Akt activation reduces cardiomyocyte death and induces cardiac hypertrophy. To help identify effector mechanisms, gene expression profiles in hearts from transgenic mice with cardiac-specific expression of activated Akt (myr-Akt) were compared with littermate controls. 40 genes were identified as differentially expressed. Quantitative reverse transcription-PCR confirmed qualitative results of transcript profiling for 9 of 10 genes examined, however, there were notable quantitative discrepancies between the quantitative reverse transcription-PCR and microarray data sets. Interestingly Akt induced significant up-regulation of insulin-like growth factor-binding protein-5 (IGFBP-5), which could contribute to its anti-apoptotic effects in the heart. In addition, Akt-mediated down-regulation of peroxisome proliferator-activated receptor (PPAR) γ co-activator-1 (PGC-1) and PPAR-α may shift myocytes toward glycolytic metabolism shown to preserve cardiomyocyte function and survival during transient ischemia. IGFBP-5 transcripts also increased after adenoviral gene transfer of myr-Akt to cultured cardiomyocytes, suggesting that this represents a direct effect of Akt activation. In contrast, substantial induction of growth like growth factor-binding protein-5 (IGFBP-5), which intriguingly Akt induced significant up-regulation of insulin-transcription-PCR and microarray data sets. Interest-
ingly Akt induced significant up-regulation of insulin-like growth factor-binding protein-5 (IGFBP-5), which could contribute to its anti-apoptotic effects in the heart. In addition, Akt-mediated down-regulation of peroxisome proliferator-activated receptor (PPAR) γ co-activator-1 (PGC-1) and PPAR-α may shift myocytes toward glycolytic metabolism shown to preserve cardiomyocyte function and survival during transient ischemia. IGFBP-5 transcripts also increased after adenoviral gene transfer of myr-Akt to cultured cardiomyocytes, suggesting that this represents a direct effect of Akt activation. In contrast, substantial induction of growth like growth factor-binding protein-5 (IGFBP-5), which intriguingly Akt induced significant up-regulation of insulin-transcription-PCR and microarray data sets. Interest-

The serine-threonine kinase Akt (or protein kinase B) has well documented anti-apoptotic effects in many systems (1–3). We have shown that expression of a constitutively active mutant of Akt (myr-Akt) is sufficient to block apoptosis in hypoxic neonatal rat cardiomyocytes in vitro (4) and in vivo prevents cardiac injury while preserving heart function during ischemia-reperfusion injury (5). The downstream targets of Akt that mediate cell survival in the heart remain poorly characterized. Indeed some Akt substrates (e.g. Bad, glycogen synthase kinase-3, and Bcl-2) identified in other cell types appear to be either expressed at very low levels or not phosphorylated by Akt in cardiomyocytes (5, 6). These data suggest that additional Akt-dependent phosphorylation, translation, and/or transcription events may be required for Akt-mediated cytoprotection in the heart.

Translational effects of Akt involve the phosphorylation and activation of the mammalian target of rapamycin (mTOR) that in turn phosphorylates 4E-BP1 and p70S6 kinase (7). The net effect of these phosphorylation events is enhanced translation of specific mRNA subset(s), which is bound by the initiation factor eIF-4F and/or the ribosomal S6 subunit. In contrast, the translational effects of Akt are less well defined, although the importance of these events may be greater than initially realized (8, 9). Akt-regulated gene transcription has been described for Gliut-1 (10), vascular endothelial growth factor (11), and Bcl-2 (12), and a number of Akt-regulated transcription factors have been identified. Akt directly phosphorylates Forkhead box transcription factors, class O (FOXOs) (13–15) and may also regulate, through direct and/or indirect mechanisms, AP-1, cAMP-response element-binding protein, and NF-xB (16–19).

To examine the translational effects of Akt in the heart we analyzed the changes in global gene expression in transgenic mice with cardiac-specific expression of myr-Akt using DNA microarrays. This approach enabled the quantitation of the effects of Akt activation on ~11,000 genes. Results of interest were validated by quantitative RT-PCR (QRT-PCR). Here we identify genes differentially regulated by chronic Akt activation in the heart and demonstrate that modulated transcripts represent a combination of primary and secondary effects. The importance of confirming microarray results of interest using additional, complimentary techniques is discussed.
Transcriptional Effects of Chronic Akt Activation in Heart

Both lines express myr-Akt specifically in the heart at levels 5–7-fold higher than the endogenous molecule and exhibit a substantial increase in Akt activation as measured by both in vitro kinase assays and in vivo phosphorylation of known substrates (20).

Preparation of cRNA for Microarray Analysis—Total RNA was extracted from 5–7-old, 20 line mouse hearts using TRIzol (Invitrogen) according to the manufacturer’s recommendations. RNA was resuspended in diethyl pyrocarbonate-treated H2O and further purified using the Qiagen (Chatsworth, CA) RNeasy total RNA isolation kit according to the manufacturer’s instructions. RNA was quantified, and samples (n = 2–5 hearts) were pooled such that pooled RNA represented equal amounts (10 μg) of cardiac ventricles of Sprague-Dawley neonates as described previously (29). Following purification RNA was quantified in triplicate using a NanoDrop spectrophotometer (NanoDrop, Wilmington, DE). Samples were treated (10 min at 20 °C) such that multiple transcripts could be amplified grade DNAse 1 (Invitrogen) following which the DNase 1 was heat-inactivated (5 min at 75 °C). QRT-PCR was performed in duplicate using the Brilliant One-Step QRT-PCR kit (Stratagene, La Jolla, CA) containing SYBR Green I (1:30,000, Sigma), forward and reverse primers (50 nm each), and sample RNA (90 ng). Primers were designed to be compatible with a single QRT-PCR thermal cycle (95 °C for 10 min, 40 cycles for 10 s and 60 °C for 1 min) such that multiple transcripts could be analyzed simultaneously. Accumulation of PCR product was monitored in real time (Mx4000, Stratagene), and the crossing threshold (Ct) was determined using the Mx4000 software. For each set of primers, a no template control and a no reverse amplification control were included. Postamplification dissociation curves were performed to verify the presence of a single amplification product in the absence of DNA contamination. -Fold changes in gene expression were determined using the ΔCt method with normalization to total RNA (21, 22).

Adenoviral Vectors (Ads)—Ad-EGFP β-gal contains cytomegalovirus-driven expression cassettes for β-galactosidase and enhanced green fluorescent protein (EGFP) (5). Ad-Akt(AA) utilizes a similar viral backbone but encodes a dominant-negative Akt mutant and was kindly provided by Dr. Wataru Ogawa, Kobe University, Japan (23). Ad-myr-Akt and Ad-EGFP mediate expression of hemagglutinin-tagged constitutively active Akt or EGFP, respectively, and have been described previously (5). Ads were amplified in 293 cells, the particle count was estimated from A260, and the litter was determined by plaque assay. Wild-type adenovirus contamination was excluded by the absence of PCK-1 staining (region 1 E1 (21)).

In Vitro Studies of myr-Akt Expression—Primary cultures of neonatal rat ventricular cardiomyocytes (NRVMs) were prepared from the cardiac ventricles of Sprague-Dawley neonates as described previously (5). To study the effects of transient transgene expression, myocytes were infected with adenoviral vectors at a multiplicity of infection of 100 for 24 h in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum. Cells were subsequently serum-starved for 24 h prior to RNA extraction. RNA was extracted, purified, and quantified as described above.

Immunoblotting—Hearts from littermate control and myr-Akt-expressing mice were removed from deeply anesthetized animals, snap frozen, and crushed under liquid nitrogen before tissue was homogenized in cold lysis buffer (20 mM Tris-HCl (pH 7.6), 150 mM NaCl, 1% Triton X-100, 2 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, 1 mM sodium orthovanadate, 1 μg/ml leupeptin, 1 μg/ml aprotinin). Proteins from NRVMs were extracted by scraping cells directly into cold lysis buffer as described previously (4). Protein concentration was measured using a Bio-Rad protein assay (Bio-Rad). Protein samples (30 μg) were separated by SDS-PAGE on 12% separation gels and transferred to nitrocellulose membranes (Schleicher & Schuell) by semi-dry transfer. Blots were incubated with anti-Akt (1:1000, Cell Signaling) overnight at 4 °C and subsequently incubated with horseradish peroxidase-conjugated secondary antibody (1:5000, Dako). Immunoreactive bands were detected by enhanced chemiluminescence (Cell Signaling).

Statistics—Data are represented as mean ± S.E. Data were compared by two-tailed Student’s t test. The null hypothesis was rejected for p < 0.05.

RESULTS

Effects of myr-Akt Expression on Gene Expression in the Heart—To identify genes differentially regulated by Akt in the heart we examined the gene expression profiles of mice with cardiac-specific expression of myr-Akt (20 line) compared with TG-negative littermate controls. The experiment was repeated three times to reduce erroneous data that can arise when pooled RNA alone is used as a substitute for experimental replication (25). Genes of interest were identified using the described filtering protocols and examined for statistically significant differences in expression. These analyses revealed that expression of myr-Akt in the heart resulted in the differential regulation of 40 (21 up-regulated and 19 down-regulated) of the ~11,000 genes examined (Tables I and II).

It is surprising to observe that the two genes with the greatest -fold changes in expression are not usually expressed in the heart. Myosin alkali light chain 1 fast/fast (MLC1F3/F, up-regulated 11.8-fold) is predominantly expressed in skeletal muscle (26) and the ovary testis transcribed (OTT, up-regulated 11.1-fold) gene is usually only expressed in the ovary or the testis (27). Induction of insulin-like growth factor-binding protein-5 (IGFBP-5) by insulin-like growth factor-1 (IGF-I) via phosphatidylinositol 3-kinase and mTOR has been observed previously (28), although a direct connection to Akt has not been reported. Some genes of related function were coordinately regulated by chronic Akt expression. For instance, the potent inhibitor of angiogenesis pigment epithelium-derived factor was up-regulated 2.6-fold, while the angiogenic factor vascular endothelial growth factor was down-regulated 1.8-fold. In addition, transcripts for peroxisome proliferator-activated receptor α (PPAR-α) and peroxisome proliferator-activated receptor γ coactivator-1 (PGC-1), both involved in fatty acid metabolism, were down-regulated.

Validation of Microarray Data for myr-Akt-expressing Mice by QRT-PCR—The differential expression of six up-regulated and four down-regulated genes, identified by microarray analysis, were validated by QRT-PCR. Relative transcript levels were determined in F3, 20 line TG-positive males compared with TG-negative male littermate controls (Fig. 1). QRT-PCR analysis confirmed 7 of the 10 genes were statistically differentially regulated (p < 0.05) in the 20 line. Cardiac ankyrin repeat protein, pigment epithelium-derived factor (PAREX1), although differentially regulated in accordance with microarray data, did not achieve statistical significance. Cardiac ankyrin repeat protein and pigment epithelium-derived factor were subsequently confirmed as differentially regulated (p < 0.05) in the 564 line. Although the -fold change of some genes (IGFBP-5, pigment epithelium-derived factor, PGC-1, PPAR,
and vascular endothelial growth factor), as determined by QRT-PCR analysis, correlated with the fold change reported by microarray analysis there were three major discrepancies. The greatest discrepancy was observed in the expression levels of OTT, which was reported as 11.1-fold up-regulated by microarray analysis compared with 675-fold by QRT-PCR (Table I and Figs. 1 and 2). The second major discrepancy was seen in the expression levels of growth differentiation factor-8 (GDF-8), which was reported as 5.1-fold up-regulated in TG20-positive hearts by microarray analysis compared with 18.4-fold

| Gene name | Fold change | p       | GenBank™  |
|-----------|-------------|---------|-----------|
| MLC1F/MLC3F | 11.8       | <0.01   | X12973    |
| Ovary testis transcribed | 11.1       | <0.01   | X96603    |
| Insulin-like growth factor-binding protein-5 | 5.4       | <0.05   | L12447    |
| Growth differentiation factor-8 | 5.1       | <0.01   | U84005    |
| FXD ion transport regulator 5 | 3.6       | <0.05   | U72880    |
| Procollagen, type VIII, α1 | 2.8       | <0.05   | X66976    |
| Lysozyme P | 2.8       | <0.01   | X15457    |
| Golgi SNAP receptor complex member 2 | 2.7       | <0.05   | A1847904  |
| Pigment epithelium-derived factor | 2.6       | <0.05   | AF036164  |
| Cardiac ankyrin repeat protein | 2.5       | <0.01   | AF041847  |
| Receptor activity modifying protein 1 | 2.1       | <0.05   | AF250489  |
| Complement component 1q | 2.1       | <0.01   | X66295    |
| Peroxisomal biogenesis factor 11a | 2.0       | <0.05   | AF093669  |
| Odorant-binding protein 1b | 2.0       | <0.05   | AW046850  |
| Lα-associated invariant chain | 1.9       | <0.05   | X00496    |
| Heterogeneous nuclear ribonucleoprotein L | 1.6       | <0.01   | A8009392  |
| Procollagen C-proteinase enhancer protein | 1.6       | <0.05   | X57337    |
| 4 ESTs | (3.0–1.8) | <0.05   | |
demonstrates that the accumulated product has a single melting point in accordance with that predicted for the specific OTT amplicon. Minimal confirming the absence of DNA contamination. Nonspecific product was observed in the no template control after 33 cycles. No amplification was observed in the no amplification control (curves analysis. A no template control (NTC) were included to confirm accumulation of a single PCR product (TG and littermate (LM) controls and subjected to QRT-PCR analysis of OTT mRNA levels using gene-specific primers and postamplification melt curve analysis. A no template control (NTC) and a no amplification control (NAC) were included to confirm accumulation of a single PCR product of the predicted melting temperature in the absence of DNA contamination. A, amplification: amplified product was detected after an average of 18.7 cycles of PCR in TG hearts compared with an average of 28.1 cycles in littermate control hearts (n = 3 in both groups). Accumulation of nonspecific product was observed in the no template control after 33 cycles. No amplification was observed in the no amplification control confirming the absence of DNA contamination. B, melting point analysis: the first derivative of the postamplification dissociation curve demonstrates that the accumulated product has a single melting point in accordance with that predicted for the specific OTT amplicon. Minimal nonspecific primer-dimer was observed in the no template control, and no DNA-derived product was observed in the no amplification control.

Comparison of Differential Gene Expression between Two myr-Akt TG Lines—To control for differences in transgene insertion, expression, and activity, we determined the relative expression of the 10 genes examined by QRT-PCR in the 20 line in a second myr-Akt-expressing line, the 564 line (Fig. 1). For all genes except OTT, the pattern of differential expression observed in TG20 mice was confirmed in TG564 mice, although the fold-change in expression was significantly greater in the 564 line for GDF-8 and IGFBP-5 (64.9 versus 18.4, p < 0.01 and 6.0 versus 3.8, p < 0.05, respectively; Fig. 1A). Although OTT mRNA was detected in the TG564 hearts, there was no difference in the low level of expression between TG-positive and -negative littersates.

Effects of Transient myr-Akt Expression on IGFBP-5 and GDF-8 Transcript Levels in Vitro—We next examined whether IGFBP-5 and/or GDF-8 were directly regulated by acute Akt activation in cardiomyocytes using an in vitro system (4). NRVMs were infected with Ad-EGFP, Ad-myr-Akt, or Ad-Akt(AA) (multiplicity of infection = 100 for all), and total RNA or protein was extracted after 24 h in serum-free medium. A, expression of IGFBP-5 and GDF-8 mRNA: relative expression levels of IGFBP-5 and GDF-8 were determined by QRT-PCR using gene-specific primers. Ad-myr-Akt increased the expression of IGFBP-5 by 7.2-fold relative to Ad-EGFP, whereas Ad-Akt(AA) did not. In contrast, Ad-myr-Akt had no effect on expression levels of GDF-8. Data are expressed as mean ± S.E. (**, p < 0.01; n = 3 in all groups). B, immunoblots of myr-Akt expression in vivo and in vitro: the expression levels of myr-Akt and endogenous Akt were determined to validate the comparison between in vivo and in vitro QRT-PCR data. Proteins (30 μg) from hearts or cultured NRVMs were separated by SDS-PAGE, and Akt expression was determined by immunoblotting. Top panel, 20 line littermate controls (lanes 1 and 2) and TG positives (lanes 3 and 4). Middle panel, 564 line littermate controls (lanes 1 and 2) and TG positives (lanes 3 and 4). Bottom panel, uninfected NRVMs (lanes 1 and 2) and NRVMs infected with Ad-myr-Akt (multiplicity of infection = 100) for 24 h (lanes 3 and 4).

Ad-myr-Akt did not alter the expression level of GDF-8 at 24 h and had no effect on GDF-8 expression at either 48 or 72 h (data not shown).

DISCUSSION

Akt protects the heart from ischemia-reperfusion injury (5, 29), although it does not appear to phosphorylate many of its potential downstream targets, including Bad, when expressed in neonatal or adult cardiomyocytes (5). Thus, the mechanisms of Akt cardioprotection remain incompletely defined and may include transcriptional effects. The recent identification of Akt-dependent transcripts (e.g. Glut-1, Bcl-2, and Fas ligand) (10–12) and Akt-modulated transcription factors (e.g. FOXOs, AP-1, and cAMP-response element-binding protein) (13–17), which
are expressed in the heart, supports this hypothesis. We characterized the transcriptional effects of myr-Akt expression in the heart using DNA microarrays.

It has been suggested that DNA microarray experiments should be repeated with at least three replicates (25) and that the resulting data sets should be filtered and validated to minimize erroneous data. Indeed, as much as one-third of the variation seen during an experimental comparison may be attributable to variations intrinsic to the arrays themselves (30). However, data filters should be used with caution as they can increase the number of false negative results. Thus changes in important, low copy transcripts, which are excluded from analysis by virtue of their low AvDiffs and/or their increased propensity to be called “absent,” may be missed. We observed significant changes in the expression of 40 (−0.4%) of the genes examined in myr-Akt-expressing hearts (Tables I and II). Of note, the two transcripts with the greatest -fold changes, OTT and GDF-8, were in the group of genes identified using the “low stringency” filter. This finding illustrates how potentially important data may be missed if too stringent a filter is applied to microarray data sets.

We have demonstrated that Akt activation increases the transcription of IGFBP-5 in the heart. IGFBP-5 may have direct and/or indirect anti-apoptotic activity (31–34). Therefore, IGFBP-5 up-regulation, in an Akt-dependent manner, may be of particular importance to the cardioprotective effects of Akt. In the light of previous studies, Akt-dependent IGFBP-5 up-regulation in the heart is likely to be mediated through mTOR (28). It is therefore interesting to note that rapamycin, an mTOR inhibitor, can dramatically attenuate the protective effects of insulin, which activates Akt, in the heart (29). In this study, we have also shown that Akt down-regulates PGC-1 and PPAR-α in the heart. This may shift cardiomyocyte metabolism away from fatty acid metabolism in favor of glycolysis, which has been shown to protect cardiomyocytes during transient ischemia (35, 36).

Confirmation of microarray data by a previously validated and established technique should be performed for a selection of differentially regulated genes and in particular for genes of specific interest. Of the 10 genes analyzed by QRT-PCR, nine were confirmed in one or both of the transgenic lines as significantly differentially expressed in keeping with the microarray data. However, the degree of differential regulation of OTT, GDF-8, and MLC1F/3F determined by QRT-PCR differed markedly from microarray results (Tables I and II and Fig. 1). OTT mRNA has been described only in the testis and ovary (27), and it was initially unclear why this gene should be up-regulated by Akt activation in the heart. As the inheritance in the 20 line is X-linked and OTT is encoded on the X chromosome (27), we hypothesize that the up-regulation of OTT may be an insertional effect of the transgene construct. Consistent with this hypothesis, OTT was not differentially regulated in the 564 line in which the low level of expression was similar to that seen in transgene-negative littermates from both lines and wild-type controls (data not shown). The possibility that the discrepancy between the two lines represents an insertional effect on an autosome in the TG564 mice (for example in a trans-acting element regulating OTT expression) appears less likely but has not been formally excluded. As microarray characterization of transgenic mice becomes more common and the murine physical map better characterized, the hitherto latent frequency of insertional events may become more apparent.

The disparity between microarray and QRT-PCR data for the expression levels of GDF-8 and MLC1F/3F highlights two other important limitations of microarray data: dynamic range and sequence specificity. The -fold change in expression of GDF-8 in TG20 hearts, compared with littermate controls, was reported as 5.1-fold up-regulated by microarray analysis. In contrast, analysis of GDF-8 expression in the 20 line by QRT-PCR, likely a more accurate means of quantifying mRNA levels, revealed that GDF-8 was up-regulated by 18.4-fold. This underestimation of -fold change was even greater for OTT, which was found to be 11.1-fold up-regulated by microarray analysis compared with 675-fold by QRT-PCR (Table I and Figs. 1 and 2). The problem of false positive results reported by microarray analysis was illustrated by the MLC1F/3F data, reported as 11.8-fold up-regulated by microarray analysis compared with 1.7-fold (20 line) and 1.4-fold (564 line) by QRT-PCR (Table I and Fig. 1). This false positive result could reflect an error in the sequences on the microarray, the occurrence of which was dramatically demonstrated when up to one-third of the sequences on one set of mouse arrays were found to be wrong (37). Other possibilities for this type of error include cross-hybridization by splice variants, related genes, and/or pseudogenes.

The Akt/mTOR pathway has been identified as the crucial regulator of skeletal muscle and pancreatic islet cell hypertrophy in vivo (38, 39). In both our myr-Akt-expressing mouse lines cardiac hypertrophy, with no evidence of decompensation, was observed at 6 weeks (20). Akt therefore promotes both skeletal and cardiac muscle hypertrophy. As Akt promotes cardiac hypertrophy, we hypothesize that the observed up-regulation of GDF-8, a negative regulator of muscle growth, acts as part of a negative feedback loop limiting heart size. The phenomenon of negative feedback and activation of adaptive mechanisms is recognized but infrequently described in transgenic and knockout mice (40, 41). GDF-8, also termed myostatin, is highly conserved across species, and although first characterized in skeletal muscle (42, 43) it has also been identified in the heart (44). The hypothesis that GDF-8 up-regulation is a secondary event is supported by our in vitro experiments where expression of myr-Akt, at levels similar to those seen in TG mice (Fig. 3B), resulted in the up-regulation of IGFBP-5 but not GDF-8 (Fig. 3A). It remains unclear whether GDF-8 expression is related to myocyte size or organ mass (24).

In summary, these data demonstrate that chronic Akt activation results in the differential regulation of ≥40 genes in the heart. Several of the observed changes generate intriguing hypotheses regarding the effects of Akt in the heart and possible mechanisms underlying Akt-mediated cardioprotection. Akt-dependent up-regulation of the anti-apoptotic molecule IGFBP-5 may be of particular importance and could contribute to the observed cytoprotective effects of Akt in the heart. Similarly Akt down-regulation of PGC-1 and PPAR-α could shift myocytes toward glycolytic metabolism previously shown to help preserve cardiomyocyte function and survival during transient ischemia (35, 36). Chronic Akt activation in the heart was associated with the differential regulation of a subset of genes that are dissimilar to those observed with acute Akt activation in other cell types, emphasizing the tissue and temporal specificity of changes in transcription profiles (9). In the myr-Akt mice, some changes (e.g. IGFBP-5) appear to be direct consequences of Akt activation and were recapitulated in cardiomyocytes in vitro, while other transcripts (e.g. GDF-8) were not induced by acute Akt activation in vitro and therefore likely represent an indirect effect of the transgene. Given the role of GDF-8 in limiting skeletal muscle growth, we hypothesize that the dramatic up-regulation of GDF-8 observed in hypertrophied hearts may represent a negative feedback mechanism. However, additional studies will be necessary to demonstrate the functional relevance of the observed alterations in transcript levels. Finally, while our transcript profiling and QRT-
PCR data were generally concordant, there were some striking discrepancies in the quantitative assessment of mRNA changes, underscoring the importance of validation of DNA microarray results through additional independent techniques.

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