Role of heat shock factor-1 activation in the doxorubicin-induced heart failure in mice

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Vedam K, Nishijima Y, Druhan LJ, Khan M, Moldovan NI, Zweier J.J., Ilangoivan G. Role of heat shock factor-1 activation in the doxorubicin-induced heart failure in mice. Am J Physiol Heart Circ Physiol 298: H1832–H1841, 2010. First published April 2, 2010; doi:10.1152/ajpheart.01047.2009.—Treating cancer patients with chemotherapeutics, such as doxorubicin (Dox), cause dilated cardiomyopathy and congestive heart failure because of oxidative stress. On the other hand, heat shock factor-1 (HSF-1), a transcription factor for heat shock proteins (Hsps), is also known to be activated in response to oxidative stress. However, the possible role of HSF-1 activation and the resultant Hsp25 in chemotherapeutic-induced heart failure has not been investigated. Using HSF-1 wild-type (HSF-1+/+) and knockout (HSF-1−/−) mice, we tested the hypothesis that activation of HSF-1 plays a role in the development of Dox-induced heart failure. Higher levels of Hsp25 and its phosphorylated forms were found in the failing hearts of Dox-treated HSF-1−/− mice. More than twofold increase in Hsp25 mRNA level was found in Dox-treated hearts. Proteomic analysis showed that there is accumulation and aggregation of Hsp25 in Dox-treated failing hearts. Additionally, Hsp25 was found to coimmunoprecipitate with p53 and vice versa. Further studies indicated that the Dox-induced higher levels of Hsp25 trans-activated p53 leading to higher levels of the pro-apoptotic protein Bax, but other p53-related proteins remained unaltered. Moreover, HSF-1−/− mice showed significantly reduced Dox-induced heart failure and higher survival rate, and there was no change in Bax upon treating with Dox in HSF-1−/− mice. From these results we propose a novel mechanism for Dox-induced heart failure: increased expression of Hsp25 because of oxidant-induced activation of HSF-1 trans-activates p53 to increase Bax levels, which leads to heart failure.

heat shock factor-1; Hsf-1; Hsp25; oxidative stress; p53

ANTHRACYCLINES and tyrosine kinase inhibitor-based cancer therapeutics cause heart failure among cancer patients who are treated with these drugs (12, 13, 19, 26). Particularly, doxorubicin (Dox) or its other formulations induce cardiotoxicity, leading to congestive heart failure among cancer patients, because of irreversible structural changes in the myocardium (13). Although many approaches have been developed to minimize these serious side effects, they are still clinically problematic (13). Dox is known to kill cancer cells by DNA intercalation and Topoisomerase II inhibition. However, the loss of cardiomyocytes in the heart has been attributed to oxidative stress, caused by oxidants such as oxygen-derived free radicals (O₂•−, OH•) and generation of H₂O₂ (22). This difference in the mechanism gives hope that if the cell death in the heart is avoided by selectively targeting the cardiomyocyte death pathways, the intended outcome of Dox treatment could be greatly improved. Indeed supplementation of antioxidants or specific overexpression of endogenous antioxidants have been found to be a very effective in alleviating Dox-induced heart failure in animal models (18). However, some clinical trials with antioxidants such vitamin E did not show any significant improvement (13). Thus to realize the full spectrum of antioxidant-based therapy, the complete mechanism of cardiomyocyte death needs to be understood.

In addition to oxidative stress, Dox may trigger other signaling cascades that may have relevance to the observed toxicity. Heat shock factor (HSF) activation is one such consequence. Heat shock proteins (Hsps) are expressed in eukaryotic tissues by activation of the HSFs (HSF-1, HSF-2, HSF-3, and HSF-4) that are known to respond to various stresses. HSF-1 has been found to be a “multifaceted factor” involving in various pathogenesis (4). Upon oxidative stress, HSF-1 is trimerized and translocated to nucleus to transcribe of various Hsps. HSF-1 was found to be essential for early development of embryos because the complete knockdown of HSF-1 was found to increase prenatal mortality (2). In another study, it was shown that Hsp25 knockout in mice did not affect embryo development or impair normal physiological function of any organ (17). Thus Hsp25 is an essential constitutive protein, but with either increased expression or decreased degradation due to the exerted stress, it causes a redox imbalance leading to tissue damage, especially in the heart (24). Many studies have explored the role of Hsp27 (the Hsp25 ortholog) in the heart (10). In our previous studies we found that treating immortalized cardiac H9c2 cells (derived from neonatal rat heart) with Dox increased Hsp27 expression (31). It was also found that Hsp25 regulates transcriptional activity of p53 in these cells, leading to the notion that there may be an interplay between Dox-induced Hsp25 and p53 activation in the heart (30). In cardiac H9c2 cells and fibroblasts, higher Hsp25 was observed to enhance the induction of p21, a cell-cycle arrest protein, and cell survival. However, cardiomyocytes in the heart are terminally differentiated, and it is not known whether p53 activation leads to death signaling instead of survival pathway. There are many other reports that overexpression of small Hsps, such as Hsp27, protects against oxidative stress, apoptosis, and other cell death pathways, in many cell types, including cardiomyocytes (10, 16). Paradoxically, however, there is compelling evidence that elevated level of Hsp27 is present in failing hearts (8). Thus it is not clear whether this observed increase in Hsp27 is the result of an unsuccessful protective mechanism or whether the increased expression of Hsp27 indeed potentiated the loss of myocytes in the heart. In the present work, we report that HSF-1 activation indeed plays a major role in the observed
cardiotoxicity of Dox. We have found that an HSF-1 activation result in increased expression of Hsp25 and accumulation of Hsp25 in the heart transactivates p53, a tumor suppressor protein, leading to an increase in pro-apoptotic protein Bax.

METHODS

Animals and Treatment

HSF-1−/− mice were crossed with wild-type Balb/c mice to generate heterozygous HSF-1−/+ mice, which were further crossed and back crossed to obtain homozygous HSF-1−/− mice with Balb/c background. Eight-week-old HSF-1 wild-type (HSF-1+/+) and knock-out (HSF-1−/−) mice were used in the present study. Mice, both HSF-1−/+ and HSF-1−/−, were divided into two groups. In the first group, doxorubicin hydrochloride (6 mg/kg) was administered to mice once a week for 3 wk by intraperitoneal injection, and in the second group saline was injected as placebo (control group). This study was reviewed and approved by the Institutional Laboratory Animal Care and Use Committee at The Ohio State University, carried out according to the approved guidelines, and conforms with the Guidelines for the Care and Use of Laboratory Animals published by the National Institutes of Health.

Echocardiographic and Electrocardiographic Evaluation

Conventional two-dimensional (2D), M-mode echo was performed on control and Dox-treated mice (sedated with isoflurane, 0.5–1%) restrained in the left lateral decubitus position on a customized table with heating pad. 2D short-axis echo was performed at the level of the midpapillary muscle. All measurements were averaged from a minimum of three cardiac cycles. LV end-diastolic and end-systolic internal diameter (LVIDd and LVIDs, respectively) were measured. LV fractional shortening (%FS) was calculated as described in supplemental methods (6). In addition to echo measurements, ECG of these animals was measured once a week throughout the study. The ECG data were recorded for 2 min, and the ST interval duration was measured.

Real-Time RT-PCR For HSP25 mRNA Determination

The mRNA levels of Hsp25 in control and Dox-treated hearts were determined by real-time RT-PCR. Animals were treated with either Dox or saline for 3 wk as described above. At the end of 8 wk, the heart failure was confirmed by echo and the hearts were isolated. Total RNA was extracted using the standard procedure provided by the manufacturer (see Supplemental methods).

Immunostaining of HSP25 in Heart Tissues

Control and Dox-treated failing hearts were collected and stored in formalin and then embedded in paraffin. Tissue sections of 4 μm were cut and stained with a primary anti-Hsp25 antibody (Stressgen) as described in the supplementary methods.

Proteomics

Heart tissue lysates were prepared for proteomic analysis as detailed out in the supplementary methods. Immunoprecipitation and immunoblotting and electrophoretic mobility super shift assay (EMSSA) were carried out as described in the supplementary methods.

Two-dimensional Western blot analysis and mass spectral proteomic analysis. Both control and Dox-treated protein samples were subjected to 2D gel electrophoresis (supplemental methods). The protein spots from 2D gel, confirmed as Hsps by matrix-assisted laser desorption/ionization (MALDI), were subjected to liquid chromatography coupled tandem mass spectrometry (LC/MS/MS). Peptide Mass Tolerance was chosen ±2 Da and Fragment Mass Tolerance was chosen ±0.6 Da. ESI TRAP was the instrument type selected. Selected tandem mass spectrometry (MS/MS) spectra were searched against the data base. To confirm the presence or absence of phosphate, search parameters included the differential modification of +80 Da on Ser, Thr, and Tyr.

Death Curve and Weight Loss

A set of animals Balb/C (n = 20) and HSF-1−/− (n = 20) were used for the study. These mice were further divided into two groups (control and Dox-treated). The Dox-treated group was injected with 6 mg/kg body wt of Dox once a week for 3 wk and monitored for loss in body weight and survival time of the animals (3). The Kaplan-Meier curve was plotted to study the survival rate in the animals belonging to different groups.

Statistics

Data are presented as means ± SE. Statistical analysis was performed using Student’s t-test and one-way ANOVA. The general acceptance level of significance was P < 0.05. Kaplan-Meier curves were analyzed using Log-Rank test to determine the significance of the difference in survival.

RESULTS

Hsp25 Aggregation in Dox-Treated Failing Hearts

Hemodynamic and contractile function in Dox-treated mice were subjected to be measured once a week for 8 wk. From the third week onwards a decrease in cardiac function was observed (see Supplemental data) (11). Figure 1A shows Western blots of Hsp25 and s-15 and s-86 phosphorylated Hsp25 obtained from control (placebo treated) and Dox-treated failing heart tissue extracts. More than a sixfold increase in Hsp25 was observed in Dox-treated failing hearts when compared with saline-treated control hearts, and similar increases in s-15 and s-86 phosphorylated Hsp25 (more than four- and threefolds, respectively) were observed. However, the ratio of both s-15 p-Hsp25 to total and s-86 p-Hsp25 to total Hsp25 was close to unity, indicating that there was no net increase in phosphorylated Hsp25 in the Dox-treated hearts (Fig. 1B). The densometric intensity plots of total Hsp25 and s-15 and s-86 phospho Hsp25 are shown in Fig. 1 (n = 5 in each group). Effect of Dox treatment on other major Hsps was also analyzed. There was no significant difference in the inducible Hsp70 (Fig. 1), which is known to be induced by oxidative stress between control and Dox-treated groups. However, Hsp90 level was found to increase by Dox treatment. Both Hsp25 and Hsp90 are present in normal hearts, but in pathological hearts these proteins are known to be higher due to stress-induced activation of HSF-1 (24). Figure 1, C and D, shows immunohistochemical tissue staining for Hsp25 in control and Dox-treated heart tissues, respectively. When compared with control group (Fig. 1C), the Dox-treated heart tissue (Fig. 1D) showed more tissue damage and higher Hsp25 compared with control group. Figure 1, E and F, are the negative controls (i.e., void of Hsp25 antibody) for Fig. 1, C and D, respectively. This is confirmed in a magnified view of striated tissue and demonstrating that the expression was in cardiomyocytes.

To determine whether there is higher transcription of this protein, HSF-1 binding activity (by super shift assay) and Hsp25 mRNA measurements (real-time RT-PCR) were carried out, and the results are shown in Fig. 2. Nuclear protein extracts of control and Dox-treated mice hearts were analyzed for binding with heat shock element (HSE) consensus sequence. As shown in Fig. 2A, increased binding was observed in Dox-treated group when compared with control. This was further confirmed by adding HSF-1...
antibody, where a positive super shift was observed (Fig. 2A). However, upon preincubation with HSE without biotin label (cold HSE) did not show any band (Fig. 2A). Real-time RT-PCR showed that there was a twofold increase in mRNA in Dox-treated group, showing that higher Hsp25 mRNA is transcribed upon treatment with Dox (Fig. 2B). The sixfold increase of Hsp25 protein level in Dox-treated hearts (Fig. 1A), despite only about twofold increase in mRNA (Fig. 2B), indicates there could be accumulation of Hsp25 in Dox-treated hearts.

Proteomics of Hsp25 in Dox-Treated Failing Hearts

Detailed studies on Dox-induced Hsp25 and its correlation to cardiac dysfunction were carried out by using proteomics. Both control and Dox-treated heart tissue extracts were subjected to 2D electrophoresis. Figure 3A shows a colloidal Coomassie blue-stained 2D gel image (left) of the Dox-treated heart tissue extracts. More than 500 proteins spots were observed in an individual gel image of heart tissue extracts. Magnified view of the gel around 25-kDa molecular mass ranges is shown in Fig. 3B. Spots in the 25-kDa range \( n = 13 \) were found to correspond to Hsp25 with \( Z \) scores higher than 2.3 (i.e., 99% probability), and these spots are marked as 1–6 in the magnified view of the gel image given in Fig. 3B. Western transfer of 2D gel was carried out, the membrane was probed with Hsp25 antibody, and the results of control and Dox-treated heart tissue extracts are shown in Fig. 3, C and D. The Western blot for the saline-treated group showed three prom-

Fig. 1. Heat shock protein (Hsp)25 overexpression in doxorubicin (Dox)-treated failing hearts of mice. A: representative Western blots of total, s-15-, and s-86-phosphorylated Hsp25, inducible Hsp70, and Hsp90. B: quantitative densitometric plots of the ratio of s-15- and s-86-phosphorylated Hsp25 to total Hsp25, normalized with respect to GAPDH \( n = 5 \). The error bars are means \( \pm \) SE. C and D: immunohistochemical staining of Hsp25 in control and Dox-treated failing heart tissues. When compared with control, in Dox-treated hearts, the Hsp25 is higher in cardiomyocytes (inset). E and F: negative controls (void of primary Hsp25 antibody).

Fig. 2. Increased heat shock factor-1 (HSF-1) binding to heat shock element (HSE) and Hsp25 mRNA generation in Dox-treated mouse hearts. A: nuclear extracts were prepared from control and Dox-treated mouse heart tissues and incubated with HSE tagged with biotin. Binding was analyzed by Western blotting of biotin. Column 1, HSE alone; column 2, HSE and HSF-1 antibody; column 3, HSE and nuclear extract of saline-treated mouse heart (control); column 4, HSE and nuclear extracts of Dox-treated mouse; column 5, HSE, nuclear extract of Dox-treated mouse and HSF-1 antibody; column 6, Dox-treated house heart nuclear extracts incubated with cold HSE (not labeled with HSE) and then loaded into gel. When compared with control, HSF-1 binding to HSE is higher in Dox-treated mouse hearts. Consistently addition of HSF-1 antibody (ab), shifted the band. B: mRNA level of Hsp25 in control and Dox-treated failing hearts \( n = 3 \), normalized with 18s mRNA.
inent spots at pI 4.99, 5.39, and 5.97 (Fig. 3C), corresponding to three (namely 1, 4, and 6) of the six Hsp25 spots identified on the Coomassie-stained gel (Fig. 3B), whereas the Dox-treated sample demonstrated a continuum of spots immunopositive for Hsp25 (Fig. 3D), including all the six spots (1 to 6) observed on the Coomassie-stained gel (Fig. 3A). Comparison of pI for control and Dox-treated samples showed that the spots 1, 4, and 6 are observed as common in both control and Dox-treated samples, whereas other spots in the Dox-treated sample appear as additional protein spots. The additional spots and the continuum in the Western blots of Dox-treated hearts indicated that there is an increased accumulation and aggregation of Hsp25 in Dox-treated hearts, likely as response to the oxidative stress caused by the redox cycling of Dox, which in turn induced the activation of HSF-1 (23). Previous studies using 2D Western blots of Hsp27 in pathogenic human hearts have assigned the three spots (at pI 4.99, 5.39, and 5.97) to biphosphorylated Hsp27 (s-15 and s-82), monophosphorylated Hsp27 (s-82), and unphosphorylated Hsp27, respectively (5).

However, the additional spots, namely 2, 3, and 5 (and the other unnumbered spots in Fig. 3D) have not been previously reported.

Additional experiments were carried out to determine whether the multiple spots correspond to different phosphorylation status of Hsp25. Figure 3E shows the 2D Western blots obtained for control and Dox-treated heart tissue extracts, probed with s-15 and s-86 phospho-Hsp25 (probed with phospho-specific antibodies) in control and Dox-treated failing hearts. F: schematics of Hsp25 oligomerization/deoligomerization upon change in pH and phosphorylation status.
case of Dox-treated samples, all the three spots appeared when probed for phospho s-15 antibody. However, intensity of these spots was higher than the control but less intense than total Hsp25 (Fig. 3D). It appears that the additional spots in the case of Dox-treated sample are due to distribution of Hsp25 aggregates of different sizes, which are separated in first dimension due to pH gradient. Also, these aggregates have both phospho and unphosphorylated Hsp25. Intensity differences show that the phosphorylated fraction is less than the unphosphorylated fractions in these aggregates. Figure 3F shows a schematic illustration how phosphorylation/dephosphorylation leads to association/dissociation of Hsp25 as reported previously (15). The present results show that change in pH can also trigger such an aggregation/disaggregation. Thus the spots distribution in 2D Western blots seems not solely governed by phosphorylation status of Hsp25 alone, as previously thought, but also by other factors such as pH depended oligomerization of these proteins. Previous studies have shown that Hsp25 aggregation did indeed depend on pH (21) especially at pH values <6. However, independent and elaborate studies are native gels/ sucrose gradients are required for analysis of the aggregation/dissociation analysis.

Further studies using LC/MS/MS analyses were carried out to characterize posttranslational modifications of Hsp25 in Dox-treated heart tissue. All six spots of the 2D gel identified as Hsp25 by MALDI-TOF were digested by using trypsin, purified, and analyzed by MS/MS. All the six spots yielded peptide sequences matching Hsp25 (70%). In all tryptic digests from the Dox-treated heart tissues, a doubly charged ion at m/z 538.5 ± 0.3 was observed, which was shown by collisional fragmentation and MS/MS to correspond to QLSSGVEISR representing residues 84 to 93 of Hsp25 (Fig. 4). Based on previous studies of Hsp27, this m/z is for the nonphosphorylated peptide (5, 29). Mass difference between COOH terminal containing fragments y6 and y7 as well as y7 and y8 is close to 87 (Fig. 4), confirming that there is no phosphorylation of serine residues in this peptide. There was no detectable peak with 95 m/z increase (corresponding to PO4 group mass) for any of the six spots studied in Dox-treated heart tissue extracts, indicating that none of the spots correspond to phosphorylated protein as a major constituent. This is consistent with the Western blots presented in Fig. 3, C–E, that the distribution of Hsp25 is due to Hsp25 aggregates of different size, and these aggregates have more nonphosphorylated Hsp25 and contain only a small fraction of phospho-Hsp25 (Fig. 3, C and D). Similarly, a doubly charged ion at m/z 503.53 was observed in all the six spots of Dox-treated heart tryptic digests, which was shown by MS/MS to correspond to SPSWEPFR, residues 13 to 20 of Hsp25. From the m/z, it is clear that s15 is not phosphorylated [expected m/z for SPSWEPFR is 543.5 (5, 29)].

**Hsp25 Binding to P53 and Regulation of Its Transcriptional Activity**

Another important result of the present study is the finding that Hsp25 interacts with p53 and regulates its transcriptional activity in the Dox-treated heart. First, we determined the p53 levels in Dox-treated mouse hearts. There was an elevated level of p53 in Dox-treated animals (Fig. 5A), agreeing with recent report (34). Upon immunoprecipitation (IP) of p53 from control and Dox-treated heart tissue extracts, we found that Hsp25 coimmunoprecipitates with p53. Figure 5B shows a representative gel of a p53 IP from control and Dox-treated heart tissue extracts. In addition to the 53-kDa band corresponding to p53, there is a distinct band at 25 kDa in control and Dox-treated heart tissue extracts. For identification of this protein, two different approaches were applied. First, we carried out MALDI-TOF fingerprint printing of the identified 25-kDa protein extracted from the IP gel. These results definitively identified this protein as Hsp25 (with z score >2.3). Second, immunoblotting was carried out for quantitative determination. Immunoblotting with anti-Hsp25 antibodies confirmed that this protein was Hsp25 (Fig. 5C) and indicated that intensity of the Hsp25 band in immunoblots was higher in Dox-treated samples (Fig. 5C). This band was absent when antibody alone immunoprecipitated without lysate, ruling out the possibility that this band corresponds to IgG light chain. Moreover, p53 intensity was also higher for the immunoprecipitates from samples with the same amount of total protein (1 mg) in Dox-treated tissue extracts, indicating that Hsp25 binding potentially stabilized p53. However, the magnitude Hsp25 association seen in the p53 immunoprecipitates was less than the sixfold increase observed with Western blots of whole cell lysates (Fig. 1A). When Hsp25 was immunoprecipitated with Hsp25 monoclonal antibody, p53 was found to coimmunoprecipitated (Fig. 5F).

Next, the question of whether nonphosphorylated or phosphorylated Hsp25 binds to the p53 was addressed. The p53 immunoprecipitates of control and Dox-treated heart tissue extracts were immunoblotted with phospho s-15- and s-86-specific antibodies, and the results are shown in Fig. 5D. The s-15 blots did not show any significant band intensity compared with the s-86 bands (Fig. 5D). Moreover the s-86 band of Dox-treated samples showed great than fivefold higher intensity compared with that of the control in whole cell lysate Western blots (Fig. 1A). These results together indicated that s-86 phosphorylated Hsp25 binds with p53 upon treating with Dox. However, there is no change in the Hsp90 association with p53 (Fig. 5F), although there was about sixfold increase.
in Western blots (Fig. 1A). Further studies focused on how Hsp25 binding to p53 alters its function in Dox-treated samples. To address this issue, the expression of p53 target proteins such as Bax, Bcl2, and p21 MDM2 was determined in control and Dox-treated tissue lysates; the results are summarized in Fig. 5G. Whereas there was no significant change in p21, MDM2, and Bcl2 expressions, Bax expression was observed to be more than 10-fold higher in Dox-treated hearts than control hearts. In summary, these results indicate that Dox-activated mitogen-activated protein (MAP) kinase activated protein-2 (MAPKAP-2) phosphorylates Hsp25 at s-86 in response to oxidative stress and the phospho-Hsp25 transactivates p53, leading to the expression of Bax, which induces the apoptotic death of cardiomyocytes in the Dox-treated hearts. To confirm the apoptosis due to higher Bax in Dox-treated samples, the poly(ADP-ribose) polymerase-1 (PARP-1) poly(ADP-ribose) polymerase-1 (PARP-1) (89 kDa band) was observed to be higher in Dox-treated heart lysates due to increased caspase-3 activity, consistent with higher Bax.

**HSF-1 Knockout Improved Heart Function and Survival Against Dox**

To further confirm the interplay between Hsp25 and p53 in the Dox-induced cardiotoxicity, the following experiments were carried out. 2D M-mode echocardiograms (Fig. 6A) showed that contractility of Dox-injected HSF-1−/− mice was higher than that in Dox-treated HSF-1+/+ mice. The quantitative data on fractional shortening (%FS) and LV mass, as shown in Fig. 6, B and C, indicate a significant improvement upon treatment for Dox-treated HSF-1−/− mice compared with HSF-1+/+ mice (Fig. 6, B and C). Additionally, significant differences in weight loss were noticed, following a similar pattern among these groups (Fig. 6E). Whereas Dox-only-injected HSF-1+/+ mice showed at least a 30% loss in weight...
in the third week, there was no significant change in weight for Dox-treated HSF-1\(^{-/-}\) mice (Fig. 6E). The body weight loss upon treatment with Dox has been attributed to systemic in vivo toxicity (7). A previous study with Balb/c mice showed 33.5% loss of body weight (7), which is consistent with our present observation (Fig. 6E). However, it is important to note that the body weight loss was 5% in HSF-1\(^{-/-}\) group, indicating less systemic toxicity by Dox on this group. Figure 6F illustrates the survival of both HSF-1\(^{+/+}\) mice and Dox-treated HSF-1\(^{-/-}\). In wild-type mice, 50% survival was observed at 21 ± 3 days after Dox treatment. However, for HSF-1\(^{-/-}\) mice, the 50% survival was found to be beyond 33 ± 2 days. In this case, 40% of the animals survived until the study was completed (100 days), unlike the other case where almost all the animals died by end of the study (Fig. 6F). In Dox-treated HSF-1\(^{-/-}\) hearts, there was no increase in Hsp25. In HSF-1\(^{-/-}\) mice there was no increase in the association of Hsp25 with p53 (Fig. 6D). Similarly, there was no change in other proteins such as Bax, Bcl2, and PARP-1 (Fig. 6D). These results show that HSF-1 activation and Hsp25 induction is integral part of Dox-induced heart failure.

**DISCUSSION**

The primary finding in the present study is that Dox treatment increases accumulation of Hsp25 in the heart due to activation of HSF-1, and the accumulated Hsp25 transactivates p53 to increase the transcription of pro-apoptotic protein Bax. Previous studies have revealed several aspects of the mechanism of Dox-induced heart failure, using various cellular and animal models and human heart studies (22). In another study, ERK activation and upregulation of Hsp60 in Dox-treated rat hearts were found to protect the heart against ischemia-reperfusion injury (25). However, none of these studies have revealed the role of HSF-1 activation and dynamic induction of Hsp25 in Dox-treated hearts, as a response to the oxidative stress, although in a recent study Hsp20 has been found to increase in mouse hearts upon treatment with Dox (9). The present study is the first to demonstrate a link between HSF-1 activation and Dox-induced heart failure and that Hsp25 could be triggering a signaling cascade causing the loss of cardiomyocytes. More importantly, the present study shows that targeting HSF-1 or suppression of Hsp25 accumulation may be a potential therapeutic approach to prevent heart failure in Dox-treated patients.
The role of small Hsp such as Hsp27 in cardiovascular disease has been controversial. Earlier studies indicated that overexpression of human Hsp27 (homologue of murine Hsp25) is beneficial in terms of ischemia-reperfusion injury (16). However, contrary to this, deletion of CRYB and HSPB2 double knockout was recently found to be protective against acute ischemia reperfusion injury in aged mice (1). In the present work, we found that Dox treatment increased the accumulation of Hsp25 in the heart, which potentiates heart failure in a chronic model, where the Dox injection was spread over 3 wk. Previous studies with rats also reported increased Hsp25 during ischemic heart failure (27). Overall, it appears that the response of overexpressed Hsp25 or Hsp27 to acute stresses such as acute ischemia-reperfusion injury is different from that of a chronic oxidative stress response via HSF-1 activation and subsequent slow and steady accumulation of Hsp25. This finding is further supported by a recent study, showing that Hsp25 accumulation in the heart may alter the redox balance, making the heart more susceptible to cardiovascular disease (24). Other heart failure models have also shown higher levels of Hsp25 in failing hearts, although its role in the pathogenesis of heart failure has not been elucidated. For example, double transgenic mice overexpressing human rennin and angiotensinogen die due to terminal congestive heart failure, and these hearts were shown to express higher amounts of Hsp27 (32). Similarly, coronary artery ligation-induced heart failure in rats showed higher Hsp27 compared with that in control animals (28), and in explanted human hearts with dilated cardiomyopathy, more than a twofold increase in Hsp27 was noticed (20). Our results in the present study are consistent with these published reports that there is increased Hsp25 in failing hearts. However, for the first time, we have defined the pathological role of the higher level of Hsp25 that increased Hsp25 potentiates the heart failure by transactivating p53 (Fig. 6).

Proteomic analyses of Hsp25 have shown that extensive oligomerization of Hsp25 occurs when the protein is over accumulated due to activation of HSF-1 (Figs. 2 and 3). Phosphorylated Hsp25 is increased in Dox-treated hearts (Fig. 1); however, the ratio of phosphorylated Hsp25 to nonphosphorylated Hsp25 is not altered. At the same time various MAP kinases are also expressed due to oxidative stress, and the p38MAPK has been shown to phosphorylate the downstream target MAPKAP-2, which in turn phosphorylates Hsp25(see supplementary data) (33). Although the aggregation/disaggregation behavior of Hsp25 has been found to be regulated by phosphorylation of Hsp25, the observation of six spots in the 2D Western blots (Fig. 3) separated by very narrow pI (observed almost as continuum) cannot be attributed by phosphorylation status alone. Similar to our Dox-induced heart failure, Dohke et al. (8) observed an increase in the number of Hsp27 spots in 2D Western blots of congestive failure hearts lysates in dog, caused by increased pacing (tachycardia). Prominently, three spots were observed in control hearts. These three spots are assigned to nonphosphorylated, monophosphorylated, and diphosphorylated Hsp27 (5, 29). In some studies Pro-Q staining (a marker of phosphorylated proteins) has stained all the three spots, and these studies have described this as mono-, di-, and triphosphorylated Hsp27 (8). However, our present study using MS/MS and phospho-specific antibodies has confirmed that all the three spots were predominantly nonphosphorylated, rather only a fraction of each spot represents a phosphorylated species in these aggregates (Figs. 3 and 4). This indicates that separation of Hsp25 in the first dimension (i.e., pH gradient) of the 2D gel is primarily determined by Hsp25 concentration and local pH-dependent aggregation [especially at pH < 6 (21)] than phosphorylation status. Indeed, previous studies on the aggregation pattern of this protein have reported that aggregation behavior is dependent on both the pH and phosphorylation status of the protein. Aggregation has been found to be pH independent about pH 7, but at lower pH ranges the aggregation depended on pH (21). Moreover, the aggregates were also found with phospho- and nonphospho-Hsp25. Thus the different spots observed in the 2D Western blots are aggregates of...
Hsp25, of different sizes, and with both phosphorylated and nonphosphorylated Hsp25.

Another important finding in the present work is that Hsp25 transactivates p53, a protein that controls the transcription of many survival as well as pro-apoptotic proteins. We have shown that p53 and s-86-phospho Hsp25 communoprecipitate, indicating that phosphorylation of Hsp25 induces an interaction with p53 and in Dox-treated hearts, the association was higher (Fig. 5, B and C). Although nothing is previously known about such a Hsp25/p53 interaction in the heart, recently it was proved that HSF-1 knockout suppressed cutaneous tumorgenesis (4) and proposed that HSF-1 may regulate p53 transcriptional activity. Recently, our group demonstrated that pifithrin-α treatment protected immortalized cardiac cells from Dox-induced toxicity, in a Hsp25- p53-dependent manner (30). Although these results are, in general, in agreement with the perception that Dox-induced apoptosis is primarily responsible for the loss of cardiomyocytes in the heart and eventually to the heart failure, recently, activation of mammalian target of rapamycin (mTOR) has been reported to protect the heart from Dox-induced heart failure. Indeed these authors have proposed that the apoptosis could only be a secondary effect to the mTOR signaling in the overall pathogenesis (34). However, there is no study reported in the literature so far linking Hsp25 to mTOR function, and hence further studies required to delineate the relationship between the Hsp25 and mTOR. Similarly, various in vitro studies have shown that Hsp27 overexpression attenuated Bax activation (14). Thus the difference in the function of Hsp27/bax in vivo and in vitro has not been fully understood. Also, it is possible that Hsp25 stabilized p53 can interact either with MDR1 and MDR2, multidrug resistance genes.

From the results of our present study, we propose a novel mechanism of Dox-induced toxicity illustrated in Fig. 7. Dox-induced oxidative stress activates the HSF-1 producing more Hsp25, which in turn binds and transactivates p53, resulting in the transcription of Bax. Bax targets mitochondria to trigger apoptosis and produces a slow loss of cardiomyocytes in the heart. Moreover, phosphorylation status of p53 and its transcription from either nucleus or cytoplasm to mitochondria may also be an important factor for complete understanding of the role of p53 in the Dox-induced cardiotoxicity. Overall, the present study has found that the increased induction of Hsp25 in Dox-treated hearts sensitizes the cardiomyocyte death. Immunoprecipitation and immunoblotting have confirmed the association of Hsp25 and p53. Survival also increased in the HSF-1−/− mice (Fig. 6E), where there was no significant accumulation of Hsp25. Hence, Dox-induced oxidative stress and HSF-1 activation plays an important role in the development of heart failure in Dox-treated mice.

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DISCLOSURES

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

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