Role of the estrogen/estrogen-receptor-beta axis in the genomic response to pressure overload-induced hypertrophy

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Institute of Gender in Medicine and Center for Cardiovascular Research, Charite University Hospital, Berlin, Germany; Department of Biosciences and Nutrition, Karolinska Institutet, Huddinge, Sweden; and Center for Nuclear Receptors and Cell Signaling, Department of Cell Biology and Biochemistry, University of Houston, Houston, Texas

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Kararigas G, Fliegner D, Gustafsson JÅ, Regitz-Zagrosek V. Role of the estrogen/estrogen-receptor-beta axis in the genomic response to pressure overload-induced hypertrophy. Physiol Genomics 43: 438–446, 2011. First published February 15, 2011; doi:10.1152/physiolgenomics.00199.2010.—Cardiac hypertrophy, the adaptive response of the heart to overload, is a major risk factor for heart failure and sudden death. Estrogen (E2) and estrogen receptor beta (ERbeta) offer protection against hypertrophy and in the transition to heart failure. However, the underlying pathways remain incompletely defined. We employed a publicly available microarray dataset of female wild-type (WT) and ERbeta knockout (BERKO) mice subjected to pressure overload-induced hypertrophy to perform a systematic investigation of the mechanisms involved in the protection conferred by the E2/ERbeta axis. We show that considerably more genes were modulated in response to pressure overload in BERKO mice than in WT mice. The majority of the identified candidates in BERKO mice were induced, while those in WT mice were repressed. Pathway analysis revealed a similar pattern. This study is the first to demonstrate that the lack of ERbeta led to a significant increase of inflammatory pathways. Mitochondrial bioenergetics- and oxidative stress-related pathways were also modulated. In conclusion, ERbeta acquires the role of gatekeeper of the genomic response of the heart to pressure overload-induced hypertrophy. This may offer the molecular explanation for its cardioprotective role. We consider the present study to be a useful resource and that it will contribute to downstream functional analysis and to the characterization of pathways with previously unknown role in hypertrophy.

gene regulation; hormones; receptors

THE ADAPTIVE RESPONSE of the heart to overload is hypertrophy, which leads to changes in heart morphology and geometry. Cardiac hypertrophy is a major cause of heart failure and sudden death (23). To investigate the occurrence of hypertrophy in animal studies, pressure overload has been simulated using the transverse aortic constriction (TAC) model (8, 33). During the development of hypertrophy, the transcriptional activation of a number of genes increases dramatically leading to the reactivation of the fetal gene program (16). However, there is a lack of understanding of how the pathology of the disease progresses and of the molecular mechanisms involved. Consequently, in recent years a considerable attempt has been made to identify the genomic changes that underlie the development of TAC-induced hypertrophy (17, 40, 42).

Estrogen (E2) signaling is fundamental in many different tissues. The main actions of E2 are mediated by the classical estrogen receptors alpha and beta, which belong to the large family of ligand-activated transcription factors. Following binding, the ligand/receptor complex translocates to the nucleus to interact with specific DNA elements in the regulatory region of target genes resulting in the modulation of transcription. However, rapid E2 effects have also been identified that do not require transcriptional activation. Furthermore, E2 receptors can interact with other nuclear factors to signal downstream. These different modes of actions reflect the complexity of E2 physiology and the large range of the effects E2 exerts in target tissues.

In particular, a number of studies have shown that E2 can attenuate or inhibit the development of hypertrophy (2, 7, 29, 30, 38). Along this line, estrogen receptor beta (ERbeta) has been demonstrated to mediate the cardioprotective effects of E2 in pressure overload (10, 31, 34). However, the molecular pathways that may be modulated by the E2/ERbeta axis require further exploration, because they are not completely understood and may lead to new strategies with therapeutic potential. To address this issue, we embarked on a systematic investigation of the role of E2 and ERbeta in the genomic response of the heart to TAC-induced hypertrophy in female mice with intact ovaries. We used a public microarray dataset, and we offer novel insight into how the particular axis might confer protection in this disease.

METHODS

Microarray dataset. The deposited dataset with accession number (GEO:GSE18224) stemming from a 9 wk TAC model of male and female wild-type (WT) and ERbeta knockout (BERKO) mice (10) was employed.

Microarray data analysis. Raw CEL files of female WT and BERKO mice were imported into the statistical programming environment R (18, 32) version 2.8.1 for analysis with the Bioconductor packages (12) as described recently (37). Following background correction, expression data were normalized with the variance stabilization and normalization algorithm (15) and log2 transformed using the median polish algorithm of robust multiarray average (19). At this step, the complete dataset was subjected to nonspecific filtering with the only criterion that each feature should have an Entrez Gene ID annotation. Furthermore, control probes were removed and genes represented by more than one
controls (Fig. 1) reported recently (10). The female mice that were subjected to TAC-induced hypertrophy and their sham microarray data corresponding to female WT and BERKO

RESULTS AND DISCUSSION

Real-time RT-PCR analysis. To verify the modulation of candidate genes, real-time RT-PCR was performed. Total RNA was isolated as previously described (10) and 0.5 μg of total RNA was reverse transcribed with the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems) according to the manufacturer’s instructions. Quantitative real-time RT-PCR reactions were performed in 25 μl final volume using SYBR Green (Applied Biosystems) in a 7000 ABI Prism Instrument (Applied Biosystems). Reactions where RNA or reverse transcription had previously been omitted during reverse transcription were used as negative controls. The levels of all candidate genes were normalized to Hprt1 (housekeeping gene) mRNA levels. Primers were designed by the Primer3 software. Primer sequences and product sizes were:

- Actn1
- Fcer1g
- Itgb2
- Mmp2
- Hprt1
- Tarc
- Vav2

Statistical analysis. Quantitative real-time PCR data were analyzed by two-way ANOVA and Tukey’s post hoc test adjusting for multiple comparisons with the R version 2.11.0 software. Data are shown as mean ± SE. A value of P < 0.05 was considered statistically significant.

RESULTS AND DISCUSSION

Study design and dataset quality. We extracted the raw microarray data corresponding to female WT and BERKO mice subjected to TAC-induced hypertrophy and their sham controls (Fig. 1) reported recently (10). The female mice that were used had their ovaries intact. Hence, we were able here to perform a comprehensive analysis of the role of E2 signaling through ERbeta to identify the protective mechanisms associated with the E2/ERbeta axis in hypertrophy. As described previously, 9 wk following TAC, the development of pressure overload-induced hypertrophy was more pronounced in female BERKO compared with WT mice (10).

In addition to focusing on female mice only, the approach of the present analysis included the gene set enrichment analysis method (25, 36), which explores differential expression at the pathway level. This allowed us to make an integrative analysis of the microarray dataset with aim of obtaining novel findings.

In the first instance, we ensured adequate quality of the data. Quality metrics and graphical tools demonstrated consistently high quality for the current dataset (Fig. 2). Assessment of the median signal intensities revealed that these were similar among individual arrays (Fig. 2A). Hence, there were no outliers, neither due to biological nor to technical reasons. Furthermore, the median of the standard deviation of intensities, a significant quality parameter, did not show any trend, demonstrating the high quality of the data (Fig. 2B).

Single probe set differential expression. Following quality assessment, we analyzed the data for differences in the expression of individual probe sets between TAC and sham mice and we then compared these differences between the two genotypes, i.e., WT and BERKO. In WT mice, TAC led to a modulation of 555 probe sets in total (adjusted P = 0.05) compared with sham mice (Fig. 3A). On the other hand, the same surgical intervention had a larger effect on the genomic response of the heart to pressure overload in BERKO than in WT mice, as 1,287 probe sets were differentially expressed (adjusted P = 0.05) between the TAC and sham conditions (Fig. 3A). Nevertheless, the expression of 365 probe sets diverged between the two conditions in both genotypes (Fig. 3A). This demonstrates that these probe sets, ultimately genes,
are associated with the development of pressure overload-induced hypertrophy independent of ERbeta.

In addition, a remarkable novel observation, which was not made or discussed in the initial study of the microarray dataset, is that of all the probe sets differentially expressed solely in WT mice, 66% of these were repressed in TAC (Fig. 3C). On the other hand, ~75% of the probe sets modulated only in BERKO mice was induced in TAC (Fig. 3B). This indicates that the presence of ERbeta is essential for the strict control of the induction of numerous genes in pressure overload. This finding is of great importance and strongly suggests a regressive role of gene expression for ERbeta, as it is also in complete agreement with previous studies. In particular, it was shown that in mouse aortas treated with E2, of all the ERbeta-specific genes identified, 87% of them were repressed (28).

Among all genes that were differentially expressed between WT and BERKO mice in pressure overload, we identified Arhgap26, a GTPase-activating molecule, which was repressed in WT but induced in BERKO mice after TAC (Supplementary File). On the other hand, we found that there was a 40% decrease in the level of Corin in WT mice after TAC, which is involved in atrial natriuretic peptide hormone processing, while this gene was not regulated in BERKO mice (Supplementary File). It would therefore be very useful to uncover the biological processes that the identified genes are involved. In the Supplementary File, we have included the biological process term(s) of Gene Ontology (1) for each identified probe set. A significant number of genes are involved in processes such as cell death, regulation of cytoskeleton, and ion homeostasis.

In contrast to the previous analysis, here we performed hierarchical clustering of the identified probe sets. This approach revealed that the two genotypes have distinct transcrip-

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1 The online version of this article contains supplemental material.
tional profiles, as expected (Fig. 4, A and B). Of great importance is the fact that pressure overload had the major effect on cardiac gene expression in both genotypes, resulting into two separate clusters, i.e., TAC and sham (Fig. 4, A and B). Another observation of high interest is that the vast majority of modulated probe sets exhibited a relatively small fold change in their expression. Recently, we suggested that the heart is a highly regulated environment, where minor changes may have major effects (21). To this extent, small fold changes from E2 effects have been broadly reported (24, 28, 40, 41).

In conclusion, the absence of ERβ led to a dramatic number of modulated probe sets in response to pressure overload. This indicates that ERβ is required for the strict regulation of cardiac gene expression that may offer protection against the progression of hypertrophy.

Pathway analysis. After the data analysis on the individual probe set level, we explored the data further with the gene set enrichment analysis approach (25, 36), using the Kyoto Encyclopedia of Genes and Genomes (20). This ensured a more comprehensive and biologically integrative analysis of the data compared with the initial study (10), as we aimed at identifying entire pathways that responded to pressure overload differentially between the two genotypes. Similarly to single probe sets, the absence of ERβ led to a higher number of pathways modulated in response to pressure overload than in WT mice (Fig. 5A). In particular, 33 pathways \( P < 0.05 \) were modulated in TAC vs. sham BERKO mice, while 26 pathways \( P < 0.05 \) were modulated in WT mice in response to pressure overload (Fig. 5A). Equally, ERβ was critical for the suppression of whole pathway expression, as 62% of the pathways responding to pressure overload in WT mice were repressed, while only 42% of the identified pathways were repressed in BERKO mice (Supplementary File). It must also be noted that the present analysis identified previously unrelated pathways to the development of pressure overload-induced hypertrophy, which are discussed below. We believe that several of these pathways deserve further functional analysis to identify their possible role in hypertrophy and to determine whether their exploitation can lead to reversal of the disease.

Once the significantly modulated pathways have been identified, it is useful to assess how much overlap there is between them. Consequently, we calculated the overlap index of the identified pathways for each genotype (Fig. 5, B and C), which is a powerful graphical tool for the visualization of overlap among pathways. This revealed that there is some overlap between a few pathways, as expected. With the help of further statistical analysis, the pathways that were redundant or those that were selected due to their extensive overlap with truly modulated pathways can be excluded. The ultimate goal of this approach is to reduce the list of pathways. However, here we provide only an overview of the extent of overlapping pathways.

On the other hand, the mean plot is also a very useful graphical tool, which can lead to the rapid and accurate overview of the behavior of a pathway in a given condition. On this basis, we calculated mean plots for pathways of major relevance that were modulated in WT and/or BERKO mice in response to pressure overload (Fig. 6). As already observed, these plots demonstrate the small fold changes in cardiac gene expression due to pressure overload. Small fold changes have been commonly challenged in terms of relevance of their effect in a biological system. However, as it is broadly acknowledged, the overall shift in the expression pattern of a whole pathway is expected to have major effects on biological processes in health and disease.

When these results are combined together, it is clear that in both genotypes there is a shift in energy utilization from fatty acid to glucose metabolism after TAC (Fig. 6A and Supplementary File). Furthermore, there is a decrease in

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Fig. 4. Hierarchical clustering of differentially expressed probe sets. Hierarchical clustering of probe set expression (vertical) and surgical intervention, i.e., TAC or sham, of the RNA sample (horizontal) in WT (A) and BERKO (B) mice. Blue indicates low expression and yellow high expression. The colored horizontal side bar is used to annotate the RNA sources: dark green, WT sham; dark red, WT TAC; light green, BERKO sham; pink, BERKO TAC.
PPAR signaling in both genotypes following TAC (Supplementary File), and the reduction in the expression of genes comprising the citrate cycle (Supplementary File) indicates impaired mitochondrial function (3). In addition, the poor prognosis and outcome for BERKO mice might be reflected by the repression of pyruvate metabolism (Fig. 6D), which does not occur in WT mice in response to pressure overload. Taken together, these findings reveal a potentially important role of ERbeta in the modulation of mitochondrial bioenergetics and function.

At the same time, however, the presence of ERbeta was necessary for the decrease in the expression of genes involved in oxidative phosphorylation in response to pressure overload (Fig. 6B). It must be noted that a similar reduction...
in this biological process was described in mouse aortic tissue treated with E2, which was attributed to the effects of ERbeta (28). The negative effects of high levels of reactive oxygen species are well described. They can lead to oxidative stress, and they are mainly produced as a result of oxidative phosphorylation. Along this line, it has been suggested that the reduction in oxidative phosphorylation gene expression might decrease overall levels of cellular reactive oxygen species (28).

In addition, we identified in the present study that mice with their ovaries and ERbeta intact were able to decrease the expression of genes annotated at two pathways related to the activity of cytochrome P450 in response to pressure overload (Fig. 6C and Supplementary File). A similar reduction was not identified in mice lacking ERbeta. Of great relevance and importance, it has already been well described that cytochrome P450 generates reactive oxygen species as a product of drug and xenobiotic metabolism and possibly of other pathways (13). Furthermore, we propose that overall reduced reactive oxygen species may also contribute to prevent an increase in the inflammatory response associated with the development of hypertrophy. In line with our hypothesis, a recent study showed that reactive oxygen species generation causes NLRP3 inflammasome activation (44). Taken together, these results indicate another molecular mechanism by which the E2/ERbeta axis might confer protection in hypertrophy.

Moreover, in contrast to WT mice, BERKO mice seem to be going through apoptosis, as demonstrated by the increase in the p53 signaling pathway (Fig. 6E). Programmed cell death has been shown to be associated with the transition of hypertrophy to heart failure (26, 39). On the other hand, activation of ERbeta by specific agonists has demonstrated significant utility in models of noncardiac disease with an inflammatory component (6, 14). Based on this, it was suggested that one function of ERbeta may be to modulate the immune response and that ERbeta-selective ligands may be therapeutically useful agents to treat chronic inflamma-
Consequently, a mechanism that has been generally suspected to be involved in the protection of the E2/ERbeta axis in hypertrophy is the regulation of inflammation. Nevertheless, there has been scarce, if any, evidence concerning this association. To this extent, studies on the effects of E2 in the endothelium revealed that E2 improved endothelial function by attenuating recruitment and adhesion of leukocytes exerting an anti-inflammatory effect (5, 27).

Our current study, however, is the first to demonstrate that the deletion of ERbeta leads to an increased inflammatory response, as reflected by the induction in the natural killer cell-mediated cytotoxicity (Fig. 6F) and leukocyte transendothelial migration (Supplementary File) pathways in BERKO mice after TAC. This finding indicates that ERbeta may exert a direct regulatory action on the expression of inflammatory factors, thus modulating the inflammatory response. Among such candidates, there was a twofold increase in the Mmp2 gene levels and a 50% induction in the expression of Cd99, Tyrobp, an immune cell receptor-associated tyrosine kinase, and a subunit of the IgE Fc receptor, i.e., Fcer1g, which regulates several aspects of the immune response. To verify modulation of selected genes involved in inflammatory pathways, we performed quantitative real-time RT-PCR analysis. For this purpose, we analyzed genes that exhibited an induction of 40% or higher in the microarrays. Similarly to the microarray data analysis, we found that after TAC the expression levels of all selected genes were significantly higher in BERKO mice (Fig. 7, right column) but did not change in WT mice (Fig. 7, left column).

Increased inflammation is known to be detrimental to the heart (4, 9, 43). In particular, activation of the immune response and proinflammatory factors lead to the inhibition of cardiac contractility (4, 9). Impaired contractile function of the heart is a major risk factor for heart failure and sudden death. Therefore, during the development and progression of hypertrophy, ERbeta could be beneficial through the suppression of the associated inflammation thought to contribute to the pathogenesis of the disease.

Collectively, these data indicate the mechanisms that may be central for the protection of the heart in hypertrophy associated with E2 and ERbeta.

Conclusions

We conclude that ERbeta is crucial for the operation of protective mechanisms in hypertrophy, as it is necessary for the strict control of cardiac gene expression in this disease and it acquires the role of gatekeeper of the genomic response of the heart to pressure overload. In addition, we have identified pathways whose potential role in the development of hypertrophy has been previously unrecognized. Along these lines, we have elucidated pathways that may be central in the attenuation of the development and progression of cardiac hypertrophy mediated by the E2/ERbeta axis. Further exploration of their therapeutic potential is necessary.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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