

The Vascular Ectonucleotidase ENTPD1 Is a Novel Renoprotective Factor in Diabetic Nephropathy

David J. Friedman,¹ Helmut G. Rennke,² Eva Csizmadia,³ Keiichi Enjyoji,⁴ and Simon C. Robson^{3,4}

Ectonucleoside triphosphate diphosphohydrolase 1 (ENTPD1) (also known as CD39) is the dominant vascular ectonucleotidase. By hydrolyzing ATP and ADP to AMP, ENTPD1 regulates ligand availability to a large family of P2 (purinergic) receptors. Modulation of extracellular nucleotide metabolism is an important factor in several acute and subacute models of vascular injury. We hypothesized that aberrant nucleotide signaling would promote chronic glomerular injury in diabetic nephropathy. Inducing diabetes in ENTPD1-null mice with streptozotocin resulted in increased proteinuria and more severe glomerular sclerosis compared with matched diabetic wild-type mice. Diabetic ENTPD1-null mice also had more glomerular fibrin deposition and glomerular plasminogen activator inhibitor-1 (PAI-1) staining than wild-type controls. In addition, ENTPD1-null mice showed increased glomerular inflammation, in association with higher levels of monocyte chemoattractant protein-1 (MCP-1) expression. Mesangial cell PAI-1 and MCP-1 mRNA expression were upregulated by ATP and UTP but not ADP or adenosine in vitro. The stable nucleotide analog ATP γ S stimulated sustained expression of PAI-1 and MCP-1 in vitro, whereas the stable adenosine analog NECA [5'-(N-ethylcarboxamido)adenosine] downregulated expression of both genes. Extracellular nucleotide-stimulated upregulation of MCP-1 is, at least in part, protein kinase C dependent. We conclude that ENTPD1 is a vascular protective factor in diabetic nephropathy that modulates glomerular inflammation and thromboregulation. *Diabetes* 56:2371–2379, 2007

From the ¹Renal Division, Department of Medicine, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, Massachusetts; the ²Department of Pathology, Brigham and Women's Hospital, Harvard Medical School, Boston, Massachusetts; the ³Transplantation Division, Department of Medicine, Beth Israel Deaconess Medical Center, Boston, Massachusetts; and the ⁴Gastroenterology Division, Department of Medicine, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, Massachusetts.

Address correspondence and reprint requests to David J. Friedman, Department of Medicine, Beth Israel Deaconess Medical Center, Harvard University, Research North 359, 99 Brookline Ave., Boston, MA 02215. E-mail: dfriedma@caregroup.harvard.edu. Or to Simon C. Robson, Department of Medicine, Beth Israel Deaconess Medical Center, Harvard University, Research North 301, 99 Brookline Ave., Boston, MA 02215. E-mail: srobson@bidmc.caregroup.edu.

Received for publication 14 November 2006 and accepted in revised form 24 April 2007.

Published ahead of print at <http://diabetes.diabetesjournals.org> on 1 May 2007. DOI: 10.2337/db06-1593.

BIM, bisindolylmaleimide I; ENPP1/PC-1, ectonucleotide pyrophosphatase/phosphodiesterase-1; ENTPD1, ectonucleoside triphosphate diphosphohydrolase 1; HPLC, high-performance liquid chromatography; MCP-1, monocyte chemoattractant protein-1; NECA, 5'-(N-ethylcarboxamido)adenosine; PAI-1, plasminogen activator inhibitor-1; PAS, periodic acid Schiff; STZ, streptozotocin.

© 2007 by the American Diabetes Association.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Diabetic nephropathy is the leading cause of renal failure in the U.S. (1). Currently, >160,000 Americans suffer from end-stage renal disease due to diabetes, with the 5-year mortality in these patients approaching 70% (1,2). Why only one in three patients with diabetes develop nephropathy is unknown. Hypertension and glycemic control are important risk factors, but genetic predisposition may be the most powerful predictor of end-stage renal disease from diabetes (3–5). Mouse models allow candidate susceptibility genes to be independently evaluated while preserving the complex architecture of the mammalian glomerulus.

Ectonucleoside triphosphate diphosphohydrolase 1 (ENTPD1) (also known as CD39) is a vascular ectoenzyme expressed by endothelial cells and smooth muscle (6,7). This nucleotidase metabolizes extracellular ATP and ADP to AMP (as well as UTP and UDP to UMP), which is subsequently converted to adenosine by the ectoenzyme CD73 (8). By controlling extracellular nucleotide levels, ENTPD1 regulates the activation of type 2 purinergic receptors (P2X and P2Y; ligand-gated and G-protein-coupled receptors, respectively) involved in many cellular processes, including thrombosis, inflammation, angiogenesis, cellular proliferation, and apoptosis (9–12). ENTPD1 is highly expressed in the kidney, including the larger vessels, afferent arterioles, and glomeruli (13). We have previously shown that ENTPD1 is a protective factor in several acute and subacute vascular injury models such as renal ischemia reperfusion (14,15) and transplant rejection (16,17).

In this study, we hypothesized that aberrant nucleotide signaling in the absence of ENTPD1 might promote chronic microvascular injury in diabetic nephropathy. Our hypothesis was bolstered by the previous finding that ectonucleotide pyrophosphatase/phosphodiesterase-1 (ENPP1/PC-1), another ectonucleotidase expressed in the kidney phosphodiesterase-1 (ENPP1/PC-1) that metabolizes ATP to AMP plus pyrophosphate, has been associated with diabetic nephropathy in human genetic association studies (18,19). ENTPD1 similarly hydrolyzes the proinflammatory molecule ATP and initiates an ectoenzymatic cascade that leads to generation of adenosine, which has predominantly anti-inflammatory properties. Interestingly, adenosine A2A agonists such as ATL 146e appear protective in diabetic nephropathy models in mice (20).

Numerous pathways have been associated with development of diabetic nephropathy in rodents and humans. Among the most intriguing are the interrelated pathways of inflammation and thromboregulation. Absence of ENTPD1 in null mice results in increased tissue factor

expression in the vasculature and increased fibrin deposition in multiple organs including the kidney (21). ENTPD1-null mice also are more prone to inflammation in the basal state and injury models (22). We suspected that any tipping of the purinergic balance toward inflammation and fibrin deposition would result in heightened sensitivity to renal injury in the context of hyperglycemia from superimposed diabetes. We tested this hypothesis using the streptozotocin (STZ)-induced model of type 1 diabetes in wild-type mice and mice null for ENTPD1.

RESEARCH DESIGN AND METHODS

STZ-induced diabetes. ENTPD1-null mice were originally generated on a mixed C57BL6/129S background (21) and subsequently backcrossed more than six times onto a C57BL6 background. Eight-week-old ENTPD1-null male mice and age-, sex-, and weight-matched wild-type mice on the C57/BL6 background were used in these experiments. Mice were given two consecutive daily intraperitoneal injections of 75 mg/kg STZ in 50 mmol/l citrate buffer or citrate buffer alone (control groups) after a 3-h fast. Glucose was checked 3 weeks after STZ injection, and mice with glucose levels between 300 and 550 mg/dl were selected for the experiment. Glucose measurements were made with Gluco-elite. Urine was collected at 12 and 24 weeks after STZ injection. Blood pressure was measured by tail cuff manometry (BP-2000 Blood Pressure Analysis System; Visitech Systems) for 7 consecutive days; >10 measurements over a 20-min period were averaged per day. The first 5 days constituted the training period, and the reported blood pressure is the average of the final two sessions (days 6 and 7). Animals were killed at 24 weeks and blood and kidney tissue harvested. Animal studies were conducted in accordance with National Institutes of Health guidelines and approved by the institutional animal care and use committee at Beth Israel Deaconess Medical Center.

Measurement of albumin and creatinine. Serum creatinine was measured by high-performance liquid chromatography (HPLC) at the Mouse Metabolic Phenotyping Center at Vanderbilt University. Urinary albumin was measured by mouse-specific enzyme-linked immunosorbent assay (Albuwell M; Exocell). Urinary creatinine was measured by colorimetric reaction (Creatinine Companion; Exocell).

Histology. Mouse kidneys were fixed in 10% buffered formalin followed by embedding in paraffin. Three-micrometer sections were stained with periodic acid Schiff (PAS). Sections were read in a blinded manner by a nephrologist and renal pathologist. Six to 11 mice were scored per group. Fifty glomeruli were scored per mouse and averaged. A sclerosis index ranging from 0 to 4 was developed to assess diabetic glomerular injury, as follows: 0, normal glomeruli; 1, mild mesangial expansion/injury (<20% PAS positive); 2, moderate injury (20–40% PAS positive); 3, severe injury (40–60% PAS positive); and 4, global sclerosis (>60% PAS positive).

Immunohistochemistry. Tissue was harvested and frozen in liquid nitrogen/isopentane (for ENTPD1, fibrin, and CD31) or fixed for 36 h in a zinc-based fixative (for plasminogen activator inhibitor-1 [PAI-1]) (23). For frozen tissue, 5- μ m sections were fixed in 4°C acetone for 10 min for light microscopy or fixed in 2% paraformaldehyde for 15 min for immunofluorescence. Zinc-fixed tissue for light microscopy was postfixed in 2% paraformaldehyde for 15 min. PAI-1 antibody (sheep anti-mouse, 1:750) was purchased from American Diagnostica. Fibrin antibody (rabbit anti-mouse, 1:15,000) was purchased from Dako and required citrate buffer or Triton pretreatment. ENTPD1 antibody (C9F, 1:1,000) is a rabbit anti-mouse polyclonal antibody generated by cDNA immunization in our laboratory. CD31 antibody (rat anti-mouse, 1 mg/ml) was purchased from BD Pharmingen. Biotinylated secondary antibodies for light microscopy (goat anti-rabbit, 2 mg/ml; rabbit anti-sheep, 2 mg/ml) were purchased from Vector. For light microscopy, after initial blocking with serum, tissue was incubated overnight in primary antibody at 4°C. Endogenous peroxidases were blocked with 1% H₂O₂. Sections were incubated in secondary antibody for 1 h at room temperature and then incubated in Avidin-Biotin Complex (Dako) for 30 min. Staining was performed with diaminobenzidine using a kit from Vector. For immunofluorescence microscopy, sections were incubated overnight at 4°C with primary antibody and for 1 h at room temperature for secondary antibody. Fluorescent-labeled secondary antibodies were purchased from Molecular Probes: Red CY5 (donkey anti-rat, 1:300) and Green FITC (donkey anti-rabbit, 1:300). Images were captured on a Zeiss ApoTome system. Nuclei were stained blue with Hoechst 33258.

PAI-1 positivity was scored for 30 consecutive glomeruli per animal from single tissue sections using an arbitrary 0 to 4 scale where 0 equaled no PAI-1 in glomerulus and 4 indicated global, heavy PAI-1 staining in glomerulus. Four mice were examined (reader blinded to grouping) per group.

Real-time PCR. RNA was isolated from renal cortex or cultured mesangial cells using the RNeasy kit from Qiagen. RNA (0.5 to 1 μ g) was reverse transcribed to cDNA using the Taqman Reverse Transcription Kit from Applied Biosystems. PAI-1 (Mm00435860_m1) and monocyte chemoattractant protein-1 (MCP-1) (Mm00441242_m1) primer-probe sets and Taqman Universal PCR Mastermix were purchased from Applied Biosystems. cDNA was amplified using an ABI PRISM 7900HT Sequence Detection System. Gene expression levels were quantified as relative expression after normalization to the 18S ribosomal RNA subunit.

Cell culture. The mouse mesangial cell line SV40 MES 13 was purchased from ATCC. Cells were grown in 3:1 DMEM:F12 media supplemented with 5% fetal bovine serum, 14 mmol/l Hepes, and penicillin/streptomycin. Media was replaced 18 h before the experiment with media containing 0.2% serum and replaced again with new 0.2% fetal bovine serum-containing media 3–4 h before the experiment. ATP, ADP, UTP, ATP- γ S, bz-ATP, 5'-(*N*-ethylcarboxamido)adenosine (NECA), and adenosine were purchased from Sigma. The global protein kinase C (PKC) inhibitor bisindolylmaleimide I (BIM) was purchased from Calbiochem and added 15 min before ATP in the inhibitor studies. Cells were harvested 1 h after treatment and RNA extracted using the RNeasy kit per the manufacturer's instructions for animal cells. For each experiment, individual conditions were performed in triplicate, with real-time PCR for each replicate performed in duplicate or triplicate and averaged. Each experiment was performed at least three times.

Statistics. Comparisons between groups were performed using ANOVA and a posttest for multiple comparisons (Tukey, Dunnett, or linear trend posttest). Error bars indicate SE.

RESULTS

Immunohistologic patterns of ENTPD1 expression in the adult mouse kidney have been described previously (13). We confirmed high levels of ENTPD1 expression in the glomeruli of wild-type mice (Fig. 1A) and its absence in ENTPD1-null mice (Fig. 1B). Double staining with ENTPD1 and the endothelial-specific marker CD31 indicated that ENTPD1 is expressed by glomerular endothelial and mesangial cells (Fig. 1C–E).

ENTPD1-null and age-, sex-, and weight-matched C57BL6 wild-type mice were injected with STZ (two consecutive daily injections of 75 mg/kg to induce diabetes) or vehicle alone at 8 weeks of age. No acute kidney injury was seen 72 h after the second injection of STZ on light microscopy (not shown).

Table 1 shows the baseline, midpoint, and end-of-experiment characteristics of the four groups of mice. Wild-type and ENTPD1-null animals had similar weights and glucose levels at each time point in both the control and diabetic groups. Kidney weight-to-body weight ratios were similar between wild-type and ENTPD1-null animals in each group. Blood pressure in the diabetic ENTPD1-null mice trended higher than in diabetic wild-type mice ($P = 0.06$). Renal function (creatinine), as measured by HPLC, was similar between wild-type and ENTPD1-null mice, both with and without diabetes.

We next measured urine for excretion of albumin as a marker for glomerular permeability indicating the severity of diabetic renal injury. At both 12 (Fig. 2A) and 24 (Fig. 2B) weeks after STZ injection, diabetic ENTPD1-null mice had 2- to 2.5-fold greater levels of albuminuria (as measured by the albumin-to-creatinine ratio) than diabetic wild-type mice ($P < 0.05$), indicating more severe injury to the glomerular filtration barrier at the functional level. Wild-type diabetic mice had small increases in albuminuria compared with nondiabetic wild-type animals, which is typical of regimens that use these relatively low doses of STZ (24).

Renal tissue was then assessed histologically for diabetes-associated vascular injury, with representative glomeruli for each group shown in Fig. 3A–D. We used a sclerosis index on PAS-stained tissue to assess mesangial expansion

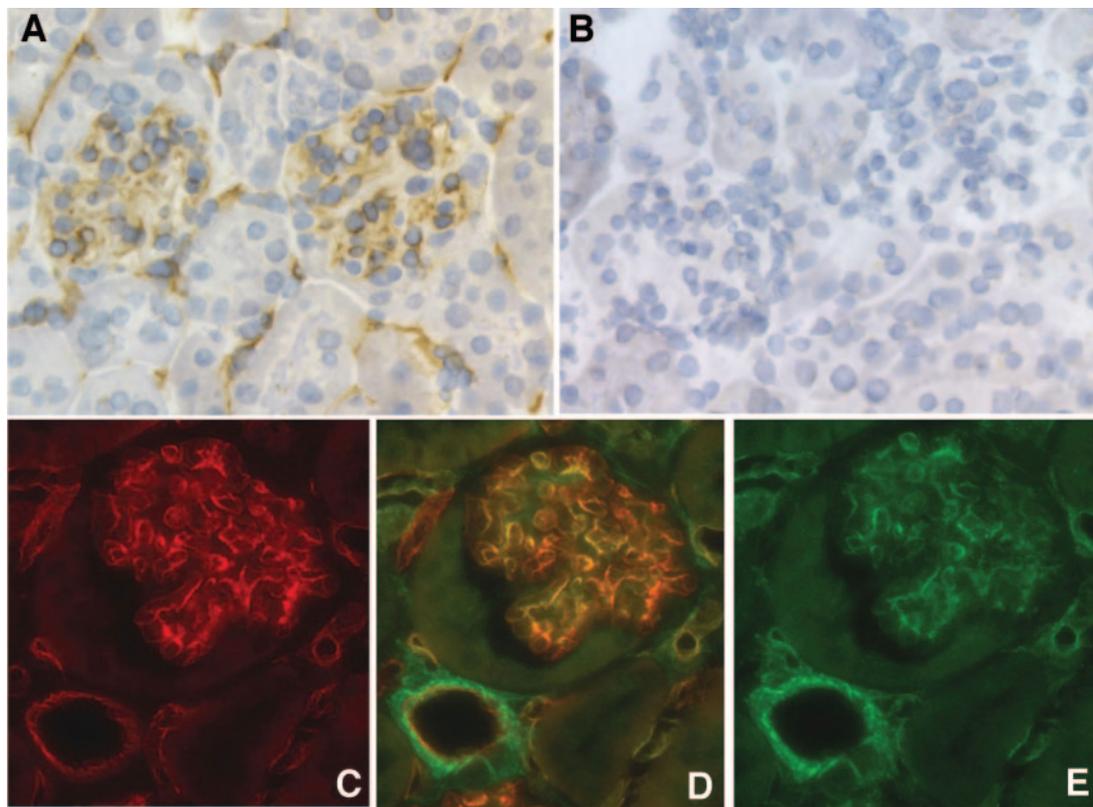


FIG. 1. Immunostaining for ENTPD1 using rabbit anti-mouse polyclonal antibody on wild-type (A) or ENTPD1-null (B) mouse kidney. Staining for the endothelial marker CD31 with CY5 red secondary antibody (C) or ENTPD1 with fluorescein isothiocyanate green secondary antibody (E). Merged image (D) shows that endothelial cells express both CD31 and ENTPD1 (yellow); areas of mesangium are positive for ENTPD1 (green) but not CD31. ENTPD1-null kidney tissue prepared in parallel showed no green staining in the glomeruli (not shown).

and loss of capillary lumen area (Fig. 3E). Fifty glomeruli from each mouse were scored in a blinded fashion on a scale of 0 to 4 (criteria detailed in RESEARCH DESIGN AND METHODS). Diabetic ENTPD1-null mice developed markedly more glomerular injury than diabetic wild-type mice at 6 months post-STZ injection (1.92 ± 0.22 vs. 1.02 ± 0.15 , $P < 0.001$).

We next examined the extent of fibrin deposition in the glomeruli using immunofluorescence microscopy. Fibrin

deposition was not evident in glomeruli from nondiabetic mice from either group (not shown), was rare and sparse in wild-type diabetic mice (Fig. 4A), but was widespread and intense in ENTPD1-null diabetic mice (Fig. 4B).

We did not note any large differences in levels of tissue factor expression in wild-type and ENTPD1-null diabetic mice on immunohistochemistry. While tissue factor activates the coagulation cascade leading to fibrin deposition, the fibrinolytic system counterbalances this process to

TABLE 1

Characteristics of experimental groups at baseline, 12 weeks post-STZ injection, and study end (24 weeks post-STZ injection)

	Wild type	Knockout	Wild type diabetic	Knockout diabetic
Weight (g)				
8 weeks	24.0 ± 0.3	24.3 ± 0.4	23.1 ± 0.3	23.9 ± 0.4
20 weeks	30.1 ± 0.6	32.2 ± 0.8	$26.4 \pm 0.7^*$	$26.0 \pm 0.7^\dagger$
32 weeks	33.5 ± 0.8	35.4 ± 1.3	$27.7 \pm 0.7^*$	$27.3 \pm 0.8^\dagger$
Glucose (mg/dl)				
11 weeks	138.9 ± 10.9	158.4 ± 6.1	$480.1 \pm 32.4^\dagger$	$431.5 \pm 24.5^\dagger$
20 weeks	152.3 ± 5.0	158.3 ± 6.9	$512 \pm 36.2^\dagger$	$532.7 \pm 21.2^\dagger$
32 weeks	150.0 ± 7.8	148.4 ± 9.5	$545.2 \pm 34.8^\dagger$	$567.0 \pm 12.2^\dagger$
Kidney weight/body weight $\times 100$	1.25 ± 0.03	1.17 ± 0.02	$1.97 \pm 0.14^\dagger$	$1.89 \pm 0.11^\dagger$
Blood urea nitrogen at 32 weeks (mg/dl)	21.0 ± 0.8	23.0 ± 1.5	31.1 ± 4.3	30.3 ± 2.9
Creatinine at 32 weeks (mg/dl)	0.094 ± 0.005	0.077 ± 0.008	0.098 ± 0.014	0.080 ± 0.005
Systolic blood pressure (mmHg)	101.8 ± 0.9	102.2 ± 2.0	101.7 ± 1.9	107.1 ± 1.6
Mean arterial pressure (mmHg)	87.7 ± 1.4	88.8 ± 1.9	84.2 ± 2.6	89.8 ± 1.2

Data are means \pm SE. Knockout denotes ENTPD1-null mice backcrossed six times onto a C57/BL6 background and wild type denotes age-, sex-, weight-, and strain-matched controls. Creatinine was measured by HPLC. Using ANOVA with Tukey posttest, no differences ($P > 0.05$) were seen between wild-type and knockout mice or between wild-type diabetic and knockout diabetic mice in any category. SBP approached statistical significance ($P \approx 0.06$) between wild-type diabetic and knockout diabetic mice. $^*P < 0.01$ and $^\dagger P < 0.001$, diabetic mice vs. nondiabetic controls ($n = 6$ –11 mice/group).

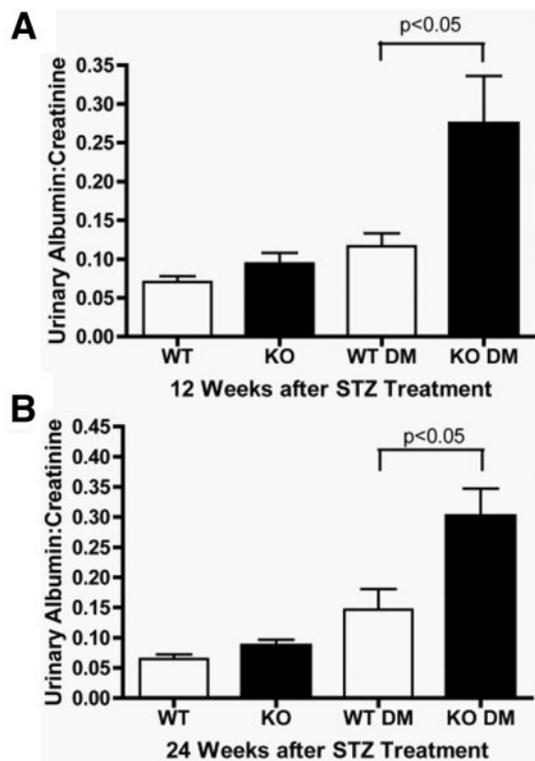


FIG. 2. Urinary albumin-to-creatinine ratio for wild-type (WT), ENTPD1-null (knockout [KO]), wild-type diabetic (WT DM), and ENTPD1-null diabetic (KO DM) mice 12 (A) or 24 (B) weeks after STZ injection ($n = 6-8$ for untreated groups and 9-11 for diabetic groups). ANOVA with Tukey posttest.

remove potentially injurious fibrin and maintain balance. Because the antifibrinolytic protein PAI-1 is upregulated in diabetes (25), promotes diabetic nephropathy (26), and is upregulated in some tissue types by extracellular nucleotides (27), we considered that increased PAI-1 expression in ENTPD1-null animals might shift the balance toward increased fibrin deposition and more severe glomerular injury. Tissue sections from each group of mice ($n = 4$ per group) were stained with anti-PAI-1 antibodies. Thirty glomeruli from each mouse were examined in a blinded fashion and graded on a scale of 0 to 4 (see RESEARCH DESIGN AND METHODS for scoring system). Increased expression of PAI-1 was seen in both nondiabetic and diabetic ENTPD1-null mice relative to their wild-type counterparts, with the highest levels observed in diabetic ENTPD1-null glomeruli (2.62 ± 0.76 vs. 0.53 ± 0.36 for diabetic wild type, $P < 0.01$; Fig. 4E). Figure 4C shows a representative diabetic wild-type glomerulus with little PAI-1 staining, whereas Fig. 4D shows two typical ENTPD1-null glomeruli with prominent PAI-1 staining.

To show that differences in extracellular nucleotide signaling specifically alter expression of PAI-1, we pursued in vitro experiments using a well-characterized mesangial cell line (28). We treated SV40 MES 13 mouse mesangial cells with ATP, ADP, UTP, and the nucleoside adenosine and measured PAI-1 mRNA expression after 1 h with TaqMan Real-Time PCR. ATP and UTP stimulation both caused strong upregulation of PAI-1 (Fig. 5A: control = 1.00 ± 0.09 ; ATP = 2.39 ± 0.22 ; UTP = 2.15 ± 0.16). Neither ADP nor adenosine administration affected PAI-1 expression. PAI-1 responded to ATP stimulation in a dose-dependent manner (Fig. 5B), indicating that regulation of ATP levels by ENTPD1 would be expected to

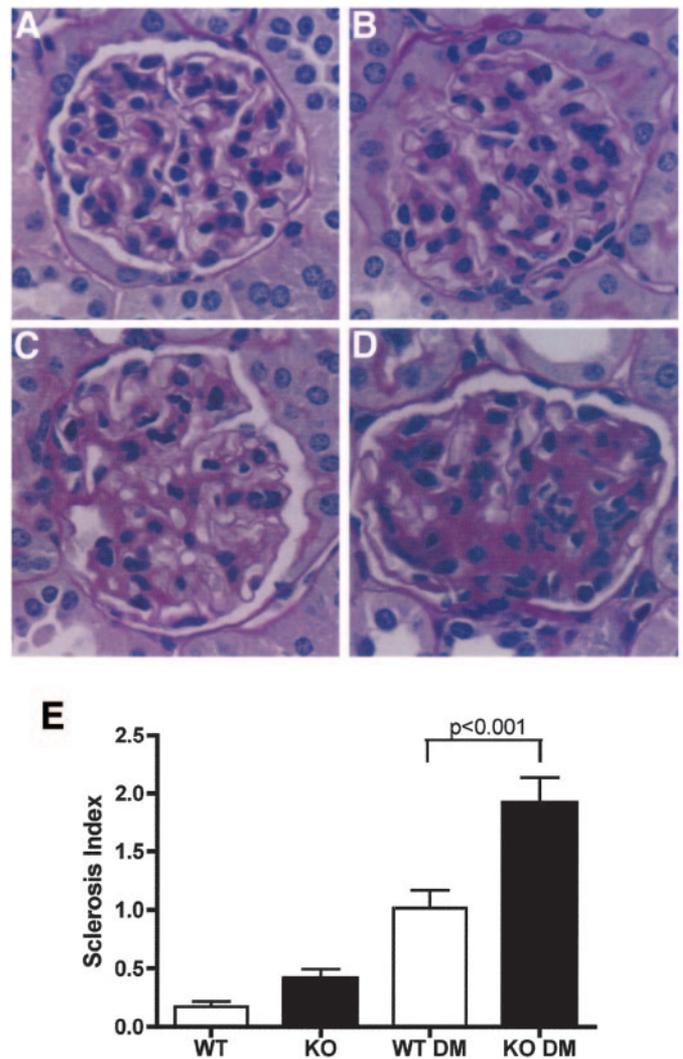


FIG. 3. Representative glomeruli from wild-type (WT) (A), ENTPD1-null (B), wild-type diabetic (C), and ENTPD1-null diabetic (D) mice after 24 weeks of diabetes. E: Glomerular sclerosis index. Fifty consecutive glomeruli per mouse were scored on a 0 to 4 scale by investigators blinded to grouping (see RESEARCH DESIGN AND METHODS) ($n = 6-9$ per group). ANOVA with Tukey posttest.

modulate expression of PAI-1, as seen in the previous in vivo experiments.

Stimulation of PAI-1 by both ATP and UTP (but not ADP) suggested a pharmacological profile consistent with a P2Y receptor such as P2Y2 or P2Y4. Because several P2Y (including P2Y2 and P2Y4) receptors exert their effects at least in part through PKC (29), which is also an important determinant of diabetic nephropathy (30), we next tested whether nucleotide-stimulated PAI-1 upregulation was PKC dependent using the PKC inhibitor BIM. However, BIM, at concentrations ≤ 150 nmol/l, had no statistically significant effect on ATP-induced PAI-1 upregulation (data not shown).

We next studied the kinetics of nucleotide stimulation on PAI-1 expression. We used the hydrolysis-resistant ATP analog ATP γ S to maintain a stable extracellular nucleotide concentration and negate the effect of adenosine generation by ectonucleotidases. We chose a dose that was equipotent to 50 μ mol/l ATP at P2Y2 receptors (31). We noted that 350 μ mol/l ATP γ S induced a progressive increase in PAI-1 expression over a 6-h time period (Fig. 5C).

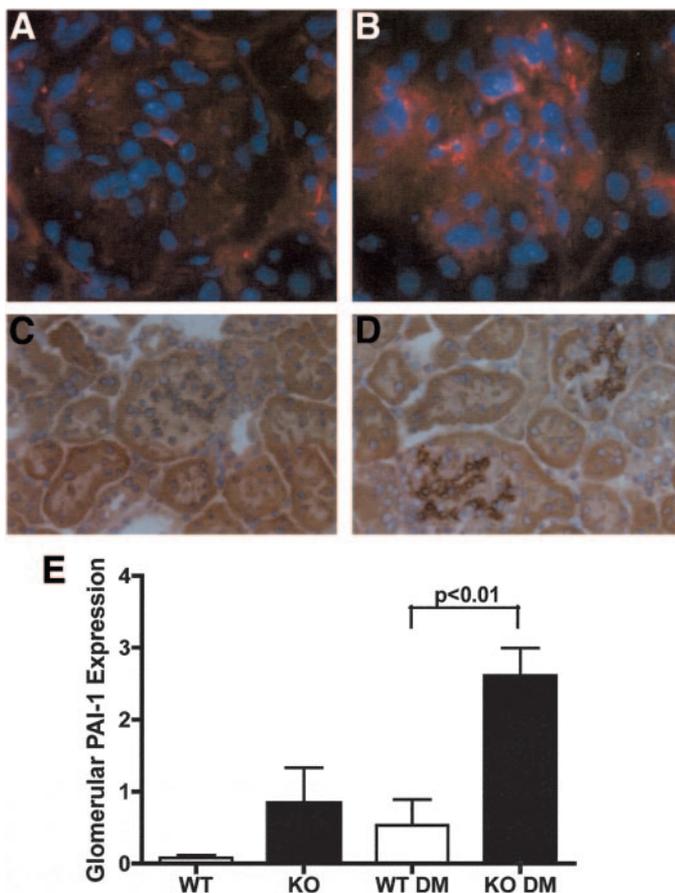


FIG. 4. Immunostaining for fibrin in renal cortex from wild-type (WT) diabetic (A) and ENTPD1-null diabetic (B) mice after 24 weeks of diabetes. Heavy fibrin staining (red) was seen in the glomeruli from diabetic ENTPD1-null mice but not from the other three groups. Immunostaining for PAI-1 in glomeruli from wild-type diabetic (C) and ENTPD1-null diabetic (D) mice after 24 weeks of diabetes. The wild-type glomerulus in C (center) shows only minimal PAI-1 staining. In contrast, the two glomeruli in D show strong PAI-1 staining in a mesangial distribution in the diabetic ENTPD1-null mice. E: Index of glomerular PAI-1 positivity. Thirty consecutive glomeruli were scored blinded to grouping on a 0 to 4 scale and averaged to give a score for each mouse ($n = 4$ mice per group). ANOVA with Tukey posttest.

While adenosine had no effect on PAI-1 expression, this may have been due to rapid metabolism and/or cellular uptake. Using the stable adenosine agonist NECA at 1 $\mu\text{mol/l}$, we noted inhibitory effects on PAI-1 expression at 6 h (Fig. 5D: control = 1.00 ± 0.05 ; NECA 1 h = 1.10 ± 0.03 , 3 h = 0.87 ± 0.73 , and 6 h = 0.64 ± 0.06 ; $P < 0.01$).

Inflammation is also a critical factor in the pathogenesis of diabetic nephropathy, and ENTPD1 is an anti-inflammatory factor in multiple conditions and experimental models (22). We noted a dramatic increase in glomerular macrophages (using the macrophage marker F4/80) in diabetic ENTPD1-null mice, and we quantified the frequency of glomerular macrophages as a proxy for glomerular inflammation in each group of mice. Glomeruli of diabetic ENTPD1-null mice were much more likely to harbor tissue macrophages than the other groups (Fig. 6A: wild-type diabetic = $5.4 \pm 0.8\%$; ENTPD1-null diabetic = $22.2 \pm 1.6\%$; $P < 0.001$), which is indicative of a heightened inflammatory state. We also investigated potential signals that could induce macrophage infiltration into the kidney using real-time PCR of renal cortex tissue. MCP-1 is a cytokine previously demonstrated to promote diabetic nephropathy in mouse models (32). MCP-1 expression was

assayed in each group by real-time PCR ($n = 6$ per group). There were no significant differences between nondiabetic wild-type and ENTPD1-null mice. However, diabetic ENTPD1-null mice expressed almost twice the level of MCP-1 relative to diabetic wild-type mice (Fig. 6B: wild-type diabetic = 0.86 ± 0.13 vs. ENTPD1-null diabetic = 1.79 ± 0.22 ; $P < 0.05$), indicating enhanced signaling for monocyte extravasation from the vascular space to become tissue macrophages.

We next determined whether upregulation of MCP-1 was nucleotide related in mesangial cell culture. We treated mesangial cells with ATP, ADP, UTP, and adenosine, and measured MCP-1 mRNA expression with Taqman Real-Time PCR (Fig. 7A). ATP and UTP stimulation both caused strong upregulation of MCP-1 (control = 1.00 ± 0.11 , ATP = 2.60 ± 0.17 , and UTP = 2.04 ± 0.23) similar to PAI-1. Adenosine, an anti-inflammatory nucleoside, had a consistent inhibitory effect on MCP-1 of $\sim 20\%$ in each of three independent experiments, but this did not reach clear statistical significance due to the relatively small difference between means. MCP-1 also responded progressively to ATP in a dose-dependent manner (Fig. 7B), again suggesting that regulation of ATP levels by ENTPD1 modulates MCP-1 expression by mesangial cells in vivo.

However, in contrast to the results seen with PAI-1, the PKC inhibitor BIM (at nanomolar concentrations specific for PKC inhibition) blunted the upregulation of MCP-1. We found that nucleotide-driven MCP-1 expression was inhibited by BIM in a dose-dependent manner, indicating that PKC activation is essential for MCP-1 upregulation by ATP (Fig. 7C). At 150 nmol/l, BIM inhibited ATP-stimulated MCP-1 expression by 80%, with no statistically significant difference remaining between control and ATP + 150 nmol/l BIM (control = 1.00 ± 0.06 , ATP = 2.30 ± 0.12 , and ATP + 150 $\mu\text{mol/l}$ BIM = 1.26 ± 0.12).

At 350 $\mu\text{mol/l}$, ATP γ S caused a sharp increase in MCP-1 expression that appeared to taper gradually over time (Fig. 7D: control = 1.00 ± 0.03 , 1 h = 2.56 ± 0.08 , 3 h = 2.20 ± 0.06 , and 6 h = 1.85 ± 0.08). Furthermore, 1 $\mu\text{mol/l}$ NECA caused a nonsignificant increase in MCP-1 expression at 1 h but a significant decrease in MCP-1 expression at 6 h (Fig. 7E: control = 1.00 ± 0.09 , 1 h = 1.24 ± 0.06 ; 3 h = 1.00 ± 0.07 , and 6 h = 0.69 ± 0.06 ; $P < 0.05$).

DISCUSSION

Extracellular nucleotides such as ATP and ADP can be released from cells in a variety of situations, including shear stress, platelet degranulation, or cellular injury, just to name a few (33–35). The vascular ectonucleotidase ENTPD1 hydrolyzes the proinflammatory molecule ATP (and the prothrombotic molecule ADP) to initiate an enzymatic cascade that ultimately produces the anti-inflammatory molecule adenosine via CD73 (35).

Here, we have shown that ENTPD1 is a critical factor preventing chronic microvascular injury in diabetes. ENTPD1-null mice developed markedly worse diabetic nephropathy than wild-type mice, as reflected by increased albuminuria and glomerular injury. Diabetic renal injury in the ENTPD1-null mice was associated with heavy glomerular fibrin deposition and increased glomerular inflammation. PAI-1 overexpression favors net fibrin accumulation by inhibiting tissue plasminogen activator. In addition, PAI-1 is a potent profibrotic agent with an inhibitory effect on matrix metalloproteinases. In both control and diabetic groups, ENTPD1 mice had more

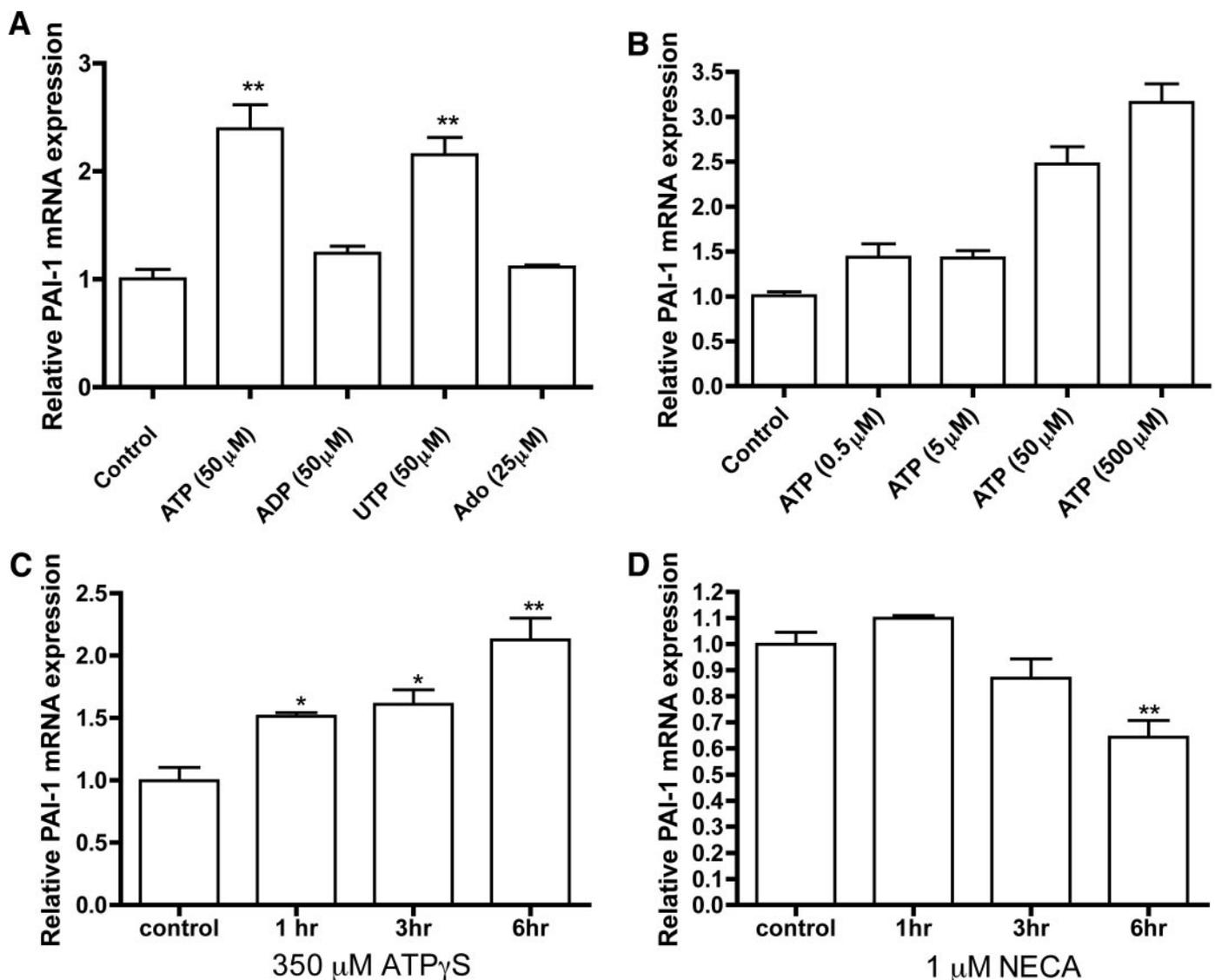


FIG. 5. *A*: Normalized PAI-1 mRNA expression (to 18S ribosomal subunit) after 1 h of nucleotide or nucleoside stimulation in mesangial cell culture by Taqman real-time PCR. ** $P < 0.001$ vs. control. ANOVA with Tukey posttest. *B*: Normalized PAI-1 mRNA expression after 1 h stimulation with increasing concentration of ATP by Taqman real-time PCR. P value for linear trend test < 0.0001 . *C*: Normalized PAI-1 mRNA expression 1, 3, and 6 h after ATP γ S stimulation. * $P < 0.05$, ** $P < 0.01$. ANOVA with Dunnett posttest. *D*: Normalized PAI-1 expression 1, 3, and 6 h after NECA stimulation. * $P < 0.01$. ANOVA with Dunnett posttest.

PAI-1 expression in the glomeruli. Because PAI-1-null mice are protected from diabetic (STZ) nephropathy (25), PAI-1 likely plays a causative role in diabetic renal injury. Increased PAI-1 expression in ENTPD1-null mice would be expected to contribute to the increased injury we observed. ACE inhibitors, one of the few agents shown to ameliorate diabetic nephropathy, downregulate PAI-1. Interestingly, ACE inhibitors also have been shown to upregulate ENTPD1 (36).

Inflammation also is an important component of diabetic renal injury (37). Glomerular macrophage infiltration serves as a useful marker for glomerular inflammation (38). Macrophages can lead to tissue injury through release of an array of cytokines and by generation of reactive oxygen species. While we saw infiltrating macrophages in only a minority of glomeruli, there was a more than fourfold increase in the frequency of glomerular macrophages in diabetic ENTPD1-null kidneys compared with diabetic wild-type kidneys. It has been shown that mice null for MCP-1 are protected from diabetic nephropathy

secondary to lower renal/glomerular inflammation (32). In addition, urinary MCP-1 levels have been shown to correlate well with degree of albuminuria in patients with diabetic nephropathy (39). Real-time PCR of whole renal cortex in our study showed upregulated expression of this inflammatory cytokine in diabetic ENTPD1-null kidneys, providing a link between extracellular nucleotide signaling and tissue inflammation.

In vitro work demonstrated a causal relationship between extracellular nucleotide signaling and both PAI-1 and MCP-1 expression. Both genes were primarily expressed in response to ATP and UTP but not ADP, a pharmacological profile consistent with a P2Y receptor such as P2Y2 or P2Y4. P2Y2 and P2Y4 have previously been described as the predominant P2Y receptors in mesangial cells (40). It is noteworthy that nucleotides caused rat mesangial cell proliferation via P2Y receptors in several published studies (12,41). While the predominant ATP/UTP upregulation of MCP-1 and PAI-1 expression points toward a P2Y2- or P2Y4-dominated effect, several

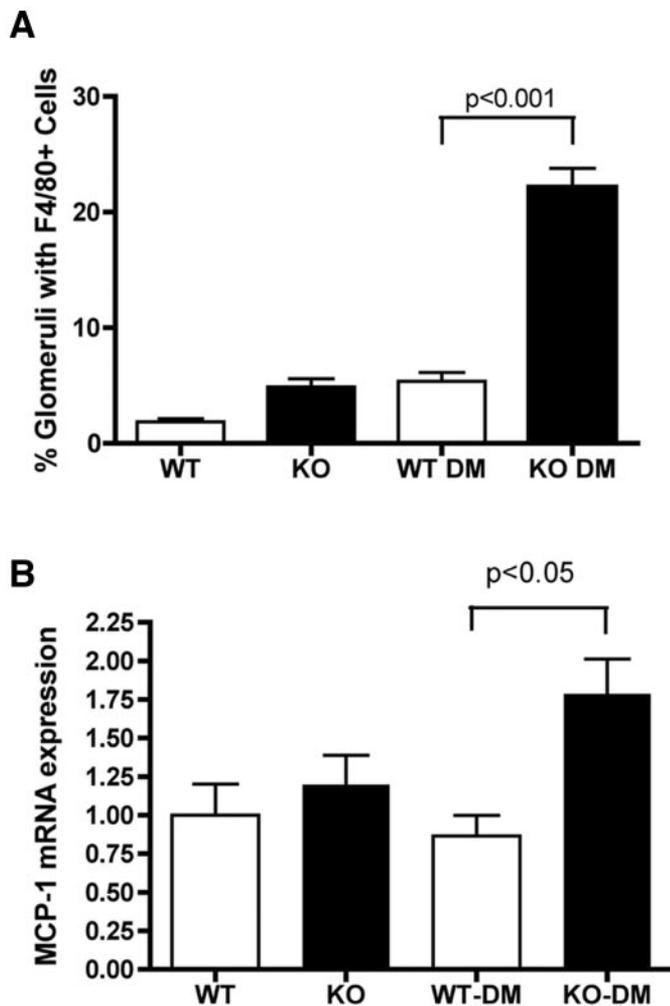


FIG. 6. A: Glomerular inflammation. Percent of glomeruli with tissue macrophages (F4/80 positive). Fifty glomeruli per mouse were assessed blinded to grouping ($n = 3$ mice per group). ANOVA with Tukey posttest. **B:** Normalized MCP-1 mRNA (to 18S ribosomal subunit) expression from renal cortex by Taqman real-time PCR ($n = 6$ mice per group). ANOVA with Tukey posttest. DM, diabetic; KO, knockout; WT, wild type.

factors suggest a more complex combination of multiple nucleotide receptors. First, the ATP effect was typically more potent than UTP in our experiments, allowing for the possibility of a small additive contribution by a P2X receptor. Second, the kinetic response to ATP γ S was somewhat different, with a slow, sustained increase of PAI-1 expression versus a rapid increase in MCP-1 expression that slowly diminished over time. The MCP-1 expression was consistent with a P2Y response (i.e., equivalent potency of 350 μ mol/l ATP γ S and 50 μ mol/l ATP as previously described for the P2Y2 receptor [31] and desensitization upon continuous stimulation). PAI-1 upregulation was less rapid but more sustained, perhaps suggesting contribution from a receptor (such as P2X4 or P2X7, both expressed in mesangial cells) that is resistant to desensitization and at which ATP γ S is less potent than at P2Y receptors.

Although no purely selective pharmacologic agonists or antagonists for P2X or P2Y receptors exist, we did observe a small (23%, $P < 0.05$; data not shown) increase in PAI-1 expression with the P2X-preferred agonist bz-ATP at low concentrations (5 μ mol/l), but no

change in MCP-1 expression, again suggesting a minor P2X component of PAI-1 upregulation. Recently, Rivera et al. (42) evaluated nucleotide-mediated calcium flux in this cell line and found that ATP and UTP had by far the strongest effect. However, small effects also were seen for ATP γ S, bz-ATP, and ADP. Though a complex system of purinergic signaling is likely, especially in vivo, ENTPD1 regulates all of these receptors by hydrolyzing ATP, UTP, ADP, and other nucleotides. We propose that ENTPD1 is at the center of this signaling system composed of any combination of P2 receptors.

An adenosine analog (NECA) caused downregulation of both MCP-1 and PAI-1 at the mRNA level. Since ENTPD1 is a crucial enzyme in the cascade that leads to adenosine production, ENTPD1-null mice would be expected to have more pericellular ATP and generate less adenosine. The presence of ENTPD1 could downregulate PAI-1 and MCP-1 expression by two mechanisms (i.e., diminished ATP and UTP signaling through P2 receptors and more adenosine signaling via P1 [adenosine] receptors).

After binding extracellular nucleotides, P2Y receptors can mediate their influence through activation of phospholipase C, which can activate multiple effector mechanisms including PKC (29). PKC activation is known to promote diabetic microvascular injury (30), so we tested whether nucleotide-regulated expression of PAI-1 and MCP-1 were orchestrated through PKC. Interestingly, the PKC inhibitor BIM showed no effect on PAI-1 expression, but it strongly inhibited MCP-1 expression in a dose-dependent fashion. PAI-1 may be activated through IP $_3$ /calcium flux, phosphatidylinositol 3/Akt, src kinase, or other G-protein-coupled receptor-regulated effectors in the P2Y signaling pathway (43). Previous (44) and ongoing (NCT00297401; see <http://clinicaltrials.gov>) trials of PKC inhibitors to treat diabetic microvascular disease highlight the potential importance of our finding that nucleotide-regulated MCP-1 expression is PKC dependent.

A minor limitation of our study was that ENTPD1-null mice did not progress to overt renal failure. However, renal failure is not a cardinal feature of murine STZ diabetic nephropathy when creatinine is measured accurately by HPLC rather than colorimetric creatinine assays (creatinine is falsely elevated by interfering chromogens) (24,45). Longer duration of diabetes might lead to outright renal failure in ENTPD1-null mice given the marked increase in glomerular sclerosis severity at 6 months.

The importance of extracellular nucleotide metabolism has been further suggested by association between diabetic nephropathy and the ectonucleotidase ENPP1/PC-1 in two independent studies in humans (18,19). ENPP1/PC-1 hydrolyzes ATP directly to AMP and pyrophosphate, whereas ENTPD1 hydrolyzes ATP and ADP to AMP and either one or two inorganic phosphate molecules. The net result in both cases is decreased extracellular ATP and ultimately increased extracellular adenosine by CD73. Notably, administration of the adenosine agonist ATL146e has been shown to ameliorate diabetic nephropathy in mice (20).

Though understanding of the role of nucleotide and nucleoside signaling in renal disease is in its infancy, our study represents an important advance in the investigation of a pathway with potential therapeutic implications at both the enzyme and receptor level.

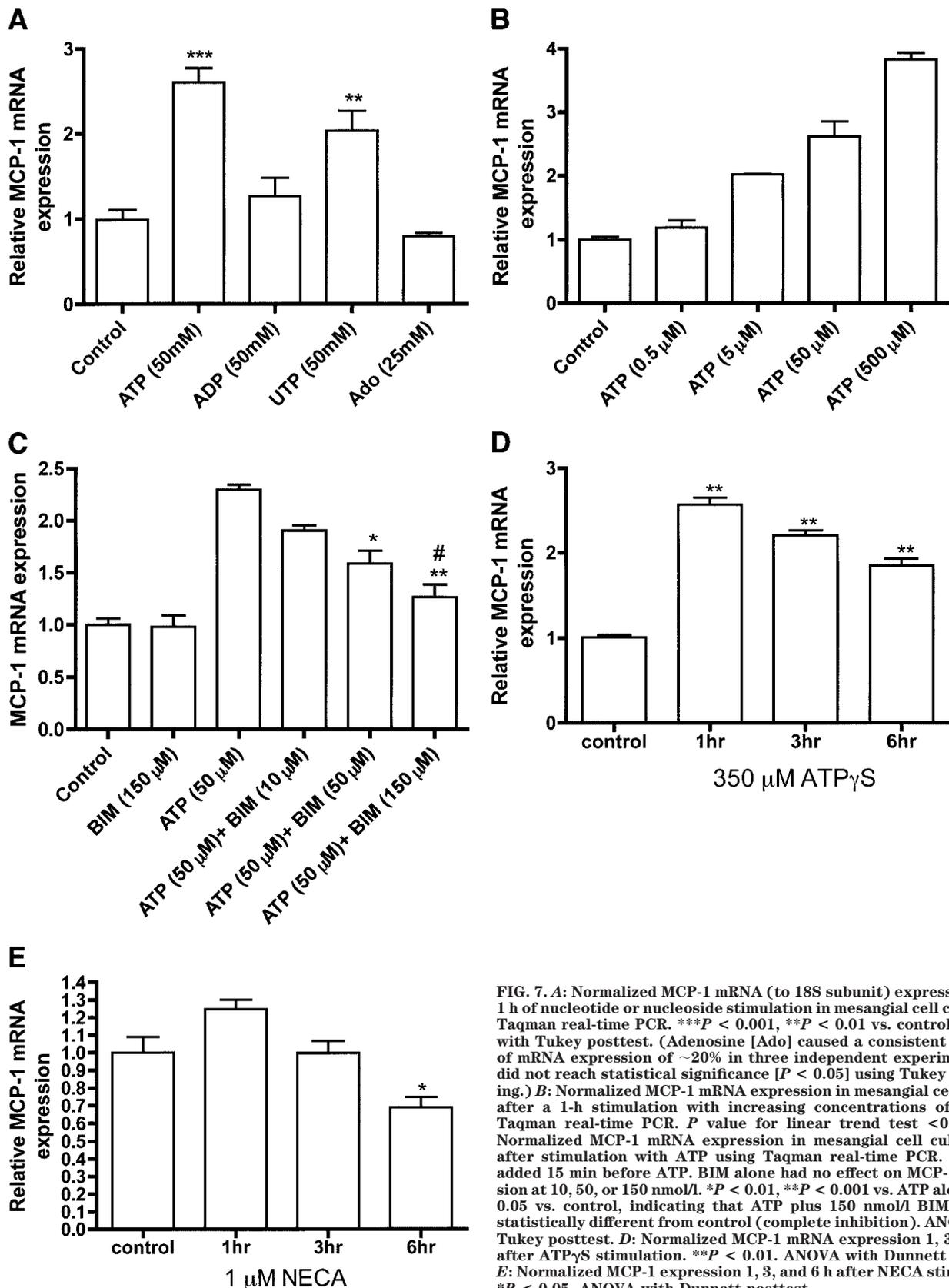


FIG. 7. *A*: Normalized MCP-1 mRNA (to 18S subunit) expression after 1 h of nucleotide or nucleoside stimulation in mesangial cell culture by Taqman real-time PCR. ****P* < 0.001, ***P* < 0.01 vs. control. ANOVA with Tukey posttest. (Adenosine [Ado] caused a consistent decrease of mRNA expression of ~20% in three independent experiments but did not reach statistical significance [*P* < 0.05] using Tukey posttesting.) *B*: Normalized MCP-1 mRNA expression in mesangial cell culture after a 1-h stimulation with increasing concentrations of ATP by Taqman real-time PCR. *P* value for linear trend test <0.0001. *C*: Normalized MCP-1 mRNA expression in mesangial cell culture 1 h after stimulation with ATP using Taqman real-time PCR. BIM was added 15 min before ATP. BIM alone had no effect on MCP-1 expression at 10, 50, or 150 nmol/l. **P* < 0.01, ***P* < 0.001 vs. ATP alone. #*P* > 0.05 vs. control, indicating that ATP plus 150 nmol/l BIM was not statistically different from control (complete inhibition). ANOVA with Tukey posttest. *D*: Normalized MCP-1 mRNA expression 1, 3, and 6 h after ATPγS stimulation. ***P* < 0.01. ANOVA with Dunnett posttest. *E*: Normalized MCP-1 expression 1, 3, and 6 h after NECA stimulation. **P* < 0.05. ANOVA with Dunnett posttest.

ACKNOWLEDGMENTS

This work was supported by HL57307 (to S.C.R.), HL63972 (to S.C.R.), and HL076540 (project to S.C.R.) from the National Institutes of Health (NIH). D.J.F. was supported by an NIH T32 training grant in nephrology awarded to

Vikas Sukhatme, Renal Division, Department of Medicine, Beth Israel Deaconess Medical Center.

We thank Wanda Snead at the Mouse Metabolic Phenotyping Center (Vanderbilt University) for help with HPLC creatinine determination.

REFERENCES

- National Institute of Diabetes and Digestive and Kidney Disease: 2004 Annual Data Report: Atlas of End-Stage Renal Disease in the United States. Bethesda, MD, National Institutes of Health, National Institute of Diabetes and Digestive and Kidney Disease, 2004
- Locatelli F, Pozzoni P, Del Vecchio L: Renal replacement therapy in patients with diabetes and end-stage renal disease. *J Am Soc Nephrol* 15 (Suppl. 1):S25–S29, 2004
- Freedman BI, Soucie JM, McClellan WM: Family history of end-stage renal disease among incident dialysis patients. *J Am Soc Nephrol* 8:1942–1945, 1997
- Seaquist ER, Goetz FC, Rich S, Barbosa J: Familial clustering of diabetic kidney disease: evidence for genetic susceptibility to diabetic nephropathy. *N Engl J Med* 320:1161–1165, 1989
- Satko SG, Freedman BI, Moossavi S: Genetic factors in end-stage renal disease. *Kidney Int Suppl* 94:S46–S49, 2005
- Sevigny J, Sundberg C, Braun N, Guckelberger O, Csizmadia E, Qawi I, Imai M, Zimmermann H, Robson SC: Differential catalytic properties and vascular topography of murine nucleoside triphosphate diphosphohydrolase 1 (NTPDase1) and NTPDase2 have implications for thromboregulation. *Blood* 99:2801–2809, 2002
- Kaczmarek E, Koziak K, Sevigny J, Siegel JB, Anrather J, Beaudoin AR, Bach FH, Robson SC: Identification and characterization of CD39/vascular ATP diphosphohydrolase. *J Biol Chem* 271:33116–33122, 1996
- Zimmermann HBN, Heine P, Kohring K, Marxen M, Sevigny J, Robson SC: *Ecto-ATPases and Related Nucleotidases*. Maastricht, the Netherlands, Shaker, 2000
- Jacobson KA, Mamedova L, Joshi BV, Besada P, Costanzi S: Molecular recognition at adenine nucleotide (P₂) receptors in platelets. *Semin Thromb Hemost* 31:205–216, 2005
- Kaczmarek E, Erb L, Koziak K, Jarzyna R, Wink MR, Guckelberger O, Blusztajn JK, Trinkaus-Randall V, Weisman GA, Robson SC: Modulation of endothelial cell migration by extracellular nucleotides: involvement of focal adhesion kinase and phosphatidylinositol 3-kinase-mediated pathways. *Thromb Haemost* 93:735–742, 2005
- Burnstock G: Purinergic signaling and vascular cell proliferation and death. *Arterioscler Thromb Vasc Biol* 22:364–373, 2002
- Harada H, Chan CM, Loesch A, Unwin R, Burnstock G: Induction of proliferation and apoptotic cell death via P₂Y and P₂X receptors, respectively, in rat glomerular mesangial cells. *Kidney Int* 57:949–958, 2000
- Kishore BK, Isaac J, Fausther M, Tripp SR, Shi H, Gill PS, Braun N, Zimmermann H, Sevigny J, Robson SC: Expression of NTPDase1 and NTPDase2 in murine kidney: relevance to regulation of P₂ receptor signaling. *Am J Physiol Renal Physiol* 288:F1032–F1043, 2005
- Candinas D, Koyamada N, Miyatake T, Siegel J, Hancock WW, Bach FH, Robson SC: Loss of rat glomerular ATP diphosphohydrolase activity during reperfusion injury is associated with oxidative stress reactions. *Thromb Haemost* 76:807–812, 1996
- Robson SC, Kaczmarek E, Siegel JB, Candinas D, Koziak K, Millan M, Hancock WW, Bach FH: Loss of ATP diphosphohydrolase activity with endothelial cell activation. *J Exp Med* 185:153–163, 1997
- Imai M, Takigami K, Guckelberger O, Enjoji K, Smith RN, Lin Y, Csizmadia E, Sevigny J, Rosenberg RD, Bach FH, Robson SC: Modulation of nucleoside [correction of nucleotide] triphosphate diphosphohydrolase-1 (NTPDase-1)cd39 in xenograft rejection. *Mol Med* 5:743–752, 1999
- Koyamada N, Miyatake T, Candinas D, Hechenleitner P, Siegel J, Hancock WW, Bach FH, Robson SC: Apyrase administration prolongs discordant xenograft survival. *Transplantation* 62:1739–1743, 1996
- Canani LH, Ng DP, Smiles A, Rogus JJ, Warram JH, Krolewski AS: Polymorphism in ecto-nucleotide pyrophosphatase/phosphodiesterase 1 gene (*ENPP1/PC-1*) and early development of advanced diabetic nephropathy in type 1 diabetes. *Diabetes* 51:1188–1193, 2002
- De Cosmo S, Trevisan R, Dalla Vestra M, Vedovato M, Argiolas A, Solini A, Saller A, Damone F, Tiengo A, Trischitta V, Fioretto P: PC-1 amino acid variant Q121 is associated with a lower glomerular filtration rate in type 2 diabetic patients with abnormal albumin excretion rates. *Diabetes Care* 26:2898–2902, 2003
- Awad AS, Huang L, Ye H, Duong ET, Bolton WK, Linden J, Okusa MD: Adenosine A_{2A} receptor activation attenuates inflammation and injury in diabetic nephropathy. *Am J Physiol Renal Physiol* 290:F828–F837, 2006
- Enjoji K, Sevigny J, Lin Y, Frenette PS, Christie PD, Esch JS 2nd, Imai M, Edelberg JM, Rayburn H, Lech M, Beeler DL, Csizmadia E, Wagner DD, Robson SC, Rosenberg RD: Targeted disruption of cd39/ATP diphosphohydrolase results in disordered hemostasis and thromboregulation. *Nat Med* 5:1010–1017, 1999
- Robson SC, Wu Y, Sun X, Knosalla C, Dwyer K, Enjoji K: Ectonucleotidases of CD39 family modulate vascular inflammation and thrombosis in transplantation. *Semin Thromb Hemost* 31:217–233, 2005
- Beckstead JH: A simple technique for preservation of fixation-sensitive antigens in paraffin-embedded tissues. *J Histochem Cytochem* 42:1127–1134, 1994
- Breyer MD, Bottinger E, Brosius FC 3rd, Coffman TM, Harris RC, Heilig CW, Sharma K: Mouse models of diabetic nephropathy. *J Am Soc Nephrol* 16:27–45, 2005
- Nicholas SB, Aguiniga E, Ren Y, Kim J, Wong J, Govindarajan N, Noda M, Wang W, Kawano Y, Collins A, Hsueh WA: Plasminogen activator inhibitor-1 deficiency retards diabetic nephropathy. *Kidney Int* 67:1297–1307, 2005
- Lyon CJ, Hsueh WA: Effect of plasminogen activator inhibitor-1 in diabetes mellitus and cardiovascular disease. *Am J Med* 115 (Suppl. 8A):62S–68S, 2003
- Bouchie JL, Chen HC, Carney R, Bagot JC, Wilden PA, Feener EP: P₂Y receptor regulation of PAI-1 expression in vascular smooth muscle cells. *Arterioscler Thromb Vasc Biol* 20:866–873, 2000
- MacKay K, Striker LJ, Elliot S, Pinkert CA, Brinster RL, Striker GE: Glomerular epithelial, mesangial, and endothelial cell lines from transgenic mice. *Kidney Int* 33:677–684, 1988
- von Kugelgen I, Wetter A: Molecular pharmacology of P₂Y-receptors. *Naunyn Schmiedeberg Arch Pharmacol* 362:310–323, 2000
- Schena FP, Gesualdo L: Pathogenetic mechanisms of diabetic nephropathy. *J Am Soc Nephrol* 16 (Suppl. 1):S30–S33, 2005
- Lazarowski ER, Watt WC, Stutts MJ, Boucher RC, Harden TK: Pharmacological selectivity of the cloned human P₂U-purinoceptor: potent activation by diadenosine tetraphosphate. *Br J Pharmacol* 116:1619–1627, 1995
- Chow FY, Nikolic-Paterson DJ, Ozols E, Atkins RC, Rollin BJ, Tesch GH: Monocyte chemoattractant protein-1 promotes the development of diabetic renal injury in streptozotocin-treated mice. *Kidney Int* 69:73–80, 2006
- Bodin P, Bailey D, Burnstock G: Increased flow-induced ATP release from isolated vascular endothelial cells but not smooth muscle cells. *Br J Pharmacol* 103:1203–1205, 1991
- Hechler B, Cattaneo M, Gachet C: The P₂ receptors in platelet function. *Semin Thromb Hemost* 31:150–161, 2005
- Chizh BA, Illes P: P₂X receptors and nociception. *Pharmacol Rev* 53:553–568, 2001
- Kishi Y, Ohta S, Kasuya N, Sakita SY, Ashikaga T, Isobe M: Perindopril augments ecto-ATP diphosphohydrolase activity and enhances endothelial anti-platelet function in human umbilical vein endothelial cells. *J Hypertens* 21:1347–1353, 2003
- Navarro JF, Mora C: Role of inflammation in diabetic complications. *Nephrol Dial Transplant* 20:2601–2604, 2005
- Kluth DC, Erwig LP, Rees AJ: Multiple facets of macrophages in renal injury. *Kidney Int* 66:542–557, 2004
- Banba N, Nakamura T, Matsumura M, Kuroda H, Hattori Y, Kasai K: Possible relationship of monocyte chemoattractant protein-1 with diabetic nephropathy. *Kidney Int* 58:684–690, 2000
- Schiebert EM, Kishore BK: Extracellular nucleotide signaling along the renal epithelium. *Am J Physiol Renal Physiol* 280:F945–F963, 2001
- Schulze-Lohoff E, Zanner S, Ogilvie A, Sterzel RB: Extracellular ATP stimulates proliferation of cultured mesangial cells via P₂-purinergic receptors. *Am J Physiol* 263:F374–F383, 1992
- Rivera I, Zhang S, Fuller BS, Edwards B, Seki T, Wang MH, Marrero MB, Incho EW: P₂ receptor regulation of [Ca²⁺]_i in cultured mouse mesangial cells. *Am J Physiol Renal Physiol* 292:F1380–F1389, 2007
- Erb L, Liao Z, Seye CI, Weisman GA: P₂ receptors: intracellular signaling. *Pflugers Arch* 452:552–562, 2006
- Tuttle KR, Bakris GL, Toto RD, McGill JB, Hu K, Anderson PW: The effect of ruboxistaurin on nephropathy in type 2 diabetes. *Diabetes Care* 28:2686–2690, 2005
- Breyer MD, Bottinger E, Brosius FC, Coffman TM, Fogo A, Harris RC, Heilig CW, Sharma K: Diabetic nephropathy: of mice and men. *Adv Chronic Kidney Dis* 12:128–145, 2005