Cardiac myocyte gene expression profiling during H$_2$O$_2$-induced apoptosis

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Clerk A, Kemp TJ, Zoumpoulidou G, Sugden PH. Cardiac myocyte gene expression profiling during H$_2$O$_2$-induced apoptosis. Physiol Genomics 29: 118–127, 2007. First published December 5, 2006; doi:10.1152/physiolgenomics.00168.2006.—High levels of oxidative stress promote cardiac myocyte death, though lower levels are potentially cytotoxic/apoptotic. We examined the changes in gene expression in rat neonatal cardiac myocytes exposed to apoptotic (0.2 mM) or nontoxic (0.04 mM) concentrations of H$_2$O$_2$ (2, 4, or 24 h) using Affymetrix microarrays. Using U34B arrays, we identified a ubiquitously expressed, novel H$_2$O$_2$-responsive gene [putative peroxide-inducible transcript 1 (Perit1)], which generates two alternatively spliced transcripts. Using 230 2.0 arrays, H$_2$O$_2$ (0.04 mM) promoted significant changes in expression of only 32 genes, all of which were seen with 0.2 mM H$_2$O$_2$. We failed to detect any increase in the rate of protein synthesis in cardiac myocytes exposed to <0.1 mM H$_2$O$_2$, further suggesting that global, low concentrations of H$_2$O$_2$ are not anabolic in this system. H$_2$O$_2$ (0.2 mM) promoted significant (P $<$ 0.05, >1.75-fold) changes in expression of 649 mRNAs and 187 RNAs corresponding to no established gene. Of the mRNAs, 114 encoded transcriptional regulators including Krüppel-like factors (Klf's). Quantitative PCR independently verified the changes in Klf expression. Thus, H$_2$O$_2$-induced cardiac myocyte apoptosis is associated with dynamic changes in gene expression. The expression of these genes and their protein products potentially influences the progression of the apoptotic response.

Microarrays; Krüppel-like factors; Perit1; oxidative stress

Contractile cardiac myocytes in the heart withdraw from the cell cycle in the perinatal period and become terminally differentiated. Although recent evidence indicates that stem cells (either resident in the heart or from the circulation) can be recruited to repair and/or regenerate the heart (24), such endogenous repair mechanisms are insufficient to maintain cardiac function. Much attention has therefore focused on the role of apoptosis and/or necrosis of contractile cardiac myocytes in the development of cardiac pathologies (19). One of the principal pathophysiological insults encountered by cardiac myocytes is oxidative stress. Neonatal or adult ventricular myocytes exposed to H$_2$O$_2$ (as a physiologically relevant and easily manipulatable form of oxidative stress) (1, 3, 12, 43) and adult myocytes subjected to hypoxia/reoxygenation (18) undergo apoptosis or necrosis. Whereas concentrations of H$_2$O$_2$ >0.1 mM promote cardiac myocyte apoptosis, at high concentrations (>1 mM) the process becomes unregulated and myocyte death becomes necrotic (22). In contrast, low levels of H$_2$O$_2$ or other forms of oxidative stress may be involved in the cytotoxic protection afforded by ischemic preconditioning (36, 38, 40, 42). Indeed, some groups report that low concentrations of H$_2$O$_2$ (7, 22, 39) or low levels of alternative oxidative stresses (33) may even promote cardiac myocyte growth (e.g., protein synthesis/accumulation).

Many studies have examined the apoptotic mechanisms in cardiac myocytes. Oxidative stresses, whether directly applied in the form of H$_2$O$_2$ (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 H$_2$O$_2$ 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 NH$_2$-terminal kinase and p38 mitogen-activated protein kinase cascades 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 H$_2$O$_2$ 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 H$_2$O$_2$-responsive gene, putative peroxide-inducible transcript 1 gene (Perit1). Further analysis of the effects of H$_2$O$_2$ 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 Na$_2$HPO$_4$, 5.6 mM glucose, 5.4 mM KCl, 0.8 mM MgSO$_4$. 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 $\times$ 10$^4$ cells/mm$^2$ 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-MIMR-3).

For the identification of Peri1, 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 t-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 Peri1 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 AI044947 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 (Qiaglick 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′-AGTGACTGTGAAGCTCCCGT-3′) was designed for 5′-rapid amplification of cDNA ends (RACE), and the products were sequenced using the antisense primer 5′-GCTCTTGTACTCTATTTAGT-3′.

Semiquantitative RT-PCR and quantitative PCR. Ratiometric RT-PCR was performed as previously described (20). Primers were designed for the study of full-length Peri1 (sense primer: 5′-CAGCAGAAGGACGAGGATCAGC-3′; antisense primer: 5′-CAGCAGAACTGTGTAGTTG-3′; 557 bp product) with the sense primer in the region deleted from the short form. To study expression of the short form of Peri1 an alternative forward primer was used with a reverse primer designed across the novel splice site (sense primer: 5′-CGCGGAGAGCCCTGG-3′; antisense primer: 5′-GACATTTCCTTATCAGTCCAC-3′; 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′-ACCAAGCTTCCAGCACTAC-3′; antisense primer: 5′-TCCACACACCTGTTGCTGTA-3′; 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 mM each of dATP, dCTP, dGTP, dTTP, using 1.25U Taq polymerase. The following conditions were used: 95°C, 3 min followed by 21 (Gapdh), 27 (full-length Peri1) or 32 (short form Peri1) 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 detector. 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 Peri1 (see above). Probes were labeled with [α-32P]dCTP by 10.220.33.4 on June 20, 2017 http://physiolgenomics.physiology.org/ Downloaded from
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 2× 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).

**Immunoblot 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 [l-³H]phenylalanine (2 μCi/ml) in the absence or presence of H₂O₂ for 2 h (the assay is linear with respect to incorporation of [l-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.

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**Identification and characterization of Perit1.** The changes in expression of ESTs induced by 0.2 mM H₂O₂ were examined using U34B arrays. The expression of AI044947 (corresponding to a 432-bp rat EST) was increased 3.2-fold and 3.6-fold at 2 and 4 h, respectively. The gene corresponding to AI044947 was elucidated in silico, the cDNA sequence was cloned, and the mRNA coding sequence of the gene was confirmed by sequencing of overlapping PCR and RACE products. The sequence was submitted to NCBI (accession no. AI621831) originally as oxidative-stress-responsive 1 (Osr1), but this was changed to putative peroxide-inducible transcript 1 (Perit1) to avoid confusion with other genes abbreviated to Osr1 (Odd-skipped related 1; oxidative stress-responsive kinase 1). Rat Perit1 is on chromosome 3, with human and mouse orthologs on chromosomes 20 (C20orf111) and 2 (C20orf111 homolog), respectively. The rat gene comprises four exons across ~23 kb (Fig. 2A) with a putative initiation codon in exon 2 and polyadenylation signal in exon 4. The full-length 1,571-base mRNA contains an 873-base open reading frame encoding a 291-amino acid protein (Fig. 2B). One clone represented an alternatively spliced form (908-base mRNA, 213-base open reading frame, 71-amino acid protein) in which an alternative donor site in exon 3 was spliced to an alternative acceptor site in exon 4 (Fig. 2, A and B). The increase in expression of full-length Perit1 was confirmed by semiquantitative RT-PCR (using a primer to a region not present in the short form) and was dependent on the concentration of H₂O₂ (maximal effective concentrations 0.1–0.3 mM, Fig. 2C) and time (maximal expression at 1–4 h, Fig. 2D). To confirm that the short form represented a bona fide gene product, RT-PCR was performed using a primer designed across the exon 3/4 splice site. The expression of the smaller mRNA was upregulated in cardiac myocytes exposed to 0.2 mM H₂O₂ in a similar manner to the originally identified mRNA, with maximal expression at 2–4 h (Fig. 2D). Full-length Perit1 mRNA was detected in all tissues studied (Northern blotting) as a band of ~1.5 kb with high levels of expression in testis and placenta (Fig. 2E). A smaller band of ~1.0 kb was also detected in testis and un pregnant uterus, presumably corresponding to the smaller gene product we detected in cardiac myocytes exposed to H₂O₂. Thus, Perit1 represents a novel gene that generates at least two genuine mRNAs in cardiac myocytes and other cells and that is responsive to H₂O₂.

**Global effects of H₂O₂ on the cardiac myocyte gene expression profile.** Since oxidative stress is increasingly implicated as a signaling intermediate that may promote cell growth (35), it seemed surprising that [in our studies with U34A and U34B arrays (20)] 0.04 mM H₂O₂ did not induce substantial changes in gene expression. This may have reflected the limited nature of the arrays we used and/or (because we used single samples) the relatively high filter on the degree of change (≥2.53 fold) that was necessary to have confidence in the data. We therefore undertook a larger study of the effects of toxic (0.2 mM) and nontoxic (0.04 mM) concentrations of H₂O₂ on the cardiac myocyte gene expression profile using the 230 2.0 arrays (n = 3). Variation of up to 1.25-fold was detected in control genes, so, to retain confidence in the data, genes were selected with >1.75-fold change in expression. Although Affymetrix annotate the probe sets, some annotations are incorrect, and all sequences were confirmed by BLAST search. Some genes were represented by multiple probe sets [e.g., nuclear factor IA (online supplement Table S1) is represented by four probe sets; the online version of this article contains supplemental material]. In these cases, the changes in expression detected by the different probe sets were largely similar.

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, 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 Krüppel-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 Klf in cardiac myocytes, we focused on specific Klf 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/H11022 Klf5/H11022 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 4 h; set 5 (190 probe sets) represented genes that were upregulated at 2 and 4 h, but not 24 h; set 6 (123 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., upregulated 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 approxi-
mately equivalent in number (though different in identity) to
those we detect with a potent hypertrophic stimulus, endothe-
ilin-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 expres-
sion 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 H2O2 are terminal deoxy-
nucleotidyl 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 me-
diated by low levels of oxidative stress, has led to the proposal
that low levels of oxidative stress per se are cytotoxic.
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 H2O2 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 H2O2 (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 H2O2 (<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 oxy-
gen species [e.g., resulting from NADP(H) oxidase activity]
may have growth-promoting effects.

Classification of identified genes: function in cardiac myo-
cyte 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 (AAI10227, 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, Mkpl (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: Egr1, Mkp1, c-Jun, Pc3 (B-cell translocation gene 2, Btg2), Copeb (Klf6), Alf639167 (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$_2$, other studies 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 p21Cip1/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 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 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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