Promotion of CHIP-Mediated p53 Degradation Protects the Heart From Ischemic Injury


Rationale: The number of patients with coronary heart disease, including myocardial infarction, is increasing and novel therapeutic strategy is awaited. Tumor suppressor protein p53 accumulates in the myocardium after myocardial infarction, causes apoptosis of cardiomyocytes, and plays an important role in the progression into heart failure.

Objectives: We investigated the molecular mechanisms of p53 accumulation in the heart after myocardial infarction and tested whether anti-p53 approach would be effective against myocardial infarction.

Methods and Results: Through expression screening, we found that CHIP (carboxyl terminus of Hsp70-interacting protein) is an endogenous p53 antagonist in the heart. CHIP suppressed p53 level by ubiquitinating and inducing proteasomal degradation. CHIP transcription was downregulated after hypoxic stress and restoration of CHIP protein level prevented p53 accumulation after hypoxic stress. CHIP overexpression in vivo prevented p53 accumulation and cardiomyocyte apoptosis after myocardial infarction. Promotion of CHIP function by heat shock protein (Hsp)90 inhibitor, 17-allylamino-17-demethoxy geldanamycin (17-AAG), also prevented p53 accumulation and cardiomyocyte apoptosis both in vitro and in vivo. CHIP-mediated p53 degradation was at least one of the cardioprotective effects of 17-AAG.

Conclusions: We found that downregulation of CHIP level by hypoxia was responsible for p53 accumulation in the heart after myocardial infarction. Decreasing the amount of p53 prevented myocardial apoptosis and ameliorated ventricular remodeling after myocardial infarction. We conclude that anti-p53 approach would be effective to treat myocardial infarction. (Circ Res. 2010;106:00-00.)

Key Words: myocardial infarction • CHIP • p53 • hypoxia

The number of patients with coronary heart disease has been increasing and cardiovascular diseases are the leading cause of deaths in the Western world. Despite the development of pharmacological and nonpharmacological interventions, 33% of the men and 43% of the women die within 5 years after myocardial infarction (MI). Therefore, a novel therapeutic approach against coronary heart disease is awaited.

Apoptosis of cardiomyocytes is accompanied with acute coronary occlusion. Because apoptotic loss of cardiomyocytes causes heart failure, inhibition of apoptosis has been suggested as an additional therapeutic approach to coronary heart disease. In mice, overexpression of antiapoptotic Bcl-2 protein or genetic deletion of proapoptotic Bax protein have been reported to prevent apoptosis and reduce infarct size, implicating that antiapoptotic approach is effective for prevention of ventricular remodeling after myocardial infarction.

The tumor suppressor p53 is an important transcription factor that regulates cell cycle progression, cellular senescence, and apoptosis. Under physiological condition, p53 protein level is maintained low, but is elevated when cells are stressed or damaged. The mechanism for keeping p53 protein level low involves several E3 ubiquitin ligases such as MDM2, COP1, and Pirh2. Importantly, the expression of these proteins were positively regulated by p53, suggesting the role for negative-feedback loop against p53 elevation.

Protein level of p53 is also kept low in the heart but it is elevated when cardiac cells are exposed to hypoxia.
We have recently reported that elevation of p53 causes the development of pressure overload-induced heart failure. We have also observed the elevation of p53 protein levels after myocardial infarction and shown that p53 gene deletion improved cardiac function after myocardial infarction, suggesting that the inhibition of p53 might become a novel therapeutic strategy for ischemic heart diseases.

As an initial approach for the investigation of anti-p53 therapy, we searched for an endogenous p53 antagonist in the heart. Through expression screening, we found that CHIP (carboxyl terminus of Hsp70-interacting protein) is an endogenous p53 antagonist that keeps p53 level low in the heart. We also found that CHIP downregulation is involved in the mechanism of p53 accumulation in the heart after myocardial infarction. Facilitating CHIP-mediated p53 degradation prevented apoptosis of cardiomyocytes and ameliorated ventricular remodeling in the postinfarct heart. The present study revealed the mechanism of p53 accumulation in the heart after myocardial ischemia and suggested that anti-p53 approach would be effective to treat myocardial infarction.

Methods

Expression Cloning

Expression cloning was performed as described previously using PG13-Luc (kind gift from B. Vogelstein) as a reporter plasmid. Initially, cDNA expression library from human heart (Invitrogen) was separated into small pools that contain ~100 clones each. cDNA clones that downregulated PG13 activity were isolated by sib-selection. Individual cDNA clone that downregulates the PG13 activity was identified by sib-selection. One of the molecules that was highly expressed in the heart (Figure 1A, in the Online Data Supplement, available at http://circres.ahajournals.org) was CHIP (also called STUB1 [Stip1 homology and U-box containing protein]), a chaperone-interacting protein with E3 ubiquitin ligase activity. Transfection of CHIP suppressed endogenous and exogenous (by overexpression of p53) PG13 activity (Figure 1A) and decreased the protein levels of p53 (Figure 1B) in a plasmid dose-dependent manner in COS7 cells. Direct interaction between CHIP and p53 was confirmed both at the exogenous level in COS7 cells (Online Figure I, A) and at the endogenous level in cardiomyocytes (Online Figure I, C). Western blotting using anti-ubiquitin antibody after immunoprecipitation with p53 revealed that overexpression of CHIP increased poly-ubiquitinated p53 (which appears as a smear) (Online Figure I, E), indicating that CHIP directs p53 for proteasome-mediated degradation. When CHIP was knocked down in cardiomyocytes using small interfering (si)RNA, p53 expression was upregulated (Figure 1C), and p53 protein levels following CHIP knockdown were comparable to those induced by the knockdown of MDM2, a well known E3 ubiquitin ligase for p53 (Figure 1D). CHIP protein level was not changed by knockdown of MDM2 (Figure 1D).

Statistical Analysis

Data are expressed as means±SE. The significance of differences among means was evaluated using analysis of variance (ANOVA), followed by Fisher’s protected least significant difference test and Dunnett’s test for multiple comparisons. Significant differences were defined as P<0.05.

Results

Identification of CHIP As a Novel p53 Antagonist From Heart cDNA Library

To elucidate novel p53 antagonists in the heart, we performed expression screening by expressing cDNA pools in COS7 cells together with a reporter plasmid, PG13-luciferase, which contains 13 copies of p53 binding site upstream of luciferase gene and responsive to wild-type p53 dependent transcription. From the screening of 500 cDNA pools, each containing around 100 individual cDNA clones obtained from human heart cDNA library, we found 5 pools that suppress the PG13 activity. Individual cDNA clone that downregulates the PG13 activity was identified by sib-selection. One of the molecules that was highly expressed in the heart was CHIP (Online Figure I, A). Transfection of CHIP suppressed endogenous and exogenous (by overexpression of p53) PG13 activity (Figure 1A) and decreased the protein levels of p53 (Figure 1B) in a plasmid dose-dependent manner in COS7 cells. Direct interaction between CHIP and p53 was confirmed both at the exogenous level in COS7 cells (Online Figure I, A) and at the endogenous level in cardiomyocytes (Online Figure I, C). Western blotting using anti-ubiquitin antibody after immunoprecipitation with p53 revealed that overexpression of CHIP increased poly-ubiquitinated p53 (which appears as a smear) (Online Figure I, E), indicating that CHIP directs p53 for proteasome-mediated degradation. When CHIP was knocked down in cardiomyocytes using small interfering (si)RNA, p53 expression was upregulated (Figure 1C), and p53 protein levels following CHIP knockdown were comparable to those induced by the knockdown of MDM2, a well known E3 ubiquitin ligase for p53 (Figure 1D). CHIP protein level was not changed by knockdown of MDM2 (Figure 1D). p53 protein levels were also markedly elevated in the heart of CHIP heterozygous mice (Figure 1E). These results suggest that CHIP induces degradation of wild-type p53 protein in cardiomyocytes, which is consistent with previous reports in other cells (H1299 cells and U2OS cells). In addition, we revealed that CHIP is a crucial negative regulator that keeps p53 protein levels low in the heart under physiological conditions.
As CHIP regulates p53 status in the heart, we speculated that CHIP might be involved in the molecular mechanism of hypoxia-induced p53 accumulation in the heart.

To investigate why CHIP is downregulated after hypoxic insult, we tested whether HIF-1 mediates hypoxia-induced downregulation of CHIP, because HIF-1 is known to downregulate some of its target genes through hypoxia-responsive element (HRE). Human CHIP promoter (from −329 bases upstream of transcription start site to +39 bases downstream of transcription start site) that contains a conserved HRE at −49 (Figure 2C) was cloned upstream of luciferase reporter gene (pGL4-CHIP). pGL4-CHIP activity was significantly suppressed by both CoCl₂ treatment (24 hours) and HIF-1α overexpression in COS7 cells (Figure 2D). When a mutation was introduced into HRE at −49 (pGL4-CHIP-mutHRE), the luciferase activity was no longer responsive to hypoxic stress or HIF-1α overexpression (Figure 2D), suggesting that CHIP gene expression is downregu-
translated by HIF-1 at the transcriptional level through HRE. Real-time PCR analysis revealed that exposure of cardiomyocytes to CoCl₂ (24 hours) and adenoviral overexpression of constitutively active HIF-1/α led to marked downregulation of CHIP mRNA levels (Figure 2E), further supporting our data that hypoxic stress downregulates CHIP levels. HIF-1/α gene is both required and sufficient for hypoxic stress-induced CHIP downregulation and p53 accumulation because knockdown of HIF-1/α attenuated the effects of CoCl₂ treatment on expressions of p53 and CHIP (Online Figure III, A), and overexpression of constitutively active HIF-1α suppressed CHIP expression and increased p53 expression in cardiomyocytes (Online Figure III, B). Furthermore, downregulation of CHIP protein levels after MI was attenuated in cardiac-specific inducible HIF-1α conditional knockout mice (Online Figure III, C). Collectively, these findings suggest that CHIP transcription is directly downregulated by hypoxia through HIF-1.

CHIP Protects Cardiomyocytes From Hypoxia-Induced p53-Mediated Apoptosis of Cardiomyocytes

Because hypoxia or p53 overexpression induces apoptotic cell death in cultured cardiomyocytes, we next examined whether hypoxia-induced cardiomyocyte apoptosis is mediated by the HIF-1α–CHIP–p53 pathway. CoCl₂ treatment (24 hours) induced p53 accumulation and promoted apoptosis of cardiomyocytes as assessed by cleaved poly (ADP-ribose) polymerase (PARP) expression (Figure 3A), Annexin V staining (Figure 3B and 3C), and caspase-3 activity (Figure 3D). CoCl₂-induced apoptosis was p53-dependent, because knockdown of p53 in CoCl₂-treated cardiomyocytes attenuated hypoxia-induced cell death (Figure 3A through 3D). We next assessed whether overexpression of CHIP could rescue CoCl₂-induced apoptosis. Adenovirus-mediated overexpression of CHIP in cardiomyocytes markedly downregulated p53 expression and attenuated apoptosis in CoCl₂-treated
cardiomyocytes (Figure 4A through 4C). These results underscore our hypothesis that downregulation of CHIP is responsible for p53 accumulation after hypoxic stress. Moreover, forced expression of CHIP prevented hypoxia-induced cardiomyocyte apoptosis by inducing degradation of p53, suggesting that CHIP-mediated p53 degradation is a potential therapeutic target.

17-AAG Protects Cardiomyocytes From Hypoxia-Induced Apoptosis

Inhibitors for heat shock protein (Hsp)90 have been shown to promote proteasomal degradation of CHIP client proteins and to be effective for the diseases caused by the accumulation of CHIP substrates.\textsuperscript{31,32} We therefore examined whether an Hsp90 inhibitor 17-allylamino-17-demethoxy geldanamycin (17-AAG) induces degradation of p53 protein and protects cardiomyocytes from hypoxic stress. In cardiomyocytes treated with CoCl\textsubscript{2}, 17-AAG downregulated p53 expression (Figure 4D). 17-AAG treatment also suppressed hypoxia-induced cardiomyocyte apoptosis in a CHIP-dependent manner, because CHIP knockdown attenuated the protective effects of 17-AAG (Figure 4E through 4G). These results suggest that 17-AAG protects cardiomyocytes from hypoxic stress by promoting CHIP-mediated p53 degradation.

Interestingly, protein level of CHIP was increased by 17-AAG treatment (Figure 4E). As mRNA level of CHIP was not changed by 17-AAG treatment (Online Figure IV, A), we speculated that protein stability was affected by 17-AAG treatment. When protein translation was inhibited by cycloheximide, 17-AAG treatment dramatically extended the protein half-life of CHIP (Online Figure IV, B and C). 17-AAG also upregulated the protein stability of other proteins, Hsp70 and HSF-1 (Online Figure IV, B and C). Because 17-AAG exerted some antiapoptotic effects even in the cells of negligible CHIP protein level (Figure 4E and 4F), upregulation of these cardioprotective proteins\textsuperscript{33,34} might mediate part of the effects of 17-AAG. It remains to be determined how 17-AAG prolongs protein half-life of certain kinds of proteins.

CHIP and 17-AAG Prevent Apoptosis and Ventricular Remodeling After Myocardial Infarction

We next examined whether promotion of CHIP-mediated p53 degradation could attenuate ischemic cardiac injury also in vivo. For this purpose, transgenic mice which overexpress CHIP specifically in the heart (CHIP-Tg) (Figure 5A) were subjected to permanent coronary artery ligation. In CHIP-Tg mice, elevation of p53 protein levels (Figure 5B) and apoptotic cardiomyocyte death in the border zone of the infarct area (Figure 5B and 5C) were attenuated compared to wild-type littermates at 24 hours after the MI operation. Apoptotic death of the cardiomyocytes in the remote zone of the infarct was not changed between littermates (data not shown). We next examined whether this decrease in apoptotic cell death leads to attenuation of cardiac ventricular remodeling. At day 14, CHIP-Tg mice exhibited smaller heart weight/body weight (HW/BW) ratio, better contractility and less ventricular remodeling (Figure 5D and 5E) compared to wild-type littermates. These results provide an evidence for our hypothesis that CHIP downregulation is responsible for p53 accumulation after myocardial infarction, and suggests that CHIP overexpression is protective for the heart by preventing p53 accumulation and cardiomyocyte apoptosis after myocardial infarction.

![Figure 3. CHIP overexpression and 17-AAG treatment protects cardiomyocytes from hypoxic stress. A through C, CoCl\textsubscript{2} treatment induces p53 accumulation (A) and apoptotic cell death in cardiomyocytes. Cardiomyocytes were treated with CoCl\textsubscript{2} and analyzed 24 hours (A and C) or 6 hours (B) later. Apoptosis is assessed by cleaved PARP expression (A), Annexin V staining (B), and caspase-3 activity (C). D and E, p53 expression was knocked down using siRNA against p53. Knockdown of p53 expression was assessed by cleaved PARP expression (D) and Annexin V staining (E). *P<0.01 vs control (Con); **P<0.01 vs Co; n=5.](image-url)
We also examined whether treatment with 17-AAG exerts similar cardioprotective effects. 17-AAG (10 mg/kg) or vehicle was intraperitoneally injected immediately after permanent coronary artery ligation. This single injection of 17-AAG effectively suppressed the elevation of p53 protein levels and apoptotic cell death in the border zone of the infarct area at 24 hours after the operation (Figure 6A and 6B). As p53 protein level was kept elevated even 4 and 7 days after MI (Figure 2B), 17-AAG was injected every other days and we assessed whether 17-AAG treatment also leads to attenuation of ventricular remodeling, as observed in CHIP-Tg mice. At day 14, mice treated with 17-AAG exhibited smaller HW/BW ratio, better contractility,
and less ventricular remodeling (Figure 6C and 6D). Interestingly, the effects of 17-AAG were greater than CHIP overexpression (compare Figures 5 and 6), suggesting that 17-AAG possesses cardioprotective activities that do not involve CHIP-mediated p53 degradation. As protein stability of cardioprotective proteins such as Hsp70 and HSF-1 was increased in vitro (Online Figure IV, B and C), we have examined the expression of these proteins in 17-AAG–treated mice. As expected, expression of these two proteins were increased by 17-AAG treatment (Online Figure IV, D), indicating that 17-AAG exerts its antiapoptotic effects by at least two mechanisms, one by inducing CHIP-mediated p53 degradation and the other by increasing cardioprotective heat shock proteins.

Finally, we examined the contribution of CHIP-mediated p53 degradation on the cardioprotective effects of 17-AAG. For that purpose we used CHIP heterozygous mice. There were no differences in cleaved PARP level (Figure 7A; compare WT MI and Het MI) and increased TUNEL positive cells (Figure 7B). The level of p53 accumulation was comparable following myocardial infarction between wild-type and CHIP heterozygous mice, suggesting the presence of p53 independent mechanisms for enhanced apoptosis caused by CHIP haploinsufficiency. Chronically, CHIP heterozygous mice showed worse cardiac function and worse ventricular remodeling compared with wild-type mice (Figure 7C and 7D). 17-AAG treatment was less effective to reduce p53 protein level, cleaved PARP level (Figure 7A; compare Het MI and Het MI 17-AAG), and TUNEL positive cardiomyocytes in CHIP heterozygous mice, possibly as a result of CHIP haploinsufficiency. 17-AAG treatment had minimal effects on improvements of cardiac function and ventricular remodeling on CHIP heterozygous mice also in the chronic phase (Figure 7C and 7D).

However, we must emphasize that the effects of 17-AAG were not fully attributable to CHIP-mediated p53 degradation.

Figure 5. Overexpression of CHIP attenuates ischemic cardiac injury in vivo. A, Cardiac-specific expression of HA-tagged human CHIP in CHIP-Tg mice. B and C, p53 accumulation (B) and apoptosis 1 day after MI are reduced in CHIP-Tg mice. Apoptosis was assessed by cleaved PARP expression (B) and TUNEL staining (C). Cleaved PARP level was assessed by densitometric analysis on band intensity of cleaved PARP over uncleaved PARP. *P<0.05 vs WT; n=3. WT indicates wild-type mice. D and E, Postinfarct cardiac remodeling is attenuated in CHIP-Tg mice (n=15). HW/BW ratio (D, left), contractile function (D, right), and percentage fibrotic area (E). *P<0.01 vs WT (n=30).
because upregulation of heat shock proteins by 17-AAG was also impaired in CHIP heterozygous mice (Figure 7A; compare WT MI 17-AAG and Het MI 17-AAG). Therefore, it would be fair to conclude that 17-AAG exerts multiple cardioprotective effects after myocardial infarction and at least one of its effects were mediated by promotion of CHIP-mediated p53 degradation.

Discussion
In the present study, we found that accumulation of p53 protein after myocardial ischemia is initiated by HIF-1-dependent downregulation of CHIP level. We have found that CHIP overexpression decreased the amount of p53 and prevented myocardial apoptosis and ameliorated ventricular remodeling after myocardial infarction. We have also found that Hsp90 inhibitor, 17-AAG, exerted similar antiapoptotic and cardioprotective effects after myocardial infarction and showed that these effects of 17-AAG was at least in part mediated by promotion of CHIP-mediated p53 degradation.

Although hypoxic stimuli have been reported to raise p53 protein levels in a variety of cell types, molecular mechanisms of p53 accumulation have been largely unknown. In the present study, we unveiled that downregulation of CHIP protein is critically involved in this process. We found that CHIP expression was downregulated after hypoxic stress through HIF-1-mediated suppression of CHIP promoter (Figure 2). We also found that overexpression of CHIP attenuated the p53 accumulation after hypoxic stress (Figures 4A and 5B). These results
Figure 7. The effect of 17-AAG was dependent on CHIP-mediated p53 degradation and upregulation of heat shock proteins. A, 17-AAG–induced reduction of p53 accumulation and PARP expression was not observed in the heart of CHIP heterozygous mice (Het). Notably, upregulation of heat shock proteins by 17-AAG was ameliorated in CHIP heterozygous mice. B, Apoptotic cardiomyocytes on 1 day after MI was reduced in 17-AAG–treated mice, but this antiapoptotic effect of 17-AAG after MI was ameliorated in CHIP heterozygous mice. Apoptosis was assessed by cleaved PARP expression (A) and TUNEL staining (B). *P<0.01 vs WT+MI+DMSO; **P<0.05 vs WT+MI+17-AAG; ***P=NS vs Het+MI+DMSO n=5. WT indicates wild-type mice; Het, CHIP heterozygous mice. C, 17-AAG–induced attenuation of postinfarct cardiac remodeling is less in CHIP heterozygous knockout mice than in wild-type mice. HW/BW ratio (C, left), contractile function (C, right), and fibrotic area (D). *P<0.001; **P<0.05 vs WT+MI+DMSO; ***P=NS vs Het+MI+DMSO. WT+MI+DMSO: n=30; WT+MI+17-AAG: n=20; Het+MI+DMSO: n=15; Het+MI+17-AAG: n=15. WT indicates wild-type mice.
suggestion in myocardial infarction.

In conclusion, our observations indicate that investigation of novel anti-p53 approach would open a way toward new strategies to prevent cardiac ischemia-reperfusion injury. Further studies on the role of p53 in ischemia-reperfusion injury are needed.
We found that CHIP downregulation is critically involved in the molecular mechanisms for p53 elevation after myocardial infarction.

We identified CHIP as the endogenous p53 antagonist expressed in the heart.

We showed several possibilities of the anti-p53 treatment after myocardial infarction.

Accumulation of tumor suppressor protein p53 in the myocardium causes the transition from adaptive cardiac hypertrophy to heart failure. However, the mechanisms of p53 accumulation in the heart and its therapeutic implications have been elusive. Here we show that downregulation of the chaperone-associated E3 ubiquitin ligase CHIP (carboxyl terminus of Hsp70-interacting protein) mediates hypoxia-induced p53 accumulation in the heart and that promotion of CHIP-induced p53 degradation protects the heart from ischemic injury. Under physiological conditions, CHIP limited the p53 protein amount at low levels by inducing proteasomal degradation of p53.

Under hypoxic conditions, hypoxia inducible factor-1 (HIF-1) downregulated CHIP, resulting in the accumulation of p53. Overexpression of CHIP or administration of an Hsp90 inhibitor promoted CHIP-mediated p53 degradation and attenuated ischemic cardiac injury. These results indicate that CHIP is a crucial negative regulator of p53 in the heart and suggest that promotion of CHIP-mediated p53 degradation could be a novel therapeutic strategy for heart diseases.
Promotion of CHIP-Mediated p53 Degradation Protects the Heart From Ischemic Injury


Circ Res. published online April 22, 2010;

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2010 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/early/2010/04/22/CIRCRESAHA.109.214346.citation

Data Supplement (unedited) at:
http://circres.ahajournals.org/content/suppl/2010/04/22/CIRCRESAHA.109.214346.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation Research can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation Research is online at:
http://circres.ahajournals.org/subscriptions/
**Detailed Methods**

**Expression Cloning.**

Expression cloning was performed as described previously \(^1\) using PG13-Luc (A kind gift from B. Vogelstein) as a reporter plasmid. Initially, cDNA expression library from human heart (Invitrogen) was separated into small pools that contain ~100 clones each. cDNA clones that downregulate PG13 activity were isolated by sib-selection.

**Plasmids and Transfection assay.**

Transfection into COS7 cells were performed using Fugene HD (Roche). HA-tag was introduced to the N-terminus of human CHIP using PCR and subcloned into pCAGGS vector \(^2\). Flag-tagged p53 was from B. Gellersen \(^3\). Adenoviral vector of HA-tagged CHIP was created using AdEasy Vector System (Qbiogen) and infected as described previously \(^4\). Human CHIP promotor (-329 to +39 from TSS) was cloned into pGL4-basic (Promega). Mutations of the HRE site (CACGTG to CTGGCG) were introduced using the QuickChange Site-Directed Mutagenesis Kit (Stratagene). Stealth siRNA against rat CHIP, MDM2, HIF-1\(\alpha\), and p53 were designed and purchased from Invitrogen. Sequences for each siRNAs are; rat CHIP-1: ugguguaguacacugccacaagugg; rat CHIP-2: cucaucuaacucucuccauucucucucagc; rat MDM2-1: agcuaaggaauuuucaggaucuccc; rat MDM2-2: auagucgucacucuccugugacagg; rat HIF1\(\alpha\): uagugcuuccacagaagcauugc; rat Tp53: uuaagggugaaauauucuccaucga. Negative controls for siRNA was purchased from Invitrogen (Medium GC). Transfection of siRNA was performed using Lipofectamine RNAiMax (Invitrogen).

**Cell culture.**

COS7 and HEK293 cells are from ATCC and cultured in DMEM containing 10% FBS (Invitrogen). Neonatal rat cardiomyocytes were isolated from 1-day old Wistar rats and
cultured as described previously. Cardiomyocytes were exposed to hypoxic stress by culturing under CoCl₂ (250 µM) or by culturing in hypoxic chamber (<1% O₂, PO₂ 18~20mmHg).

**Evaluation of apoptosis**

Caspase-3 activity was examined by using CaspACE™ Assay System, Colorimetric (Promega). Annexin V staining was performed as previously described using Annexin V-Cy3 apoptosis detection kit (Bio Vision). TUNEL staining on fresh frozen section of the heart was performed using In situ apoptosis detection kit (TaKaRa) and fluorescence was enhanced using anti-fluorescein rabbit IgG Alexa488 conjugated (Molecular Probe). The section was counterstained with α-sarcomeric actinin (SantaCruz) and DAPI. We have performed TUNEL staining 24 hours after coronary artery ligation because myocardial apoptosis was most prominent 24 hours after the operation (unpublished observations).

**Luciferase assays**

The reporter plasmid, PG13-Luc, pGL4-CHIP, or pGL4-CHIP-mutHRE was transfected into COS7 cells using Fugene HD (Roche) or to neonatal rat cardiomyocytes using Lipofectamine 2000 (Invitrogen). pRL-tk encoding Renilla luciferase was co-transfected as an internal control. Luciferase assay was carried out 24 hours after transfection of the effector plasmids or addition of drugs using dual-luciferase assay system (Promega).

**RNA analysis**

Total RNA extraction and DNase treatment was performed using SV total RNA isolation Kit (Promega). RNA was DNase treated and reverse transcribed using QuantiTect Reverse Transcription Kit (QIAGEN). Real time quantitative PCR was performed using
Universal Probe Library (UPL) (Roche) and Light Cycler TaqMan Master kit (Roche). Relative levels of gene expression were normalized to the rat GAPDH (for rat cardiomyocytes) or human β-actin (for HEK293 cells) expression using the comparative Ct method according to the manufacturer’s instructions. Primer sequences and UPL number were; rat GAPDH: Fwd aatgtatccgttgtggatctga, Rev gcttcaccacctcttctgtgt and UPL No. 80; Human beta actin: Fwd ggaatctgtcgtgacatatta, Rev ccgtcaggcagctctctgtag and UPL No. 80; rat CHIP: Fwd ctcaaggagcagggaaacc, Rev aagtgggttccgggtgat and UPL No. 70; human p21: Fwd cgaagtcagttccttgtggag, Rev catgggttcttgacggacat and UPL No. 82; human PUMA: Fwd gacctcaagcagcctacgca, Rev gagattgtcaggacccctcca and UPL No. 68; human MDM2: Fwd tctgatagtatttttctttttg, Rev tgttcaaccttaccagcactaa and UPL No. 21.

**Protein analysis.**

Whole cell lysates (50-100 µg) were separated by SDS-PAGE. Proteins were transferred to polyvinylidene difluoride membrane and incubated with primary antibodies followed by incubation with a HRP-conjugated secondary antibody (Jackson). Membrane was developed using ECL-Plus (Amersham). Densitometric analysis for the band intensity was performed using ImageJ. Immunoprecipitation was performed using specific antibodies and Protein A/G agarose (SantaCruz). Antibodies used were; anti-CHIP (CHEMICON), anti-p53 (1C12) (Cell Signaling), anti-HA (Roche), anti-Flag (M2) (Sigma), anti-MDM2 (R&D), anti-ubiquitin (CHEMICON), anti-HIF-1α (Novus), anti-PARP (Cell signaling), and anti-actin (Sigma).

HIF-1α activity was determined using TransAM™ HIF-1 (ActiveMotif).

**Animals**

All protocols were approved by Chiba University review board. CHIP heterozygous mice
and cardiac-specific inducible HIF-1 knockout mice were described \(^6\)\(^8\). Unlike the CHIP-deficient mice described by Dai et al. \(^9\), our CHIP heterozygous mice were maintained on a pure C57BL/6 background. Very few homozygous knockout were obtained (2.9% of the alive embryo) but all of them were small in size and died before 1 week after birth. The reason for perinatal lethality in our mouse strain remains to be elucidated. Cardiac-specific CHIP transgenic mice were generated by pronuclear injection of \(\alpha\)MHC-HA-CHIP transgene construct. Two independent lines that express HA-tagged CHIP to the same level were obtained and both lines showed similar results. Cardiac specific expression of HA-tagged CHIP was confirmed by Western blotting. The basal cardiac parameters of the mice used in this study (CHIP heterozygous knockout mice and cardiac specific CHIP overexpressing transgenic mice) were shown in Table 1.

Permanent coronary artery ligation was performed on 10-week old male mice as described previously \(^4\). We anesthetized mice by intraperitoneally injecting a mixture of 100 mg/kg ketamine and 5 mg/kg xylazine. 17-AAG (Alexis) was dissolved in DMSO and injected intraperitoneally right after coronary artery ligation.

**Physiological analysis and histological analysis.**

Echocardiography was performed as described previously \(^8\). Paraffin-embedded heart samples were sectioned and stained with Masson’s Trichrome staining as previously described \(^4\).

**Statistical analysis.**

Data are expressed as mean±SE. The significance of differences among means was evaluated using analysis of variance (ANOVA), followed by Fisher’s PLSD test and Dunnett’s test for multiple comparisons. Significant differences were defined as \(P<0.05\).
Supplemental References


Online Figure II

A

Relative expression level

CHIP/actin

p53/actin

Myocardial Infarction

Sham Day 1 Day 4 Day 7

B

Relative expression level

CHIP/actin

Myocardial Infarction

Sham Day 1 Day 4 Day 7
Online Figure III

A

B

C

Con Co CoSHIF-1

Ad GFP Ad HIF-1a

WT Sham WT MI HIF1 CKD MI

WT Sham HIF1 CKD MI

WT MI HIF1 CKD MI

WT MI
Online Figure IV

A

![Bar graph showing relative CHIP mRNA expression in DMSO and 17-AAG conditions.](image)

B

![Western blot images of CHIP, Hsp70, and HSF-1 under DMSO and 17-AAG conditions.](image)

C

![Line graphs showing band intensity compared with 0 hr for CHIP, Hsp70, and HSF-1 under DMSO and 17-AAG conditions.](image)

D

![Western blot images of Hsp70, HSF-1, and actin under Sham, MI, and MI 17-AAG conditions.](image)
Figure Legends for Supplementary Figures

Online Figure I. (A) Tissue distribution of endogenous CHIP. (B) CHIP interacts with p53 in COS7 cells transfected with HA-CHIP and Flag-p53 as revealed by immunoprecipitation/western blot analysis. IP, immunoprecipitation. (C) Endogenous CHIP interacts with endogenous p53 in neonatal rat cardiomyocytes. (D) CHIP induces ubiquitination of p53 in cultured cardiomyocytes. Ubiquitinated p53 appears as a smear. MG132 was used as a positive control because it inhibits proteasomal degradation, leaving ubiquitinated protein undegraded. (E) MG132 prevents CHIP-induced p53 degradation in cultured cardiomyocytes. (F) Both CoCl$_2$ treatment and hypoxia induces p53 upregulation and CHIP downregulation in cardiomyocytes. Con, control; Co, CoCl$_2$. (G) HIF-1$\alpha$ activity was determined to confirm that HIF-1$\alpha$ was actually active by CoCl$_2$ treatment. Con, control; Co, CoCl$_2$.

Online Figure II. Densitometric analysis for the Western blot shown in Figure 2B. p53 protein level (A), and CHIP protein level (B).

Online Figure III. (A) Knockdown of HIF-1$\alpha$ by siRNA reverses the effect of CoCl$_2$ in cardiomyocytes. (B) Overexpression of HIF-1 downregulates CHIP expression and induces accumulation of p53 in cardiomyocytes. (C) Wild type and cardiac-specific inducible HIF-1$\alpha$ knockout mice were subjected to coronary artery ligation. HIF-1 deletion attenuates downregulation of CHIP expression induced by myocardial infarction. WT, wild type mice; CKO, cardiac-specific inducible HIF-1$\alpha$ knockout mice.

Online Figure IV. (A) 17-AAG treatment showed no effect on CHIP mRNA transcription. (B) Representative blots for the expression level of CHIP, Hsp70 and HSF-1 after treatment with cycloheximide 100 $\mu$g/mL, with or without 17-AAG. (C) Kinetics of protein stability obtained from densitometric analysis. (D) 17-AAG treatment
up-regulates the protein level of Hsp70 and HSF-1 after myocardial infarction.