HAX-1 regulates cyclophilin-D levels and mitochondria permeability transition pore in the heart

Chi Keung Lam1, Wen Zhao1, Guan-Sheng Liu, Wen-Feng Cai, George Gardner, George Adly, and Evangelia G. Kranias2

Department of Pharmacology and Cell Biophysics, University of Cincinnati College of Medicine, Cincinnati, OH 45267-0575

Edited by Andrew R. Marks, Columbia University College of Physicians & Surgeons, New York, NY, and approved October 21, 2015 (received for review May 5, 2015)

The major underpinning of massive cell death associated with myocardial infarction involves opening of the mitochondrial permeability transition pore (mPTP), resulting in disruption of mitochondria membrane integrity and programmed necrosis. Studies in human lymphocytes suggested that the hematopoietic-substrate-1 associated protein X-1 (HAX-1) is linked to regulation of mitochondrial membrane function, but its role in controlling mPTP activity remains obscure. Herein we used models with altered HAX-1 expression levels in the heart and uncovered an unexpected role of HAX-1 in regulation of mPTP and cardiomyocyte survival. Cardiac-specific HAX-1 overexpression was associated with resistance against loss of mitochondrial membrane potential, induced by oxidative stress, whereas HAX-1 heterozygous deficiency exacerbated vulnerability. The protective effects of HAX-1 were attributed specific down-regulation of cyclophilin-D levels leading to reduction in mPTP activation. Accordingly, cyclophilin-D and mPTP were increased in heterozygous hearts, but genetic ablation of cyclophilin-D in these hearts significantly alleviated their susceptibility to ischemia/reperfusion injury. Mechanistically, alterations in cyclophilin-D levels by HAX-1 were contributed by the ubiquitin-proteosomal degradation pathway. HAX-1 overexpression enhanced cyclophilin-D ubiquitination, whereas proteosomal inhibition restored cyclophilin-D levels. The regulatory effects of HAX-1 were mediated through interference of cyclophilin-D binding to heat shock protein-90 (Hsp90) in mitochondria, rendering it susceptible to degradation. Accordingly, enhanced Hsp90 expression in HAX-1 overexpressing cardiomycocytes increased cyclophilin-D levels, as well as mPTP activation upon oxidative stress. Taken together, our findings reveal the role of HAX-1 in regulating cyclophilin-D levels via an Hsp90-dependent mechanism, resulting in protection against activation of mPTP and subsequent cell death responses.

HAX-1 | necrosis | mitochondrial permeability transition | cyclophilin-D | heat shock protein-90

The dynamics of contraction and relaxation in the heart are highly regulated by a finely tuned cross-talk between demand by the periphery and energy production by the mitochondria. The heart relies on mitochondria for fueling ATP on a beat-to-beat basis and for adjusting mitochondrial metabolism to meet the increases in cardiac output during exercise and flight-or-fight responses (1, 2). Mitochondria are also essential in controlling cell death through apoptosis, necrosis, and autophagy (3, 4). Indeed, disruption of mitochondrial integrity underlies cardiomyocyte death associated with myocardial infarction (5) that attributes to one of every six deaths in the United States (6).

The mitochondrial hematopoietic-substrate-1 associated protein X-1 (HAX-1), a 35-kDa protein has recently emerged as a critical regulator of cell survival in various tissues (7–9). Indeed, global ablation of HAX-1 was shown to result in death by 14 wk of age due to neuronal degeneration from excessive cell death (10). In addition, a recent study demonstrated that degradation of HAX-1 triggers cell death in human B-cell lymphoma, further supporting the pivotal role of HAX-1 in dictating cell survival (11). Although HAX-1 has been studied in various cell types since its discovery in 1997 (7), its presence in cardiomyocytes was only recently identified. Interestingly, cardiac HAX-1 localizes not only to mitochondria but also the endo/sarcoplasmic reticulum (ER/SR) and it binds to phospholamban, increasing inhibition of the SR calcium transport ATPase and reducing SR calcium cycling. As cardiomyocyte contraction-relaxation is coupled to calcium oscillation, HAX-1 is an important regulator of cardiomyocyte contractility (12, 13). HAX-1 can also modulate ER stress responses by inhibiting the inositol requiring enzyme-1 (IRE-1) signaling arm, which diminishes cell death through reduction of proapoptotic transcription factor expression and caspase activation in an ischemic insult, leading to improved contractile recovery during reperfusion (14). However, HAX-1 also localizes to mitochondria in the heart (13), but its role in this organelle is unknown. Importantly, human mutations in HAX-1 have been linked to mitochondrial function. These mutations, resulting in loss of protein or activity, were discovered in a subgroup of severe neutropenia patients (8, 15, 16) and they were associated with loss of mitochondrial membrane potential in the neutrophils of the human carriers (16). Thus, HAX-1 deficiency may cause a defect in the mitochondrial membrane.

One of the mechanisms that regulate mitochondrial membrane integrity is the mitochondria permeability transition pore (mPTP). Thus, it is important to understand how this pore is regulated to prevent cell death. In this study, we reported that hematopoietic-substrate-1 associated protein X-1 (HAX-1) is an inhibitor of the pore and promotes cell survival. HAX-1 works through recruitment of a chaperone protein called Hsp90 from cyclophilin-D, a major component of the pore. Displacement of Hsp90 from cyclophilin-D promotes cyclophilin-D degradation, resulting in inhibition of pore opening and cell death. Given the opening of the mitochondrial permeability transition pore contributes to various diseases, our findings have broader applications reaching beyond the heart.

Significance

The massive cell death, associated with a heart attack, is mainly due to disruption of mitochondrial membrane integrity upon activation of the mitochondrial permeability transition pore. Thus, it is important to understand how the pore is regulated to prevent cardiac cell death. In this study, we reported that hematopoietic-substrate-1 associated protein X-1 (HAX-1) is an inhibitor of the pore and promotes cell survival. HAX-1 works through recruitment of a chaperone protein called Hsp90 from cyclophilin-D, a major component of the pore. Displacement of Hsp90 from cyclophilin-D promotes cyclophilin-D degradation, resulting in inhibition of pore opening and cell death. Given that the opening of the mitochondrial permeability transition pore contributes to various diseases, our findings have broader applications reaching beyond the heart.

Author contributions: C.K.L., W.Z., and E.G.K. designed research; C.K.L., W.Z., G.-S.L., W.-F.C., G.G., and G.A. performed research; C.K.L., W.Z., and G.S.L. contributed new reagents/analytic tools; C.K.L., W.Z., and G.S.L. analyzed data; and C.K.L. and E.G.K. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

1C.K.L. and W.Z. contributed equally to this work.
2To whom correspondence should be addressed. Email: litsa.kranias@uc.edu.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1508760112/-/DCSupplemental.

E6466–E6475 | PNAS | Published online November 9, 2015

www.pnas.org/cgi/doi/10.1073/pnas.1508760112
cardiac injury (5), because it allows solutes and ions that should be excluded from mitochondria to infuse into their matrix. Concomitantly, influx of water results in swelling of the originally compacted cristae structure, leading to complete mitochondria membrane failure and subsequent activation of massive necrotic cell death and fibrosis (5, 17). This injury is irreversible, posing greater damage to organs that possess limited regenerative capacity, such as the heart (5). Although the mitochondrial permeability transition pore was first described in 1976 (18), the full identity of its protein components remains obscure. Several constituents have been proposed (17), but only cyclophilin-D (Cyp-D), a peptidyl-prolyl cis-trans isomerase (PPI) in the mitochondrial matrix, has been a genetically proven regulator of mPTP (19). Ablation of this protein by gene targeting or pharmacological inhibition by cyclosporine A can effectively prevent the opening of the pore and inhibit the activation of cell death during ischemia/reperfusion injury (17, 19), demonstrating the crucial role of Cyp-D function in cell survival. Furthermore, Cyp-D activity in cancer cells can be regulated by a mitochondrial chaperone network that involves the cytosolic heat shock protein-90 (Hsp90) and its mitochondria analogs, which are crucial in inducing cancer cell death (20).

Based on our recent observations that Hsp90 is a newly identified binding partner of HAX-1 (14), it was intriguing to speculate that the Hsp90/HAX-1 interaction may be also involved in regulation of Cyp-D function in the heart. Thus, the current study was designed to address this hypothesis by using mouse models with alterations in HAX-1 levels. Our findings indicate that HAX-1 could specifically reduce Cyp-D levels by enhancing its ubiquitination and proteosomal degradation, which, in turn, protected mitochondria from oxidative stress and calcium overload. These beneficial effects of HAX-1 were mediated through Hsp90, which appeared to stabilize Cyp-D protein in cardiomyocytes. Taken together, our results reveal a novel mechanism by which the HAX-1/Hsp90 complex may inhibit activation of the mPTP via regulation of Cyp-D levels, and promote cardiomyocyte survival upon oxidative stress and calcium overload in ischemia/reperfusion injury.

Results

HAX-1 Preserves Mitochondria Membrane Integrity and Inhibits Cell Death. The integrity of the mitochondrial membrane is crucial in maintaining cardiomyocyte function and survival. Increases in the generation of reactive oxygen species or mitochondrial calcium overload during cardiac stress, such as ischemia/reperfusion injury, can disrupt mitochondrial membrane integrity, dissipate membrane potential for ATP production, and cause massive cell death (5). HAX-1 has recently emerged as a regulator of cardioprotection, and we have shown that it localizes to mitochondria besides ER/SR (13). To further address its localization, we isolated mitochondria (Fig. S1) and subjected them to protease digestion. HAX-1 was present in the inner mitochondrial membrane (Fig. S2), consistent with previous reports (10, 21). Thus, we sought to investigate whether HAX-1 may regulate mitochondria membrane potential and control activation of cell death. To examine this hypothesis, cardiomyocytes were isolated from HAX-1 overexpressing (HAX-OE, 2.5-fold expression; Fig. S3) and wild-type (WT), and HAX-1 heterozygous knockout (HAX−/+).

**Fig. 1.** Overexpression of HAX-1 protects mitochondrial membrane integrity against oxidative stress and calcium overload, whereas heterozygous ablation of HAX-1 has opposite effects. (A) HAX-1 protein expression in HAX-OE and HAX−/+ hearts. CSQ was used as loading control. (B and C) Isolated WT, HAX-OE, and HAX−/+ cardiomyocytes were loaded with mitochondrial membrane potential fluorescent dye, TMRE. Upon 2 mM H$_2$O$_2$ administration for 20 min, membrane potential was diminished in all groups, but this effect was most pronounced in the heterozygous cells. HAX-OE exhibited more than 80% preserved TMRE signals. (D) HAX-1 overexpressing mitochondria demonstrated resistance to swelling induced by 50 μM calcium. n = 3 hearts for each group. (E) HAX-1 overexpression in cardiomyocytes prevented cell death due to loss of plasma membrane integrity (E) and apoptosis (F) after 20 min of 2 mM H$_2$O$_2$ treatment, whereas HAX-1 heterozygous ablation increased cell death. n = 8 hearts for HAX-OE, 12 hearts for WT, and 13 hearts for HAX−/+ (>10,000 cells per heart). *P < 0.05 vs. WT at 2 mM H$_2$O$_2$. Data are expressed as mean ± SEM.

Lam et al. PNAS Published online November 9, 2015 E6467
(HAX+/−, 40% protein expression; Fig. 1A) (13) mice, and they were loaded with a mitochondrial localized fluorescent membrane potential indicator, TMRE, allowing us to monitor the membrane potential before and after hydrogen peroxide challenge, using confocal microscopy. Under basal conditions, the percentage of TMRE-positive cardiomyocytes was similar among the three groups (Fig. 1B and C). However, after administration of 2 mM hydrogen peroxide to induce oxidative challenge, both WT and HAX heterozygous cardiomyocytes lost some fluorescence, and by 20 min, only 30% of the heterozygous cells maintained the TMRE signal, compared with 50% of WT. In contrast, more than 70% of the HAX-overexpressing cardiomyocytes were resistant to 20 min of oxidative challenge (Fig. 1B and C). These findings suggest a protective role of HAX-1 in maintaining mitochondrial membrane potential during oxidative stress.

The protective effects of HAX-1 on mitochondrial integrity were also evidenced upon calcium overload, a situation that induces the opening of the permeability transition pore (mPTP), leading to failure of osmotic pressure control, water influx, and swelling of mitochondria (17). Isolated WT cardiac mitochondria demonstrated a steady loss of light scattered by the mitochondrial membrane structure after the addition of 50 mM calcium. However, HAX-1 overexpressing mitochondria were resistant under the same conditions and showed no sign of swelling (Fig. 1D). In contrast, isolated HAX-1 heterozygous-deficient mitochondria exhibited significant swelling under basal conditions. These findings together with the results from the hydrogen peroxide treatment indicate that HAX-1 may protect the mitochondrial membrane integrity.

To determine whether the protection of mitochondrial membrane by HAX-1 would translate to improved cell survival, we used isolated cardiomyocytes and assessed the degree of propidium iodide (PI) inclusion as an indicator of necrotic cell death. Treatment of cells with either 1 or 2 mM hydrogen peroxide for 20 min resulted in increases in Propidium iodide-positive cells. Notably, 2 mM hydrogen peroxide, which was associated with loss of mitochondria membrane integrity (Fig. 1C), resulted in a 15% increase in PI-positive WT cardiomyocytes, but this increase was significantly blunted in HAX-1 overexpressing cells. However, heterozygous loss of HAX-1 exacerbated the increase in cells with propidium iodide inclusion (Fig. 1E), consistent with the findings in the TMRE experiments. Similar results were obtained, when the expression of cell surface annexin V was quantified, as a marker of apoptotic cell death. Increasing HAX-1 levels effectively reduced apoptosis, whereas loss of HAX-1 elevated its activation under hydrogen peroxide stress (Fig. 1F). Thus, our data suggest that HAX-1 maintains the mitochondrial membrane potential and protects membrane integrity, resulting in reduced apoptosis and necrotic cardiomyocyte death.

HAX-1 Regulates the Opening of Mitochondrial Permeability Transition Pore. It has been demonstrated that mitochondrial membrane integrity highly depends on the function of mPTP. The opening of this pore is a key mediator of cardiac ischemia/reperfusion injury because it facilitates loss of mitochondrial membrane potential, induces swelling, and leads to the rupture of mitochondrial membrane and activation of cell death (5). To determine whether the HAX-1 regulatory effects in mitochondria are mediated through an mPTP-dependent mechanism, we assessed the effects of mPTP inhibition in cardiomyocytes. To this end, we treated...
cardiomyocytes with cyclosporine A (CsA), an mPTP inhibitor (17). CsA resulted in preservation of mitochondrial membrane potential in both WT and HAX-1 heterozygous cardiomyocytes exposed to hydrogen peroxide (Fig. 2A and B). Importantly, the levels of TMRE in these treated groups were similar to those in HAX-1 overexpressing cells (Fig. 1B), suggesting that the protective effects of HAX-1 may be mediated through the mPTP. To further confirm that the observed protection against loss of mitochondrial membrane potential by HAX-1 overexpression was not due to impaired function of the mPTP, we used a high calcium concentration (375 mM) in the swelling assay to induce mPTP opening in isolated mitochondria. Under these conditions, the swelling response of the HAX-1 overexpressing mitochondria was similar to that of WT mitochondria (Fig. 2C), suggesting that HAX-1 regulates the sensitivity of the mPTP opening. To further address this hypothesis, we determined the dose of cyclosporine A required for complete inhibition of mitochondrial swelling, induced by 375 mM calcium. Both WT and HAX-1 overexpressing mitochondria were resistant to swelling in the presence of 1 μM cyclosporine A. However, upon dose reduction to 0.5 μM, WT mitochondria exhibited swelling in a time-dependent manner, whereas HAX-1 overexpressing mitochondria maintained their resistance (Fig. 2D). These findings further suggest that expression of HAX-1 reduces the sensitivity of mPTP opening in cardiomyocytes.

We also applied cyclosporine A before hydrogen peroxide challenge and assessed the degree of cell death in isolated cardiomyocytes to confirm that the prosurvival effects of HAX-1 overexpression (15-fold increase in HAX-1 levels) were due to complete inhibition of mitochondrial swelling, induced by 375 mM calcium. Both WT and HAX-1 overexpressing mitochondria were resistant to swelling in the presence of 1 μM cyclosporine A. However, upon dose reduction to 0.5 μM, WT mitochondria exhibited swelling in a time-dependent manner, whereas HAX-1 overexpressing mitochondria maintained their resistance (Fig. 2D). These findings further suggest that expression of HAX-1 reduces the sensitivity of mPTP opening in cardiomyocytes.

HAX-1 Regulates Cardiac Cyclophilin-D Protein Levels. Because cyclophilin A is a direct pharmacological inhibitor of cyclophilin-D (17), an important regulator of the permeability transition pore, we hypothesized that the reduced dose of CsA required for complete blockade of swelling in HAX-1 overexpressing mitochondria may be associated with decreases in Cyp-D levels. Indeed, quantitative immunoblotting indicated that the Cyp-D levels in HAX-1 overexpressing hearts were reduced by 40% compared with WT hearts (Fig. 3A and B). Interestingly, the effects of HAX-1 appeared specific for Cyp-D, because the expression levels of other proposed mPTP components, namely the voltage-dependent anion channel (VDAC) and adenine nucleotide translocase (ANT1), were not altered (Fig. 3A). Furthermore, the expression levels of Bak and Bak, which were recently suggested to govern the porosity of the mitochondrial outer membrane (22), were not altered by HAX-1 overexpression either. These findings in cardiac homogenates were also similar to those obtained in isolated mitochondria (Fig. 3C and D). Thus, we speculated that if HAX-1 can negatively regulate Cyp-D expression, then loss of HAX-1 should have a positive effect on Cyp-D levels. Indeed, heterozygous-deficient hearts with 60% loss of HAX-1 (13) exhibited a 70% increase in Cyp-D levels (Fig. 3E and F). Similar findings were observed with isolated mitochondria (Fig. 3G and H). There were no alterations in the levels of VDAC, ANT1, Bak, and Bak (Fig. 3E and F), similar to HAX-1 overexpression. Thus, the protective effect of HAX-1 on mPTP opening may be attributed to the reduction of Cyp-D expression in the mitochondria.

To exclude the possibility that reduction of Cyp-D level was an indirect compensatory response to chronic HAX-1 overexpression in vivo, we used adenoviral delivery to acutely increase HAX-1 levels in cardiomyocytes. At 24 h after infection, HAX-1 overexpression (15-fold increase in HAX-1 levels) was associated with a 40% decrease in Cyp-D levels (Fig. 3A and B). Accordingly, acute down-regulation of HAX-1 by 55%, using an antisense approach, led to a 60% increase in Cyp-D expression levels in cardiomyocytes (Fig. 3C and D). The acute alterations in Cyp-D levels reflected parallel changes in the TMRE responses upon hydrogen peroxide challenge. Cardiomyocytes

![Fig. 3.](image.png)

**Fig. 3.** HAX-1 specifically alters cyclophilin-D levels in the heart. (A and B) Cyp-D levels were reduced in HAX-OE cardiac homogenates, n = 4 hearts for each group. (C and D) Cyp-D levels were reduced in HAX-OE cardiac mitochondrial fraction, n = 5 hearts for HAX-OE, 6 hearts for WT. (E and F) Cyp-D levels were increased in HAX−/− cardiac homogenates, n = 6 hearts for each group. (G and H) Cyp-D levels were increased in the HAX−/− mitochondrial fraction, n = 9 hearts for each group. *P < 0.05 vs. WT. Data are expressed as mean ± SEM.
with increased HAX-1 levels showed enhanced resistance to loss of mitochondria membrane potential, whereas down-regulation of HAX-1 led to increased dissipation of proton gradient (Fig. S3 E and F). These findings coupled with the ones above in animal models suggest that HAX-1 may be a regulator of cardiac Cyp-D expression levels.

To further delineate the contribution of reduced Cyp-D levels in the protective effects of HAX-1 on mPTP opening and membrane potential, we used two complementary approaches and examined the effects of altered Cyp-D expression on the TMRE responses upon hydrogen peroxide challenge. First, we infected adult mouse WT and HAX-1 overexpressing cardiomyocytes with an adenovirus-encoding Cyp-D cDNA. Increased Cyp-D protein levels to a similar extent between WT and transgenic cells (Fig. 4 A) effectively abolished the protective effects of HAX-1 overexpression on mPTP opening (Fig. 4 B and C). Second, we isolated cardiomyocytes from the Cyp-D knockout (or PPIF null) mouse and infected them with adenoviruses carrying HAX-1 sense or antisense cDNA (Ad.HAX-1 or Ad.HAX-AS) to alter HAX-1 expression levels (Fig. 4D). In the absence of Cyp-D, the alterations in HAX-1 levels did not affect the TMRE response to hydrogen peroxide challenge (Fig. 4 E and F). Collectively, the regulatory effects of HAX-1 on mPTP sensitivity appear to be mediated through alterations of Cyp-D levels.

We previously reported that reduction in HAX-1 levels have detrimental effects in the heart, when subjected to ischemia/reperfusion injury. Because mPTP has also been shown to play an important role in this injury, we examined whether the effects of HAX-1 may be mediated through Cyp-D. To this end, we crossed the Cyp-D knockout mouse with the HAX-1/− model (CypD-KO/HAX-1/) and obtained HAX-1 heterozygous mice that were also deficient in Cyp-D. Hearts from the WT, HAX-1/−, CypD-KO, and the cross model were then subjected to global no-flow ischemia (40 min), followed by reperfusion (60 min) in the Langendorff perfusion mode. This protocol induced a 26% infarct size in WT hearts (Fig. 4 G and H), consistent with previous studies (14). Heterozygous loss of HAX-1 increased myocardial infarction to 40%, whereas ablation of Cyp-D significantly diminished it to 11% (Fig. 4 G and H). Importantly, ablation of Cyp-D in HAX-1/− hearts resulted in significant reduction of infarct size from 40 to 18% (Fig. 4 G and H), suggesting that mPTP plays a critical role in the protective effects of HAX-1 in cardiac ischemia/reperfusion injury.

HAX-1 Regulates Cyclophilin-D Levels Through Hsp90. To determine whether the effects of HAX-1 were mediated at the transcriptional level, we assessed cyclophilin-D (or ppif) mRNA expression in WT, HAX-OE, and HAX-1/− cardiac homogenates (Fig. 5A). There were...
no alterations among the three groups, indicating that HAX-1 does not regulate Cyp-D transcription. We then hypothesized that HAX-1 modulates the Cyp-D protein levels specifically through its degradation by the proteosomal pathway. To test this hypothesis, we acutely increased HAX-1 expression in adult rat cardiomyocytes by adenoviral delivery (Ad.HAX-1) and subsequently treated the cells with bortezomib, the well-recognized proteosomal inhibitor in cancer cells (23, 24). HAX-1 overexpression decreased Cyp-D levels as expected, but the effect was abolished by bortezomib, suggesting that HAX-1 regulates proteosomal degradation of Cyp-D (Fig. 5B). Because protein degradation is known to be controlled by ubiquitination (25), we sought to examine whether HAX-1 may affect Cyp-D ubiquitination. Thus, HAX-OE or HAX+/− cardiac homogenates were subjected to immunoprecipitation studies, using either ubiquitin or Cyp-D as a bait. Indeed, HAX-1 overexpression markedly increased Cyp-D ubiquitination (Fig. 5C and D), whereas heterozygous ablation of HAX-1 resulted in less ubiquitinated Cyp-D, compared with WTs (Fig. 5E and F). These findings indicate that HAX-1 regulates the degradation of Cyp-D protein through the ubiquitination-proteosomal pathway.

Previous studies in mitochondria isolated from various cell types have shown that Cyp-D interacts with the heat shock protein 90 (Hsp90) in the matrix (20). Interestingly, we also recently found that Hsp90 is a binding partner of HAX-1 in the heart (14). These findings prompted us to investigate whether the observed effects of HAX-1 on Cyp-D levels may involve Hsp90. Because the role of Hsp90 on Cyp-D is not known, we acutely overexpressed Hsp90 in adult cardiomyocytes and determined the expressions of Cyp-D. We observed a significant increase in Cyp-D protein levels (Fig. S4) and reduction in its ubiquitination (Fig. S5), suggesting that Hsp90 may serve as a chaperone for Cyp-D and prevent its degradation. This notion was further supported by immunoprecipitation studies in cardiac homogenates from HAX-1 heterozygous-deficient hearts, which exhibit increases in Cyp-D levels. Using Cyp-D as bait, there was an increase in the amount of Hsp90 associated with Cyp-D, compared with WTs (Fig. 6A). A similar increase in the Cyp-D levels was observed when Hsp90 was used as bait (Fig. 6B). On the contrary, overexpression of HAX-1 resulted in reduced Hsp90/Cyp-D association, using either Hsp90 or Cyp-D as bait in the cardiac homogenates (Fig. 6C and D). These alterations appear to occur in mitochondria, because similar findings were observed when isolated mitochondrial fractions from HAX-OE and HAX+/− hearts were used for immunoprecipitation experiments (Figs. S6 and S7). Notably, the changes in Hsp90/Cyp-D complex formation were not associated with alterations in cardiac or mitochondrial Hsp90 levels in either HAX-1 overexpressing or heterozygous-deficient hearts (Figs. S8 and S9). Taken together, these findings suggest that HAX-1 may directly displace Cyp-D from binding to Hsp90 in the mitochondria.

To further examine whether HAX-1 physically hinders the interaction of Cyp-D with Hsp90, we performed an in vitro competitive binding ELISA, using recombinant proteins. We observed a progressive reduction of the Cyp-D/Hsp90 binding in the presence of increasing HAX-1 protein levels (Fig. 6E). Thus, increases in HAX-1 appear to reduce the levels of mitochondrial Hsp90 that bind to Cyp-D, leading to degradation of Cyp-D.

To further test whether the effect of HAX-1 overexpression on decreasing the Cyp-D levels is mediated through reduction of the Hsp90/Cyp-D association, we acutely overexpressed Hsp90 to restore complex formation. Indeed, Hsp90 overexpression in the setting of HAX-1 overexpression resulted in markedly enhanced Cyp-D levels (Fig. 6F), abolishing the protective effects of HAX-1 and exacerbating loss of membrane potential upon hydrogen peroxide treatment (Fig. 6G and H).

Fig. 5. HAX-1 enhances the degradation of cyclophilin-D. (A) Cyp-D mRNA transcript, or ppif levels were similar in WT, HAX-OE, and HAX+/− hearts. n = 4 hearts for WT, and 5 hearts for each of the HAX-OE and HAX+/− hearts. (B) Proteosomal inhibitor, Bortezomib, restored Cyp-D levels in HAX-1 overexpressing cells. Glycer-aldehyde 3-phosphate dehydrogenase (GAPDH) was used as a loading control. n = 4 hearts for each group; *P < 0.05 vs. Ad.GFP. (C and D) Ubiquitination of Cyp-D was reduced in HAX-OE hearts. Three independent experiments were performed. n = 4 hearts for each group. (E and F) Ubiquitination of Cyp-D was reduced in HAX+/− hearts. Three independent experiments were performed. n = 4 hearts for each group. Data are expressed as mean ± SEM.
provide further evidence that the cardioprotective effects of HAX-1 are mediated through regulation of mPTP function and specifically through recruitment of Hsp90 from the Hsp90/Cyp-D complex, contributing to degradation of Cyp-D protein and increased survival.

**Discussion**

In this study, we identified HAX-1 as a regulator of mPTP and mitochondrial membrane integrity, impacting cell survival in the heart. Increases in HAX-1 expression protected mitochondrial membrane from damage associated with oxidative stress and calcium overload, whereas decreases in HAX-1 levels exacerbated loss of membrane integrity. The underlying mechanisms involved the ability of HAX-1 to sequester Hsp90 from Cyp-D, which then rendered Cyp-D prone to ubiquitination and degradation. Thus, HAX-1 inhibits the opening of mPTP and prevents loss of membrane integrity and cell death activation during oxidative stress or mitochondrial calcium overload. These findings, coupled with our recent observations, indicating that HAX-1 can suppress ER stress-induced apoptosis (14), reveal a previously unidentified paradigm of cell death regulation by HAX-1 in two different cellular organelles.

Growing evidence demonstrates an axiomatic prosurvival role of HAX-1, and human mutations of this protein have been shown to associate with diseases characterized of altered cell viability, such as congenital neutropenia (15), neurodevelopmental retardation (26), and mantle cell lymphoma (11). Although HAX-1 has been known to predominantly localize to mitochondria in various tissues and cell types (8), and mitochondria are known for their gate-keeping role in cell death activation, it is surprising that there are only a few studies addressing the function of HAX-1 in regulation of cell death by this organelle. One study reported that mitochondrial HAX-1 interacts with the high temperature requirement protein-2 (HtrA2) and abrogation of this complex by HAX-1 ablation in mice contributes to Bax accumulation, neurodegeneration, and a short lifespan (14 wk). However, ablation of Bax in HAX-1–deficient mice could only prolong survival by 9 wk without fully rescuing the phenotype (10), suggesting that the HtrA2/Bax regulation is not the sole anti-apoptotic mechanism mediated by HAX-1. Our findings herein suggest that mitochondrial localized HAX-1 has a direct protective effect on the membrane integrity of this subcellular organelle by regulating the mPTP opening.

We previously showed that cardiac ischemia/reperfusion injury is associated with loss of HAX-1 and overexpression of this protein conferred protection by reducing infarct size, limiting troponin release and inhibiting DNA fragmentation. On the contrary, heterozygous loss of HAX-1 led to exacerbated cardiac injury (14). Particularly, leakage of intracellular content, such as troponin proteins, is a sign of plasma membrane rupture and necrotic cell death (27, 28), which is mainly caused by failure to maintain plasma membrane potential due to loss of ATP supply (3, 4). The diminished energy production reflects compromised mitochondrial function associated with mPTP opening during ischemia/reperfusion injury (5). Although we previously showed that HAX-1 inhibits apoptosis mediated by the ER stress response, this effect would not account for the reduction in cardiac troponin I release during ischemia/reperfusion injury. Thus, the current identification of HAX-1 as a regulator of Cyp-D levels and an inhibitor of mPTP activation provides additional insights.
to its cardioprotective mechanisms. The role of Cyp-D was further elucidated by generation of a cross model with ablation of this protein in the HAX-1 heterozygous the deficient hearts, which were characterized by increased levels of this protein and mPTP activity. The cross model exhibited diminished myocardial infarction, although its infarct size was not reduced to the level of Cyp-D knockout hearts, suggesting that inhibition of both mPTP and ER stress contributes to the cardioprotective effects of HAX-1. Given that myocardial infarction is characterized by a continuum of apoptosis and necrosis, our data demonstrate that HAX-1 orchestrates a dual protective mechanism by inhibiting a mitochondrial-based necrotic activation, in addition to the previously reported ER-based apoptotic cell death program.

The activation of mitochondrial permeability transition pore has been shown to be a main driver in the development of myocardial infarction upon ischemia/reperfusion injury (5). Although the genetic identity of the pore is unknown, it has been suggested to consist of two complexes located on the outer and inner mitochondrial membrane. Although it was originally indicated that VDAC constitutes the outer membrane portion, a recent report suggested that VDAC is dispensable in mPTP activation (29) and outer membrane porosity is induced by the oligomerization of Bax/Bak (22). Furthermore, HAX-1 homozgyous ablation was shown to facilitate Bax accumulation, and cause neurodegeneration (10). However, in both HAX-1 overexpressing and heterozygous-deficient hearts, the levels of Bax and Bak were not altered in either the homogenates or mitochondrial fractions, suggesting that the alterations of mPTP in our models were independent of Bax/Bak accumulation.

The only genetically proven regulator of the mPTP is Cyp-D, which is located within the matrix of the mitochondria. Either ablation or pharmacological inhibition of this protein results in significant reduction of infarct size after ischemia/reperfusion injury (17, 19). However, increasing the expression of Cyp-D results in enhanced mPTP activity and mitochondrial swelling (19, 30). Thus, regulation of Cyp-D expression level becomes of paramount importance in the control of cell survival. To this end, our study presents the first evidence to our knowledge that Cyp-D levels can be posttranslationally regulated by HAX-1 and the chaperone protein Hsp90. Chaperone proteins have been well characterized in ER stress and facilitate protein turnover, directing unfolded or unwanted proteins to the ubiquitination/proteosomal degradation pathway. We previously demonstrated that HAX-1 works with Hsp90 to control the activity of IRE-1, one of the ER stress response signaling components, suggesting that HAX-1 may function as a co-chaperone protein for Hsp90 (14). This chaperone/cochaperone regulation appears to exist also in mitochondria, controlling the ubiquitination and degradation of Cyp-D. Increases in HAX-1 expression can sequester Hsp90 from the Cyp-D/Hsp90 complex, promoting Cyp-D ubiquitination and degradation. In turn, the reduced Cyp-D levels confer resistance to mPTP opening and subsequent membrane potential loss upon stress stimuli. These findings are consistent with previous observations in breast carcinoma, colon cancer, and HEK293 cell lines, which showed that cleavage of HAX-1 by granzyme B leads to loss of mitochondrial membrane potential (20). Thus, it was proposed that both Hsp90 and TRAP-1 serve to antagonize Cyp-D function. Based on this hypothesis, it is expected that displacement of Hsp90 from Cyp-D by HAX-1 overexpression would increase Cyp-D activity and promote mPTP opening. However, we did not observe a reduction of mPTP activation when HAX-1 was overexpressed. Moreover, we found that overexpression of Hsp90 in either WT or HAX-OE cardiomyocytes resulted in increased Cyp-D expression (Fig. 6F) and exacerbated loss of mitochondrial membrane potential after hydrogen peroxide challenge (Fig. 6G and H). These results suggest that Hsp90 promotes Cyp-D function in cardiac cells. The apparent discrepancy between our current results and previous reports in tumor cell lines may be due to the high dependency of Hsp90 function on the availability and combination of its cochaperone proteins, which may differ between cell types. It is likely that tumor cells have a remodeled mitochondrial Hsp90 chaperone network to enhance their survival and proliferation. In support of this notion, we failed to observe any significant association between HAX-1/TRAP-1 (Fig. S10), suggesting that the Hsp90 interaction with HAX-1 in cardiac cells may not be associated with TRAP-1. Indeed, mitochondrial targeted Hsp90 inhibitors have been shown to exhibit excellent specificity toward tumor cells, but not normal cells (20, 32). These reports support our results and point to a complex regulation of Hsp90 function by its cochaperones and the intracellular environment.

Our findings on the newly discovered function of HAX-1 in controlling Cyp-D levels and activation of the mitochondrial permeability transition pore are not only critical in heart function and survival, but also provide significant insights in other diseases, such as immunodeficiency, tumorgenesis, and neurodegeneration. Studies in patients with HAX-1 mutation, exhibiting severe congenital neutropenia, showed that their neutrophils had low mitochondrial membrane potential compared with the healthy controls (16). However, elevated HAX-1 levels are associated with various types of cancer (8), where the mPTP activity can be inhibited for permissive survival benefits (33).

In summary, this is the first study to demonstrate that HAX-1 and specifically the HAX-1/Hsp90 complex promote Cyp-D ubiquitination and degradation. This regulatory mechanism confers resistance to mPTP opening and prevents cell death against oxidative stress and mitochondrial calcium overload (Fig. S11). Thus, HAX-1 and Hsp90, which are ubiquitously expressed, serve as common nodal points in both apoptotic and necrotic cell death. Because mPTP opening is implicated in the pathogenesis of various diseases, our findings suggest that the HAX-1/Hsp90 complex may be potentially exploited as a feasible therapeutic target.

Materials and Methods

Animal Models. HAX-1 transgenic (HAX-DE, FVB/N) (13), HAX-1 knockout heterozygous (HAX+/−, FVB/N) (13), Cyp-D knockout (B6129s) (19), the cross of CypD-KO/HAX+/− (B6129s) mice, and their wild-type littermates were used in this study. To avoid the complication of gender differences, only male mice at 3 mo of age were used for these studies, according to the National Institutes of Health Publication No. 8523: Guide for the Care and Use of Laboratory Animals (34).

Mouse Cardiomyocyte Isolation and Virus Infection. Mouse myocytes from WT and HAX-1 OE hearts were isolated as described (14). Briefly, adult mouse hearts were excised following mouse anesthesia with sodium pentobarbital (70 mg/kg, i.p.) and cannulated on a Langendorff system. Ca-free Tyrode solution (113 mM NaCl, 4.7 mM KCl, 0.6 mM CaCl2, 0.06 mM MgCl2, 5.5 mM glucose, pH 7.4) was used to perfuse the heart for 3 min at 37 °C. The perfusion was then switched to a digestion solution containing 0.25% Liberase Blendzyme (Roche). Following digestion, the left ventricular tissue was excised, minced, and dissociated into a cell suspension. Calcium
was serially added to the cellular suspension until final calcium concentra-
tion in the Tyrode solution was 1.2 mM/L. Cardiomyocytes were pelleted by
gravity, resuspended in plating media (DMEM with 5% (v/v) FBS, 10 mM 2,3-butanediol monoxime, 100 units/mL penicillin streptomycin and 2 mL
× 105 mCi). After 15 min at room temperature, fluorescence signals from TMRE excited at 560 nm were collected at emmission of 610 nm, using live-cell confocal imaging. To induce cardiomyo-
tical membrane potential before and after oxidative challenge. There was no significant loss of mitochondrial membrane observed until the 15th–18th minute of the treatment, suggesting that primary cardiomyocytes are resistant to cell death initiation.

Mitochondrial Membrane Potential. At 24 h after adenosine exposure, mouse cardiomyocytes were stained with TMRE dye (ebioscience) for 15 min at room temperature. Fluorescence signals from TMRE excited at 560 nm were collected at emission of 610 nm and using live-cell confocal imaging. To induce mitochondrial membrane potential transition, stained mouse cardiomyocytes were cultured with 2 mM hydrogen peroxide, in the absence or presence of cyclosporin A (Sigma). The TMRE signal was recorded every minute for a period of 20 min to calculate the percentage of cells with preserved mitochondrial membrane potential before and after oxidative challenge. There was no significant loss of mitochondrial membrane observed until the 15th–18th minute of the treatment, suggesting that primary cardiomyocytes are resistant to cell death initiation.

Mitochondria Isolation and Swelling. Mouse heart mitochondria were iso-
lated by homogenization followed by different centrifugation. Briefly, mouse hearts were homogenized in Preco 24 Lysis and homogenization machine at 5,000 cpm for 10 s with 750 μL of 2.3-mm Zirconia/silica beads and an equal amount of homogenization buffer containing 250 mM sucrose, 10 mM Tris at pH 7.4, and 1 mM EDTA. The homogenates were spun at 1,300 × g for 10 min at 4 °C to pellet nuclei and cell debris. The supernatant was then spun at 12,000 × g for 30 min at 4 °C to pellet the mitochondria. After washing twice in homogenization buffer (minus EDTA), the mitochondria were resuspended in buffer containing 120 mM KCl, 10 mM Tris at pH 7.4, and 5 mM KH2PO4 for the swelling assay. Mitochondrial swelling was induced by 50 or 375 μM, where the light-scattering of 250 μg of mitochondria in a 1-mL volume was measured at 540 nm for 10 min.

Propidium Iodide and Annexin V Assays. Cells were gently washed once and stained with annexin V-specific APC dye (ebioscience) for 20 min. Various concentrations of hydrogen peroxide were applied at the beginning of annexin V measurement to assess the percent of Annexin V-positive population as an indication of apoptosis. FlowJo software (Tree Star) was used to generate the diagram of cell distribution according to fluorescence intensity and to calculate the percent of Annexin V-positive population as an indication of apoptosis.

Quantitative Immunoblot Analysis. Hearts were snap frozen in liquid nitrogen at the end of the Langendorff perfusion period and homogenized in 1× Cell Lysis Buffer (Cell Signaling Technology) supplemented with 1 mM PMFS and complete protease inhibitor mixture (Roche Applied Science). For each protein, equal amounts of samples (5–120 μg) from each heart were an-
alyzed by SDS/PAGE, as described (13). After transfer to membranes, im-
munoblotting analysis was performed with the corresponding primary antibodies (Bax, Bak, and ubiquitin from Cell Signaling; Cyp-D, COX4, VDAC, ANT1, Hsp90, and TRAP-1 from Abcam; HAX-1 from BD Biosciences; Hsp90, Hsp70, and TRAP from CAYMAN Chemical). The cell phenotype and morphology remained similar among noninfected and adenosine-infected groups after 24 h of infection.

Global Ischemia/Reperfusion Injury. Myocardial infarction was induced by using an isolated perfused heart model, as described (14). Briefly, hearts were exposed in a Langendorff apparatus, and perfused with Krebs-Henseleit

Medium was replaced with infection medium (plating medium containing no
bombardment cuvette. For each experiment, the densitometric values from pre-IR WT controls were arbitrarily converted to 1.0, and the values of samples from the other groups were normalized accordingly.
One hundred microliters of protein G PLUS agarose beads (Santa Cruz Bio-technology) were added into the mixture and incubated for an additional 5 h. Agarose beads were sedimented and washed six times with the cell lysis buffer. Beads-bound proteins were dissociated in 2x SDS at room temperature for 30 min with vortexing at 5-min intervals. The identification of the associated proteins was detected by Western blots. Homogenates from WT hearts were used as positive controls.

**Competitive Protein Binding ELISA.** Competitive binding assay using recominant proteins was performed as described (35). Briefly, each well on a 96-well high affinity binding plate (BD Falcon catalog no. 351172) was coated with 200 ng of recombinant Hsp90 protein (StressMarq) or GST protein (Novus Biologicals, as control) in 100 μL of coating buffer (85 mmol/L NaHCO3 and 15 mmol/L Na2CO3, pH 9.5) overnight at 4 °C. After five washes with PBS and 0.05% Tween-20 (PBS-T), blocking solution (PBS containing 1% BSA) was added for 1 h at room temperature. Following another five PBS-T washes, 400 ng of Cyp-D protein (Abnova) was added along with 400 ng of “competition protein mixture.” This mixture contained various amounts of HAX-1 (Abnova) supplemented with GST to reach a total of 400 ng of protein/reaction. Incubation proceeded for 2 h at room temperature. Each well was then washed with PBS-T six times, and the HAX-1 antibody, which was dissolved in blocking solution, was added for 2 h at room temperature. After six washes with PBS-T, the secondary antibody conjugated with horseradish peroxidase was added for 1 h at room temperature. Following eight PBS-T washes, TMB substrate reagent (BD Biosciences) was applied to develop signal in blue color. After color was developed, 1 mol/L HCl was added to stop the reaction. The colorimetric intensity of each well was read at 450 nm with a microplate reader.

**Statistical Analysis.** Data were expressed as the mean ± SEM. Comparisons between the means of two groups were performed by unpaired Student’s t test. Multiple groups were analyzed by using one-way ANOVA with a Bonferroni test for post hoc analysis. Results were considered statistically significant at P < 0.05.

**ACKNOWLEDGMENTS.** We thank Dr. J. Molkentin for providing the Ad.Cyp-D virus; Dr. W. Sessa for providing the Ad.Hsp90 plasmid; Drs. A. Maloyan and J. Robbins for assistance with the mitochondrial swelling assay; and Drs. J. Molkentin and J. Karch for helpful discussions.