Activation of endoplasmic reticulum stress response during the development of ischemic heart disease

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Abstract

Endoplasmic reticulum (ER) stress has been found to be associated with neurodegenerative diseases and diabetes mellitus. Whether ER stress is involved in the development of heart disease is not known. Cardiac-specific expression of monocyte chemoattractant protein-1 (MCP-1) in mice causes the development of ischemic heart disease. Here we report that microarray analysis of gene expression changes in the heart of these transgenic mice revealed that a cluster of ER stress-related genes was transcriptionally activated in the heart during the development of ischemic heart disease. The gene array results were verified by quantitative real-time PCR that showed highly elevated transcript levels of genes involved in unfolded protein response such as ER and cytoplasmic chaperones, oxidoreductases, protein disulfide isomerase (PDI) family, and ER-associated degradation system such as ubiquitin. Immunoblot analysis confirmed the expression of chaperones, PDI, and ubiquitin. Immunohistochemical analyses showed that ER stress proteins were associated mainly with the degenerating cardiomyocytes. A novel ubiquitin fold modifier (Ufm1) that has not been previously associated with ER stress and not found to be induced under any condition was also found to be upregulated in the hearts of MCP mice (transgenic mice that express MCP-1 specifically in the heart). The present results strongly suggest that activation of ER stress response is involved in the development of ischemic heart disease in this murine model.

Keywords

monocyte chemoattractant protein-1; transgenic; myocardial inflammation

A variety of human disorders, including neurodegenerative diseases and diabetes mellitus, are known to involve endoplasmic reticulum (ER) stress. ER stress leads to accumulation of unfolded proteins in ER (17, 31), which in turn evokes the unfolded protein response (UPR) (33). This response attempts to reduce the amount of misfolded proteins by inducing the production of the ER chaperones, such as BiP/Grp78 and Grp94, that promote protein folding, and by reducing general protein synthesis (12) and enhancing the degradation of misfolded proteins via a ubiquitin-proteasome system termed ER-associated degradation (ERAD) (3, 39). The persistent accumulation of misfolded proteins beyond the capacity of ER quality control causes cellular dysfunction and cell death involved in several human disorders. Involvement of this process in heart disease has not been extensively studied (41), and whether it is involved in ischemic heart disease is unknown.

Ischemic heart disease, a leading cause of death, is widely recognized to be an inflammatory disease, and infiltration of monocytes/macrophages is thought to make a significant contribution to the pathophysiology of this disease (16). The major chemotactic factor for monocytes/macrophages, monocyte chemoattractant protein-1 (MCP-1), a member of the small inducible chemokine gene family, plays a role in the recruitment of monocytes to the sites of injury and infection. Recent studies revealed that MCP-1 plays an important role in the development of ischemic heart disease (6, 7, 14). However, the molecular changes induced by MCP-1 during the development of the disease are not known. The progression of such molecular changes can be best studied in an animal model. To investigate the molecular mechanisms involved in the induction of ischemic heart disease by the chronic inflammatory processes, we developed transgenic mice that express MCP-1 specifically in the heart (MCP mice; 18). The MCP mice manifest myocardial inflammation and develop thrombolic vasculopathy, cardiac dilatation, interstitial fibrosis, myocyte vacuolization, and mortality with congestive heart failure by ~6 mo of age (18, 23). The clinical, histological, and molecular features that occur in the hearts of these animals closely resemble the changes observed in human ischemic cardiomyopathy (1, 18, 23). This model is therefore suitable for elucidating how chronic inflammation contributes to the development of ischemic heart disease and to investigate whether ER stress response is involved in this process.

The aim of this study was to investigate whether the gene expression changes associated with ER stress are involved in the development of ischemic heart disease by using the MCP mouse model. Here we report that DNA microarray analysis of gene expression profile changes revealed transcriptional activation of a set of ER stress-related genes during the development of heart disease in this model. A recently identified ubiquitin fold modifier 1 (Ufm1) gene that had not been previously reported to be induced by any condition was also found to be upregulated. Quantitative real-time PCR validated the conclusions reached from the gene array analyses. The expression of ER stress-related proteins was also demonstrated by immunoblotting and immunohistochemical studies. These findings, for the first time, strongly suggest that ER stress response activation is involved in the development of ischemic heart disease induced by chronic inflammation.

MATERIALS AND METHODS

Animals used

The animal protocols used in this study were approved by the Animal Care and Use Committee of University of Central Florida and the Ohio State University. Hearts from five male animals for both wild-type and MCP mice from each age of 2, 4, 8, 16, and 24 wk were used. The hearts were removed, rinsed free of blood, and immediately either snap frozen in liquid nitrogen or fixed with buffered 10% (vol/vol) formalin for histopathological analyses. Frozen samples then underwent RNA and protein isolation for further microarray, real-time PCR, and Western blot analysis.

DNA microarray analysis

Total RNA was extracted from left ventricular specimens with RNA Kit (Invitrogen, Carslbad, CA). Generation of cDNA for Affymetrix arrays and hybridization conditions were set up according to the manufacture's protocol (Affymetrix, Santa Clara, CA). Briefly, 10 μg of total RNA were used to synthesize double-stranded cDNA with a SuperScript synthesis kit (Bio-Rad, Hercules, CA), incorporating a T7 promoter sequence by using oligo(dT)-T7 primer. Biotin labeling of cDNA and hybridization with Murine Genome-U74A GeneChip [which has 6,000 functionally characterized sequences and 6,000 expressed sequence tags from the UniGene database (Affymetrix)] was done as recommended by the manufacturer. Data analysis

was performed by using Affymetrix GeneChip 3.1 software under default parameter setting at the Heart and Lung Institute at Ohio State University.

Real-time quantitative PCR analysis

Total RNA samples used in microarray analysis were also used for quantitative real-time PCR. Primers used for real-time RT-PCR are listed in Table 1. The relative expression levels of each targeted gene were normalized by subtracting the corresponding mouse β-actin threshold cycle (C_T) values by using the $\Delta\Delta C_T$ comparative method (26).

Protein extraction and immunoblot analysis

The heart samples were homogenized in a buffer containing 20 mM Tris · HCl, pH 6.8, 1 mM EDTA, 1% SDS, 1 mM PMSF, and 1× protease inhibitor cocktail (Roche, Mannheim, Germany). Equal amounts of protein from each sample were separated by 10% (Grp78 and Grp94) or 12.5% [protein disulfide isomerase (PDI), 25-kDa heat shock protein (HSP25), 40-kDa heat shock protein (HSP40)] SDS-PAGE. The amount of proteins loaded was HSP40, 100 μg; Grp78, Grp94, ubiquitin, and PDI, 50 μg; HSP25, 10 μg. Percentage gel used and amount of protein loaded were dependent on the molecular weight of the protein and the sensitivity of the antibody, respectively. The separated proteins were transferred to polyvinylidene difluoride membranes (Immobilon-P, Millipore, MA), and the membranes were incubated with polyclonal antibody to HSP25, 40-kDa heat shock protein (HSP40), Bip/Grp78, Grp94, PDI (Stressgene Biotechnologies, Canada), or ubiquitin (Dako, Carpinteria, CA) followed by horseradish peroxidase-conjugated secondary antibodies. Immunoreactive bands were visualized with the SuperSignal West Pico enhanced chemiluminescence kit according to the manufacturer's specifications (Pierce, Rockford, IL). Equal protein loading was confirmed by staining the gel with Coomassie blue and probing with GAPDH antibody (Novus Biological, Littleton, CO) because this protein level did not change. Band intensities were analyzed using a densitometer equipped with a multianalyst software program (Alpha Innotech, San Leandro, CA).

Immunohistochemistry

Hearts from MCP mice and wild-type mice were cut into three slices from the base to the apex and embedded in paraffin by using standard protocols. The transverse sections (5 μm) were incubated with antibodies against HSP25, HSP40, HSP70, Grp78, Grp94, PDI (Stressgene Biotechnologies), or ubiquitin (Dako). The sections were then incubated with horseradish peroxidase-conjugated secondary antibodies followed by diaminobenzidine and counterstained with hematoxylin.

Statistical analysis

The experimental data were analyzed by using SPSS 10.0 software under Windows XP. At least five animals were analyzed for each data point, and each experiment was repeated at least three times. All values are presented as means ± SE. The statistical significance of differences between wild-type control and MCP-1 transgenic group was calculated by using Student's *t*-test. A value of $P < 0.05$ was considered significant.

RESULTS

Gene expression profile changes reveal induction of ER stress-associated genes during the development of ischemic heart disease

To identify the gene expression changes that occur during the development of ischemic heart disease caused by chronic inflammation, gene expression profile changes of the hearts of the MCP mice were examined during progression of the disease. RNA from hearts of MCP mice

of 2, 4, 8, 16, and 24 wk of age with age and sex-matched wild-type controls were used for this analysis using Murine Genome-U74A chips. Among the genes whose expression was upregulated was a set of genes that are thought to be associated with ER stress (Table 2). The largest group of upregulated genes was represented by genes encoding ER resident chaperones and disulfide isomerases such as Cai, endoplasmic reticulum oxidoreductase (ERO1), Bip/Grp78, and PDI. The second major group of upregulated genes includes genes involved in protein transport. This group includes members of the Sec61 complex (Sec51 and Sec63) and translocation-associated protein. Asparagine synthetase was also significantly induced. Genes involved in the regulation of ER Ca^{2+} homeostasis such as calumenin and ribophorin were also induced. The transcription factors included two established UPR genes: C/EBP homologous protein (CHOP) and X box-binding protein 1 were persistently activated in the hearts of MCP mice. Upregulated genes also included genes involved in ER-associated degradation such as ubiquitin-conjugating enzyme and proteosome 26S subunit. The expression of the chaperone HSP40 was also upregulated in the hearts of MCP mice. These data show that ER stress response pathway members were upregulated at the transcript level in the hearts of MCP mice as the myocardial pathology and dysfunction developed, indicating that ER stress was activated in this mouse model of ischemic heart disease.

Real-time PCR verification of upregulation of the ER stress-associated genes in the hearts of MCP mice

To test for the validity of the microarray findings, the mRNA levels of some of the upregulated ER stress-associated genes were measured by quantitative real-time PCR. Consistent with the results obtained by the microarray analysis, mRNA levels of BiP/ Grp78, Grp94, PDI, Cai/ERp72, asparagine synthetase, ribophorin, and ERO1, CHOP, activating transporter factor (ATF6), and *tribbles*-related protein 3 (TRB3) were found to be significantly upregulated in the hearts of the MCP mice compared with age- and sex-matched wild-type controls (Fig. 1). The mRNA for BiP/Grp78, Grp94, PDI, ERO1, asparagine synthetase, and ribophorin were at high levels at 2 mo of age with subsequent decreases at 4 and 6 mo of age. At all time points their expression levels were significantly higher than that in the age- and sex-matched wild-type controls. Consistent with the gene array data, mRNA levels of cytosolic chaperones HSP25, HSP40, and HSP70 were also found to be upregulated in the hearts of MCP mice (Fig. 2). These results demonstrate activation of ER stress response in the hearts of MCP mice during the development of myocardial deterioration and dysfunction.

Immunoblot analysis of ER stress-associated proteins in the hearts of MCP mice

To test whether the transcript level changes observed in the hearts of MCP mice were reflected in ER stress-associated protein levels, the hearts of MCP mice and age- and sex-matched control mice were examined by immunoblot analysis. The levels of Bip/Grp78, Grp94, and PDI were elevated in the hearts of MCP mice compared with wild-type controls (Fig. 3, *A–D*), indicating that ER stress response was activated in the hearts of MCP mice. The expression of cytosolic chaperones HSP25 and HSP40 were also induced (Fig. 3, *A, E,* and *F*). Because these chaperones play crucial roles in protein folding and are induced on stress, it is clear that inflammatory changes in the heart of MCP mice elicited a stress response not only in the ER but also in the cytosol.

Immunohistochemical evidence for increased expression of ER chaperones in the cardiomyocytes in the hearts of MCP mice

To determine the localization of ER stress-associated proteins in the hearts of MCP mice, immunohistochemical staining was performed. Strong staining for Grp78 was observed in the hearts of MCP mice, with intense staining found in cardiomyocytes undergoing degenerative changes as indicated by the presence of vacuoles. The cardiomyocytes with less visible

indications of degeneration showed weaker immunoreactivity, whereas the hearts of wild-type mice showed a much weaker staining for Bip/Grp78 in both myocardium and interstitial infiltrating inflammatory cells (Fig. 4, *A* and *D*). A similar pattern of immunoreactivity for Grp94 was observed in the hearts of MCP mice (Fig. 4, *B* and *E*). A strong staining for PDI was found in many cardiomyocytes of MCP mice but not in wild-type controls (Fig. 4, *C* and *F*). The strongest staining for PDI was found in cardiomyocytes undergoing degenerative changes such as vacuolation with weaker staining in cardiomyocytes with less obvious degenerative changes (Fig. 4*F*). The expression of cytosolic chaperones HSP25, HSP40, and HSP70 were also markedly elevated in the cardiomyocytes of MCP mice compared with age- and sex-matched wild-type controls (Fig. 5, *A–F*). The degenerating vacuolated cardiomyocytes showed the strongest staining.

Induction of ER-associated protein degradation in the hearts of MCP mice

A severe form of ER dysfunction would result in accumulation of misfolded proteins, which tend to form toxic aggregates. Besides UPR, the ERAD is known to be induced to activate degradation of unfolded or misfolded proteins via the ubiquitinproteasome system (17, 24). To test for such a possibility, we examined the accumulation of ubiquitinylated proteins in the hearts of MCP mice by immunohistochemistry and Western blot analysis. The immunoreactivity for ubiquitin was evident in the cardiomyocytes of MCP mice but not in the wild-type controls, demonstrating induction of ER stress in the cardiomyocytes (Fig. 6, *A* and *B*). Western blotting revealed marked accumulation of ubiquitinylated proteins in the hearts of the MCP mice compared with the wild-type controls (Fig. 6*C*). This implies that the accumulation of unfolded proteins induced by ER dysfunction exceeds the capacity of the proteasome to degrade them, strongly suggesting that ER functioning is disturbed in the hearts of MCP mice that are undergoing the development of ischemic heart disease.

One of the transcriptionally upregulated genes found in the MCP mice would encode a small protein that had not been previously found in any organism but shows homology to a recently identified human ubiquitin fold modifier designated Ufm1 that was not induced by any condition (19). Quantitative real-time PCR analysis confirmed that this murine Ufm1 was transcriptionally upregulated just before the onset of the clinical symptoms of left ventricular dysfunction (Fig. 6*D*).

DISCUSSION

In this study we found that two major components of the ER stress response pathway, UPR and ERAD, were highly activated in the hearts of MCP mice during the development of ischemic heart disease. Two main groups of genes that belong to UPR were upregulated in the MCP mice. One group includes peptide-binding molecular chaperones Bip/Grp78 and Grp94 (17, 24). Bip/Grp78 and Grp94, the most well characterized among them, interact transiently with protein-folding intermediates to prevent aggregation of a protein by keeping it in a folding-competent state (17, 21, 24). Interaction between the chaperones and proteins ensures that only proteins that are properly assembled and folded leave the ER compartment and thus attempt to alleviate the threat of cell death (17). The second group that was upregulated during the development of ischemic heart disease is composed of the disulfide isomerase family, including PDI, Cai/ERp72, ERp59, and ERp29 (17). These are involved in protein folding by functioning as oxidoreductases in the formation/isomerization of disulfide bonds and thereby increase the rate at which proteins attain their final folded conformation (9, 21). In addition, PDI, Cai/ERp72, ERp59, and ERp29 also have peptide-binding activity (9, 21). Cai is an ER luminal protein that is both a stress protein and a member of the protein disulfide isomerase (PDI) family of proteins (35). ERO1, which was found to be significantly upregulated in the present study at 2 mo in transgenic mice, is probably involved in the delivery of oxidizing equivalents

required for the ER-located PDI (8). In humans, Ero1α and Ero1β are upregulated by hypoxia, suggesting that the ERO1 protein undergoes functional specialization in response to the different demands of oxygen tension and high-throughput protein folding. A variety of endoplasmic proteins including ERO1 and chaperones are induced by energy depletion caused by severe cellular hypoxia (anoxia) or by glucose deprivation (2). Induction of hypoxia-associated genes observed in the present study is consistent with the finding that MCP mice develop vasculopathy and ischemic degeneration of cardiomyocytes (23). It has been observed that CHOP sensitizes cells to ER stress-mediated death by directly regulating target genes in the nucleus. By activating ERO1α, CHOP promotes oxidizing conditions in the ER, which contribute to death (22). ATF6, which is a resident transmembrane protein of ER, has been found to be upregulated in the MCP transgenic mice. It has been proposed that ER stress signaling, especially that mediated by ATF6, plays a decisive role in the induction of apoptosis in muscle tissues through activation of caspase-12 (27). TRB3 (*tribbles*-related protein 3) was found to be up-regulated in the present study; it has been reported that TRB3 plays a important role in cell death in conjunction with CHOP where CHOP is also induced by other stress signals such as oxidative stress, amino acid deprivation, and hypoxia. Under prolonged ER stress, the excess TRB production probably would ultimately lead to cell death, as previously suggested (29). The present results are consistent with the conclusion that MCP mice develop ischemic heart disease.

ER-associated degradation (ERAD) constitutes further evidence for the ER stress response. Accumulation of unfolded or misfolded proteins impairs the uniquitin-proteasome protein degradation system, which sensitizes cells to ER stress (5, 37). Hyperubiquitination of proteins have been observed in human dilated cardiomyopathy (5) and was also found in mutant KDEL receptor transgenic mice, in which the ER quality control system was impaired and the mice developed dilated cardiomyopathy (11). We observed increased ubiquitination of proteins in the hearts of the MCP mice with disease progression, and the ubiquitinylated proteins were located primarily in those cardiomyocytes showing marked vacuolization. This finding suggests that increased levels of unfolded or misfolded proteins probably saturated the uniquitin-proteasome system and caused prolonged ER stress in cardiomyocytes that were no longer able to escape degeneration and death.

One of the genes transcriptionally activated during the development of inflammation-induced heart failure encodes a novel protein, Ufm1, that was recently identified in HEK293 cells and mouse tissues (19). This protein was suggested to function with a unique set of alternate ubiquitin conjugating enzyme complexes, although the biological role of this protein is not known (19). Agents known to induce ER stress, and other stress inducers such as high temperature and heavy metals, were reported to be unable to induce this protein. The present inflammation-induced heart disease represents the first instance of induction of this newly discovered member of the ubiquitin family of proteins.

The magnitude of the ER reorganization upon massive accumulation of unfolded proteins invokes not only ER-specific but also general cellular stress mechanisms (4, 40). HSPs are molecular chaperones in the cytosol and are involved in various cellular metabolic processes, including protein synthesis, folding, assembly, and degradation, thereby preventing intracellular accumulation of unfolded or misfolded proteins (25, 32). In the present study, we observed that expression of HSPs, such as HSP70, HSP40, and HSP25, was also markedly increased at both mRNA and protein levels in the hearts of MCP mice compared with aged-matched wild-type controls. Even though the elevated levels of these proteins may assist the folding of proteins into appropriate conformations, re-fold misfolded proteins, and rescue previously aggregated proteins (10, 15), in the face of prolonged stress caused by chronic myocardial inflammation, these efforts fail to protect the cardiac cells.

Although the precise molecular mechanisms that lead to the development of ischemic heart disease in the MCP mouse model are not well understood, all of the data so far obtained indicated the following sequence of events. Cardiac-specific MCP-1 expression induces the infiltration of monocytes/macrophages within myocardium where they undergo apoptosis with the release of Fas ligand (FasL) that cause death of vascular endothelial and smooth muscle cells, leading to thrombotic occlusive arteriolar vasculopathy that results in ischemia, which triggers a series of events (28). The cardiomyocytes become exposed to proinflammatory cytokines, altered redox state, and oxidative stress, which in turn triggers the stress response in the ER as well as in the cytosol. ER stress proteins and HSPs are generated to protect the cells from the adverse effect of stress. However, with the chronic inflammation, these efforts fail, despite the production of the high levels of such proteins, and the cells succumb to the death-inducing processes. Thus, in the early stages of disease, the MCP mice hearts show high levels of expression of ER stress proteins throughout the myocardium. As the protection fails, some of cardiomyocytes are unable to withstand the prolonged stress and undergo degenerative process that results in the formation of highly vacuolated cardiomyocytes. As the degenerative process is in its advanced stages, the ER stress proteins synthesis no longer stays at high levels. Thus ER stress is involved in the initiation of the disease.

Neurodegenerative diseases are known to involve cell death initiated by ER stress and are thus regarded as ER stress-associated diseases or conformational diseases (13, 20, 34, 36). However, the involvement of ER stress in heart disease has not been studied extensively (41). Studies in transverse aortic constriction (TAC) mice have demonstrated that pressure overload by TAC induces prolonged ER stress, which contributes to cardiac myocyte apoptosis during progression of cardiac hypertrophy to failure (30). The present report that activation of ER stress is associated with the development of ischemic heart disease, and further studies on the association of stress proteins with ischemic heart disease in other models and humans, may reveal early biomarkers for the ischemic heart disease.

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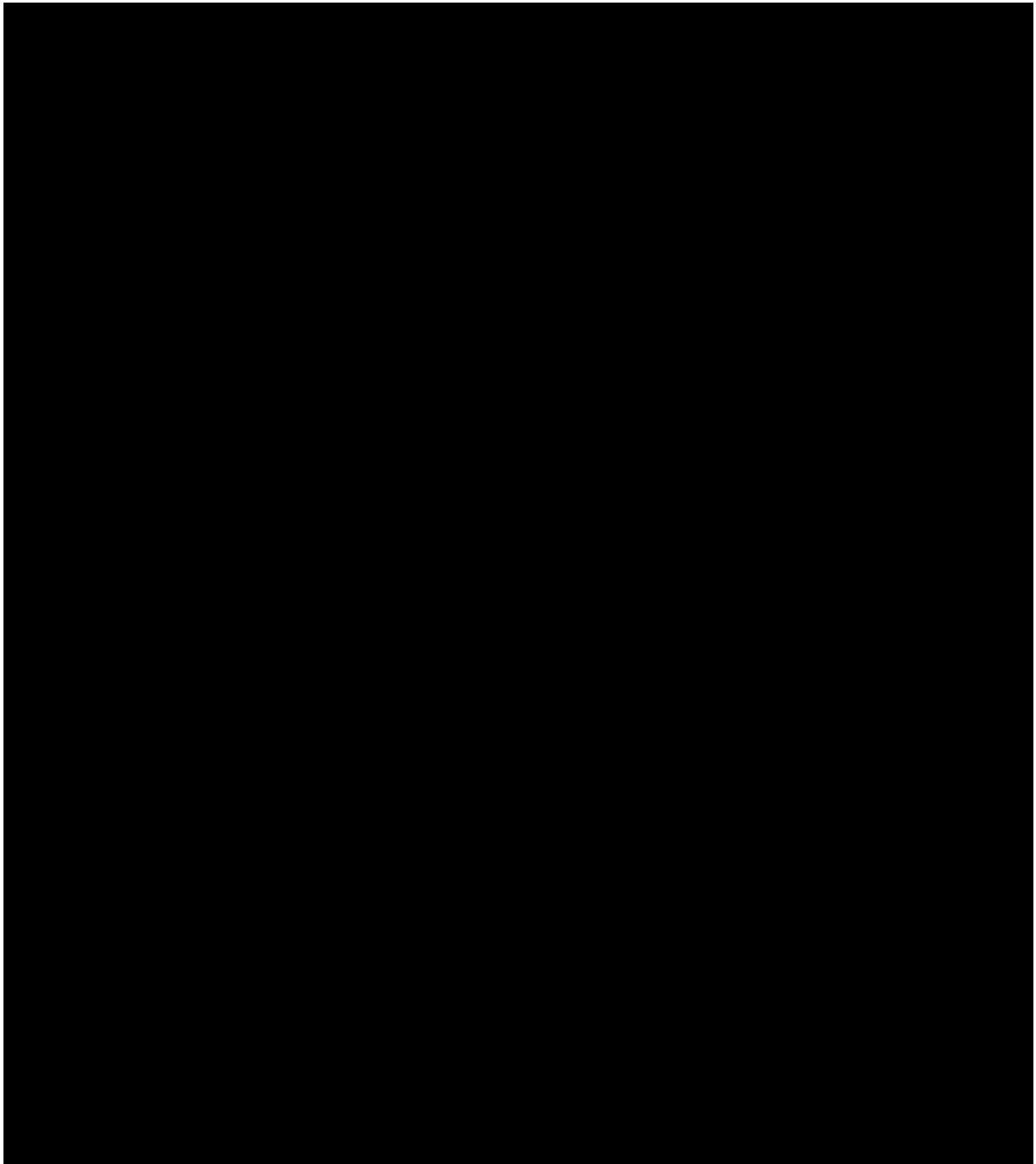
Fig. 1.
Elevated levels of transcripts of endoplasmic reticulum (ER) stress-associated genes in the hearts of transgenic mice that express monocyte chemoattractant protein-1 (MCP-1) specifically in the heart (MCP mice). Relative mRNA levels of ER stress-associated genes Bip/Grp78 (*A*), Grp94 (*B*), protein disulfide isomerase (PDI; *C*), ribophorin (*D*), asparagine synthetase (*E*), Cai (*F*), endoplasmic reticulum oxidoreductase (ERO1; *G*), C/EBP homologous protein (CHOP; *H*), ATF6 (*I*) and *tribbles*-related protein 3 (TRB3; *J*) were measured by quantitative real-time PCR. A total of 5 samples for each time point was used, and each sample was run in triplicate for real-time PCR. Expression level of all the samples was normalized by β-actin. *Significantly different from wild-type controls (*P* < 0.05).
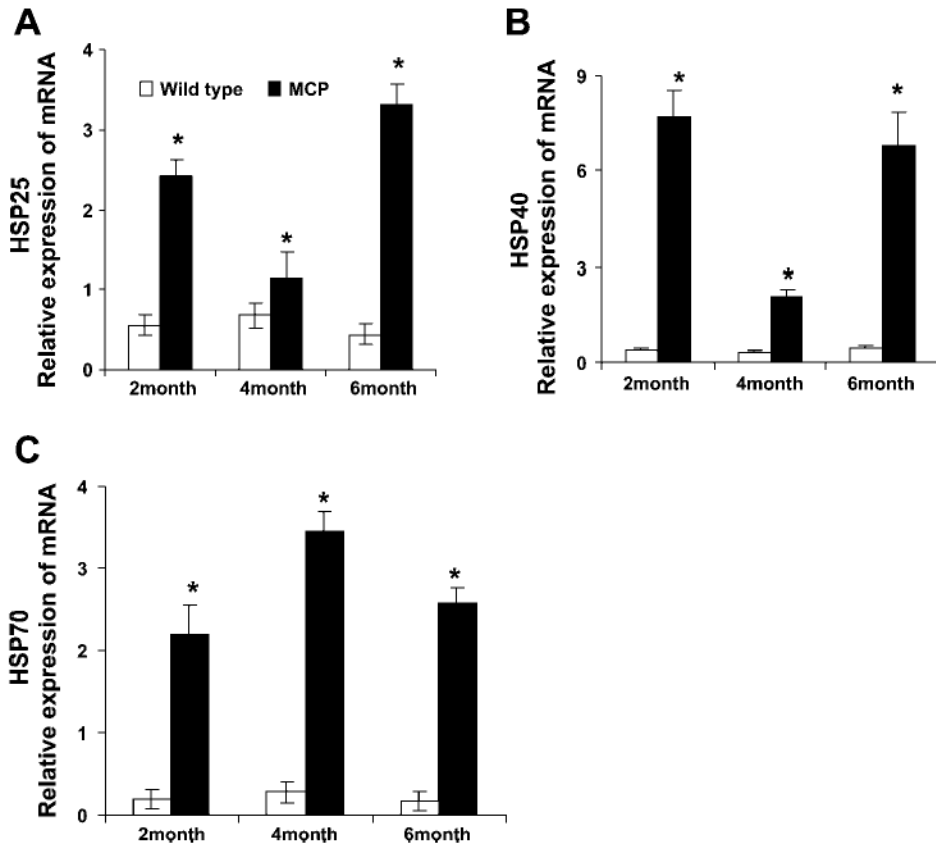
Fig. 2.
Elevated levels of transcripts of cytosolic chaperones in the hearts of MCP mice. Relative mRNA levels of cytosolic chaperones, 25-kDa heat shock protein (HSP25; *A*), 40-kDa heat shock protein (HSP40; *B*), and 70-kDa heat shock protein (HSP70; *C*), were measured by quantitative real-time PCR. A total of 5 samples for each time point was used, and each sample was run in triplicate for real-time PCR. The expression level of all the samples was normalized by β-actin. *Significantly different from wild-type controls ($P < 0.05$).
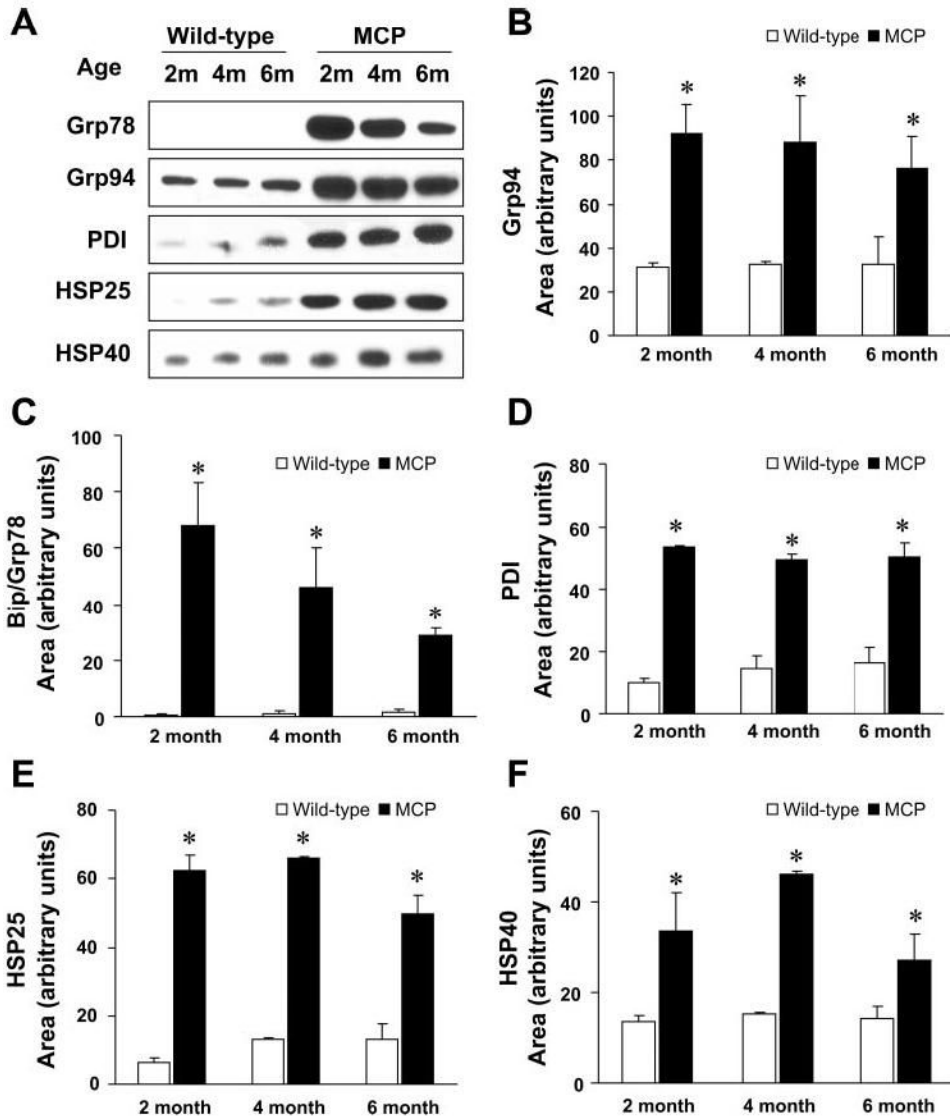
Fig. 3.
Immunoblot analysis of ER resident chaperones and cytosolic chaperones in the hearts expressing MCP-1. *A*: Western blots showing levels of BiP/Grp78, Grp94, PDI, HSP25, and HSP40 proteins in the hearts of 2-, 4-, and 6-mo-old wild-type controls and MCP mice. The expression level of all the samples was normalized to GADPH (*B–F*). Quantitative analyses of levels of Bip/Grp78, Grp94, PDI, HSP25, and HSP40 proteins by densitometry. *Significantly different from wild-type controls ($P < 0.05$).
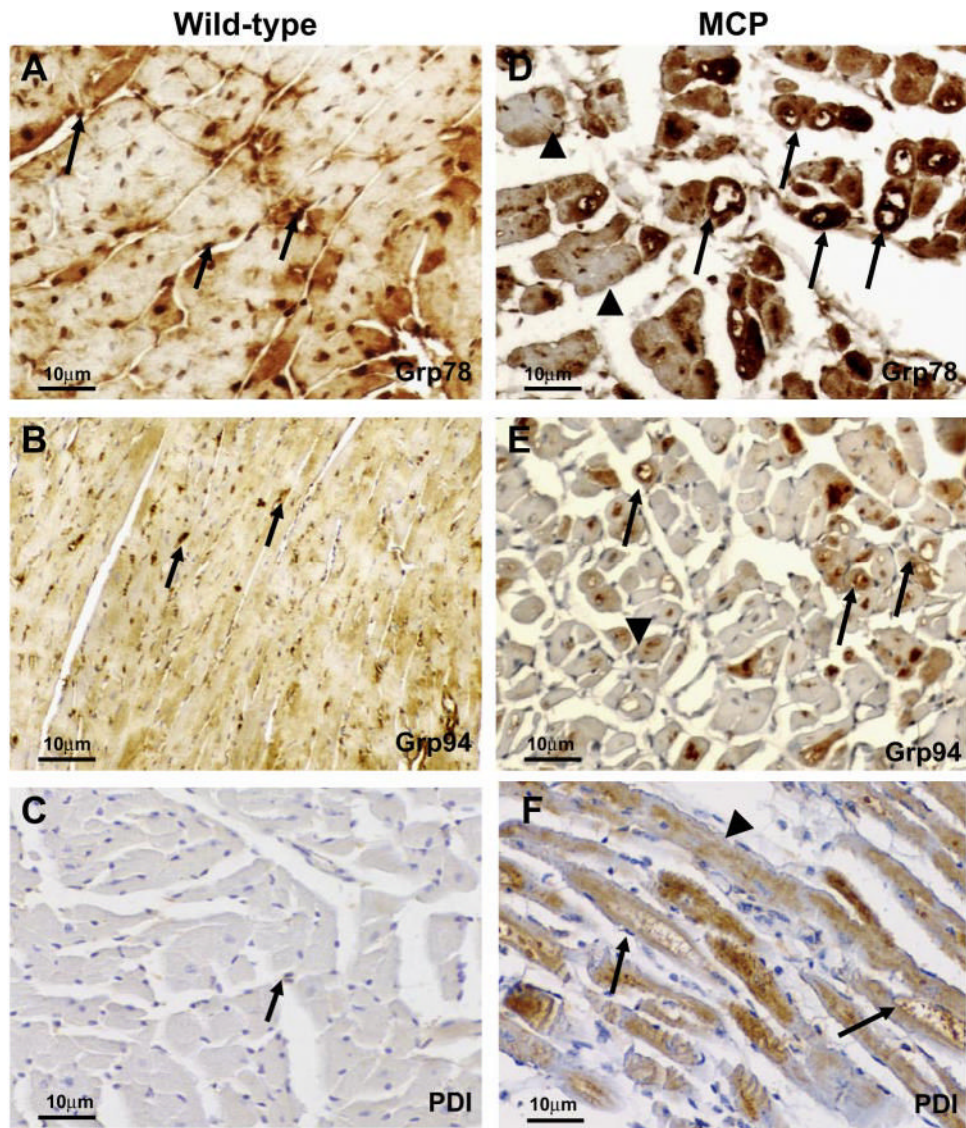
Fig. 4.
Immunohistochemical staining of ER-stress associated proteins Bip/Grp78, Grp94, and PDI in the hearts of wild-type controls and MCP mice of 6 mo of age. *A–C*: immunoreactivity (brown) in myocardium from wild-type controls. Note the absence or very weak cytoplasmic staining in cardiomyocytes and positive staining of Grp78 or Grp94 in interstitial infiltrating cells (arrow). *D–F*: immunoreactivity (brown) in myocardium from MCP mice. Note the strong staining in cardiomyocytes undergoing degenerative changes such as vacuolation (arrow) and the weaker immunoreactivity in cardiomyocytes with normal appearance (arrowheads). Original magnification, × 400.

Fig. 5.
Immunohistochemical staining of heat-shock proteins HSP25, HSP40, and HSP70 in the hearts of wild-type controls and MCP mice of 6 mo of age. *A–C*: immunore-activity (brown) in myocardium from wild-type controls. Note the absence or very weak cytoplasmic staining in cardiomyocytes. *D–F*: immunoreactivity (brown) in myocardium from MCP mice. Note the strong staining in cardiomyocytes undergoing degenerative changes such as vacuolation (arrow) and the weaker immunoreactivity in cardiomyocytes with less degeneration (arrowheads). Original magnification, ×400.
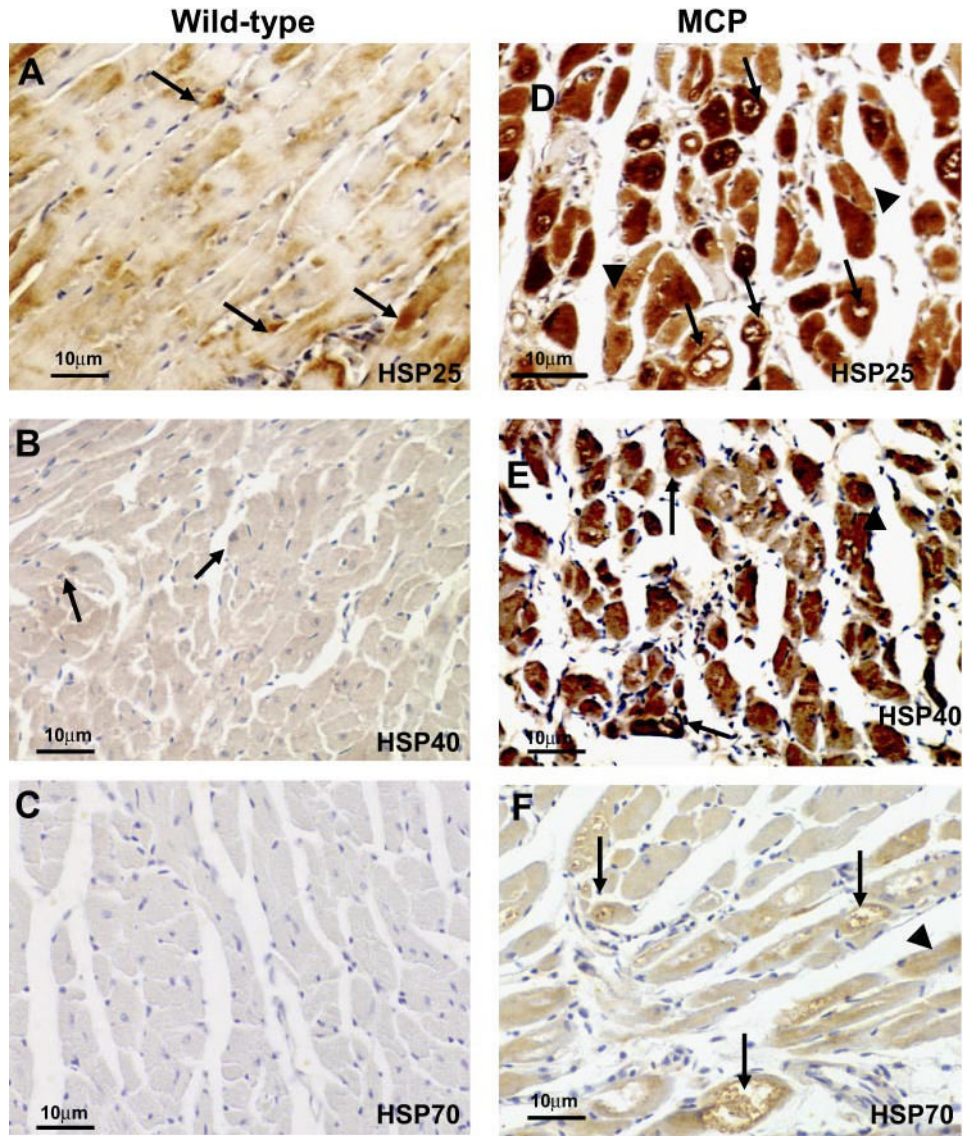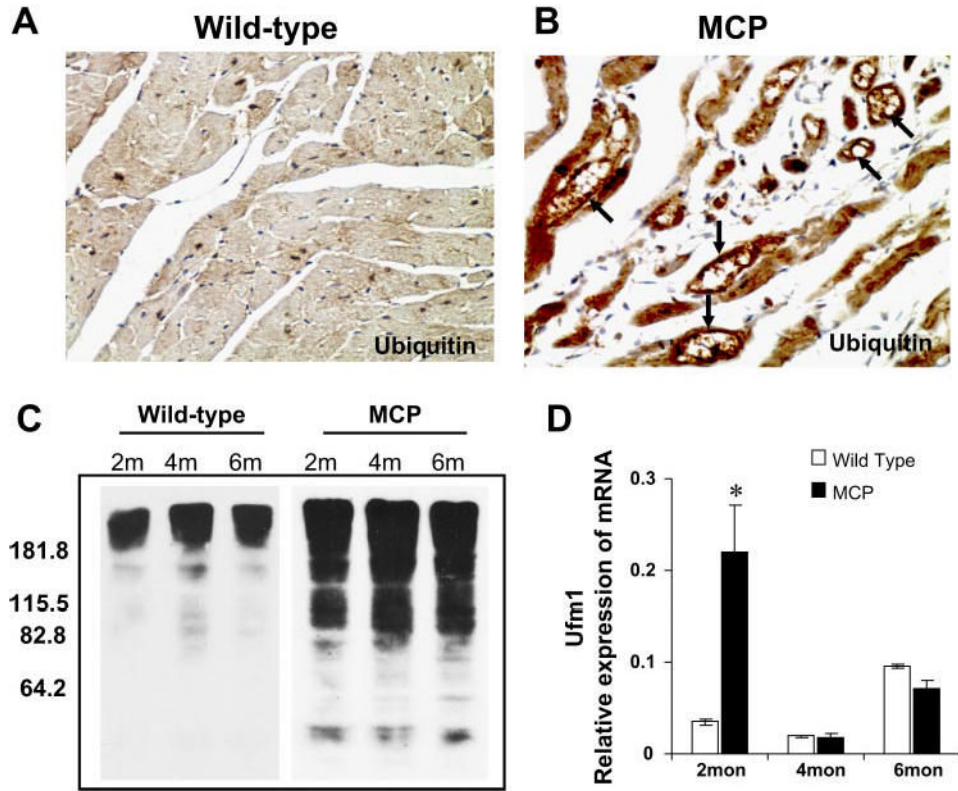
Fig. 6.
Accumulation of ubiquitinylated proteins in the hearts of wild-type and MCP mice. Immunohistochemical staining of ubiquitin in the hearts of wild-type controls (*A*) and MCP (*B*) mice of 6 mo of age. Note the absence or weaker cytoplasmic staining (brown) in cardiomyocytes of wild-type controls and the strong staining in cardiomyocytes undergoing degenerative changes such as vacuolation (arrow). Original magnification, ×400. *C*: Western blotting of heart homogenates from wild-type controls and MCP mice of 2, 4, and 6 mo of age showing marked accumulation of ubiquitinylated proteins in the hearts of MCP mice. *D*: elevated expression of ubiquitin fold modifier 1 (Ufm1) in the hearts of MCP mice determined by quantitative real-time PCR. *Significantly different from wild-type controls ($P < 0.05$).

Table 1

Primer sequences used in real-time PCR

Sequence No.	Gene Name	Primer Sequences (Mouse)	Accession No.	Product Size, bp
1	Asparagine synthetase	Forward: GAACACAGACAGCGTGGTG Reverse: CGGAGAACATCAAACAGGT	NM_012055	152
2	PDI	Forward: AACGGGAGAAGCCATTGTA Reverse: AGGTGTCATCCGTCAGCTCT	AW045202	156
3	Ribophorin	Forward: GCCAGGAAGTGGTGTTTGTT Reverse: CCAGAGGATTGGGTTCTTCA	D31717	158
4	Cai	Forward: CTCTCCGGGAATTTGTCACA Reverse: ATGTCGTTGGCAGTAGCATC	NM009787	280
5	ERO1	Forward: AGCAAACAGCACCAAAGAA Reverse: TGGTCCTGCGAATCATCATA	NM026184	129
6	CHOP	Forward: TATCTCATCCCCAGGAAACG Reverse: CTGCTCCTTCTCCTTCATGC	NM007837	258
7	ATF6	Forward: TGGGAGTGAGCTGCAAGTGT Reverse: ATAAGGGGGAACCGAGGAG	XM129579	276
8	TRB3	Forward: CGCTTTGTCTTCAGCAACTG Reverse: CAAGTCGCTCTGAAGGTTCC	AF358868	354
9	BiP/Grp78	Forward: ACCTGGGTGGGGAAGACTTT Reverse: TCTTCAAATTTGGCCGAGT	NM022310	232
10	Grp94	Forward: TGGGTCAAGCAGAAAGGAG Reverse: TCTCTGTTGCTTCCCGACTT	NM011631	212
11	HSP25	Forward: CCTCTTCGATCAAGCTTTCG Reverse: GCCTTCCTTGGTCTTCACTG	NM013560	276
12	HSP40	Forward: CGGTTCCGAATCAAAGTTGT Reverse: GGGAAATATGGACCTTGTG	NM018808	148
13	HSP70	Forward: TGCAGCAGGACATCAAGTTC Reverse: TACGCCTCACGCAGTCTCCTT	AJ002387	157
14	Ufm1	Forward: CCGTTCACGCAGTGCTAAA Reverse: CCGTGCTTCAGGAAAACA	NM026435	130
15	β-Actin	Forward: GAAACAGCTTCAGCCCCAAA Reverse: ACGTCAGGTGACTGCTGAA	NM_007393	216

PDI, protein disulfide isomerase; ERO1, endoplasmic reticulum oxidoreductase; CHOP, C/EBP homologous protein; ATF6, activating transporter factor; TRB3, *tribbles*-related protein 3; HSP25, HSP40, HSP70, 25-kDa, 40-kDa, and 70-kDa heat shock protein; Ufm1, ubiquitin fold modifier 1.

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Table 2

ER stress-related genes with fold changes in the hearts of MCP mice

Gene Name	Accession No.	*Fold Change with Age				
		2 wk	1 mo	2 mo	4 mo	6 mo
CHOP	X67803	1.8	6.7	6.5	3.9	3.2
BiP/GRP78	AJ002387	4.2	112	3.3	2.4	2.1
PDI	AW045202	6.3	24	6	4.3	3.4
PDI-related protein P5	A1842377	3.2	9.5	4.3	4.2	3.6
DnaJ (HSP40) homologous protein	AW120711	5.3	17	8.1	3.9	2.5
Similar to ER-associated DnaJ	AW122551	2.5	7.6	4.9	3.3	2.4
Calumenin	U81829	1.1	31	1.7	1.7	1.5
Asparagine synthetase	U38940	1.4	23	7.6	3.7	2.1
ATF6	AB012276	1.7	11	10	5.4	2.7
X-box binding protein	AW123880	1.8	13	2.1	1.1	1.8
Defender against apoptotic death	U81052	1.3	47	1.7	1.4	1.6
Stress associated ER protein 1	A1843466	4.5	12.2	4.2	3.9	7.7
Ribophorin	D31717	1.8	14	2.6	1.8	1.5
Ribophorin 1	A1846708	3.7	6.8	3.1	2.4	2.4
Oligosccharyl transferase	D89063	4.1	8.6	3.1	2.5	2.3
Translocation-associated protein	AW227650	1.8	22	2.2	1.8	2.1
ER-integral membrane protein P34	AW125178	1.1	17	2.2	1.9	1.9
Secretary complex (Sec61)	U11027	1.5	31	2.5	1.9	1.5
Secretary complex (Sec53)	AF007267	1.3	3	5.2	7.1	5
Secretary complex (sec23b)	A1848343	2.8	5.1	6.7	7.3	3.7
Ubiquitin-conjugating enzyme	X92665	2.1	3.3	2	1.3	1
Proteosome 26S subunit, non-ATPase	M64298	−1	15	1.8	2.2	2.5
Lysozyme M	M21050	5	15	1.8	2.2	2.5
Lysozyme P	X51547	10	26	3.7	4.5	7.5
Cai/ERp72	NM_009787			4.86	1.18	2.18
ERO1	NM_026184		3.83	3.92	1.75	1.92
Ufm1	NM_026435			4.08	1.51	1.15

ER, endoplasmic reticulum; MCP mice, transgenic mice that express monocyte chemoattractant protein-1 specifically in the heart.

*Fold change expression in the MCP mice/expression in the wild-type mice.