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Early molecular changes associated with streptozotocin-induced diabetic bladder hypertrophy in the rat

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Summary. We describe early alterations in rat bladder gene expression which may relate to the development of diabetic bladder dysfunction in a streptozotocin model of inducible diabetes. Utilizing cDNA probes, the gene products sulfated glycoprotein-2 (SGP-2), transforming growth factor-beta (TGF-β), beta-actin (β-actin), N-*ras* and beta nerve growth factor (β-NGF), were quantitated in bladders of male Sprague-Dawley rats at 1, 2, 4 and 6 weeks after induction of diabetes with streptozotocin (STZ). β-actin and SGP-2 expression were transiently increased at 1 and 4 weeks after induction, respectively. TGF-β was not altered over the period of the study. N-*ras* was reduced at all times compared with control rat bladders. Transcripts encoding β-NGF were dramatically increased in STZ-treated rats at 4 weeks. None of these changes were seen in diuresis control group fed 5% sucrose. Our results suggest that during the early stages of diabetes, cellular hypertrophy, growth and remodeling are occurring concomitantly with cellular injury and programmed cell death. Furthermore, the transient increase in expression of β-NGF mRNA may represent a compensatory response to the diabetic condition in an attempt to attract further innervation and revascularization.

Key words: Bladder – Gene expression – Diabetes – Streptozotocin

Autonomic neuropathy is one of the major sequelae of diabetes [6]. Diabetic cystopathy is the most prominent manifestation of this neuropathy in the human urinary tract [7, 10]. Several urodynamic studies have defined clinical parameters with which to measure bladder dysfunction in diabetes. These studies have suggested that the earliest defects occur in the sensory nerves of the bladder, resulting in bladder distension, while the later stages of atony and impaired detrusor contractility are a result of defects in muscle function within the bladder [10, 16]. The etiology of these defects at the cellular and molecular level remains elusive, especially because of the difficulty in studying humans over long periods of time and because the parameters being measured are usually end effects.

Animal models, particularly streptozotocin-induced diabetes in rats, have provided a well-accepted method for experimentally examining the effects of diabetes on the function of many different organ systems [19, 27]. The advantage of using such an animal model is the ability to follow changes from the initial onset of the disease to the first signs of organ dysfunction. Hence, this allows for the determination of any "critical" event in the development of symptoms of diabetes. Previous work has demonstrated the morphologic, biochemical and functional alterations in experimental diabetes induced by streptozotocin. These changes include an increase in bladder mass secondary to muscle hypertrophy and extensive collagen deposition [16, 26]. Other studies have demonstrated diabetes-induced alterations in the cholinergic innervation of the bladder which is presumably responsible for the decreased contractile response of bladder smooth muscle [17].

The work presented here is modeled after previous molecular studies of progressive diseases of the kidney, heart and liver [5, 11, 15, 25]. Each of these studies has demonstrated a distinct pattern of gene activity which accompanies both the development and the progression of functional organ impairment. In order to establish a more temporal relationship between the onset of diabetes and consequent development of bladder dysfunction in the rat, we analyzed for the expression of several gene products during the early phase of diabetes. Bladders from control (untreated) rats, rats subjected to streptozotocin-induced diabetes, and rats subjected to equivalent diuresis produced by 5% sucrose feeding were utilized to identify genetic changes consistent with the onset of diabetes. Our experiments represent a preliminary attempt to use molecular analysis for identifying the basis of structural and neurochemical changes occurring in bladders exposed to diabetes.

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As a first endeavor into the use of these methods a number of specific gene products were examined, including genes associated with cell death and tissue remodeling [sulfated glycoprotein-2 (SGP-2) and transforming growth factor-beta (TGF-β)], cell structure (β-actin) and neuronal survival and growth (beta nerve growth factor, β-NGF). In addition, we examined for the activity of a proto-oncogene, N-*ras*, because of previous work demonstrating that expression of this gene product is increased during obstructive rabbit bladder hypertrophy [2]. The results of this survey have identified several gene products in the bladder which undergo a significant change in expression concomitant with the onset and early progression of diabetes.

Materials and methods

Experimental animals and tissue

Male Sprague-Dawley rats (350–400 g) were obtained from Camm Industries (Wayne, N.J.). All animals were housed in groups of two per cage and received food and water ad libitum except when indicated. To induce diabetes rats were first fasted for 24 h. A single injection of streptozotocin (STZ, 65 mg/kg intraperitoneally; Sigma, St. Louis, M.) dissolved in citrate buffer 20 mM, pH 4.2 was then administered. Due to the instability of STZ in aqueous media, all solutions for injection were made immediately before administration. Age-matched rats were injected with streptozotocin (STZ group) or with similar volumes of citrate buffer (VEH group). For the first 48 h after treatment (STZ or VEH), the water supply was supplemented with 5% sucrose to minimize deaths resulting from acute hypoglycemia. One sucrose-fed control group (CON-S group) consisted of rats that received 5% sucrose in water for 48 h but no injection of STZ or vehicle. After 48 h, all rats received food and water ad libitum. An additional group of rats (FED-S group) was given a 5% sucrose solution ad libitum as the only source of drinking water for the duration of the study. It has been reported that this treatment results in a large fluid intake with concomitant nonosmotic diuresis and does not lead to glucosuria [4]. A final control group (CON group) received the usual food and water. These rats did not receive sucrose supplementation or injection.

Confirmation of diabetes was by measuring body weight (which decreases within 48 h after injection) and by measuring blood glucose at the time the animals were killed. The blood glucose concentration was measured using Chemstrip bG and Accu-Chek II (Boehringer Mannheim, Indianapolis, Ind.). All the STZ-treated rats that showed decrease in body weight had blood glucose concentrations greater than 17 mM at the time they were killed. Twenty-four hour water intake and urinary output were also measured for the various groups using metabolic cages. At 1, 2, 4 and 6 weeks after induction, STZ, VEH and FED-S rats were killed by lethal injection of pentobarbital sodium. The entire bladder was then removed, weighed (free of urine) and immediately frozen in liquid nitrogen.

Extraction and analysis of mRNA

Polyadenylated mRNA (Poly(A)+ mRNA) was extracted from frozen rat bladders using Fast Track mRNA Isolation Kit (Invitrogen, San Diego, Calif.) as directed by the manufacturer. Frozen tissue was pulverized unter liquid nitrogen and homogenized with a Polytron homogenizer in a lysis buffer provided in the kit. Digestion with RNase and proteinase preceded oligo(dT) cellulose chromatography for adsorption. Poly(A)+ mRNA was eluted in a low-salt buffer and its concentration quantified by spectrophotometry at 260 nm. Five-microgram aliquots of poly(A)+ mRNA were denatured in MOPS buffer (30 nM 3-[*N*-morpholino]propanesulfonic acid, pH 7.0, and 5 mM EDTA), 2.2 M formaldehyde, and 50% deionized formamide at 55°C for 5 min. Samples were applied to a denaturing 1.2% agarose gel (in MOPS buffer, 2.2 M formaldehyde) and electrophoresed at 100 V for 2.5 h at room temperature. The integrity of RNA was assessed from the ethidium bromide staining pattern of the 28S and 18S ribosomal bands visualized under ultraviolet (UV) light. After electrophoresis, the gel was soaked in ×20 SSC (×1 SSC = 0.015 M sodium citrate, 0.15 M NaCl, pH 8.0) for 1 h. RNA was transferred to a charge-modified nylon filter (Bios, New Haven, Conn.) by the Northern blotting method [21]. RNA was then fixed to the filter by UV cross-link.

Preparation of ^{32}P-labeled probes and Northern blot hybridization

cDNA probes for β-actin were obtained from Donald Cleveland, Johns Hopkins University, Baltimore, Md.; N-*ras* from Oncor, Gaithersburg, Md.; TGF-β and β-NGF from the American Type Culture Collection, Rockville, Md.; SGP-2 from Ralph Buttyan, Columbia University, New York, N.Y.; 18S rRNA from Dr.Ramreddy Guntaka, University of Missouri, Columbia, Mo. All probes labeled with [α-^{32}P]dCTP (Amersham, Arlington Heights, Ill.) by a random primer technique [3] using the Random Primed DNA Labeling Kit Boehringer, Mannheim. Labeled fragments were chromatographed on Sephadex G-50 and denatured by boiling. A typical incorporation procedure yielded probes with specific activities of 5×10^9 cpm/μg DNA.

Blots were prehydridized in a solution of ×6 SSC, 0.5% SDS, 5 mM EDTA, ×5 Denhardt's solution (1% Ficoll, 1% polyvinylpy-'rolidine, 1% bovine serum albumin), 50% deionized formamide and 200 μg/ml denatured salmon sperm DNA for 4 h. Denatured labeled probe was added and hybridization continued at 39°C or 42° C for 18–24 h. The blots were washed in a successive series of solutions containing 0.1% SDS, 1 mM EDTA and SSC (from ×2 to ×0.1) at 55°C with shaking and were exposed to X-ray film for autoradiography.

Quantitation of mRNA expression

X-ray autoradiographs were scanned on a Molecular Danymics scanning laser densitometer to produce an image. Selected areas of the computer image containign individual bands of hybridization were then identified for densitometric quantitation by means of a "grid" selection program. This program allowed equivalent areas to be assigned to each band on the autoradiograph. The densities obtained were corrected for the film background as measured from an empty grid section adjacent to the transcript bands. For each probe, hybridization intensity was controlled for deviations in mRNA loading by comparing each probe with the respective hybridization to the 18S rRNA probe.

Statistical analysis

Statistical analysis was performed using one-way analysis of variance (ANOVA), and Student's *t*-test.

Results

Induction of the diabetic state

As expected, adaptations to diabetes in the STZ group, including weight loss (229 ± 44 g compared with 424 ± 17 g

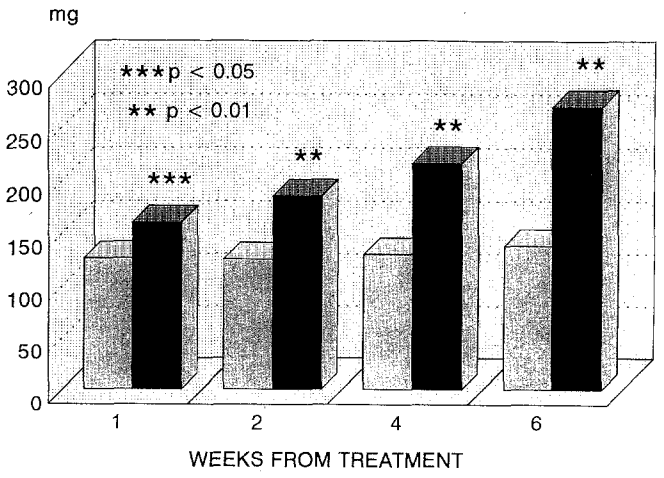
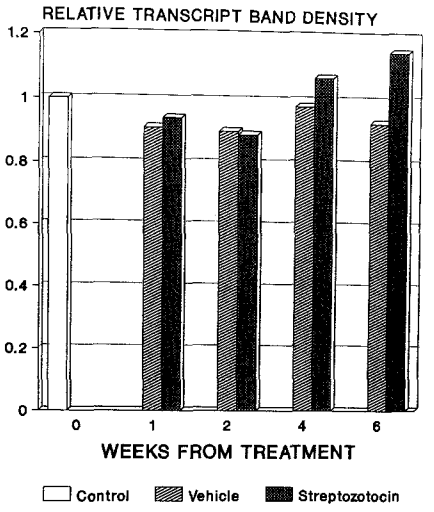


Fig. 1. Bladder weights of rats treated with streptozotocin (STZ) and vehicle. At each of the time points (1, 2, 4 and 6 weeks) following injection, there were significant differences in mean bladder weight ($P > 0.05$) in the STZ rats when compared with vehicle-treated (VEH) rats. □ Vehicle, ■ streptozotocin



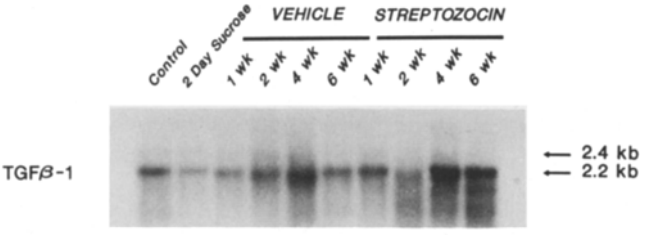


Fig. 2. Expression of transforming growth factor-beta (TGF-β) from rat bladder mRNA at 1, 2, 4 and 6 weeks following induction with STZ. Poly(A)+ mRNA was extracted from pooled, homogenized rat bladders. Five-microgram aliquots of mRNA from each specimen were co-electrophoresed in adjacent lanes of a denaturing agarose gel. Gels were blotted onto nylon filter paper and the resulting Norther blots were hybridized to ^{32}P-labeled cDNA probes. For each probe, hybridization intensity was controlled for deviations in mRNA loading by comparing the hybridization in each lane with the respective hybridization to the 18S rRNA probe. The relative band intensities obtained by quantitative densitometry are shown graphically. The 2.2 kb transcript encoding TGF-β was not significantly altered over the 6-week period of this study in either STZ- or vehicle-treated animals

in control rats, $P < 0.001$), increased blood glucose (22.1 ± 0.8 mM compared with 7.3 ± 0.5 mM in controls, $P < 0.001$) and increases in bladder weight (216 ± 18 mg compared with 130 ± 18 mg in controls, $P < 0.01$) were seen and were significnatly different from the findings in the CON, CON-S and VEH groups (Fig. 1). FED-S bladders also showed an increase in bladder weight (data not shown), but no difference in body weight and blood glucose compared with controls. By 2 weeks, STZ rats showed an approximately sixfold increase in fluid intake as compared with VEH rats. The urine output in STZ rats also increased to nearly tenfold when compared with VEH rats. There was no significant difference in fluid intake or urine output among CON, CON-S and VEH groups. In the FED-S group, polydipsia (200–210 ml/24 h) and polyuria (120–150 ml/h) were seen comparable to those in the diabetic group and eightfold higher than in normal rats.

Northern blot analysis of mRNA extracted from rat bladders

Northern Blot analysis was used to study mRNA extracted from bladders from each group at 1, 2, 4 and 6 weeks. The blots were then subsequently probed for a series of gene products of interest and compared. One of the gene products that we analyzed, the 2.2 kb transcript encoding TGF-β, was not significantly altered over the 6-week period of this study in either STZ- or vehicle-treated animals (Fig. 2). We had chosen this growth factor as one of our primary genes of interest because of its known role in stimulating collagen formation [21]. Extensive collagen deposition is characteristic of end-stage diabetic cystopathy. In contrast to TGF-β, expression of the 1.7 kb mRNA encoding β-actin was selectively elevated (twofold) in diabetic rat bladders compared with control, vehicle-treated and sucrose-fed animals early (by the first week) and returned to control levels by 4 weeks after treatment (Fig. 3). This gene product provided the earliest change to be detected in the STZ group. SGP-2 is complement inhibitor substance that is constitutively expressed in some tissues and highly induced in cells undergoing programmed cell death [3]. We utilized this probe to determine whether programmed cell death might be a factor in the developmental stages of diabetes-induced bladder changes. As with certain other tissues, the SGP-2 transcript (2.0 kb) was expressed constitutively in our bladder RNA specimens. However, in the STZ group there were slight, yet consistent elevations in the level of this transcript by the fourth week, continuing through the sixth week after treatment (Fig. 4).

Diabetic cystopathy is associated with changes in autonomic nerve function; we therefore wanted to determine whether there was any alteration in the expression of nerve growth factor associated with streptozotocin treatment. Following hybridization with our β-NGF cDNA

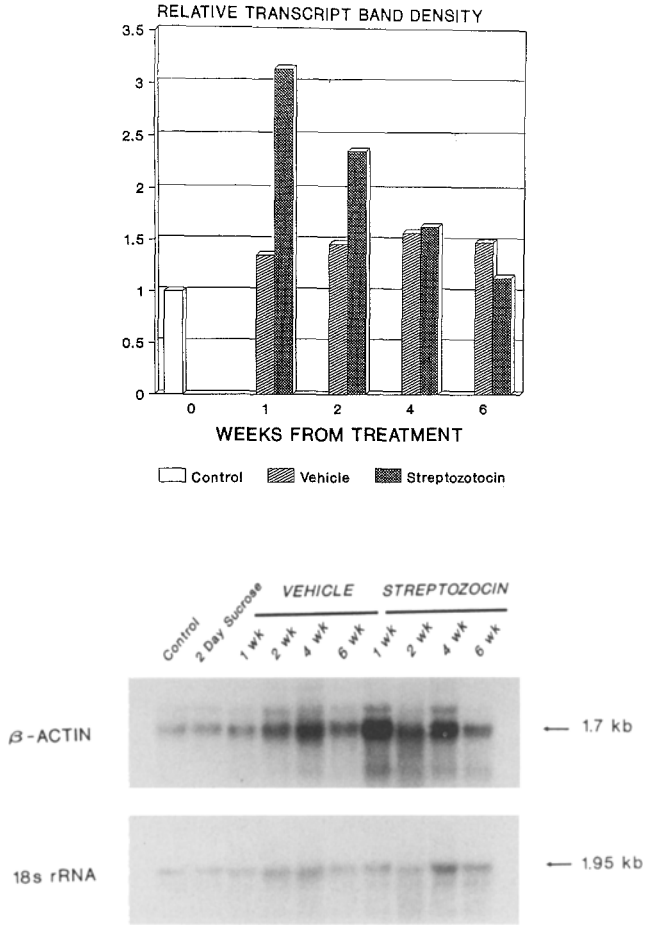


Fig. 3. Expression of β-actin from rat bladder mRNA at 1, 2, 4 and 6 weeks following induction with STZ. The 1.7 kb mRNA encoding β-actin was selectively elevated (twofold) early in STZ rat bladders compared with control (CON) and VEH, and returned to control levels by the fourth week following treatment. The *bottom panel* shows the autoradiographs from hybridization with β-actin and 18S rRNA respectively

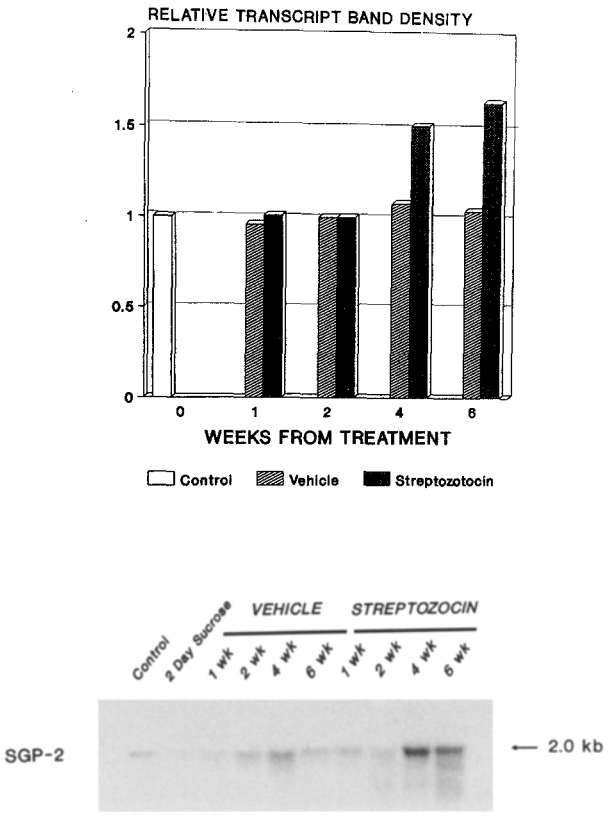


Fig. 4. Expression of sulfated glycoprotein-2 (SGP-2) from rat bladder mRNA at 1, 2, 4 and 6 weeks following induction with STZ. The 2.0 kb transcript encoding SGP-2 was expressed constitutively in all bladder mRNA specimens. In the STZ group there were slight, yet consistent elevations in the level of this transcript by the fourth week which continued through the sixth week

probe, transcripts encoding β-NGF were undetectable in CON, CON-S, VEH, FED-S and early STZ bladder mRNA. However, by the fourth week following induction of diabetes β-NGF transcripts (1.7 kb and 2.5 kb) were dramatically elevated and subsequently decreased in the 6-week STZ bladder (Fig. 5).

We further chose one memeber of the proto-oncogene *ras* family, N-*ras*, for this analysis, on the basis of a prior study in which this gene was shown to be active during the early period of obstructive rabbit bladder hypertrophy [2]. However, as demonstrated by our results shown in Fig. 6, and, in contrast to all the other gene products that we analyzed, the 1.55 kb transcript encoding N-*ras* demonstrated significntly reduced levels at all time points in the STZ-treated group when compared with vehicle-treated and control rat bladders.

When the same series of transcripts were analyzed in RNA extracted from bladders at 1, 2, 4 and 6 weeks in rats fed 5% sucrose there were no differences from the VEH and CON-S control groups. Therefore, diuresis alone is not sufficient to induce changes in the expression of β-NGF, SGP-2, β-actin and N-*ras*.

Discussion

Cells respond to adverse changes in their environment by altering their pattern of gene expression to adjust to stress. This has been studied extensively for a variety of tissues undergoing compensatory hypertrophy [2]. The use of a diabetic rat bladder model will allow for the study of the cascade of gene activity during both a hypertrophic stage (cell growth) and the later stage of cellular damage. Our ability to identify a reproducible pattern of gene activity may help in formulating a hypothesis for the basis of bladder cell hypertrophy and cellular damage induced during diabetes. The use of STZ-induced diabetic rat model has allowed for a more detailed morphologic, functional, biochemical and, as we show here, molecular characterization of the diabetic disease process. In this

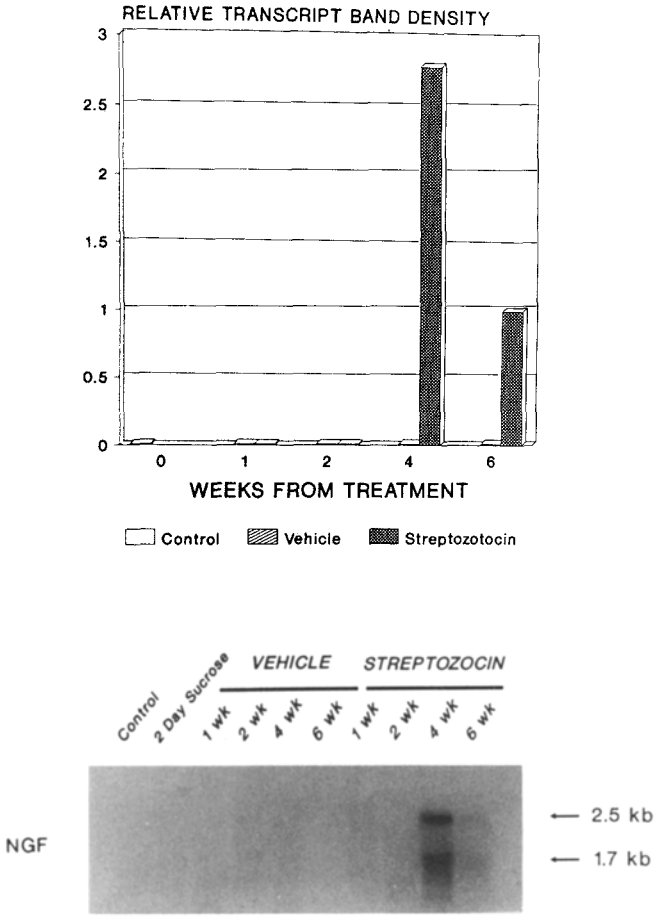


Fig. 5. Expression of beta nerve growth factor (β-NGF) from the rat bladder mRNA at 1, 2, 4 and 6 weeks following induction with STZ. Transcripts encoding NGF were undetectable in CON, CON-S, VEH and early STZ bladder mRNA. However, by the fourth week following induction of diabetes, NGF transcripts (1.7 kb and 2.5 kb) were dramatically elevated and subsequently decreased in the 6-week STZ bladders

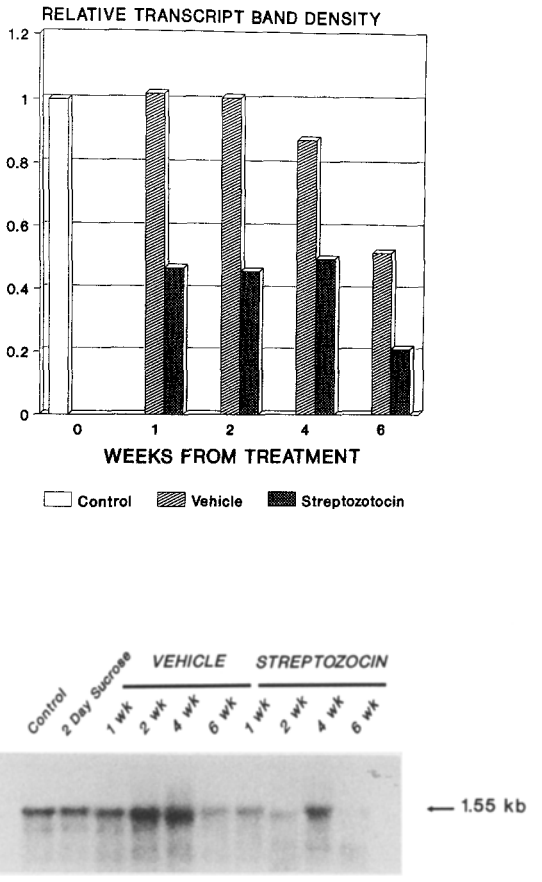


Fig. 6. Expression of N-*ras* from rat bladder mRAN at 1, 2, 4 and 6 weeks following induction with STZ. In contrast to all the other gene products that were analyzed, the 1.55 kb transcript encoding N-*ras* demonstrated significantly reduced levels at all time points in the STZ group whencompared with CON and VEH groups

study acute alterations in gene expression in the diabetic bladder cell were evaluated using a series of molecular probes.

Our findings of increased bladder weight and distension are in agreement with reports from other investigators who have examined rat bladders at times ranging from 2 to 16 weeks following STZ treatment [16, 26]. Moreover, Kudlacz et al. [13] have shown that there are concomitant increases in bladder protein and DNA content in proportion to the increase in bladder weight sustained in STZ-treated rats. Thus, this weight increase is a net growth and is not due to tissue water accumulation. The increase in fluid intake and urinary output in the STZ rats are also consistent with other functional studies in diabetic rats which have shown increased urine output, micturition volume, urinary capacity and bladder compliance [1, 18].

Despite a similar polydipsia and polyuria seen in the sucrose-fed (FED-S) group when compared with the diabetic group (STZ), altered expressions of mRNA encoding β-actin, SGP-2, β-NGF and N-*ras* were not observed. This is significant because sucrose feeding results in measurable changes in rat urinary bladder function [13]. Therefore, the use of these controls is important for distinguishing alterations produced by diabetes-induced metabolic effects on gene expression from those produced simply by processing a prolonged, excessively large urine volume. Even though many effects of diabetes on the bladder have been attributed to the extensive diuresis associated with this condition [14], our results suggest that certain changes in the bladder are occur subsequent to metabolic alterations specific to the diabetic state. With this inducible model of diabetes (STZ-directed), the potential for a mutational or carcinogenic effect from the direct action of STZ must be considered. However, if this were the case, one would expect there to be a more global induction of proto-oncogenes in the rat bladder tissues. We examined one such proto-oncogene, N-*ras*, which was shown actually to decrease in expression following STZ treatment. Moreover, the chronic time period over which these changes in gene activity occurred are more consistent with progressive tissue stresses asso-

ciated with the onset of diabetes rather than an acute insult from the STZ treatment.

There was an early gene response to the diabetic state as evidenced by the increased (greater than twofold) expression of β-actin transcripts in the STZ rat bladders as early as the first week following induction. The β-actin transcript levels subsequently declined to the levels in the VEH and CON bladders by 4 weeks.It is interesting to note that an increase in activity of a structural gene (β-actin) was detected by the first week while the earliest histologic changes in STZ-exposed bladders are not seen until about the second week following treatment [16]. The transient induction of this transcript during the early period following STZ treatment and its subsequent reduction may signify the arrest of the rapid growth and hypertrophy stage.

SGP-2 is a gene product that has received an extensive amount of attention recently because it is highly induced in dying cells and in atrophic tissues [3]. It is important to note that this gene product is synthesized constitutively in certain tissues including testis, brain and eye [1, 18]. On the basis of our results, SGP-2 is normally expressed in the rat bladder. The significant elevation of SGP-2 expression at the fourth week following induction in the STZ group suggests cell death associated with the progression of diabetic cystopathy.

Polypeptide growth factors have recently emerged as a distinct group of gene products that have been proven to regulate the onset of cellular change during regeneration or diseased states. By definition, growth factors control cellular proliferation, but they have also ben shown to regulate many other cellular processes such as differentiation, survival and the deposition of basement substances. β-NGF plays a vital role in both the maintenance and survival of sensory and sympathetic neurons. β-NGF has been shown to be synthesized and released in limiting amounts by the target tissues of the peripheral sympathetic and neural-crest-derived sensory nervous system. It is then taken up by the innervating nerve terminals via specific receptors and transported retrograde to the corresponding perikarya (i.e. sympathetic and sensory nerve cell bodies) where it exerts most of its neurotrophic action by an unknown second messenger mechanism [8]. The density of sympathetic innervation is tightly correlated with the amount of β-NGF mRNA produced in target tissue [9, 12, 24]. Sympathetic and neural-crest-derived sensory neurons, consisting of unmyelinated and small myelinated fibers, are affected at an early stage in diabetes mellitus [20]. Since these peripheral neurons are NGF-sensitive and need β-NGF for development and maintenance, changes in endogenous β-NGF levels could be of relevance for the pathogenesis of diabetic neuropathy. Moreover, in experimental animal models of diabetes, impairment of retrograde axonal transport of several molecules including experimentally administered β-NGF has been observed in vivo and in vitro [22, 23]. However, although β-NGF is the best characterized neurotrophic factor so far, little is known about its role in the pathogenesis of any disease(s).

In this study, endogenous β-NGF is likely to be synthesized at such low levels in the normal rat bladder that it is undetectable by the usual Northern blot technique. However, the bladders from the STZ group at 4 weeks demonstrated a dramatic increase in NGF transcript level (1.3 kb) which subsequently declined at 6 weeks. These findings are similar to those of Hellweg and Hartung [8] who measured endogenous β-NGF levels in STZ-induced diabetic rats.

Diabetes is known to cause both a peripheral and an autonomic neuropathy. The increased β-NGF levels in the STZ rats are secondary either to increased β-NGF synthesis by the target cells in the bladders or to reduced removal of β-NGF by the degenerating neurons. It has been shown by other investigators that after surgical or pharmacologic denervation of axonal transport mechanisms β-NGF levels rise several-fold in the corresponding target tissues [8]. The transient burst in β-NGF expression in STZ rat bladders and in the denervated nerve model may represent a compensatory response by target cells in an attempt to attract further innervation.

The use of a STZ-induced diabetic model allows for the study of gene activity during the early phases of inducible diabetic cystopathy. The animal model makes it possible to follow the changes from initial diagnosis to the first signs of organ impairment. Collectively, these changes demonstrate many similarities to those in humans. Some investigators have questioned whether bladder alterations seen in diabetic animal models are secondary to diabetes or to the physiologic response of the bladder to chronic overdistension [14]. Here again, the importance of using a diuretic group to exclude this as a reason for bladder dysfunction is clear. We have shown in this study that although a similar volume of urine is processed by rats ingesting 5% sucrose, they do not exhibit the same pattern of altered gene expression that diabetic rats do.

The goal of this study was to provide a new direction in the elucidation of the complex pathogenesis of diabetic cystopathy. While further exploration of gene products during both the early and late phases of diabetes is under way, these preliminary results suggest that there are a host of interactive forces directing cellular hypertrophy, remodeling and growth, as well as cell death in the early phase of diabetes. The use of in situ hybridization techniques using specific gene probes will make it possible to study the location of these processes. In addition, the molecular events in subpopulations of these cells remain to be determined.

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