Increased Expression of Ornithine Decarboxylase in Distal Tubules of Early Diabetic Rat Kidneys

Are Polyamines Paracrine Hypertrophic Factors?

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Polyamines are small biogenic molecules that are essential for cell cycle entry and progression and proliferation. They can also contribute to hypertrophy. The activity of ornithine decarboxylase (ODC), the rate-limiting enzyme in polyamine biosynthesis, increases in the early diabetic kidney to enable renal hypertrophy. Inhibition of ODC in early diabetes attenuates diabetic renal hypertrophy and glomerular hyperfiltration. The current studies examine the temporal profile of renal ODC protein expression and localization, intrarenal polyamine levels, and sites of proliferation in kidneys of rats during the first 7 days of streptozotocin diabetes. ODC mRNA and protein content were increased in diabetic kidneys. High-performance liquid chromatography analysis showed increased intrarenal polyamine concentrations peaking after 24 h of diabetes. A subsequent increase in the number of proliferating proximal tubular cells was detected by in vivo 5-bromodeoxyuridine (BrdU) incorporation on day 3. Surprisingly, immunohistochemical studies revealed that increased ODC protein was apparent only in distal nephrons, whereas the main site of diabetic kidney hypertrophy is the proximal tubule. These findings raise the possibility that polyamines produced in the distal nephron may mediate the early diabetic kidney growth of the proximal tubules via a paracrine mechanism. *Diabetes* 52: 1235–1239, 2003

In the very early phases of human and experimental diabetes, the kidney becomes large, mainly due to growth of the proximal tubule (1–3). Because renal enlargement and accompanying glomerular hyperfiltration contribute to the later development of overt diabetic kidney disease (4), it is worthwhile to understand what causes the kidney to grow in diabetes. One characteristic of diabetic renal growth is an association with increased activity of the enzyme, ornithine decarboxylase (ODC). ODC is the initial and rate-limiting enzyme in the biosynthetic pathway of polyamines (putrescine, spermidine, and spermine) that are required for cell growth. Previous studies by us and others (5,6) have demonstrated that inhibition of ODC activity with difluoromethylornithine (DMFO) blunts renal enlargement and hyperfiltration (6) in early streptozotocin (STZ) diabetes. The present studies investigate expression of ODC protein and its cellular location and polyamine levels within the early diabetic kidney. We find that ODC expression increases with diabetes, as would be anticipated from the previously reported increases in activity. However, this expression is not localized to the proximal tubules, the major site recognized for hypertrophy and proliferation in this model, but rather to the distal nephron.

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DMFO, difluoromethylornithine; HPLC, high-performance liquid chromatography; ODC, ornithine decarboxylase; PBST, PBS containing Tween; STZ, streptozotocin; TGF, tubuloglomerular feedback.

RESEARCH DESIGN AND METHODS

Animals. Animal experimentation was conducted according to National Institutes of Health guidelines for the care of experimental animals. Adult male Wistar rats (Harlan, Indianapolis, IN) weighing of 230 ± 5 g (mean ± SE) were randomized into four groups (control, diabetes, diabetes + DMFO, and androgen treatment) matched for age and body weight at the start of the experiment. Diabetes was induced by injection of STZ (65 mg/kg body wt i.p.; ICN Biomedicals, Aurora, OH) in sodium citrate buffer (pH 4.2). DMFO (200 mg/kg i.p.) was given immediately after the injection of the STZ and every 12 h thereafter. Twenty-four hours after administration of STZ, the blood glucose concentration was determined in tail-nick blood samples by a Glucometer Elite Blood Glucose Meter (Bayer, Berkeley, CA). Only animals with blood glucose levels >300 mg/dl were utilized in the study. Diabetic rats were treated daily with protamine zinc insulin (0.5–1.5 I.U. s.c. q.d.; Anpro Pharmaceutical, Arcadia, CA) to adjust blood glucose levels to ~350 mg/dl throughout the experiment. As a positive control for ODC immunostaining, a group of female Wistar rats were injected with the androgen 19-nor-Δ^4-androstene-17-β-0l-3-1-decanoate (Organon, West Orange, NJ) in sesame oil, subcutaneously, at a dose of 0.4 mg/100 g body wt for 5 days (7). At the end of the experiment the animals were anesthetized with sodium pentobarbital (50 mg/kg body wt), the kidneys quickly removed, and the rats killed.

RT-PCR for ODC mRNA. The expression of ODC mRNA in diabetic kidneys versus controls was examined by RT-PCR. Total RNA was isolated from kidney cortex with Qiagen total RNA isolation kit (cat. no. 74104; Qiagen) following the manufacturers instructions. cDNA was generated by reverse transcription of 1 μg of DNAse-treated total RNA in a reaction volume of 20 μl using oligo-dT as a primer and a commercially available reverse transcription system kit (SuperScript First Strand Synthesis System for RT-PCR, cat. no. 11904-018; Gibco). The reaction mixture, consisted of 0.5 μg oligo (dT), 0.4 mmol/l dNTP, and 200 units of SuperScript II RT. The primer pair for PCR was designed to cover ODC cDNA from 424 to 1122 (accession no. M16982), producing a band of 698 bp. The forward primer sequences are 5 AT GG GCA GCT TTA CTA AGGAAGAG-3. The backward primer sequences are 5 GT CAA GC AG AT AC ATGCTGAAACC-3. PCR was carried out in a final volume of 50 μl consisting of two units of Platinum *Taq* DNA polymerase (cat. no. 10966-1-018; Gibco), 2 μl of RT product, 0.4 mmol/l dNTP, and 1 μmol/l of each forward and backward ODC primer. The PCR protocol was as follows: a start

of 94°C for 3 min followed by 35 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min. Both RT and PCR share the same buffer provided from the kit (cat. no. 11904-018; Gibco).

ODC protein immunoblotting. ODC protein was evaluated in early diabetic kidney versus controls by Western blotting using a mouse monoclonal anti-rat ODC IgG (clone MP16-2, cat. no. MS-464-P1; NeoMarkers, Fremont, CA) against an epitope in amino acid 355-366 (IWGPTC) of the ODC, producing a band with a size of 53 kDa (8). Kidney cortex was sliced from cold PBS-perfused kidney and homogenized in lysis buffer (50 mmol/l Tris, 0.3% SDS, pH 7.2) with protease inhibitor cocktail tablet (cat. no. 1697498; Boehringer Mannheim, Mannheim, Germany). The lysate was centrifuged at 14,000*g* at 4°C for 20 min. The supernatant was used for Western blotting. Protein determination was also performed on an aliquot of the supernatants (Bio-Rad kit cat. no. 500-0114; Bio-Rad). A total 60 μg of each sample was denatured in sample buffer for 5 min at 100°C before loading on a 4–12% Bis-Tris Gel, running in MOPS buffer with a constant voltage of 200 V for 50 min. The samples are then transferred to a nitrocellulose membrane at 25 V for 1 h in transfer buffer. All gels, buffers, and membranes used in the electrophoresis and transfer were purchased from Invitrogen (Carlsbad, CA). The protein samples immobilized on the nitrocellulose blot are then blocked in 5% milk protein in PBS containing 0.1% Tween-20 (PBST) for 2 h before overnight incubation with the primary antibody (2 μg/ml) at 4°C. After washing three times with PBST, the membrane was incubated with horseradish peroxidase–conjugated anti-mouse IgG (1:2,000; Santa Cruz Biotechnologies, Santa Cruz, CA). The immunoblot was visualized by chemiluminescence, ECL Plus reagents (Amersham Pharmacia Biotech, Piscataway, NJ).

Intrarenal polyamine measurement. Intrarenal levels of polyamines (putrescine, spermidine, and spermine) were measured using high-performance liquid chromatography (HPLC) separation of extracts from control and early diabetic kidney with or without DMFO treatment, as previously described (9).

5-Bromodeoxyuridine incorporation. To determine the number of proliferating kidney cells, rats were given a single dose of bromodeoxyuridine (BrdU) (50 mg/kg i.p.; Sigma, Saint Louis, MO) 24 h before tissue harvesting. Immunohistochemical staining for BrdU utilized an indirect, two-step labeling technique with the streptavidin-biotin peroxidase complex. Kidney sections were fixed in 10% formalin or methacarn overnight and embedded in paraffin wax. Kidney that was to be compared with one another was embedded together in the same block. After slicing and rehydrating, endogenous peroxidase was blocked by immersion in 3% H_2O_2 for 20 min. Then DNA was denatured in 1 N HCl for 30 min at 37°C. After neutralization in 0.1 mol borate buffer, sections were incubated with monoclonal primary BrdU antibody (1:1,000; Sigma, Saint Louis, MO). The sections were washed in PBS and incubated with biotinylated anti-mouse IgG (Vectastain ABC kit; Vector Laboratories, Burlingame, CA) diluted 1:250 for 30 min at room temperature and then in Vectastain ABC reagent diluted 1:250 for 30 min. Tissue-bound peroxidase was visualized using 0.05% 3 diaminobenzidine and 0.003% hydrogen peroxide in 0.1 mol/l Tris HCl buffer (pH 7.6) for 50 min under visual control. Several cortical sections were photographed from each kidney and BrdU-positive nuclei counted by three observers who were blinded as to the experimental groups. Sections taken from the gut of each animal, where cell turnover is rapid, served as positive controls.

ODC protein immunohistochemistry. In vivo expression of ODC protein in early diabetic kidneys was compared with nondiabetic male and androgen-treated female rat kidneys by immunohistochemistry. Kidney tissues were fixed in Bouin fixative for 5 h and then paraffin-embedded. Sections of 4-μm thickness were mounted on object slides, dewaxed in xylene, passed in a decreasing series of ethanol solutions, and finally rehydrated in distilled water. The kidney sections were soaked in 3% H_2O_2 for 20 min to block endogenous peroxidase. Then antigen retrieval was done by boiling the slides in 0.01 mol/l sodium citrate, pH 6.0, for 20 min in a steam cooker. After nonspecific blocking in PBS containing 2% BSA the slides were incubated with the same primary antibody used for Western blot in PBS containing 1% BSA, 0.1% Tween-20, and 0.01% SDS for 3 h at room temperature and then overnight at 4°C in a humidified chamber. After three washes in PBS the sections were incubated with secondary antibody, biotinylated anti-mouse IgG (2.5 μg/ml; Vector Laboratories) at room temperature for 1 h. The amplified (TSA Biotin System; NENLife Science Products, cat. no. NEL700A) bound antibodies were visualized with ABC Elite Vectastain reagents (Vector Laboratories). Tissue sections were lightly counterstained with hematoxylin blue.

RESULTS

RT-PCR. Both normal and early diabetic kidney cortex expressed ODC mRNA. A clear band of appropriate size was present in early diabetic (7-day) kidney cortex samples with a band of lesser intensity in normal control (Fig. 1).

Western blotting. The 53-kDa band representing ODC protein in renal cortex was substantially intensified in early diabetes (7-day) (Fig. 2). By densitometry the band was a threefold denser in diabetic kidneys versus controls.

HPLC quantification of intrarenal polyamines. By ANOVA, there was no significant effect of DFMO, diabetes, or the ANOVA cross-term, diabetes*DFMO, on the kidney content of arginine or ornithine (Fig. 3). In other words, there was no confounding of the polyamine content by differing amounts of polyamine precursors. Diabetes as a categorical variable (four levels for ANOVA; control, 1, 3, and 7 days) affected putrescine content ($P = 0.0002$). Putrescine doubled by day 1 ($P < 0.01$ by ANOVA with Tukey's test) and then gradually returned to normal by day 7. Consistent with its role as ODC inhibitor, DFMO reduced putrescine by approximately two-thirds in each group ($P < 0.00005$). At day 1, there was a tendency for DFMO to suppress spermine and spermidine contents, but this did not achieve statistical significance ($P = 0.13$). At subsequent time points there was even less evidence that spermine and spermidine could be affected by DFMO.

BrdU incorporation and BrdU labeling index. BrdU uptake was quantified as the number of nuclei staining positive per high-powered field. Ten fields were photographed from the cortex of each kidney. These were assessed by three observers who were blinded to treatment. Kidneys fixed in formalin contained more BrdU-positive cells than those in methacarn. Control kidneys manifest little or no BrdU uptake. In diabetes at day 1, there were occasional BrdU-positive nuclei apparent in proximal tubules. By day 3 of diabetes, BrdU staining was increased 10-fold. By day 7 the increase in BrdU staining had subsided (Fig. 4) While clearly more prevalent than in control kidneys, BrdU staining was far less in the diabetic kidney than in the normal gut. DFMO treatment prevented the increase in BrdU staining in 3-day diabetic kidneys and in the gut of these animals. (Reduced BrdU staining in gut of DFMO-treated animals was previously reported [10].)

Immunohistochemical localization of ODC protein. An increase in ODC protein abundance in the diabetic kidney was detected by immunostaining. By day 3 the staining was most abundant. Surprisingly, ODC staining was evident in the distal tubules but not proximal tubules of diabetic kidneys. To confirm that the failure to detect ODC in proximal tubules was not due to a problem with the immunostaining method, positive controls (7,8) were generated in the form of androgen-treated female rats. In these rats, abundant staining was observed in proximal tubules (Fig. 4). This manifestation of ODC expression was still in evidence at day 7, by which time the increase in BrdU uptake and tissue polyamine content had both subsided.

DISCUSSION

Early in diabetes the kidney becomes large and GFR increases. This is due to hypertrophy (1–3) and hyperplasia of the proximal tubule (11), although experts disagree as to the relative importance of these two basic processes (11–13). In either case, kidney growth in diabetes is commonly viewed as a downstream response to the in-

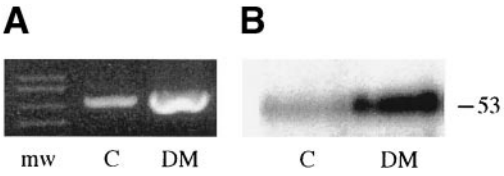

FIG. 1. Diabetes increases ODC expression. *A*: RT-PCR analysis of ODC mRNA from renal cortex. Samples from untreated control (C) and STZ-induced diabetic (DM) rats yield a single band of appropriate size. mw, molecular weight marker. *B*: Western blotting of renal cortex for ODC protein expression demonstrates a 53-kDa band corresponding to ODC. DM at 3 and 7 days post-STZ administration for RT-PCR and Western blotting, respectively. This is representative of three separate experiments.

crease in GFR in which growth becomes necessary in order for tubular reabsorption to keep up with the filtered load. However, we have recently shown that the increase in proximal tubular reabsorption that accompanies kidney hypertrophy in early diabetes is too great to arise from hyperfiltration (6). In fact, growth and hyperfunction of the proximal tubule actually fuel glomerular hyperfiltration by reducing the signal for tubuloglomerular feedback (TGF), a system which operates in the juxtaglomerular apparatus of each nephron and causes GFR to vary reciprocally in response to the amount of salt reaching the macula densa. Despite glomerular hyperfiltration, the amount of salt reaching the macula densa is reduced in diabetes and the stimulus for TGF is reduced below normal. Thus, the increase in tubular reabsorption in diabetes cannot be explained by normal glomerulotubular balance upstream from the macula densa and must result from a primary increase in reabsorptive capacity or avidity of those tubular segments for salt (14–18). Because glomerular hyperfiltration appears to be more a consequence than a cause of kidney hypertrophy, it is reasonable to examine factors other than hyperfiltration that might contribute to kidney hypertrophy, since such factors might provide a common point for attacking the functional as well as structural changes that befall the kidney in diabetes.

The present studies evaluate some aspects of the arginine-ornithine-polyamine axis in this context. Most work in the field of ODC and polyamines has focused on these molecules in the context of cell proliferation. ODC converts ornithine to putrescine and is the initial and rate-limiting enzyme in the biosynthetic pathway of the other polyamines, spermidine and spermine (19). The biological activity of ODC is rapidly induced in response to virtually all agents known to promote cell proliferation, including hormones, drugs, growth factors, mitogens, and tumor promoters, leading to the elevations of polyamine levels and then cell growth. Many protein kinases, transcription factors, and protooncogenes, which are involved in the signal transduction pathway, are activated by polyamines. Spermidine and putrescine at micromolar concentrations stimulate the transcription and translation of nuclear protooncogenes, c-*myc*, c-*fos*, and c-*jun*, in rat kidney epithelial cells (20).

However, ODC expression is a mid-G1 event that can also mediate hypertrophy. ODC-mediated hypertrophy may not reflect any special actions of the polyamines distinct from their actions during cell proliferation but might arise when cells in G1 phase of the cell cycle are restricted by another factor from entering S-phase (21,22). In its earliest stages, the diabetic kidney appears to exemplify ODC-mediated renal growth. The importance of ODC in this process was established in previous studies by using a bioassay for ODC activity (liberation of $^{14}CO_2$ from labeled ornithine) in cells or cell lysates from harvested kidneys and from the ability of the ODC blocker (DFMO) to blunt hypertrophy, hyperreabsorption, and hyperfiltration (5,6).

Pursuant to these previous functional studies, the present experiments were performed to confirm the presence of ODC enzyme and identify its location, as well as to measure its substrate and product within the early diabetic kidney. The observed increase in ODC protein content was expected based on previous experience with the ODC bioassay (6). The studies also confirm a temporal pattern

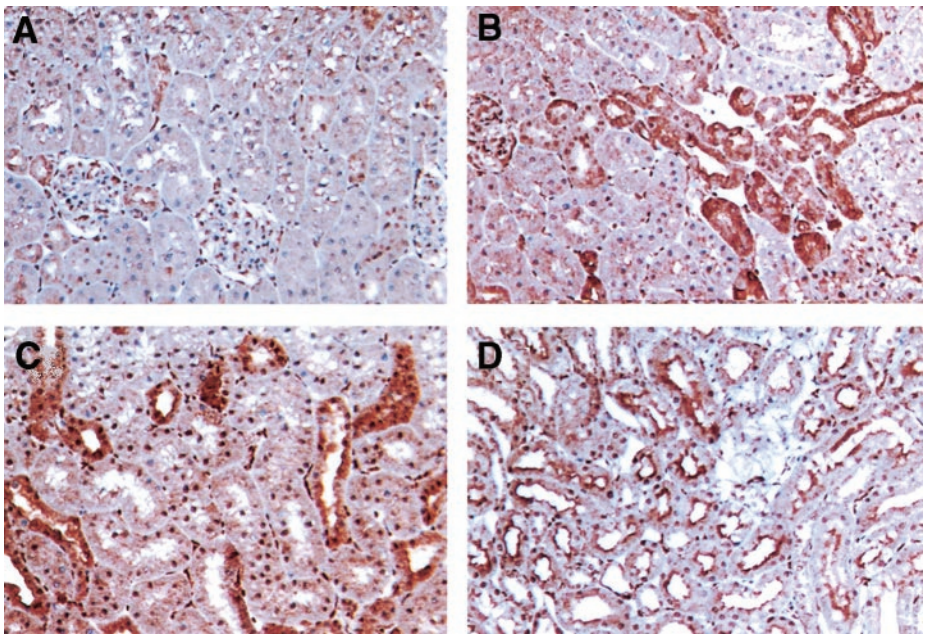

FIG. 2. Immunohistochemical location of ODC protein in rat kidney cortex. Normal rat kidney (*A*); 3-day diabetic rat kidney (*B*); 7-day diabetic kidney (*C*); and kidney from androgen-treated female rat (*D*).

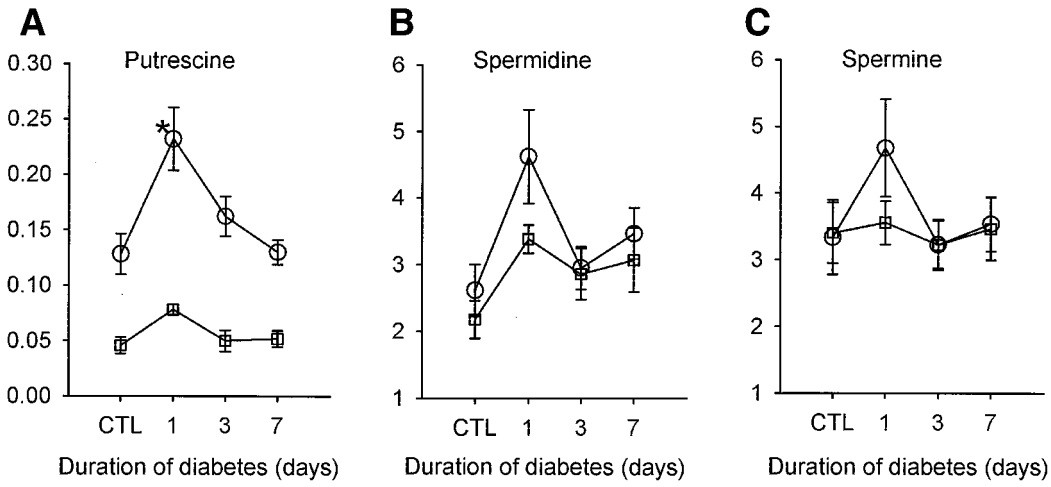

FIG. 3. HPLC determination of polyamine levels in kidney cortex as functions of time after initiation of STZ-induced diabetes for putrescine (*A*), spermidine (*B*), and spermine (*C*). All values are nmol/100 μg protein. □, diabetes; ○, diabetes + DFMO. **P* < 0.05 vs. control by Tukey's test.

of BrdU uptake previously noted by others (23). However, the apparent localization of new ODC to the distal nephron in diabetes is surprising for two reasons. First, ODC blockade in early diabetes interferes with growth of the proximal tubule (6). Second, the proximal tubule accounted for most DFMO-inhibited BrdU staining in the present study. This suggests that polyamines made in the distal nephron may act on the proximal tubule in a paracrine fashion. Also, the contrast between the sites of increased ODC expression in rats with early diabetes and those nondiabetic rats treated with androgen infers specificity to the mechanism whereby ODC expression is upregulated.

The factors that trigger ODC to increase in diabetes remain to be identified, as does the specific downstream mechanism whereby polymamines cause the diabetic tubule to grow. ODC enzyme is mainly regulated at the levels of translation and protein degradation. For example, translational regulation occurs via extensive secondary structure of the 5′ untranslated region and its interaction with endogenous factors (known to exist, yet remaining to be characterized), regulation of translation initiation factors,

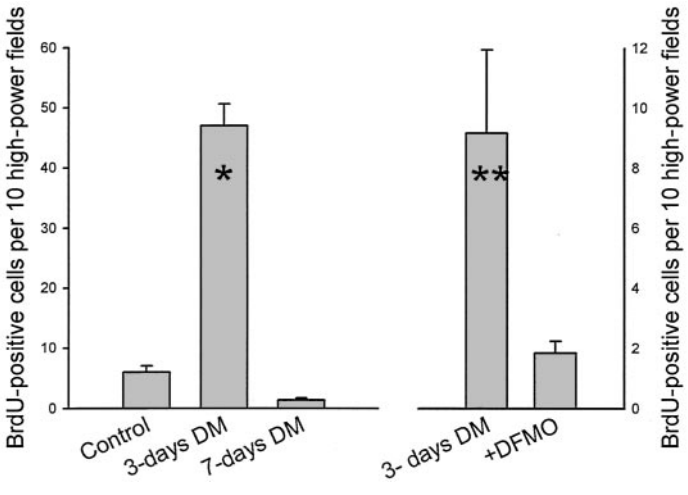

FIG. 4. Number of BrdU-positive cells per 10 high-power fields. *Left*: with formalin-fixation tissue. *Right*: with methacarn fixation. Data show ODC-dependent cell-proliferation increased on day 3 and normalized by day 7 of diabetes. **P* < 0.005 vs. other control and 7-day diabetes; ***P* < 0.05 vs. DFMO-treated diabetes. Statistics by Kruskal-Wallis test.

binding to ribosomes (insulin affects ODC this way), and biphasic regulation by polyamines. Due to these factors, translation can vary many-fold. (rev. in 24). Degradation of ODC enzyme is also variable and highly regulated, mainly through the protein antizyme, which binds to ODC and renders it susceptible to degradation by the 26S proteasome. Antizyme itself is highly regulated by polyamines, agmatine, and antizyme inhibitory proteins. In fact, antizyme inhibitory proteins can rescue ODC from degradation, even after it has been tagged by antizyme (25).

Considering possible connections to what else is known about diabetes, one comes naturally to growth factors and transforming growth factor-β which feature prominently in many investigations of the diabetic kidney (12,26). These molecules may stimulate polyamines (27) which, in turn, may affect these same molecules and their receptors (28) and alter phosphorylation of retinoblastoma protein (29), which is integral to the G1-S phase transition (22). Furthermore, transforming growth factor-β might also inhibit phosphorylation of retinoblastoma protein, thereby arresting progression through the cell cycle and converting hyperplasia into hypertrophy (22). As noted, most polyamine research has been performed in proliferating cells, so the lessons drawn from that research may or may not apply to the situation of diabetic hypertrophy. Perhaps closest to the present situation is the observation that uptake of nonhydrolyzable glucose analogs by sodium cotransort stimulates ODC transcription in a proximal tubular cell line (30), although ODC is usually regulated other than by transcription (vide supra), and we find ODC to be increased mainly in the distal tubule. ODC is also known to mediate certain actions of peptide growth factors receiving attention in diabetes (rev. in 12), including IGF-1 (31) and hepatocyte growth factor (32).

In this study, the kidney content of putrescine was elevated in early diabetes, likely due to increased ODC activity. However, the increase in putrescine did not translate into statistically significant increases in ODC-dependent spermine or spermidine content. One would expect the polyamines to change in parallel, albeit not necessarily in constant proportion. It may be that we failed to detect this due to the signal to noise ratio of our method and the lability of these compounds. Two enzymes working in sequence, spermine/spermidine N1-acyltransferase (SSAT) and polyamine oxidase (PAO), govern the poly-

amine back conversion pathway from spermine to spermidine to putrescine. The expression and activities of these enzymes have not been examined in diabetes. We also do not know how the polyamines partition between cells and interstitium or how much of each polyamine species is required to cause tubular hypertrophy in vivo. Furthermore, cellular spermine content is often unaffected by DFMO administration, and spermidine may be utilized as the sole substrate in the conversion of a lysine residue on eIF-5A to hypusine, which is required for proliferation (33,34).

It is apparent from the present data that ODC helps initiate early growth of the diabetic kidney but that ODC immunoreactivity persists after the coincident spikes in polyamine content and BrdU uptake subside. Therefore, the abundance of ODC cannot be the sole determinant of polyamine content. Also, ornithine availability does not appear to be rate limiting. Therefore, the kidney must invoke a compensatory mechanism to buffer the impact of ODC on polyamine content and cell proliferation. In any event, the prospect that a single transient spike in tissue polyamines that occurs very early in diabetes may be sufficient to set into motion the events that result in a persistently large kidney is not encouraging vis-à-vis the polyamine axis as a target for therapy in diabetes, since the timing of such treatment would be problematic.

To summarize, we have confirmed that polyamines formed by ODC contribute to proliferative growth of the proximal tubule in early diabetes, although the distal nephron is the main site of increased ODC immunoreactivity in this setting.

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