Cardiac myocyte gene expression profiling during H₂O₂-induced apoptosis

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Many studies have examined the apoptotic mechanisms in cardiac myocytes. Oxidative stresses, whether directly applied in the form of H₂O₂ (12) or generated intracellularly by small molecules such as doxorubicin (21), diethyldithiocarbamate (34), or chelerythrine (45) stimulate apoptosis through the mitochondrial death pathway with release of cytochrome c from the mitochondria followed by cleavage and activation of caspases 9 and 3 (1, 3, 9, 12, 43, 45). However, concentrations of H₂O₂ that promote cardiac myocyte cell death activate intracellular signaling pathways. These include the extracellular signal-regulated kinases 1/2 (ERK1/2) (1, 11) and protein kinase B/Akt cascades (28), which are usually associated with the regulation of gene expression, protein synthesis and cytoprotection. Other signaling pathways such as the c-Jun NH2-terminal kinase and p38 mitogen-activated protein kinase cascade are also activated by oxidative stress (11) and probably regulate gene expression. This raises the question of whether changes in gene and protein expression are integral to the apoptotic process. We previously reported the changes in the cardiac myocyte gene expression profile induced by H₂O₂ over 2–4 h using Affymetrix Rat Genome U34A arrays (representing ~8,000 genes) (20). We expanded this study to examine changes using U34B arrays, which represent less well-characterized genes (A. Clerk and T. J. Kemp, unpublished data), and, here, we report the identification and characterization of a novel H₂O₂-responsive gene, putative peroxide-inducible transcript 1 gene (Perit1). Further analysis of the effects of H₂O₂ on the cardiac myocyte gene expression profile over 24 h using high-density Affymetrix Rat Genome 230 2.0 arrays (>31,000 sequences represented) emphasized that oxidative stress-induced cardiac myocyte apoptosis is associated with dynamic changes in gene expression. We propose that the expression of these genes and their protein products influences the cardiac myocyte apoptotic response.

METHODS

Cardiac myocyte culture. Primary cultures of neonatal ventricular myocytes from 2-day Sprague-Dawley rats were prepared as described (5, 17). In brief, ventricles were digested with collagenase (0.4 mg/ml) and pancreatin (0.6 mg/ml) in 116 mM NaCl, 20 mM HEPES (pH 7.35), 0.8 mM Na₂HPO₄, 5.6 mM glucose, 5.4 mM KCl, 0.8 mM MgSO₄. Cells were recovered by centrifugation (5 min, 60 g), resuspended in plating medium (68% (vol/vol) Dulbecco’s modified Eagle’s medium, 17% (vol/vol) M199, 10% (vol/vol) horse serum, 5% (vol/vol) fetal calf serum, 100 units/ml penicillin and streptomycin) and preplated on uncoated tissue culture dishes (37°C, 30 min) to remove nonmyocytes. The nonadherent cardiac myocytes were plated at 1.4 × 10⁴ cells/mm² on 60-mm Primaria culture dishes precoated in 1% (vol/vol) gelatin. After plating them in serum-containing medium for 18 h, we cultured myocytes in serum-free media for 24 h prior to experimentation.
RNA preparation and microarray analysis. Total RNA was extracted, and cRNA was synthesized from 10 μg total RNA and purified as described (20). To minimize the effects of variation between separate myocyte preparations, RNA from four independent experiments was pooled prior to cRNA synthesis. For studies with Affymetrix rat genome U34B arrays, we used the same samples as previously [two controls, 0.2 mM H₂O₂, 2 and 4 h (20)]. For studies with Affymetrix rat genome 230 2.0 arrays, three separate experiments were performed for each condition (i.e., 12 myocyte preparations in total) with two separate controls for each experiment. Fragmentation of antisense cRNA and hybridization to Affymetrix arrays were performed at the CSC Microarray Centre according to their protocol (http://microarray.csc.mrc.ac.uk). The MIAME-compliant data were exported to ArrayExpress (ArrayExpress ID E-MIRR-3).

For the identification of Perit1, the data were analyzed as previously described (20). For analysis of the Affymetrix 230 2.0 microarray data, hybridization data were generated by MicroArray Suite 5.0. Raw data were imported into GeneSpring 7.0 (Agilent Technologies) as tab-delimited text files. Log₁₀ values were used for subsequent analysis and values were set to a minimum of 0.01. The data were normalized per array (to the 50th percentile) and per gene (values in the treated samples were normalized to the mean of their corresponding controls). The error model was based on deviation from 1 (this assumes that most genes in the array will not change). A confidence filter was applied whereby genes were selected if present or marginal in all controls or all of any of the treatments. One-way nonparametric r-tests were performed for each selected transcript for each condition relative to the appropriate controls. The false discovery rate was set to <0.05, and multiple testing correction was performed with the Benjamini and Hochberg false discovery rate algorithm. Transcripts were filtered on the basis of fold stimulation >1.75. All genes were confirmed by basic local alignment search tool (BLAST) search and were correct as of 1st January 2006. Genes were classified as far as possible according to biochemical function using National Center for Biotechnology Information (NCBI) Entrez Gene (www.ncbi.nlm.nih.gov/entrez) and literature searches.

Elucidation of Perit1 gene structure, mRNA sequence, and predicted protein sequence. BLAST searches were used to identify additional expressed sequence tags (ESTs) and cDNA clones with sequence identity to A1044947 EST. Two rat ESTs, AW140640 and BF525204, contained additional sequence, and the combined sequence was used to identify a mouse cDNA clone (BG518521) with high homology. This was used to identify another rat cDNA clone (CB814496), and a full putative sequence (1,771 bases) was constructed. The putative translated product had 93% identity with the hypothetical mouse protein, C20orf111 homolog, and 85% identity with the human hypothetical protein C20ORF111 (i.e., open reading frame 111 on chromosome 20). From the full-length human mRNA sequence, additional rat ESTs were identified for the 5’-region (BF523769, AI576603) and used to construct the putative full-length rat mRNA. Overlapping primers (full details available on request) were designed for reverse transcriptase-polymerase chain reaction (RT-PCR) to amplify the rat mRNA from cardiac myocytes exposed to 0.2 mM H₂O₂ (4 h). PCR products were purified (Qiagen Quick PCR Purification Kit, Qiagen) and cycle sequenced (Advanced Biotechnology Centre, Imperial College London, UK). To obtain the sequence of the 5’-untranslated region, a gene-specific antisense primer (5’-AGTGAGTCTCTCCTGCCGACGAGACGCTGGG3’; 557 bp product) with the sense primer in the region deleted from the short form. To study expression of the short form of Perit1 an alternative forward primer was used with a reverse primer designed across the novel splice site (sense primer: 5’-CGGGAGAGACGCTGGG3’; antisense primer: 5’-GACATTTCCTTATGCGGACAGACGCTGGG3’; 293 bp product). The positions of these primers are shown in Fig. 1B. The expression of the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (Gapdh) was monitored (sense primer: 5’-ACCAAGTTCAGACATCCAGACGCTGGG3’; antisense primer: 5’-TCCACCACCTTGTGGTCTGTA3’; 452 bp product). PCR reactions were carried out in 50 μl containing 200 ng cDNA, 50 pmol of each primer, 20 mM (NH₄)₂SO₄, 75 mM Tris·HCl (pH 8.8 at 25°C), 1.5 mM MgCl₂, 0.01% (vol/vol) Tween 20, and 0.2 μM each of dATP, dCTP, dGTP, and dTTP, using 1.25U Taq polymerase. The following conditions were used: 95°C, 3 min followed by 21 (Gapdh), 27 (full-length Perit1) or 32 (short form Perit1) cycles of denaturation (95°C, 30 s), annealing (59°C, 30 s), and extension (72°C, 50 s). The resulting PCR products were analyzed by ethidium bromide-agarose gel electrophoresis, and the bands were captured under UV illumination. Bands were subjected to densitometric analysis and were normalized to Gapdh.

For quantitative PCR, reagents were from Applied Biosystems. Preoptimized primer and TaqMan probe mix were obtained for Kifs 4, 5, 9, and 10 (inventoried assay on demand; fluor = FAM) and were multiplexed with primers for 18S rRNA (fluor = VIC). Amplification [20 μl: 10 μl 2× TaqMan universal PCR mastermix (Applied Biosystems catalogue no. 4324018), 1 μl 20× primer and probe assay mix, 1 μl 18S primer and probe mix, 2 μl cDNA, 6 μl H₂O] involved heating (50°C, 2 min; 95°C, 10 min) followed by 40 cycles of 95°C (15 s) and 60°C (1 min). Reactions were duplicated, and products were detected with an ABI Prism 7700 sequence detection system. There was no detectable interference by genomic DNA (determined by omission of the reverse transcriptase step, data not shown). Analysis was with Sequence Detection Software (Applied Biosystems) and the level of expression of mRNA normalized to 18 S rRNA (User Bulletin # 2, Applied Biosystems).

Northern blotting. The probe was prepared from PCR products for full-length Perit1 (see above). Probes were labeled with [α-³²P]dCTP

Fig. 1. Concentration-dependent effects of H₂O₂ on cardiac myocyte apoptosis and protein synthesis. Cardiac myocytes were unstimulated (Control) or exposed to the indicated concentrations of H₂O₂. A: after 6 h, myocyte apoptosis was assessed by immunoblotting of extracts with antibodies specific for cleaved (i.e., activated) caspase 3 (~17 kDa). The experiment was repeated with similar results. B: the rate of protein synthesis was assessed by the incorporation of [1-³¹P]phenylalanine into myocyte protein over 2 h. Results are expressed relative to unstimulated controls and are means ± SE for 3 independent myocyte preparations.
by a random prime labeling system (Rediprime II, GE Healthcare Life Sciences). Unincorporated label was removed using G-50 columns (ProbeQuant, GE Healthcare Life Sciences). Radiolabeled probe [2–10 ng/ml ~ 10⁶ cpm] was added to Rapid-hyb buffer (GE Healthcare Life Sciences) and hybridized overnight at 65°C with rat multiple tissue northern blots of poly(A)+ RNA (RNWAY Laboratories, Korea). Blots were washed twice (20 min, 65°C) with 2X SSC (30 mM sodium citrate, 300 mM NaCl) containing 0.1% (wt/vol) SDS and subjected to autoradiography. Quantification was performed using Image master 1D prime version 3.0 (GE Healthcare Life Sciences).

*Immuno blot analysis.* Cardiac myocyte extracts were prepared and immunoblotted essentially as described (41). Proteins (15 μg) were separated using 15% polyacrylamide gels and blots were probed with antibodies to cleaved caspase 3 (Asp175) (Cell Signaling, 1:500 dilution). Primary antibodies were detected using goat anti-rabbit immunoglobulins coupled to horseradish peroxidase (Dako, 1:5,000 dilution), and bands were detected by enhanced chemiluminescence (Santa Cruz Biotechnology).

**Rate of protein synthesis.** Myocytes were incubated with [3H]phenylalanine (2 μCi/ml) in the absence or presence of H2O2 for 2 h (the assay is linear with respect to incorporation of [3H]phenylalanine over at least 4 h). Myocytes were washed with PBS (2× 1 ml, 4°C) and dissolved in 1 ml of NaOH (0.2 mM). A sample was taken for total protein assay by the Bio-Rad Bradford method. Bovine serum albumin (0.1 ml, 100 mg/ml) was added to the remaining sample, and proteins were precipitated with 5% (wt/vol) trichloroacetic acid (8 ml). Samples were centrifuged (2,500 g, 5 min, 4°C), and the precipitates were washed in 5% (wt/vol) trichloroacetic acid (3× 6 ml). The precipitates were dissolved in Soluene tissue solubilizer prior to liquid scintillation counting. Experiments were performed in duplicate, and the mean taken. Each experiment was repeated three times with independent myocyte preparations.

**RESULTS**

**Effects of H2O2 on protein synthesis and apoptosis in cardiac myocytes.** It is widely accepted that high concentrations of H2O2 or high levels of oxidative stress promote myocyte death, but some groups have reported that lower, nontoxic concentrations of H2O2 promote cytoprotection or growth (22, 35). We reexamined the concentration-dependent effects of H2O2 on apoptosis (assessed by cleavage of caspase 3) and on the rate of protein synthesis (as an index of hypertrophy) in cardiac myocytes. Consistent with our previous studies (12, 28), 0.2 or 0.5 mM H2O2 increased the rate of apoptosis in serum-starved myocytes (Fig. 1A), and concentrations >0.3 mM inhibited the rate of protein synthesis (Fig. 1B). However, lower levels of H2O2 (<0.1 mM) had no effect on caspase 3 cleavage and did not increase the rate of protein synthesis in our system.

**Identification and characterization of Perit1.** The changes in expression of ESTs induced by 0.2 mM H2O2 were examined not to increase the rate of protein synthesis in our system. With 0.2 mM H2O2, we identified significant (P < 0.05) changes in expression of 649 established genes (782 probe sets) and 187 RNAs corresponding to no established gene at 2, 4, and/or 24 h (Fig. 3A; online supplement Tables S1–S13). Approximately 69% of these were upregulated. Perit1 was represented on the 230 2.0 arrays (probe set 1374911_at) although the fold stimulation of this gene was less than that detected on the U34B arrays (online supplement Table S12: 1.74-fold at 2 h; 1.92-fold at 4 h). This may reflect differential coverage of the mRNA by the probe sets on the U34B vs. 230 2.0 arrays. Of the probe sets corresponding to no established gene at 2, 4, and/or 24 h (Fig. 3A; online supplement Tables S1–S13). Approximately 69% of these were upregulated. Perit1 was represented on the 230 2.0 arrays (probe set 1374911_at) although the fold stimulation of this gene was less than that detected on the U34B arrays (online supplement Table S12: 1.74-fold at 2 h; 1.92-fold at 4 h). This may reflect differential coverage of the mRNA by the probe sets on the U34B vs. 230 2.0 arrays. Of the probe sets corresponding to no established gene at 2, 4, and/or 24 h (Fig. 3A; online supplement Tables S1–S13). Approximately 69% of these were upregulated. Perit1 was represented on the 230 2.0 arrays (probe set 1374911_at) although the fold stimulation of this gene was less than that detected on the U34B arrays (online supplement Table S12: 1.74-fold at 2 h; 1.92-fold at 4 h). This may reflect differential coverage of the mRNA by the probe sets on the U34B vs. 230 2.0 arrays. Of the probe sets corresponding to no established gene at 2, 4, and/or 24 h (Fig. 3A; online supplement Tables S1–S13). Approximately 69% of these were upregulated. Perit1 was represented on the 230 2.0 arrays (probe set 1374911_at) although the fold stimulation of this gene was less than that detected on the U34B arrays (online supplement Table S12: 1.74-fold at 2 h; 1.92-fold at 4 h). This may reflect differential coverage of the mRNA by the probe sets on the U34B vs. 230 2.0 arrays. Of the probe sets corresponding to no established gene at 2, 4, and/or 24 h (Fig. 3A; online supplement Tables S1–S13). Approximately 69% of these were upregulated. Perit1 was represented on the 230 2.0 arrays (probe set 1374911_at) although the fold stimulation of this gene was less than that detected on the U34B arrays (online supplement Table S12: 1.74-fold at 2 h; 1.92-fold at 4 h). This may reflect differential coverage of the mRNA by the probe sets on the U34B vs. 230 2.0 arrays. Of the probe sets corresponding to no established gene at 2, 4, and/or 24 h (Fig. 3A; online supplement Tables S1–S13). Approximately 69% of these were upregulated. Perit1 was represented on the 230 2.0 arrays (probe set 1374911_at) although the fold stimulation of this gene was less than that detected on the U34B arrays (online supplement Table S12: 1.74-fold at 2 h; 1.92-fold at 4 h).
gene, 115 localized to predicted genes/hypothetical proteins on the rat and/or mouse genome, but 72 corresponded to no gene whatsoever (online supplement Table S13). Consistent with our previous study (20), 0.04 mM H2O2 promoted significant changes in expression of very few genes (32 RNAs, 37 probe sets), the expression of each of which was upregulated or downregulated to a similar or greater extent by 0.2 mM H2O2 (Fig. 3B).

The genes were classified as far as possible according to known or probable function (Fig. 4A, online supplement Tables S1–S13). The general significance of the groupings is considered below (see DISCUSSION), but two families appeared to be particularly responsive to H2O2: the Kru¨ ppel-like transcription factors (Klf, online supplement Table S1) and dual-specificity phosphatases (Dusp, online supplement Table S2). Since little is known of the expression of Klfs in cardiac myocytes, we focused on specific Klfs for validation of the data. From the microarrays, Klf4, Klf5, and Klf10 were upregulated in cardiac myocytes exposed to 0.2 mM H2O2, but to differing extents (Klf4/H2O2 Klf5/H2O2 Klf10, Fig. 4B). Klf9 was also represented on the arrays (probe sets 1370209_at, 1387882_at) and was expressed in cardiac myocytes, but there was no significant change in expression at 2, 4, or 24 h in response to 0.2 mM H2O2 (data not shown). Using quantitative PCR, we confirmed the upregulation of Klf4, Klf5, and Klf10, and the absence of any change in expression of Klf9 (Fig. 4C). We also demonstrated that,
whereas Klf4 and Klf10 were upregulated within 1 h of stimulation of 0.2 mM H$_2$O$_2$, Klf5 was maximally upregulated at 2 h.

The RNAs with significant changes in expression in cells exposed to 0.2 mM H$_2$O$_2$ clustered into six groups with K-means clustering, essentially according to the time at which expression changed (Fig. 5, A and B). Thus, set 1 (117 probe sets) represented genes with maximal downregulation at 24 h, with lesser downregulation at 2–4 h; set 2 (178 probe sets) represented genes with maximal downregulation at 2–4 h; set 3 (183 probe sets) represented genes that were upregulated at 2, 4, and 24 h; set 4 (177 probe sets) represented genes that were upregulated primarily at 2 h; set 5 (190 probe sets) represented genes that were upregulated at 2 and 4 h, but not 24 h; set 6 (133 probe sets) represented genes that were upregulated primarily at 2 h with a significant decrease by 4 h. The differences in time courses for gene expression were further highlighted by comparing the levels of stimulation of individual genes in the whole group at 2 and 24 h (Fig. 5C). Thus, 275 probe sets showed an absolute change (i.e., unregulated or downregulated) of >1.75-fold at 2 h relative to 24 h, and 26 probe sets showed an absolute change of >1.75-fold at 24 h relative to 2 h. Examples of different time courses of gene expression are shown in the inset panels in Fig. 5C. Whereas, for some genes, the changes in expression persisted throughout the 2- to 24-h time period [e.g., ATP-binding cassette, subfamily B (MDR/TAP), member 1, Abcb1], for others, the changes occurred primarily at 2 h [e.g., early growth response-1 (Egr1)], at 2–4 h [e.g., G0/G1 switch gene 2 (G0s2)], or at 24 h (e.g., epoxide hydrolase, Ephx1).

DISCUSSION

Gene expression in oxidative stress-induced cardiac myocyte apoptosis. It is now well-recognized that specific cell death machinery is activated during apoptosis and, in cardiac myocytes, oxidative stress activates the mitochondrial death pathway (1, 3, 9, 12, 43, 45). This commitment to a cell death program is probably influenced by the genes and proteins expressed in the myocyte prior to and during the insult. Because oxidative stress is associated with the activation of intracellular signaling pathways known to influence gene and protein expression, we hypothesized that cardiac myocyte apoptosis is associated with such changes that influence the response. Consistent with this, 0.2 mM H$_2$O$_2$ did indeed induce substantial changes in gene expression (Fig. 3A, online sup-
plement Tables S1–S13), and, at 2–4 h, these were approximately equivalent in number (though different in identity) to those we detect with a potent hypertrophic stimulus, endothelin-1 (A. Clerk, T. J. Kemp, and P. H. Sugden, unpublished data). Over 24 h, the gene expression profile changed further (Fig. 5), presumably a consequence of the changes in expression of transcriptional regulators at 2–4 h (online supplement Table S1). By 16–24 h, 25–50% of neonatal rat cardiac myocytes exposed to 0.1–0.2 mM H$_2$O$_2$ are terminal deoxynucleotidyl dUTP nick-end labeling positive (12), indicative of DNA fragmentation and a relatively late stage of the apoptotic response. The changes in gene expression at 24 h are therefore likely to be associated with surviving cells and could confer protection or simply reflect a particular stage of apoptosis in these cells.

Although high levels of oxidative stress promote cardiac myocyte death, the significance of low levels is unclear. The phenomenon of ischemic preconditioning, which may be mediated by low levels of oxidative stress, has led to the proposal that low levels of oxidative stress per se are cytoprotective. Although some studies support this (36, 38, 40, 42), other effects of the preconditioning ischemia (e.g., changes in pH and calcium fluxes) potentially also participate. In other cells, generation of intracellular oxidative stress may facilitate cell proliferation and growth (4, 15), and some studies in cardiac myocytes suggest that low concentrations of H$_2$O$_2$ promote growth (7, 22, 39). Evidence has also been presented that a number of agonists (angiotensin II, endothelin-1, α-adrenergic agonists) or mechanical strain that promote cardiac myocyte hypertrophy achieve this by inducing intracellular oxidative stress (2, 8, 26, 30, 37). Here, we identified changes in only a few genes in cardiac myocytes exposed to a low, nontoxic concentration of H$_2$O$_2$ (0.04 mM, Fig. 3B), and we have failed to detect any increase in the rate of protein synthesis (Fig. 1B) or accumulation of total protein (A. Clerk and P. H. Sugden, unpublished data) in myocytes exposed to low concentrations of H$_2$O$_2$ (<0.1 mM). This contrasts with genuine hypertrophic stimuli (e.g., endothelin-1 or phenylephrine) that promote changes in expression of hundreds of genes (A. Clerk, T. J. Kemp, and P. H. Sugden, unpublished data) and increase the rate of protein synthesis (27). However, it should be noted that our study was directed toward the effects of global oxidative stress as occurs during, for example, myocardial infarction. It is possible that local production of intracellular reactive oxygen species [e.g., resulting from NADP(H) oxidase activity] may have growth-promoting effects.

**Classification of identified genes: function in cardiac myocyte apoptosis?** Given the advanced stage of sequencing for the rat and mouse genomes, it seems surprising that ~20% of RNAs that we detected with significant changes in expression could not be localized to established genes, and many did not localize to any hypothetical gene/protein on the rat or mouse genome. Classically, proteins are considered the fundamental regulatory components of cell function, but an increasing...
number of nonprotein-coding RNAs appear to regulate mRNA and protein expression. Thus, natural antisense RNAs (23) and microRNAs (29) may regulate the accumulation and translation of specific mRNAs. The sequences for the 72 RNAs with no associated gene may lie in this category of nonprotein-coding gene, and one additional probe set (1382882_x_at) corresponds to an established nonprotein-coding RNA identified in brain (online supplement Table S12). Even of the mRNAs with established protein-coding genes, many have no established function (online supplement Table S12) and, whereas 13 are associated with cell death (online supplement Table S11), their function is not understood. We therefore have no understanding of >30% of the RNAs we identified, highlighting our lack of fundamental knowledge of the regulation of cardiac myocyte responses. In this context, it is extremely difficult to assess the contribution of an individual gene in the global response, although consideration of the types of genes that are altered can provide an overview of the cardiac myocyte response.

A large number of genes that we identified were associated with transcriptional regulation or intracellular signaling (Fig. 4A, online supplement Tables S1 and S2) presumably to elicit the global myocyte response. It was of particular interest that several dual-specificity phosphatases were upregulated since these may antagonize signaling through mitogen-activated protein kinases (ERK1/2, c-Jun NH2-terminal kinases, and p38 mitogen-activated protein kinases) (14) activated by H2O2 in cardiac myocytes (11). The changes in expression of genes...
encoding metabolic enzymes with, as might be expected, an increase in a number of genes associated with cellular antioxidants (online supplement Table S3) and in genes that would influence ion fluxes in the cell (online supplement Table S5) indicate substantial alterations in the internal environment presumably to "manage" the cellular stress and maintain cell function. Since 0.2 mM H₂O₂ is expected to cause cellular damage, it was not surprising to detect increased expression of genes encoding chaperones to refold damaged proteins (e.g., Bag3, Hsp70, Hsp40) and enzymes associated with the ubiquitin proteolysis system (ubiquitin conjugating enzymes; ubiquitin-specific proteases) to degrade proteins damaged beyond repair (online supplement Table S4). We also detected changes in genes associated with DNA structure, management, and repair (online supplement Table S7) that may represent an attempt to maintain the structure of the DNA (e.g., by upregulating histone expression) but that may alternatively facilitate DNA fragmentation during the later phases of apoptosis. Changes in expression of genes/proteins that regulate RNA synthesis and translation (online supplement Table S8) were also detected. Surprisingly, we detected changes in expression of relatively few genes encoding components of the myofibrillar apparatus or internal cytoskeleton (online supplement Table S9), suggesting that this was not a priority for cardiac myocytes in this situation.

Overall, a global picture emerges of cardiac myocytes actively responding to oxidative stress by changes in metabolism and ion fluxes, management of the increased oxidative stress, and repair of internal damage. These potentially enable the myocyte to maintain function as far as possible. A crucial aspect of this function is probably to maintain energy production that is not only required to maintain contractility, but also to ensure that cell death is regulated rather than necrotic, which, in vivo, would cause substantial global damage to the heart. In addition to regulation of the internal cellular environment, we detected significant changes in genes encoding cell surface receptors and receptor agonists (online supplement Table S6), cell adhesion molecules and extracellular matrix (on-line supplement Table S10), plus enzymes that generate extracellular matrix components (e.g., hyaluronan synthase 2, online supplement Table S3). Thus, following exposure to H₂O₂, cardiac myocytes not only alter their responsiveness to their immediate environment but may also influence cells in their vicinity and the extracellular matrix around them. This suggests that the myocytes themselves potentiate remodeling of the heart.

In other cells, it is clear that gene and protein expression does not always correlate (16) and activation of ERK1/2 and/or protein kinase B/Akt can influence the rate of translation of specific mRNAs (32). Further studies are clearly required to determine which transcripts are actively translated and the mechanisms involved. However, it should be borne in mind that any transcripts that are not translated immediately are clearly not necessarily degraded and are potentially available for translation as the cell recovers.

Identification of novel genes: Perit1. As discussed above, ~20% of RNAs detected were associated with no established gene, and much work remains to be done in this area. When we initiated this study, the rat Perit1 gene had not been predicted, the human gene was known only as an open reading frame (C20ORF111), and the mouse ortholog of this had been predicted. Here, we report that this is a bona fide gene with high homology between rat, mouse, and human, which generates a protein-coding mRNA, is upregulated by H₂O₂, and exists as two alternatively spliced isoforms (Fig. 2). Orthologs are present in other genomes including orangutans (CAH90349, 89% homology with the rat protein), cow (AA110227, 90% homology with rat protein), Rhesus monkeys (AAZ81015, 91% homology with rat protein), chicken (NP_001025152 XP_417380, 73% homology with rat protein), and Xenopus (AAH61626, 69% homology with rat protein). The conservation between species and the broad tissue distribution (Fig. 2E) suggest that Perit1 plays an important role in cellular responses. The Osr1/Perit1 gene (GeneID: 296346) has been classified as a serine-type endopeptidase on the basis of a study by Puente and Lopez-Otin (31). However, the basis of this is not clear to us since there is no obvious protease domain (the only conserved protein domain is a DUF776 motif of unknown function) and we cannot find any mention of Perit1 or a protein of equivalent sequence in any of the data provided. Further studies of Perit1 are required to determine its function in cardiac myocyte apoptosis.

Other studies of oxidative stress-induced changes in gene expression. It is currently difficult to compare published data from different microarray studies, partly because of the different platforms used (e.g., oligonucleotide arrays vs. cDNA arrays). For example, some studies use custom DNA microarrays, and it is difficult to know whether a gene we identified with the Affymetrix arrays was represented on the DNA array and whether the sequence could detect a specific alternatively spliced product. Nevertheless, DNA arrays have been used to demonstrate that H₂O₂ or cigarette smoke upregulates expression of Mdm2, Mkp1 (dual specificity phosphatase 1, Dusp1), Hsp40 (Dnajb1), Hsp70, and glutaredoxin (46) consistent with effects of H₂O₂ in cardiac myocytes. As can be seen from even this small list, another problem encountered when comparing microarray data is that the genes may be renamed. Even with a single commercial platform, the content varies according the array. This is highlighted by comparing our data here using high-density Affymetrix rat genome 230 2.0 arrays with those from a previous study on the effects of H₂O₂ on cardiac myocyte gene expression using U34A arrays (20). Of the 22 upregulated genes that we identified previously, all but three were identified in this study. Two genes (superoxide dismutase 1 and dynorphin) were represented by extremely short sequences (32 and 36 bp) on the U34B arrays, and the newly designed probe sets on the 230 2.0 arrays covering a longer sequence showed no change (superoxide dismutase 1) or showed great variability with some “absence” calls resulting in lack of selection (dynorphin). For the third gene (glutathione synthetase), the new probe sets also showed great variability between the three experiments. Of the 27 downregulated genes previously identified, only five were identified as downregulated in this study. Of the rest, nine were not represented on the 230 2.0 arrays, and the remaining 13 had newly designed probe sets. Of these 13, five were called “absent” in some controls and were therefore excluded, and the rest showed variability in the response. Overall, allowing for the different probe sets on the arrays, the data in the two studies, particularly for upregulated gene expression, are reasonably consistent.

Further difficulties arise when comparing data with studies in other cells by other groups that have all used varying
conditions in either the concentration and/or duration of exposure to H$_2$O$_2$. Probably the most closely related published study of effects of H$_2$O$_2$ and gene expression profiling used PC-12-D$_2$R cells exposed to 0.2 mM H$_2$O$_2$ for 1 h with Affymetrix U34 arrays (25). Of the 10 genes reported for H$_2$O$_2$ in PC12 cells, all but one were also identified in our study: Eg1, Mk1p, c-Jun, Pc3 (B-cell translocation gene 2, Btg2), Copeb (Klf6), Al639167 (probe set 1375374_at), c-fos, RhoB, and Hsp70. Other studies are less comparable with ours with respect to methodology. For example, in a study of human breast cancer MCF7 cells exposed to a 30-min pulse of 0.1 mM H$_2$O$_2$, using Affymetrix human U133a arrays, fewer genes were identified than in our study (either because of reduced numbers of probe sets on the arrays and/or the pulsed nature of the stress) (10). Of the 33 genes with clear identities, 21 were detected in our study, including p21$^{	ext{Cip1/Waf1}}$, Btg2, Mdm2, and prostate differentiation factor (Gdf15). It is not clear if the absence of the other 12 from our list reflects a difference in duration of the stimulus or the cells or may be a technicality because of the different arrays and species used. In relation to this last point, it should be noted that, during 2005, we found a surprisingly large number of the Affymetrix annotations of their probe sets to be incorrect (unpublished data), and others suggest that the proportion of discrepancies, particularly in older literature, could be much higher (30–50%) (13). For probe sets for which there is no known identity at the time of publication, the problem is exacerbated. For Peri1, the rat sequence and annotation were released in January 2004, and it was represented on the Affymetrix U34B arrays (for which few people have published data) as an EST. However, the human ortholog C20ORF111 was represented on Affymetrix human arrays and is upregulated in colorectal carcinoma cells exposed to a lipid peroxidation product, 4-hydroxy-2-nonenal (44). Of 103 established genes identified in the carcinoma cells, 28 were also identified in our study of cardiac myocytes exposed to H$_2$O$_2$. As more studies are published, and the full data-sets become available for further analysis, it should become possible to distinguish core responses of any cell to oxidative stress from the response to a specific stressor or of a specific cell.

Relevance of the neonatal cardiac myocyte model. There is continued debate about the most relevant experimental model for the study of cardiac myocyte responses. Ideally, human cardiac myocytes should be used, but the terminally differentiated nature of the cells makes this impossible. For this type of study of the temporal regulation gene expression, it is essential to use a well-characterized, robust system in which the cells can be synchronized with respect to response (by serum starvation) and exhibit appropriate phenotypic changes. The only cells that meet these criteria for cardiac myocytes are primary cultures of rat neonatal ventricular myocytes, which we have used here. Although these cells are from a neonatal animal, the intracellular signaling pathways that we have studied are operative in adult myocytes and in isolated perfused hearts, and, where it has been possible to compare the systems, we have detected few differences.

Other groups suggest that adult myocytes are more representative of the adult phenotype and should be used preferentially and/or propose that the only relevant experimental systems are in vivo animal models. Adult rat or mouse ventricular myocytes can be cultured for ~24 h at best with 25–50% loss of viability over this time (6, 47). They also dedifferentiate in longer term culture (suggesting that there are fundamental phenotypic changes even at early times), they cannot be cultured at confluence with the intercellular contacts and communication channels that occur in the heart, and they do not beat spontaneously (as neonatal myocytes do). It should also be noted that the isolation procedure for adult myocytes activates stress-responsive signaling pathways (A. Clerk and P. H. Sugden, unpublished data) that would be expected to modulate gene expression. In any whole heart system (e.g., isolated perfused hearts), nonmyocytes (e.g., endothelial cells, fibroblasts) constitute up to 70% of the total cell number in the heart. Until the likely response of the cardiac myocyte is established, it will be difficult to dissect the contribution of different cell types and the impact of a multicellular system. Nevertheless, it will be important in future studies to determine whether acute changes in gene expression that we detect in neonatal myocytes occur in whole heart preparations and how great an impact nonmyocytes may have on the cardiac myocyte response.

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