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EFFECTS OF OSMOTIC STRESS AND HYPERGLYCEMIA ON ALDOSE REDUCTASE GENE EXPRESSION IN HUMAN RENAL PROXIMAL TUBULE CELLS

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Sorbitol levels in proximal tubule cells cultured for 96 h in the presence of 16.5 mM and 27.5 mM glucose were significantly elevated when compared to cells cultured in 5.5 mM glucose. No changes in the levels of aldose reductase activity, mRNA and immunoreactivity were observed in cells cultured for up to 168 h in media containing either 5.5 mM or 27.5 mM glucose. In contrast, cells cultured in the presence of hypertonic media (600 mosmol/kg) containing either 5.5 mM or 27.5 mM glucose contained markedly elevated aldose reductase activity, mRNA and immunoreactivity. These results demonstrate that exposure of human renal proximal tubule cells to elevated glucose for up to 168 h does not result in enhancement of aldose reductase gene expression at transcriptional, translational or post-translational levels. However, exposure to a hyperosmotic milieu causes dramatic induction of aldose reductase gene expression.

Aldose reductase (ALR2 ; E.C.1.1.21), the first and rate-limiting enzyme of the polyol pathway, catalyzes the NADPH-dependent reduction of hexose or pentose sugars to their corresponding alcohols or polyols (1). The action of ALR2 has been implicated in the etiology of many complications of diabetes mellitus including cataract, retinopathy, neuropathy, and nephropathy. Elevated polyol levels are characteristically found in diabetic and galactosemic human and animal tissues and may be associated with biochemical and functional abnormalities that develop in affected tissues (2). Factors controlling expression of the ALR2 gene have been previously studied using cells derived from the renal inner medulla (3,4), where ALR2-derived sorbitol synthesis probably serves an osmoprotective function. Accumulation of the osmolytes sorbitol, betaine, and glycerophosphorylcholine in these cells is thought to balance the hyperosmolarity of the extracellular environment and maintain cell volume while preserving the intracellular ionic milieu (5). The relationship between sorbitol accumulation and ALR2 gene

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Abbreviations used: ALR2, aldose reductase; HPT, human proximal tubule; SDS, sodium dodecyl sulfate; sorbinil, [*S*-6-fluoro-spiro-chroman-4,4'-imidazolidine)-2",5"-dione].

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expression in cells exposed to both hypertonic and hyperglycemic conditions currently remains unclear. The present study investigates this relationship utilizing cultured renal human proximal tubule (HPT) cells.

MATERIALS AND METHODS

Cell Culture Stock cultures of human proximal tubule (HPT) cells were grown as described previously (6). Cells were fed fresh growth medium every three days, and when confluent were subcultured using trypsin-EDTA (0.05%, 0.02%). For these studies, the cells were utilized at passage 7.

To assess the effect of elevated glucose concentrations on sorbitol accumulation and ALR2 gene expression, the growth medium was adjusted to contain 5.5 mM, 16.5 mM or 27.5 mM glucose. Control medium was prepared by the addition of sterile sucrose to growth medium containing 5.5 mM glucose until the final osmolarity equaled that of growth media containing 27.5 mM glucose (290 mosmol/kg). To determine the effect of hyperosmolarity, NaCl was added to normal glucose containing medium (5.5 mM glucose; 0.14 M NaCl) and elevated glucose containing medium (27.5 mM; 0.14 M NaCl) to achieve an osmolarity of 500 mosmol/kg. This resulted in a final growth medium concentration of 0.3 M NaCl. The precise osmolarities and glucose concentrations of all media were determined by use of a vapor pressure osmometer (Wescor, Inc., Logan UT) and a glucose analyzer II (Beckman, Fullerton CA), respectively. Experimental protocols were initiated by subculturing confluent stock cultures grown on 5.5 mM glucose at a 1:2 subculture ratio into a series of 75 cm^2 T-flasks or 60 mm tissue culture dishes. The cells were fed fresh growth medium every three days until confluent. At confluency, the cells were changed to fresh growth medium containing either elevated glucose, elevated salt, normal glucose/normal salt, or high glucose/high salt and were cultured for the appropriate time periods.

Intracellular Sorbitol Determination The measurements of sorbitol were performed on neutralized perchloric acid extracts of the HPT cells using ovine liver sorbitol dehydrogenase (E.C. 1.1.1.14; Sigma, St. Louis MO) as described previously (7).

Aldose Reductase Activity Determination Aldose reductase activity was measured in HPT cell homogenates (6) using 10 mM DL-glyceraldehyde as enzyme substrate (8). One unit of enzyme oxidized one μmole NADPH/min at 37°C. Michaelis constants were determined in the same buffer containing various concentrations of substrate (DL-glyceraldehyde).

Isolation and Quantitation of ALR2 mRNA Total RNA was extracted from HPT cells in a buffer containing guanididium isothiocyanate (9). For Northern blot analysis, RNA samples (20 μg) were denatured by treatment with glyoxal (10) and electrophoresed through 1% agarose gels in parallel with similarly treated RNA size standards (BRL, Bethesda MD). RNAs were transferred to nylon hybridization membranes as described previously (10) and were probed with either human ALR2 cDNA (11) or human actin cDNA probes (12). Preliminary studies demonstrated that the human ALR2 and actin cDNA probes hybridized specifically with 1.4 kb and 2.0 kb RNA transcripts, respectively, the sizes expected for their respective mRNA transcripts and that cross-hybridization of the aldose reductase probe with aldehyde reductase sequences did not occur (data not shown). No size differences were noted in ALR2 transcripts extracted from cells grown in isosmotic or hyperosmotic culture media.

For quantitating ALR2 mRNA, HPT cell RNA was treated as described (10) and serial dilutions (1:1) applied directly to nitrocellulose slot blots with 20 μg RNA in the uppermost slot. Filter blots were prehybridized for 2 h at 42° in 50% formamide containing 5x Denhardts solution (1x Denhardts solution contains 0.2 g/l each of bovine serum albumin, polyvinyl pyrrolidone, and Ficoll), 5x SSPE (1x SSPE contains 0.18 M NaCl, 0.01 M NaH_2PO_4, 0.01 M EDTA, pH 7.4), 0.1% SDS and 0.1 μg/ml salmon sperm DNA. The filters were then hybridized for 12-16 h at 42° in a freshly prepared quantity of the above solution also containing [^{32}P]-labeled cDNA probes. After hybridization, filters were washed for 30 min with 1x SSPE containing 0.1% SDS at 37° and were then exposed at -80°C to Kodak XAR-5 film for 1-7 days.

Filters containing HPT RNA samples representing 4 culture time points under 5 different culture conditions were pooled and subjected to hybridization analysis simultaneously. Filters were initially probed with [^{32}P]-labeled human ALR2 cDNA using hybridization and post-hybridization washing conditions described above. After autoradiography, the filters were stripped by treatment for 30-120 min at 65°C with 50% formamide containing 6x SSPE and exposed to film as described above to ensure that all the signal had been removed. Thereafter, the filters were reprobed with [^{32}P]-labeled actin cDNA using hybridization conditions described above.

SDS-PAGE and Western Immunoblotting For Western immunoblots, proteins (30 μg) from representative HPT cultures were subjected to SDS-PAGE through 12% gels in parallel with molecular weight standards (BioRad, Richmond CA) and purified recombinant human ALR2 (11). After electrophoresis, proteins were visualized by staining with Coomassie Brilliant Blue G-250 or were transferred by electroblotting to nitrocellulose membranes and incubated with antibodies to bovine lens aldose reductase as described (13). Immune complexes were visualized by treatment with [^{125}I]-protein A (>30 mCi/μg, 8-10 μCi/filter, Amersham, Arlington Heights IL) followed by autoradiography at -80°C for 36-72 hr. No cross-reactivity was observed between these antibodies and purified human aldehyde reductase (H. Parekh, personal communication).

RESULTS

Sorbitol levels were markedly elevated in HPT cells cultured for 96 h in the presence of isosmotic medium (290 mosmol/kg) containing elevated glucose and in cells exposed to hypertonic media (600 mosmol/kg) with or without glucose-enrichment (Figure 1). Sorbitol levels in cells cultured in the presence of isosmotic media containing 16.5 mM and 27.5 mM glucose were increased 2.9-fold and 3.5-fold control, respectively at 24 h and 4.4-fold and 10-fold, respectively, after 96 h. Sorbitol levels in cells cultured for 24 h in the presence of isosmotic (290 mosmol/kg)

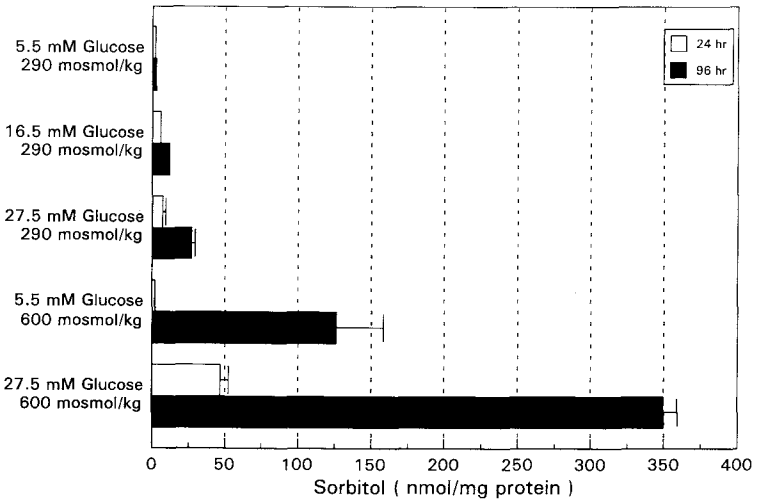

Figure 1. Sorbitol accumulation in HPT cells. Cells were cultured for 24h and 96h in the presence of 5.5 mM, 16.5 mM or 27.5 mM glucose in either isosmotic (290 mosmol/kg) or hyperosmotic (600 mosmol/kg) culture media. Data are mean ± SEM for triplicate determinations.

or hypertonic (600 mosmol/kg) culture media containing 5.5 mM glucose were virtually identical. However, after 96 h sorbitol levels in cells cultured in hypertonic medium containing 5.5 mM glucose were increased approximately 47-fold that of cells in isosmotic medium. Sorbitol accumulation was further enhanced in HPT cells cultured in the presence of hypertonic medium containing 27.5 mM glucose, reaching a level almost 3-fold that of cells cultured for 96 h in the presence of hypertonic medium containing 5.5 mM glucose.

To determine if changes in aldose reductase levels could explain the enhanced sorbitol accumulation observed in cells cultured under hypertonic conditions, enzyme activity levels were determined. Aldose reductase activity levels were virtually unchanged in cells incubated for up to 168 h in isosmotic media containing 16.5 mM or 27.5 mM glucose (Figure 2). In contrast, activity levels increased steadily in cells cultured in hypertonic media containing either 5.5 mM or 27.5 mM glucose, reaching levels at 168 h >15- and 10-fold control, respectively. The $K_{m\ DL\text{-}glyceraldehyde}$ (0.37 mM) and IC_{50} sorbinil (3.5 μM) of the reductase activity indicated that most of the reductase activity in the induced cultures could be attributed to aldose reductase rather than aldehyde reductase.

To determine if elevated glucose or osmolarity caused transcriptional induction of the ALR2 gene, RNA slot blot analysis was carried out on total RNA extracted from HPT cells at various time points. No differences in ALR2 mRNA transcript levels were detected in cells cultured for up to 168 h in the presence of increasing concentrations of glucose (Figure 3). However, cultur-

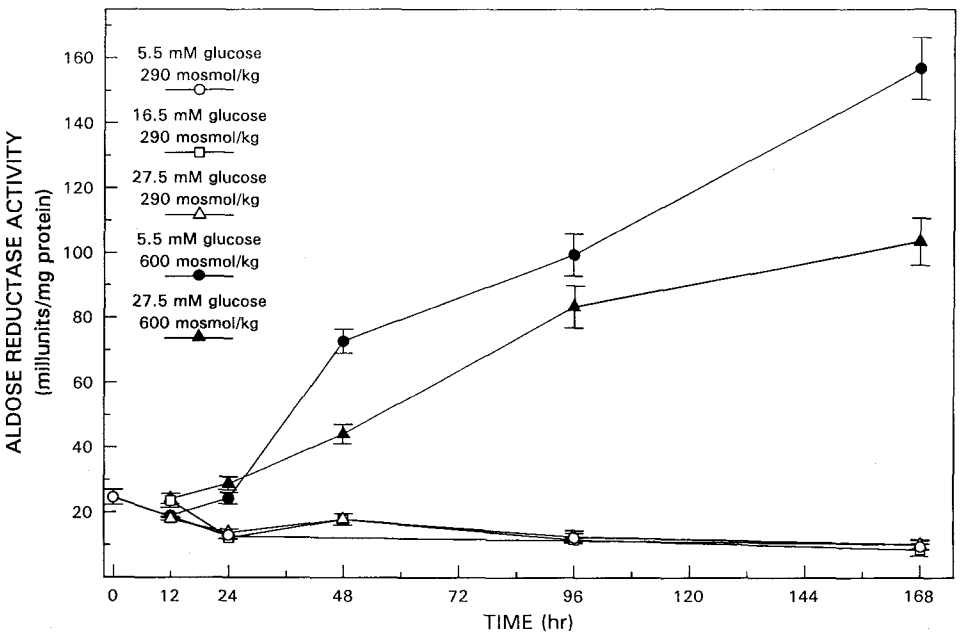

Figure 2. Aldose reductase activity in HPT cells. Cells were cultured for up to 7 days (168 h) in the presence of 5.5 mM, 16.5 mM or 27.5 mM glucose in either isosmotic (290 mosmol/kg) or hyperosmotic (600 mosmol/kg) culture media. Data are mean ± SEM for triplicate determinations.

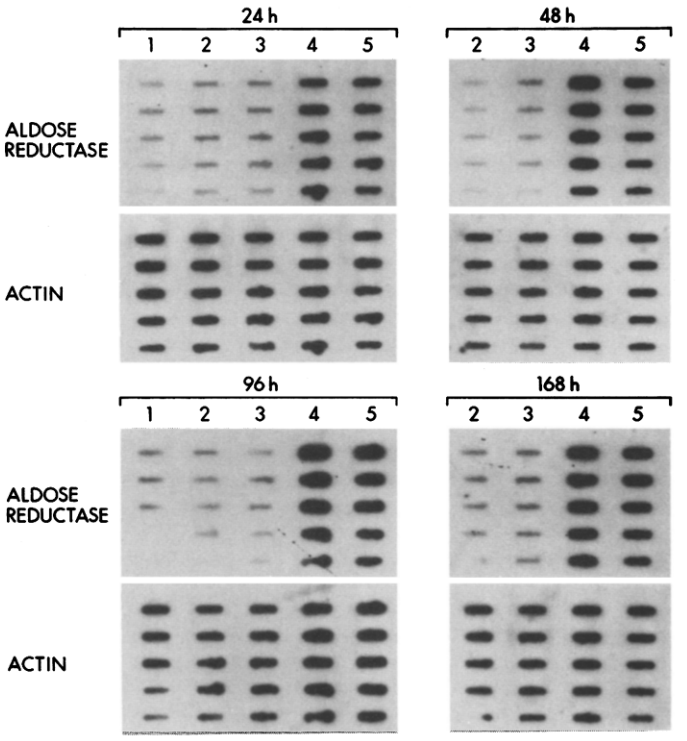

Figure 3. Slot blot quantitation of ALR2 mRNA in HPT cells. Cells were cultured in the presence of 5.5 mM, 16.5 mM or 27.5 mM glucose in either isosmotic (290 mosmol/kg) or hyperosmotic (600 mosmol/kg) culture media. Filters were probed with [^{32}P]-labeled human ALR2 cDNA (11), stripped, then re-probed with human actin cDNA (12). Lanes: 1) 5.5 mM glucose, 290 mosmol/kg; 2) 16.5 mM glucose, 290 mosmol/kg; 3) 27.5 mM glucose, 290 mosmol/kg; 4) 5.5 mM glucose, 600 mosmol/kg; 5) 27.5 mM glucose, 600 mosmol/kg.

ing in the presence of hypertonic media resulted in a dramatic increase in ALR2 mRNA levels. Within 24 h, ALR2 mRNA was increased >30-fold in cells cultured in hypertonic media containing either 5.5 mM or 27.5 mM glucose and remained elevated for the duration of the study (Figure 3). After 48 h, ALR2 mRNA levels in cells cultured in hypertonic medium containing 27.5 mM glucose were approximately 2 to 4-fold lower than those cultured in hypertonic medium containing 5.5 mM glucose.

The abundance of ALR2 protein detected by immunoblotting was unchanged in HPT cells cultured for up to 96 h in isosmotic media containing increasing amounts of glucose (Figure 4), but increased in cells cultured in hypertonic media containing either 5.5 mM or 27.5 mM glucose, respectively. By 96 h, ALR2 protein was increased approximately 4- to 8-fold in cells cultured in hypertonic media, roughly paralleling the increase in ALR2 activity at this time point.

DISCUSSION

Increased synthesis and accumulation of sorbitol are thought to be linked to pathogenesis in many tissues affected by chronic hyperglycemia such as in diabetes mellitus. Because elevated

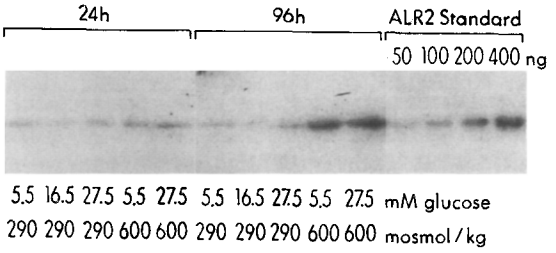

Figure 4. Immunodetection of ALR2 in HPT cells. Cells were cultured for 24h or 96h in the presence of 5.5 mM, 16.5 mM or 27.5 mM glucose in either isosmotic (290 mosmol/kg) or hyperosmotic (600 mosmol/kg) culture media. Western blots contained HPT cell proteins (30 μg/lane) and purified recombinant human ALR2 (11). Data represent immunoreactive bands observed at a position corresponding to M_r~36,000 on the western blot. No other significant immunoreactive bands were observed when western blots were probed with pre-immune serum (data not shown).

sorbitol levels are characteristically observed in diabetic tissues, investigators have sought to establish if ALR2 synthesis is enhanced in patients with diabetes mellitus. Increased levels of ALR2 activity have been measured in tissues from diabetic patients (8,14), and may result from post-translational activation of endogenous stores of the enzyme (15). Expression of the ALR2 gene in rat lenses is reportedly induced by excess accumulation of the ALR2 substrate galactose (16), although the specificity of this induction has been questioned (17). Studies first conducted with a cell line derived from the rabbit kidney papillary epithelium demonstrated that culturing in the presence of hypertonic media resulted in dramatic transcriptional induction of the ALR2 gene (4,18). Hyperosmotically-induced transcriptional of the ALR2 gene has since been observed in several other cell lines (19-21).

The purpose of the present investigation was to evaluate the relative contributions of hyperglycemia and hyperosmolarity on induction of the ALR2 gene. HPT cells responded dramatically when cultured in the presence of hyperosmotic medium as evidenced by marked increases in sorbitol and ARL2 activity, immunoreactivity and mRNA transcript levels. When cultured in isosmotic media containing increasing concentrations of glucose, the HPT cells accumulated increasing amounts of sorbitol. However, ARL2 activity, immunoreactivity and mRNA transcript levels remained unchanged from control. These results demonstrate that exposure of HPT cells to glucose concentrations up to 27.5 mM for at least 7 days does not result in enhancement of ALR2 gene expression at transcriptional, translational or post-translational levels. These observations are consistent with those of Lightman and coworkers, who observed no enhancement of ALR2 gene transcription in the lenses of streptozocin-injected rats with hyperglycemia of 3 week's duration (16). However, as other investigators have reported enhanced ALR2 gene expression in diabetic rat tissues after 3 months of hyperglycemia (22,23), it is possible that exposure of cells to chronic hyperglycemia for periods longer than used in the present study may be required to observe glucose-induced enhancement of ALR2 gene expression. It is also possible that the homogeneous cell population used in the present study does not adequately reflect the complex cellular interactions that occur in diabetic tissues. Other cellular and metabolic changes secondary to

chronic hyperglycemia and/or insulin deficiency may also have some role in modulating ALR2 gene expression.

HPT cells cultured simultaneously under hyperglycemic (27.5 mM glucose) and hyperosmotic (600 mosmol/kg) conditions contained elevated ARL2 activity and mRNA transcript levels, but at levels moderately lower than cells cultured in hyperosmotic media containing 5.5 mM glucose. This apparent attenuation in ARL2 gene induction may result from slightly decreased transcriptional efficiency or mRNA stability in cells cultured simultaneously under hyperglycemic and hyperosmotic conditions. Alternatively, intracellular accumulation of sorbitol may feed back to suppress the induction of aldose reductase gene transcription (4).

Although most evidence linking chronic hyperglycemia and nephropathy comes from study of the diabetic glomerulus, evidence for non-glomerular involvement in diabetic nephropathy is emerging. Elevated glucose exerts a direct effect on the structure and function of proximal tubule cells (24), and leads to ultrastructural alterations and modification of intracellular junctions (25). Our results demonstrate that exposure of HPT cells to elevated ambient glucose concentrations for up to 7 days does not result in enhancement of ALR2 activity at either transcriptional or post-translational levels.

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