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Enhanced Galactosyltransferase Expression in the Failing Hearts of Spontaneously Hypertensive Rats

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To examine differences in cardiac gene expression between spontaneously hypertensive rats with and without heart failure, we have used subtractive hybridization to identify differentially expressed genes. After subtraction, cDNAs were amplified by PCR, cloned and sequenced. One of 36 independent cDNAs was found to be 86% homologous to murine UDP-galactose:*N*-acetylglucosamine β-1,4-galactosyltransferase. RNA blot analysis confirmed the $\sim$4.0 kb rat galactosyltransferase transcript was increased in failing hearts relative to non-failing hearts. Biochemical assay also showed increased galactosyltransferase activity in the failing hearts.

Heart failure continues to be a progressive and fatal disease. Despite extensive research, an understanding of mechanisms underlying its development remains elusive. Molecular biology has led to the ability to detect cellular responses in terms of changes in gene expression. To date, many of the responses studied have largely followed changes associated with preconceived concepts of factors underlying or associated with failure (1–3).

The availability of animal models of heart failure and subtractive hybridization technology allows the screening of mRNAs for differences in gene expression between failing hearts and controls. Such studies may facilitate the detection of unanticipated responses and permit the emergence of new concepts for mechanisms underlying the transition from compensated hypertrophy to failure. Therefore, we subtracted biotinylated mRNA obtained from the non-failing heart of the spontaneously hypertensive rat (SHR) from cDNAs synthesized from mRNA obtained from the heart of the SHR with heart failure. After subtraction, the remaining cDNAs were amplified by PCR, cloned, and sequenced. Here we report our initial studies showing for the first time that UDP-glucosaminyl β-1,4-galactosyltransferase gene expression and activity is increased in the failing heart.

MATERIALS AND METHODS

Animal model. Male spontaneously hypertensive rats (SHR) and Wistar-Kyoto (WKY) rats were purchased from Taconic (Germantown, N.Y.). All rats were housed two per container and fed regular rat chow and water. After age 18 months, animals were observed several times a week and studied when they developed tachypnea and labored respirations. Twelve month old SHR-NF (which clearly have compensated hypertrophy) and age-matched 18–24 month SHR-F and WKY were studied. After sacrifice, hearts were quickly removed, and placed in oxygenated physiologic salt solution at 28°C. The thorax was examined for the presence of pleuropericardial effusions, and the heart for atrial thrombi. The right and left ventricles were carefully dissected. Tissues were gently blotted and weighed. Left and right ventricular wet weight normalized by body weight (LV/BW and RV/BW) were used as indices of ventricular hypertrophy. Heart failure was considered present in animals with pleuropericardial effusions, atrial thrombi and right ventricular hypertrophy (Table 1). These pathological findings are similar to those observed in previous studies of the 18–24 month SHR where we have documented the presence of impaired myocardial function with heart failure (4–6). After weighing, LV tissues were stored in liquid nitrogen for subsequent analysis as described below.

Subtraction hybridization. RNA was isolated from age matched non-failing and failing SHR rats by the acid guanidinium procedure (7). Poly(A) mRNA was selected with oligo dT spin columns (Pharmacia). A subtractive hybridization kit was purchased from Invitrogen and used essentially as directed. Non-failing heart mRNA (10 μg) was labelled with biotin using a 150 W reflector bulb for 20 min at 0°C; failing heart mRNA (1 μg) was used to synthesize a first strand cDNA with oligo GACTCGAGTCGACATCGA(T_{17}). The biotinylated RNA and cDNA were hybridized for 48 h at 68°C. After extraction

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TABLE 1
Animal Weight, Chamber Hypertrophy, and pathological Features of Heart Failure

	WKY	SHR-NF	SHR-F
n	6	6	6
BW	617 ± 26*	419 ± 22#	380 ± 7
LV/BW	1.88 ± 0.11*	2.92 ± 0.16#	3.77 ± 0.12
RV/BW	0.45 ± 0.02*	0.63 ± 0.02#	1.34 ± 0.04
Effusions	0/6	0/6	6/6
Atrial Thrombi	0/6	0/6	6/6

Data are means ± SEM. BW = body weight (g); LV/BW = left ventricular weight/BW (mg/g); RV/BW = right ventricular weight/BW (mg/g).

* $p < 0.01$ WKY vs SHR-NF and SHR-F.

# $p < 0.01$ SHR-NF vs SHR-F.

with strepavidin and precipitation, the isolated cDNA was amplified by polymerase chain reaction (PCR) using the oligonucleotide GACTCGAGTCGACATCG. Cycle conditions were 94°C for 30 sec, 40°C for 45 sec, and 72°C for 1 min for 5 cycles followed by another 30 cycles with an annealing temperature of 55°C. The amplified DNA was subcloned into pCRII (Invitrogen) and sequenced using the dideoxy chain termination method (8) with Sequenase (United States Biochemical) and 35S[dATP] (DuPont-New England Nuclear). Sequence alignments were performed with Geneworks software (Intelligenetics).

Expression of galactosyltransferase. RNA (3 μg mRNA) from frozen left ventricular samples of SHR-F, SHR-NF and WKY was denatured with formamide at 65°C and fractionated on a 1.2% agarose-formaldehyde gel prior to transfer to Duralon nylon membranes (Stratagene). Membranes were prehybridized for 20 min in Quickhyb (Stratagene) at 68°C prior to adding 32P[dCTP] (DuPont-New England Nuclear) labelled random primed probes for galatosyltransferase (GalT) atrial natriuretic factors (ANF) or cardiac α-actin. After 2 h, membranes were three times with 2X SSC (1X SSC is 0.15 M NaCl/0.015 M sodium citrate) containing 0.1% SDS for 10 min at 42°C. This was followed by a single stringency wash with 0.2 × SSC containing 0.1% SDS for 60 min at 60°C.

Galactosyltransferase activity was determined from frozen left ventricle tissue samples of 12 month SHR-NF and age matched 18–24 month SHR-F and WKY rats. Samples were powdered in liquid nitrogen using a mortar and pestal, then homogenized on ice in 1 ml of 10 mM HEPES buffer containing 10 mM magnesium chloride, 5 mM calcium chloride and 1% Triton X-100. The extraction was completed by incubation of the homogenizate in extraction buffer for an hour on ice. Insoluble material was removed by centrifugation at 15,000 × g for 15 min.

The enzymatic activity was determined by the incorporation of tritium labelled galactose into ovalbumin (9). The assay mixture contained 50 mM MES buffer (pH 6.5), 10 mM manganese chloride, 1% Triton X-100, 40 mg/ml ovalbumin, 1 mM UDP-[^{3}H]-galactose (8.85×10^5 cpm) and 6.5 μl Triton extracted tissue homogenizate in a final volume of 25 μl. The mixture was incubated at 37°C for 90 minutes, the reaction was stopped by the addition of 5 μl 0.5 M EDTA solution, and applied to Whatman No. 1 chromatography paper. The chromatograms were developed for 18 h by descending chromatography in ethanol: 1 M ammonium acetate, pH 7.8 (5:2, v/v). The amount of labelled ovalbumin was determined by counting the origin of each lane in a liquid scintillation analyzer. Galactosyltransferase activity was expressed as cpm tritium incorporated into ovalbumin per mg of total protein in the extract as determined by the BCA protein assay (Pierce).

Statistical analysis. A two-way analysis of variance was used to examine group and treatment effects. The Newman-Keuls multiple-sample comparison test was used to localize differences where appropriate. Data from the SHR-F, SHR-NF and WKY groups were compared using one-way analysis of variance with replication. Data are expressed as means ± SEM.

RESULTS AND DISCUSSION

We have previously observed changes in cardiac gene expression during the transition from stable hypertrophy to heart failure in the SHR. Atrial natriuretic factor (ANF), collagen I and III, and fibronectin mRNA levels were increased while cardiac actin remained unchanged (5). Many of these findings are consistent with our current limited understanding of the pathogenesis of heart failure. As an approach to understanding early events leading to heart failure, we have begun to examine differences between mRNAs in hypertrophied and failing hearts.

We subtracted mRNA obtained from the hypertrophied non-failing SHR heart from cDNAs made to mRNA isolated from the failing heart of a second SHR. The remaining cDNAs were amplified by PCR using arbitrary priming and subcloned into a TA vector. Sequencing of 36

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          Gly Thr Pro Arg
Rat       GGG ACA CCA AGA TAGCA-TTTTGGTACAAATAAGAGACCCGAGGATGGC   48
          ||| ||| ||  ||| ||||| |||| |||| |||||||||||| ||| |||||
Mouse     GGG ACA CCG AGA TAGCATTTTTAGTACCAATAAGAGACCTGAGAATGGC

CAGAGACCTCAGATGTGTGTCTCTGCCAGTCGACTGGGCTGGTCCCTCTCATTTGTTCAG  108
| |||||||||||| |||||||||||||||| |||||||||||||||||||||||||| |||
CGGAGACCTCAGATATGTGTCTCTGCCAGTTGACTGGGCTGGTCCCTCTCATTTGTACAG

TCTGAATGATAGTTCTCCTTCCTTACCATTCAGACATCTTTCCAGATGCCCAGGGTGAAT  168
|||||||||  ||||||      |||| |||||||||  ||  |||||||||||||||||  |
TCTGAATGACAGTTCT----TCTTATCATTCAGACGTCCCTCCAGATGCCCAGGGTGAGT

GTCACGTTTACCCACAACCTGGCTCGGCACTGGGTGAAATTCTACAAGGTGTTTATCAGT  228
||  ||  |||||||||||||||||||||||||||| ||||||||||||||||   |   |||
GTAACATTTACCCACAACCTGGCTCGGCACTGGATGAAATTCTACAAGGTGAGT-GGAGT

GTAAAAACGGTCAGCCTTTGGAGAGGTTTCTTGGACACGTCACCCCCAAAGAGTCGGAAC  288
||||||   ||||||||  ||||||||   |||||||     |||||||||||||||| ||||
GTAAAACTGGTCAGCCCTTGGAGAGACTTCTTGGTTGTGTCACCCCCAAAGAGTCAGAAC

TGTGCCCAGCTCCAACCTTAGTGACTGTGGGTCATAGTCCCAGTGCTGAAACTGCTGAAG  348
|||  |  |||  ||  ||  |||||||||||||||||  |  |  |||  ||  |||||||||| ||
TGTACACAGTTCAAAACTTAGTGACTGTGGGTCACA-TTCCACTGTTGAAACTGCTAAAT

TGTGACATGGGTAAGAGCTTTGCTTTAGTCGG                              380
||||||  ||||  |||   ||||||||||||||
TGTGACCTGGGGAAGGACTTTGCTTTAGTCGG
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FIG. 1. Alignment of murine and rat UDP-galactose:*N*-acetylglucosamine β-1,4-galactosyltransferase cDNAs. The cDNA sequence of rat subtraction clone 6 was aligned with the corresponding region of the murine galactosyltransferase cDNA. The last 4 amino acids of the galactosyltransferase protein are underlined. Identical residues are indicated (&mvb;).

independent clones and search against Genebank demonstrated four cDNAs with significant homology to sequences contained in Genebank; these were murine UDP glucosaminyl β-1,4 galactosylransferase (10,11), rat phosphatidylethanolamine-binding protein (12), human protein phosphatase-2A regulatory alpha-subunit (13), and rat cardiac actin (accession number X80130). Alignment of the nucleotide sequences of rat and murine UDP glucosaminyl β-1,4 galactosyltransferase is shown in Fig. 1 The isolated cDNA was 86% homologous to the mouse sequence, and included nucleotides encoding the last 4 amino acids of galactosyltransferase and 368 nucleotides of the 3′ untranslated region. The other three cloned cDNAs were subsequently shown not to be increased in the SHR-F (see below), and their alignment is not shown.

While subtractive hybridization technology has enabled enriching cDNA clones of differentially expressed genes, incomplete subtraction of cDNAs may result in the isolation of false positive

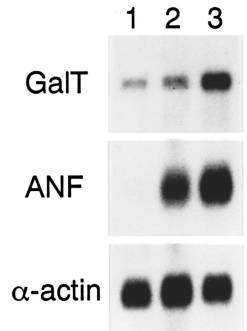

FIG. 2. Expression of UDP-galactose:*N*-acetylglucosamineβ-1,4-galactosyltransferase. A RNA blot containing heart mRNA (3 μg) from WKY (lane 1), SHR-NF (lane 2), and SHR-F (lane 3) was hybridized sequentially with ^{32}P-labelled galactosyltransferase (GalT), atrial natriuretic factor (ANF), and cardiac α-actin DNA fragments.

TABLE 2
UDP *N*-Acetylglucosamine β-1,4-Galactosyltransferase Activity in WKY, SHR-NF, and SHR-F Hearts: ^{3}H-Galactose Incorporated (cpm × 10^{-3})/mg Protein

WKY	SHR-NF	SHR-F
5.3 + 0.9	7.5 + 1.4	14.7 + 2.9*

* $p < 0.01$ SHR-F vs WKY and SHR-NF.

cDNAs. This maybe especially true of relatively abundant cDNAS such as cardiac actin which has previously been shown to be unchanged with failure (5). Therefore, RNA blots were hybridized with 32P labelled DNA fragments to determine whether the identified cDNAs were increased in the failing heart. As shown in Fig. 2, there was an increase in galactosyltransferase mRNA in the hearts from a failing SHR relative to WKY and non-failing hypertrophied SHR. The blot was also hybridized with cDNAs for ANF and cardiac actin; as previously demonstrated (5), ANF mRNA levels were greatly increased in the hearts from both SHR-NF and SHR-F compared to WKY. Cardiac actin mRNA levels were used to control for loading. Levels of transcripts for phosphatidylethanolamine-binding protein and phosphatase-2A regulatory alpha-subunit transcripts were found to be unchanged in the failing heart relative to SHR-NF or WKY (data not shown) and were not further evaluated.

Galactosyltransferase enzyme activity was measured in six hearts each from WKY and non-failing and failing SHR rats to determine if the enzyme activity was also increased in failing hearts, and to determine if this was a finding associated with heart failure or was unique to the animals used to isolate the RNA for these experiments. Galactosyltransferase activity was slightly, but not significantly, increased in the nonfailing SHR relative to WKY controls while it was increased three-fold in hearts from failing SHR over controls (Table 2, $p < 0.001$). Thus, both mRNA and enzyme activity are elevated in failing SHR hearts.

UDP N-acetylglucosamine β-1,4 galactosyltransference is a widely distributed enzyme which catalyzes the transfer of galactose to N-acetylglucosamine residues of glycoproteins and glycolipids. The enzyme is found mainly as a resident of the trans-Golgi cisternae of cells, but has also been detected on the plasma membrane of some cells (14,15). The intracellular enzyme is involved in glycoprotein and glycolipid synthesis while the cell surface enzyme has been implicated in the migration of fibroblasts and modification of the extracellular matrix (16). The significance of increased β-1,4 galactosyltransferase activity in the failing heart is not known at this time, but increased UDP N-acetylglucosamine β-1,4 galactosyltransferase may result in increased terminal galactose residues on glycoproteins and glycolipids. Considering the importance of terminal carbohydrate sequences in cell-cell interactions and adhesion, increased galactosyltransferase activity may reflect important pathophysiologic events in the failing heart. Increased galactosyltransferase activity may also be consistent with the marked upregulation of extracellular matrix gene expression (4) and connective tissue response (17) observed in the SHR heart during the transition from compensated hypertrophy to failure.

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