Vol. 118, No. 1, 1984 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
January 13, 1984 Pages 304-309

ACCUMULATION OF GALACTONATE IN LIVER OF SUCKLING RATS PERFUSED WITH GALACTOSE

Shirley Rogers, Gary Lichtenstein, Diane Gentile and Stanton Segal

Division of Biochemical Development and Molecular Diseases, Children's Hospital of Philadelphia, and Department of Pediatrics and Medicine, University of Pennsylvania School of Medicine, Philadelphia, Pa., USA 19104

Received December 7, 1983

SUMMARY: Livers of 14-day-old suckling rats were perfused for 30 min in the nonrecirculating (once-through) mode with media containing 0-60 mM galactose. Galactonate was detected in livers perfused with as little as 1 mM galactose and increased with higher perfusate galactose to 178 nmoles per gram of liver at 60 mM hexose. Tissue galactonate levels were as high as 60% of galactose-1-phosphate which also accumulated under the same conditions. Galactonate was also found to be present in the effluent perfusate. The perfused suckling rat liver appears to be a physiological model for assessing the role of galactose oxidation to galactonate as an alternate metabolic pathway of galactose metabolism.

In 1966 Cuatrecasas and Segal (1) reported a pathway of galactose conversion to xylulose via oxidation to galactonate in rat liver as an alternative route of galactose disposition. Support for such a metabolic sequence came from studies in patients with galactose-1-phosphate uridyltransferase deficient galactosemia who were shown to excrete excessive amounts of urinary galactonate (2) and who preferentially oxidized [1-^{14}C]galactose to $^{14}CO_2$ compared to [2-^{14}C]galactose (3). Galactonate has also been found in the liver of a patient with transferase deficiency and has been shown to be present in the tissue of animals fed a high galactose diet (4). In the present studies galactonate has been shown to accumulate in galactose-perfused livers of suckling rats, thus adding support to the growing evidence that galactose oxidation to galactonate is a significant alternate pathway of galactose metabolism. The present findings indicate that the perfused liver may provide an easily manipulated model system to examine the regulatory

Abbreviations. GLC, gas liquid chromatography; TMS, Trimethylsilylating agent; Gal-1-P, galactose-1-phosphate.

0006-291X/84 $1.50

features of the reaction and, indeed, the extent of the function of the pathway of galactose conversion to pentose (1).

MATERIALS AND METHODS

Animals. Fifteen-days-pregnant rats of the Sprague-Dawley strain were purchased from Charles River, Boston, MA. On the fifth day of life, litters were mixed and reduced to 9 pups each to minimize biologic variability. Eighteen hours prior to perfusion, animals were placed in a warm, quiet environment for fasting. The mean fasted body weight for a 14-day-old suckling rat was 30.9 ± 1.4 g. Anesthesia was induced prior to perfusion by the intraperitoneal injection of sodium pentobarbital at a dose of 50 mg/kg.

Perfusion. The technique for the in situ perfusion of suckling-rat-livers has been described in our previous publications (5,6). The apparatus designed by Mortimore (7) permits up to six livers to be perfused simultaneously with warmed, oxygenated media at 2.5 ml per min. Krebs-Ringer-bicarbonate buffer (pH 7.4) containing 3% bovine serum albumin (Fraction V, Miles Laboratories, Elkhart, IN) was the basic media employed. Sufficient galactose that was essentially glucose-free was added to adjust concentrations to 0-60 mM. At 30 min of a nonrecirculating perfusion and while the media was still passing through the liver, the entire liver was rapidly excised and freeze-clamped in tongs pre-cooled in liquid nitrogen. The frozen livers were kept at -80° until analysis was performed.

Materials. Calcium galactonate and sodium gluconate came from Pfanstiehl Laboratories, Inc., Waukegan, IL. AG 1-X8 anion exchange resin, 200-400 mesh in the bicarbonate form and glass barrel columns (20 x 0.7 cm) were products of Bio Rad Laboratories, Richmond, CA. Trimethylsilyating agent and butyl boronic acid came from Pierce Chemical Company, St. Louis, MO. Dextrose, iodine (resublimed), ammonium bicarbonate, ethyl acetate, pyridine, Whatman 3 M paper and o-phenylenediamine were purchased from Fisher Scientific Company, King of Prussia, PA. DEAE cellulose was a product of Eastman Kodak Co., Rochester, NY. Ethyl ether was obtained from Burdick and Jackson Laboratories, Inc., Muskegon, MI. and the bovine albumin (Fraction V) from Miles Laboratories Inc., Miles Research Products, Elkhart, IN. Ribonolactone, galactose and xylulose were products of Sigma Chemical Company, St. Louis, MO. The galactose, supplied by Sigma, was essentially glucose-free and did not contain detectable levels of galactonate. [^{14}C]Galactose (55.7 mCi/mM) and [^{14}C]glucose (59 mCi/mM) were from New England Nuclear, Boston, MA. Traces of glucose were removed from the [^{14}C]galactose by treatment with hexokinase and subsequent chromatography on a DEAE cellulose column (8), and the purified galactose was used to prepare radiolabelled galactonate by the method of Moore and Link (9). All other chemicals were of the finest purity commercially available from local suppliers.

Assay Procedures. Galactonate was isolated from other nonacidic sugars found in rat livers by the ion exchange chromatographic procedure of Rancour et al (4) employing 5 and 100 mM ammonium bicarbonate to separate these compounds. To determine the amount of galactonate lost during the preparative procedure, a known quantity of labeled galactonate was added during the initial homogenization of the tissue. After ion exchange chromatography, none of the [^{14}C]galactonate appeared in the 5 mM (neutral sugar) fraction and 77% was recovered in the 100 mM NH_3HCO_3 fraction. Sugar contamination of the 100 mM fraction and recovery in the 5 mM fraction was determined with [^{14}C]galactose and [^{14}C]glucose. There were no sugar contaminants found in the 100 mM fraction and 90% was recovered in the 5 mM fraction. GLC was performed on a Perkin-Elmer 3920 gas chromatograph fitted with a 6 foot x 4 mm glass column packed with 3% OV-1 on Gas Chrom. Q, 100-120 mesh. The initial temperature was 180° for 4 min followed by an 8° rise/min to a final temperature of 250°

for 4 min. TMS derivatives were prepared as described by Sweeley et al (10). With this derivative we are able to detect levels of galactonate as low as 200 picomoles but were unable to separate galactonate from gluconate since the retention times of the hexonates were equal. Derivatization with butylboroacetic acid (11) resulted in lower sensitivity, but produced different retention times for galactonate and gluconate. In order to measure the level of gluconate present in galactose-perfused liver, a butylboroacetate and a TMS derivative were prepared from equal portions of the hexonate fraction (100 mM) from a liver perfused with 60 mM galactose for 30 min. No gluconate was detected with the butylboroacetate derivative. Mass spectrometric analysis performed on the TMS derivative revealed hexonic acid chiefly comprised of galactonate and very little gluconate indicating that peaks obtained from TMS derivatives represented galactonate. The data presented, therefore, was obtained with the TMS derivative which provided greater sensitivity and all values were corrected for the 23% loss incurred during the preparative procedure.

For media [^{14}C]galactonate analysis, the media from perfusion with radiolabelled 60 mM galactose was collected, the galactonate fraction obtained by ion exchange chromatography, as described above, concentrated and two-50 µl aliquots were chromatographed on Whatman 3M paper with ethyl acetate/pyridine/water (12:5:4 by vol) as the solvent phase (12). The area corresponding to galactonate was identified by chromatographing a [^{14}C]galactonate standard. The segment corresponding to galactonate of one aliquot was cut out for radioactive counting in scintillation fluid. The same area of the other aliquot was removed, the galactonate eluted, 100 mg of unlabeled galactonate added to the eluant, the galactonate precipitated with methanol (13), collected on a tared filter paper, weighed and the radioactive content determined. Galactonate was calculated by isotope dilution analyses from the specific activity of the perfused galactose.

The coupled enzyme assay previously described (5,6) was employed to measure the Gal-1-P content of the perfused livers. The 5mM NH_3HCO_3 fraction containing the neutral sugars from liver perfused with 20 mM galactose was analyzed by GLC for xylulose content subsequent to glucose removal by treatment with hexokinase, which eliminated the "overlap" between the xylulose and glucose peaks.

RESULTS AND DISCUSSION

The accumulation of galactonate in livers perfused for 30 min with varying amounts of galactose is shown in Figure 1. No galactonate is detectable in liver perfused with hexose-free buffer. Galactonate was found in the tissue when 1 mM galactose was perfused and the amount appeared to increase in a linear fashion up to 20 mM galactose. The curve has the configuration of a saturable process with leveling off of tissue galactonate at higher concentration.

Figure 1 also shows the accumulation of Gal-1-P during galactose perfusions. Gal-1-P was found in the absence of medium galactose and increased with galactose exposure. Previous indication that the Leloir pathway is functioning maximally in the perfused liver at medium galactose

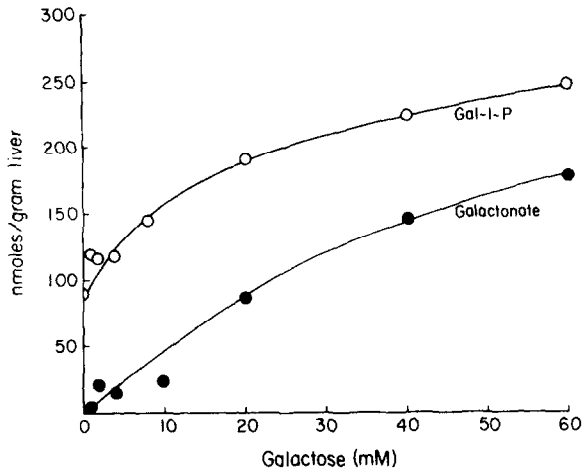

Figure 1 Accumulation of galactonate and galactose-1-phosphate by perfused rat liver. Galactonate (•) and galactose-1-phosphate (o) levels are plotted as a function of nonrecirculating perfusion media galactose concentration (mM). Each point is the average of results from 2-3 livers of suckling rats perfused with galactose at the concentrations indicated. Data are expressed as nmoles per gm of liver and experimental conditions are described in the text.

levels below 1 mM is consistent with Gal-1-P increases seen at that level (14). It thus appears that galactonate is formed when the capacity of the sugar nucleotide route of galactose metabolism is limited and is unable to handle the galactose load. At medium galactose concentrations below 10 mM, tissue galactonate is 10-15% of the Gal-1-P concentration; whereas at higher galactose perfusion levels, the amount of galactonate was about 60% of the Gal-1-P. This comparably high level of galactonate in relation to Gal-1-P may be due to the fact that galactokinase is both substrate and product inhibited (15) so that the level of Gal-1-P is better regulated than that of galactonate.

Since hepatic galactonate is readily formed in this system and the liver has the highest tissue level in rats fed a high galactose diet (4), perhaps this metabolite in conjunction with Gal-1-P (16) may be related to hepatic toxicity seen in galactose-1-P uridyltransferase deficiency galactosemia. Galactonate thus joins galactitol (17, 18) as a metabolite that should be considered a factor in toxicity syndromes associated with inability to metabolize galactose via the sugar nucleotide pathway. Galactitol, however, has not been found in large amounts in livers of galactose toxic rats (19) and

although it has been shown to play a role in galactose-induced cataracts (20) it would not seem to be a factor in hepatic toxicity.

Galactonate was detected in effluent medium collected during the 30 min perfusion. At 60 mM galactose, 34.5 ± 5.5 μmoles (M ± SEM, n = 4) of the metabolite was found in the 75 ml perfusate. Galactonate formed in hepatic cells was thus able to exit into the medium. Little is known of the process involved except that in vitro galactonate has been shown to enter liver cells (21). The ability of galactonate to efflux into plasma from tissues where it is formed underlies the urinary excretion of galactonate by transferase deficient patients (2).

In the alternate pathway described by Cuatrecasas and Segal (1) galactose is converted to D-xylulose by oxidation to galactonate which via a β-keto-D-galactonic acid intermediate loses CO_2 to form D-xylulose. It is the preferential loss of the C-1 as CO_2 in this pathway which would explain the marked difference in $^{14}CO_2$ expired by transferase deficient patients given [1-^{14}C]galactose and [2-^{14}C]galactose (3) and gives credence to the operation of the pathway. In the present studies the focus is on galactonate formation. In a gas chromatographic analysis of the TMS derivatives prepared from the neutral sugar fraction of three 20 mM galactose-perfused livers, peaks corresponding to xylulose were observed while none was present in liver perfused with buffer alone. Compared to a xylulose standard, the average was 63 nmoles/gm liver. This provides tentative evidence that in the perfused liver system there is further metabolism of galactonate as previously proposed by Cuatrecasas and Segal (1).

The original postulate of a soluble galactose dehydrogenase mechanism (22,23) to explain the hexose conversion to hexonate has been questioned (24). More recently, Rancour et al. have reported a liver microsomal system for oxidation of galactose to galactonate producing hydrogen peroxide with a K_m of 39 mM. Whatever the mechanism, the evidence is now substantial that galactose conversion to galactonate does occur under a variety of conditions. The perfused suckling rat liver provides a model physiological system which by

altering the conditions of perfusion may aid in the assessment of the underlying mechanisms for galactose oxidation to galactonate.

ACKNOWLEDGEMENTS

This research was supported by National Institutes of Health, Grant HD 08536.

REFERENCES

1. Cuatrecasas, P., and Segal, S. (1966) Science 153, 549-551.
2. Bergren, W.R., Ng, W.G., and Donnell, G.N. (1972) Science 176, 683-684.
3. Segal, S. and Cuatrecasas, P. (1968) Amer. J. Med. 44, 340-347.
4. Rancour, N.J., Hawkins, E.D. and Wells, W.W. (1979) Arch. Biochem Biophys. 193, 232-241.
5. Berman, W., Rogers, S., Bautista, J. and Segal, S. (1978) Metabolism 27, 1721-1731.
6. Berman, W., Rogers, S., Bautista, J. and Segal, S. (1979) Am. J. Physiol. 236, E633-E637.
7. Mortimore, G. (1961) Am. J. Physiol. 200, 1315-1319.
8. Beutler, E., Paniker, N.V. and Trinidad, J. (1971) Biochem. Med. 5, 325-332.
9. Moore, S. and Link, K.P. (1940) J. Biol. Chem. 133, 293.
10. Sweeley, C.C., Bentley, R., Makita, M., and Wells, W.W. (1963) J. Am. Chem. Soc. 85, 2497-2507.
11. Wiecko, J. and Sherman, W.R. (1976) J. Amer. Chem. Soc. 98, 7631-7637.
12. Shih, V.E. (1973) Laboratory Techniques for the Detection of Hereditary Metabolic Disorders. p. 83, CRC Press, Cleveland.
13. Blair, A. and Segal, S. (1960) J. Lab. Clin. Med. 55, 959-964.
14. Keiding, S. (1973) Scand. J. Clin. Lab. Invest 31, 319-325.
15. Cuatrecasas, P. and Segal, S. (1965) J. Biol. Chem. 240, 2382-2388.
16. Schwarz, V., Goldberg, S., Komrower, G.M. and Holzel, A. (1956) Biochem. J. 62, 34.
17. Wells, W., Pittman, J., and Egan J. (1966) Am. J. Dis. Child. 112, 477.
18. Gitzelmann, R., Curtius, H.C. and Muller, M. (1966) Biochem. Biophys. Res. Commun. 22, 437.
19. Quan-Ma, R., and Wells, W. (1965) Biochem. Biophys. Res. Commun. 20, 486.
20. Reddy, V.N., Schwass, D., Chakrapan, B. and Lim, C.P. (1976) Exp. Eye Res. 23, 483-493.
21. Crawhall, J.C. and Segal, S. (1968) Biochim. Biophys. Acta 163, 163-170.
22. Cuatrecasas, P. and Segal, S. (1966) J. Biol. Chem. 241, 5904-5909.
23. Cuatrecasas, P. and Segal, S. (1966) J. Biol. Chem. 241, 5910-5918.
24. Beutler, E. (1967) Science 156, 1516.