Glomerular expression of p27^{Kip1} in diabetic *db/db* mouse: Role of hyperglycemia

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Glomerular expression of p27^{Kip1} in diabetic *db/db* mouse: Role of hyperglycemia. Early diabetic nephropathy is characterized by glomerular hypertrophy. Previous studies *in vitro* have demonstrated that mesangial cells exposed to high glucose are arrested in the G$_1$-phase of the cell cycle and express increased levels of the cyclin-dependent kinase inhibitor p27^{Kip1}. The present study was performed to investigate the renal expression of p27^{Kip1} in *db/db* mice, a model of diabetes mellitus type II. Glomerular p27^{Kip1} protein, but not mRNA expression, was strongly enhanced in diabetic *db/db* mice compared with non-diabetic *db/+* littermates. Immunohistochemical studies revealed that this stimulated expression was mainly restricted to the nuclei of mesangial cells and podocytes, but glomerular endothelial cells occasionally also stained positively. Quantification of p27^{Kip1} positive glomerular cells showed a significant increase of these cells in *db/db* mice compared with non-diabetic *db/+* animals. Although tubular cells revealed a positive staining for p27^{Kip1} protein, there was no difference between *db/+* and *db/db* mice. Immunoprecipitation experiments revealed that p27^{Kip1} protein associates with Cdk2 and Cdk4, but not with Cdk6. To test for the influence of hyperglycemia on cell cycle arrest and p27^{Kip1} expression, mesangial cells were isolated from *db/+* and *db/db* mice. There was a similar basal proliferation when these cells were grown in normal glucose-containing medium (100 mg/dl). However, raising the glucose concentration to 275 to 450 mg/dl induced cell cycle arrest in *db/+* as well as *db/db* mesangial cells. Increasing the medium osmolarity with D-mannitol failed to induce p27^{Kip1} expression in mesangial cells. Transfection of cells with p27^{Kip1} antisense, but not missense, phosphorothioate oligonucleotides facilitated cell cycle progression equally well in *db/+* and *db/db* mesangial cells. Furthermore, p27^{Kip1} expression was comparable in both cell lines in normal glucose, but increased in high glucose medium. Our studies demonstrate that p27^{Kip1} expression is enhanced in diabetic *db/db* animals. This induction appears to be due to hyperglycemia. Expression of p27^{Kip1} may be important in cell cycle arrest and hypertrophy of mesangial cells during early diabetic nephropathy.

Diabetic nephropathy is a major complication of diabetes mellitus and is currently one of the major causes of chronic renal failure. After an initial phase of glomerular hypertrophy associated with an increase in glomerular filtration rate, the natural history of diabetic nephropathy is characterized by the development of irreversible glomerulosclerosis and tubulointerstitial fibrosis causing end-stage renal disease [1–3]. Although originally considered to be only a feature of insulin-dependent diabetes mellitus type I (IDDM), there is now ample evidence that similar features of diabetic nephropathy also occur in patients with diabetes mellitus type II (NIDDM) [4–6]. In fact, it has been claimed that patients suffering from NIDDM may even have an earlier onset of nephropathy than subjects with IDDM [5]. The pathological lesions are quite similar in NIDDM and IDDM, underscoring the pathophysiological role of hyperglycemia and/or substances modified by high glucose such as advanced glycation end products and Amadori products [7–9].

Mesangial cells (MC) play a key role in the glomerular hypertrophy of early diabetic nephropathy [2]. These cells are also important in the secretion of extracellular matrix proteins that eventually contribute to the development of glomerulosclerosis. We and others have previously demonstrated that MC, grown in high ambient glucose, are growth arrested in the G$_1$-phase of the cell cycle and undergo cellular hypertrophy [10–12]. This growth inhibitory effect of high glucose on MC is mediated by synthesis and bioactivation of transforming growth factor-β (TGF-β) [10]. Furthermore, the high glucose-mediated synthesis of collagen type IV is also mediated by TGF-β [13, 14]. In a recent series of experiments we have investigated the role of the cyclin-dependent kinase (Cdk) inhibitor p27^{Kip1}, a protein that binds to cyclin-Cdk complexes and inhibit their kinase activity, in high glucose-mediated cell cycle arrest in MC [15]. These studies revealed that high glucose increased p27^{Kip1} protein in MC [15]. Interference with high glucose-induced p27^{Kip1} expression applying oligonucleotide antisense technology abolished cellular hypertrophy and facilitated G$_1$-phase exit indicating a pivotal role of p27^{Kip1} in MC growth regulation under conditions of high glucose [15].

The current study was performed to test whether similar mechanisms may be operative in the kidneys of *db/db* mice, a well-characterized model of NIDDM in which early glomerular expansion resembling that found in human diabetes has been documented [16–19]. Our data demonstrate that p27^{Kip1} protein, but not mRNA, is enhanced in glomeruli of diabetic *db/db* mice. Glomerular MC are a likely source of this increase. However, transfer of isolated MC from *db/db* mice to normal glucose containing-cell culture medium reduces the p27^{Kip1} expression to

similar levels as MC derived from non-diabetic heterozygotic *db/+* controls. These findings indicate that the increased p27[Kip1] expression in kidneys of *db/db* mice is solely mediated by hyperglycemia.

METHODS

Animal experiments

Heterozygotic C57BL/Ks-*db/+* (*db/+*) mice were obtained from Bomholtgard Breeding Center (Ry, Denmark) and a breeding colony was established in our animal facility. Homozygotic *db/db* offspring can be identified on the basis of appearance of obesity around four to six weeks of age [16, 17]. Diabetic *db/db* mice as well as their non-diabetic *db/+* littermates were studied at seven weeks of age. The body wt was determined and blood was drawn from anesthetized animals for the determination of blood glucose. Kidneys were weighed, pooled from three mice and kept on ice until further processing. Blood glucose was measured with B-Glucose analyzer (HemoCue, Ängelholm, Sweden).

For selected experiments, insulin-dependent diabetes mellitus (IDDM) was induced in seven-week-old BALB/c mice (Charles River, Wiga, Germany) after overnight fasting by the intraperitoneal injection of a single dose of 200 mg/kg body wt streptozotocin (STZ; Sigma, Deisenhofen, Germany) [20] dissolved in citrate buffer (pH 4.5). Blood was obtained from anesthetized mice for measurement of glucose concentration after injection of STZ. Only mice with a glucose concentration of 200 to 300 mg/dl after 24 hours were used. At different time points (24 hr to 1 week), kidneys were removed and glomeruli were isolated by differential sieving as established in this laboratory [21, 22].

Isolation of glomeruli and cell culture studies

Renal cortices were separated from the medulla, the kidneys were pooled, minced, and glomeruli were isolated by differential sieving exactly as previously described [22]. The resulting preparation contained > 80% glomeruli as judged by light microscopy.

For the establishment of MC cultures, glomeruli were treated with 0.1% collagenase for 30 minutes, extensively washed, and plated in small culture flasks (50 ml; Nunc, Roskilde, Denmark) in Dulbecco's modified Eagle's medium (DMEM; Gibco-BRL, Eggenstein, Germany) containing 100 mg/dl D-glucose. This medium was supplemented with 10% fetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM glutamine. The glomeruli were cultured at 37°C in 5% CO_2, and outgrowth of spindle-like MC could be observed after three to five days. Cells were passaged after eight days and some cells were grown in glass slide chambers for further characterization. The identity of mesangial origin of the cells was established by positive staining for desmin and vimentin, but failure of binding to antibodies generated against factor VIII and proximal tubular cell 3M-1 antigen (gift of Dr. Eric Neilson, University of Pennsylvania, Philadelphia, PA, USA).

For ^{3}[H]thymidine incorporation experiments, 10^4 MC from either *db/+* or *db/db* mice were plated into each well of a 96-well culture plate (Nunc) and rested for 24 hours in serum-free DMEM with 100 mg/dl D-glucose. Cells were then incubated for another 48 hours in serum-free medium with 100 mg/dl (normal glucose) or 450 mg/dl (high glucose) D-glucose. Cells were pulsed with 1 μCi ^{3}[H]thymidine (5 Ci/mmol; Amersham, Braunschweig, Germany) during the last six hours of culture. After pulsing, MC

were washed twice with PBS, trypsinized for 10 minutes at room temperature, and finally collected on glass-fiber paper with an automatic cell harvester. Radioactivity of dry filters was measured by liquid scintillation spectroscopy. Experiments were independently repeated with three different primary MC cultures and four duplicates for each experiment.

Proliferation experiments were also performed in the presence of p27[Kip1] antisense and missense phosphorothioate oligonucleotides [23]. The following murine sequences were used [24]: antisense p27[Kip1], 5′UGGCUCUCCUGCGCC3′; and missense p27[Kip1], 5′UCCCUUUGGCGCGCC3′. Cells were transfected in serum-free normal glucose medium with 1 μM of either p27[Kip1] antisense or missense oligonucleotides using lipofectin [24, 25]. After another 12 hours, the medium was changed to serum-free normal glucose or high glucose for another 48 hours and cells were pulsed with ^{3}[H]thymidine as described above.

For Western blots, a total of 10^6 of primary cultures of MC derived from either *db/+* or *db/db* mice were incubated for 24 hours in serum-free DMEM with 100 mg/dl D-glucose. Cells were subsequently incubated for 48 hours in serum-free medium with different concentrations (100, 275, 333, and 450 mg/dl) of glucose. In addition, the osmolarity of normal glucose medium was adjusted with D-mannitol (Sigma), so that the final osmolarity was equal to high glucose. After washing in ice-cold PBS, cell monolayers were directly lysed in lysis buffer (2% SDS, 60 mM Tris-HCl [pH 6.8], 100 mM dithiothreitol). Lysates were centrifuged and the supernatants were stored at −20°C until further processing. Western blots of primary cultures of MC were repeated twice from independent isolates.

Western blots and immunoprecipitations

Isolated glomeruli from *db/+*, *db/db*, or STZ-injected BALB/c mice (200 μl of glomerular suspension) were lysed in lysis buffer. After centrifugation the supernatants were transferred to new tubes. Protein content was measured in all supernatants including those from lysed MC by a modification of the Lowry method that is insensitive to the used concentrations of SDS and dithiothreitol [23]. Protein concentrations were adjusted to 80 μg/sample and 5% glycerol/0.05% bromophenol blue was added. After boiling for five minutes and centrifugation, supernatants were loaded onto a denaturing 12% SDS-polyacrylamide gel. Prestained low molecular weight markers (Amersham), which comprise 2,350 to 45,000 Daltons, served as the molecular weight standards. After completion of electrophoresis, proteins were electroblotted onto a nitrocellulose membrane (High-bond-N; Amersham) in transfer buffer [50 mM Tris-HCl (pH 7.0), 380 mM glycine, 20% methanol]. Filters were stained with Ponceau S to control for equal loading and transfer. Blocking and detection of p27[Kip1] were performed exactly as previously described [15, 23]. A 1:1000 dilution of a mouse monoclonal anti-p27[Kip1] antibody (Transduction Laboratories, Lexington, MA, USA) was used as primary antibody. This antibody reacts with mouse and rat p27[Kip1] [23]. The ECL system (Amersham) was used for the luminescence detection of horseradish peroxidase-conjugated second antibody. Western blots were independently performed three times from independent experimental series with qualitatively similar results. Exposed films were scanned with a laser densitometer (Hoefer Scientific Instruments, San Francisco, CA, USA), and the area under the curve was determined by Gaussian integration with the computer program GS 365W from Hoefer. Signals obtained from *db/+*

glomeruli or cells grown in normal glucose were assigned as a relative value of one.

To determine which Cdk may associate with $p27^{Kip1}$, immunoprecipitation experiments were performed on glomerular lysates of *db/+* and *db/db* mice. A total of 100 μl lysate, 400 μl sterile water, 500 μl 2× immunoprecipitation buffer (1 × immunoprecipitation buffer: 1% Triton X-100, 150 mM NaCl, 10 mM Tris-HCl (pH 7.4); 2 mM EDTA, 0.2 mM sodium vanadate, 0.2 mM phenyl methylsulfonylfluoride, 0.5% NP-40], and 5 μg antibody were mixed and incubated on a shaking-platform on ice for 60 minutes. Monoclonal anti-Cdk2 and anti-Cdk4 antibodies (both from Transduction Laboratories) or a polyclonal anti-Cdk6 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were used. All these antibodies react with the putative Cdks. To facilitate immunoprecipitations, 5 μg polyclonal rabbit anti-mouse IgG (Transduction Laboratories) was added to the mixtures in which monoclonal antibodies were used and tubes were incubated for an additional 30 minutes on ice. For immunoprecipitation, 50 μl *S. aureus* Cowan strain (Calbiochem-Novabiochem, Bad Soden, Germany) was added, and tubes continued shaking for another 60 minutes. Precipitates were formed by centrifugation, washed three times in ice-cold 1× immunoprecipitation buffer, and the pellets were resuspended in 40 μl SDS-electrophoresis buffer (2% SDS, 60 mM Tris-HCl; pH 6.8; 100 mM dithiothreitol, 5% glycerol, 0.03% bromophenol blue). After boiling for 10 minutes, tubes were shortly centrifuged, and the supernatants were finally loaded onto a 12% SDS-polyacrylamide gel. Western blotting and detection of $p27^{Kip1}$ were performed as described above. Immunoprecipitation experiments with subsequent Western blotting were independently performed twice with qualitatively similar results.

Isolation of RNA and cDNA amplification

To detect minor changes in $p27^{Kip1}$ mRNA expression, semi-quantitative cDNA amplification after reverse transcription of total RNA was performed exactly as previously described using the housekeeping RNA of β-actin as an internal control [23]. Isolated glomeruli from six kidneys obtained from *db/+* as well as *db/db* mice were directly lysed in 4 M guanidinium isothiocyanate, 25 mM sodium citrate (pH 7.0), 0.5% sarcosyl, and 0.1 M 2-mercaptoethanol. Isolation of total and reverse transcription of total RNA was performed exactly as previously described [23]. For cDNA amplification, the following primers were used: $p27^{Kip1}$ (5′GTCTAACGGGAGCCCGAGCCTGG3′; 5′GAAGGCCGG-GCTTCTTGGGCG3′); β-actin (5′GGCCAAGTCATCACTAT-TGG3′, 5′GGACTCATCGTACTCCTGC3′). A total of 150 ng of the primers was used. The complete amplification mix without the primers was equivalently distributed to separate tubes containing either $p27^{Kip1}$ or β-actin primers. Reactions were performed using the GeneAmp™ kit (Perkin Elmer Cetus, Überlingen, Germany). Reactions were performed for 30 cycles with an annealing temperature of 60°C for 1.5 minutes, an extension step at 72°C for 1.5 minutes, and a denaturation step at 92°C. Ten microliters of the reaction product were run on a 1.5% agarose gel containing 0.5 μg/ml ethidium bromide. Bands of the predicted size ($p27^{Kip1}$, 560 bp; β-actin, 360 bp) were photographed with Polaroid 55 negative film, and the reaction products on the film were scanned by laser densitometry. Reverse transcription and cDNA amplification were independently performed three times.

Table 1. Body weight, kidney weight, and blood glucose levels of *db/+* and *db/db* mice 7 weeks after birth

	db/+	*db/db*
Body weight *g*	23.8 ± 1.1	51.5 ± 4.3[a]
Kidney weight *g* (left kidney)	0.18 ± 0.01	0.30 ± 0.02[a]
Blood glucose *mg/dl*	103 ± 9.3	251 ± 18.3[a]

$N = 8$.
[a] $P < 0.001$

Immunohistochemistry

Small pieces of kidneys were fixed in Methyl Carnoy's solution, embedded in paraffin, and 2 μm thick sections were prepared. For immunohistochemistry demonstration of $p27^{Kip1}$, a rabbit polyclonal anti-mouse $p27^{Kip1}$ antibody (Santa Cruz) was used. This rabbit antiserum was selected instead of the mouse monoclonal anti-$p27^{Kip1}$ antibody to prevent non-specific binding of secondary anti-mouse antibody to nonspecifically trapped IgG in diabetic mice [26]. Sections were dewaxed, rehydrated and microwave pretreated. Tissue sections were incubated with a 1:20 dilution of the rabbit polyclonal anti-$p27^{Kip1}$ antibody. As additional controls, slides were incubated with normal rabbit serum. The APAAP complex was used for the visualization of the primary antibody. Evaluation of $p27^{Kip1}$-positive cells were performed by an investigator blinded to the experimental protocol. $p27^{Kip1}$-positive cells were counted in at least 10 glomeruli from three independent animals.

Statistical analysis

Results are expressed as means ± SEM. Statistical significance was tested with the Wilcoxon-Mann-Whitney test. A value of $P < 0.05$ was considered significant.

RESULTS

db/db and *db/+* mice

As predicted, *db/db* mice at seven weeks after birth revealed a higher body and kidney weight as well as significantly higher blood glucose concentrations compared with non-diabetic, age-matched *db/+* littermates (Table 1).

As shown in Figure 1, glomeruli from *db/db* mice at seven weeks of age expressed easily detectable amounts of $p27^{Kip1}$ protein whereas $p27^{Kip1}$ expression was hardly detectable in non-diabetic *db/+* littermates (Fig. 1). However, in contrast to protein expression, there was no difference in glomerular mRNA expression between *db/db* and *db/+* mice as determined by cDNA amplification of reverse-transcribed RNA (Fig. 2). Quantification of $p27^{Kip1}$ expression by laser densitometry is given in Table 2.

Immunohistochemistry staining was performed to obtain further information on the morphological localization of the increased $p27^{Kip1}$ protein expression in *db/db* mice. Since diffuse linear localization of IgG along the glomerular capillary wall has been observed in diabetic nephropathy which may be non-specifically detected by a secondary anti-mouse antibody [26], we used for our indirect immunohistochemistry studies a rabbit polyclonal anti-$p27^{Kip1}$ antiserum with an anti-rabbit secondary antibody. Furthermore, sections were microwave pretreated which destroys potential mouse IgG. $p27^{Kip1}$ staining was principally localized to nuclei. Only a few, mainly podocytes and
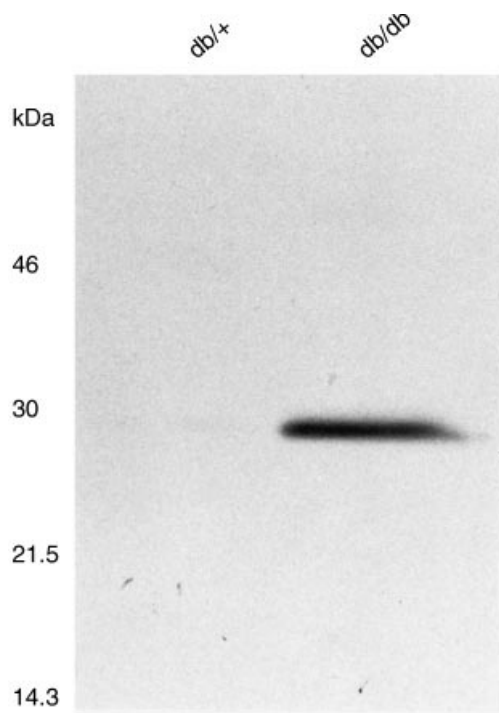
Fig. 1. Western blot for p27^{Kip1}. Isolated glomeruli from seven-week-old *db/+* or *db/db* mice were lysed, and 80 µg total protein were separated on an SDS-polyacrylamide gel. There was only very weak expression of p27^{Kip1} protein in *db/+* mice. In contrast, a strong band was detected in glomerular lysates from diabetic *db/db* mice. This blot is representative of three independent experiments using isolated glomeruli of separate animals.

endothelial cells, stained positively for p27^{Kip1} in *db/+* mice (Fig. 3A). In contrast, a significant increase in the number of p27^{Kip1}-positive cells were observed in *db/db* mice (Fig. 3B). These positive cells were mainly found in mesangial areas, but occasionally nuclei of endothelial cells were also positive. Quantification of glomerular p27^{Kip1}-positive cells revealed a significant increase in *db/db* mice compared with non-diabetic littermates (*db/+* mouse, 1.8 ± 0.4; *db/db* mouse, 5.7 ± 1.3 positive cells per glomerulus, $N = 30$, $P < 0.01$). Although a few nuclei of mainly distal tubuli irregularly stained positive for p27^{Kip1}, there was no significant difference between *db/+* and *db/db* mice (data not shown). No staining was detected when the primary antibody was substituted with normal rabbit serum, demonstrating the specificity of the detection (data not shown).

To gain insight into which Cdks may associate with the increased p27^{Kip1} expression in *db/db* mice, immunoprecipitation experiments using specific antibodies against various Cdks with subsequent Western blotting for p27^{Kip1} were performed. As shown in Figure 4A, p27^{Kip1} associates with Cdk2 as well as with Cdk4. However, there was no appreciable difference in the amount of p27^{Kip1} protein bound to these Cdks in *db/+* and *db/db* mice. In contrast, p27^{Kip1} failed to associate with Cdk6 in *db/+* as well as *db/db* mice (Fig. 4B).

Mesangial cell cultures

Since we have previously demonstrated that increasing the glucose concentration of the culture medium stimulates p27^{Kip1}
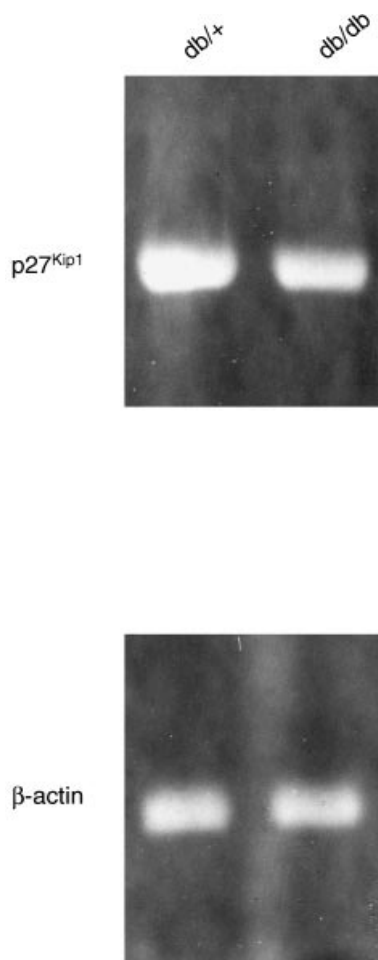

Fig. 2. Semiquantitative cDNA amplification of reversed transcribed total RNA. In contrast to p27^{Kip1} protein expression, there was no change in glomerular transcript abundance between *db/+* and *db/db* mice. Parallel amplification of the housekeeping gene β-actin served as an internal control. Isolation of total RNA, reverse transcription, and cDNA amplification were independently performed three times using isolated glomeruli from different animals.

Table 2. Quantitative analysis of Western blots by laser densitometry

Experiment	Relative densitometric values		Significance	N
Figure 1	*db/+*	*db/db*		
	1.0 ± 0.3	13.5 ± 3.8	$P < 0.01$	3
Figure 5	MC *db/+* G100	MC *db/+* G450		
	1.0 ± 0.3	12.0 ± 4.0	$P < 0.01$	2
	MC *db/db* G100	MC *db/db* G450		
	1.0 ± 0.5	9.0 ± 2.8	$P < 0.01$	2
Figure 6	Control	STZ		
	1.0 ± 0.7	6.2 ± 2.2	$P < 0.05$	3

expression in mouse MC cell line, we were interested in whether the glomerular (mainly mesangial) expression of p27^{Kip1} in kidneys of *db/db* mice may be due to hyperglycemia or is induced by different mechanism. To this end, primary cultures of MC from *db/+* and *db/db* mice were established in normal glucose-containing medium. Outgrown cells exhibited the typical characteristics
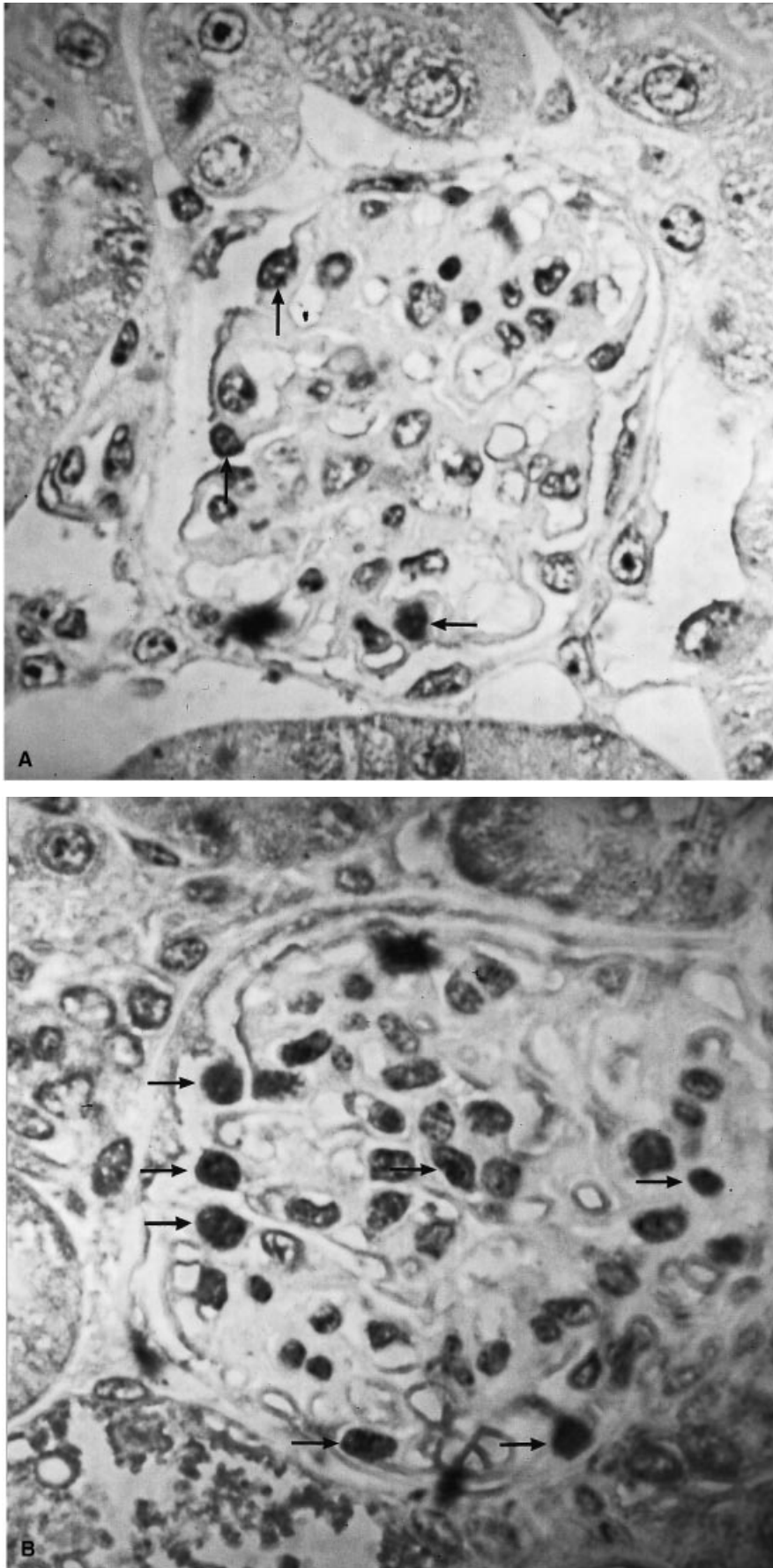
Fig. 3. Immunohistochemistry staining for p27^Kip1^ on kidney sections from seven-week-old *db*/+ and *db*/*db* mice. (*A*) p27^Kip1^ expression was principally localized to nuclei. In *db*/+ mice only a few glomerular cells stained positively for p27^Kip1^ (arrows). (*B*) In contrast, the number of glomerular p27^Kip1^-positive cells increased in the kidney of *db*/*db* mice (arrows). There was only occasional positive staining of tubular nuclei (magnification ×800).
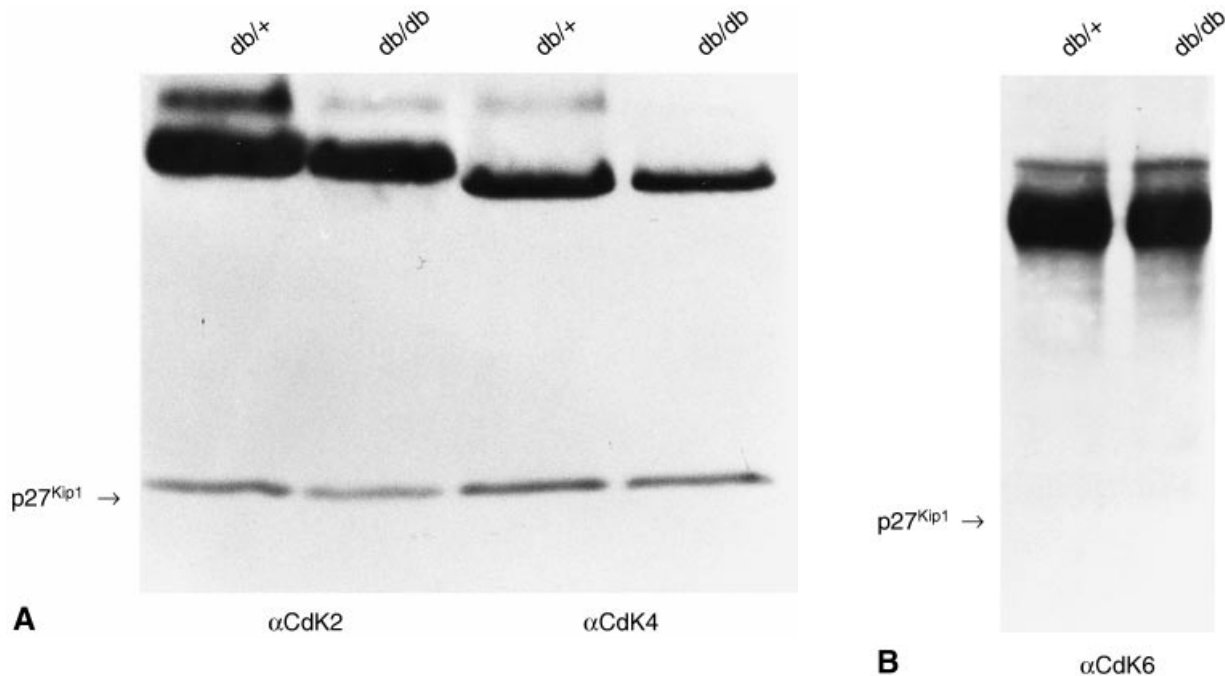
Fig. 4. Immunoprecipitation experiments using anti-Cdk2, 4 and 6 antibodies with subsequent Western blotting and detection of p27^{Kip1}. (*A*) Glomerular lysates were immunoprecipitated with anti-Cdk2 or anti-Cdk4 antibody. Although p27^{Kip1} associates with Cdk2 and Cdk4, there was no major difference in the amount of p27^{Kip1} bound to these Cdks between *db/+* and *db/db* mice. (*B*) There was no detection of p27^{Kip1} after immunoprecipitation with an anti-Cdk6 antibody, indicating that p27^{Kip1} does not associate in this system with Cdk6. This blot is representative of two experiments with qualitatively similar changes.

Table 3. ^{3}[H]thymidine incorporation into mesangial cells isolated from *db/+* and *db/db* mice

	db/+	*db/db*
G 100 + no oligonucleotides	0.8 ± 0.03	1.0 ± 0.02
G 450 + no oligonucleotides	0.5 ± 0.02[a]	0.6 ± 0.05[a]
G 100 + p27^{Kip1} missense	1.1 ± 0.03	0.9 ± 0.07
G 450 + p27^{Kip1} missense	0.4 ± 0.05[a]	0.5 ± 0.03[a]
G 100 + p27^{Kip1} antisense	1.5 ± 0.07[b]	1.6 ± 0.04[b]
G 450 + p27^{Kip1} antisense	2.8 ± 0.06[c]	3.2 ± 0.05[c]

Effect of p27^{Kip1} antisense and missense phosphorothioate oligonucleotides on proliferation in either normal (G 100) or high glucose (G 450) medium. Data are × 10^3 cpm, *N* = 12.

[a] *P* < 0.01 vs. G100
[b] *P* < 0.05 vs. G 100 + no oligonucleotides
[c] *P* < 0.01 vs. G 450 + no oligonucleotides

of MC including positive staining for desmin and vimentin, but failed to bind antibodies generated against factor VIII and 3M-1 proximal tubular cell antigen (data not shown). MC from *db/+* and *db/db* mice showed a similar degree of proliferation in normal glucose (Table 3). In addition, increasing the ambient glucose for 48 hours suppressed proliferation in both cell lines to a similar extent (Table 3). Treatment of cells with antisense p27^{Kip1}, but not with missense, oligonucleotides facilitated cell cycle progression in high glucose medium to a similar extent in MC obtained from *db/+* and *db/db* (Table 3). There was a comparable baseline expression of p27^{Kip1} protein when primary MC cultures from both mouse strains were cultured in normal glucose medium (Fig. 5A). However, raising the glucose content of the serum-free medium to 275 to 450 mg/dl stimulated p27^{Kip1} protein expression in MC from *db/+* as well as *db/db* mice (Fig. 5B; quantification of

values are provided in Table 2). However, raising the osmolarity of normal glucose medium (100 mg/dl) with D-mannitol failed to induce p27^{Kip1} expression in primary MC cultures from *db/+* as well as *db/db* mice (Fig. 5B).

Glomerular p27^{Kip1} expression in streptozotocin-induced diabetes mellitus

To investigate whether glomerular p27^{Kip1} expression may also exist in animals with IDDM, we performed some limited studies with STZ-induced diabetes mellitus in mice. As shown in Figure 6, glomerular p27^{Kip1} expression was strongly stimulated in diabetic mice 48 hours after injection of a single dose of STZ (quantification of densitometric values are provided in Table 2). Diabetic mice 48 hours after STZ-injection exhibited a blood glucose of 290 ± 23.5 mg/dl compared with 87 ± 6.7 mg/dl of non-diabetic controls (*N* = 6). Time-course studies revealed that this increased expression was not present 24 hours after STZ injection; expression remained elevated one week after administration of STZ (data not shown).

DISCUSSION

Mesangial matrix expansion and thickening of capillary basement membranes are hallmarks of diabetic nephropathy [2, 27]. Mesangial cell hypertrophy has been linked to the progression of renal disease in diabetes mellitus [28]. There is strong evidence that hyperglycemia significantly contributes to these structural abnormalities [29, 30]. Cell culture studies have provided considerable insight into the mechanisms of how hyperglycemia *per se* mediates pathophysiological changes in renal cells. For example, increasing the glucose concentration of culture medium has a
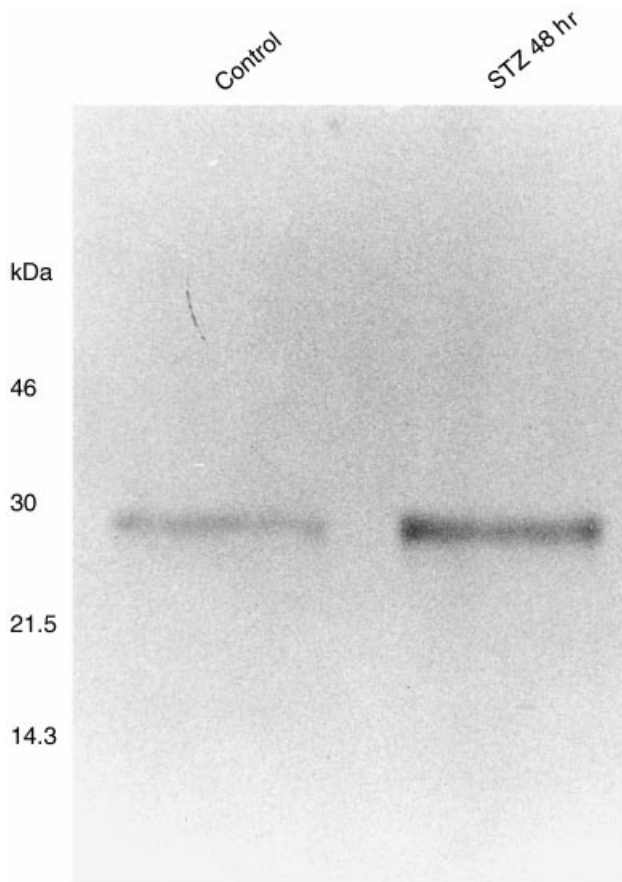
A

B
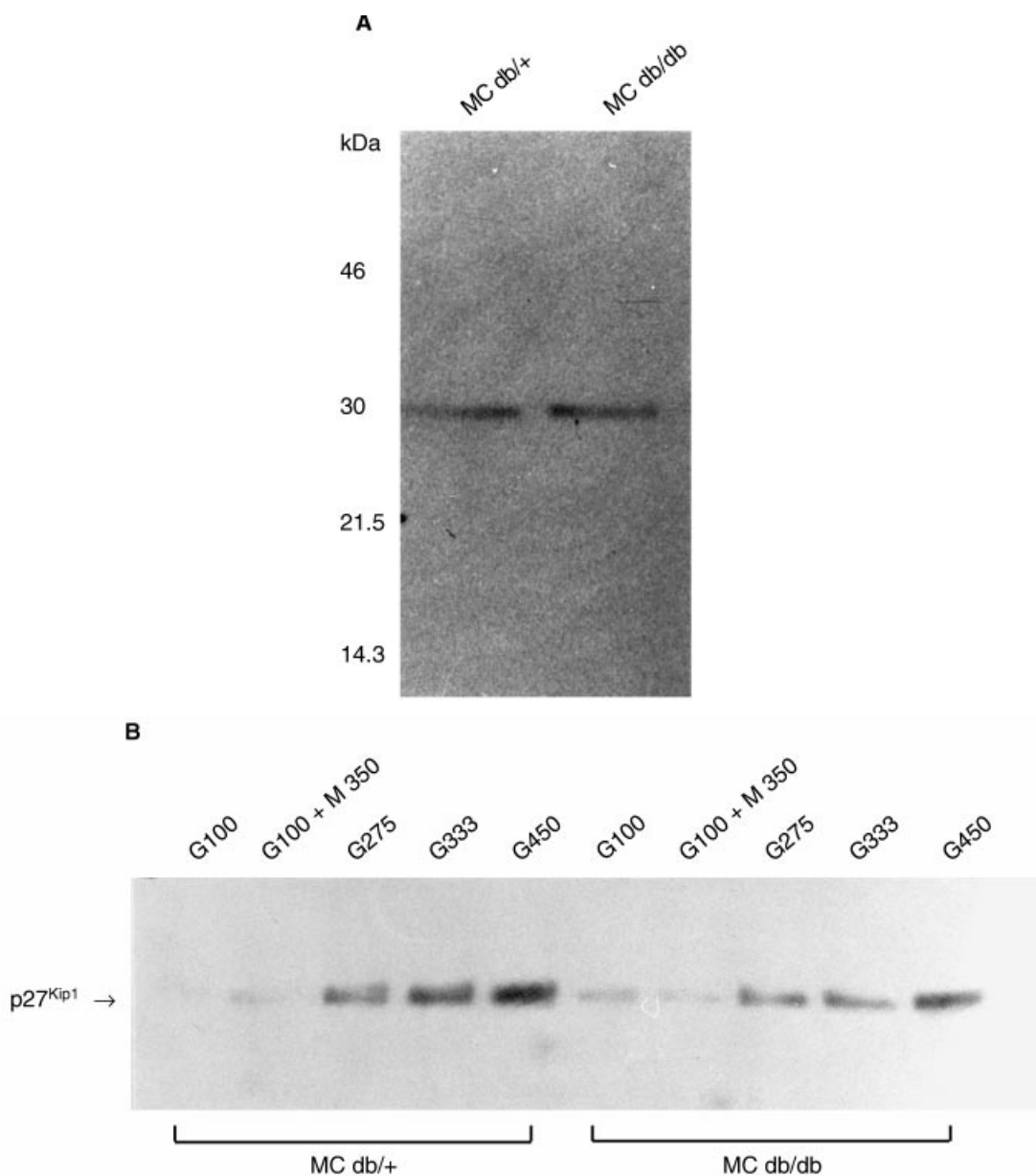
Fig. 5. Western blots of mesangial cell (MC) lysates for p27^{Kip1}. Primary MC cultures were established in normal glucose medium from *db/+* and *db/db* mice. (*A*) There was similar expression of p27^{Kip1} when cells from either group of mice were grown in serum-free normal glucose-containing medium (G100). (*B*) Incubation of MC for 48 hours in different glucose concentrations (275 to 450 mg/dl, G257-G450) readily induced p27^{Kip1} expression in MC from both *db/+* and *db/db* mice compared with normal glucose (100 mg/dl, G100). However, raising the osmolarity of normal glucose medium with 350 mg/dl D-mannitol (G100 + M 350) failed to stimulate p27^{Kip1} expression. This blot is representative of two experiments using primary cultures isolated from different animals.

biphasic effect on the growth of MC [10]. After an initial proliferation, MC are growth arrested in the G$_1$-phase of the cell cycle, enlarge, and produce more extracellular matrix proteins [10–14]. We and others have previously demonstrated that the high glucose-induced cell cycle arrest is mediated by TGF-β [10, 30]. A similar sequence of events (initial proliferation with subsequent G$_1$-phase arrest) has been described *in vivo* in the STZ-induced model of IDDM in rats [31]. Additional factors such as angiotensin II may contribute to the growth stimulation mediated by hyperglycemia [32].

Cell growth is controlled by the complex mechanisms that

constitute the cell cycle [33–35]. This cell cycle is divided into four sequential phases. The period associated with DNA synthesis, the so-called S-phase, is preceded by the G$_1$-phase in which the cell prepares its machinery for the subsequent replication of DNA. During the last decade a universal paradigm has emerged from genetic studies with yeast as well as information derived from experiments with marine invertebrates, amphibians, and mammalian cells that the key transitions in the cell cycle are controlled by the activation of a special family of protein kinases called the cyclin-dependent kinases (Cdk) [reviewed in 33, 34]. One essential step in Cdk activation is the assembly of an appropriate cyclin with

medium strongly stimulated p27^{Kip1} expression, but failed to influence mRNA abundance [15]. Furthermore, these effects were independent of the osmolarity of the medium and involved activation of protein kinase C [15]. p27^{Kip1} antisense, but not missense, oligonucleotides inhibited in these *in vitro* studies high glucose-stimulated protein synthesis and facilitated G$_1$-phase exit indicating the central role of p27^{Kip1} in high glucose-induced MC hypertrophy [15]. Since potential factors and mechanisms stimulating growth of cultured cells may be fundamentally different from the *in vivo* situation, the present study was undertaken to test whether increased glomerular expression occurs in *db/db* mice, a model of NIDDM.

The mutation in the *db/db* mouse occurred spontaneously in mice of the C57BL/KsJ strain [17]. Diabetes mellitus in the *db/db* mouse is initially expressed as hyperinsulinemia, followed by hyperphagia and progressive obesity, resulting finally in hypoinsulinemia and early death [39]. The *db/db* mouse is characterized by a mutation in the leptin receptor (abnormal splicing) with a missing cytoplasmic tail resulting in an interrupted signaling pathway [40, 41]. Thus, despite high leptin levels, food consumption is not suppressed in *db/db* mice. This hyperphagia leads to a marked increase in the body wt that can be recognized as early as five weeks of age. The consequence of this increased food consumption, obesity, and the insulin resistance caused by high leptin levels, is a syndrome remarkably similar to NIDDM of humans. Although there are no initial structural abnormalities between *db/db* mice and their heterozygotic *db/+* littermates, *db/db* mice develop a progressive nephropathy during the course of the disease with early glomerular hyperfiltration associated with mesangial hypertrophy, and subsequent increases in mesangial synthesis of extracellular matrix proteins such as collagen type IV and fibronectin causing further expansion of the glomerular tuft and thickening of the peripheral capillary basal lamina [16, 18, 19, 42–44]. Finally, *db/db* mice die of renal failure induced by diffuse glomerulosclerosis and nodular thickening of the glomerular basement membrane. The accumulation of extracellular matrix proteins in kidneys of *db/db* mice is partly mediated by increased glycation of serum albumin because an antibody against Amadori-glycated albumin prevents the development of nephropathy [16].

The present study provides clear evidence that p27^{Kip1} protein expression is strongly stimulated in glomeruli of seven-week-old diabetic *db/db* mice compared with non-diabetic age-matched *db/+* heterozygotes. At this time point, *db/db* mice can be clearly distinguished from their littermates by their obesity, and they exhibit hyperglycemia and an increase in serum insulin levels. At seven weeks after birth, *db/db* mice also have glomerular hyperfiltration and display glomerular hypertrophy, but they have not yet developed frank diabetic nephropathy as characterized by increased extracellular matrix deposition [18, 19, 43]. Since endogenous immunoglobulins may be deposited in the glomerulus during the course of diabetic nephropathy [26], great care was taken in selecting antibodies which do not cross-react with mouse immunoglobulins indicating the specificity of the observed staining pattern. In addition to *db/db* mice, we also observed a similar glomerular distribution of p27^{Kip1} staining in kidneys from mice after one week of STZ-induced IDDM (data not shown).

Our immunohistochemistry experiments demonstrated that p27^{Kip1} expression was principally localized to cell nuclei. Quantification of p27^{Kip1}-positive glomerular cells revealed a significant

Fig. 6. Western blot for p27^{Kip1} in glomerular lysates obtained from BALB/c mice made diabetic with streptozotocin (STZ). In contrast to controls, there was an increase in p27^{Kip1} expression in diabetic mice 48 hours after injection of STZ. This increase was not present at 24 hours, but increased expression remained up to one week (data not shown). Qualitatively similar results were obtained in two additional experiments.

its putative Cdk, since only this dimer has the necessary kinase activity for passing restriction points that induce cell cycle progression [33, 34]. Cyclins are a family of proteins that are periodically activated during the cell cycle, and specific cyclin/Cdk dimers are assembled and activated at different stages in the cell cycle. The signaling pathways that regulate cell cycle progression seem to be primarily associated with the G$_1$-phase of the cell cycle. During this phase, at least two types of cyclins, cyclin D and E, bind to and activate Cdks during G$_1$, and both are necessary for S phase to begin. In addition to being pivotal for G$_1$/S-phase transit, cyclin D/Cdk4 and cyclin E/Cdk2 complexes are both rate-limiting for completion of the G$_1$-phase. p27^{Kip1} is a Cdk inhibitor that binds to the various G$_1$ cyclin/Cdks complexes and inhibits their kinase activity, presumably by interacting with the catalytic cleft for ATP [35]. An increase in p27^{Kip1} expression has been associated with cell cycle arrest in various circumstances and overexpression of p27^{Kip1} leads to G$_1$-phase arrest in all cell lines that have been tested so far [36–38].

Since MC that are cultured in high glucose medium are arrested in the G$_1$-phase, we have recently investigated whether high glucose-containing medium may influence p27^{Kip1} expression in cultured MC. These studies demonstrated that high glucose

increase in *db/db* mice compared to non-diabetic *db/+* littermates. In the glomerulus, mainly mesangial cells and podocytes stained positive, but occasionally glomerular endothelial cells (particularly in *db/db* mice) exhibited p27^{Kip1} expression. Although tubular cells randomly disclosed p27^{Kip1} positive nuclei, there was no significant difference between *db/+* and *db/db* animals. Our study is in agreement with recent observations by Shankland and co-workers who found p27^{Kip1} protein expression mainly in nuclei of mesangial cells, but glomerular endothelial cells also revealed a positive staining [45].

It is generally acknowledged that isolating a pure glomeruli preparation from mice is more demanding than from rats. Although our preparation revealed > 80% glomeruli, there was some tubular contamination. One may therefore ask whether tubular contamination contributes to the increase p27^{Kip1} expression *db/db* mice. We don't think that this is likely since only a few tubular cells stained positively for p27^{Kip1} and the immunohistochemistry revealed no difference between *db/+* and *db/db* mice. Along this line, it also seems improbable that size effects (larger glomeruli from diabetic animals) may explain the difference in p27^{Kip1} expression because equal concentrations of protein were loaded for the Western blot experiments. Assuming that larger glomeruli from *db/db* mice would be preferentially harvested, more control glomeruli would be needed to obtain the same protein concentration.

In contrast to increased p27^{Kip1} protein expression, there was no change in transcript levels as detected by semiquantitative RT-PCR between *db/db* mice and their *db/+* littermates. This is in good agreement with previous studies in several cell types demonstrating that changes in p27^{Kip1} expression may be primarily regulated by post-transcriptional mechanisms [23, 37, 38]. Although this post-transcriptional regulation of p27^{Kip1} protein levels is currently not well understood, studies on colorectal carcinomas in which p27^{Kip1} expression is reduced, demonstrated an increased proteasome-dependent degradation of this Cdk-inhibitor [46]. Selective proteolysis of Cdk-inhibitors has been also suggested as one major pathway inducing cell cycle progression in non-transformed cells [47, 48]. Since a reduction of various renal proteases has been described in diabetes mellitus [49], such a decrease in p27^{Kip1} turnover may contribute to the enhanced glomerular expression in *db/db* mice.

We further tested whether mesangial expression of p27^{Kip1} in *db/db* mice is due to hyperglycemia or other factors that may be present in the leptin resistance background. Mesangial cells were isolated from *db/+* and *db/db* mice, and p27^{Kip1} expression was comparably weak between MC from *db/+* and *db/db* mice when cells were grown in medium with normal (100 mg/dl) D-glucose. However, in agreement with previous studies performed in a mesangial cell line, a strong p27^{Kip1} protein expression could be induced by transferring MC into medium with increasing concentrations of glucose (275 to 450 mg/dl). An increase in p27^{Kip1} expression compared with normal glucose was already induced by an increase in medium glucose concentration (275 to 333 mg/dl) that is comparable to the blood glucose measured in the diabetic mice. High glucose-induced p27^{Kip1} expression was independent from the medium osmolarity because raising the osmolarity with D-mannitol failed to stimulate expression of this Cdk inhibitor. Furthermore, MC from *db/+* and *db/db* mice grow equally well in medium with normal glucose concentration, but cell cycle arrest occurred in medium with high glucose. These findings strongly suggest that mesangial p27^{Kip1} expression in *db/db* mice *in vivo* is due to hyperglycemia and not due to other factors. Moreover, limited experiments also demonstrated that glomerular p27^{Kip1} was strongly enhanced in mice with STZ-induced IDDM indicating that hyperglycemia *per se* and not the type or model of diabetes is pivotal for this stimulation of the Cdk inhibitor.

We have previously shown that high glucose medium induces p27^{Kip1} protein in cultured MC and that this induction depended, at least to some extent, on the endogenous stimulation of TGF-β [15]. Thus, it is tempting to assume that the cell cycle arrest caused by high glucose in MC *in vivo* and *in vitro* may be mediated by TGF-β-stimulated expression of p27^{Kip1}. However, recent studies have clearly demonstrated that p27^{Kip1} is not necessary for the antiproliferative effects mediated by TGF-β in MC [50]. In addition, cell cycle arrest of lymphocytes mediated by TGF-β remains intact in transgenic mice lacking p27^{Kip1} [51]. However, TGF-β may exert antimitogenic effects by several other mechanisms including inhibition of G$_1$-phase Cdk and cyclins and/or transcriptional activation of IκBα inducing apoptosis [52–55].

It has been previously suggested that p27^{Kip1} binds to Cdk2, Cdk4, or Cdk6 with their respective cyclins, and inhibits kinase activity [36–38]. However, our present immunoprecipitation experiments, although demonstrating binding of p27^{Kip1} to Cdk2 and Cdk4, but not to Cdk6, failed to show a difference in the amount of p27^{Kip1} bound to each Cdk between glomeruli from *db/+* and *db/db* mice. The reason for this observation is currently unknown, but some suggestions may be made. The current picture of Cdk inhibitors suggests that they set an adjustable threshold for cyclin-dependent activation of the various Cdks. According to this hypothesis, p27^{Kip1} is sequestered by Cdk4/cyclin D complexes, which are needed to overcome a p27^{Kip1} threshold to become active when cells progress through the G$_1$-phase [36, 37]. For example, p27^{Kip1} may contribute to TGF-β-mediated G$_1$ arrest by interfering with Cdk2/cyclin E complexes as a result of its release from cyclin Cdk4/cyclin D complexes due to the TGF-β-mediated inhibition of Cdk4 production [55]. To explain our findings, one may assume that in addition to p27^{Kip1}, the synthesis of Cdk2 or Cdk4 is enhanced in the diabetic environment. Thus, not the absolute level of p27^{Kip1} protein, but rather the relationship among various Cdk-inhibitors and their putative Cdk/cyclin complexes may ultimately regulate cell cycle progression.

In summary, we found that glomerular, mainly mesangial, p27^{Kip1} expression is enhanced in *db/db* mice, a model of NIDDM. This stimulation is due to hyperglycemia. Since p27^{Kip1} overexpression is associated with cell cycle arrest, this finding may explain the glomerular hypertrophy observed in early diabetes. However, whether this protein is indeed involved in this lesion will await studies in which induction of diabetes is produced in p27^{Kip1} negative mice.

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REFERENCES

1. CHOWDHURY TA, BARNETT AH, BAIN SC: Pathogenesis of diabetic nephropathy. *Trends Endocrinol Metab* 7:320–323, 1996
2. IBRAHIM HN, HOSTETTER TH: Diabetic nephropathy. *J Am Soc Nephrol* 8:487–493, 1997
3. PERNEGER TV, BRANCATI FL, WHELTON PK, KLAG MJ: End-stage renal disease attributable to diabetes mellitus. *Ann Intern Med* 121:912–918, 1994
4. NELSON RG, BENNETT PH, BECK GJ, TAN M, KNOWLER WC, MITCH WE, HIRSCHMAN GH, MYERS BD: Development and progression of renal disease in Pima Indians with non-insulin-dependent diabetes mellitus. *N Engl J Med* 335:1636–1642, 1996
5. RITZ E, KELLER C, BERGIS KH: Nephropathy of type II diabetes mellitus. *Nephrol Dial Transplant* 11(Suppl 9):38–44, 1996
6. PAGTALUNAN ME, MILLER PL, JUMPING-EAGLE S, NELSON RG, MYERS BD, RENNKE HG, COPLON NS, SUN L, MEYER TW: Podocyte loss and progressive glomerular injury in type II diabetes. *J Clin Invest* 99:342–348, 1997
7. PORTE D, SCHWARTZ MW: Diabetes complications: Why is glucose potentially toxic? *Science* 272:699–700, 1996
8. BUCALA R, VLASSARA H: Advanced glycosylation end products in diabetic renal and vascular disease. *Am J Kidney Dis* 26:875–888, 1995
9. COHEN MP, ZIYADEH FN: Role of Amadori-modified nonenzymatically glycated serum proteins in the pathogenesis of diabetic nephropathy. *J Am Soc Nephrol* 7:183–190, 1996
10. WOLF G, SHARMA K, CHEN Y, ERICKSEN M, ZIYADEH FN: High glucose-induced proliferation in mesangial cells is reversed by autocrine TGF-β. *Kidney Int* 42:647–656, 1992
11. COSIO FG: Effects of high glucose concentrations on human mesangial cell proliferation. *J Am Soc Nephrol* 5:1600–1609, 1995
12. NAHMAN NS, LEONHARDT KL, COSIO FG, HEBERT CL: Effects of high glucose on cellular proliferation and fibronectin production by cultured human mesangial cells. *Kidney Int* 41:396–402, 1992
13. ZIYADEH FN, SHARMA K, ERICKSEN M, WOLF G: Stimulation of collagen gene expression and protein synthesis in murine mesangial cells by high glucose is mediated by autocrine activation of transforming growth factor-β. *J Clin Invest* 93:536–542, 1994
14. AYO SH, RADNIK RA, GLASS WF II, GARONI JA, RAMPT ER, APPLING DR, KREISBERG JI: Increased extracellular matrix synthesis and mRNA in mesangial cells grown in high-glucose medium. *Am J Physiol* 260:F185–F191, 1991
15. WOLF G, SCHROEDER R, ZIYADEH FN, THAISS F, ZAHNER G, STAHL RAK: High-glucose stimulates expression of p27^Kip1 in cultured mouse mesangial cells: Relationship to hypertrophy. *Am J Physiol* 273:F348–F356, 1997
16. COHEN MP, SHARMA K, JIN Y, HUD E, WU VY, TOMASZEWSKI J, ZIYADEH FN: Prevention of diabetic nephropathy in *db/db* mice with glycated albumin antagonists. A novel treatment strategy. *J Clin Invest* 95:2338–2345, 1995
17. VELASQUEZ MT, KIMMEL PL, MICHAELIS OE: Animal models of spontaneous diabetic kidney disease. *FASEB J* 4:2850–2859, 1990
18. LIKE AA, LAVINE RL, POFFENBARGER PI, CHICK WL: Studies in the diabetic mutant mouse. VI. Evolution of glomerular lesions and associated proteinuria. *Am J Pathol* 66:193–224, 1972
19. GÄRTNER K: Glomerular hyperfiltration during the onset of diabetes mellitus in two strains of diabetic mice (C57BL/6J *db/db* and C57BL/KsJ *db/db*). *Diabetologia* 15:59–63 (1978)
20. ITAGAKI SI, NISHIDA E, LEE MJ, DOI K: Histopathology of subacute renal lesions in mice induced by streptozotocin. *Exp Toxic Pathol* 47:485–491, 1995
21. THAISS F, WOLF G, ASSAD N, ZAHNER G, STAHL RAK: Angiotensinase A gene expression and enzyme activity in isolated glomeruli of diabetic rats. *Diabetologia* 39:275–280, 1996
22. WOLF G, HABERSTROH U, NEILSON EG: Angiotensin II stimulates the proliferation and biosynthesis of type I collagen in cultured murine mesangial cells. *Am J Pathol* 140:95–107, 1992
23. WOLF G, STAHL RAK: Angiotensin II-stimulated hypertrophy of LLC-PK$_1$ cells depends on the induction of the cyclin-dependent kinase inhibitor p27^Kip1. *Kidney Int* 50:2112–2119, 1996
24. COATS S, FLANGAN WM, NOURSE J, ROBERTS JM: Requirement of p27^Kip1 for restriction point of the fibroblast cell cycle. *Science* 272:877–880, 1996
25. BENNETT CF, CHIANG MY, CHAN H, SHOEMAKER JE, MIRABELLI CK: Cationic lipids enhance cellular uptake and activity of phosphorothioate antisense oligonucleotides. *Mol Pharmacol* 41:1023–1033, 1992
26. WESTBERG NG, MICHAEL AF: Immunohistopathology of diabetic glomerulosclerosis. *Diabetes* 21:163–174, 1972
27. KREISBERG JI, AYO SH: The glomerular mesangium in diabetes mellitus. *Kidney Int* 43:109–113, 1993
28. KLEINMAN KS, FINE LG: Prognostic implications of renal hypertrophy in diabetes mellitus. *Diab Metabol Rev* 4:179–189, 1988
29. WOLF G, THAISS F: Hyperglycemia-pathophysiological aspects at the cellular level. *Nephrol Dial Transplant* 10:1109–1112, 1995
30. MOGYORÓSI A, ZIYADEH FN: Update on pathogenesis, markers and management of diabetic nephropathy. *Curr Opin Nephrol Hypertens* 5:243–253, 1996
31. YOUNG BA, JOHNSON RJ, ALPERS CE, ENG E, GORDON K, FLOEGE J, COUSER WG: Cellular events in the evolution of experimental diabetic nephropathy. *Kidney Int* 47:935–944, 1995
32. WOLF G, ZIYADEH FN: The role of angiotensin II in diabetic nephropathy: Emphasis on nonhemodynamic mechanisms. *Am J Kidney Dis* 29:153–163, 1997
33. OKAYAMA H, NAGATA A, JINNO S, MURAKAMI H, TANAKA K, NAKASHIMA N: Cell cycle control in fission yeast and mammals: Identification of new regulatory mechanisms. *Cancer Res* 69:17–62, 1996
34. ELLEDGE SJ: Cell cycle checkpoints: Preventing an identiity crisis. *Science* 274:1664–1672, 1996
35. RUSSO AA, JEFFREY PD, PATTEN AK, MASSAGUÉ J, PAVLETICH NP: Crystal structure of the p27^Kip1 cyclin-dependent-kinase inhibitor bound to the cyclin A-cdk2 complex. *Nature* 382:325–331, 1996
36. SHERR CJ, ROBERTS JM: Inhibitors of mammalian G$_1$ cyclin-dependent kinases. *Genes Develop* 9:1149–1163, 1995
37. GRANA X, REDDY EP: Cell cycle control in mammalian cells: Role of cyclins, cyclin dependent kinases (CDKs), growth suppressor genes and cyclin-dependent kinase inhibitors (CKIs). *Oncogene* 11:211–219, 1995
38. ROBERTS JM, KOFF A, POLYAK K, FIRPO E, COLLINS S, OHTSUBO M, MASSAGUÉ J: Cyclins, Cdks, and cyclin kinase inhibitors. *Cold Spring Harb Symp Quant Biol* 59:31–38, 1994
39. BERGLUND O, FRANKEL BJ, HELLMAN B: Development of the insulin secretory defect in genetically diabetic (*db/db*) mouse. *Acta Endocrinol* 87:543–551, 1978
40. LEE GH, PROENCA R, MONTEZ JM, CARROLL KM, DARVISHZADEH JG, LEE JI, FRIEDMAN JM: Abnormal splicing of the leptin receptor in diabetic mice. *Nature* 379:632–635, 1996
41. CHEN H, CHARLAT O, TARTAGLIA LA, WOOLF EA, WENG X, ELLIS SJ, LAKEY ND, CULPEPPER J, MOORE KJ, BREITBART RE, DUYK GM, TEPPER RI, MORGENSTERN JP: Evidence that the diabetes gene encodes the leptin receptor: Identification of a mutation in the leptin receptor gene in *db/db* mice. *Cell* 84:491–495, 1996
42. CARO JF, SINHA MK, KOLACZYNSKI JW, ZHANG PL, CONSIDINE RV: Leptin: The tale of an obesity gene. *Diabetes* 45:1455–1462, 1996
43. LEE SM, BRESSLER R: Prevention of diabetic nephropathy by diet control in the *db/db* mouse. *Diabetes* 30:106–111, 1981
44. ZHANG B, GRAZIANO MP, DOEBBER TW, LEIBOWITZ MD, WHITE-CARRINGTON S, SZALKOWSKI DM, HEY PJ, WU M, CULLINAN CA, BAILEY P, LOLLMANN B, FREDERICH R, FLIER JS, STRADER CD, SMITH RG: Down-regulation of the expression of the *obese* gene by an antidiabetic thiazolinedione in Zucker diabetic fatty rats and *db/db* mice. *J Biol Chem* 271:9455–9459, 1996
45. SHANKLAND SJ, HUGO C, COATS SR, NANGAKU M, PICHLER RH, GORDON KL, PIPPIN J, ROBERTS JM, COUSER WG, JOHNSON RJ: Changes in cell-cycle protein expression during experimental mesangial proliferative glomerulonephritis. *Kidney Int* 50:1230–1239, 1996
46. LODA M, CUKOR B, TAM SW, LAVIN P, FIORENTINO M, DRAETTA GF,

JESSUP JM, PAGANO M: Increased proteasome-dependent degradation of the cyclin-dependent kinase inhibitor p27 in aggressive colorectal cracinomas. *Nature Med* 3:231–234, 1997

47. KING RW, DESHAIES RJ, PETERS JM, KIRSCHNER MW: How proteolysis drives the cell cycle. *Scinece* 274:1652–1659, 1996
48. UDVARDY A: The role of controlled proteolysis in cell-cycle regulation. *Eur J Biochem* 240:307–313, 1996
49. OLBRICHT CJ, GEISSINGER B: Renal hypertrophy in streptozotocin diabetic rats: Role of proteolytic lysosomal enzymes. *Kidney Int* 41:966–972, 1992
50. SHANKLAND SJ, PIPPIN J, FLANAGAN M, COATS SR, NANGAKU M, GORDON KL, ROBERTS JM, COUSER WG, JOHNSON RJ: Mesangial cell proliferation mediated by PDGF and bFGF is determined by levels of the cyclin kinase inhibitor p27^{Kip1}. *Kidney Int* 51:1088–1099, 1997
51. NAKAYAMA K, ISHIDA N, SHIRANE M, INOMATA A, INOUE T, SHISHIDO N, HORII I, LOH DY, NAKAYAMA KI: Mice lacking p27^{Kip1} display increased body size, multiple organ hyperplasia, retinal dysplasia, and pituitary tumors. *Cell* 85:707–720, 1996
52. REDDY KB, HOCEVAR BA, HOWE PH: Inhibition of G$_1$ phase cyclin dependent kinases by transforming growth factor β1. *J Cell Biochem* 56:418–425, 1994
53. GENG Y, WEINBERG RA: Transforming growth factor β effects on expression of G$_1$ cyclins and cyclin-dependent protein kinases. *Proc Natl Acad Sci USA* 90:10315–10319, 1993
54. ARSURA M, WU M, SONENSHEIN GE: TGFβ_1 inhibits NF-κB/Rel activity inducing apoptosis of B cells: Transcriptional activation of IκBα. *Immunity* 5:31–40, 1996
55. EWEN ME, SLUSS HK, WHITEHOUSE LL, LIVINGSTON DM: TGF-β inhibition of cdk4 synthesis is linked to cell cycle arrest. *Cell* 73:487–497, 1993