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Combined acute hyperglycemic and hyperinsulinemic clamp induced profibrotic and proinflammatory responses in the kidney

Meenalakshmi M. Mariappan,[1,3] Kristin DeSilva,[1] Gian Pio Sorice,[2] Giovanna Muscogiuri,[2] Fabio Jimenez,[1,3] Seema Ahuja,[1,3] Jefferey L. Barnes,[1,3] Goutam Ghosh Choudhury,[1,3] Nicolas Musi,[2,3] Ralph DeFronzo,[2,3] and Balakuntalam S. Kasinath[1,3]

[1]*Division of Nephrology, Department of Medicine, University of Texas Health Science Center, San Antonio, Texas;* [2]*Division of Diabetes, Department of Medicine, University of Texas Health Science Center, San Antonio, Texas; and* [3]*South Texas Veterans Health Care System, San Antonio, Texas*

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Mariappan MM, DeSilva K, Sorice GP, Muscogiuri G, Jimenez F, Ahuja S, Barnes JL, Choudhury GG, Musi N, DeFronzo R, Kasinath BS. Combined acute hyperglycemic and hyperinsulinemic clamp induced profibrotic and proinflammatory responses in the kidney. *Am J Physiol Cell Physiol* 306: C202–C211, 2014. First published October 9, 2013; doi:10.1152/ajpcell.00144.2013.—Increase in matrix protein content in the kidney is a cardinal feature of diabetic kidney disease. While renal matrix protein content is increased by chronic hyperglycemia, whether it is regulated by acute elevation of glucose and insulin has not been addressed. In this study, we aimed to evaluate whether short duration of combined hyperglycemia and hyperinsulinemia, mimicking the metabolic environment of prediabetes and early type 2 diabetes, induces kidney injury. Normal rats were subjected to either saline infusion (control, $n = 4$) or 7 h of combined hyperglycemic-hyperinsulinemic clamp (HG+HI clamp; $n = 6$). During the clamp, plasma glucose and plasma insulin were maintained at about 350 mg/dl and 16 ng/ml, respectively. HG+HI clamp increased the expression of renal cortical transforming growth factor-β (TGF-β) and renal matrix proteins, laminin and fibronectin. This was associated with the activation of SMAD3, Akt, mammalian target of rapamycin (mTOR) complexes, and ERK signaling pathways and their downstream target events in the initiation and elongation phases of mRNA translation, an important step in protein synthesis. Additionally, HG+HI clamp provoked renal inflammation as shown by the activation of Toll-like receptor 4 (TLR4) and infiltration of CD68-positive monocytes. Urinary F2t isoprostane excretion, an index of renal oxidant stress, was increased in the HG+HI clamp rats. We conclude that even a short duration of hyperglycemia and hyperinsulinemia contributes to activation of pathways that regulate matrix protein synthesis, inflammation, and oxidative stress in the kidney. This finding could have implications for the control of short-term rises in blood glucose in diabetic individuals at risk of developing kidney disease.

hyperglycemia; hyperinsulinemia; renal fibrosis; TGF-β; mTOR; laminin

CHRONIC ELEVATION of blood glucose levels is known to be the primary initiating factor in the gradual development of diabetic complications of organ systems, including the kidney (44, 45). It is not known whether metabolic defects associated with prediabetic and early diabetic states set the stage for these diabetic complications (4, 16, 25). Acute elevation of plasma glucose concentration triggers an array of tissue responses that may contribute to the development of diabetic complications. Growing evidence suggests that postprandial hyperglycemia is important in the development of cardiovascular disease (1, 5, 36). Atherosclerotic changes start to develop in the prediabetic state when postprandial blood glucose levels are only moderately and briefly elevated (19). In addition, hyperinsulinemia is provoked by acute hyperglycemia and its role in pathogenesis of diabetic target tissue injury is of great interest. Elevated circulating insulin levels have been identified as an important risk factor for atherosclerotic lesions (48).

In normal humans, acute combined hyperglycemic and hyperinsulinemic clamp modulates proinflammatory gene expression and cytokine production following LPS stimulation (47); it also stimulates accumulation of myocardial lipids and leads to alterations in myocardial function (51). Acute hyperglycemia has been reported to elicit an injurious response in the kidney. A 2-h hyperglycemic insult is sufficient in normal humans to induce an increase in urinary excretion of transforming growth factor-β1 (TGF-β1) and isoprostane (33). However, whether such acute transient changes are sufficient for stimulating regulatory mechanisms of protein synthesis resulting in increased extracellular matrix (ECM) proteins in the kidney tissue is not known; this is a matter of significance considering the central role of ECM accumulation in renal pathology in diabetes.

Since chronic hyperglycemia-induced progressive accumulation of extracellular matrix is strongly implicated in failure of clearance function of the kidney in diabetes (11), it is important to know whether acute elevation in plasma glucose and insulin levels contributes to this process. Even a brief exposure of proximal tubular epithelial cells and glomerular epithelial cells, in vitro, to high glucose or high insulin for 5–15 min stimulates synthesis of laminin. Laminins, a major component of kidney ECM (29, 32), are glycoprotein chains expressed primarily in basement membrane, which is important in maintaining the integrity of the glomerular filtration barrier and normal renal function. This rapid stimulation occurs by a nontranscriptional mechanism that involves activation of mRNA translation under the control of mammalian target of rapamycin (mTOR) complex 1 (mTORC1). The purpose of the present study was to determine whether acute hyperglycemia in combination with

Address for reprint requests and other correspondence: M. M. Mariappan, Dept. of Medicine, MC 7882, Univ. of Texas Health Science Center at San Antonio, 7703 Floyd Curl Drive, San Antonio, Texas 78229 (e-mail: malini@uthscsa.edu).

Table 1. *Blood glucose, plasma insulin, ratio of kidney weight to body weight, and urinary F2t isoprostanes in saline-infused control rats and rats subjected to combined high glucose and high insulin clamp for 7 h*

	Saline Control	HG+HI Clamp	*P* Value
Blood glucose, mg/dl	89.25 ± 0.63	342.1 ± 5.12	<0.0001
Plasma insulin, ng/ml	0.54 ± 0.18	16.36 ± 1.66	<0.0001
Kidney wt/body wt, % of control	100 ± 1.65	102 ± 2.87	NS
Urinary isoprostanes, ng/mg creatinine	1.15 ± 0.76	2.45 ± 1.14	0.027

Values are means ± SD for 4 to 6 rats in each group. HG+HI, high glucose and high insulin; NS, not significant.

hyperinsulinemia induces pro-fibrotic and pro-inflammatory responses in the kidney of normal rats and to identify if it leads to urinary excretion of F2t isoprostane as a marker of oxidative stress.[1]

MATERIALS AND METHODS

Animals. Male Sprague-Dawley rats weighing 300–350 g were purchased from Harlan (Indianapolis, IN) and maintained on standard rodent chow. The Institutional Animal Care and Use Committee at our institution approved all studies.

Surgical preparation. After a 1-wk acclamation period, rats underwent surgical placement of catheters (polyethylene 50) into the left carotid artery and the right internal jugular vein under pentobarbital sodium (60 mg/kg body wt) anesthesia; the catheters were externalized to the back of the neck. All study animals regained their presurgical body weight before clamp studies, which were performed 5–7 days after surgery (41).

High glucose + high insulin clamp study design. Prior to study, 12 h-fasted rats underwent a 5-h acclimation to the procedure room, following which a 7 h-clamp procedure was performed (41). Rats received *1*) saline infusion (4 to 5 μl/min; $n = 4$) or *2*) a primed-continuous infusion of insulin (4 $mU \cdot kg^{-1} \cdot min^{-1}$; $n = 6$) (Humulin R; Eli Lilly, Indianapolis, IN) along with a bolus of a 45% glucose solution (0.75 g/kg body wt) followed by a variable infusion of a 20% dextrose solution (Sigma-Aldrich, St. Louis, MO) that was periodically adjusted to clamp the plasma glucose at ~325 to 350 mg/dl. Blood samples (10 μl) for determination of glucose were collected every 30 min during the clamp. Arterial blood glucose level was measured by glucose oxidase method on a Beckman Glucose Analyzer II (Beckman, Fullerton, CA). Blood samples for determination of plasma insulin were obtained at 60-min intervals before and during the study, and insulin levels were measured by ELISA (Crystal Chem, Downer's Grove, IL). Urine was collected during clamp by placing the rats in metabolic cages, and F2t isoprostane (Crystal Chem), a prostaglandin metabolite employed as a marker of oxidative stress, was measured. At the end of the study, animals were euthanized and

[1] This article is the topic of an Editorial Focus by Seung Hyeok Han and Katalin Susztak (19a).

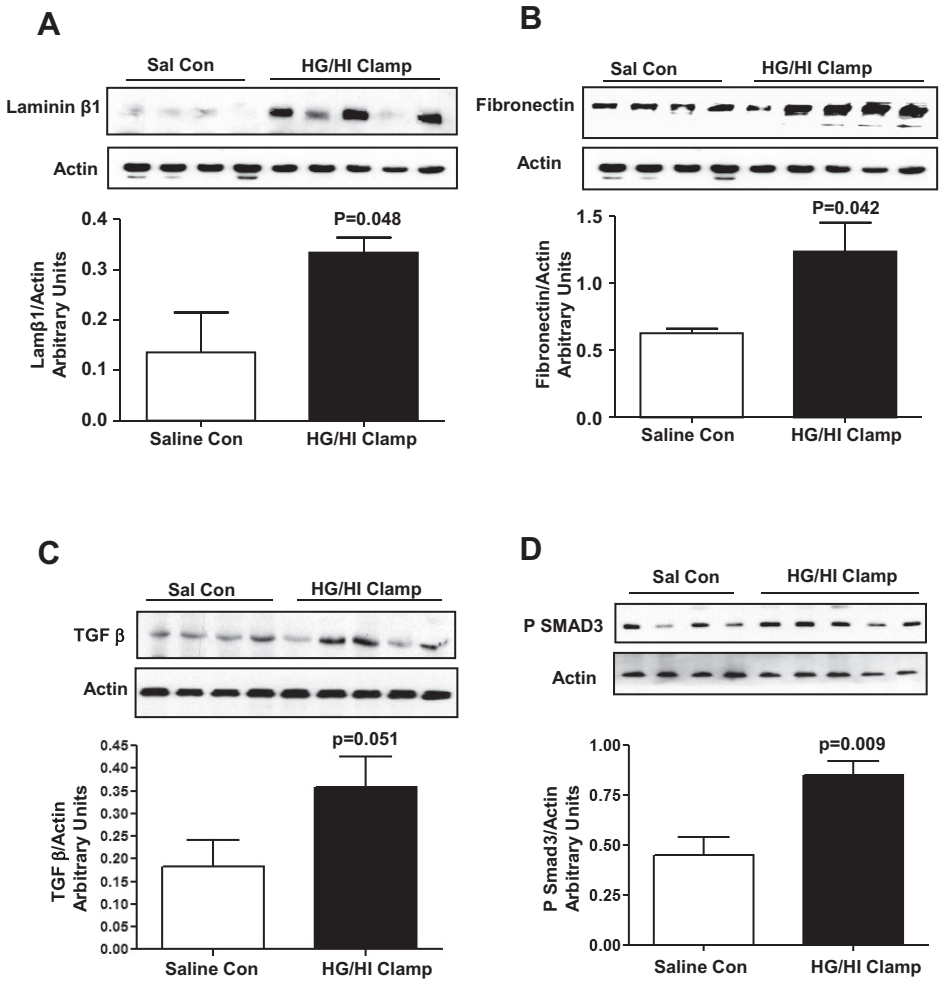

Fig. 1. High glucose and high insulin (HG+HI) clamp increases renal extracellular matrix proteins and induces renal transforming growth factor-β (TGF-β) activity. Immunoblotting was done on renal cortical homogenates to assess changes in laminin β1 (Lamβ1; *A*), fibronectin (*B*), TGF-β (*C*), and phosphorylation of Smad3 (*D*). Actin expression was used to assess loading. Histograms show means ± SD for 4 to 6 animals in each group. Sal Con, saline control.

kidney, liver, and skeletal muscle tissues were flash frozen for further analysis.

Immunohistochemistry and histomorphometric analysis. Briefly, 8-mm OCT-embedded sections were air dried and fixed in cold acetone. Endogenous peroxidase was inactivated using 3% H_2O_2 in 1% NaN_3 for 30 min, and nonspecific binding was blocked using 5% BSA + 1% normal goat serum. CD68 antibody (Clone ED1, AbD Serotec, Oxford, UK) was used at a concentration of 1:100. Following overnight incubation, sections were washed and incubated with a F(ab')$_2$ goat anti-mouse IgG-horseradish peroxidase (Jackson Immunolabs, West Grove, PA) secondary antibody at a concentration of 1:300 for 45 min at room temperature. Next, sections were washed and color reaction was developed with Vector NovaRed (Vector Labs, Burlingame, CA), according to the manufacturer's instructions. Finally, sections were counterstained with 1% methyl green (Sigma, St. Louis, MO) and mounted permanently with mounting media. All washes were done with PBS-Tween (0.05%). Percentage of stained area positive for CD68 was determined from 10–30 fields per slide, using ImageJ software (National Institutes of Health, Bethesda, MD).

Immunoperoxidase histochemistry. Fresh frozen cortical sections (6 μm thick) were fixed and permeabilized in acetone for 10 min and then rehydrated in PBS-0.1% BSA for 15 min. Sections were incubated with 0.6% hydrogen peroxide in methanol to block nonspecific peroxidase activity and 0.01% avidin, 0.001% biotin to block localization of endogenous activity before the addition of the appropriate blocking immunoglobulin for 15 min. Sections were incubated with antibody to laminin β1 (Biomeda, Foster City, CA) and mouse monoclonal antibodies specific for the alternatively spliced extra domain (EIIIA) of fibronectin (clones 3E2, Sigma and IST-9, Serotec, Harlan Bioproducts for Science, Indianapolis, IN) for 30 min in a humidified chamber at room temperature. They were then washed three times in PBS-0.1% BSA and then incubated with biotinylated secondary anti-mouse IgG for 30 min at room temperature. Bound antibody was identified by immunoperoxidase avidin-biotin complex staining following the manufacturer's instructions (Vector). The sections were then dehydrated and mounted with Permount (Sigma) and viewed by bright-field microscopy.

Immunoblotting. Equal amounts of proteins from liver, skeletal muscle, and kidney cortical homogenates were processed for Western blotting as described earlier (29, 32). In brief, equal amounts of kidney extract protein (10–50 μg) were mixed with sample loading buffer and separated under reducing conditions on SDS-PAGE. Proteins were electro-transferred to a nitrocellulose membrane. Membrane was probed with primary antibody overnight and blocked for an hour in 5% nonfat dry milk. Following washes in Tris-buffered saline containing 0.1% Tween 20, the membrane was incubated with respective antibodies against phospho- and unmodified proteins (Cell Signaling Technology, Beverly, MA), actin (Sigma-Aldrich), and laminin β1 (US Biologicals, Swampscott, MA) at 1:1,000 dilution for 3 h. The membranes were then washed and incubated with secondary antibodies linked to horseradish peroxidase (Santa Cruz Biotechnology, Santa Cruz, CA). The reactive bands were detected by chemiluminescence using the ECL system (Thermo-Pierce, Rockford, IL). Signal intensity was assessed by densitometric analysis (Scion Image software).

Immunoprecipitation. Equal amounts of kidney tissue homogenate protein were immunoprecipitated with eukaryotic Initiation factor 4A (eIF4A) antibody by rotating overnight at 4°C. Protein G agarose bead slurry (Santa Cruz Biotechnology) was added on the following morning and rotated for a further 2 h. The tubes were then centrifuged and the beads were washed 2× with RIPA buffer followed by 1× with PBS; 2× sample buffer was added to the beads, boiled for 6 min, and Western blotting was performed as described above.

Akt kinase assay. Activity of Akt kinase was measured using a nonradioactive immunoprecipitation kinase assay kit (catalog no.

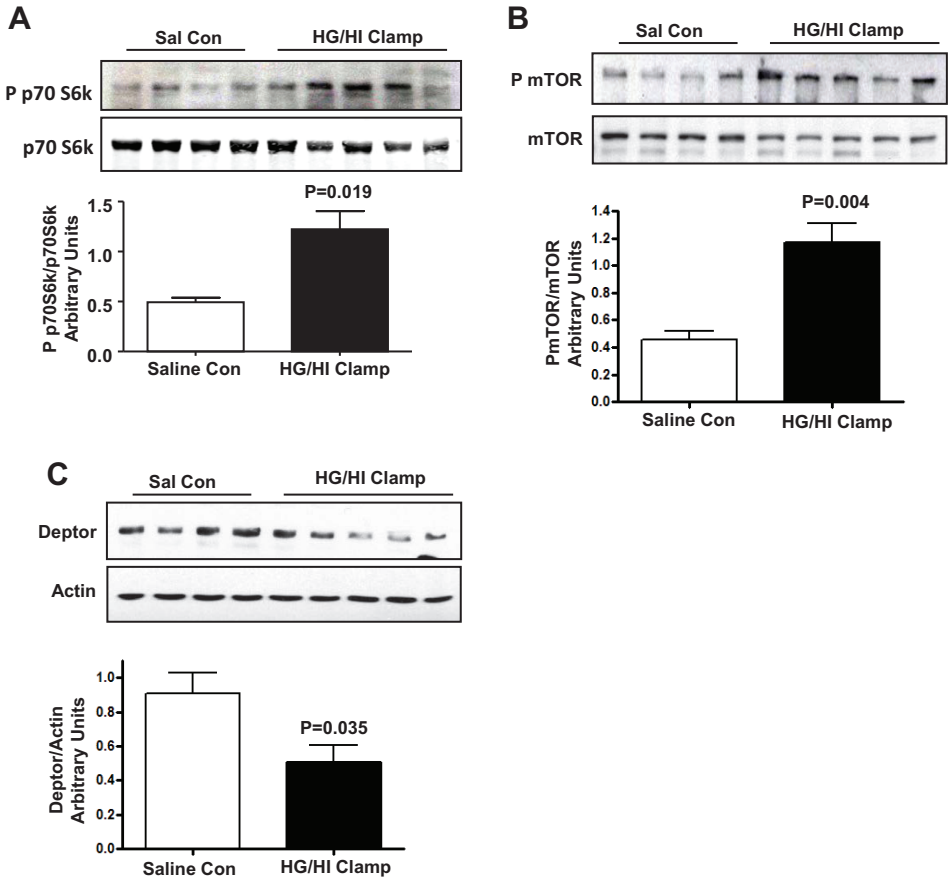

Fig. 2. Activation of mammalian target of rapamycin (mTOR)/p70S6 kinase (S6k) pathway in the kidney by HG+HI clamp. *A*: mTOR complex 1 activity was assessed in the renal cortex by phosphorylation (P) of its substrate, p70S6 kinase, on Thr389 by immunoblotting. *B*: p70S6 kinase activation was measured by immunoblotting with phospho-specific mTOR antibody. *C*: changes in the content of deptor, an mTOR-associated inhibitory protein, were assessed by immunoblotting. Histograms present combined data from 4 to 6 rats in each group.

9840, Cell Signaling, Danvers, MA). Briefly, kidney homogenates were centrifuged for 10 min and the supernatant was used for the assay following protein estimation. Equal amounts of protein were immunoprecipitated with immobilized phospho-Akt (Ser473) antibody bead slurry. The pellets were then washed 2 times with 1× lysis buffer followed by 1× kinase assay buffer. The pellet was then suspended in 1× kinase assay buffer with 10 mM ATP and GSK 3β fusion protein as kinase substrate. Reaction was terminated by adding 3× sample buffer followed by centrifugation. The samples were boiled for 5 min and loaded on SDS-PAGE, transferred, and probed with horseradish peroxidase-conjugated phospho-GSK 3β antibody.

NF-κB activity assay. The DNA-binding activity of NF-κB was assayed using a nonradioactive ELISA-based colorimetric assay kit (item no. 10011223; Cayman Chemical, Ann Arbor, MI). The assay was done according to the manufacturer's protocol. In brief, equal amounts of protein (50 μg) from nuclear extracts from saline control and high glucose and high insulin (HG+HI) clamp kidney cortices were added to 96-well plates coated with dsDNA sequence containing the NF-κB (p50/p65 Combo) response element. Complete transcription buffer provided in the kit was added and incubated overnight at 4°C. NF-κB contained in a nuclear extract binds specifically to the NF-κB response element. NF-κB p50 and p65 were detected by addition of specific primary antibodies directed against either NF-κB p50 or p65 to all of the wells including the blank wells that did not receive nuclear extracts and incubated for 1 h at room temperature. A secondary antibody conjugated to horseradish peroxidase was added followed by addition of developing solution and stop solution to provide a sensitive colorimetric readout at 450 nm. The percentage changes in activity of NF-κB in HG+HI clamp kidney extracts with respect to saline control were determined after subtracting the blank values.

Statistical analysis. Values are expressed as means ± SD. Student's *t*-test (unpaired) was used to compare the two groups ($n = 4–6$ animals per group) using GraphPad Prism 4 software. Results were considered statistically significant at $P < 0.05$.

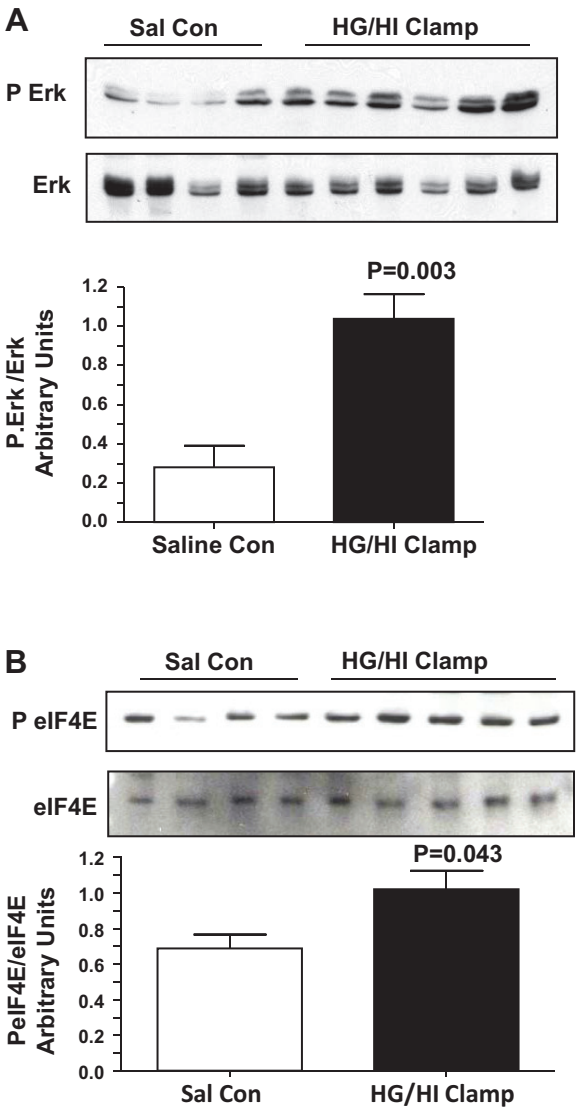

Fig. 3. Activation of ERK/eukaryotic initiation factor 4E (eIF4E) pathway in the kidney by HG+HI clamp. Phosphorylation of ERK (*A*) and eIF4E (*B*) was analyzed by immunoblotting of renal cortical homogenates. Histograms show quantification of data from 4–6 rats from each group.

RESULTS

Clinical parameters. During the combined hyperglycemic-hyperinsulinemic (HG+HI) clamp, the mean blood glucose concentration was 342 mg/dl ($P < 0.0001$) and the mean plasma insulin concentration was 16.36 ng/ml ($P < 0.0001$) compared with 89.25 mg/dl and 0.54 ng/ml, respectively, in the saline-infused control rats (Table 1). There was no difference in the kidney-to-body weight ratio between the saline control and HG+HI clamp rats. The mean urinary isoprostane concentration, which is indicative of renal oxidative stress, was increased nearly twofold after HG+HI clamp ($P = 0.027$) (Table 1).

HG+HI clamp increases the expression of matrix proteins and induces TGF-β activation in the kidney. We examined whether short-term HG+HI clamp affects matrix content in the kidney. Immunoblotting showed that the renal cortical content of laminin β1 chain (Fig. 1*A*, $P = 0.048$) and fibronectin (Fig. 1*B*, $P = 0.042$) was increased by more than twofold in the HG+HI rats when compared with saline-infused controls. Immunoperoxidase staining with specific antibodies showed that while the increment in laminin β1 was seen in both glomerular and tubular compartments, the increment in fibronectin was more evident in the glomerular compartment (data not shown).

TGF-β promotes extracellular matrix synthesis in kidney cells and is a key mediator of renal fibrosis in diabetic nephropathy (3, 42). The kidney is a site of TGF-β production and a target of TGF-β action; mRNA for all three TGF-β isoforms and receptors and the active form of TGF-β have been identified in all cell types of the glomerulus and in the proximal tubular cells (20). Immunoblotting showed a 2.5-fold increment in the expression of TGF-β in the renal cortex of HG+HI clamp rats (Fig. 1*C*, $P = 0.051$). We examined whether the increment in TGF-β was associated with activation of its canonical signaling pathway. here was a significant increase in Ser423/425 phosphorylation of Smad3 (Fig. 1*D*; $P = 0.009$), demonstrating that HG+HI clamp promotes both the expression and the activation of TGF-β.

Short-term HG+HI clamp stimulates mTORC1 activation and events in mRNA translation. Translation of mRNA is a rate-limiting step in gene expression culminating in protein synthesis; it occurs in three phases: initiation, elongation, and termination (17, 22, 46). Augmented synthesis of matrix proteins including laminin is partly regulated by mTORC1 by stimulation of mRNA translation in the kidney in type 2

diabetes (29). mTORC1 controls both the initiation phase and the elongation phase of mRNA translation (46). Thr389 phosphorylation of p70S6 kinase, a direct substrate of mTORC1, was increased in the renal cortex of HG+HI clamp rats ($P = 0.019$), demonstrating mTORC1 activation (Fig. 2*A*). p70S6 kinase is also known to stimulate Ser2448 phosphorylation of mTOR (9), which indicates activation of mTORC1. HG+HI induced Ser2448 phosphorylation of mTOR ($P = 0.004$), providing functional evidence for p70S6 kinase activation (Fig. 2*B*). Deptor is a scaffolding protein that is an inhibitory component of both mTORC1 and mTORC2; to facilitate activation of mTOR, deptor detaches from mTOR and is degraded (18, 24, 39). The content of deptor was reduced by >50% in the renal cortex of HG+HI clamp rats (Fig. 2*C*) ($P = 0.035$), consistent with activation of mTORC1. These data showed that a short duration of hyperglycemia and hyperinsulinemia is able to activate mTORC1 in the kidney.

The initiation phase of translation is governed by mRNA cap binding protein, eIF4E, which is phosphorylated on Ser209 under the control of ERK (28). A multimeric eIF4F complex composed of eIF4E, eIF4G, and eIF4A is formed on the mRNA cap, which augments the efficiency of translation. Phosphorylation of ERK ($P = 0.003$) and eIF4E ($P = 0.043$) was significantly augmented in the renal cortex of HG+HI clamp rats (Fig. 3). Programmed cell death protein (PDCD4) keeps eIF4A in an inactive binary complex in the resting cell; when a stimulus for increased protein synthesis is received, PDCD4 undergoes sequential phosphorylation by p70S6 kinase and degradation by the proteasomal pathway, releasing eIF4A to bind eIF4G and eIF4E and facilitate translation initiation (13). High glucose stimulation of laminin synthesis in renal podocytes is associated with decrease in PDCD4 (27). HG+HI clamp resulted in nearly 50% reduction in the expression of PDCD4 in the renal cortex ($P = 0.032$, Fig. 4*A*) and increase in the binding of eIF4A with eIF4E (Fig. 4*B*, $P = 0.001$), consistent with augmented formation of the eIF4F complex. During the initiation phase of translation, eIF4A functions as a helicase unwinding secondary structure in the 5′-untranslated region of the mRNA and facilitates scanning for the translation competent AUG codon by the 40S ribosomal subunit; it is assisted by eIF4B in this task (38). Activation of eIF4B can occur by phosphorylation on Ser422 by p70S6 kinase or ERK1/2 MAP kinase via p90 ribosomal s6 kinase (43). Immunoblotting showed that Ser422 phosphorylation of eIF4B was increased following HG+HI clamp ($P = 0.054$, Fig. 4*C*).

During the elongation phase of mRNA translation, amino acids are added to the nascent peptide chain in accordance with the sequence of codons in the mRNA and it is facilitated by dephosphorylation of Thr56 of eukaryotic elongation factor 2 (eEF2). The mTORC1 substrate p70S6 kinase phosphorylates eEF2 kinase on Ser366 and inhibits its activity, thus contributing to reduction in the phosphorylation of eEF2 (22). HG+HI clamp was associated with >70% reduction in eEF2

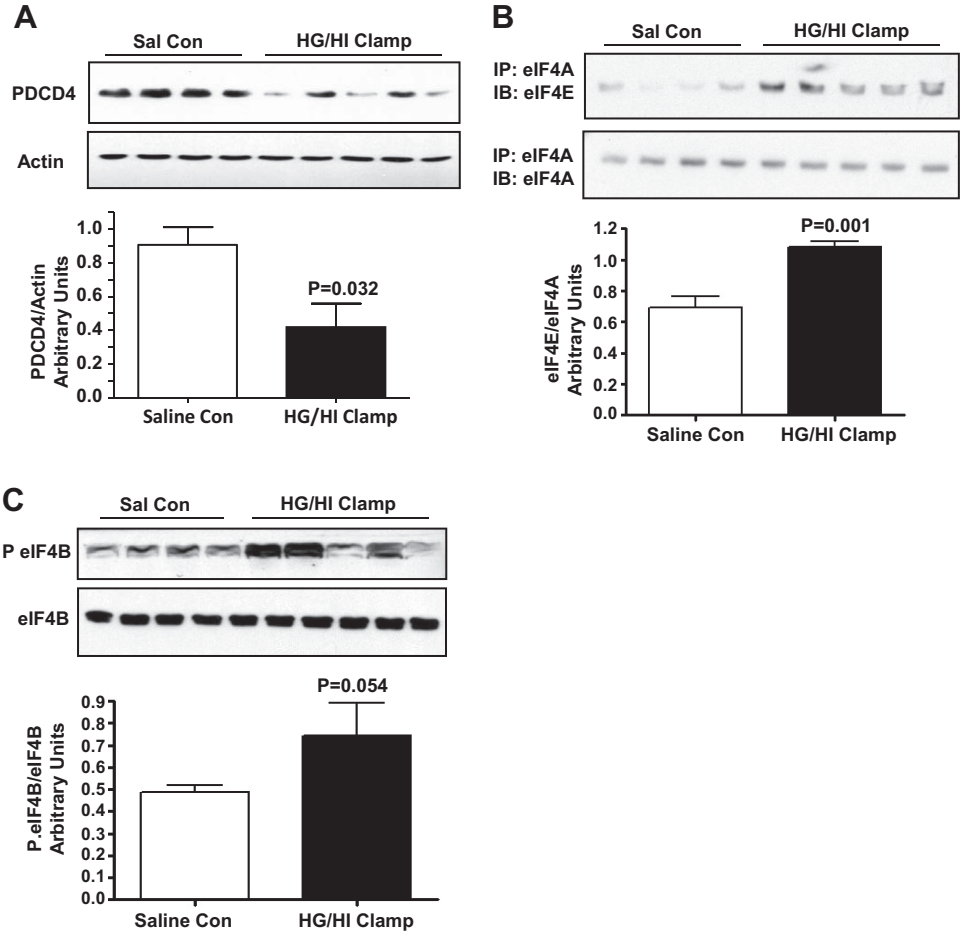

Fig. 4. Activation of the initiation phase of mRNA translation in the kidney by HG+HI clamp. *A*: immunoblotting was done on renal cortical preparations to assess changes in programmed cell death protein (PDCD4). *B*: renal cortical homogenates were immunoprecipitated (IP) with antibody against eIF4A and immunoblotted (IB) with antibodies against eIF4E or eIF4A. *C*: immunoblotting was done to assess changes in phospho-eIF4B. Histograms depict combined data from 4 to 6 animals in each group.

phosphorylation ($P = 0.031$) implying its activation and nearly twofold increase in the phosphorylation of eEF2 kinase ($P = 0.008$), indicating its inactivation (Fig. 5). Taken together, these data show that HG+HI clamp for a short duration stimulates both the initiation phase and the elongation phase of mRNA translation, promoting synthesis of proteins.

HG+HI clamp regulates signaling events upstream and downstream of mTOR. Stimulation of protein synthesis and matrix formation in the kidney by hyperglycemia is associated with activation of Akt and inactivation of GSK 3β (2, 23, 26). We employed an activity assay for Akt using GSK 3 fusion protein as a substrate and phosphorylation of GSK 3 was measured by Western blotting, using phospho-GSK 3α/β (Ser21/9) antibody. HG+HI clamp stimulated Akt activity by nearly twofold (Fig. 6*A*, $P = 0.036$). mTOR, existing in two complexes, is regulated by several factors that facilitate activation of itself and its downstream targets. Full activation of Akt requires phosphorylation of Thr308 by PDK1 and of Ser473 by mTORC2 (22). HG+HI clamp augmented the phosphorylation of Akt at Ser473, suggesting mTORC2 activation (Fig. 6*A*, *middle*; $P = 0.006$). Stimulation of protein synthesis by high glucose requires inactivation of GSK 3β by Ser9 phosphorylation, an Akt-responsive site (23). HG+HI clamp induced increase in Ser9 phosphorylation of endogenous GSK 3β protein ($P = 0.032$, Fig. 6*B*), consistent with its inactivation. The mechanism by which GSK 3β inhibits protein synthesis involves phosphorylation and inactivation of eIF2Bε, an important factor that is involved in the assembly of the preinitiation complex. When the initiation phase of mRNA translation is stimulated, eIF2Bε is activated by dephosphorylation (22, 23). Immunoblotting showed nearly 50% reduction in phosphorylation of eIF2Bε (Fig. 6*C*, $P = 0.018$), providing evidence for reduction in GSK 3β activity and stimulation of the initiation phase of mRNA translation. These data suggest that stimulation of Akt may lead to mTORC1 activation, and induction of mTORC2 in turn augments Akt activity in the kidney following short duration of hyperglycemia and hyperinsulinemia.

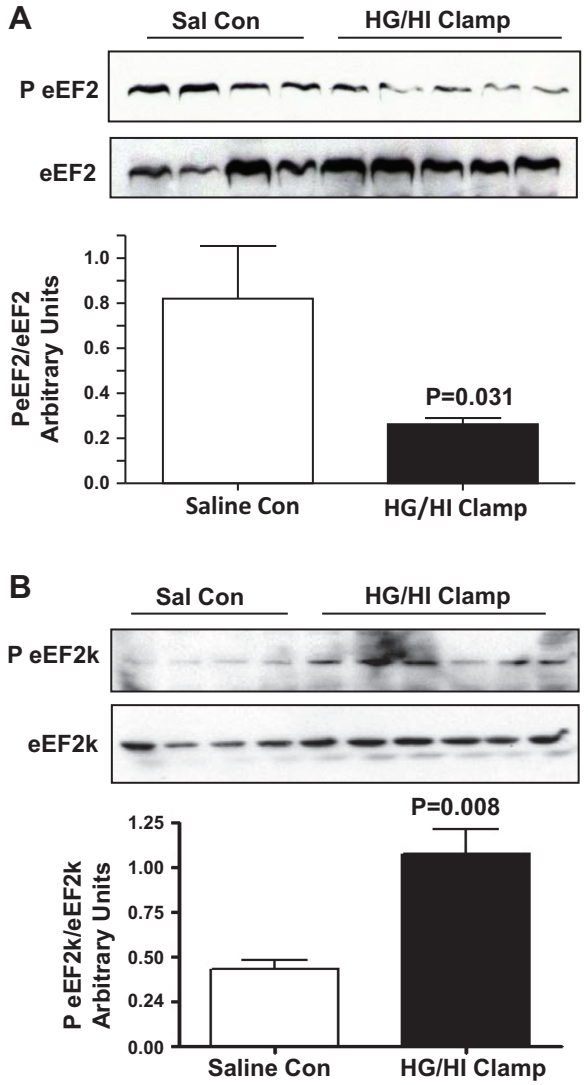

Fig. 5. Activation of the elongation phase of mRNA translation in the kidney by HG+HI clamp. Immunoblotting of renal cortical homogenates showed decreased phosphorylation of eukaryotic elongation factor (eEF2; *A*), indicating its activation, and, increased phosphorylation of eEF2 kinase (eEF2k; *B*), indicating its inactivation. Composite data from 4–6 rats from each group are represented in the histograms.

HG+HI clamp promotes inflammation in the kidney. Inflammation characterized by NF-κB activation and monocyte infiltration is an integral part of renal injury in diabetes and occurs in conjunction with increased expression of TGF-β and fibronectin (31). Nonradioactive colorimetric assay showed increase in the activation of NF-κB in the nuclear extracts from renal cortices of HG+HI clamp rats ($P = 0.044$, Fig. 7*A*). In addition to regulating the expression of growth factors and fibronectin, NF-κB is also involved in generating cytokines that attract circulating monocytes to enter the renal parenchyma (7, 34). CD68 staining showed a significant increase in the number of macrophages in the renal parenchyma of rats undergoing HG+HI clamp (Fig. 7*B*, $P = 0.034$). We next examined an upstream mechanism that may lead to NF-κB activation and monocyte infiltration. Pathogen-associated molecular patterns associated with viral, bacterial, and endogenous factors activate Toll-like receptors (TLRs). The kidneys express several TLRs including TLR4, which is prominently expressed in the renal cortex (8). When a ligand such as lipopolysaccharide binds to TLR4, two distinct intracellular signaling pathways are recruited, leading to activation of NF-κB; one of the signaling pathways involves binding of MyD88 to the intracellular domain of TLR4 (49). Coimmunoprecipitation showed increased association between TLR4 and MyD88, providing evidence of TLR4 activation in the kidneys of rats subjected to HG+HI clamp (Fig. 7*C*, $P = 0.045$); however, there was no change in the expression of TLR4. These data demonstrate that TLR4 activation, NF-κB activation, and monocyte infiltration can be induced by acute hyperglycemia and hyperinsulinemia, similar to that shown in chronic hyperglycemia (8).

To assess whether HG+HI clamp affected organ systems other than the kidney, the liver and skeletal muscle were examined. Immunoblotting showed that fibronectin, TGF-β, and mTOR phosphorylation at Ser2448 were unchanged in both the liver and the skeletal muscle. Phosphorylation changes suggestive of activation were detected with eIF4E, eEF2, and Akt in the liver. Phosphorylation of eIF4E and eEF2 was unchanged in the skeletal muscle although Akt appeared to be activated (data not shown). It is possible that changes in the

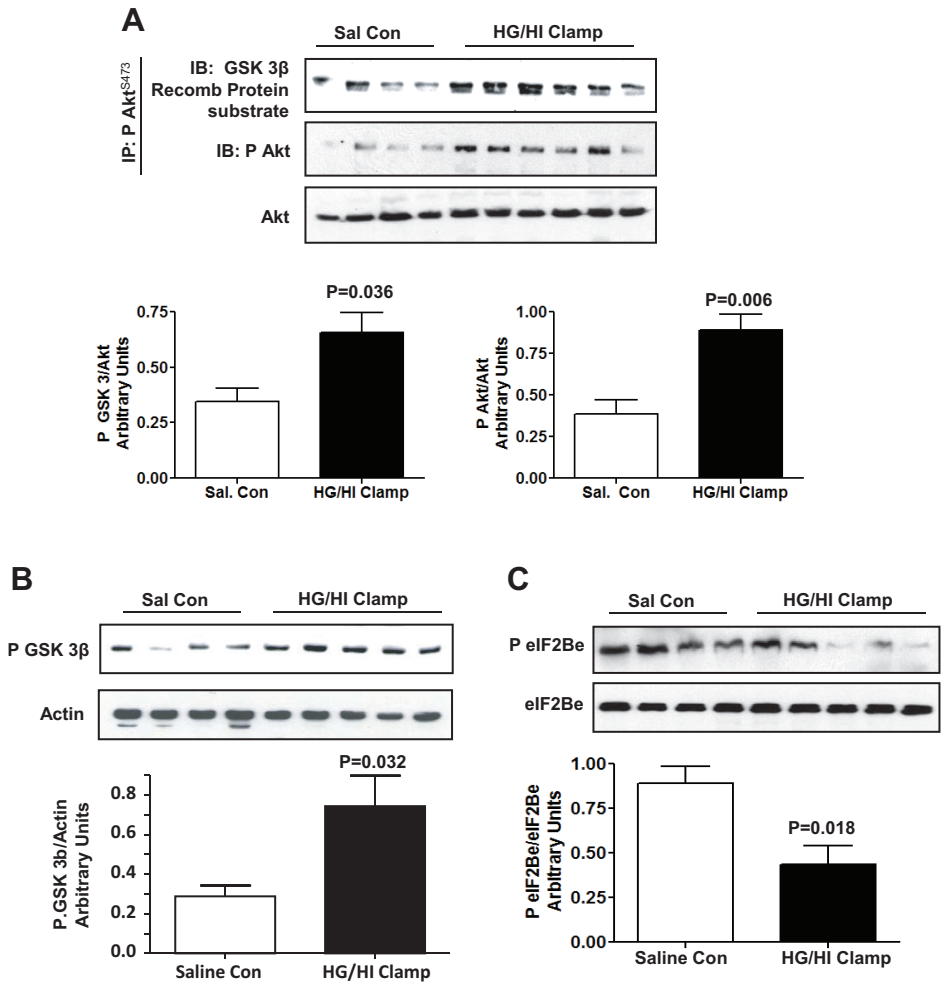

Fig. 6. Activation of Akt, mTOR complex 2, and, inhibition of GSK 3β in the kidney by HG+HI clamp. *A*: Akt activity in renal cortical homogenates was assessed by an in vitro kinase reaction using GSK 3β recombinant protein as substrate followed by immunoblotting using phospho-specific GSK 3β antibody (*top*). *Middle* blot shows phosphorylation of Akt at Ser473 that is indicative of mTOR complex 2 activation. *B* and *C*: immunoblotting of kidney cortical homogenates using phospho-specific antibody for GSK 3β (*B*) and eIF2Be (*C*) was done to assess their inactivation and activation, respectively. Histograms depict combined data from 4 to 6 animals in each group.

liver are related to alterations in glucose metabolism induced by the HG+HI clamp.

DISCUSSION

This study demonstrates for the first time that exposure to a short 7-h combined HG+HI is sufficient to activate TGF-β, a key mediator in renal fibrosis, increase matrix protein expression, and incite inflammation in the kidney in normal rats. The mechanism appears to involve activation of initiation and elongation phases of mRNA translation regulated by mTORC1, mTORC2, and ERK. Urinary F2t isoprostane content, a marker of renal oxidative stress, was increased significantly. Figure 8 provides a schematic for possible interaction of various pathways culminating in increased matrix protein synthesis and inflammatory response in the kidneys following HG+HI clamp. In contrast to the kidney, there was no change in TGF-β and matrix protein content in the liver and skeletal muscle during the clamp. HG+HI clamp promoted activation of eIF4E, eEF2, and Akt in the liver; these changes may be related to changes in glucose metabolism induced by the clamp. Except for Akt activation we did not find any significant change in the aforementioned signaling parameters in the skeletal muscle (data not shown). Thus, HG+HI clamp appears to affect tissues selectively, with more pronounced effect on signaling pathways related to protein synthesis and matrix metabolism in the kidney relative to liver and skeletal muscle.

Acute glycemic excursions significantly contribute to microvascular end-organ injury, including in the endothelial cells (40); however, the mechanisms have not been well understood. Our study suggests that stimulation of mRNA translation via activation of Akt, mTORC1, mTORC2, and ERK may constitute an important mechanism by which combined acute HG+HI causes renal injury. Short duration of hyperglycemia in our study resembles postprandial surges in blood glucose in diabetic subjects. In vitro studies in renal fibroblasts have shown that 2-h surges in medium glucose have greater effect on production of matrix proteins compared with sustained elevation (40); however, whether this occurs in vivo in the kidney was not studied. Previous studies have documented that the renal expression of TGF-β and type IV collagen can be increased within 3 days of onset of hyperglycemia in rats (37). Our data show that even a few hours of combined hyperglycemia-hyperinsulinemia are sufficient to promote renal TGF-β expression and matrix protein content in the rat. Inflammation was manifested as TLR4 activation, NF-κB activation, and increase in renal parenchymal macrophages, which are an integral part of renal pathology in diabetes (15). Transient elevation of plasma glucose in normal humans and in subjects with impaired glucose tolerance, including diabetes, is associ-

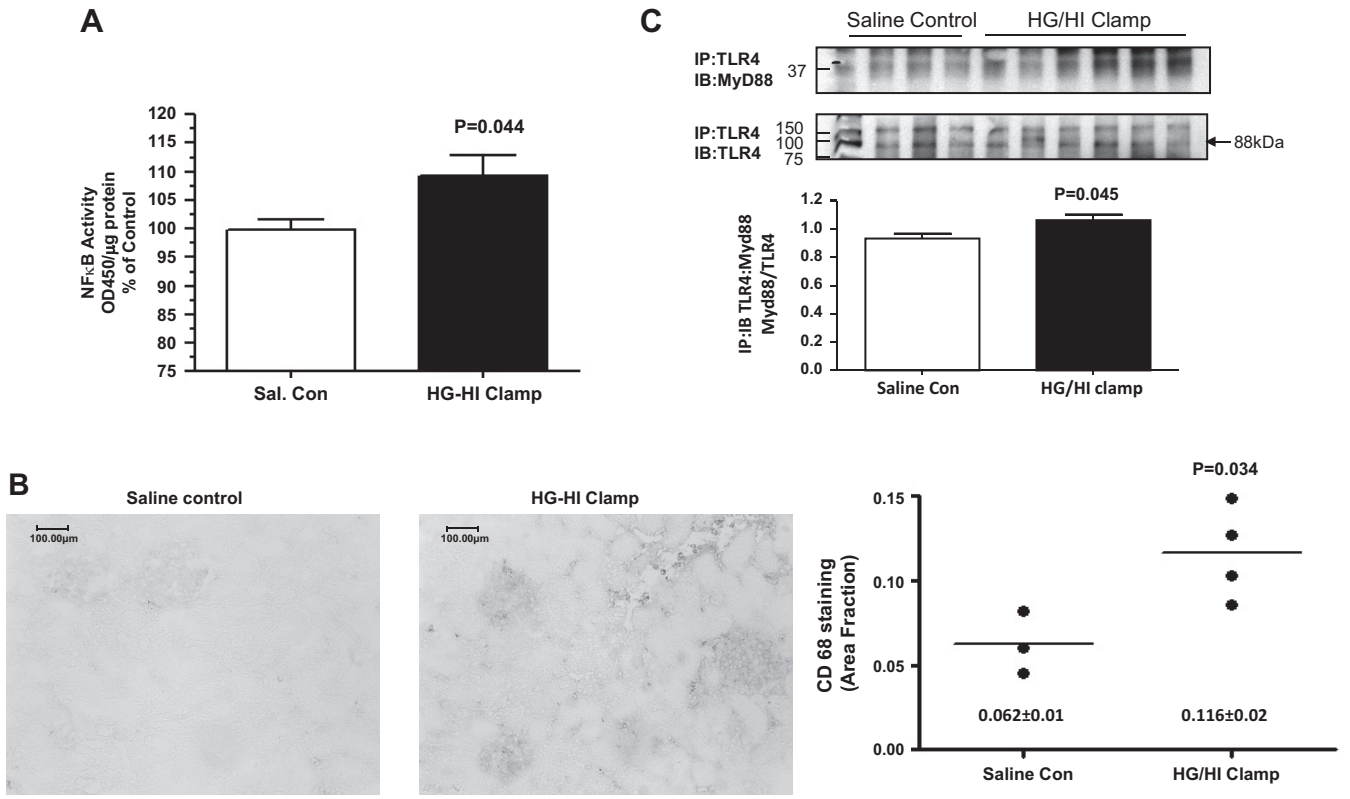

Fig. 7. Induction of inflammation in the kidney by HG+HI clamp. *A*: NF-κB activity was measured in the nuclear extracts from the renal cortices by ELISA. *B*: monocyte infiltration into renal parenchyma was assessed by immunostaining for CD68; quantification shows data from 3 to 4 animals in each group. *C*: renal cortical homogenates were immunoprecipitated with Toll-like receptor 4 (TLR4) antibody and immunoblotted with MyD88 antibody to evaluate TLR4 activation. Histogram shows quantification of the data from 4 to 6 animals in each group.

ated with spikes in circulating inflammatory cytokines (8, 16). In our study, a few hours of modest hyperglycemia were enough to promote inflammation in the kidney. The mechanism may involve activation of TLR4 leading to NF-κB activation, which in turn promotes production of cytokines that attract monocytes to the kidney. The ligands that activate TLR4 in the HG+HI environment need to be identified. An epigenetic mechanism involving Set-7-mediated histone methylation of the NF-κB promoter leading to increase in its expression in endothelial cells has been reported in mice (14). Hyperglycemic spikes also induce oxidative stress and subsequent endothelial dysfunction (6, 35). Our finding of increased urinary F2t isoprostane is consistent with these observations.

In contrast to hyperglycemia, the role of hyperinsulinemia in target organ injury in states of insulin resistance is debated. Combined hyperglycemia and hyperinsulinemia occurs in response to glucose loads in early type 2 diabetes (50) and qualitatively corresponds to combined HG+HI clamp employed in our studies. Hyperinsulinemia in states of impaired glucose tolerance and type 2 diabetes is due to insulin resistance with respect to the stimulation of glucose uptake. However, it is possible that some tissues, such as the kidneys, retain sensitivity to other metabolic actions of insulin, e.g., protein synthesis, and hyperinsulinemia may amplify such reactions. In support of this concept, we have demonstrated that insulin regulation of protein anabolism is intact in insulin-resistant type 2 diabetic patients (30) and that the growth-promoting atherogenic effects of insulin are not impaired in diabetic individuals (12). Furthermore, in *db/db* mice

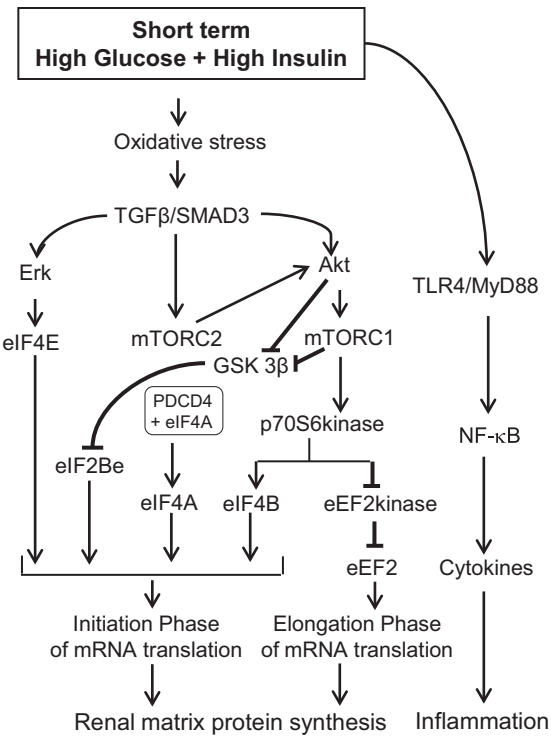

Fig. 8. Schematic for the possible mechanism of extracellular matrix protein increment and inflammation induced by short-term course HG+HI clamp in rat kidneys.

with obesity, insulin resistance, and type 2 diabetes, insulin receptor is activated in the kidney in contrast to the liver, a site of insulin resistance (2). Since insulin receptor activation in the *db/db* mouse kidney coincides with renal hypertrophy and onset of matrix protein accumulation (2, 10), hyperinsulinemia could play a contributory role in the development of diabetic nephropathy by promoting protein synthesis. The protein synthesis-promoting actions of insulin may also be operative in the clamp setting reported in our study. This is further supported by a previous report in which insulin stimulated mRNA translation to augment matrix protein synthesis in cultured proximal tubular epithelial cells (32), similar to the data in the present study. Hyperinsulinemia also has been implicated in promoting TGF-β expression in prediabetic subjects (21) and may serve to promote matrix protein synthesis in the kidney.

The limitations of the present study include the inability to assess the independent effects of high glucose and high insulin; future studies are needed to address individual effects of the two variables. However, the conditions employed in our study correspond to the combined hyperglycemic-hyperinsulinemic state encountered in states of impaired glucose tolerance and early type 2 diabetes. There could be an issue with the relevance of our data obtained in normal rats to states of insulin resistance. However, the use of normal rats allowed us to elucidate the role of two variables, whereas in rodents with obesity and/or type 2 diabetes we would have to account for many more variables including changes in lipids. The implication of our findings is that even transient elevations in circulating glucose and insulin, as opposed to the well-known pathogenetic effect of chronic hyperglycemia, can also lead to significant renal parenchymal injury and increase in matrix content. Conceivably, repeated excursions of plasma glucose and insulin, as occur following meals, may result in stimulation of synthesis of matrix proteins, which, over time, may contribute to renal fibrosis and loss of renal clearance function. Thus, a case may be made for stricter control of postprandial excursions of glucose and insulin, even in early stages of impaired glucose tolerance and type 2 diabetes. It also paves the way for further investigations to possibly identify meaningful early urinary biomarkers that will allow identification of patients who have prediabetes characterized by impaired fasting blood glucose and/or hyperinsulinemia and are at risk for developing renal complications.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

M.M.M., R.D., and B.S.K. conception and design of research; M.M.M., K.D., G.S., G.M., and F.J. performed experiments; M.M.M., S.A., J.L.B., N.M., R.D., and B.S.K. analyzed data; M.M.M. interpreted results of experiments; M.M.M. prepared figures; M.M.M. drafted manuscript; M.M.M., S.A., G.G.C., N.M., R.D., and B.S.K. edited and revised manuscript; M.M.M. and B.S.K. approved final version of manuscript.

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