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Maturational Changes in Gene Expression for Adenine Nucleotide Translocator Isoforms and βF_1-ATPase in Rabbit Heart

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Maturational changes in myocardial respiratory control have been related to postnatal accumulation of adenine nucleotide translocator (ANT) in the inner mitochondrial membrane. Alternatively alterations in relative isoform distribution for this nuclear-encoded gene during myocardial maturation might be responsible for changing the kinetics of respiratory control. Rabbit hearts were analyzed for adenine nucleotide translocator isoform (ANT1, ANT2, ANT3) gene expression and distribution at four ages (fetal, 29/31 days of gestation; 1 h postnatal; 9 days postnatal; and 3–4 months postnatal). Transcript levels for the coordinately expressed βF_1-ATPase were also examined in these hearts. These studies demonstrated that mRNA expression for ANT1 in coordination with βF_1-ATPase increased substantially after 9 days of age in rabbit hearts. Expression of the minor isoform ANT3 parallels ANT1, though no change in expression of the kidney-specific isoform ANT2 occurs in heart during this developmental period. Previous work has demonstrated that ANT protein accumulation is closely coordinated with mRNA expression for ANT1. These results support previous studies, which indicate that the operational mode of myocardial respiratory control depends on adenine nucleotide mRNA expression. Changes in relative adenine nucleotide translocator isoform distribution do occur during fetal to mature transition and may contribute to observed changes in the mode of respiratory control. © 1999 Academic Press

Key Words: **mitochondria; energy metabolism; oxidative phosphorylation; F1-F0-ATPase; adenine nucleotide translocator.**

The adenine nucleotide translocator (ANT) resides in the inner mitochondrial membrane and represents the most abundant protein within that organelle (1). This nuclear-encoded protein exchanges ATP synthesized within the mitochondrial matrix for cytosolic ADP. The protein functions in coordination with oxidative phosphorylation and the mitochondrial proton translocating ATPase (F_1-F_0-ATPase). Linkage of mitochondrial oxidative phosphorylation to cytosolic energy utilizing processes in heart requires rapid and unlimited exchange by ANT. In mature myocardium *in vivo,* highly efficient ADP/ATP exchange maintains steady-state cytosolic ADP and ATP levels during substantial elevations in ATP hydrolysis at the myofibrils (2). Thus, the relationship between ADP and oxidative phosphorylation rate does not conform to kinetic models that suggest ANT regulates myocardial respiration (2). However, steady-state ADP levels do increase during concomitant elevations in oxidative phosphorylation in immature myocardium, and the relationship conforms to kinetic models of respiratory regulation involving ANT (2).

Myocardial respiratory control through ANT appears to decrease after birth in association with mitochondrial ANT accumulation (2,3). This observation implies that a specific number of mitochondrial ANT carrier sites are required to achieve the non-ADP-regulated respiration apparent in mature myocardium. Possible alterations in the characteristics of ANT during postnatal development offer an alternative hypothesis for maturational changes in kinetics of respiratory control. Kinetic differences might be due to developmentally related changes in ANT isoform expression, as opposed to total mitochondrial ANT content (4). Three distinct human

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ANT isoforms have been cloned, ANT1, ANT2, and ANT3 (4–6). These correspond to isoforms in other mammalian species (4). Each ANT isoform demonstrates a similar tissue-specific expression pattern in different mammals, which has been related to tissue-specific energy requirements (4). These isoforms are also differentially regulated during muscle cell differentiation.

In this study we examined relative ANT isoform gene expression in rabbit heart during prenatal and postnatal development. In other tissues and species, ANT gene expression is closely coordinated with gene expression for another nuclear-encoded mitochondrial membrane protein, the β-subunit of the F_1-ATPase (βF_1-ATPase) (2,5). Steady-state mRNA levels for this specific protein subunit have been used as markers for mitochondrial biogenesis (7,8). Therefore, we have also analyzed developmental expression of this gene in the current model.

MATERIALS AND METHODS

White New Zealand rabbits in four age groups were used for these studies: newborn (Nb) age 1 h; neonatal (N) age 9 days; and mature (M) age 5 months. Rabbits received heparin (700 U/kg, intravenously or intraperitoneally) and were anesthetized with sodium pentobarbital (45 mg/kg, intravenously or intraperitoneally). Fetal (F) rabbits were obtained by Caesarian section at 29/31 days of gestation after the doe received the heparin and pentobarbital.

RNA isolation. After median sternotomy, the hearts were rapidly excised. After rinsing with 4°C saline and removal of extracardiac fat and connective tissues, the left ventricular wall was briefly blotted on nine-layer gauze and then immediately frozen in liquid nitrogen and stored at −70°C. Approximately 100 mg of the frozen tissue was pulverized and homogenized and total RNA was extracted with a RNA isolation kit (Ambion Inc., Austin, TX). RNA samples were tested by ultraviolet absorption at $A_{260\ nm}$ to determine the concentration. The quality and concentration of the RNA samples were further confirmed by electrophoresis on denatured 1% agarose gels.

Northern blot analysis. RNA (15 μg) was denatured and electrophoresed in a 1% formaldehyde agarose gel and then transferred to a nitrocellulose transfer membrane (Micron Separations Inc., Westboro, MA) and cross-linked to the membrane with short-wave ultraviolet cross linker. The prehybridizing and hybridizing solutions contained 50% formamide, 1× Denhardt's solution, 6× SSPE, and 1% sodium dodecyl sulfate (SDS). Complementary DNA (cDNA) probes were labeled with [^{32}P]dCTP by random primer extension (PRIME-IT II, Stratagene, La Jolla, CA) and added to the hybridizing solution to a specific activity. Hybridization was carried out at 42°C for 18 h. The blots were then washed several times with a final wash in 1–2× standard sodium citrate and 0.1% SDS at 65°C. The relative amount of mRNA was evaluated using a PhosphorImager (Model 400S, Molecular Dynamics, Sunnyvale, CA). The same size area at each band was taken to measure the intensity and the same size area at the closest upstream position of each band was taken as the background of the image, respectively. The blots were also exposed on Kodak X-omat film (Eastman Kodak Co., Rochester, NY) at −70°C. The following probes were used to detect mRNA for the three adenine nucleotide translocase isoforms: a 1.4-kb ANT1 cDNA fragment cloned from a human skeletal muscle library (ATCC, Rockville, MD) and a 1.5-kb ANT3 fragment cloned from a human liver library. A human 363-bp ANT2 fragment probe was synthesized by PCR amplification method (9) with a pair of primers (F1, 5′GCATGATCACTGCAGATAAGCA3′; and R1, 5′ATCTTAACCAGGCAGTCACCGAG3′). The fragment corresponds to part of exon 2 of the human ANT2 gene (10). βF_1-ATPase mRNA levels were detected using a 1.8-kb βF_1-ATPase cDNA fragment cloned from a human HeLa cell line library (ATCC). To compare different mRNA levels in the same myocardial sample, aliquots of 15 μg of total RNA from the myocardium were analyzed by sequentially reprobing the membranes with 28S, ANT1, βF_1-ATPase, ANT3, and ANT2 cDNA probes.

Statistical analyses. Values reported are means ± standard error in the text and figures. The Statview 4.5 (FPV) Program (Abacus Concepts, Inc., Berkeley, CA, 1995) was used for statistical analyses. Data were evaluated with single factor analysis of variance across groups. When significant F values were obtained, Scheffe's test was used to distinguish which groups differed from each other significantly. The criterion for significance was taken to be $P < 0.05$ for all comparisons.

RESULTS

Semiquantitative analyses of steady-state mRNA levels for ANT1 and βF_1-ATPase were performed for

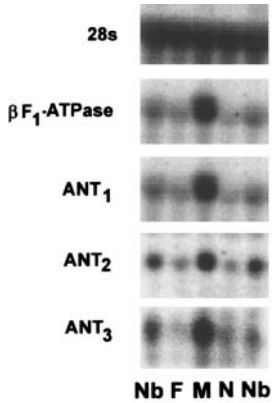

FIG. 1. A representative Northern blot. Each lane was loaded with 15 μg of total RNA from ventricular myocardium and probed specifically for 28S, βF_1-ATPase, and adenine nucleotide translocator isoforms ANT1, ANT2, and ANT3. Each lane represents a different age group: F, fetal (29/31 days of gestation); Nb, newborn (age 1 h); N, neonatal (age 9 days); and M mature (age 5 months).

five hearts from each of the four age groups: F, Nb, N, and M. Northern blots were performed for four samples obtained from each heart. Hybridization of the βF_1-ATPase, ANT1 and ANT3 mRNA probes was similar to that previously observed in human, mouse, and rabbit tissues. A representative Northern blot is illustrated in Fig. 1.

The mRNA levels for ANT1 and βF_1-ATPase genes normalized to 28S ribosomal RNA intensity are shown in Fig. 2. Across individual membranes, there were no significant changes in the 28S band intensity (Fig. 1). Statistical analyses reveal that steady-state mRNA levels for ANT1 and βF_1-ATPase genes remain constant from late fetal age until at least 9 days after birth. A near three- to fivefold increase in these mRNA levels occurs between postnatal day 9 and mature age.

ANT1, ANT2, and ANT3 mRNA intensities relative to 28S ribosomal RNA intensity for a specific lane were normalized to the mean mature lane intensity across the membrane. The mean normalized isoform intensities for each age group are illustrated in Fig. 3. These values do not represent absolute signal intensity ratios, but instead provide an assessment for changes in isoform distribution during cardiac maturation. Statistical analyses demonstrate that there is no significant change in the expression of ANT2 during the transition from late fetal to the mature age. ANT3 expression closely parallels ANT1 expression. These results imply that expression of ANT1 and ANT3 increases relative to ANT2 after 9 days of age in rabbit heart.

DISCUSSION

Results of the present study conform to previous work performed in lamb myocardium, which demonstrated that ANT1 gene expression coordinate with βF_1-ATPase increases substantially after 1 week of age (2). Regulation of these two genes is mediated through a shared muscle-specific promoter element located upstream from the putative transcription start sites as well as a tissue ubiquitous overlapping repressor region (5,6). Specific inhibition of the repressor element occurs through binding of environmental factors including thyroid hormone, which surges in the postnatal period *in vivo* (5,11). Removal of transcriptional inhibition by such environmental factors after birth might explain the coordinate increase in transcript levels for these genes.

Several investigators have described ANT1 as the specific and dominant isoform in striated muscle (4,5). Confirmation for this specificity in rabbit is provided in Fig. 4, which shows relative mRNA expression of ANT1 and ANT2 in various tissues. ANT1 signal is abundant in heart and skeletal muscle, though absent in kidney and liver. As previous investigators have established the predominance of ANT1 in cardiac muscle, we did not find it necessary to determine absolute mRNA levels for these isoforms. Rather, we examined relative changes in expression of the three isoforms, which might occur

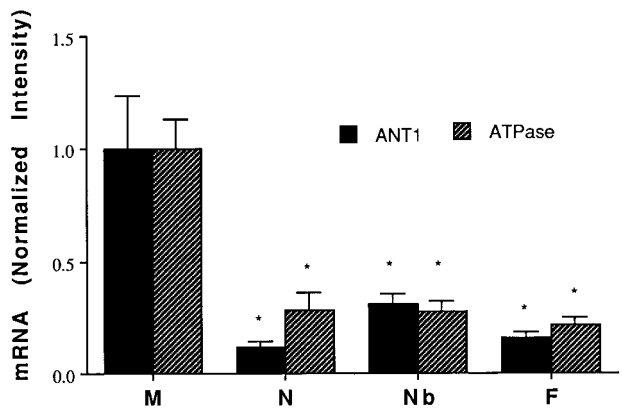

FIG. 2. Densitometric band intensities for ANT1 and βF_1-ATPase relative to 28S and normalized to the mean (M) intensity are shown for each age group. Abbreviations are as noted in the legend to Fig. 1. *$P < 0.05$ versus M. No significant differences among F, Nb, or N occurred. These steady-state mRNA levels are substantially higher in M.

during the transition from the late fetal to the mature developmental state. The observation that ANT1 expression increased over ANT2 and ANT3 during myoblast to myotube differentiation suggests that relative expression might change with further development and continue through the postnatal period (4). Our results show that ANT1 and ANT3 gene transcript levels increase during this period, while ANT2 does not change significantly. Other workers have already demonstrated that the ubiquitous isoform ANT3 contributes a relatively minor fraction of the total ANT expression in heart (4,12). These observations indicate that changes in the kinetics of respiratory control attributed to ANT accumulation (2,3) during maturation are related to increases in total and relative ANT1 expression.

Protein expression was not examined during this study. However, previous work has demonstrated that postnatal myocardial ANT protein expression increases coordinately with steady-state ANT1 mRNA levels (2). This response is relatively specific since βF_1-ATPase does not demonstrate similar coordination. Altered translational efficiency might explain the differences in mRNA protein coordination in these two genes, which demonstrate coordinate expression at the transcriptional level.

In summary, this study demonstrates that the muscle-specific isoform ANT1 accumulates after birth and expression is increased relative to ANT2, the kidney-specific isoform. Thus, maturational alterations in the kinetics of myocardial respiratory control may be caused in part by changes in ANT isoform distribution, as well as increases in total steady-state ANT1 mRNA accumulation. These results imply though do not prove that increased ANT protein expression and activity noted during postnatal development is regulated in part at the transcriptional level.

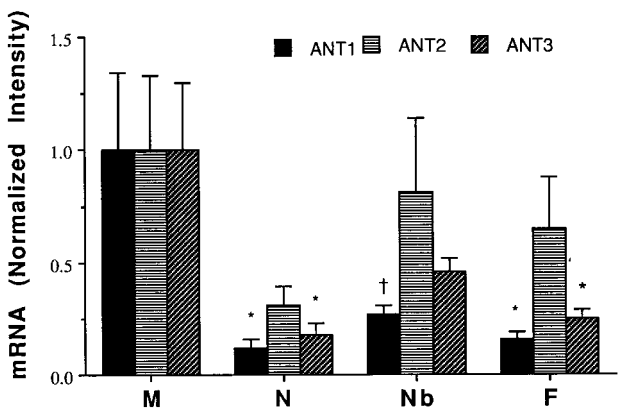

FIG. 3. Signal intensities for the three isoforms ANT1, ANT2, and ANT3 are shown in a format similar to Fig. 2. *$P < 0.05$ versus M; †$P = 0.06$.

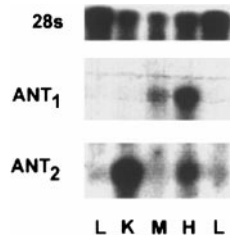

FIG. 4. Northern blot exhibits relative intensities of ANT1 and ANT2 in liver (L), kidney (K), muscle pectoralis major (M), and heart (H).

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