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Role of Heparanase-Driven Inflammatory Cascade in Pathogenesis of Diabetic Nephropathy



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Renal involvement is a major medical concern in the diabetic population, and with the global epidemic of diabetes, diabetic nephropathy (DN) became the leading cause of end-stage renal failure in the Western world. Heparanase (the only known mammalian endoglycosidase that cleaves heparan sulfate) is essentially involved in DN pathogenesis. Nevertheless, the exact mode of heparanase action in sustaining the pathology of DN remains unclear. Here we describe a previously unrecognized combinatorial circuit of heparanase-driven molecular events promoting chronic inflammation and renal injury in individuals with DN. These events are fueled by heterotypic interactions among glomerular, tubular, and immune cell compartments, as well as diabetic milieu (DM) components. We found that under diabetic conditions latent heparanase, overexpressed by glomerular cells and posttranslationally activated by cathepsin L of tubular origin, sustains continuous activation of kidney-damaging macrophages by DM components, thus creating chronic inflammatory conditions and fostering macrophage-mediated renal injury. Elucidation of the mechanism underlying the enzyme action in diabetic kidney damage is critically important for the proper design and future implementation of heparanase-targeting therapeutic interventions (which are currently under intensive development and clinical testing) in individuals with DN and perhaps other complications of diabetes.

Heparanase is the only known mammalian endoglycosidase capable of degrading heparan sulfate (HS) glycosaminoglycan,

a highly sulfated polysaccharide that is ubiquitously found in the extracellular matrix (ECM) and at the cell surface (1,2). HS chains bind to and assemble ECM proteins, thus playing important roles in the structural integrity and barrier function of the ECM (in particular, basement membranes) (1,2). In addition, HS chains regulate the activity of a wide variety of bioactive molecules (i.e., cytokines, growth factors) at the cell surface and in the ECM (1,3). Enzymatic degradation of HS by heparanase profoundly affects numerous physiological and pathological processes, including morphogenesis, neovascularization, and tumorigenesis (1,4,5), and, more recently, immune reactivity and inflammation (6,7).

Involvement of heparanase in diabetic nephropathy (DN) was suggested more than a decade ago, owing to elevated levels of the enzyme in the kidneys and urine of DN patients, induction of renal heparanase in murine DN models, as well as in vitro studies demonstrating the upregulation of heparanase in kidney-derived cell lines by hyperglycemic conditions and DN mediators (i.e., albumin and advanced glycation end products [AGEs]) (8–12).

The essential role for heparanase in DN was recently demonstrated using heparanase-null (*Hpse-KO*) mice, which, unlike their wild-type (WT) littermates, failed to develop albuminuria, mesangial matrix expansion, and tubulointerstitial fibrosis in response to streptozotocin (STZ)-induced diabetes (13). Furthermore, a lower degree of albuminuria was detected in type 1 and type 2 diabetic

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mice treated with the heparanase inhibitor compared with mice treated with vehicle alone (13).

Although these data validate a causal role of heparanase in sustaining the pathology of DN, a uniform model for the mode of heparanase action in DN remains incomplete. Previously, it was generally accepted that elevated levels of glomerular heparanase contribute to DN progression primarily through the degradation of HS in the glomerular basement membrane (GBM). Indeed, GBM (along with fenestrated glomerular endothelium and podocyte foot processes/slit diaphragms) represents the major functional component of the kidney filtration barrier, with HS being the principle polysaccharide of the GBM (14,15). For more than 2 decades, the HS contribution to GBM perm-selective filtration and the loss of HS in the GBM were linked to the pathophysiology of DN. More recently, several reports (16,17) challenged the notion that heparanase-mediated loss of HS in the GBM interferes with GBM barrier properties and represents the primary mechanism implicating the enzyme in DN. In parallel, the view on DN as a consequence of solely metabolic/hemodynamic alterations has been transformed in recent years, with clear evidence indicating that the activation of innate immunity and chronic inflammation plays a significant role in the pathogenesis of both diabetes and its complications, including DN (18,19). Immunocytes (primarily macrophages) (19–23) and numerous inflammatory molecules such as nuclear factors (i.e., nuclear factor- κ B [NF- κ B]) and cytokines (i.e., tumor necrosis factor- α [TNF- α]) have been implicated in diverse pathogenic pathways related to DN (for review, see Navarro-González et al. [18] and Lim and Tesch [19]). Macrophages are considered to be the major immune cells infiltrating the diabetic kidney and critically contributing to the development of renal damage (23,24). In the diabetic kidney, macrophages, activated by various elements of the diabetic milieu (DM) (e.g., high glucose [25], AGE [26,27], albumin [28], free fatty acids [29]), release reactive oxygen species and proinflammatory cytokines (e.g., TNF- α and interleukin-6), which cause injury to podocytes and tubular cells (18,24,30,31).

Interestingly, the emerging data linking heparanase to inflammatory responses suggest a previously unrecognized mode of the enzyme action in DN pathogenesis. Heparanase was recently shown to create a chronic inflammatory microenvironment, preserving abnormal activation of macrophages and preventing inflammation resolution in chronic colitis (32) and psoriasis (33). These findings, together with the preferential overexpression of heparanase in diabetic kidneys, led us to assume that in the DN setting heparanase facilitates inflammatory responses induced by the DM, sustaining continuous activation of kidney-damaging macrophages, thus fostering DN development and progression.

RESEARCH DESIGN AND METHODS

Animals

Hpse-KO mice and their WT littermates (34), db/db (Lepr^{db}/db) mice, their control nonobese db/m mice on

C57BLKS/J background, and C57BL/6J mice were kept under pathogen-free conditions; all experiments were performed in accordance with the Hebrew University Institutional Animal Care and Use Committee.

STZ-Induced DN Model

Hpse-KO and WT mice were injected intraperitoneally with 40 mg/kg body wt STZ in 100 mmol/L citrate buffer (pH 4.6) for 5 consecutive days (after an overnight fast). By week 2 of the experiment, 100% of the WT and KO mice developed diabetes, as revealed by increased blood glucose levels (>300 mg/dL). Blood glucose levels were maintained at <350 mg/dL by treatment with insulin (0.4 units/mouse), administered every other day for 16 weeks. Body weight and blood glucose concentrations measured throughout the experiment, as well as the amount of insulin required to maintain glycemic control, did not differ between *Hpse-KO* and WT mice. Urinary albumin levels were measured using an ELISA kit (Bethyl Laboratories). Creatinine levels were measured using VITROS 5,1 FS Chemistry System (Johnson & Johnson). As reported previously (13), *Hpse-KO* mice do not develop DN in response to STZ-induced diabetes, and their 24-h urinary albumin excretion rate (quantified as micrograms of albumin per milligram of creatinine) before the onset of diabetes (14.5 ± 2.4) did not differ significantly from that observed on experimental week 16 (16.7 ± 1.9 μ g albumin/mg creatinine). In contrast, a significant increase in urinary albumin excretion was detected in WT mice on week 16 of STZ-induced diabetes (98.1 ± 37.5 vs. their nondiabetic controls 18.8 ± 4.2 μ g albumin/mg creatinine, $P < 0.05$) (13). Animals were killed at indicated time points, and half of each kidney was snap-frozen for RNA preparation/protein extraction and the other half processed for histology and immunostaining.

Type 2 DN Model

Lepr^{db}/db mice and control nonobese db/m mice on C57BLKS/J background were purchased from The Jackson Laboratory (Bar Harbor, ME); all db/db mice involved in experiments exhibited increased blood glucose levels (>300 mg/dL) at 6 weeks of age. Twofold to threefold increases in 24-h urine albumin excretion ($P < 0.05$) were detected on experimental weeks 14 and 18 in db/db mice, while in db/m animals urine albumin excretion remained unchanged.

Cell Culture

Isolation of murine peritoneal macrophages was performed as previously described (32). HK-2 human proximal tubule epithelial cells (12) were grown in DMEM supplemented with 1 mmol/L glutamine, 50 μ g/mL streptomycin, 50 units/mL penicillin, and 10% FCS (Biological Industries, Beit HaEmek, Israel) at 37°C and 8% CO₂. At 60–80% confluence, cells were maintained for 24 h in serum-free low-glucose (1 g/L) DMEM, then remained untreated or incubated with high-glucose (4.5 g/L) DMEM, AGE (catalog #JM-2221–10; MBL International Corporation), BSA (Sigma), and interferon- γ (IFN- γ)

(Pepro Tech). The final endotoxin levels in experimental media containing AGE/BSA were 0.024–8 pg/mL, which were significantly lower than the concentrations typically found in diabetic patients (35), or than those required to activate Toll-like receptor (TLR) 4 or the classic NF- κ B pathway (36,37). Cell lysates were analyzed by immunoblotting and heparanase activity assay. In some experiments, cells were cultured on glass coverslips (12 mm; Carolina Biological Supply), fixed with 100% ice-cold methanol and processed for immunofluorescent staining.

Antibodies

Immunoblot analysis and immunostaining were carried out with the following antibodies: anti-F4/80 (AbD Serotec); anti TNF- α (R&D Systems); anti-cathepsin-L (CatL; R&D Systems and Sigma); anti-phospho NF- κ B p65 (Cell Signaling Technology); anti-actin (Santa Cruz Biotechnology); and anti-heparanase monoclonal antibody 01385–126, recognizing both the 50-kDa subunit and the 65-kDa proheparanase (32), which was provided by Dr. P. Kussie (ImClone Systems).

Immunohistochemistry

Paraffin-embedded slides were deparaffinized and incubated in 3% H₂O₂. Antigen unmasking was carried out by heating (20 min) in a microwave oven in 10 mmol/L Tris buffer containing 1 mmol/L EDTA. Slides were incubated with primary antibodies diluted in CAS-Block (Invitrogen) or with CAS-Block alone, as a control. Appropriate secondary antibodies (Nichirei) were then added, and slides were incubated at room temperature for 30 min. Mouse stain kit (Nichirei) was used when primary mouse antibodies were applied to stain mouse tissues. Color was developed using the DAB Substrate Kit (Thermo Scientific) or Zymed AEC Substrate Kit (Zymed Laboratories), followed by counterstaining with Mayer's Hematoxylin. Controls without addition of primary antibody showed low or no background staining in all cases. Immunohistochemistry was scored based on staining intensity and the percentage of positive cells, as described in figure legends.

Immunofluorescence

For immunofluorescence analysis, DyLight 488 donkey anti-rat and DyLight 549 donkey anti-goat and anti-mouse (The Jackson Laboratory) antibodies were used as secondary antibodies. Nuclear staining was performed with 1,5-bis[[2-(di-methylamino)ethyl]amino]-4,8-dihydroxyanthracene-9,10-dione (DRAQ5) (Cell Signaling Technology). Images were captured using a Zeiss LSM 5 confocal microscope and analyzed with Zen software (Carl Zeiss).

Analysis of Gene Expression by Quantitative Real-Time PCR

Total RNA was isolated from snap-frozen tissue samples using TRIzol (Invitrogen), according to the manufacturer's instructions. After oligo (dT)-primed reverse transcription of 500 ng total RNA, the resulting single stranded cDNA was amplified using quantitative real-time PCR (qRT-PCR) as described previously (32). Actin primers were used as an

internal standard. The following primers were used: mouse β -actin, sense: 5'-ATGCTCCCCGGGCTGTAT-3', antisense: 5'-CATAGGAGTCCTTCTGACCCATTC-3'; mouse TNF- α , sense: 5'-CATCTTCTCAAATTCGAGTGACAA-3', antisense: 5'-TGGGAGTAGACAAGGTACAACCC-3'; mouse heparanase, sense: 5'-ACTTGAAGGTACCGCCTCCG-3', antisense: 5'-GAAGCTCTGGAAGTCCGGCAA-3'.

TNF- α ELISA

TNF- α levels were assayed with Mouse TNF- α ELISA MAX Kit (BioLegend).

Immunoblotting

Kidney tissue samples or HK-2 whole-cell lysates were homogenized in lysis buffer containing 1% Triton X-100, 150 mmol/L NaCl, 20 mmol/L Tris-HCl, pH 7.5, supplemented with a mixture of protease inhibitors (Sigma). Equal protein aliquots were subjected to SDS-PAGE (10% acrylamide) under reducing conditions, and proteins were transferred to a polyvinylidene difluoride membrane (Millipore). Membranes were blocked with 1% skim milk for 1 h at room temperature and probed with the appropriate antibody, followed by horseradish peroxidase-conjugated secondary antibody (KPL) and a chemiluminescent substrate (Biological Industries). Band intensity was quantified by densitometry analysis using Scion Image software.

Heparanase Activity Assay

Measurements of heparanase enzymatic activity were carried out as described previously (32,38,39). Briefly, conditioned medium or cell lysates were incubated (16–36 h, 37°C, pH 6.2) on dishes coated with sulfate-labeled ECM. Sulfate-labeled material released into the incubation medium was analyzed by gel filtration on a Sepharose 6B column (38,39). Nearly intact HS proteoglycans are eluted just after the void volume (peak I, variation in elution position [K_{av}] < 0.2, fractions 1–10) and HS degradation fragments are eluted later with $0.5 < K_{av} < 0.8$ (peak II, fractions 15–35) (38,39). These fragments were shown to be degradation products of HS as they were fivefold to sixfold smaller than intact HS side chains, resistant to further digestion with papain and chondroitinase ABC, and susceptible to deamination by nitrous acid (39). Each experiment was performed at least three times, and the K_{av} values did not exceed $\pm 15\%$.

Statistical Analysis

Values are expressed as mean \pm SE. Statistical analysis was performed using a *t* test or Mann-Whitney test. A *P* value of <0.05 was considered to be significant.

RESULTS

Effects of Heparanase on Macrophage Activation in Diabetic Kidney

Macrophage activation and recruitment is a characteristic feature of diabetic kidney disease, associated with both experimental and human DN (20–23,27). We previously reported a fivefold increase (*P* = 0.001) in macrophage infiltration in the kidney cortex of diabetic WT mice after

16 weeks of STZ-induced diabetes, while no statistically significant change in macrophage infiltration was detected in the kidney of diabetic *Hpse-KO* mice (13). Importantly, diabetic *Hpse-KO* mice, unlike their WT littermates, did not develop DN in response to STZ-induced diabetes (13), consistent with the key role of macrophages in DN pathogenesis (19–23). Macrophages activated by DM components represent the important cellular source of TNF- α (a key cytokine implicated in renal injury) (30) in mouse and human diabetic kidneys (18). In agreement with this notion, a ~15-fold induction of TNF- α expression was detected by qRT-PCR in the renal tissue of diabetic versus nondiabetic WT mice (Fig. 1A). This increase in TNF- α mRNA levels was invariably associated with elevated heparanase expression in the kidney tissue of WT diabetic mice (Fig. 1B). However, under the same diabetic conditions, no increase in TNF- α mRNA levels was detected in the kidneys of diabetic versus nondiabetic *Hpse-KO* mice (Fig. 1A). Additionally, immunofluorescent analysis revealed a significant increase in TNF- α -overexpressing cells both in the cortical tubulointerstitium and glomeruli of WT versus *Hpse-KO* diabetic mice (Fig. 1C and D). Of note, the pattern of TNF- α staining strongly resembled the pattern of macrophage infiltration previously reported in STZ-induced diabetes (27), further suggesting that heparanase may affect macrophage activation and TNF- α production in the diabetic kidney.

To test *in vitro* whether heparanase is capable of modulating macrophage activation in the setting of DN, we isolated primary mouse macrophages (as described by Lerner et al. [32]) and compared production of TNF- α in the presence of recombinant active heparanase alone or in combination with several key components of DM that are commonly present in the diabetic kidney and are known to affect macrophage responses (i.e., high glucose levels, AGE, albumin, IFN- γ) (25–27,40) to recapitulate conditions that are typical for DN. As shown in Fig. 2A, heparanase strongly augmented macrophage activation by various DM components, as evidenced by a marked increase in TNF- α secretion in DM-stimulated macrophages in the presence of the enzyme. Of note, heparanase alone exerted only a modest stimulatory effect on macrophages, which was dependent on heparanase enzymatic activity, since neither heat-inactivated heparanase (iHpa) (Fig. 2A) or proteinase K-digested (not shown) heparanase had any effect on macrophage activation.

Enhanced NF- κ B signaling is the hallmark of macrophage activation and chronic inflammation (41), which is strongly implicated in DN pathogenesis (31,42,43). We therefore compared activation of the NF- κ B pathway in resting macrophages and macrophages stimulated by DM components in the absence or presence of enzymatically active heparanase, applying immunofluorescent staining with anti-phospho-p65 antibody. NF- κ B is normally sequestered in the cytoplasm by means of its association with an inhibitory protein (I κ B α). Activation of NF- κ B involves stimulation of the inhibitor of nuclear factor

κ B kinase (IKK) complex, which triggers I κ B α degradation and translocation of the active (phosphorylated) NF- κ B subunit p65 into the nucleus. As shown in Fig. 2B and C, heparanase markedly enhanced NF- κ B signaling in DM-stimulated macrophages, as indicated by significantly increased nuclear localization of phospho-p65 (Fig. 2C).

Next, to confirm *in vivo* the role of heparanase in the activation of macrophages residing in the diabetic kidney, we applied double-immunofluorescent staining with antibodies directed against F4/80 (a mouse macrophage specific marker) (44) and TNF- α in the kidney tissue samples derived from healthy and diabetic (STZ-treated) WT and *Hpse-KO* mice. As shown in Fig. 3A and B, macrophages in specimens harvested after 16 weeks of STZ-induced diabetes from the kidneys of WT mice (compared with *Hpse-KO* mice) exhibited exacerbated inflammatory phenotype, evidenced by significantly increased numbers of F4/80⁺/TNF- α ⁺ double-positive cells, both in tubulointerstitial (Fig. 3B, top) and glomerular (Fig. 3B, bottom) compartments. With respect to observations in the glomerular compartment, it should be noted, however, that under pathological conditions some podocytes are known to undergo macrophage-like phenotypic changes (i.e., expressing macrophage-associated markers and cytokines) (23,45,46). Although we validated *in vitro* that the anti-F4/80 antibody does not stain podocytes cultured *in vitro* (Supplementary Fig. 1), it still cannot be ruled out that some of the F4/80⁺ cells found in glomeruli (Fig. 3A) may be of podocyte origin. In agreement with these *in vivo* findings, primary macrophages isolated from WT mice expressed significantly higher levels of TNF- α in response to stimulation with DM components, compared with *Hpse-KO*-derived primary macrophages (Fig. 3C).

Tubular Cells Supply CatL Responsible for Posttranslational Activation of Heparanase

The heparanase gene encodes a latent 65-kDa proenzyme, whose activation involves proteolytic cleavage, brought about predominantly by CatL, yielding an enzymatically active heterodimer composed of 8- and 50-kDa subunits (47,48) (Supplementary Fig. 2). Thus, in addition to transcriptional regulation of the heparanase gene in diabetic kidneys (i.e., activation of *Hpse* promoter by glucose) (11,13), posttranslational processing may represent a key regulatory mechanism (47,48), which is highly relevant to the role of the enzyme in macrophage activation in the DN setting.

Indeed, along with elevated expression of heparanase in the diabetic kidney (Supplementary Fig. 3A), as shown in previous reports (9,11,13), immunoblot analysis revealed that the majority of heparanase protein in diabetic kidneys was present as a proteolytically activated 50-kDa form (Supplementary Fig. 3B), which is at least 100-fold more active than the 65-kDa proenzyme (38). The ratio of activated 50-kDa heparanase to latent 65-kDa proenzyme (calculated by densitometric analysis of immunoblot) was 1.8-fold higher in the diabetic kidney than in the nondiabetic

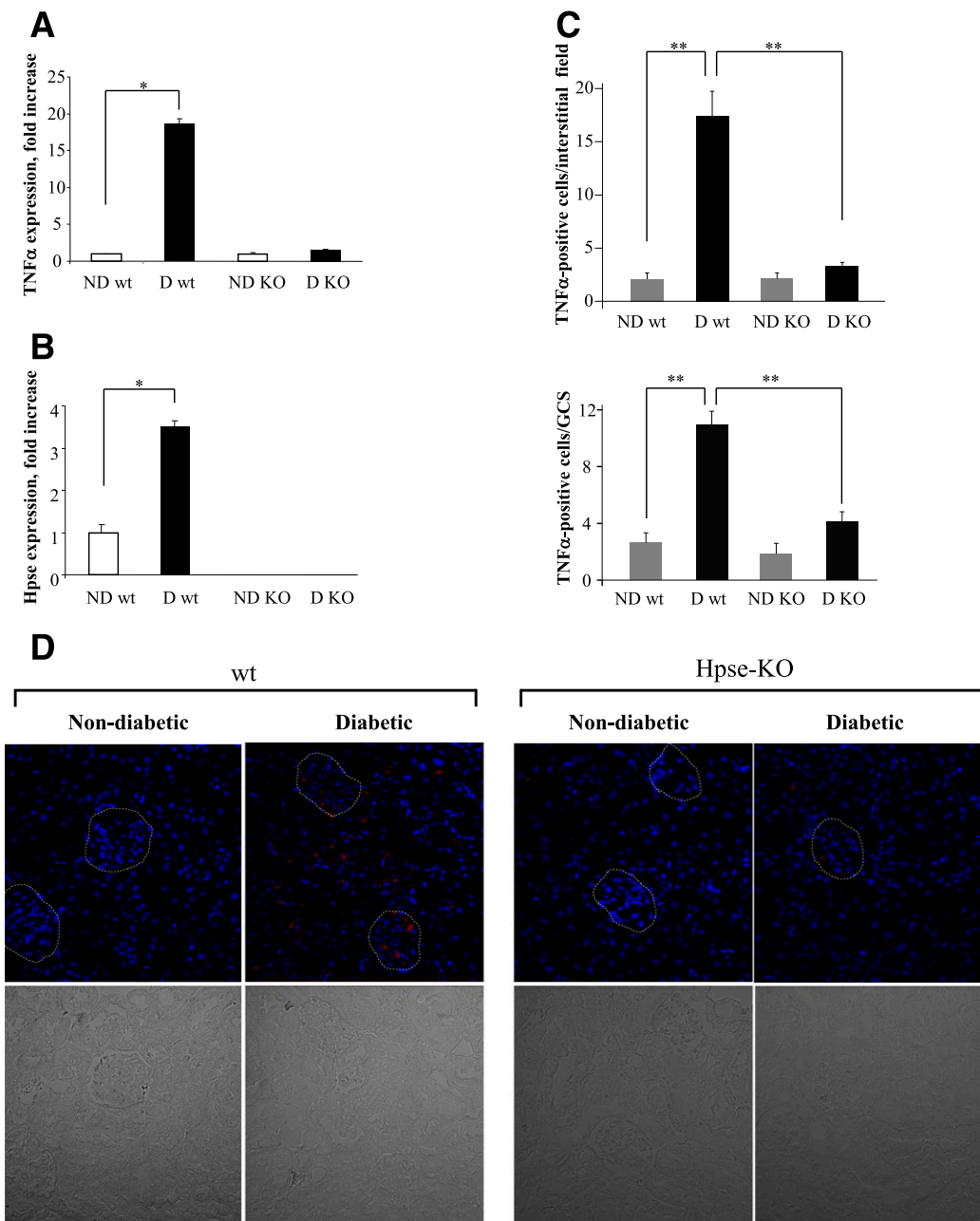


Figure 1—Induction of TNF- α in the kidneys of diabetic mice correlates with the overexpression of heparanase. Kidney tissue was harvested from nondiabetic and diabetic WT and *Hpse*-KO mice 10 weeks after the induction of diabetes by STZ, lysed, and analyzed for TNF- α (A) and heparanase (Hpse) (B) expression by qRT-PCR. At least three mice per condition were analyzed. Error bars represent \pm SE. C and D: The distribution and number of TNF- α ⁺ cells in the tubulointerstitial and glomerular compartments of nondiabetic and diabetic mice were determined using immunofluorescent staining with anti-TNF- α (red) antibody. C, top: TNF- α ⁺ cells in the cortical interstitial compartment were quantified per microscopic field (0.07 mm²), based on six sections from four independent mice in each group, under $\times 400$ magnification. C, bottom: The number of TNF- α ⁺ cells per glomerular cross-section (GCS) was counted in ≥ 40 glomeruli per animal. Data are the mean \pm SE, $n = 4$ mice per condition. * $P < 0.01$, ** $P < 0.001$. D: Representative immunofluorescent (top) and light microscopy (bottom) images are shown, $\times 400$ magnification. Glomeruli are delineated by dashed lines. D, diabetic; KO, knockout; ND, nondiabetic.

kidney ($P = 0.04$, not shown). In accordance with this, increased levels of CatL protein were detected in protein extracts from both type 1 and type 2 diabetic mouse kidney tissues, compared with nondiabetic kidney tissues (Fig. 4A and B). Densitometric quantification of immunoblot data revealed a 2.3-fold increase in CatL levels in type 1 diabetic

kidney and a threefold increase in type 2 diabetic kidney ($P < 0.002$; data not shown). CatL immunostaining identified the tubular compartment as a primary source of CatL in both type 1 and type 2 diabetic mouse kidney, and quantification studies confirmed a statistically significant increase in staining intensity/percentage of CatL⁺ cells (Fig.

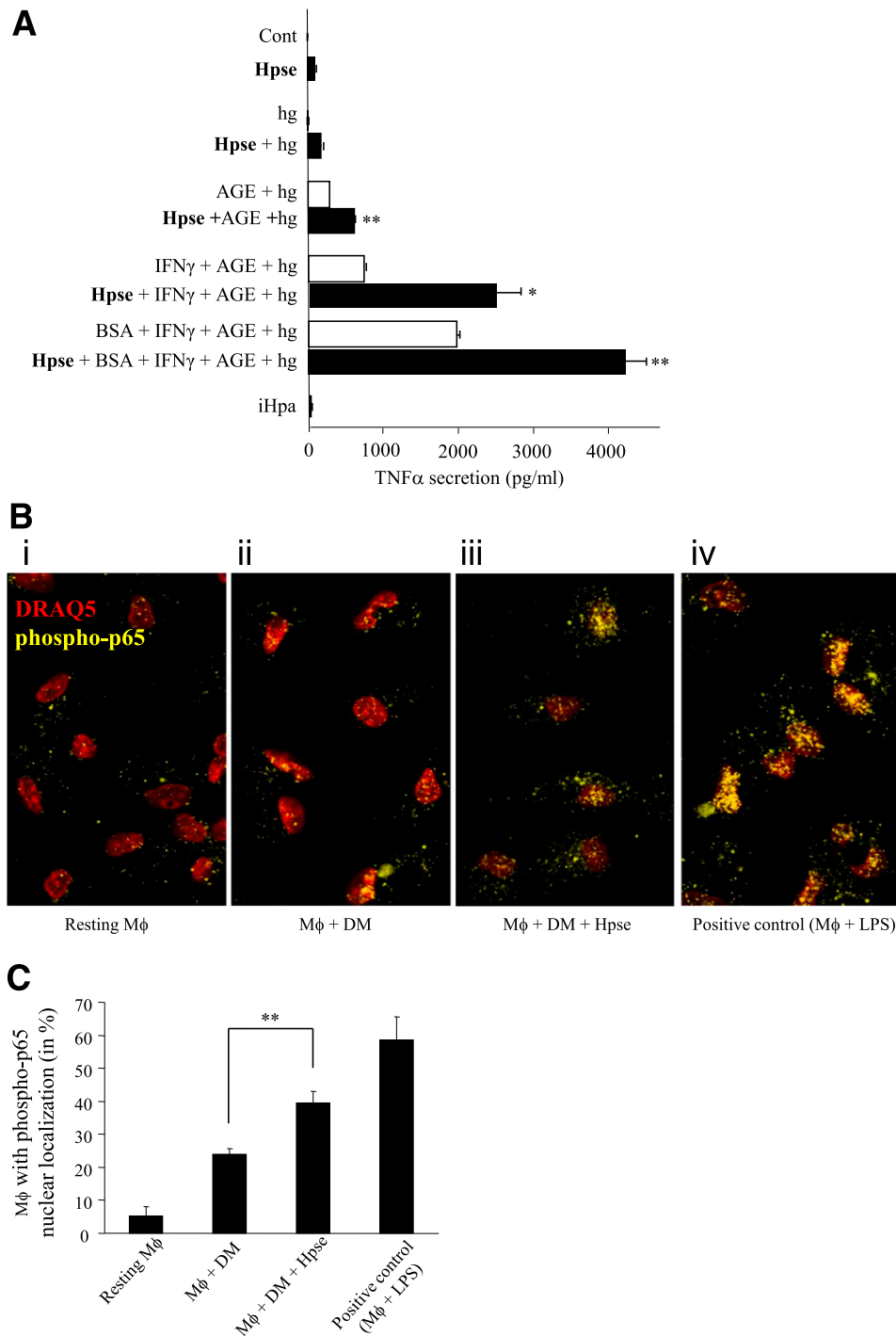


Figure 2—Heparanase sensitizes macrophages to activation by DM components in vitro. *A*: Mouse peritoneal macrophages were plated in medium containing low glucose (1.0 g/L) and either remained untreated (Cont) or incubated (2 h, 37°C) with the following: active recombinant heparanase (Hpse; 0.8 μg/mL); high glucose (hg; 4.5 g/L); Hpse + hg; AGE (60 μg/mL) + hg; Hpse + AGE + hg; IFN-γ (100 units) + AGE + hg; Hpse + IFN-γ + AGE + hg; albumin (BSA, 30 μg/mL) + IFN-γ + AGE + hg; Hpse + BSA + IFN-γ + AGE + hg; and iHpa. TNF-α secretion was evaluated by ELISA of the conditioned medium. Note that active heparanase enzyme, purified as described by Blich et al. (52), markedly enhanced the production of TNF-α by macrophages activated by various combinations of DM components. *B*: Induction of NF-κB signaling in macrophages activated by the combination of kidney DM components (glucose 4.5 g/L; AGE 60 μg/mL; albumin 30 μg/mL) in the absence and the presence of heparanase (Hpse; 0.8 μg/mL) was assessed by immunofluorescent analysis using anti-phospho-p65 (yellow) antibodies. Macrophage nuclei were counterstained with DRAQ5 (red). *Bi*: Resting macrophages (Mφ). *Bii*: Mφ activated by DM. *Biii*: Mφ activated by DM in the presence of Hpse. *Biv*: Mφ activated by LPS (100 ng/mL) served as a positive control. *C*: Quantification of the effect of heparanase on the nuclear localization of phospho-p65 in macrophages activated by DM. The nuclear accumulation of p65 in ≥100 cells/condition was analyzed. The percentage of cells with nuclear p65 is indicated. Error bars represent SE. **P* < 0.04, ***P* < 0.02.

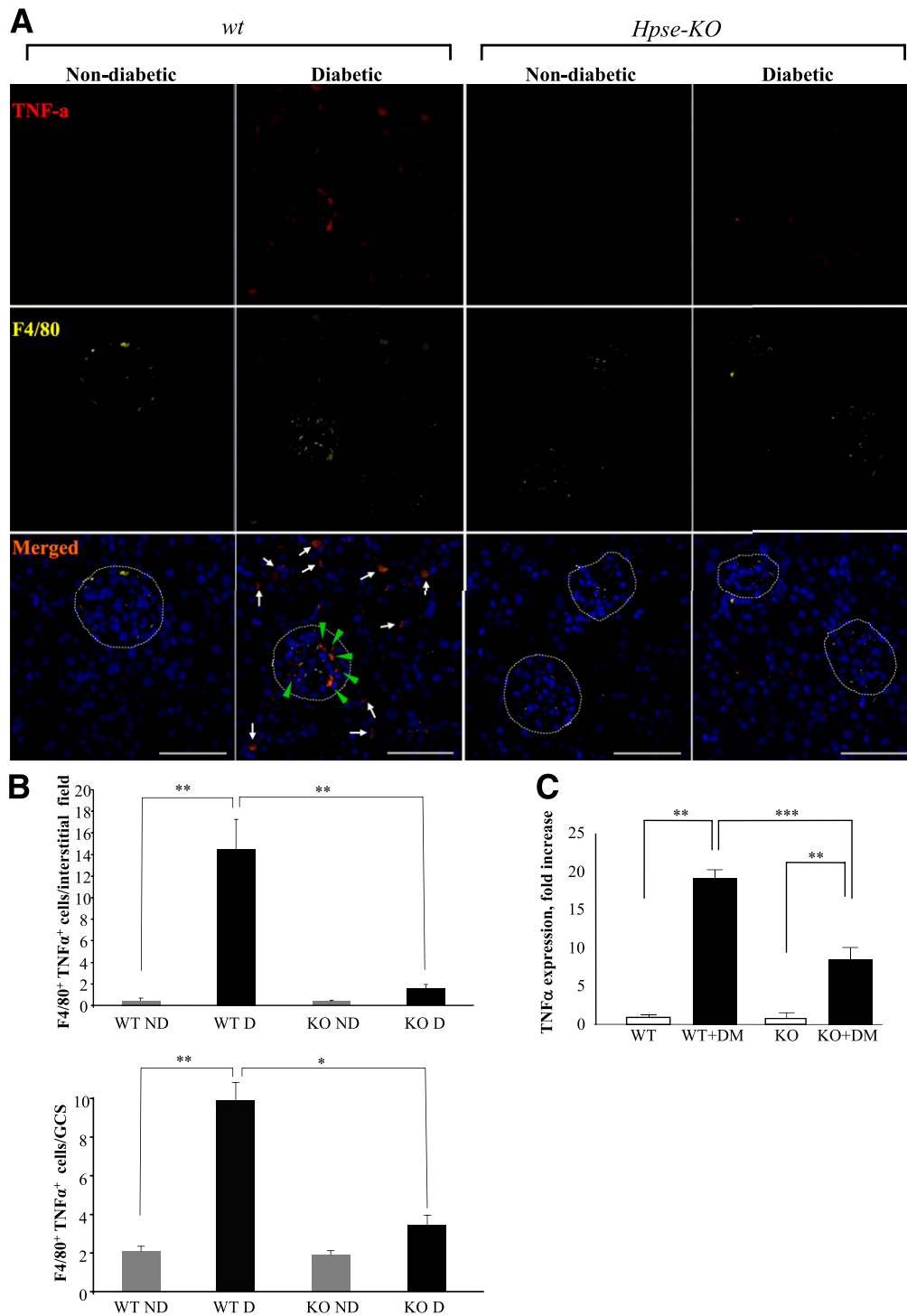


Figure 3—A and B: Increased number of TNF- α -expressing macrophages in diabetic kidneys of WT mice compared with *Hpse-KO* mice. **A:** Diabetes was induced by STZ, as described in RESEARCH DESIGN AND METHODS. On experimental week 16, nondiabetic (ND) and diabetic (D) WT and *Hpse-KO* (KO) mice were killed, and their kidney tissue was processed for double-immunofluorescent analysis with anti-F4/80 (yellow) and anti-TNF- α (red) antibodies. Cell nuclei were counterstained with DRAQ5 (blue). Representative images are shown. F4/80⁺TNF- α ⁺ double-positive cells in the tubulointerstitial (white arrows) and glomerular (green arrowheads) compartments of diabetic kidneys in WT mice are indicated. Glomeruli are delineated by dashed lines. Scale bars, 50 μ m. **B, top:** F4/80⁺TNF- α ⁺ double-immunostained cells in the tubulointerstitial compartment were counted per microscopic field (0.07 mm²), based on six sections from four independent mice of each group, under \times 400 magnification. **B, bottom:** The number of double-immunostained glomerular cells per glomerular cross-section (GCS) was counted in \geq 40 glomeruli per animal. Data are the mean \pm SE, $n = 4$ mice per condition. * $P < 0.01$, ** $P < 0.001$. **C:** Macrophages from WT mice express higher levels of TNF- α compared with *Hpse-KO* mice. Peritoneal macrophages derived from WT and *Hpse-KO* mice were either untreated or incubated (2 h, 37°C) with DM components (120 μ g/mL AGE and BSA, 100 units IFN- γ). TNF- α expression was evaluated by qRT-PCR. ** $P < 0.001$, *** $P < 0.008$.

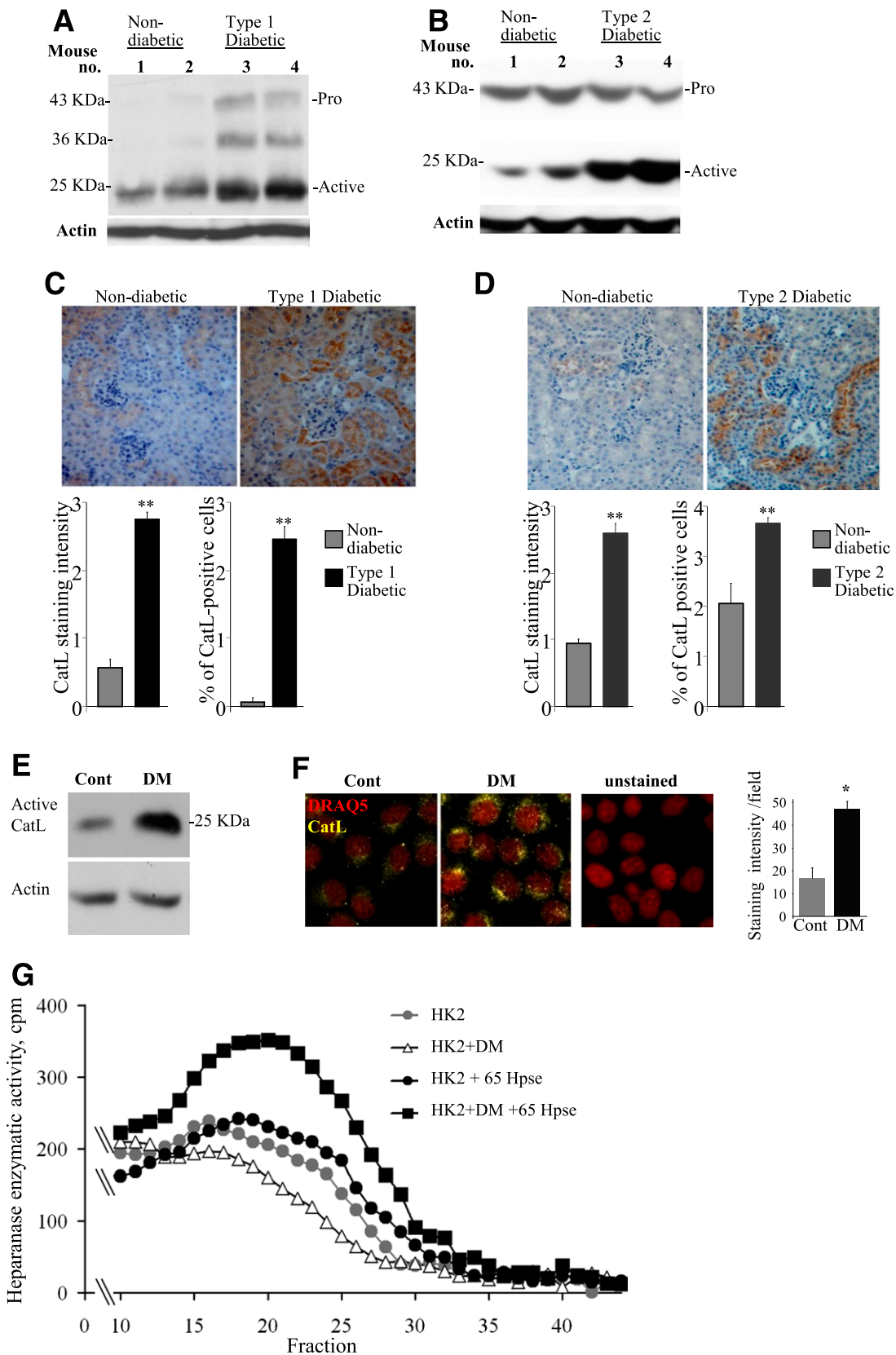


Figure 4—Induction of CatL under diabetic conditions in vivo and in vitro. *A–D*: CatL protein levels in kidneys from nondiabetic and diabetic mice. Kidneys were harvested from STZ-treated type 1 diabetic mice (*A* and *C*) and db/db type 2 diabetic mice (*B* and *D*) and were analyzed for CatL protein levels by immunoblotting showing 43-kDa procathepsin L (Pro) and 25-kDa (active) forms of the enzyme (*A* and *B*) and immunostaining (*C* and *D*). *C* and *D*, top: Note the CatL staining (brown) in the tubular compartment of diabetic kidney (original magnification $\times 200$). *C* and *D*, bottom: Specimens were scored according to CatL immunoreactive intensity (0–3) and distribution (0–4). The intensity

4C and D). Moreover, the induction of CatL in the tubular compartment under diabetic conditions (i.e., by DM components) was further confirmed in vitro by incubation of proximal tubular cell line HK-2 with high glucose levels, AGE, BSA, and IFN- γ , followed by immunoblot/immunofluorescent staining (Fig. 4E and F). A statistically significant increase in CatL levels in HK-2 cells after incubation with DM components was revealed by both densitometric analysis of immunoblot ($P < 0.025$; data not shown) and quantification of immunofluorescent data (Fig. 4F, right panel).

To further confirm that tubular cells are capable of proteolytic processing and activation of proheparanase, we incubated purified 65-kDa heparanase precursor with untreated or DM-treated HK-2 cells and examined the resulting heparanase enzymatic activity. As shown in Fig. 4G, a marked increase in heparanase activity became evident upon incubation of the 65-kDa precursor with DM-stimulated HK-2 cells, while stimulation of HK-2 cells with DM in the absence of the 65-kDa precursor or incubation of the 65-kDa precursor with unstimulated HK-2 cells resulted in little or no increase in heparanase activity.

Collectively, these findings indicate that, in addition to the ability to upregulate heparanase gene expression in renal tissue (11–13), DM decisively contributes to proteolytic activation of latent heparanase proenzyme via induction of CatL in the tubular compartment of diabetic kidneys. It should be noted that different cellular origins of heparanase and CatL imply that proteolytic activation of heparanase by CatL can occur extracellularly, which is in agreement with the previously reported secreted nature of both proteins in kidney (10,49).

DISCUSSION

With the global epidemic of diabetes, DN has become the leading cause of end-stage renal failure in the Western world (50,51). Thus, identifying new cellular/molecular pathways responsible for renal injury in diabetes is highly important. While both causal involvement of heparanase in DN and the molecular mechanism underlying heparanase induction by hyperglycemic conditions/diabetic mediators were previously described by the present authors and others (11–13), the exact mode of heparanase action in DN pathogenesis has not been fully

elucidated. Apart from heparanase-mediated loss of HS in the GBM, it was suggested that heparanase contributes to the development of DN by inducing changes in glomerular cell-GBM interactions due to the loss of HS; through the release of HS-bound growth factors, cytokines, and bioactive HS fragments in glomeruli; or by activating signaling cascades that alter cell properties and lead to proteinuria (16,17).

Here we emphasize a novel function of heparanase in coupling macrophage activation, chronic inflammation, and renal injury under diabetic conditions. While DN was not traditionally considered an inflammatory condition, recent studies clearly indicate the importance of chronic inflammation in diabetic renal injury, highlighting the role of kidney-infiltrating macrophages (18,19,23,24). We demonstrated that heparanase sensitizes macrophages to stimulation by DM components in vitro, as manifested by a significant increase in the production of TNF- α , a key inflammatory mediator in the pathogenesis of DN (18,19,23,24,30). Importantly, elevated levels of TNF- α were detected in renal tissue of diabetic WT mice (which developed albuminuria and renal damage after 16 weeks of STZ-induced diabetes) and correlated with increased expression of heparanase (Fig. 1). In contrast, no increase in TNF- α was detected in renal tissue of *Hpse-KO* mice, which failed to develop DN in response to STZ-induced diabetes. In agreement, increased numbers of TNF- α -producing macrophages were found in diabetic kidneys of WT mice, but not *Hpse-KO* mice (Fig. 3A and B). Consistent with in vivo findings, TNF- α expression in response to in vitro stimulation by DM components was significantly higher in WT macrophages than in *Hpse-KO*-derived macrophages (Fig. 3C).

The emerging role of heparanase in modulating macrophage activation is further supported by recent findings in additional inflammatory models other than DN, including inflammatory bowel disease (32), atherosclerotic plaque progression toward vulnerability (52), and psoriasis (33). While the exact mechanism underlying heparanase-dependent activation of macrophages is not fully understood, the stimulation of pattern recognition receptors, such as TLRs, is among the candidate pathways. TLRs, best known for their role in host defense from infection because of their ability to recognize highly conserved microbial structures, can also undergo activation

was scored as follows: 0, no staining; 1, weak staining; 2, medium staining; 3, strong staining. The percentage of positive cells was scored as "0" (<5%), "1" (5–25%), "2" (25–50%), "3" (50–75%), and "4" (>75%). The data shown are the mean \pm SE of staining scores, $n = 4$ mice per condition. ** $P < 0.001$. E and F: Induction of CatL in proximal tubuli cell line HK-2 by DM components. HK-2 cells were either untreated (Cont) or incubated (24 h, 37°C) with DM components (4.5 g/L glucose, 120 μ g/mL AGE and BSA, 100 units IFN- γ) and processed for Western blot analysis (E) and immunofluorescent staining (F). F, right panel: Staining intensity was quantified using Zen software (Carl Zeiss) per microscopic field (0.012 mm²), based on 40 fields per condition. Data shown are the mean intensity \pm SE. * $P = 0.006$. G: Activation of latent heparanase by DM-stimulated HK-2 cells. Heparanase enzymatic activity was examined using sulfate-labeled ECM as the substrate (32,38,39) in lysates of HK-2 cells, either untreated (HK2) or stimulated by DM components (HK2 + DM), as described above, in the absence or presence of 65-kDa heparanase precursor (65 Hpse) purified as shown previously (32). Note the increased enzymatic activity upon incubation of 65 Hpse with DM-stimulated HK-2 cells (HK2 + DM + 65 Hpse).

by endogenous molecules in noninfectious inflammatory conditions, including DM components (e.g., AGE) or ECM-derived products (53–55). HS, the enzymatic substrate of heparanase, can modulate TLR4 signaling as well (56,57); although intact extracellular HS inhibits TLR4 signaling and macrophage activation, its removal relieves this inhibition (57). In fact, the functional importance of heparanase enzymes in macrophage activation by a specific TLR4 ligand, lipopolysaccharide (LPS), was recently demonstrated in the setting of chronic colitis (32). Heparanase activity reduces the amount of HS on the macrophage cell surface (32) and facilitates ligand binding to TLRs (E.H., unpublished data), suggesting that enzymatic degradation of cell surface HS may increase accessibility of the TLRs to their ligands. This assumption is also consistent with the recently reported ability of heparanase to increase innate immunocyte binding to the adhesion molecules presented on blood vessel walls (58).

Moreover, soluble HS, which is released from the cell surface upon heparanase activation, was found to stimulate TLR4 (55–57). Thus, in the diabetic kidney increased levels of heparanase, acting synergistically with DM-related TLR4 ligands, and/or generating by itself soluble HS fragments capable of stimulating TLR4, may sustain macrophage inflammatory responses and renal damage.

Although other heparanase-related factors (i.e., HS degradation in the GBM, altered interactions between glomerular cells and GBM, or release of HS-bound bioactive molecules) (16,17) may also contribute to DN development, our recent observations unravel what we believe to be a previously unrecognized combinatorial circuit of heparanase-dependent molecular events powering chronic inflammation and kidney injury in individuals with DN (Fig. 5). These events appear to be driven by heterotypic interactions among various cell types and DM components present in the renal tissue in the diabetic state: increased glucose levels induce heparanase gene expression by glomerular cells (11) via an early growth response 1–dependent mechanism (13) (Fig. 5A and B). Of note, heparanase expression is not limited solely to glomeruli, and was reported in the tubulointerstitial compartment as well (for review, see Szymczak et al. [16]). The resulting latent 65-kDa proheparanase is then processed into its enzymatically active (8- plus 50-kDa) form by CatL, which is supplied by tubular cells activated by DM components (Fig. 5C). Active heparanase, in turn, governs macrophage activation by DM (Fig. 5D), leading to increased levels of TNF- α (Fig. 5E), thus creating chronic inflammatory conditions in the diabetic kidney and fostering macrophage-mediated renal injury (Fig. 5F).

The emerging link between heparanase action and kidney-damaging inflammation in DN implies that the

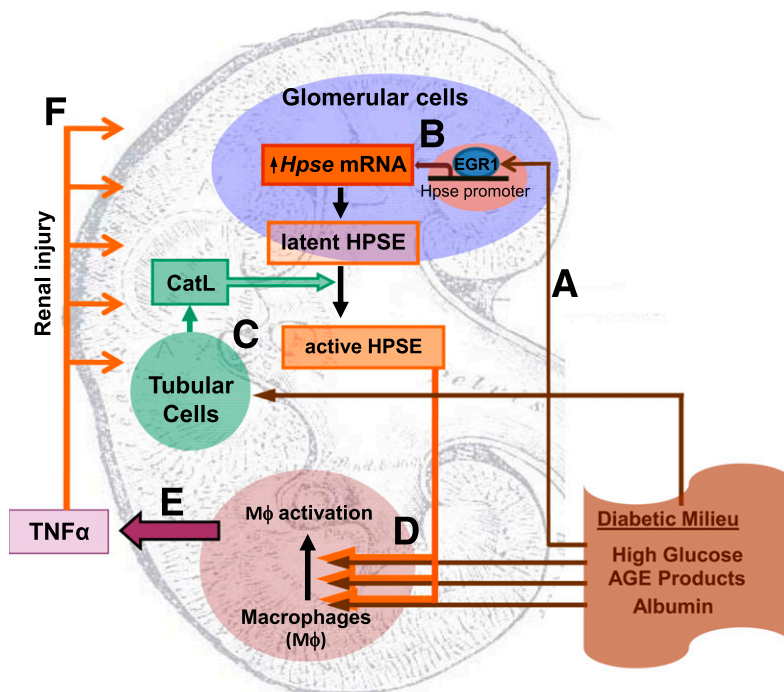


Figure 5—A model of a heparanase-driven sequence of events powering chronic inflammation and kidney injury in DN. Increased glucose levels induce heparanase gene expression in diabetic kidney (A) (acting via an early growth response 1 [EGR1]-dependent mechanism, among other pathways; B). C: The overexpressed 65-kDa heparanase proenzyme (latent HPSE) is processed into its enzymatically active (8- + 50-kDa) form (active HPSE) by CatL, which is supplied by tubular cells activated by DM components. D: Active heparanase sustains macrophages stimulation by DM, resulting in increased production of kidney-damaging cytokines (i.e., TNF- α ; E), thus fostering DN development and progression (F).

enzyme inhibitors (currently being clinically tested in malignant diseases) (59) may be highly beneficial in DN. One such inhibitor, SST0001 (modified glycol split heparin), has had beneficial effects in mouse models of DN, although albuminuria and renal damage were not completely halted by this compound (13). Investigating the reasons for the somewhat limited effects of SST0001 in experimental DN, we assumed that this HS analog, which is similar to soluble HS (57), may facilitate TLR-dependent activation of macrophages. Indeed, ELISA analysis of TNF- α levels secreted by macrophages stimulated with LPS in the presence of SST0001 resulted in a threefold increase in TNF- α secretion compared with LPS alone (data not shown), leading to the conclusion that independently of its heparanase-inhibiting action, SST0001 mimics soluble HS and hence stimulates macrophage activation. This assumption may also explain the failure of a recent clinical trial using sulodexide, a drug composed of a mixture of glycosaminoglycans including small-molecule heparin, in preventing proteinuria (60).

Thus, a systematic search for heparanase-inhibiting compounds lacking macrophage-activating properties and their analysis in the setting of DN are important procedures in the development of effective therapeutic strategies aiming to disrupt heparanase-driven heterotypic interactions among glomerular, tubular, and immune cells in the diabetic kidney.

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