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Inhibitor of differentiation 3, a transcription factor regulates hyperlipidemia associated kidney disease

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Abstract

Background—Lipoprotein abnormalities are associated with a rapid decline in renal function in patients of chronic kidney disease. In addition, hyperlipidemia is associated with an increased risk of developing renal insufficiency. The underlying molecular mechanisms for these clinical findings are unclear. We have previously reported a role for inhibitor of differentiation 3 (ID3), a transcription factor, in regulating kidney disease in hyperlipidemia. Introducing a genetic deficiency of *Id3* in spontaneously hyperlipidemic Apolipoprotein E knockout (*Apoe*$^{-/-}$) mice led to accelerated mesangio-proliferative glomerulonephritis. The present study was carried out to further investigate the contribution of ID3 in hyperlipidemia associated kidney disease.

Methods—Female C57BL/6 mice that were ID3 sufficient wild type (WT) or ID3 deficient (*Id3*$^{-/-}$) were fed a western diet and evaluated for proteinuria, glomerular pathology and immune infiltrating cells. Primary mesangial cell lines were generated from both mouse strains and stimulated with oxidized phospholipids. Cytokines and chemokines produced were measured by multiplex assays, ELISA, and QPCR. Glomerular isolates were studied for CXCL1 expression by QPCR.

Results—*Id3*$^{-/-}$ mice on a western diet developed accelerated proteinuria and mesangio-proliferative glomerulonephritis compared to WT controls. In vitro, *Id3*$^{-/-}$ glomerular mesangial cell lines produced higher levels of the monocyte chemoattractant, CXCL1 in response to oxidized phospholipids. This was consistent with the rapid increase in glomerular CXCL1 expression followed by macrophage infiltration in *Id3*$^{-/-}$ mice fed a western diet.

Conclusions—A functional ID3 influences susceptibility to kidney disease and prevents glomerular injury by regulating local chemokine production and inflammatory cell recruitment.

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Introduction

The association between hyperlipidemia and progression of chronic kidney disease is well established [1]. Clinical studies show that individuals with elevated cholesterol levels are at a higher risk of developing kidney disease [2, 3]. These studies, and data from mouse models suggest that the development of kidney disease in the presence of hyerplipidemia may be influenced by additional factors [4]. We have shown that introducing a deficiency of inhibitor of differentiation 3 (ID3) in spontaneously hyperlipidemic Apolipoprotein E deficient (*Apoe*$^{-/-}$) mice leads to accelerated atherosclerosis [5] and mesangio-proliferative glomerulonephritis (GN) [6]. In addition to causing hyperlipidemia, Apolipoprotein E deficiency also directly affects mesangial cell function [4, 18]. Therefore, to delineate the role of ID3 on hyperlipidemia associated kidney disease, without the effect of Apolipoprotein E deficiency, female WT and *Id3*$^{-/-}$ mice were fed an atherogenic western diet (TD.88137, 42% calories from fat) to cause diet induced hyperlipidemia. Our results suggest that the lack of ID3 facilitates the development of kidney disease, and a functional ID3 is reno-protective.

Inhibitor of differentiation 3 (ID3) is a broadly expressed transcription factor and belongs to the helix-loop-helix (HLH) family of proteins [7]. ID3 forms hetero-dimers with basic HLH transcription factors like E-proteins through the protein binding HLH domains. However, ID3 lacks the DNA binding domain and therefore, ID3-E-protein hetero-dimers fail to bind DNA. Thus, ID3 is a dominant negative regulator of transcription in multiple cell types including smooth muscle cells [7] immune cells [8], and adipocytes [9].

Oxidized phospholipids are increased in hyperlipidemic states and induce proinflammatory chemokines and cytokines in glomerular cells [10]. We hypothesized that production of inflammatory mediators is exacerbated in ID3 deficiency, resulting in recruitment of immune cells into the glomeruli. This hypothesis was tested using ID3 sufficient (WT) and ID3 deficient *(Id3*$^{-/-}$*)* mice.

Methods

All methodologies are described in details in the supplementary materials.

Mice

All procedures followed NIH guidelines for humane use of animals and were approved by the Institutional Animal Care and Use Committee. C57BL/6 wild type (WT) and *Id3*$^{-/-}$ mice [5] were fed either western diet (TD.88137, Harlan-Teklad) or mouse chow (TD 7012) starting at 6-8wks of age. Urinary albumin and creatinine levels and lipid profile analyses were measured as previously described [6].

Evaluation of renal pathology

Mice were sacrificed after either 8wks or 15wks on diet and kidneys were studied for renal pathology, fibronectin deposition, and immune cell infiltration [6, 12-14].

Treatment of mesangial cells with oxidized phospholipids

Primary mesangial cell lines were generated from glomerular isolates [15] and used after 5th passage. Cells were stimulated with oxidized phospholipids, Oxidized 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphocholine (oxPAPC), the active principle of minimally modified LDL [16]. Supernatants were collected and chemokines and cytokines were measured using the Mouse Grp1 23-plex and Grp2 9-plex Bio-Plex suspension array system (Bio-Rad). CXCL1 levels were determined by ELISA (R&D systems) for repeat experiments.

Gene expression analyses in mesangial cell lines and isolated glomeruli

CXCL1 expression in mesangial cells and isolated glomeruli were carried out as previously described [17, 18]. Data are represented as fold change over untreated B6 mesangial cell cDNA. GAPDH was used as a housekeeping gene control. For glomerular gene expression, results are presented as relative expression using GAPDH as a housekeeping control.

Statistical Analyses

Statistical analyses were carried out using one way ANOVA with Bonferroni correction for multiple comparisons, two way ANOVA, and Mann Whitney test using Graph Pad Prism 3.0 software.

Results

Diet induced hyperlipidemia leads to GN and proteinuria in Id3$^{-/-}$ mice

Both strains, WT and *Id3*$^{-/-}$ were fed a western diet for 8 wks and showed significant increases in weight, serum LDL, HDL, and total cholesterol compared to chow fed controls (Supplementary Table 1). Neither strain showed changes in blood glucose levels, in concordance with previous reports that female mice on high fat diets are resistant to metabolic syndrome-like disease [19]. Despite this, *Id3*$^{-/-}$ females developed significant proteinuria and GN after 8wks on the western diet (Figure. 1a-c). In contrast, WT mice on a western diet developed little proteinuria and showed mild glomerular pathology. Quantitative analyses showed a significant increase in glomerular area staining for fibronectin, an indicator of mesangial activation and mesangio-proliferative GN in *Id3*$^{-/-}$ mice (Figure 1d, **and** Supplementary Table 2). Representative images of glomerular pathology and fibronectin deposition are shown in Figure 2. In an additional cohort of mice sacrificed after 15wks of feeding, *Id3*$^{-/-}$ mice on the western diet showed further progression of mesangial expansion, inflammatory cell infiltration, and glomerulosclerosis (mean severity score 2.37±0.37; n=6) compared to WT mice (mean severity score 0.73±0.030; n=4; p=0.0079). WT and *Id3*$^{-/-}$ mice fed mouse chow did not develop kidney disease. Thus, diet induced hyperlipidemia resulted in mesangio-proliferative GN in *Id3*$^{-/-}$ mice.

Glomerular pathology is associated with macrophage infiltration

To identify inflammatory cell infiltrates, kidney sections were stained with antibodies to T cells (CD3, CD4), B cells (B220), dendritic cells (CD11c) and macrophages (CD68 and F4/80). Macrophages were the predominant infiltrating cells seen in mice with GN. A

quantitative analysis of CD68 positive cells within glomeruli showed a significant increase in *Id3*$^{-/-}$ mice on a western diet (Figure 1e, supplementary table 2). The CD68 cells were activated as indicated by co-expression of MHC class II (Figure 2). Glomerular infiltrates of CD68 cells were infrequent in the other mouse groups. After 15wks of western diet, increased renal pathology was associated with a further increase in glomerular CD68+ infiltration in *Id3*$^{-/-}$ mice (6.13+0.18; n=6) compared to WT mice (3.22+0.72; n=5; $p<0.001$). There was no increase in T, B, or dendritic cell infiltration in either group (data not shown). Neutrophils were not seen infiltrating the glomeruli in any of the groups. The pathology was restricted predominantly to the glomerular and peri-glomerular regions with little involvement of the tubulointerstitial regions.

Id3 regulates mesangial cell responses to oxidized phospholipids

To investigate the potential mechanism of mesangio-proliferative GN induced by ID3 deficiency, we established primary mesangial cell lines from WT and *Id3*$^{-/-}$ mice. The mesangial cell lines were checked for purity by staining cells with a panel of antibody reagents reacting with mesangial, epithelial, endothelial, podocyte, and fibroblast markers and were used after the 5th passage. All cell lines were >90% pure (Supplementary figure 1). Oxidized and minimally modified LDL are taken up by mesangial cells through the scavenger receptors and are known to modulate mesangial cell function [20]. The *Id3*$^{+/+}$ and *Id3*$^{-/-}$ mesangial cells were stimulated with oxPAPC, an inflammatory oxidized phospholipid and an active principle of minimally modified LDL [16]. Supernatants collected 24 hrs later were analyzed for chemokines and cytokines using the Mouse Grp I 23-plex and Grp II 9-plex Bio-Plex suspension array system (BioRad). Of the 32 cytokines and chemokines studied, *Id3*$^{-/-}$ mesangial cells showed higher basal CXCL1 production than WT cells. Other chemokines detected include MCSF, PDGFbb and VEGFa, and they were comparable in both *Id3*$^{-/-}$ and WT cells (supplementary figure 2). Higher CXCL1 production by Id3$^{-/-}$ mesangial cells in response to oxPAPC was confirmed by ELISA using another set of independently generated WT and Id3$^{-/-}$ cell lines and the results are shown in Figure 3a. CXCL1 production in supernatant was associated with a corresponding increase in CXCL1 gene expression and mRNA levels (Figure 3b). CXCL1 is a neutrophil and monocyte chemoattractant and has been identified as the earliest chemokine critical for arrest of circulating monocytes at the site of endothelial injury [21, 22]. In the kidney, CXCL1 production in the glomeruli has been implicated in inflammatory cell recruitment in immune complex mediated glomerulonephritis [23]. This supports our hypothesis that ID3 regulates local responses to pro-inflammatory lipids and ID3 deficiency exacerbates recruitment of macrophages into the renal glomeruli.

A rapid upregulation of glomerular CXCL1in Id3$^{-/-}$ mice on a western diet—To investigate whether hyperlipidemia affected local CXCL1 production in vivo, WT and *Id3*$^{-/-}$ mice were fed mouse chow or a western diet and CXCL1 expression in isolated glomeruli was studied 2 wks later. A significant increase in glomerular CXCL1 expression was seen in *Id3*$^{-/-}$ mice on a western diet (0.1172+0.024; n=5) compared to chow fed *Id3*$^{-/-}$ mice (0.050+0.023; n=4;) ($p=0.032$ by Mann Whitney test). Glomerular CXCL1 expression was not different between WT mice fed regular chow (0.067+0.04; n=3) or western diet

(0.060+0.04; n=3). These data are consistent with the mesangial cell results showing that ID3 deficiency is associated with CXCL1 upregulation in response to inflammatory lipids.

Discussion

In the present study, we have recapitulated the salient characteristics of kidney disease previously reported in *Apoe*$^{-/-}$*Id3*$^{-/-}$ double knockout mice; and show that ID3 per se, in hyperlipidemic mice directly influences susceptibility to kidney disease. ID3 deficiency may exacerbate CXCL1 production by glomerular cells in response to inflammatory lipids and the resulting macrophage recruitment. Since Id3 is present in multiple cell types, it is also possible that other glomerular cells lacking Id3 may contribute to cytokine production in vivo. Thus, the reno-protective effect of ID3 may be through regulation of local chemokine production. ID3 is known to directly interact with more than 30 different transcription factors [24]. Clearly, a change in the ID3 function may impact a wide range of protein-protein interactions with potentially significant consequences.

In contrast to our findings in *Apoe*$^{-/-}$*Id3*$^{-/-}$ mice with glomerulonephritis, the hyperlipidemic *Id3*$^{-/-}$ mice did not show significant increase in glomerular immune complex deposition compared to hyperlipidemic WT mice. Apoliprotein E deficiency is known to cause increased immune responsiveness [25] and these results add significance to the present study in dissecting the effects of ID3 alone on kidney disease. Clinical studies provide evidence for the relationship between lipids and chronic kidney disease. However, they fail to explain why certain individuals (in the absence of diabetes or metabolic syndrome) are likely to develop chronic kidney disease [3]. In humans, a single nucleotide polymorphism (SNP) in *ID*3 is associated with increased susceptibility to atherosclerosis [5]. Our preliminary findings in humans suggest a significant association between the same ID3 SNP and proteinuria, specifically influenced by small low density lipoproteins (p=0.0024) (Nackiewicz et al., Arthritis Rheum 2013; 65:S240 Abstract #552).

C57BL/6 male mice on high fat diets (60% calorie from fat) develop metabolic syndrome associated with obesity, elevated plasma glucose, proteinuria and GN and this may be due to decrease in renal AMP activated protein kinase, a cellular energy sensor [26]. However, C57BL/6 female mice on high fat diets develop GN and proteinuria only in the absence of ID3 suggesting distinct pathogenic mechanisms between females and males.

Investigating molecular mechanisms in mice has identified ID3 as a novel transcription factor that may contribute to kidney disease and provide mechanistic links between atherosclerosis, hyperlipidemia and kidney disease in humans.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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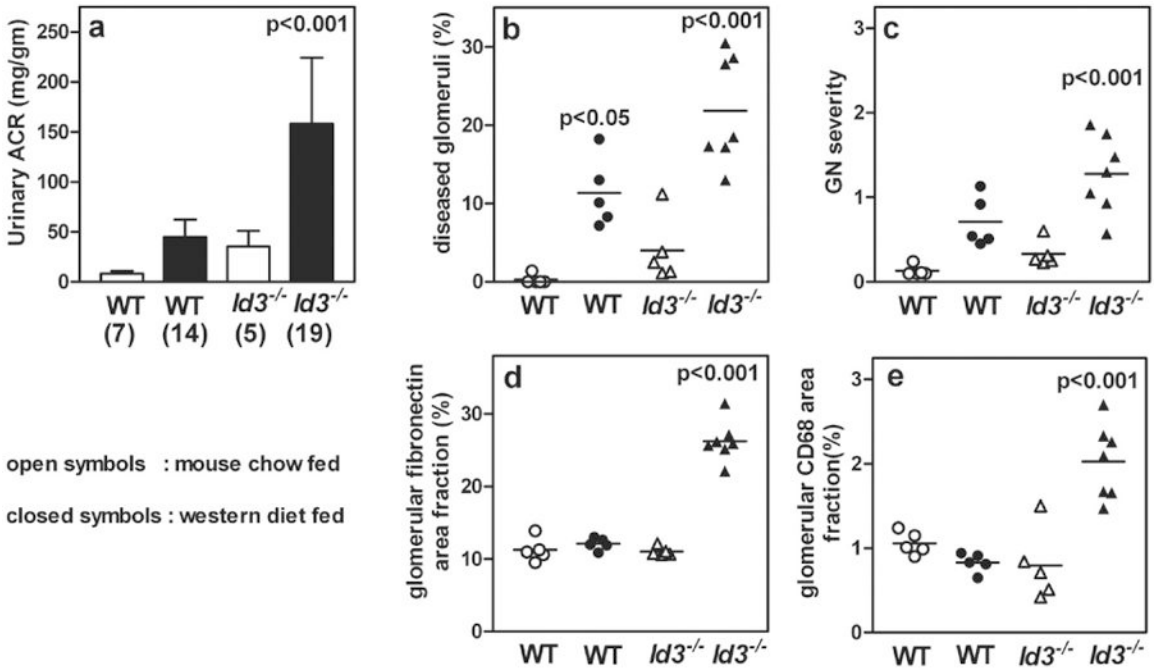

Figure 1. *Id3*$^{-/-}$ female mice develop kidney disease after 8 wks on a western diet

WT or *Id3*$^{-/-}$ female mice were fed regular mouse chow (open symbols) or western diet (closed symbols). Number of mice in each group is indicated in parenthesis. Urine samples (24 hr) were analyzed for urinary albumin to creatinine ratios (ACR) (**a**). PAS stained kidney sections were studied for frequency of glomeruli affected (**b**) and severity of GN was scored (**c**). Kidney sections were stained by immunofluorescence for fibronectin deposition and results show percent glomerular area positive for fibronectin (**d**). Inflammatory cell infiltration in the glomeruli was quantified by measuring glomerular area staining positive for CD68+ macrophages (**e**). Each data point represents one mouse. Statistical analyses by ANOVA with Bonferroni post test for multiple comparisons. p values indicate statistical significance compared with wild type controls. Detailed methodology and results of stereologic analyses for fibronectin and CD68 positive staining are presented in Supplementary Table 2.

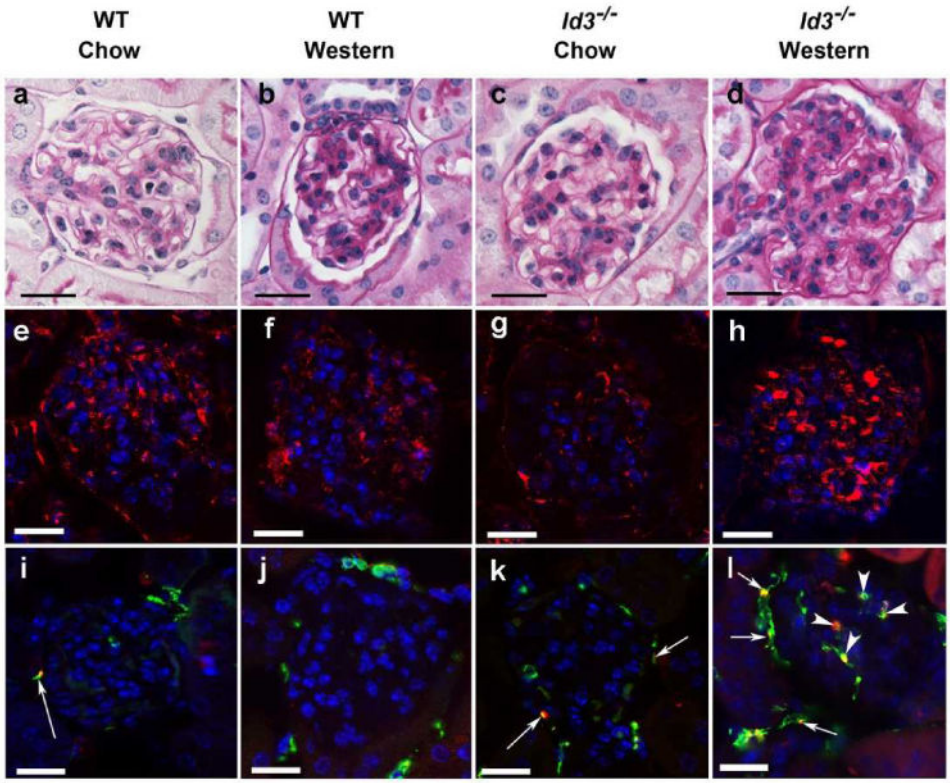

Figure 2. *Id3*$^{-/-}$ females fed a western diet develop GN, glomerular fibronectin deposits and glomerular infiltration by activated macrophages

Representative photomicrographs of paraffin embedded PAS stained kidney sections showing glomeruli from WT and *Id3*$^{-/-}$ female mice fed chow or western diets for 8 wks (**a-d**). Immunostaining for fibronectin deposits (**e-h**) showing increased glomerular fibronectin in western fed *Id3*$^{-/-}$ mice. Immunofluorescence microscopy shows macrophages CD68 (red) and MHCII (green) in periglomerular areas indicated by arrows and intra-glomerular shown by arrowheads (**i-l**). Nuclei (**e-l**) are stained blue with DAPI. Scale bar is 20 microns.

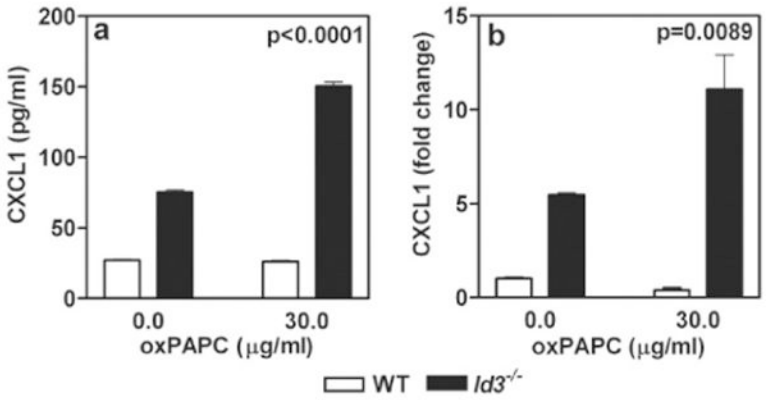

Figure 3. oxPAPC induces CXCL1 in *Id3*$^{-/-}$ mesangial cells

Primary mesangial cells (5×10^4cells/well) were incubated in RPMI medium with 0.5% low lipid fetal calf serum for 24 hrs, followed by incubation with oxPAPC (30μg/ml) for another 24 hrs. CXCL1 measured by ELISA in supernatants of cultured mesangial cells from *Id3*$^{-/-}$ and WT mice are shown (**a**). CXCL1 mRNA levels in the same cells were measured by QPCR (**b**). Similar results were obtained in additional experiments with two sets of independently generated primary mesangial cell lines from WT and *Id3*$^{-/-}$ mice. p values calculated using two way ANOVA using graph pad prism 3.0