure 9 | Expression of carbohydrate epitopes of α-DG in adriamycin nephropathy. In adriamycin nephropathy (second column), the expression of the carbohydrate epitopes of α-DG-specific MoAb IIH6 and VIA4.1 was investigated at day 28, and revealed a reduced staining with both antibodies (second and third rows). The expression of utrophin, another component of the DG complex, was unaltered (first row). In contrast to the *in vitro* observations, no protective effect of DMTU was observed on the expression of these carbohydrate epitopes of α-DG (third column, Bar = 100 μm).

glomerular α-DG. However, when bovine glomerular α-DG was immobilized on microtiter plates, the epitope for ShαRbDG 549–572 could be detected in ELISA, which enabled us to evaluate the susceptibility of the αDG 549–572 core epitope to ROS. Also, in ELISA, the carbohydrate epitopes of α-DG recognized by IIH6 and VIA4.1 are lost upon ROS treatment. Similar to the experiments on kidney sections, the epitope of antibody IIH6 is more sensitive to ROS than the epitope of VIA4.1. Inhibition of the formation of hydroxyl radicals, as well as scavenging hydroxyl radicals, prevented the deglycosilation of α-DG. As superoxide and hydrogen peroxide are still available during scavenging hydroxyl radicals by DMTU, we conclude that hydroxyl radicals are the main cause of the observed deglycosilation of α-DG. Scavenging superoxide by SOD did not completely prevent the loss of carbohydrate epitopes of α-DG. This might be explained by the fact that during the degradation of superoxide by SOD hydrogen peroxide is formed, still allowing some generation of hydroxyl radicals. We have previously shown that α-DG also covers the apical membrane of the podocyte, where it presumably plays a role in the maintenance of the filtration slit by its negative charge derived from its sialic acid residues.[1,2] Deglycosilation of α-DG by ROS will lead to loss of this negative charge and may thereby play a role in foot process effacement. On kidney sections after exposure to ROS, unpublished experiments from our laboratory indeed show a loss of staining of *Maackia amurensis lectin* and *Sambuccus nigra agglutinin* (both specific for sialic acid), and many other lectins.

DG plays a role in the binding of the podocyte to laminin and agrin in the glomerular basement membrane via its oligosaccharide side chains. Deglycosilation of α-DG may lead to a decreased binding to these ligands. Such an effect has been documented in patients with muscle–eye–brain disease, Fukuyama muscular dystrophy, or in *myd* mice, in which muscle α-DG is hypoglycosilated and the binding of α-DG to laminin and agrin is severely impaired.[12–14] Unfortunately, no information is available about the glycosilation of glomerular α-DG, kidney function, or proteinuria in these patients or in this experimental model. *In vitro* it has been shown that chemical deglycosilation of muscle α-DG can disrupt its binding to laminin.[8,30,33] Very recently, a loss of binding has been postulated after ROS exposure of muscle α-DG to immobilized laminin and agrin.[34] However, the outcome of these experiments could be flawed by the fact that MoAb VIA4.1 was used to detect binding of α-DG, while we have shown here that the epitope of VIA4.1 is sensitive to exposure to ROS. Therefore, we addressed whether the binding of glomerular α-DG to laminin and agrin is actually decreased after ROS treatment. In the ligand overlay assay, we found that laminin and agrin clearly bound to

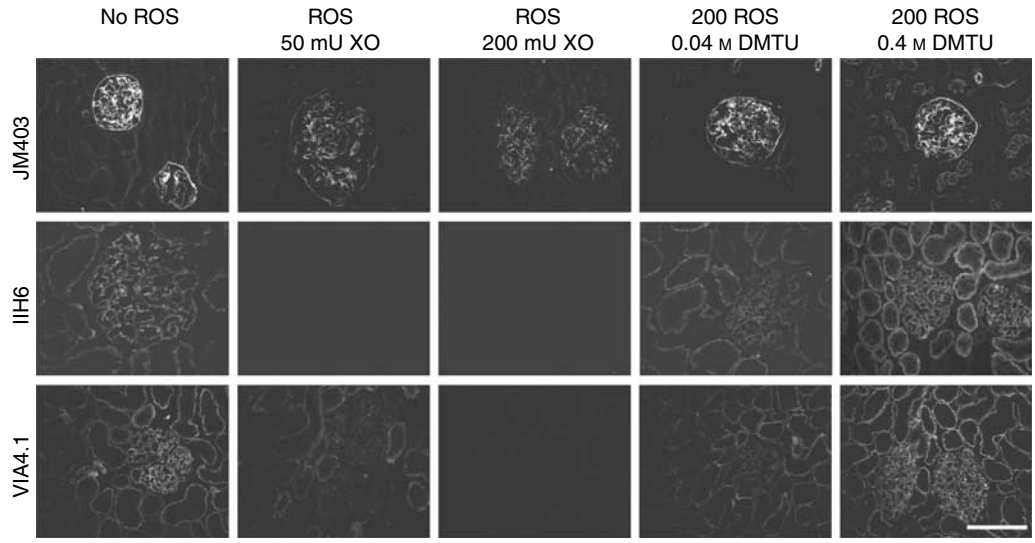

Figure 10 | Carbohydrate epitopes of α-DG are more susceptible to ROS than heparan sulfate on agrin. Rat kidney sections were exposed to ROS. The carbohydrate epitopes of α-DG IIH6 and VIA4.1 are completely lost after exposure to 200 mU XO (second and third rows), whereas the expression of heparan sulfate (JM403) is partially conserved (first row). Furthermore, the epitope of JM403 was protected against ROS at a lower concentration DMTU (fourth and fifth columns, Bar = 100 μm).

purified native α-DG. This binding was completely lost upon exposure of α-DG to ROS. In the solid-phase assay, we observed a substantial decrease, but not a complete loss, of binding of ROS-treated α-DG to both agrin and laminin. This partial loss of binding of laminin and agrin to α-DG in the solid-phase assay was prevented by scavenging hydroxyl radicals. The discrepancy of the complete loss of binding in the ligand overlay assay and the partial loss in the solid phase assay can be explained by either differences in efficacy of ROS in the fluid or solid phase, or differences in sensitivity of the solid-phase assay in comparison to the ligand overlay assay. Nevertheless, both assays show that deglycosilation of α-DG may have functional consequences for its binding to the ligands agrin and laminin in the GBM.

A decreased binding of α-DG to laminin and agrin might therefore lead to detachment of podocytes *in vivo*. Podocytes are highly specialized cells, which are unable to replicate.[27] A decrease in podocyte number has been correlated with deterioration and even with progression to end-stage renal failure in several glomerulopathies.[35–38] The number of podocytes in the urine of patients with systemic lupus erythematosus and diabetes has been shown to correlate with disease activity.[39–42] Preservation of podocyte anchorage to the GBM may be a new target in the therapy of these glomerular diseases.

In adriamycin nephropathy, we showed previously that scavenging hydroxyl radicals with DMTU can prevent the depolymerization of heparan sulfate and lowers proteinuria by 50%. Adriamycin nephropathy is a model for minimal change nephropathy, a human podocyte disease with little or no abnormalities in light microscopy. However, in electron microscopy a fusion of foot processes of podocytes is revealed, accompanied by a loss of anionic charge selectivity leading to a high level of albuminuria.[43] Previously, a decrease of the epitope of antibody VIA4.1 has been shown in patients with minimal change nephropathy.[44] In adriamycin nephropathy, we observed also a decrease of both carbohydrate epitopes of IIH6 and VIA4.1 on α-DG, whereas the expression of another component of the DG complex, utrophin, remained unaltered. However, the decrease of these α-DG carbohydrate epitopes in adriamycin nephropathy was not prevented by treatment with DMTU. We could demonstrate *in situ* that carbohydrate epitopes on rat glomerular α-DG were more susceptible to ROS than the heparan sulfate side chains on agrin. This difference may explain the failure of chosen DMTU doses to prevent *in vivo* deglycosilation of α-DG, and explain the persistence of a certain degree of albuminuria.[15] Furthermore, it might be due to the fact that after deglycosilation α-DG is endocytosed by podocytes, as was recently reported.[34] Alternatively, some carbohydrate epitopes on glomerular α-DG, other than those recognized by IIH6 and VIA4.1, may be preserved by DMTU. Nevertheless, our data from adriamycin nephropathy demonstrate that α-DG on podocytes is rather sensitive to ROS.

In conclusion, we have shown that glomerular α-DG can be deglycosilated by hydroxyl radicals, which leads to an impaired binding to laminin and agrin. Furthermore, this may lead to a loss of negative charge of the filtration slit, and thereby to foot process effacement. We speculate that the disruption of the glomerular α-DG–agrin complex may lead to proteinuria, podocyte loss, and ultimately to a decrease of renal function.

MATERIALS AND METHODS

Antibodies and immunofluorescence

ShαRbDG core protein antibodies were developed with peptides corresponding to rabbit α-DG a.a. 500–517, 517–526, and 549–572,

Table 2 | Primary and secondary antibodies in indirect immunofluorescence, ELISA and Western blot

	Primary antibody			Secondary antibody	
Antibody	Epitope	Isotype	Dilution	Conjugate	Dilution
IIH6[a]	Carbohydrates α-DG	Mouse IgM	6 μg/ml[b] 1.5 μg/ml[d]	Goat anti-mouse IgM Alexa fluor 488[b,c] Goat anti-mouse IgM peroxidase[d,e]	1:500 80 ng/ml
VIA4.1[a]	Carbohydrates α-DG	Mouse IgG1	20 μg/ml[b] 5 μg/ml[d]	Goat anti-mouse IgG Alexa fluor 488[b,c] Goat anti mouse $IgG_{(H+L)}$ peroxidase[d,f]	1:200 80 ng/ml
ShaRbDG 517–526[g]	α-DG a.a. 517–526	Sheep IgG	20 μg/ml[b] 10 μg/ml[d]	Goat anti-sheep IgG Alexa fluor 594[b,c] Rabbit anti-sheep IgG peroxidase[d,f]	1:2000 80 ng/ml
ShaRbDG 549–572[g]	α-DG a.a. 549–572	Sheep IgG	20 μg/ml[b] 10 μg/ml[d]	Goat anti-sheep IgG Alexa fluor 594[b,c] Rabbit anti-sheep IgG peroxidase[d,f]	1:2000 80 ng/ml
NCL-DRP2[h]	Utrophin	Mouse IgG	1:10	Goat anti-mouse IgG Alexa fluor 488[b,c]	1:200
MI-90[i]	Agrin core protein	Hamster IgG	1:400	Goat anti-Syrian hamster IgG FITC[b,c]	1:50
10B2[j]	Perlecan	Mouse IgG	1:250	Goat anti-mouse IgG Alexa fluor 488[b,c]	1:200
PCLP-1[k]	PCLP-1	Goat IgG	1:100	Rabbit anti-goat IgG Alexa fluor 488[b,c]	1:2000
JM403[l]	Heparan sulfate, agrin	Mouse IgM	1:100	Goat anti-mouse IgM Alexa fluor 488[b,c]	1:500
Anti-laminin[m]	EHS laminin	Rabbit IgG	0.1 μg/ml	Goat anti-rabbit IgG peroxidase[d,k]	40 ng/ml
JM170[n]	Agrin core protein	Mouse IgG	10 μg/ml	Goat anti mouse $IgG_{(H+L)}$ peroxidase[d,f]	80 ng/ml

ELISA=enzyme-linked immunosorbent assay; ShαRbDG=sheep anti-rabbit α-dystroglycan; Ig=immunoglobulin; PCLP=podocalyxin-like protein.
[a]KP Campbell.[12]
[b]Immunofluorescence.
[c]Molecular Probes, Leiden, The Netherlands.
[d]ELISA, solid-phase assay, Western blot and overlay assay.
[e]Southern Biotechnology Associates, Inc., Birmingham, AL.
[f]Jackson, Immunoresearch Laboratories, Inc., Westgrove, PA.
[g]This work.
[h]Novocastra Laboratories Ltd, Newcastle upon Tyne, UK.
[i]Berden.[24]
[j]Couchman.
[k]Santa Cruz Biotechnology, Inc. Santa Cruz, CA.
[l]Berden.[48]
[m]Sigma.
[n]Berden (unpublished results).

as described previously.[29] Antibodies were affinity purified on coupled peptides (sulfo link coupling gel, Pierce, Rockford, IL). Specificity was evaluated by peptide ELISA and immunoprecipitation with purified antibodies bound to gammabind sepharose (Pharmacia, Uppsala, Sweden) using homogenized rabbit muscle extract in 50 mM Tris[hydroxymethyl]aminomethane (Sigma, Trizma base), 100 mM NaCl, 1% Triton X-100 (Sigma, St Louis, MO), pH 7.5. SDS-PAGE and Western blot analysis were performed according to the methods of Laemmli.[45] Immunofluorescence was performed as described previously.[2,15] Antibodies and conjugates are listed in Table 2.

Purification of bovine glomerular α-DG and agrin

Glomeruli were obtained from kidneys of 4-year-old cows by gradual sieving.[46–48] Glomeruli were dissolved in 0.1% collagenase type II (Invitrogen Corporation, Paisley, UK) in 50 mM Tris, pH 7.6, incubated for 15 min at 37°C, and spun down. The pellet was resuspended in 50 mM Tris, 1 mM phenylmethylsulfonyl fluoride (Sigma), and 0.75 mM benzamidine (Sigma), pH 7.6. A pH 11 extraction was performed and subsequently α-DG was affinity purified on EHS laminin (Campro Scientific, Berlin, Germany) 4 mg coupled to 20 ml sepharose S4B (Pharmacia).[49,50]

Agrin was purified from the pellet of the pH 11 extraction by guanidine HCl extraction.[24,48] From this extract, agrin was purified with affinity chromatography using monoclonal antibody JM170.

Generation of ROS and effects on α-DG

ROS were generated as described previously (Table 1).[15,51] Either kidney sections, dissolved or immobilized purified α-DG, were incubated with ROS, after which they were thoroughly washed in phosphate-buffered saline. *In vivo*, the effect of ROS was studied in adriamycin nephropathy, performed as described.[15] All animal experiments were approved by the local ethics committee.

Purified α-DG was coated in Maxisorp plates (Nunc, VWR International, Amsterdam, The Netherlands) and exposed to ROS. Blocking was performed with 5% non-fat dry milk (Biorad, Hercules, CA) in phosphate-buffered saline. For the ELISA IIH6 or VIA4.1 was used (Table 2) in non-fat dry milk/phosphate-buffered saline. For the ligand overlay and solid-phase assays, polyvinylidene difluoride or Maxisorp-bound α-DG was incubated with 10 nM EHS laminin or purified agrin in 50 mM Tris, 137 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, pH 7.6 (laminin binding buffer), and gently shaken for 16 h at 4°C.[12] Laminin and agrin were visualized by using specific antibodies (Table 2). Chemiluminescent peroxidase substrate (Sigma) or 3,3′,5,5′-tetramethyl benzidene (SFRI Diagnostics, Berganton, France) was used as developing solution.

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