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# High Glucose Up-regulates ADAM17 through HIF-1α in Mesangial Cells*

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**Background:** ADAM17 is an important promoter of fibrosis in diabetic kidney disease.
**Results:** ADAM17 is transcriptionally up-regulated by glucose through HIF-1α in kidney mesangial cells, associated with enhanced enzymatic activity.
**Conclusion:** ADAM17 induces its own up-regulation, thereby augmenting its activity, in response to glucose.
**Significance:** These studies provide a strong foundation for further evaluation of the role of ADAM17 in the profibrotic response of kidney cells to high glucose.

**We previously showed that ADAM17 mediates high glucose-induced matrix production by kidney mesangial cells. ADAM17 expression is increased in diabetic kidneys, suggesting that its up-regulation may augment high glucose profibrotic responses. We thus studied the effects of high glucose on ADAM17 gene regulation. Primary rat mesangial cells were treated with high glucose (30 mM) or mannitol as osmotic control. High glucose dose-dependently increased ADAM17 promoter activity, transcript, and protein levels. This correlated with augmented ADAM17 activity after 24 h *versus* 1 h of high glucose. We tested involvement of transcription factors shown in other settings to regulate ADAM17 transcription. Promoter activation was not affected by NF-κB or Sp1 inhibitors, but was blocked by hypoxia-inducible factor-1α (HIF-1α) inhibition or down-regulation. This also prevented ADAM17 transcript and protein increases. HIF-1α activation by high glucose was shown by its increased nuclear translocation and activation of the HIF-responsive hypoxia-response element (HRE)-luciferase reporter construct. Assessment of ADAM17 promoter deletion constructs coupled with mutation analysis and ChIP studies identified HIF-1α binding to its consensus element at −607 as critical for the high glucose response. Finally, inhibitors of epidermal growth factor receptor (EGFR) and downstream PI3K/Akt, or ADAM17 itself, prevented high glucose-induced HIF-1α activation and ADAM17 up-regulation. Thus, high glucose induces ADAM17 transcriptional up-regulation in mesangial cells, which is associated with augmentation of its activity. This is mediated by HIF-1α and requires EGFR/ADAM17 signaling, demonstrating the potentiation by ADAM17 of its own up-regulation. ADAM17 inhibition thus provides a potential novel therapeutic strategy for the treatment of diabetic nephropathy.**

The kidney is a major site of diabetic microvascular complications, with diabetic nephropathy being the leading cause of end-stage renal disease. Glomerular sclerosis, characterized by excess accumulation of extracellular matrix, is the pathologic hallmark of diabetic nephropathy and significantly contributes to declining renal function (1). Hyperglycemia is a primary pathogenetic factor in both type 1 and type 2 diabetes, and glomerular mesangial cells (MC)[2] exposed to high glucose (HG) synthesize extracellular matrix proteins both *in vitro* (2) and *in vivo* (1, 3). However, glucose control and renin-angiotensin system inhibition, the mainstay of currently available therapy, are not sufficient to prevent the progression of disease in most patients. This necessitates a greater understanding of the pathophysiologic mechanisms underlying diabetic renal fibrosis to enable the development of novel therapeutic approaches.

We previously showed that the epidermal growth factor receptor (EGFR) is transactivated by HG in MC and mediates the profibrotic response of these cells (4, 5). In studies to elucidate how the EGFR is activated by HG, we discovered an important role for the metalloprotease ADAM17, the activation of which releases the EGFR ligand HB-EGF. Inhibition of either ADAM17 or HB-EGF prevented glucose-induced up-regulation of the profibrotic cytokine TGFβ1 (6), a major regulator of diabetic glomerular sclerosis (1). *In vivo*, short-term treatment of diabetic mice with an inhibitor of metalloproteases, including ADAM17, attenuated glomerular matrix accumulation (7). These studies suggest an important role for ADAM17 in the pathogenesis of diabetic nephropathy. However, targeting ADAM17 clinically is complicated by the lack of specificity of

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[2] The abbreviations used are: MC, mesangial cells; HG, high glucose; HRE, hypoxia-response element; HB-EGF, heparin binding EGF-like growth factor; EGFR, epidermal growth factor receptor; ADAM, a disintegrin and metalloproteinase; HIF-1, hypoxia-inducible factor-1; CREB, cAMP-response element-binding protein; con, control.

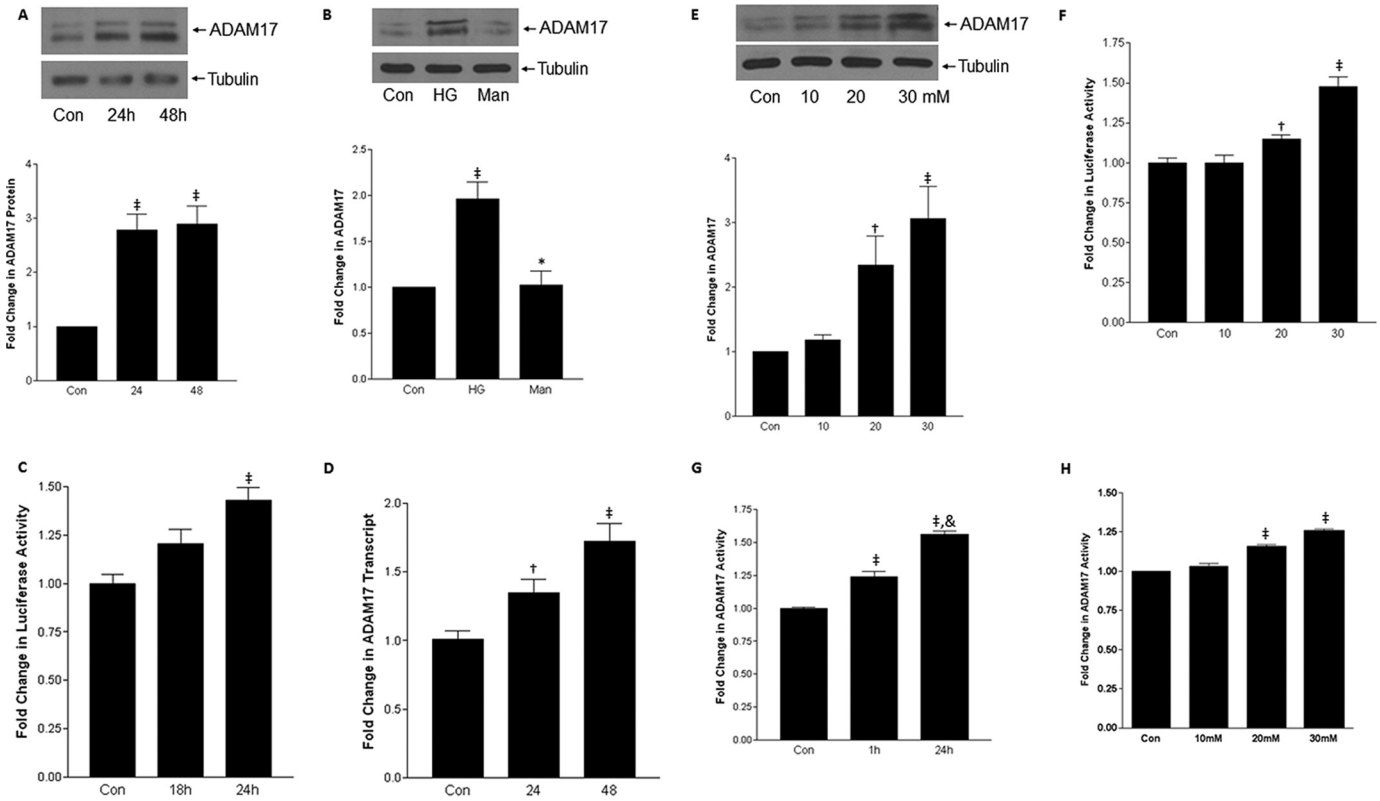


FIGURE 1. **ADAM17 is up-regulated by glucose.** *A* and *B*, MC were treated with HG for 24 or 48 h (*A*) or with HG or mannitol (*Man*) for 24 h (*B*), and ADAM17 was assessed by immunoblotting (*A*, ‡, $p < 0.001$ versus con, $n = 8$; *B*, ‡, $p < 0.001$ versus con, *, $p < 0.001$ versus HG, $n = 8$). *C*, ADAM17 promoter activation in response to HG was assessed by luciferase activity (‡, $p < 0.001$ *versus* con, $n = 6$). *D*, ADAM17 transcript levels were assessed by quantitative RT-PCR (†, $p < 0.05$ *versus* con, ‡, $p < 0.001$ *versus* con, $n = 4$). *E* and *F*, MC were treated with increasing concentrations of glucose for 24 h, and ADAM17 was assessed by immunoblotting (*E*, †, $p < 0.05$ *versus* con, ‡, $p < 0.01$ *versus* con, $n = 6$), or its promoter activity was assessed by luciferase assay (*F*, †, $p < 0.05$ *versus* con, ‡, $p < 0.001$ *versus* con, $n = 8$). *G*, ADAM17 activity was assessed after 1 or 24 h of HG as described under "Experimental Procedures" (‡, $p < 0.001$ *versus* con, &, $p < 0.001$ *versus* 1 h, $n = 8$). *H*, ADAM17 activity was assessed after 1 h of increasing concentrations of HG (‡, $p < 0.001$ *versus* con, $n = 3$). Data are presented as the mean ± S.E.

currently available inhibitors and/or their toxicity, which precludes their long-term use (8).

Interestingly, increased ADAM17 protein expression has been observed in kidneys of both diabetic humans and animal models (9, 10). The mechanism underlying this observation is unknown. ADAM17 expression may be increased at several levels, including augmented processing from the precursor form to active enzyme, prolonged half-life of the mature enzyme, and increased transcription. The transcriptional response has been seen in response to hypoxia, cytokines, and growth factors, but was also shown to be cell-specific in response to a given stimulus (11–14). For example, TNF$\alpha$ induced ADAM17 in endothelial cells, but not in monocytes (14). Whether HG increases ADAM17 expression and transcription are unknown and are addressed by our studies. Furthermore, because systemic overexpression of ADAM17 did not enhance shedding activity (15), we also investigated whether increases in ADAM17 expression in response to HG correlate with augmented activity.

In contrast to ADAM17 activation, comparatively little attention has been given to identifying the mechanism of ADAM17 gene regulation. Both hypoxia and TNF$\alpha$ were shown to induce ADAM17 transcription through hypoxia-inducible factor-1 (HIF-1) (13). This is a transcription factor composed of two subunits. HIF-1$\beta$ is constitutively expressed, whereas the expression level of HIF-1$\alpha$ is regulated. HIF-1$\alpha$ is continuously synthesized, and under normoxic conditions undergoes hydroxylation and proteasome-mediated degradation. This process is inhibited by hypoxia (16), but may also occur under normoxic conditions. Indeed, glucose was shown to activate HIF-1 in MC and in kidneys of diabetic mice (17, 18). We thus also sought to determine whether HIF-1$\alpha$ might be involved in ADAM17 regulation by glucose.

In the current study, we demonstrate the transcriptional up-regulation of ADAM17 by HG, mediated by HIF-1. Importantly, ADAM17 up-regulation is associated with greater ADAM17 activation, suggesting a potentiation of its effects on matrix production. This opens a new avenue for targeting ADAM17 synthesis as a potential therapeutic option for diabetic kidney disease.

## Experimental Procedures

*Cell Culture*—Sprague-Dawley primary rat MC (passages 6–15) were cultured in Dulbecco's modified Eagle's medium supplemented with 20% fetal calf serum (Invitrogen), streptomycin (100 $\mu$g/ml), and penicillin (100 units/ml) at 37 °C in 95% air, 5% $CO_2$. Medium contained 5.6 mmol/liter glucose. Either 24.4 mmol/liter glucose or 24.4 mmol/liter mannitol (final concentration 30 mmol/liter) was added for high glucose or osmotic control, respectively. MC were made quiescent by serum deprivation for 24 h prior to treatment. Pharmacologic inhibitors were

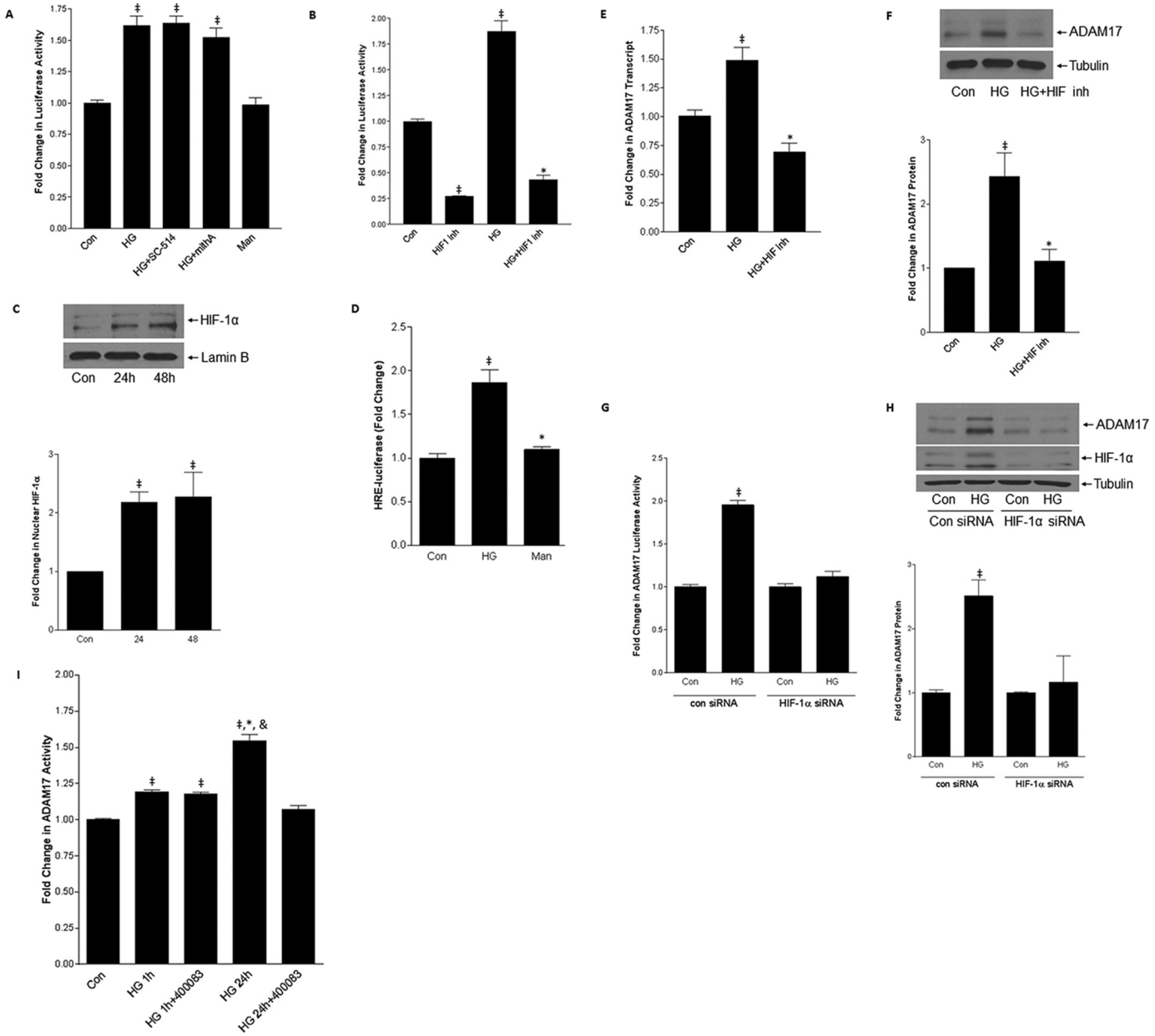


FIGURE 2. **HIF-1$\alpha$ regulates glucose-induced ADAM17 up-regulation in MC.** *A*, MC were treated with the NF-$\kappa$B inhibitor SC-514 or the Sp1 inhibitor mithramycin (*mithA*) prior to HG, or with mannitol (*Man*), and ADAM17 promoter activity was assessed (‡, $p < 0.001$ *versus* con, $n = 6$). *B*, ADAM17 promoter activation was assessed after treatment with the HIF-1$\alpha$ inhibitor (*inh*) 400083 with or without HG for 24 h (‡, $p < 0.001$ *versus* con, *, $p < 0.001$ *versus* HG, $n = 9$). *C*, HIF-1$\alpha$ activation was assessed by its nuclear levels after HG for 24 or 48 h (‡, $p < 0.05$ *versus* con, $n = 4$). *D*, HIF-1 activation was determined by activation of HRE-luciferase in response to HG or mannitol for 24 h (‡, $p < 0.001$ *versus* con, *, $p < 0.01$ *versus* HG, $n = 9$). *E* and *F*, the effects of 400083 on HG (24-h)-induced ADAM17 transcript and protein up-regulation were assessed (*E*, ‡, $p < 0.01$ *versus* con, *, $p < 0.001$ *versus* HG, $n = 6$; *F*, ‡, $p < 0.01$ *versus* con, *, $p < 0.01$ *versus* HG, $n = 5$). *G* and *H*, HG (24-h)-induced ADAM17 promoter luciferase activation and protein up-regulation were determined in MC transfected with control or HIF-1$\alpha$ siRNA. Successful down-regulation of HIF-1$\alpha$ is shown in *H* (*G*, ‡, $p < 0.001$ *versus* con, $n = 12$; *H*, ‡, $p < 0.01$ *versus* con, $n = 3$). *I*, ADAM17 activity was assessed after 1 or 24 h of HG $\pm$ the HIF-1$\alpha$ inhibitor 400083 (‡, $p < 0.001$ *versus* con, *, $p < 0.001$ *versus* HG 1 h, &, $p < 0.001$ *versus* HG 24 h +400083, $n = 4$). Data are presented as the mean $\pm$ S.E.

added prior to glucose as follows: SC514 (25 $\mu$M, 1 h, Cayman Chemical), mithramycin (150 nM, 1 h, A. G. Scientific), 400083 (2.6 $\mu$M, 1 h, EMD Millipore), PD168393 (0.5 $\mu$M, 30 min, EMD Millipore), AG1478 (1 $\mu$M, 30 min, Sigma), heparin (100 units/ml, 30 min, Pharmaceutical Partners of Canada), CRM197 (500 ng/ml, 1 h, Sigma), TAPI2 (100 $\mu$M, 1 h, EMD Millipore), GW280264 (5 $\mu$M, 1 h, generously provided by Dr. A. Ludwig, Aachen University, Germany (19)), LY294002 (10 $\mu$M, 30 min, Sigma), wortmannin (100 nM, 1 h, Sigma), Akt inhibitor VIII (10 $\mu$M, 1 h, EMD Millipore), or U0126 (10 $\mu$M, 30 min, Sigma).

*Protein Extraction and Analysis*—Cells were lysed and protein extracted as we have described previously, with the addition of BB-94 (1 $\mu$M, Tocris) to the lysis buffer (20). Cell lysates were centrifuged at 4 °C, 14,000 rpm for 10 min to pellet cell debris. Supernatant (50 $\mu$g) was separated on SDS-PAGE, and Western blotting performed. Antibodies were goat polyclonal ADAM17 (1:1000, Santa Cruz Biotechnology), monoclonal tubulin (0.5 $\mu$g/ml, Sigma), rabbit HIF-1$\alpha$ (1:1000, Sigma), and polyclonal phospho-EGFR Tyr-1068 (1:1000, Sigma).

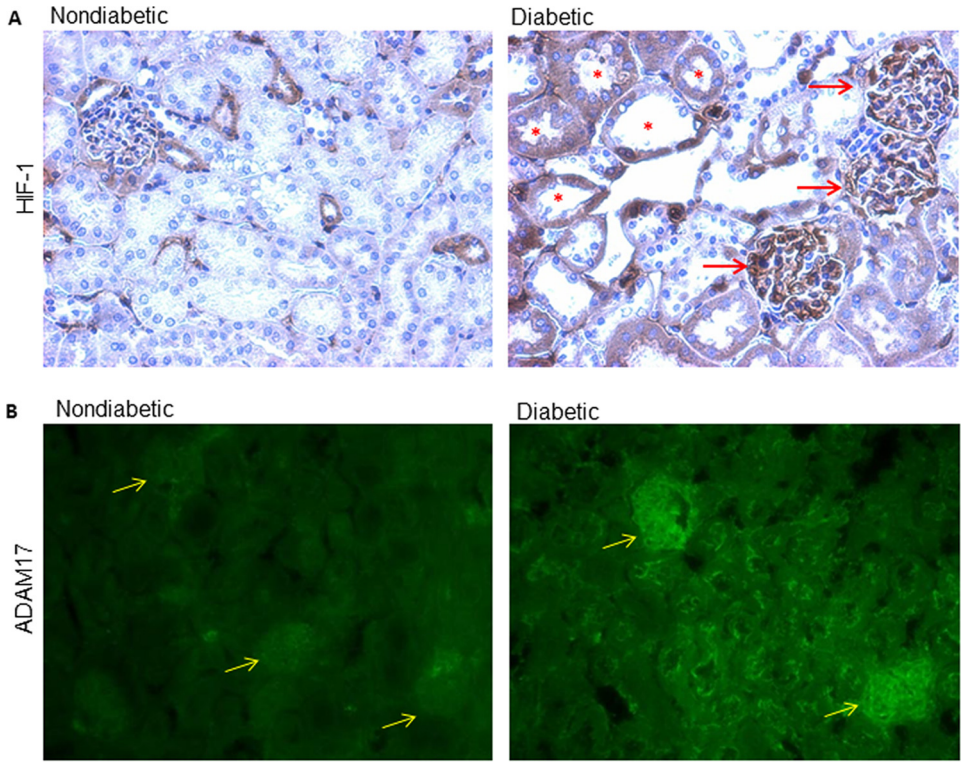


FIGURE 3. **HIF-1 and ADAM17 up-regulation are seen in diabetic kidneys.** *A* and *B*, kidneys from type 1 diabetic mice and their nondiabetic littermates were stained for HIF-1$\alpha$ by immunohistochemistry (*A*) and ADAM17 by immunofluorescence (*B*) as outlined under "Experimental Procedures." *Arrows* identify glomeruli in *A* and *B*, and *asterisks* identify tubules in *A*.

For nuclear lysates, cells were lysed in hypotonic buffer and centrifuged at 500 rpm for 10 min at 4 °C, and the pellet containing the nucleus was resuspended in buffer and sonicated. Nuclear protein loading was assessed by immunoblotting for lamin B (goat polyclonal, 1:1000, Santa Cruz Biotechnology).

*Luciferase Assay*—MC plated to 85% confluence were transfected with 0.5 $\mu$g of various luciferase constructs and 0.05 $\mu$g of pCMV-$\beta$-gal (Clontech) using Lipofectamine (Qiagen). Luciferase constructs used were the ADAM17 promoter (−2304 to −1), its various deletion constructs, and H4mut in which the hypoxia-response element (HRE) sequence at position −607 is mutated as described previously (13). The H1mut was generated by replacing the HRE motif 5′-CAAGTGTG-3′ at position −2279 by 5′-CAAGGCTG-3′ (the mutated base pairs are underlined) in the full-length promoter and confirmed by sequencing. HRE-luciferase, which contains three tandem HRE sites, was obtained from Addgene (plasmid 26731).

MC were serum-deprived overnight 24 h after transfection and then exposed to glucose for 24 h. Lysis was achieved with reporter lysis buffer (Promega) using one freeze-thaw cycle, and luciferase and $\beta$-gal activities were measured on clarified lysate using specific kits (Promega) with a Berthold luminometer and a plate reader (420 nm), respectively. $\beta$-gal activity was used to adjust for transfection efficiency.

*Quantitative Real-time PCR*—Total RNA was extracted using TRIzol according to the manufacturer's instructions (Life Technologies). Reverse transcription was performed using standard methods, and cDNA was analyzed by real-time PCR using a SYBR Green PCR master mix kit (Applied Biosystems). Amplification using specific primers for ADAM17 with 18S as an internal control was measured continuously using an ABI 7500 sequence detector (Applied Biosystems). -Fold change over control was calculated using the ΔΔCt method.

*ADAM17 Activity Assay*—After treatment, protein was extracted from MC lysed in activity assay buffer (50 mM Tris-HCl, pH 7.4, 25 mM NaCl, 4% glycerol, 10 mM $ZnCl_2$). ADAM17 activity was measured in duplicate for each sample using 20 $\mu$g of protein and the TNF-$\alpha$ converting enzyme (TACE) substrate IV (Calbiochem). Cleavage of this substrate was measured in a fluorometer at 420 nm.

*RNA Interference*—Rat HIF-1$\alpha$ siGENOME siRNA was purchased from Dharmacon. HB-EGF and ADAM17 Silencer Select siRNA and control non-targeting siRNA were purchased from Applied Biosystems. MC were transfected with 200 nM HIF-1$\alpha$ or 100 nM ADAM17 or HB-EGF using Lipofectamine RNAiMAX (Life Technologies) at 60% confluence. After 48 h, cells were serum-deprived for 24 h, and cell response to HG assessed. Protein expression was used to assess the efficacy of down-regulation by RNAi.

*ChIP*—After treatment, MC were cross-linked with 1% formaldehyde, washed, and scraped into cold PBS with protease inhibitors. After centrifugation, the cell pellet was resuspended in buffer (20 mM HEPES, pH 7.9, 25% glycerol, 420 mM NaCl,1.5 mM $MgCl_2$,0.2 mM EDTA, protease inhibitors), incubated on ice for 20 min, and centrifuged. The pellet (nucleus) was resuspended in breaking buffer (50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 150 mM NaCl, 1% SDS, 2% Triton X-100, protease inhibitors) and sonicated 5 × 10 s, and Triton buffer was added (50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 150 mM NaCl, 0.1% Triton X-100). An aliquot was reserved as the input, and the

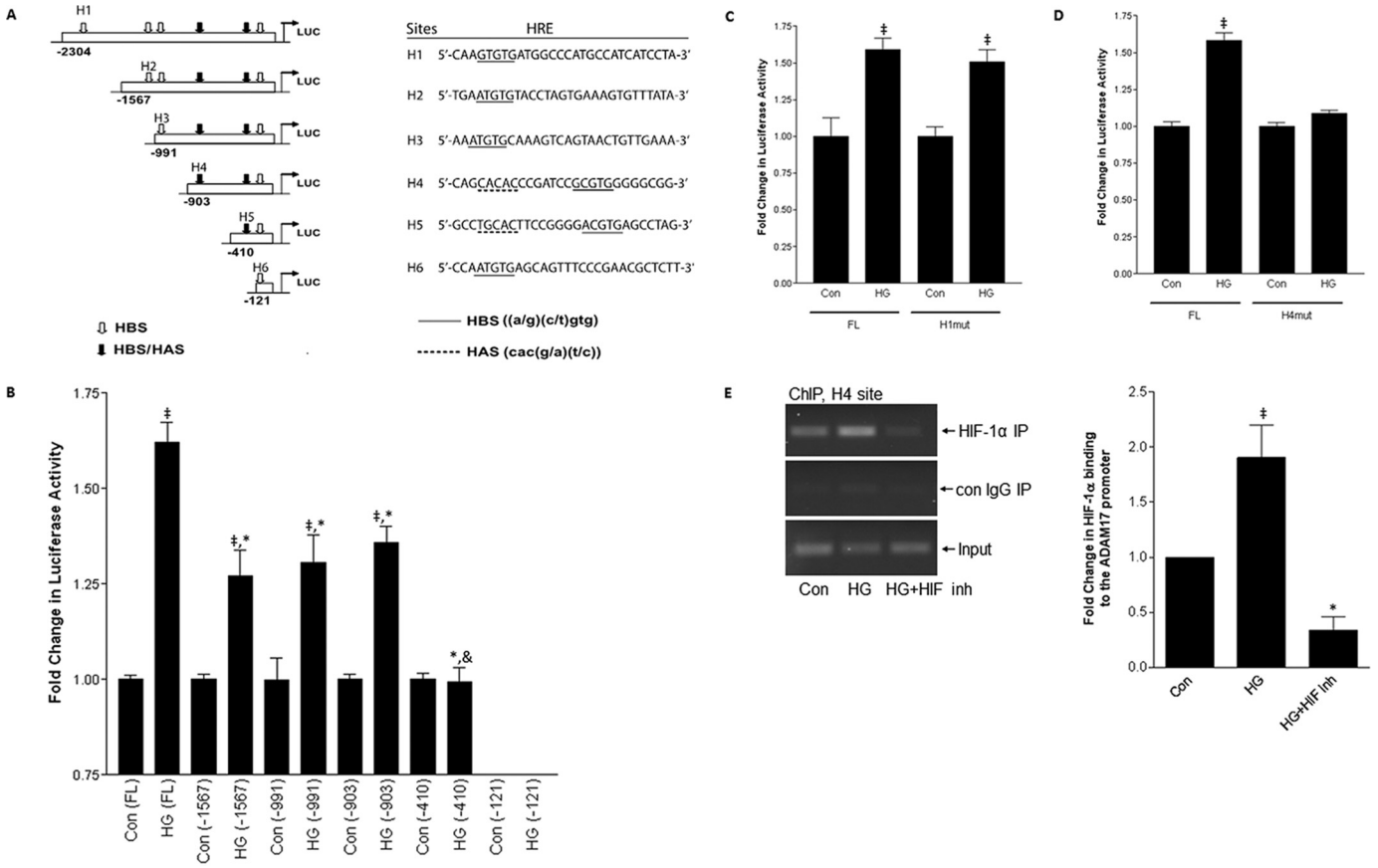


FIGURE 4. **The HRE at −607 mediates HIF-1$\alpha$ regulation of the ADAM17 promoter in response to HG.** *A*, diagram showing the successive ADAM17 promoter luciferase (*LUC*) deletion constructs used to isolate putative HIF-1 recognition sequences. Shown are the HREs composed of a HIF-1 binding site (*HBS*) alone or with a HIF-1 ancillary sequence (*HAS*). *B*, successive ADAM17 promoter deletion constructs were generated and placed upstream of the luciferase gene. The HG-induced response was attenuated with the −1567 and −410 constructs as compared with the full-length (*FL*) promoter (‡, $p < 0.01$ *versus* its own control, *, $p < 0.01$ *versus* HG (FL), &, $p < 0.001$ *versus* HG (−903), $n = 12$). These harbor the H1 and H4 HRE sites, respectively. *C* and *D*, the H1 site at −2279 and H4 site at −607 in the full-length ADAM17 promoter luciferase construct were mutated (*mut*), and their response to 24 h of HG was tested (*C*, ‡, $p < 0.01$ *versus* its own con, $n = 6$; *D*, ‡, $p < 0.001$ *versus* con, $n = 6$). *E*, ChIP was used to detect whether HIF-1$\alpha$ binding to the H4 site was induced by HG, with IgG immunoprecipitation (*IP*) serving as a control. Amplification of input DNA served as an additional control (‡, $p < 0.05$ *versus* con, *, $p < 0.01$ *versus* HG, $n = 4$). *inh*, inhibitor. Data are presented as the mean ± S.E.

remainder was divided to immunoprecipitate with control goat IgG (Jackson ImmunoResearch Laboratories) or goat HIF-1$\alpha$ (R&D Systems) antibodies followed by incubation with protein G beads. Samples were washed three times in Triton buffer, SDS buffer was added (62.5 mM Tris-HCl, pH 6.8, 200 mM NaCl, 2% SDS, 10 mM DTT, 2 $\mu$l of proteinase K (40 mg/ml)), and then samples were vortexed and incubated at 65 °C overnight to reverse cross-linking. DNA was isolated using phenol/chloroform extraction and resuspended in distilled $H_2O$. PCR was performed using the following primers to amplify the HRE site at −607: 5′-CAGTCCCCTGAGCATTTTCAGTGACAA (forward) and 5′-CGTGATTAAAGTCCCCTTCAGCGTC (reverse), at 55 °C annealing temperature for 36 cycles.

*Immunofluorescence*—MC were washed in PBS, fixed with 3.7% formaldehyde, permeabilized with 0.1% Triton X-100, blocked with 1% BSA/3% donkey serum, and incubated with mouse HIF-1 (1:50, R&D Systems). After incubation with secondary antibody (donkey Alexa Fluor 488), mounting medium was applied, and the slide was imaged.

*Diabetic Mice*—Type 1 diabetic male Akita mice (C57Bl/6) and their wild-type counterparts were housed under standard conditions with free access to regular chow and water. Procedures were approved by the Centre de Recherche du Centre Hospitalier de l'Université de Montréal (CRCHUM) Animal Care Committee and conformed to Canadian Council on Animal Care guidelines. Mice were euthanized at 20 weeks of age, and kidneys were harvested. HIF-1$\alpha$ was assessed by immunohistochemistry on formalin-fixed sections. Heat-induced epitope retrieval was used. ADAM17 was assessed on cortical sections stored in OCT by immunofluorescent staining as described (21). Primary antisera used were mouse HIF-1$\alpha$ (1:20, R&D Systems) and rabbit polyclonal ADAM17 (1:100, Enzo Life Sciences).

*Statistical Analyses*—Statistical analyses were performed with SPSS 22 for Windows using one-way analysis of variance, with Tukey's honest significant difference for post hoc analysis for experiments with more than two groups. A $p$ value $< 0.05$ (two-tailed) was considered significant. Data are presented as the mean ± S.E. The number of experimental repetitions is denoted by $n$.

## Results

*ADAM17 Is Up-regulated by Glucose*—Increased mesangial expression of ADAM17 has been noted in human diabetic

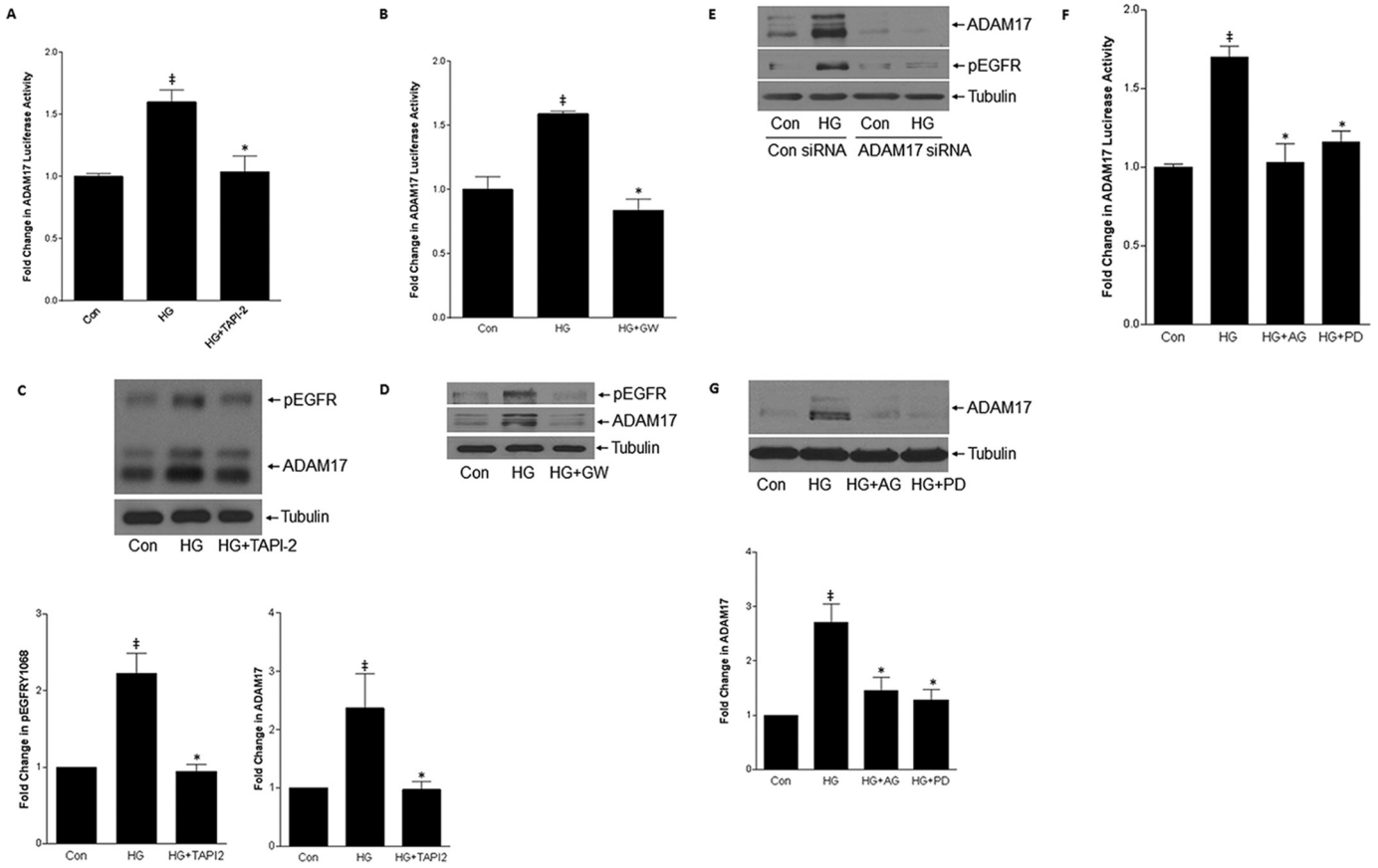


FIGURE 5. **ADAM17 up-regulation by HG occurs through a positive regulatory feedback loop involving EGFR/ADAM17 signaling.** *A* and *B*, ADAM17 promoter luciferase activity was tested in response to HG for 24 h ± the ADAM17 inhibitor TAPI-2 (*A*, ‡, $p < 0.001$ *versus* con, *, $p < 0.001$ *versus* HG, $n = 12$) or GW280264 (*GW*) (*B*, ‡, $p < 0.01$ *versus* con, *, $p < 0.01$ *versus* HG, $n = 3$). *C* and *D*, the effects of both ADAM17 inhibitors were tested on ADAM17 up-regulation by HG for 24 h. EGFR activation, determined by its autophosphorylation on Tyr-1068, was also determined (*C*, ‡, $p < 0.001$ *versus* con, *, $p < 0.001$ *versus* HG for phospho-EGFR (*pEGFR*) and ‡, $p < 0.05$ *versus* con, *, $p < 0.05$ *versus* HG for ADAM17, $n = 6$). *E*, HG (24-h)-induced EGFR activation was determined in MC transfected with control or ADAM17 siRNA. Successful down-regulation of ADAM17 is also shown. *F* and *G*, the effects of EGFR inhibition with AG1478 (*AG*) or PD168393 (*PD*) on ADAM17 promoter luciferase activation or protein induction by HG for 24 h were assessed (*F*, ‡, $p < 0.001$ *versus* con, *, $p < 0.05$ *versus* HG, $n = 4$; *G*, ‡, $p < 0.001$ *versus* con, *, $p < 0.01$ *versus* HG, $n = 7$). Data are presented as the mean ± S.E.

nephropathy (9). We thus wanted to determine whether HG can increase ADAM17 expression in primary MC. As shown in Fig. 1, *A* and *B*, 24–48 h of HG, but not 24 h of the osmotic control mannitol, increased ADAM17 protein levels. To assess whether glucose regulates ADAM17 at the transcriptional level, we tested its effects on the ADAM17 promoter using a promoter-luciferase construct. Fig. 1*C* shows increased luciferase activation by HG. ADAM17 transcript levels are correspondingly increased, as assessed by quantitative RT-PCR (Fig. 1*D*). We next tested the effects of increasing concentrations of glucose on ADAM17 regulation. Fig. 1, *E* and *F*, show the dose-dependent effects of HG on ADAM17 protein and promoter activation, respectively, with significant up-regulation seen at higher concentrations of glucose. To verify that elevated ADAM17 levels were meaningful, we tested ADAM17 activity in cell lysates. Fig. 1*G* shows significantly increased ADAM17 activity after 24 h of HG as compared with shorter-term (1-h) incubation. Fig. 1*H* demonstrates the dose-dependent effects of HG (1 h) on ADAM17 activation. Thus, HG regulates ADAM17 both through activation of the enzyme, seen with early HG responses, as well as through gene and protein up-regulation, seen with longer-term HG exposure (24 h). Furthermore, longer-term ADAM17 up-regulation is also associated with greater ADAM17 activation, suggesting a potentiation of its effects on matrix production.

*HIF-1α Regulates Glucose-induced ADAM17 Up-regulation in MC*—The ADAM17 promoter contains multiple Sp1 and HIF-1 binding sites, but no consensus TATA box or AP1 sites. HIF-1 mediated hypoxia-induced ADAM17 up-regulation, in some cases with Sp1 (13, 22–24), whereas NF-κB mediated ADAM17 up-regulation in response to TNFα (13). Using inhibitors of NF-κB (SC-514) and Sp1 (mithramycin A), we excluded a role for these transcription factors in glucose-induced ADAM17 promoter activation in MC (Fig. 2*A*). However, the HIF-1α inhibitor 400083 potently reduced both basal and glucose-induced ADAM17 promoter activity (Fig. 2*B*). HIF-1 activity is regulated by altering its expression levels (16). To confirm increased HIF-1 activation by HG in our culture conditions, MC were treated for 24–48 h, and the presence of HIF-1α in nuclear extracts was assessed. Fig. 2*C* shows increased HIF-1 at both time points. This corresponded to increased HIF-1 transcriptional activity as assessed by activation of a reporter luciferase construct containing tandem HREs (Fig. 2*D*). As a control, mannitol had no effect on HIF-1 activation. We next verified that HIF-1 inhibition also suppresses HG-induced ADAM17 transcript and protein up-regulation

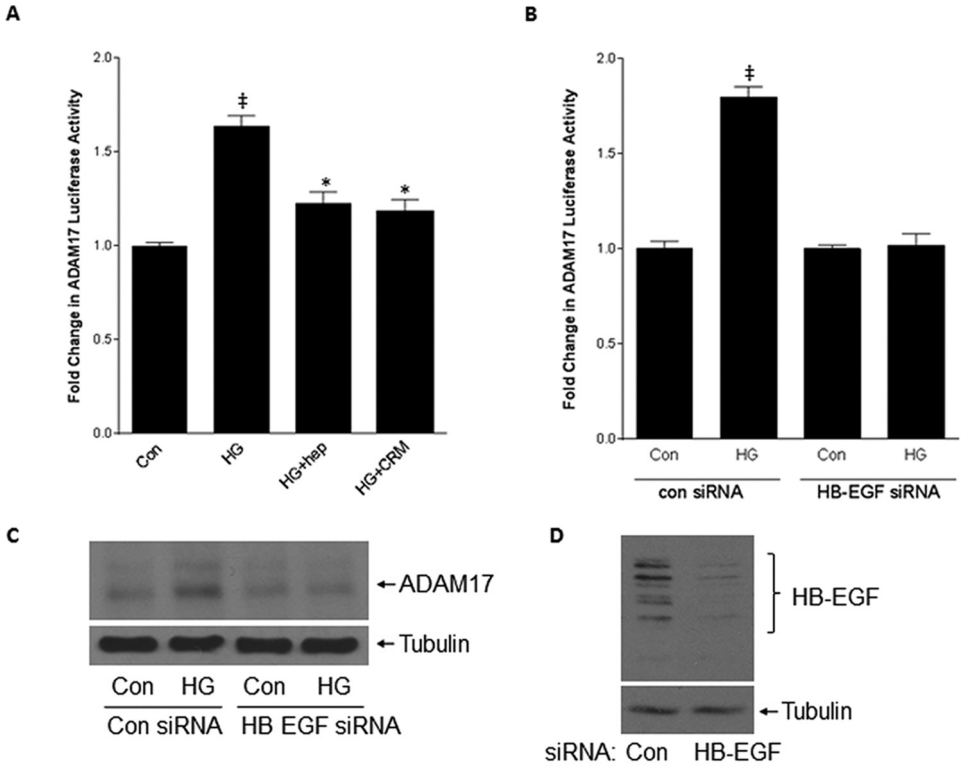


FIGURE 6. **The EGFR ligand HB-EGF mediates ADAM17 up-regulation by HG.** *A*, the effects of the HB-EGF inhibitors heparin (*hep*) and CRM197 (*CRM*) on HG (24-h)-induced activation of the ADAM17 promoter were tested (‡, $p < 0.001$ *versus* con, *, $p < 0.001$ *versus* HG, $n = 8$). *B* and *C*, HG (24-h)-induced ADAM17 promoter luciferase activation and protein up-regulation were determined in MC transfected with control or HB-EGF siRNA (*B*, ‡, $p < 0.001$ *versus* con, $n = 6$). *D*, down-regulation of proHB-EGF by siRNA was confirmed by immunoblotting. ProHB-EGF was detected as multiple bands (~20–30 kDa) representing heterogeneous translation products as reported previously (52). Data are presented as the mean ± S.E.

(Fig. 2, *E* and *F*). Furthermore, down-regulation of HIF-1α using siRNA prevented glucose-induced ADAM17 promoter luciferase activation and protein up-regulation (Fig. 2, *G* and *H*). Successful HIF-1α down-regulation by siRNA is shown in Fig. 2*H*. We next confirmed the functional role of HIF-1α in enhanced ADAM17 activation associated with its up-regulation with longer-term HG exposure. Fig. 2*I* shows that HIF-1α inhibition does not prevent ADAM17 activation in response to 1 h of HG, but does prevent the increased ADAM17 activity seen after 24 h of HG.

To determine whether increased HIF-1α and ADAM17 are also observed *in vivo*, we performed imaging on kidneys from Akita mice. These mice harbor a spontaneous mutation in the insulin 2 gene, leading to low insulin levels and the development of type 1 diabetes with significant hyperglycemia by 3 weeks of age. Kidneys from 20-week-old mice were assessed. Fig. 3*A* shows a clear increase in HIF-1α expression in diabetic glomeruli (*arrows*) as well as in some tubules (*asterisks*). ADAM17 expression is also significantly increased in diabetic glomeruli as compared with glomeruli in control kidneys (Fig. 3*B*; glomeruli are identified by *arrows*).

*Identification of the HRE That Regulates Glucose Activation of the ADAM17 Promoter*—As described and illustrated by Charbonneau *et al.* (13), the ADAM17 promoter contains six putative HIF-1-responsive sites, four of which have the HIF-1 binding sequence and two of which have the HIF-1 binding sequence in association with a HIF-1 ancillary sequence. To determine which site(s) regulate the glucose response in MC, we tested a series of ADAM17 promoter deletion constructs that successively isolate each of the putative HRE sites (Fig. 4*A*). Fig. 4*B* shows a significant reduction in the glucose response with the −1567 and −410 constructs as compared with the full-length −2304 construct. These constructs correspond to deletion of the H1 and H4 sites, respectively, at positions −2279 and −607. To determine whether loss of the HRE in these constructs was responsible for the attenuated glucose response, the H1 and H4 sites were mutated in the full-length construct and their response to HG was tested. As seen in Fig. 4, *C* and *D*, only mutation of H4 abrogated glucose-induced promoter activation. HIF-1α binding at this site was confirmed using ChIP (Fig. 4*E*). This was prevented by the HIF-1α inhibitor 400083. These data support an important role for HIF-1α in mediating HG-induced ADAM17 up-regulation in MC through its binding to the H4 site.

*ADAM17 Up-regulation by HG Occurs through a Positive Regulatory Feedback Loop Involving EGFR/ADAM17 Signaling*—Interestingly, inhibition of ADAM17 activity with TAPI-2, a broad-spectrum inhibitor of TNFα-converting enzymes (ADAM17 and other ADAMs) (25), prevented HG-induced ADAM17 promoter luciferase activation (Fig. 5*A*), suggesting that ADAM17 regulates its own synthesis. This was confirmed using a more selective ADAM17 inhibitor, GW280264 (19) (Fig. 5*B*). ADAM17 protein levels were also blocked by both inhibitors (Fig. 5, *C* and *D*). We had previously shown that ADAM17 cleavage of HB-EGF mediates early (1-h) activation of the EGFR (6). Fig. 5, *C* and *D*, confirm that both

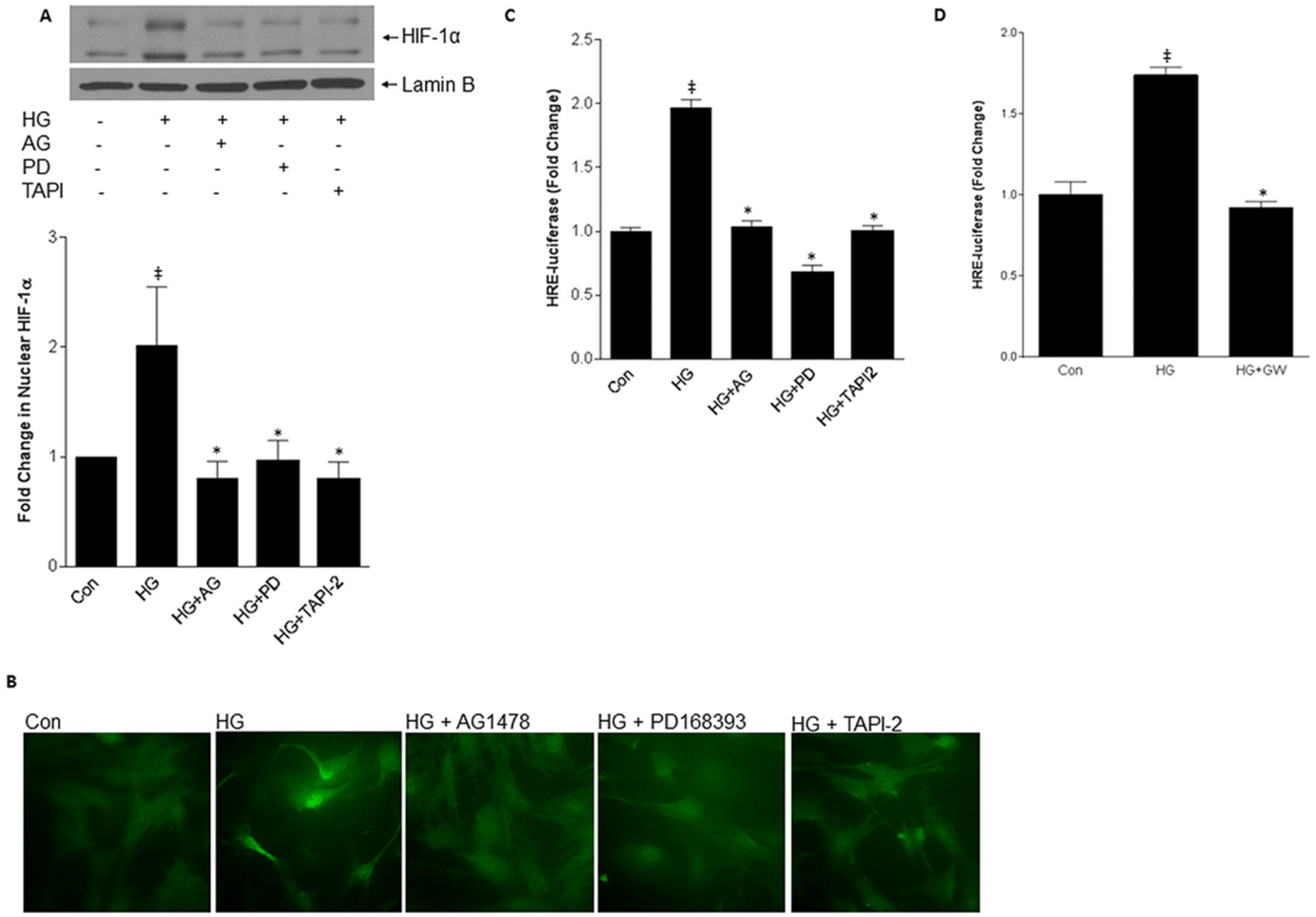


FIGURE 7. **EGFR/ADAM17 regulate HIF-1$\alpha$ activation by HG.** *A*, MC were treated with the EGFR inhibitors AG1478 (*AG*) or PD168393 (*PD*) or the ADAM17 inhibitor TAPI-2 (*TAPI*), and nuclear HIF-1$\alpha$ was assessed after 24 h of HG (‡, $p < 0.05$ *versus* con, *, $p < 0.05$ *versus* HG, $n = 4$). *B*, HIF-1 was assessed by immunofluorescence after treatment as described for *A*. *C*, the effects of EGFR and ADAM17 inhibitors on HIF-1 transcriptional activity were also determined using HRE-luciferase (‡, $p < 0.001$ *versus* con, *, $p < 0.001$ *versus* HG, $n = 6$). *D*, the effects of ADAM17 inhibition on HRE-luciferase activation by HG were confirmed using a second inhibitor GW280264 (*GW*) (‡, $p < 0.001$ *versus* con, *, $p < 0.001$ *versus* HG, $n = 3$). Data are presented as the mean ± S.E.

ADAM17 inhibitors also prevented EGFR activation after 24 h of HG. The important role of ADAM17 in longer-term EGFR activation was verified using siRNA, with Fig. 5*E* showing that ADAM17 down-regulation prevented EGFR activation by 24 h of HG. To determine whether EGFR activation was required for ADAM17 up-regulation by HG, we used two selective EGFR inhibitors, AG1478 and PD168393. Both inhibitors strongly reduced HG induction of ADAM17 promoter luciferase activity (Fig. 5*F*) and protein expression (Fig. 5*G*).

We next determined whether HB-EGF mediates HG-induced ADAM17 up-regulation. For this, we used two distinct inhibitors. Heparin competes with cell surface-associated heparan sulfate proteoglycans, coreceptors for HB-EGF binding to EGFR (26). CRM197, a mutated nontoxic form of diphtheria toxin that binds the extracellular domain of HB-EGF and blocks its biological activity, is a more specific inhibitor (27). Both inhibitors significantly blocked ADAM17 promoter luciferase activation by HG (Fig. 6*A*). We then down-regulated the expression of HB-EGF using siRNA, which we have shown previously to effectively reduce HB-EGF protein expression (6). As shown in Fig. 6, *B* and *C*, this prevented ADAM17 promoter luciferase activation and protein up-regulation by HG. Fig. 6*D* shows effective down-regulation of HB-EGF by its siRNA. Taken together, these data indicate that ADAM17 induces its own expression in response to HG through EGFR activation via HB-EGF.

*HG-induced HIF-1α Activation and ADAM17 Up-regulation Are Mediated by PI3K/Akt and Erk Signaling Downstream of the EGFR*—The EGFR has been shown to activate HIF-1$\alpha$ in other settings (28), suggesting that it might mediate HIF-1$\alpha$ activation by HG. We first tested the effects of EGFR inhibitors AG1478 and PD168393 and the ADAM17 inhibitor TAPI-2. All prevented HG-induced HIF-1$\alpha$ nuclear accumulation (Fig. 7*A*). This was also seen by immunofluorescence, shown in Fig. 7*B*. Confirming functionality, HG-induced HIF-1 transcriptional activity assessed using the HIF-1-responsive HRE-luciferase was also prevented by these inhibitors (Fig. 7*C*). The more selective ADAM17 inhibitor, GW280264, also prevented HG-induced HIF-1 transcriptional activation (Fig. 7*D*).

Both PI3K/Akt and Erk are well known downstream signaling mediators of the EGFR that we and others have shown are important in glucose-induced profibrotic responses in MC (4, 29). Although they have also been shown to regulate HIF-1$\alpha$ activation in various settings (16, 28, 30, 31), their role in HG-

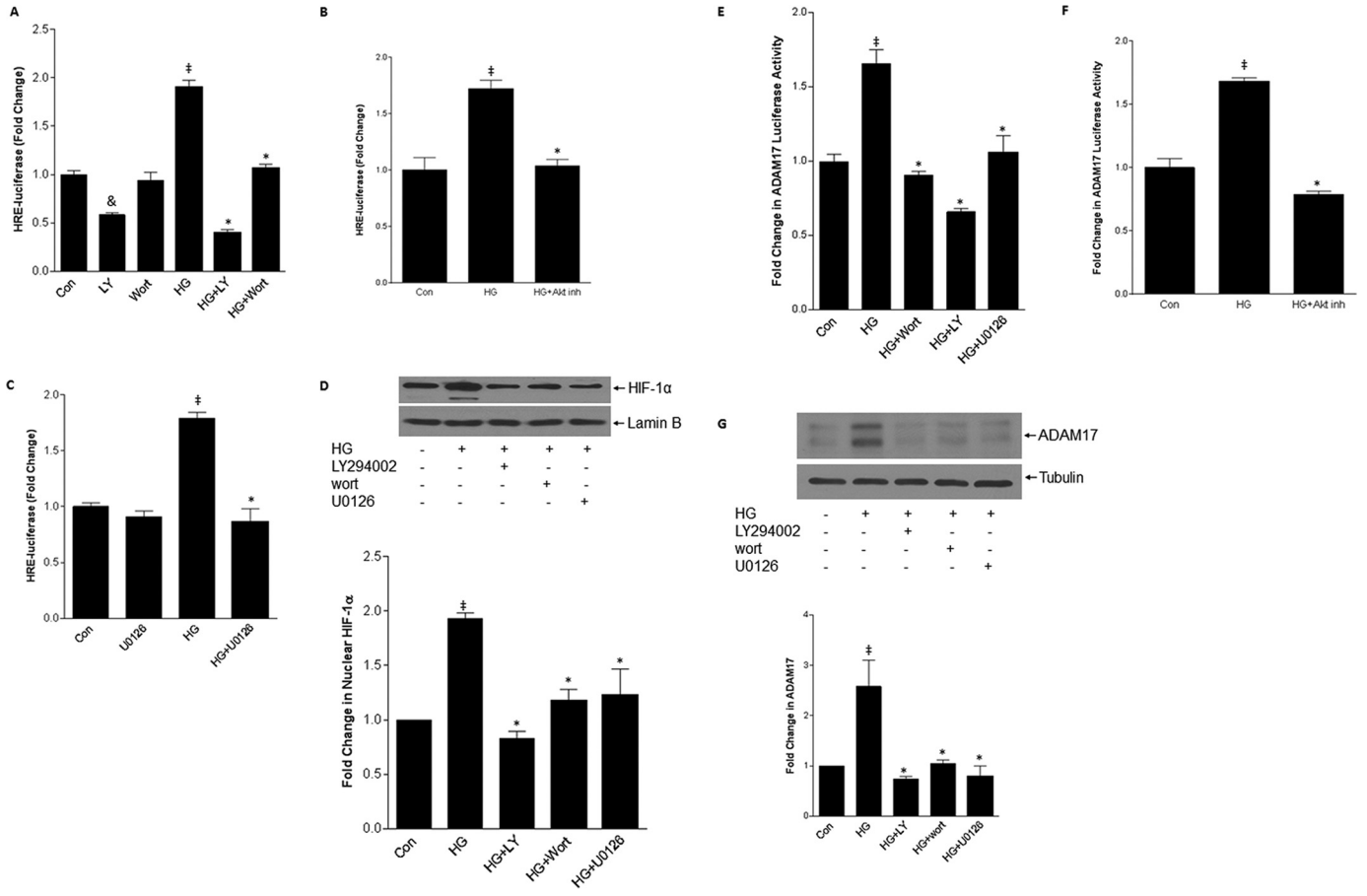


FIGURE 8. **HG-induced HIF-1$\alpha$ activation and ADAM17 up-regulation are mediated by PI3K/Akt and Erk signaling.** *A* and *B*, MC were treated with the PI3K inhibitors LY294002 (*LY*) or wortmannin (*Wort*) (*A*) or Akt inhibitor VIII (*Akt inh*) (*B*) prior to HG for 24 h, and effects on HIF-1 activation were assessed using HRE-luciferase (*A*, &, $p < 0.01$ *versus* con, ‡, $p < 0.001$ *versus* con, *, $p < 0.001$ *versus* HG, $n = 4$; *B*, ‡, $p < 0.01$ *versus* con, *, $p < 0.01$ *versus* HG, $n = 3$). *C*, MC were treated with the MEK inhibitor U0126, and HG-induced HIF-1 activation was assessed using HRE-luciferase (‡, $p < 0.001$ *versus* con, *, $p < 0.001$ *versus* HG, $n = 4$). *D*, nuclear HIF-1$\alpha$ was assessed after treatment of MC with HG ± PI3K inhibitors LY294002 or wortmannin, or MEK inhibitor U0126 (‡, $p < 0.01$ *versus* con, *, $p < 0.05$ *versus* HG, $n = 3$). *E–G*, the effects of PI3K inhibitors LY294002 or wortmannin, Akt inhibitor VIII, or MEK inhibitor U0126 on HG-induced ADAM17 induction were assessed using the ADAM17 promoter luciferase construct (*E* and *F*) or by immunoblotting (*G*) (*E*, ‡, $p < 0.001$ *versus* con, *, $p < 0.001$ *versus* HG, $n = 3$; *F*, ‡, $p < 0.001$, *, $p < 0.001$ *versus* HG, $n = 3$; *G*, ‡, $p < 0.05$ *versus* con, *, $p < 0.05$ *versus* HG, $n = 3$). Data are presented as the mean ± S.E.

induced HIF-1 activation is unknown. We thus first tested the effects of two PI3K inhibitors, LY294002 and wortmannin, and the Akt inhibitor VIII on HIF-1$\alpha$ transcriptional activation by HG. All of these inhibitors prevented HIF-1$\alpha$ transcriptional activity as assessed by HRE-luciferase activation (Fig. 8, *A* and *B*). A similar effect was seen with the inhibitor of Erk activation, U0126 (Fig. 8*C*). PI3K and Erk inhibitors also prevented HG-induced HIF-1$\alpha$ accumulation in nuclear extracts (Fig. 8*D*). In keeping with the inhibitory effects on HIF-1$\alpha$ activation, PI3K/Akt and Erk inhibition also prevented glucose-induced activation of the ADAM17 promoter luciferase construct (Fig. 8, *E* and *F*) and ADAM17 protein up-regulation (Fig. 8*G*). Thus, PI3K/Akt and Erk signaling are required upstream of HIF-1$\alpha$ activation for HG-induced ADAM17 up-regulation. The schematic summary of our findings is shown in Fig. 9.

## Discussion

Although ADAM17 has been recognized as a potentially important therapeutic target in a variety of diseases, an important role for ADAM17 in the pathogenesis of diabetic nephropathy is now emerging. We and others have shown that ADAM17 is activated by HG in MC and regulates the profibrotic response to glucose in these cells (6, 32). In diabetic mice and humans, increased renal ADAM17 expression has also been observed (7, 9, 10), and in mice, this was normalized by insulin (10). We now show that HG also induces the transcriptional up-regulation of ADAM17, and importantly that this translates to augmented ADAM17 activity, suggesting a potentiation of its effects on matrix production. We further identified a critical role for HIF-1$\alpha$ in regulating ADAM17 induction by HG. Interestingly, ADAM17 itself, through activation of the EGFR and downstream PI3K/Akt and Erk activation, induces its own up-regulation (Fig. 9). We thus describe an amplification loop that augments the fibrotic response and offers new targets for therapeutic intervention.

Although the mechanism of ADAM17 activation has been extensively studied, relatively little attention has been paid to ADAM17 gene regulation. While hypoxia, inflammatory cytokines, particularly TNF$\alpha$, and several growth factors were found to increase ADAM17 transcript levels (13, 14, 23, 24, 33, 34), no studies have as yet examined the effects of HG on ADAM17 gene regulation. We thus aimed to define this mechanism. We first assessed Sp1 because the ADAM17 promoter contains multiple Sp1 binding sites and Sp1 is known to be

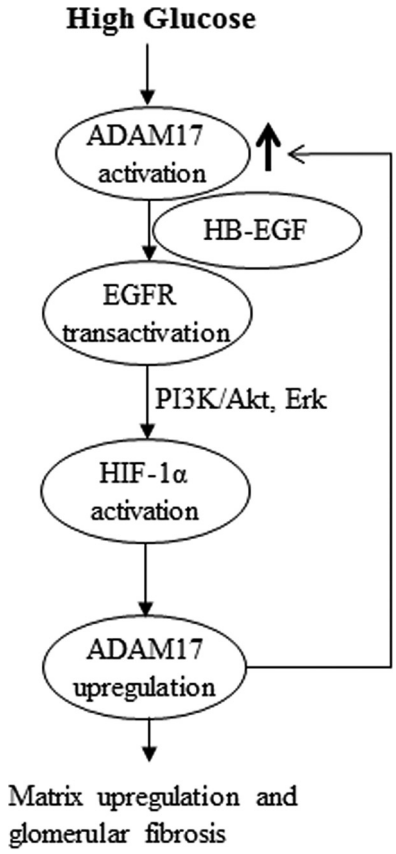


FIGURE 9. **Schematic of HG-induced ADAM17 up-regulation.** We have previously shown that HG leads to activation of ADAM17 with subsequent cleavage of the EGFR ligand HB-EGF. This activates the EGFR to increase production of matrix proteins in MC (6). We now show that active ADAM17 induces its own up-regulation through PI3K/Akt- and Erk-dependent activation of HIF-1α. Subsequent HIF-1α binding to the ADAM17 promoter drives its transcription. Up-regulation of ADAM17 leads to augmentation of ADAM17 activity beyond that of short-term HG exposure, supporting the relevance of elevated ADAM17 expression to enhanced profibrotic signaling.

activated by HG in MC (35, 36). Sp1 inhibition, however, did not affect HG-induced ADAM17 up-regulation. Our data also excluded a role for NF-κB, although this mediates ADAM17 up-regulation in response to TNFα (13), demonstrating stimulus-specific gene regulation.

We finally assessed a potential role for HIF-1α because glucose has been show to increase its activity in MC (17), and several HIF-1 binding sites have been identified in the ADAM17 promoter. These regulated hypoxia- and TNFα-induced ADAM17 synthesis (13, 23, 24). Using both an inhibitor and siRNA, we found that HIF-1α is a potent mediator of ADAM17 up-regulation in response to HG through its binding to the H4, but not H1, consensus binding sequence. This is similar to the recently reported role of HIF-1α in the induction of ADAM17 expression by angiotensin II in vascular smooth muscle cells (37). However, the preserved glucose response of the H1 mutant in the context of attenuation of promoter activity in the −1567 deletion construct suggests the presence of additional glucose-responsive transcriptional regulators of the ADAM17 promoter, the identification of which is the goal of future studies.

A role for HIF-1 in the pathogenesis of renal fibrosis including that of diabetic nephropathy has been shown by several groups (18, 38, 39), although others have recently suggested that HIF activation may protect against diabetic nephropathy (40). In this latter study, diabetic rats were treated for 4 weeks with cobalt chloride, a relatively nonspecific activator of HIF. Treatment induced significant weight loss in diabetic rats with associated lower glucose levels, and matrix deposition was not assessed. Further studies testing the role of HIF-1 in diabetic nephropathy are thus needed. Our studies support a profibrotic role for HIF-1 through its up-regulation of ADAM17.

Very little is known of the signaling leading to ADAM17 up-regulation. Of interest, TNFα, the cleavage product of the ADAM17 substrate pro-TNFα, itself induced ADAM17 up-regulation, suggesting the presence of a positive feedback loop (24). Because TNFα was also shown to be increased in diabetic kidneys (41), we assessed it as a potential mediator of ADAM17 up-regulation with longer term HG exposure. No detectable TNFα secretion into medium by MC with either short (1-h) or prolonged (24- or 48-h) HG exposure was observed using ELISA (Pierce, data not shown). Our data do, however, suggest that a positive feedback mechanism does occur whereby ADAM17 regulates persistent HG-induced EGFR transactivation through HB-EGF release to induce its own synthesis (Fig. 9). Interestingly, increased HB-EGF has also been observed in both type 1 and type 2 diabetic kidneys (42, 43), suggesting augmentation at several points in this proposed pathway. Recently, endothelial HB-EGF deletion in endothelial NOS knock-out diabetic mice was shown to protect against diabetic nephropathy (44), although whether HB-EGF inhibition would ameliorate diabetic nephropathy in mice that are not deficient in endothelial NOS remains to be tested.

Accumulating evidence supports an important role for the EGFR in diabetic renal disease. We and others showed increased glomerular and cortical EGFR transactivation in both type 1 and type 2 diabetic models (4, 43, 44). EGFR inhibition decreased renal and glomerular hypertrophy, albuminuria, and glomerular sclerosis (45–47). We previously showed that EGFR is transactivated by HG in MC and initiates signaling through PI3K/Akt to effect collagen I and TGFβ1 up-regulation (4, 5, 48). Our data now show that this signaling pathway drives the up-regulation of ADAM17, providing evidence for multiple profibrotic targets downstream of EGFR activation.

Our data also show that EGFR signaling is critical for the increased expression and activity of HIF-1α in response to HG, and that PI3K and Erk are required for this. In other cell types, PI3K/Akt and Erk mediated growth factor, cytokine, and smoke-induced HIF-1α activation not through protein stabilization, but rather through stimulation of HIF-1α translation and transcriptional activity (16, 28, 30, 31). Although PI3K signaling can stimulate HIF-1α translation (30, 49), Erk was found to regulate the transactivation activity of HIF-1α through promotion of its interaction with the cofactor p300/CREB-binding protein (CBP) (50). Erk was also shown to phosphorylate HIF-1α, although the functional impact of this is as yet unknown (16, 49, 50). In HG-treated MC, Isoe *et al.* (17) showed that HG increased HIF-1α transcription through carbohydrate-responsive element-binding protein (ChREBP). Whether EGFR/PI3K and Erk regulate carbohydrate-responsive element-binding protein remains to be determined.

With the ability of ADAM17 to cleave a variety of substrates, this enzyme is now recognized as a potential therapeutic target in multiple diseases including cancer, inflammatory, and potentially, vascular diseases. Our findings support further investigation into the efficacy of ADAM17 inhibition as a therapeutic option for diabetic kidney disease. Given that currently available inhibitors either are not specific for ADAM17 or have toxicity, which precludes their long-term use (8), targeting ADAM17 up-regulation may be an alternate effective therapeutic option that should be explored. Although we have shown an important role for HIF-1 in glucose-induced ADAM17 transcription, systemic inhibition of HIF-1 may not be feasible given its role in the regulation of a wide spectrum of hypoxia responses. These include erythropoietin production and the promotion of wound healing in skin, an important consideration in diabetic patients (51). HB-EGF is a potential alternate candidate that may more specifically target ADAM17 up-regulation and EGFR signaling. However, to broaden therapeutic options for diabetic kidney disease, further elucidating how ADAM17 is activated and better delineating the mechanism of its up-regulation are important goals to achieve.

*Author Contributions*—J. C. K. designed the study, analyzed the data, and wrote the paper. R. L. and L. U. conducted the experiments with the assistance of B. G. The ADAM17 promoter deletion constructs and H4 mutant were made by M. C. Akita experiments were carried out by Y. S. J. S. D. C. and C. M. D. provided intellectual input and assisted with manuscript revision. All authors approved the final version of the manuscript.

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