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Suppression of chaperone-mediated autophagy in the renal cortex during acute diabetes mellitus

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Background. In the renal hypertrophy that occurs in diabetes mellitus, decreased proteolysis may lead to protein accumulation, but it is unclear which proteins are affected. Because the lysosomal proteolytic pathway of chaperone-mediated autophagy is suppressed by growth factors in cultured cells, we investigated whether the abundance of substrates of this pathway increase in diabetic hypertrophy.

Methods. Rats with streptozotocin (STZ)-induced diabetes were pair-fed with vehicle-injected control rats. Proteolysis was measured as lysine release in renal cortical suspensions and protein synthesis as phenylalanine incorporation. Target proteins of chaperone-mediated autophagy were measured in cortical lysates and nuclear extracts by immunoblot analysis. Proteins that regulate chaperone-mediated autophagy [the lysosomal-associated membrane protein 2a (LAMP2a) or the heat shock cognate protein of 73 kD (hsc-73)] were measured in lysosomes isolated by density gradient centrifugation.

Results. Proteolysis decreased by 41% in diabetic rats; protein synthesis increased at 3 days, but returned to baseline by 7 days. The abundance of proteins containing that chaperone-mediated autophagy KFERQ signal motif increased 38% and individual KFERQ containing proteins [e.g., M2 pyruvate kinase, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and pax2] were more abundant. LAMP2a and hsc73 decreased by 25% and 81%, respectively, in cortical lysosomes from diabetic vs. control rats.

Conclusion. The decline in proteolysis in acute diabetes mellitus is associated with an increase in proteins degraded by chaperone-mediated autophagy and a decrease in proteins which regulate this pathway. This study provides the first evidence that reduced chaperone-mediated autophagy contributes to accumulation of specific proteins in diabetic-induced renal hypertrophy.

Growth of the mature kidney (renal hypertrophy) occurs in many disorders, but the mechanisms controlling this growth remain unclear. Protein accumulation during growth results from an imbalance between the rates of synthesis and degradation of intracellular protein. In conditions such as diabetes or acidosis, growth of the kidney occurs despite an overall increase in protein catabolism and a decrease in tissue mass in other organs [1–4]. Because growth responses in the kidney are associated with the development of chronic renal insufficiency, understanding the mechanisms underlying renal cell growth in these metabolic conditions could lead to strategies for therapeutic intervention.

Protein accumulation in growth has been traditionally thought to result from increased protein synthesis. However, in some in cell culture models of renal hypertrophy, protein degradation is suppressed and it appears that a lysosomal proteolytic system is affected [2, 5, 6]. Because growth in the diabetic kidney occurs under the influence of growth factors [7], we examined changes in proteolysis in cultured renal tubule cells in response to epidermal growth factor (EGF). EGF treatment specifically suppressed lysosomal proteolysis; EGF did not affect proteasomal and calcium-sensitive proteolytic pathways [6].

Which lysosomal pathway might be regulated in diabetes? Proteins can be degraded by lysosomes (*1*) via macroautophagy of cytosol, membrane, or organelles or (*2*) through direct import through the lysosomal membrane [8, 9]. The best-described direct import pathway is chaperone-mediated autophagy in which the heat shock cognate protein of 73 kD (hsc-73) recognizes a peptide sequence (consensus sequence Lys-Phe-Glu-Arg-Gln or KFERQ) within the protein and unfolds the target [10]. Hsc-73 also interacts with an intrinsic lysosomal membrane protein, the lysosomal-associated membrane protein 2a (LAMP2a), also called 96 kD lysosomal glycoprotein in rats. These interactions facilitate the transfer of KFERQ-containing proteins into the lysosome where they are degraded [11]. In kidney, up to 30% of cellular proteins contain a KFERQ motif [12], including many proteins involved in growth regulation [2, 13]. In cultured renal epithelial cells, chaperone-mediated autophagy is down-regulated during hyperplasia

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induced by EGF or hypertrophy induced by ammonia [14]. These data suggest that chaperone-mediated autophagy is down-regulated in conditions that are associated with renal growth, in vivo.

In this report we investigated whether chaperone-mediated autophagy is regulated in acute diabetes mellitus. In experimental rats, acute diabetes mellitus is marked by hyperphagia. This notable because both total lysosomal proteolysis and chaperone-mediated autophagy are extremely sensitive to dietary changes [8, 15–17]. In particular, chaperone-mediated autophagy in the kidney is markedly up-regulated during starvation and suppressed by feeding [12]. This sensitivity to diet makes it impossible to determine if changes in proteolysis are due to differences in food consumption or due to signals independent of protein and caloric intake unless the diet is controlled. Therefore, we studied acutely diabetic rats using a pair-feeding protocol to dissociate alterations in proteolysis that are due to increased caloric and protein intake from changes induced by diabetes. We provide evidence that the reduction in proteolysis in the renal cortex of rats with streptozotocin (STZ)-induced diabetes is correlated with alterations in markers of chaperone-mediated autophagy activity.

METHODS

Animal model

Male Sprague-Dawley rats weighing ~200 g were given STZ (in citrate buffer, 0.1 mol/L, pH 4.0; 65 or 125 mg/kg body weight) or vehicle via a single tail vein injection at 7:00 a.m. as described [18]. They were placed in individual metabolic cages and fed a 23% protein, powered chow ad libitum. During the first 24 to 48 hours after injection the diabetic rats ate less chow than their pair-fed controls, so the quantity of food eaten by the STZ-treated rats was measured and the control rats were pair-fed an equivalent amount of chow. By the end of the second day, the diabetic rats became hyperphagic and the diabetic animals were then fed only the amount of food eaten by the ad libitum–fed control animals. The animals had unlimited access to water. Glycosuria and ketonuria were measured daily using Multistix (Miles, Elkhart, IN, USA). Rats were studied at 3 or 7 days of hyperglycemia (4 or 8 days after the STZ injection). At the time of sacrifice, blood was obtained by aortic puncture for measurement of blood glucose level with One Touch Profile (Life Scan, Milpitas, CA, USA) and for arterial blood gas using OPTI 1 (AVL, Roswell, GA, USA). The protocol was approved by the Emory University Institutional Animal Care and Use Committee.

Chemicals

All chemicals or reagents were purchased from Sigma Chemical Co. (St. Louis, MO, USA) except Dulbecco's modified Eagle's media (DMEM) and MG132, which were obtained from Gibco (Grand Island, NY, USA); metrizamide was obtained from Accurate Chemical & Scientific (Westbury, NY, USA); STZ was obtained from ICN (Aurora, OH, USA) and L-[U-14C] phenylalanine was from Dupont (Boston, MA, USA); anti-M2 pyruvate kinase was from Scebo-Tech, A.G. (Giessen, Germany); anti-hsc-73 antisera was from Maine Biotechnology (Portland, ME, USA); anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was from Biodesign International (Kennebunk, ME, USA); anti-pax2 and 14–3-3ζ were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Affinity-purified antisera to the pentapeptide KFERQ and to LAMP2a were a generous gift from Dr. J.F. Dice, Tufts University, Boston, Massachusetts.

Cortical suspensions

Kidney cortices from three rats were pooled together and washed in a ultrafiltrate-like solution containing (in mmol/L) 115 NaCl, 2.1 KCl, 25 $NaHCO_3$, 1.2 $MgCl_2$, 1.2 $MgSO_4$, 1.2 $CaCl_2$, and 2.4 KH_2PO_4 that was then equilibrated with a gaseous mixture of 95% O_2/5% CO_2 at 4°C. Cortices were minced with a razor blade, then incubated in the same solution with 0.1% collagenase, 0.25% albumin, 25 mmol/L mannitol, 5 mmol/L glucose, 4 mmol/L sodium lactate, 1 mmol/L alanine, 1 mmol/L sodium butyrate under a 95% O_2/5% CO_2 atmosphere for 45 minutes in a shaker bath. Collagenase digestion was stopped by dilution in cold ultrafiltrate-like solution and the suspension was filtered through two layers of cotton gauze. The viability of the cortical tubule cells was assessed by trypan blue exclusion.

Measurement of protein synthesis and degradation in renal cortical suspensions

The cortical suspension was washed and resuspended in lysine-free, low glucose DMEM for measurement of synthesis and degradation by the method of May et al [19]. Briefly, after a 30-minute preincubation, the suspension was divided into aliquots containing 5 to 10 mg tubule protein/flask, and incubated with L-[U-^{14}C] phenylalanine to measure protein synthesis. Samples of the media were removed at 20-minute intervals and protein was precipitated by adding trichloroacetic acid (TCA) to a final concentration of 10%. After the last time point, precipitated tubule protein was dissolved overnight at 37° C in 1 mL N NaOH for protein determination to normalize results per gram of the protein.

Protein degradation was measured as the release of TCA-soluble lysine into the media. Lysine was quantified using high-performance liquid chromatography (HPLC) after precolumn derivatization with 9-flourenylmethyl chloroformate [19]. To examine the role of lysosomal

pathways, kidneys from 6 animals of each group (control and diabetes) were pooled and the resulting cortical suspensions divided into two separate suspensions. One control and one diabetic suspension had 10 mmol/L NH_4Cl added to inhibit lysosomal proteolysis and then protein degradation was measured on the suspensions as above.

Isolation of kidney lysosomes

Kidney lysosomes were isolated from a light mitochondria-lysosomal fraction in a discontinuous metrizamide density gradient as modified for kidney cells by Cuervo et al [20]. Briefly, Spraque-Dawley rats were anesthetized by intraperitoneal injection with phenobarbital solution (0.5 mg/kg) (Abbott, Abbott Park, IL, USA). Kidney cortices from two or three rats were pooled, minced, and homogenized with a Teflon homogenizer. The homogenate was filtered through double layers of gauze by adding chilled 0.25 mol/L sucrose (4 mL/g of kidney cortex) and centrifuged at 6800*g* for 15 minutes. The supernatant was centrifuged at 17,000*g* for 20 minutes. The pellet (mitochondrial-lysosomal fraction) was washed once in 0.25 mol/L sucrose and resuspended in 57% metrizamide (final concentration) and a discontinuous gradient of metrizamide was constructed (layers from the bottom to top were 57%, 32.8%, 26.3%, and 19.8%) then centrifuged 1 hour at 17000*g*. Isolated lysosomes were collected from the top layer and the 26.3%/19.8% interface. In all experiments, purity of the lysosomal fraction was determined by measuring β-N-hexosaminidase activity as described [21]. When the β-N-hexosaminidase activity in the lysosomal preparations from diabetic and pair-fed control rats was not equal, the preparations were not included in the analysis. Mitochondrial contamination was assayed by phase contrast microscopy and was not detected in any lysosomal fraction.

Western blotting

Aliquots of the lysosomal fraction or homogenized lysates were mixed with sample buffer containing 1% sodium dodecyl sulfate (SDS), 0.5% β-mercaptoethanol, and boiled for 5 minutes. Proteins were separated by SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to nitrocellulose membranes. Membranes were stained with Ponceau S Red to visualize the completeness of the protein transfer and consistency of lane loading. Membranes were blocked with either 5% powdered milk or 3% bovine serum albumin (BSA) solution added to Tris-buffered saline (TBS) containing 0.1% Tween-20. Primary antibodies (see **Materials** section) were detected using the enhanced chemiluminescence (ECL) system (Amersham, Buckinghamshire, UK) and using Kodak BCL film.

Nuclear extract preparation and electrophoretic mobility shift assay (EMSA)

Nuclear protein extracts were prepared from renal cortex by the method of Dignam [22]. Protein:DNA binding assays were performed as described [23]. Briefly, nuclear protein extracts (1 μg) were preincubated for 5 minutes on ice in a reaction buffer containing 15 mmol/L HEPES (pH 7.9), 50 mmol/L KCl, 5 mmol/L $MgCl_2$, 0.12 mmol/L ethylenediaminetetraacetic acid (EDTA), 10% glycerol, 0.25 mg/mL bovine serum albumin (BSA), 0.05% Nonidet P-40, 10 mmol/L dithiothreitol (DTT), and 200 ng of poly (dI/dC). In some instances, a double-stranded competitor probe (0.7 pmol) or an anti-pax2 or GAPDH antibody (2 μg) (Santa Cruz Biotechnology or Biodesign, respectively) was included in the preincubation mixture. Competitor probes were created by annealing complementary 40 base oligonucleotides. A [γ-^{32}P] adenosine triphosphate (ATP) 5′-end-labeled (5×10^5 dpm), gel-purified, double-stranded probe corresponding to the pax2 binding site in the murine thyroglobulin promoter was added to each binding reaction (20 μL total volume), and the mixtures were incubated on ice for an additional 25 minutes before electrophoretic separation in a 5% nondenaturing acrylamide gel. The sequence of the sense strand of the probe was 5′-TTCGTGGTCATGTAAG TTGTTTCACTTGGATGAAATCCCTCTTCAGGA GATGTT. This site does bind pax5 and pax8 as well as pax2. This DNA probe was labeled with Klenow enzyme before a binding reaction with nuclear extracts. Antibody blocking was performed by preincubating the nuclear extracts for 30 minutes at room temperature (RT) with pax2 or GAPDH (Biodesign) antibodies.

Statistics

Results are expressed as mean ± SEM. Differences between two groups were analyzed by paired *t* test, and multiple comparisons were analyzed by analysis of variance (ANOVA) and were considered significant when $P < 0.05$.

RESULTS

Food intake over the course of the experiment did not differ between rats that were injected with 125 mg/kg of STZ and their pair-fed, vehicle-injected control rats (Table 1). After 3 or 7 days of hyperglycemia, kidney weight expressed per gram of body weight was increased in diabetes rats compared to values in controls. Urine samples were positive for ketones in most diabetic rats at day 2, but were negative in most by day 3 and in all by day 4. Plasma bicarbonate values (measured in a subset of eight control and diabetic rats) were not different between diabetic and control rats after 7 days. To evaluate renal protein turnover in diabetes, we measured the rates

Table 1. Results of pair-feeding of Sprague-Dawley rats made diabetic with streptozotocin (STZ) at the indicated dose and pair-fed for the indicated time as described in the **Methods** section

Day 3 125 mg/kg ($N = 6$)	Body weight day 0 *g*	Body weight day 3 *g*	Chow/day *g*	Blood glucose *mg/dL*	Kidney weight *g*	Kidney weight/ 100 g body weight
Control	202 ± 3	220 ± 4	19 ± 1	122 ± 13	1.94 ± 0.03	0.88 ± 0.02
Diabetes	196 ± 2	206 ± 3[a]	20 ± 1	350 ± 27[a]	2.24 ± 0.1[b]	1.08 ± 0.05[a]
Day 7 125 mg/kg ($N = 18$)	Body weight day 0 *g*	Body weight day 7 *g*	Chow/day *g*	Blood glucose *mg/dL*	Kidney weight *g*	Kidney weight/ 100 g body weight
Control	203 ± 26	265 ± 29	25 ± 3	128 ± 11	2.31 ± 0.25	0.88 ± 0.09
Diabetes	199 ± 28	175 ± 18[a]	26 ± 4	391 ± 107[a]	2.33 ± 0.23	1.34 ± 0.12[a]
Day 7 65 mg/kg ($N = 18$)	Body weight day 0 *g*	Body weight day 7 *g*	Chow/day *g*	Blood glucose *mg/dL*	Kidney weight *g*	Kidney weight/ 100 g body weight
Control	241 ± 2	292 ± 4	29 ± 1	109 ± 10	2.42 ± 0.09	0.83 ± 0.03
Diabetes	240 ± 3	231 ± 7[a]	29 ± 1	159 ± 23[c]	2.39 ± 0.19	1.33 ± 0.14[a]

[a] $P < 0.01$; [b] $P < 0.05$; [c] $P = 0.06$.

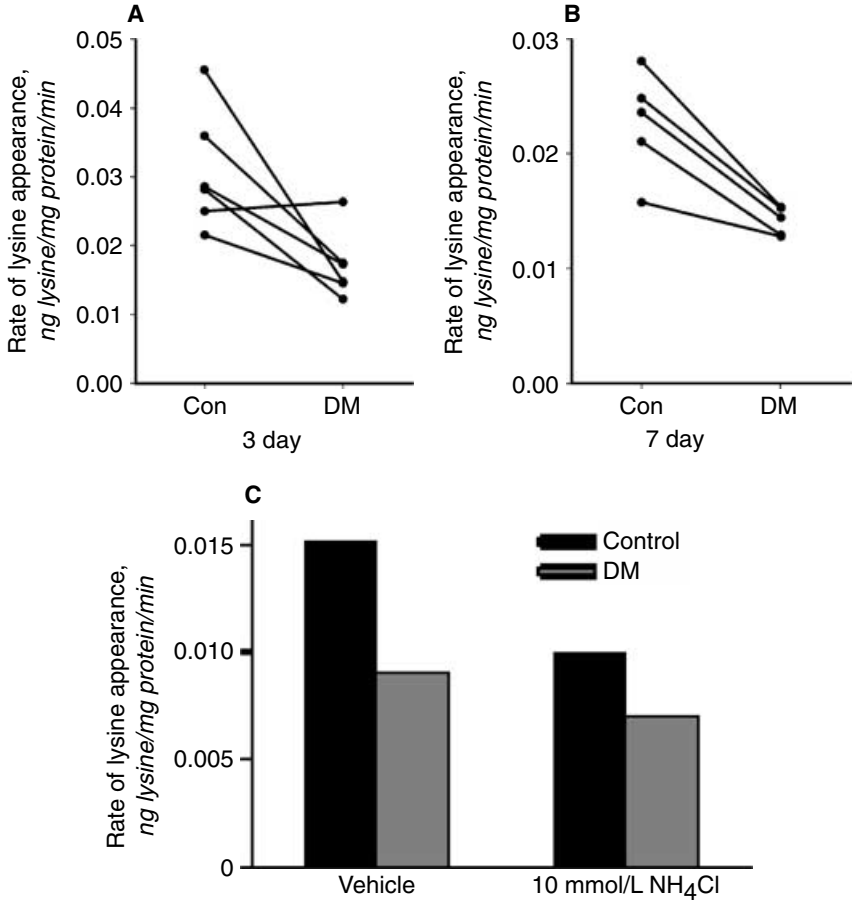


Fig. 1. Acute diabetes suppresses protein degradation in renal cortical suspensions. Sprague-Dawley rats were given a single intravenous injection of 125 mg/kg streptozotocin (DM) and then pair-fed with vehicle-injected control (Con) rats for 3 (*A*) or 7 days (*B*). The rate of protein degradation was measured as lysine release on cortical suspensions (see **Methods** section). Results for individual pair-fed animals are indicated by a line. $P < 0.05$ for both time points, $N = 6$ at 3 days, $N = 5$ at 7 days. (*C*) Six DM rats and their pair-fed controls were treated for 7 days and had their kidneys pooled for cortical suspensions. The suspensions were divided and 10 mmol/L NH_4Cl was added during the protein degradation measurement to one-half the sample. The results shown are those of a representative experiment of two repeats.

of protein degradation and synthesis in renal cortical suspensions from six diabetic and pair-fed controls. Protein degradation in the cortical suspensions from diabetic rats was decreased by 40% ± 10% at 3 days ($N = 6$, $P < 0.05$) and 41% ± 15.4% after 7 days of hyperglycemia ($N = 5$, $P < 0.05$) compared to controls (Fig. 1). In the same preparations, protein synthesis was increased by 46% ± 4% ($P < 0.05$, $N = 3$) in suspensions from diabetic rats compared to control at 3 days, but was not significantly increased at 7 days (a decrease of 8.5% ± 11%, NS, $N = 5$). To determine the proteolytic pathway that is regulated, we used 10 mmol/L NH_4Cl to inhibit lysosomal proteolysis. NH_4Cl reduced proteolysis in suspensions from both control and diabetic rats. Significantly, NH_4Cl markedly decreased the difference between control and diabetic proteolytic rates (Fig. 1C) suggesting that diabetes predominantly suppresses a lysosomal pathway.

Next, we examined whether substrates of chaperone-dependent autophagy (i.e., KFERQ-containing proteins) accumulate in renal cortical cells in response to diabetes.

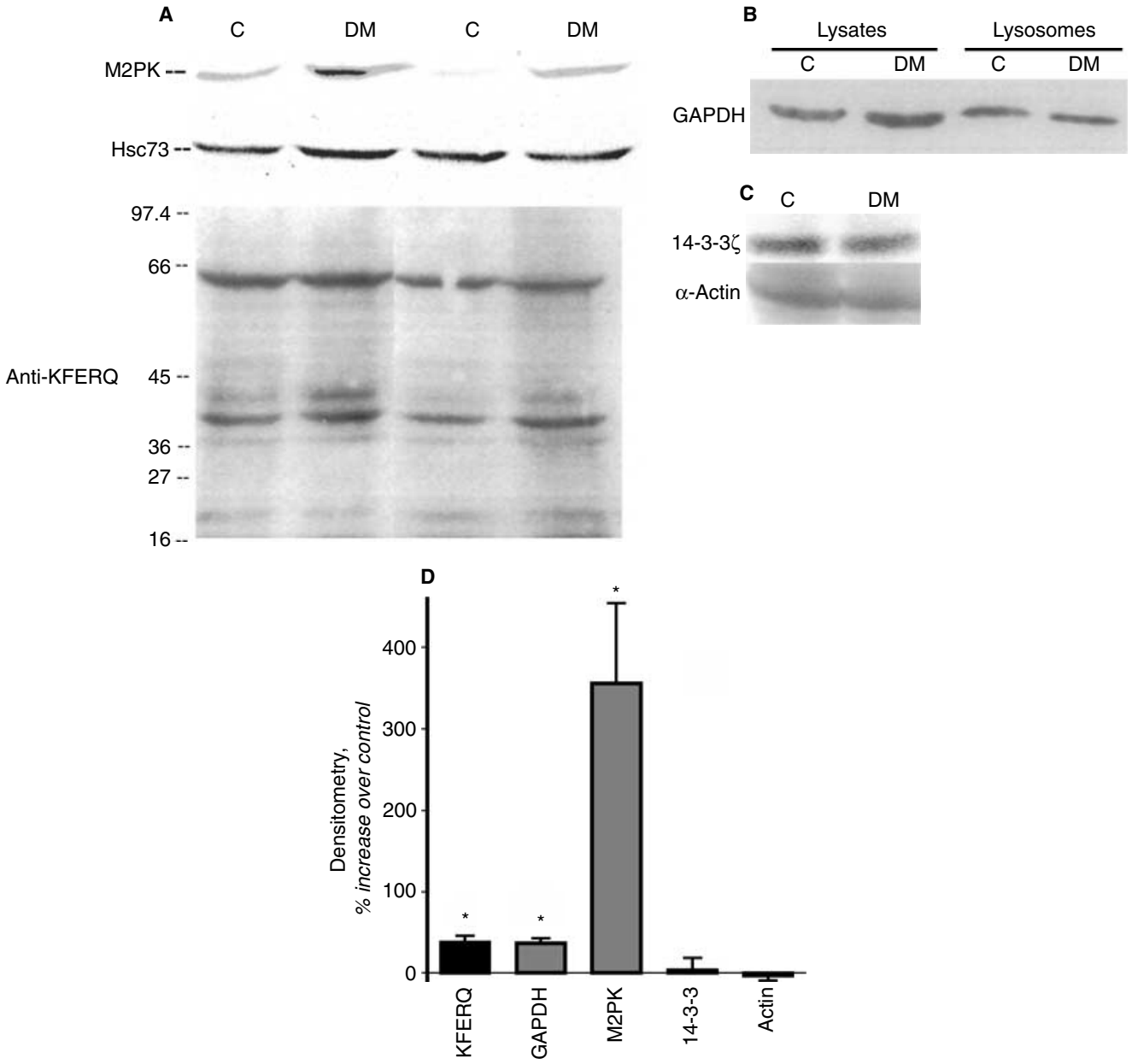


Fig. 2. Acute diabetes increases renal cortical KFERQ protein abundance. Rats were treated with 125 mg/kg streptozotocin (STZ) as in figure 1 (DM) and pair-fed with control rats (C) for 7 days. (*A*) Western blot analysis was performed with whole cell homogenates of renal cortex (40 μg protein/lane) and probed with primary antibodies to the M2 isoform of pyruvate kinase (M2PK), heat shock cognate protein of 73 kD (hsc-73), or a polyclonal antisera to the KFERQ peptide motif. The images shown are representative blots from eight pairs of diabetic and control rats. (*B*) Western blot analysis was performed with whole cell homogenates of renal cortex (40 μg protein/lane) and isolated lysosomes (6 μg protein/lane) and probed with primary antibodies to glyceraldhyde-3-phosphate dehyrodrogenase (GAPDH) (a representative blot of three repeats). (*C*) Western blot analysis was performed with whole cell homogenates of renal cortex (40 μg protein/lane) and probed with primary antibodies against 14-3-3ζ, and α-actin (a representative blot of four repeats). (*D*) Densitometry results of blots described in (A to C) plotted as change from control amount. $^*P < 0.01$ vs. control by paired *t* test ($N = 4$ to 8).

The abundance of proteins recognized by affinity-purified antibodies against the pentapeptide KFERQ motif was not significantly changed at 3 days after STZ injection (10% ± 9% increase in diabetics, $N = 5$), but the abundance of these proteins increased 38% ± 8% in 7 days diabetics vs. controls ($P < 0.05$, $N = 5$) (Fig. 2). We also examined the M2 isoform of pyruvate kinase (M2PK), a protein that contains the KFERQ motif, and it was increased by 239% ± 209% in diabetic rats at 3 days ($N = 6$, NS) and by 356% ± 98% ($N = 6$, $P < 0.05$) at 7 days (Fig. 2). GAPDH is a known substrate of chaperone-mediated autophagy [21] that is typically not regulated by

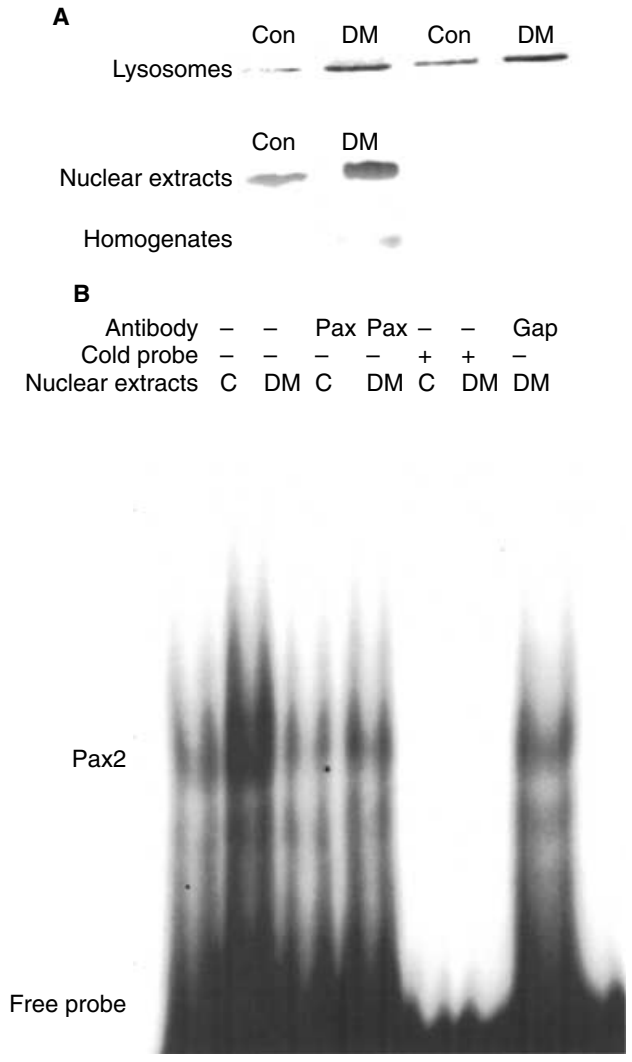


Fig. 3. Acute diabetes increases pax2 protein abundance and DNA binding. Rats were treated with 125 mg/kg streptozotocin (STZ) as in figure 1 (DM) and pair-fed with control rats (Con) for 7 days. (*A*) Western blot analysis was performed with whole cell homogenates of renal cortex (40 μg protein/lane), isolated lysosomes (6 μg protein/lane), or nuclear extracts (10 μg protein/lane) and probed with primary antibodies to pax2. The images shown are representative blots from four separate experiments. (*B*) Electrophoretic mobility shift assays were performed as described in the **Methods** section. Cold probe, pax2 antibodies, or glyceraldhyde-3-phosphate dehyrodrogenase (GAPDH) antibodies were incubated with the nuclear extract for 30 minutes prior to addition of the labeled probe. Image shown is a representative autoradiogram from four separate experiments.

transcriptional mechanisms. Its abundance was increased by 37% + 6% in cell homogenates from 7 day diabetic rats (Fig. 2B). Consistent with decreased lysosomal import, the amount of GAPDH found in lysosomes did not change.

We have suggested that chaperone-mediated autophagy may regulate growth processes by affecting the degradation of specific transcription factors [2]. To test this possibility, we examined KFERQ-containing pax2 transcription factor, because it regulates the expression of genes involved in renal growth and development [2]. Pax2 protein was undetectable in control cortex but was detectable from lysosomes and nuclear extracts from control rats (Fig. 3). With diabetes (7 days), pax2 was faintly detected in whole cortex, and was abundant in nuclear extracts. Pax8 was also more abundant in nuclear extracts from diabetic renal cortices (data not shown). To confirm the identity of pax2 in nuclear extracts, we examined its binding to the pax2 consensus site in the thyroglobulin promoter (Fig. 3). We observed enhanced pax2 binding in the nuclear extracts from the diabetic renal cortex. The binding was diminished by preincubation with pax2-specific antibodies, but not by preincubation with nonspecific GAPDH antibodies. We also evaluated the abundance of several other proteins lacking the KFERQ sequence, including 14-3-3ζ and α-actin (Fig. 2C). Diabetes (7 days) did not change their abundance in the renal cortex. These results are consistent with diabetes suppressing proteolysis via chaperone-mediated autophagy.

Table 2. Typical results of hexosaminadase activity in isolated lysosomes from the pooled renal cortices of two rats made diabetic with 125 mg/kg streptozotocin (STZ) and their pair-fed controls after 7 days of diabetes; recovery of total protein and hexosaminadase activity were measured, with no significant differences between control and diabetes by paired *t* test

	Hexosaminadase activity %	Protein %	Specific activity
Control ($N = 4$)			
Homogenate	100	100	1
Lysosomes	3.05 ± 0.84	0.65 ± 0.25	5.66 ± 0.93
Diabetes ($N = 4$)			
Homogenate	100	100	1
Lysosomes	2.95 ± 0.93	0.59 ± 0.23	5.78 ± 0.82

We also measured levels of LAMP2a and hsc-73 because their lysosomal levels correlate with the activity of chaperone-mediated autophagy [24]. No significant change in LAMP2a or hsc-73 levels was detected by Western blotting in whole cell lysates. This finding was not surprising because these proteins only control the activity of chaperone-mediated autophagy when they are associated with the lysosome [24]. We isolated lysosomes from the pooled renal cortex of two or three day 7 rats using a protocol modified for kidney tissue [20] and measured the lysosomal distribution of hsc-73 and LAMP2a. The yield and purity of lysosomes from kidney cortex from diabetic and pair-fed control rats was equal as judged by protein content and enrichment of the specific activity of the lysosomal enzyme, hexosaminadase (Table 2). LAMP2a was enriched in the lysosomes approximately sixfold over the levels in the unfractionated cell, but was not enriched in renal cortical mitochondria (Fig. 4). In the lysosomes isolated from the diabetic renal cortex, the amount of LAMP2a and hsc-73 was 25% ± 12% and 81% ± 8% (both $P < 0.01$, $N = 4$) lower than in the renal cortex of control rats (Fig. 5).

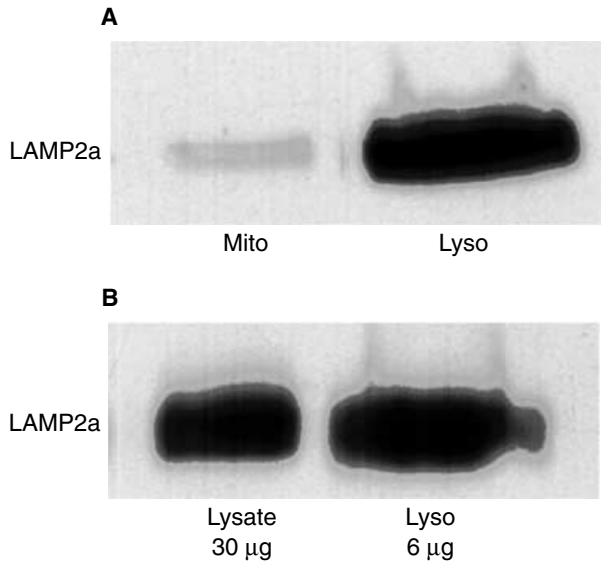


Fig. 4. Lysosomal-associated membrane protein 2a (LAMP2a) abundance in renal cortical cell fractions. Lysosomes and mitochondria were isolated from the renal cortices of two control rats (see **Methods** section) and LAMP2a was detected by Western blot analysis. The experiment was repeated three times with identical results. (*A*) Five micrograms of isolated mitochondrial (Mito) and lysosomal (Lyso) fractions. (*B*) Thirty micrograms of unfractionated cortical lysate protein (Lysate) and 6 µg of lysosomal protein (Lyso).

To determine if LAMP2a and hsc-73 respond to a less severe degree of diabetes, we examined the renal cortices of rats given a lower dose of STZ. We initially attempted to use insulin treatment (1 to 2 units of ultralente subcutaneously daily) for this purpose, but insulin treatment reduced the purity of the isolated lysosomes as measured by hexosamindase activity and microscopy (Sooparb and Franch, unpublished data) making the comparison difficult to interpret. Rats given 65 mg/kg STZ developed hyperglycemia, but urine ketones were negative at all time points. Diabetic rats had an increase in kidney weight/body weight 7 days after STZ injection (Table 1). The amount of renal cortical M2PK increased 308% ± 154% ($P < 0.01$, $N = 6$) in diabetes. Once again, there was no difference in the purity of lysosomes isolated between control and diabetic conditions as judged by protein content and enrichment of the specific activity of hexosaminadase. LAMP2a and hsc-73 levels in lysosomes isolated from the renal cortex of diabetic rats were decreased by 16% ± 7% (NS) and 57% ± 20% ($P < 0.05$), respectively ($N = 4$), (Fig. 6) compared to lysosomes isolated from cortices of their pair-fed controls.

DISCUSSION

Previous studies have suggested that renal hypertrophy is associated with decreased protein breakdown. In 1968, Coe and Korty [25] noted that uninephrectomy suppressed L-[^{14}C] leucine release from prelabeled cortical slices. Shecter, Boner, and Rabkin [26] and Fawcett et al [27] similar conclusions about the rate of protein degradation in isolated proximal tubules from rats with diabetes or metabolic acidosis. The accuracy of these methods has been questioned because branched chain amino acid (BCAA) tracers were used. BCAA are degraded by branched chain ketoacid dehydrogenase [28, 29], and the activity of this enzyme varies in the kidney under different conditions including diabetes mellitus [30, 31]. Hence, differences in the specific radioactivity of the intracellular pool of BCAA could be different in proximal tubule cells from control and diabetes rats. To avoid this complication, we used lysine appearance to measure protein degradation and synthesis because lysine is not metabolized by the kidney [19].

A second notable difference between our experimental model and those used in published reports is that we studied pair-fed diabetic and control rats to be certain that differences in food intake did not confound our results. This is important because diabetic rats consume considerably more chow than controls. Several proteolytic pathways, including lysosomal proteolysis via chaperone-mediated autophagy, are affected by protein and caloric intake [15–17, 24]. Our pair-feeding regimen resulted in a net restriction of dietary intake in the diabetic rats to avoid the hyperphagia they develop in response to diabetes. This is important because their higher food consumption could have been the basis for the reduction in proteolysis reported by others. The fact that lysosomal proteolysis was reduced in our pair-fed diabetic rats proves that the changes in cortical proteolysis results from pathologic rather than nutritional signals. In contrast to the accelerated protein degradation in muscle [18], acute diabetes decreases protein degradation in the renal cortex, and this response persists for at least 7 days even though protein synthesis falls to the level measured in control rats within this time frame. Thus, the kidney's capacity to lower its rate of proteolysis contributes to differential growth of the kidney under these intensely catabolic conditions.

An initial clue that decreased lysosomal protein degradation contributes to hypertrophy was the observation that growth stimuli (e.g., EGF or ammonia) suppressed lysosomal proteolysis, but without changing either ubiquitin-proteasome or calcium-activated proteolysis in cultured renal tubular cells [6]. Since our cell culture experiments suggested that chaperone-mediated autophagy may be involved in cell growth, we examined whether this specific pathway is regulated in the kidney by diabetes. We found that diabetes specifically decreases lysosomal proteolysis (Fig. 1C) and leads to a decrease in the amount of LAMP2a and hsc-73 associated with lysosomes in the renal cortex (Fig. 5). Since the amount of LAMP2a in lysosomes directly correlates with the activity of chaperone-mediated autophagy [24], it is likely that the decrease in protein degradation we measured in

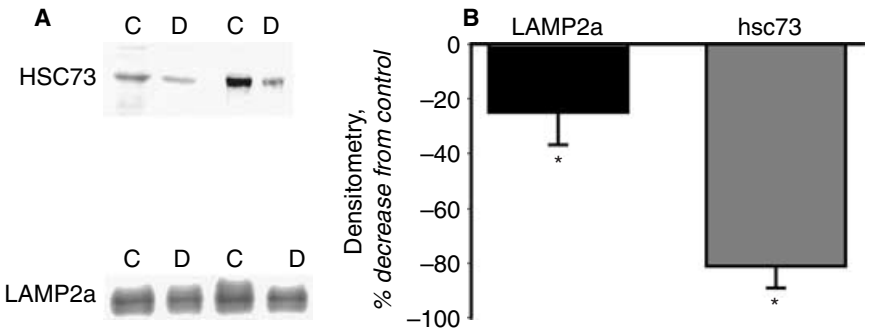
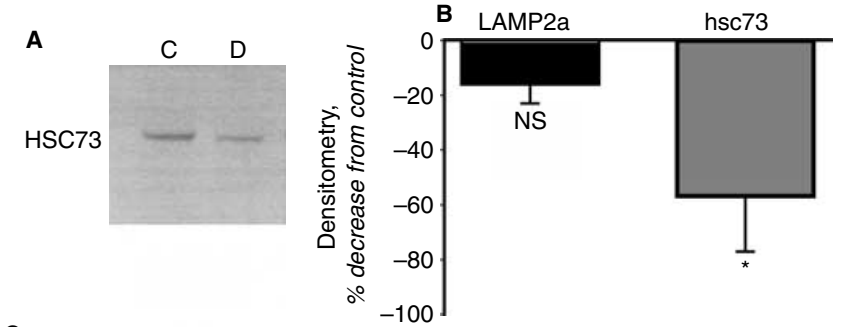


Fig. 5. Diabetes suppresses heat shock cognate protein of 73 kD (hsc-73) and lysosomal-associated membrane protein 2a (LAMP2a) in renal cortical lysosomes. Rats were treated with 125 mg/kg streptozotocin (STZ) as in figure 1 (D) and pair-fed with control rats (C) for 7 days. Lysosomes were isolated from the pooled cortices of two rats. (*A*) Western blotting was performed with 6 μg of lysosomal protein and antibodies against hsc-73 or LAMP2a. Results of two representative experiments out of six total is shown. (*B*) Densitometry of LAMP2a and hsc-73 results plotted as change from control amount. $^*P <$ 0.01 vs. control by paired *t* test.



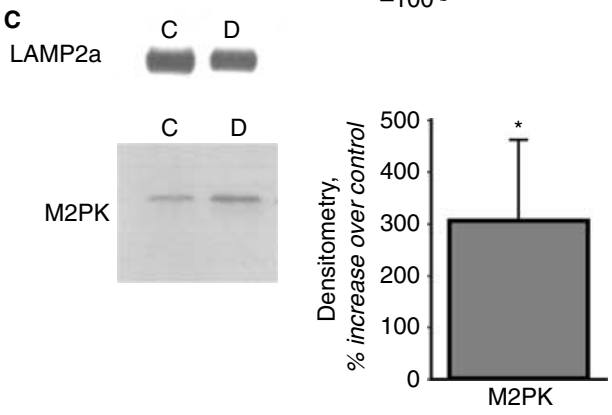


Fig. 6. Diabetes suppresses heat shock cognate protein of 73 kD (hsc-73) and lysosomal-associated membrane protein 2a (LAMP2a) in renal cortical lysosomes from nonketotic rats. Rats were treated as per figure 1 with 65 mg/kg streptozotocin (STZ) (D) and pair-fed with control rats (C) for 7 days and lysosomes were isolated from the pooled cortices of three rats. Results of a representative experiment out of four total is shown. (*A*) Western blot analysis performed with 6 μg of lysosomal protein and antibodies against hsc-73 or LAMP2a. (*B*) Densitometry of LAMP2a and hsc-73 results plotted as change from control amount. $^*P < 0.05$ vs. control by paired *t* test. (*C*) Western blot analysis (40 μg of unfractionated cortical homogenate) was performed with antibodies against the M2 isoform of pyruvate kinase.$^*P < 0.05$ vs. control by paired *t* test.

cortical suspensions in diabetes is due in part to a reduction in chaperone-mediated autophagy. Consistent with this conclusion, the amount of KFERQ-containing proteins increases in the renal cortex of diabetic rats. Although stimuli that increase lysosomal LAMP2a levels have been described [24], this is the first report to document suppression of basal levels of chaperone-mediated proteolysis in vivo.

Lysosomal hsc-73 fell to a much greater extent than LAMP2a. This finding is consistent with our previous observation that the magnitude of the change in lysosomal hsc-73 correlates closely with changes in the half-life of KFERQ containing proteins in cell culture [14]. Hsc-73 becomes associated with lysosomes during chaperone-mediated autophagy and its abundance in lysosomes is a sensitive marker for the activity of chaperone-mediated autophagy [24, 32]. The decline in lysosomal LAMP2a was much smaller in vivo than we observed in the cell culture. This may reflect heterogeneity in the response of different cell types or a smaller overall response than in the cultured cells. Nevertheless, a 25% reduction in the activity of chaperone-mediated autophagy results in significant accumulation of KFERQ-containing proteins (Fig. 2).

What is the signal that leads to suppression of chaperone-mediated autophagy in diabetes? We can exclude the potential role of protein or calorie intake in mediating the decrease in proteolysis due to diabetes based on our pair-feeding. Ketosis could possibly result in an acidosis that leads to increased ammoniagenesis in the renal cortex [33], since we have shown that elevated levels of ammonia are sufficient to increase KFERQ containing proteins and decrease lysosomal LAMP2a in cultured renal cells [14]. However, the rats were only ketotic for the first 3 of the 7-day period, had normal blood pH at 7 days, and rats given a nonketotic dose of STZ still have decreased lysosomal hsc-73 in the renal cortex (Fig. 6). Preliminary experiments with NH_4Cl-fed

animals showed that KFERQ containing proteins increase significantly in only in frankly acidotic animals (Sooparb and Franch unpublished data) suggesting that subtle acid loading does not significantly influence this pathway. Based on our findings in cultured renal tubular cells [14], a more likely mechanism is that the increase in growth factors, including EGF, in diabetic kidney drives the suppression of chaperone-mediated autophagy [7, 34].

Our findings do not rule out the possibility that diabetes induces the suppression of other lysosomal pathways, because conditions that accelerate chaperone-mediated autophagy (e.g., starvation) also enhance macroautophagy in mammalian cells [35]. They are also consistent with a report that the activity of several lysosomal proteases, (e.g., cathepsin D and L) are decreased in cortical suspensions from diabetic animals [26]. However, it is not been clear to what extent the in vitro cathepsin assay reflects lysosomal proteolysis because (*1*) the activities of cathepsins are measured at a suboptimal neutral pH when the lysosomal pH in vivo is acidic and may be regulated [9]; and (*2*) total lysosomal proteolysis is dependent on translocation of proteins into the lysosome, [8, 36]. In light of our present results, this report now can be seen to suggest a general decrease in multiple lysosomal signaling pathways.

It is unlikely that suppression of proteolysis is the sole mechanism causing protein accumulation. Protein synthesis was increased at 3 days and synthesis of specific KFERQ proteins may have been increased because we found large variations in the amounts of the KFERQ-containing proteins that accumulate in the kidney of diabetic rats relative to controls (Fig. 2). We speculate that suppression of proteolysis plays a crucial role in amplifying changes in the production of certain proteins and maintaining high levels of these long-lived proteins after normalization of protein synthesis [14]. Prolonging the half-life of transcription factors (such as pax2) may lead to increased transcription and synthesis of specific proteins, even though global protein synthesis is unchanged. Since prolonging protein half-life reduces the energy demands required for growth, a reduction in degradation may be advantageous in conditions like diabetes that cause a negative nitrogen (protein) balance [18].

A limitation of our study is the inability to study purified lysosomes from diabetic rats given replacement doses of insulin due to changes in lysosomal density. Since insulin is used to treat most cases of severe human diabetes, we must consider how insulin treatment might influence the clinical relevance of our findings. Although their methodology differs from our model, the work of Shechter, Boner, and Rabkin [26] suggests that insulin treatment partially reverses the effect of diabetes on proteolysis. This report suggests that our results may be applicable to insulin-treated humans. However, it is possible that there are significant differences as well. Certainly, we found major alterations in lysosomal density with insulin treatment, so further exploration of the effect of insulin of the lysosomal system in the kidney is required. It is also possible that protein synthesis makes a greater contribution in the growth of the kidney during insulin-treated, human diabetes than we observed in our rat model.

Proteins with the KFERQ motif play crucial roles in processes that are important for renal cell growth. For example, renal growth is accompanied by an increase in glycolysis [37] and a search of the Entrez protein database (*www.ncbi.nlm.nih.gov/entrez*) reveals that many glycolytic enzymes contain KFERQ sequences, including phosphofructokinase, glyceraldehyde 6 phosphate dehydrogenase, and pyruvate kinase. This is also true for the Kennedy pathway of phospholipid synthesis which includes choline kinase and phosphatidylcholine transferase, two substrates of chaperone-mediated autophagy. Some signaling proteins linked to growth (e.g., MARCKS protein, c-fos, and pax2) also contain KFERQ motifs and we have shown that the half-life of the paired box related transcription factor pax2 is increased by growth factors in cultured renal tubular cells [14]. The abundance of this developmentally important oncogene is also increased in the cortex of rats with STZ-induced diabetes (Fig. 3). Thus, chronic suppression of chaperone-mediated autophagy is a mechanism that can augment and maintain high levels of proteins required for renal growth.

CONCLUSION

Our findings demonstrate that an intrinsic decline in the activity of chaperone-mediated autophagy plays a role in the renal hypertrophy associated with acute diabetes mellitus.

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