Gene Expression of Antioxidative Enzymes in the Human Heart

Increased Expression of Catalase in the End-Stage Failing Heart

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Background—An increase in oxidative stress is suggested to be intimately involved in the pathogenesis of heart failure. However, gene expression of enzymes that metabolize reactive oxygen metabolites has not been investigated in the human heart.

Methods and Results—Myocardial tissue homogenates of the left ventricular wall from hearts in end-stage failure due to dilated (DCM) or ischemic (ICM) cardiomyopathy (n = 12 each), as well as from nonfailing donor hearts (n = 12), were analyzed for mRNA levels of manganese superoxide dismutase (MnSOD), copper-zinc superoxide dismutase (CuZnSOD), glutathione peroxidase (GPX), and catalase by Northern blot analyses. Protein levels of MnSOD, CuZnSOD, and catalase were determined by Western blot or ELISA. MnSOD, CuZnSOD, and GPX mRNA levels were similar in all 3 groups. In contrast, catalase mRNA levels were found to be increased by 123 ± 23% in DCM hearts and by 93 ± 10% in ICM hearts (P < 0.01 each) compared with control hearts. Likewise, catalase protein levels were found to be increased in failing hearts (DCM by 90 ± 10%, ICM by 90 ± 13%; P < 0.05 each) compared with control hearts. In addition, the observed upregulation of catalase mRNA and protein in failing hearts was attended by an increased catalase enzyme activity (DCM by 124 ± 16%, ICM by 117 ± 15%; P < 0.01 each), whereas MnSOD, CuZnSOD, and GPX enzyme activity levels were unchanged in failing compared with nonfailing myocardium.

Conclusions—Increased oxidative stress in human end-stage heart failure may result in a specific upregulation of catalase gene expression as a compensatory mechanism, whereas SOD and GPX gene expression remain unaffected. (Circulation. 2000;101:33-39.)

Key Words: antioxidants • enzymes • free radicals • heart failure • molecular biology

Increasing evidence suggests that oxidative stress mediated by the generation of oxygen free radicals plays a critical role in the pathogenesis of heart failure.1–3 The myocardium is equipped with a variety of endogenous enzymatic and nonenzymatic antioxidant systems that are sufficient to metabolize oxygen free radicals generated during normal cellular activity. In particular, dismutation of superoxide anion by cytosolic copper/zinc- and mitochondrial manganese-containing superoxide dismutase (CuZnSOD and MnSOD, respectively) and the degradation of H2O2 by glutathione peroxidase (GPX) and catalase limit the cytotoxic effects of reactive oxygen metabolites.4 During pathophysiological conditions, however, the balance between free radicals and antioxidants may shift in favor of a relative increase in free radicals, resulting in increased oxidative stress.

During the last decade, considerable research effort has been directed at the identification of changes in oxidative stress and in antioxidant enzymes as one of the mechanisms underlying the development of heart failure. It has been reported that heart hypertrophy in rats and guinea pigs is associated with a decrease in oxidative stress and an increase in antioxidant reserve,5–8 whereas heart failure under both acute and chronic conditions is associated with increased oxidative stress and a reduced antioxidant reserve.6–9–11 In humans with chronic heart failure, products of free radical reactions, ie, plasma lipid peroxides, are elevated, whereas plasma thiols, as an index of the oxidative status of the extracellular environment, are decreased.2 Expired breath pentane levels, as an index of lipid peroxidation, were found to be elevated in patients with heart failure as well.12 Furthermore, it has been reported that plasma antioxidative enzyme activities were decreased in patients with ischemic heart disease and with heart failure.13,14 However, direct proof for an increased free radical formation in the failing human heart is yet to be furnished.

In addition to the biochemical aspects of oxidative stress, gene expression of antioxidative enzymes in the heart has been investigated during the last several years. In this regard,
an increase in myocardial antioxidant enzyme gene expression has been reported after acute oxidative stress induced by endotoxin,15 cytokines,16,17 and ischemia/reperfusion.18 However, gene expression of antioxidants in the human heart with end-stage heart failure has not been investigated. In view of increasing evidence for the involvement of oxidative stress in heart failure, it is of considerable interest to examine whether changes in antioxidant enzymes at the transcriptional or translational level may exist under chronic conditions in human heart failure. Therefore, we studied gene expression of CuZnSOD, MnSOD, GPX, and catalase in human end-stage failing hearts from patients with dilated cardiomyopathy (DCM) and ischemic cardiomyopathy (ICM) compared with nonfailing (NF) control hearts.

Methods

Patients

Hearts from patients with end-stage heart failure who were undergoing cardiac transplantation because of either DCM (n=12) or ICM (n=12) were investigated. Furthermore, NF donor hearts (n=12) that were ultimately rejected for transplantation because of technical reasons were also included in this study. Hemodynamic data at the time of cardiac transplantation and age of patients are given in the Table.

The protocol of this study was reviewed and approved by the ethics committee of the University Clinics of Freiburg.

Tissue Sample Preparation

Excised hearts were rinsed immediately in cardioplegic Krebs-Henseleit solution containing 30 mmol/L 2,3-butanedione monoxime. Transmural tissue samples derived from the left ventricular free wall were frozen in liquid nitrogen and stored at −80°C until use. For Northern blot and Western blot analyses, as well as for enzyme activity assays, aliquots of ~100 mg of tissue were ground in liquid nitrogen and homogenized with an EP 120 Fast Prep Cell Disruptor (Savant Instruments) in the respective homogenization buffer.

Northern Blot Analysis

Myocardial tissue was homogenized in lysis buffer RTL (Qiagen), and total RNA was extracted by use of an RNasy Mini Kit (Qiagen) according to the manufacturer’s instructions. Eight micrograms of total RNA per lane was size fractionated on a denaturing 1% agarose-formaldehyde gel, transferred to nylon membrane (Duralon-UV, Stratagene) by overnight capillary blotting, and immobilized by ultraviolet irradiation. Specific DNA probes for detection of catalase, MnSOD, CuZnSOD, GPX, and GAPDH gene transcripts were generated by polymerase chain reaction (PCR) from a cardiac specific cDNA sample with the following primer pairs: catalase, forward 5’-TCCGGGATCTTTTTAAACGCCATG-3’, reverse 5’-TCGAGCAGCCTGAGGACACTTAC-3’ (nucleotide position 855 to 1216, according to the sequence of the human SOD2 gene20); MnSOD, forward 5’-CTCCCGGACTGGCTTACAGC-TAC-3’, reverse 5’-AAAACAGGCAAACCACCCGCTGAAG-3’ (nucleotide position 176 to 549, according to the sequence of the human SOD2 gene20); CuZnSOD, forward 5’-GTGGGAAGAGCATGCCAAGCAGGACACCTGTCGTA-3’ (nucleotide position 343 to 759, according to the sequence of the human GPX1 gene20); GAPDH, forward 5’-CCACCGAGACTGTTGGAT-3’, reverse 5’-GTGGAAGTCA-GAGGAGACCAC-3’ (nucleotide position 608 to 921, according to the sequence of the human GAPDH gene20). The identity of the PCR fragments was verified by sequencing. DNA probes were labeled with [α-32P]dCTP by random priming (DNA labeling kit, Pharmacia Biotech), and unbound radioactivity was removed by spin columns. DNA/RNA hybridization was performed with QuickHyb hybridization solution (Stratagene) for 2 hours at 68°C. Blots were washed twice in 2×SSC/0.1% SDS for 10 minutes at room temperature and in 0.1×SSC/0.1% SDS for 30 minutes at 60°C. After autoradiography, specific signal intensity was quantified by video-image analysis. GAPDH data were used as an internal standard to normalize the catalase, MnSOD, CuZnSOD, and GPX data.

Western Blot Analysis

Protein levels of catalase, MnSOD, and caseasequin were examined by Western blot analyses. Tissue samples were homogenized in ice-cold lysis buffer containing 20 mmol/L Na-HEPES (pH 7.5), 4 mmol/L EGTA, 1% Triton X-100, 2 mmol/L desoxycortolate, 1 mmol/L phenylmethylsulfonyl fluoride, 0.05 mmol/L leupeptin, 1 mmol/L iodoacetamide, 1 µg/mL aprotinin, and 20 µg/mL trypsin inhibitor. Crude homogenates were centrifuged for 5 minutes at 10 000g, and protein concentration of supernatants was determined by the BCA method (Pierce Chemical Co). Samples were denatured in electrophoresis buffer containing 100 mmol/L Tris/Cl (pH 6.8), 8% (w/v) DTT, 2% SDS, 2% glycerol, and 0.05% bromophenol blue at 95°C and subjected to SDS-PAGE. Proteins were transferred to nitrocellulose membranes by electroblocting. Membranes were blocked in 5% nonfat dry milk in Tris-buffered saline and processed for immunodetection with a rabbit polyclonal antibody specific for catalase (Paezel 38 Lorei, Hanau, Germany), a sheep polyclonal antibody specific for MnSOD (Biotrend, Koeln, Germany), and a rabbit polyclonal antibody specific for caseasequin as primary antibodies.24 The secondary antibodies were peroxidase-conjugated anti-rabbit or anti-sheep antisera. Visualization of immunoreactive bands was performed with the enhanced chemoluminescence assay (ECL, Amersham), and signal intensity was quantified as an internal standard to video-image analysis. The caseasequin data were used as an internal standard to normalize the respective catalase and MnSOD data.

CuZnSOD ELISA

Myocardial protein levels of CuZnSOD were determined with a commercially available ELISA (CuZnSOD SURALISA, Immundiagnostik GmbH). Determinations were performed in triplicate according to the manufacturer’s instructions with the same tissue homogenates that were used for Western blot analysis.

Enzyme Activity Assays

Catalase Enzyme Assay

Tissue samples were homogenized in an ice-cold isotonic 0.01 mol/L sodium phosphate buffer (pH 7.4) and centrifuged for 5 minutes at 12 000g at 4°C. Catalase activity was examined in the supernatants by use of a rapid spectrophotometric method described by Cohen et al.25 Briefly, the catalase-catalyzed decomposition of H2O2 was measured by subjecting it to reaction for 3 minutes with a standard excess of KMnO4 and by subsequent measurement of the residual KMnO4 at 480 nm. Measurements were performed in triplicate. Protein concentrations were estimated by the BCA method. Catalase activity was calculated as units per milligram of protein.

SOD Enzyme Assay

Tissue samples were homogenized in ice-cold 0.01 mol/L sodium phosphate buffer (pH 7.4) supplemented with 0.03 mol/L KCl to facilitate the recovery of MnSOD and centrifuged for 5 minutes at
12,000g at 4°C. Total superoxide dismutase (SOD) activity was examined in the supernatants according to the method described by Del Maestro and McDonald.26 This assay is based on the ability of SOD to scavenge superoxide anion radical (O$_2^-$), which decreases the overall rate of pyrogallol autoxidation. In brief, 1 mL of 0.05 mol/L Tris-HCL buffer (pH 8.2) containing 1 mmol/L DTPA was added to 40 μL of tissue sample (2 mg of total protein/mL). The reaction was initiated by the addition of 0.2 mmol/L pyrogallol, and the change in optical density at 420 nm was recorded for 3 minutes. In a separate reaction, specific MnSOD activity was measured by the addition of 1 mmol/L sodium cyanide to the tissue sample to inhibit CuZnSOD. SOD activity was calculated as units per milligram of protein, with 1 U of SOD defined as the amount that inhibited the rate of pyrogallol autoxidation by 50%. We calculated CuZnSOD activity by subtracting the value using cyanide from the total SOD value.

**GPX Enzyme Assay**

Tissue homogenates were prepared as described for the SOD assay. GPX activity was examined in the supernatants according to the method described by Del Maestro and McDonald.26 The assay is based on the oxidation of reduced glutathione by GPX coupled to the disappearance of NADPH by glutathione reductase. In brief, a 40-μL tissue sample (2 mg of protein/mL) was added to 0.9 mL of 0.05 mol/L potassium phosphate buffer (pH 7.0) with 0.5 mmol/L DTPA containing 2 mmol/L glutathione (Sigma), 1 U of glutathione reductase (Sigma), and 0.16 mmol/L NADPH (Sigma). After addition of 0.6 mmol/L tert-butyl hydroperoxide, the change in optical density at 340 nm was recorded for 3 minutes. GPX activity was calculated as units per gram of protein, with 1 U of GPX causing the oxidation of 1 μmol/L glutathione per minute in the system outlined.

**Statistical Analysis**

All experimental values represent the mean of at least 2 independent determinations. Data are expressed as mean±SEM. For statistical analyses, median values of the 3 study groups (NF, DCM, and ICM) were compared by Kruskal-Wallis 1-way ANOVA on ranks, followed by a multiple comparison procedure (Dunn’s method) to isolate study groups that differed from the others. A value of P<0.05 was considered statistically significant. For graphical reasons, NF data from Northern and Western blots were set to 100%. DCM and ICM data are expressed in percent of NF data.

**Results**

**Relative mRNA Abundance of Antioxidative Enzymes**

 Autoradiograms shown in Figure 1A indicate that specific cDNA probes for human CuZnSOD recognized a minor 0.7-kb and a minor 0.9-kb transcript, a single 4-kb transcript for human MnSOD, and a single 1-kb transcript for human GPX, respectively, in all hearts examined. The relative mRNA levels, measured as the ratios of the intensities of mRNA for antioxidative enzymes to those of GAPDH mRNA, are shown in Figure 1B. For CuZnSOD, MnSOD, and GPX mRNA contents, no significant differences could be detected between the 3 study groups.

The autoradiogram in Figure 2A indicates that the cDNA probe for human catalase recognized a single 2.5-kb transcript. In contrast to the other antioxidative enzymes examined, relative catalase mRNA contents were significantly elevated in DCM hearts (Figure 2B; 123±23% increase compared with NF; P<0.01) and in ICM hearts (93±10% increase compared with NF; P<0.01). No statistically significant difference was found between the DCM and ICM groups.

**Protein Contents of Antioxidative Enzymes**

Because mRNA levels may not necessarily reflect the corresponding protein levels, we also investigated protein contents of catalase and MnSOD by Western blot analyses. The antibodies for catalase and MnSOD specifically detected proteins with molecular sizes of 58 kDa for catalase and 21 kDa for MnSOD, respectively. To account for potential differences in extracellular matrix content in tissue homogenates from failing and nonfailing myocardium, protein data represent relative values normalized to the myocyte-specific protein calsequestrin, for which equal distribution in NF, DCM, and ICM hearts was confirmed. CuZnSOD protein contents determined by ELISA represent absolute values and are given in nanograms per milligram of total protein. Determination of human GPX protein level was not done in the present study, because the tested commercially available antibodies against GPX detected proteins of different molecular weights than the reported size for the human GPX protein.27

Analyses of antioxidative enzyme protein levels revealed similar results as for the respective mRNA levels. As shown in Figure 3, catalase protein contents normalized to calsequestrin were 67% higher in DCM hearts than in NF hearts and 11% higher in ICM hearts than in NF hearts. MnSOD protein contents showed a 38% increase in DCM hearts compared with NF hearts. CuZnSOD protein contents were not significantly different between the groups.
were significantly elevated in DCM and ICM hearts compared with NF controls (DCM 90 ± 6% increase and ICM 90 ± 13% increase, \( P < 0.05 \) each), whereas MnSOD (Figure 4A) and CuZnSOD (Figure 4B) protein levels showed no significant differences between failing and non-failing myocardium.

We normalized protein levels of catalase and MnSOD to total protein content in crude homogenates in addition to normalizing them to calsequestrin. Expressed in relative arbitrary units, protein levels of MnSOD were 1.03 ± 0.03 in NF myocardium, 1.05 ± 0.05 in DCM hearts, and 0.96 ± 0.09 in ICM hearts, with no statistically significant difference between groups. Catalase protein levels were increased in DCM and ICM hearts by 72.9 ± 7.0% and 70.4 ± 7.1% compared with NF hearts, respectively (\( P < 0.05 \) each; 0.58 ± 0.12 versus 1.01 ± 0.09 and 0.83 ± 0.08 relative arbitrary units in NF, DCM, and ICM hearts, respectively).

Enzyme Activity Levels
In accordance with mRNA and protein data, catalase activity was found to be increased in DCM and ICM hearts by 124 ± 16% and 117 ± 15%, respectively (\( P < 0.01 \) each), compared with NF controls (Figure 5A). Enzyme activity levels of MnSOD, CuZnSOD, and GPX were similar in all 3 groups (Figure 5, B through D).

**Discussion**
There is ample support for the hypothesis that an increased generation of oxygen free radicals is involved in the pathogenesis as well as the progression of heart failure. It has been reported that in various animal models of heart failure, an increase in oxidative stress may be combined with a decrease in scavenging enzyme activity, either as a cause or as a result. However, it remains unclear whether human end-stage heart failure is also associated with an altered myocardial antioxidant reserve.

We studied gene expression and enzyme activity levels of myocardial scavenger enzymes in human hearts with end-stage heart failure of different origins (DCM and ICM) compared with NF hearts. The results of our study clearly demonstrate that no differences in gene expression of MnSOD, CuZnSOD, and GPX exist between failing and NF human hearts. On the contrary, catalase gene expression was ∼2-fold higher in the failing heart than in the NF heart. We found catalase expression to be increased on both the mRNA
and protein level, with a conformable increase in catalase enzyme activity as well, whereas enzyme activities of MnSOD, CuZnSOD, and GPX were similar between NF and end-stage failing myocardium.

These findings are interesting under various aspects. It has been suggested that SOD and GPX play the major role in detoxification of reactive oxygen metabolites in the heart.\(^1\) The unchanged gene expression of MnSOD, CuZnSOD, and GPX in human end-stage failing myocardium demonstrated in the present study is in contrast with the findings of several animal studies and the report about a significant reduction in plasma scavenging enzyme activities in patients with heart failure.\(^1\) Although plasma and tissue levels of antioxidative enzymes cannot necessarily be compared. Our data indicate that the myocardial antioxidant reserve is not diminished during human end-stage heart failure, and a deficit in antioxidative capacity may therefore not play a significant pathophysiological role in human heart failure.

Interestingly, expression and activity of catalase were found to be considerably increased in the failing myocardium. Although SOD is the first line of defense against oxygen free radical–mediated damage, it acts to increase the levels of H\(_2\)O\(_2\) by virtue of catalyzing the dismutation of superoxide anion to H\(_2\)O\(_2\). The major danger of H\(_2\)O\(_2\) accumulation is the production of highly reactive hydroxyl radical, for which no physiological defense system exists.\(^4\) As a result, catalase and GPX become the most crucial antioxidative enzymes, because they act to detoxify H\(_2\)O\(_2\). It was shown that the heart contains \(<2\%\) of the catalase found in liver, yet it produces a greater amount of hydrogen peroxide per gram of tissue than any other organ.\(^29\)–\(^31\) Together with the fact that catalase has a lower affinity for H\(_2\)O\(_2\), it is therefore believed that the glutathione redox system acts as the major route for the metabolism of H\(_2\)O\(_2\) in the heart and that catalase is of little importance.\(^32\) However, catalase enables the cell to decompose H\(_2\)O\(_2\) regardless of the cellular concentration of glutathione, which may be important in light of evidence that high plasma levels of tumor necrosis factor, as found in patients with heart failure, reduce tissue glutathione levels.\(^33\)–\(^34\) Thus, catalase may be of particular relevance in the failing myocardium. Furthermore, a number of studies do suggest a significant role for endogenous catalase, and catalase mRNA is reported to be upregulated in the mammalian heart after subjection to a stress insult.\(^17\)–\(^35\) For example, it is well known that reperfusion after ischemia causes generation of oxygen free radicals and induces oxidative stress. In this regard, it has been shown that repeated ischemia and reperfusion enhanced catalase expression,\(^17\) and there is evidence that in addition to heat shock proteins, catalase is implicated in the cardioprotective effect of heat stress against reperfusion arrhythmia.\(^36\) Furthermore, pretreatment with catalase but not SOD prevented abnormalities in contraction-relaxation processes and offered essentially complete functional protection against oxygen-derived free radicals in rat papillary muscle preparations.\(^37\) Protection against a lethal oxidant injury of H\(_2\)O\(_2\) has also been demonstrated to be conferred by adenovirus-mediated gene transfer of human catalase into human umbilical vein endothelial cells.\(^38\) Finally, it has been found that under chronic oxidative stress by direct challenge of myocytes with H\(_2\)O\(_2\), catalase but not GPX was selectively induced by transcriptional activation.\(^39\)

In this context, it should be mentioned that we only analyzed gene expression and enzyme activity levels of cellular antioxidative enzymes. Expression and enzyme activity levels of extracellular SOD (EC-SOD), the third SOD isozyme, were not determined in the present study. Transgene overexpression of EC-SOD was shown to provide rabbit hearts with substantial protection against myocardial stunning without concomitant administration of catalase and to preserve myocardial function after ischemia-reperfusion injury in isolated murine hearts.\(^40\)–\(^41\) We cannot exclude that plasma levels or tissue vascular levels of EC-SOD are altered during end-stage heart failure in response to an increase in oxidative stress. For example, EC-SOD expression and activity were shown to be reduced in advanced human atherosclerotic lesions.\(^42\) However, Adachi et al\(^43\) reported similar plasma concentrations of EC-SOD in healthy individuals compared with patients with heart diseases, although only a limited number of patients with unspecified heart disease were included in that study. Furthermore, because the present study was performed in myocardial tissue samples, we cannot differentiate whether catalase was specifically increased in the myocardium, in the microvascular system, or in both. Likewise, an increased expression of SOD and GPX in cardiomyocytes could be offset by a reduced expression within the vascular system.

In summary, it appears that an increase in endogenous catalase can be regarded as an effort made by the heart to protect itself from an oxidative assault. Under physiological conditions, catalase expression seems to be much lower than with other antioxidative enzymes. However, the results of the present study underscore the importance of catalase induction
at the transcriptional level as an adaptive cardioprotective response under chronic pathophysiological conditions.

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References

Figure 5. Bar graphs showing catalase (A), MnSOD (B), CuZnSOD (C), and GPX (D) enzyme activity in NF and end-stage DCM and ICM failing hearts (n=12 each). Catalase activity was significantly increased in failing myocardium (*P<0.01 vs NF), whereas MnSOD, CuZnSOD, and GPX activities were similar in all 3 groups.


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