Involvement of GADD153 and Cardiac Ankyrin Repeat Protein in Hypoxia-Induced Apoptosis of H9c2 Cells*

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Key Words: Hypoxia, GADD153, apoptosis, CARP, H9c2 cells, cardiomyocytes

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1The abbreviation used are: TA, transactivation domain; CARP, cardiac ankyrin repeat
protein gene; DP, 2, 2’-dipyridyl; DFO, desferrioxamine; NAC, N-acetyl-L-cysteine; PI, propidium iodide; 4HPR, N-(4-hydroxyphenyl)retinamide; DAPI, diamidino-2-phenylindole; FCM, flow cytometry; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MAPK, mitogen-activated protein kinases; GRPs, glucose-regulated proteins.
ABSTRACT

Oxidative stress is the main cause of cardiac injury during ischemia/reperfusion but the molecular mechanism for this process is unclear. In this study, it was found that hypoxia induces apoptosis in rat embryonic heart derived H9c2 cells leading to the induction of GADD153, which is an apoptosis-related gene. Therefore, this study addressed the molecular role of GADD153 in hypoxia-induced apoptosis. The stable or inducible overexpression of GADD153 sensitized the H9c2 cells to apoptotic cell death. The results suggest that the transactivation domain (TA) of the GADD153 might be responsible for this cell execution and play a role in the nucleoplasmic localization of GADD153. The cells transiently transfected with the antisense GADD153 were more resistant to hypoxia-induced apoptosis than the vector control cells. Furthermore, GADD153 transcriptionally down-regulated the expression of the cardiac ankyrin repeat protein gene (CARP), which is a nuclear transcriptional co-factor that negatively regulates the expression of the cardiac gene. The ectopic expression of CARP in H9c2 cells increased the resistance to hypoxia-induced apoptosis. These results suggest that GADD153 over-expression and the concomitant downregulation of CARP might have a causative role in the apoptotic cell injury of hypoxic H9c2 cells.
INTRODUCTION

The apoptosis of cardiac myocytes is a cellular mechanism of a limited ischemic injury to the heart, while prolonged ischemia appears to cause necrosis of the cardiac myocytes (1-3). Apoptosis in the cardiac myocytes can be induced by hypoxia (4), serum withdrawal (5), or a combination of hypoxia and glucose and serum deprivation (6). Therefore, a better understanding of the mechanisms by which cardiomyocytes undergo apoptosis following oxidative stress would be helpful, and might provide additional targets for the treatment of ischemic heart disease. It has been reported that hypoxia-induced apoptosis of neonatal rat cardiomyocytes is regulated by the expression of the Fas antigen and the intracellular signaling pathways activated by p53 (7, 8). The c-Jun NH2-terminal kinase (JNK) pathway or cyclin A/cdk2 activation plays an important role in signaling oxidative stress-induced apoptosis in cardiomyocytes (9, 10). Recently, the expression of the pro-apoptotic gene, BNIP3, was shown to contribute to apoptotic cell death in primary rat neonatal cardiomyocytes (11). However, it is unclear how hypoxia actually triggers cell death in cardiomyocytes.

The GADD153 protein (also known as CHOP-10) is a member of the CCAAT/enhancer-binding protein (C/EBP) family of transcriptional factors (12) that regulate the cell cycle and apoptosis. GADD153 is transcriptionally activated. In addition, is strongly expressed after treating the cells with a variety of growth arrest and/or DNA damaging factors (13,14), such as calcium ionophores (15), glucose deprivation (16), oxidative stress (17), reductive stress (18), endoplasmic reticulum stress (19), or activation of the acute phase response (20). GADD153 has been implicated in growth arrest and cell death. A microinjection of GADD153 induces 3T3 cells to arrest at the G1/S boundary (21), while the ectopic expression of
GADD153 causes M1 myeloblast leukemia cells to undergo apoptosis (22). GADD153 was reported to sensitize cells to endoplasmic reticulum stress via mechanisms involving the down-regulation of Bcl-2 and enhanced oxidative injury (23). In particular, hypoxia/oxidative stress-induced GADD153 gene expression was observed in pulmonary artery smooth muscle cells or vascular smooth cells (24, 25). GADD153 was induced at both the mRNA and protein levels, which is in parallel with the induction of vascular smooth cell apoptosis, after treatment with the platelet-derived growth factor–BB. The overexpression of GADD153 in the vascular smooth muscle cells significantly reduced the level of cell viability and induced apoptosis. Moreover, in a rat carotid artery balloon injury model, the GADD153 protein was expressed in apoptotic vascular smooth muscle cells (25). However, recent reports do not explain whether or not GADD153 over-expression functions in hypoxic stress-induced apoptosis in cardiomyocytes. Therefore, this study showed that GADD153 is over-expressed in H9c2 cells as a result of hypoxia, which may be associated with apoptotic cell death, and proposes the molecular role of GADD153 in this type of apoptosis in cardiomyocytes.

**EXPERIMENTAL PROCEDURES**

*Cell Lines and Reagents*—The rat embryonic heart-derived H9c2 cells were obtained from the American Tissue Culture Collection (ATCC, Rockville, MD). CoCl₂, 2, 2’-dipyridyl (DP), and the desferrioxamine (DFO), N-acetyl-L-cysteine (NAC), and propidium iodide (PI) were obtained from Sigma (St. Louis, MO). The adriamycin (doxorubicin) was obtained from Sigma, and the N–(4–hydroxyphenyl)retinamide (4HPR) was a kind gift from the Johnson Pharmaceutical Research Institute (Spring House, PA).
Cell Culture and Detection of Apoptotic Cells—The cells were cultured in DMEM supplemented with 10% FBS in air containing 5% CO₂. The cells (10⁵) were plated on 6-cm dishes and transferred to a chamber. Hypoxic conditions were achieved using a BBL GasPac Plus (Becton Dickinson, San Diego), which catalytically reduced the oxygen level to 0.5% within 150 min (time 0 of the hypoxic conditions) (26). The number of viable cells was determined using trypan blue dye exclusion. The percentage of apoptotic cells was evaluated using diamidino-2-phenylindole (DAPI) staining. Briefly, the H9c2 cells were either incubated under hypoxic conditions for 16 h, or treated with the indicated concentrations of CoCl₂, DFO, or DP for a 48-h period. The cells were fixed in ice-cold methanol for 10 min and stained with the DAPI reagent (1 µg/ml). Fluorescence microscopy was performed using a Nikon diaphot microscope. Apoptotic cells were recognized by the condensed, fragmented, and degraded nuclei, or ghosts that stained only faintly. At least 200 cells were counted at each time point and all the counting was carried out in a blinded fashion.

DNA Fragmentation or Flow Cytometric Analysis for Apoptotic Quantitation—The genomic DNA was analyzed by harvesting the cells and combining them with the nonattached cells in the supernatant. The cells were resuspended in 0.5 ml of a lysis buffer (50 mM Tris-HCl, 100 mM EDTA, 0.5% SDS, pH 8.0) containing 0.1 mg/ml RNAse A. After incubation at 37°C for 30 min, the extracts were treated with 1 mg/ml proteinase K for an additional 16 h at 37°C. The DNA was extracted with phenol/chloroform, then with chloroform, and was finally precipitated with ethanol and sodium acetate. Twenty µl of each extract (dissolved in 50 µl H₂O) was then loaded on a 1.5% agarose gel and separated in the presence of 0.5 µg/ml ethidium
bromide. Flow cytometry (FCM) with a FACScan (BD bioscience, San Jose, CA) was used to count the number of cells, as described previously (27). A 630 nm-long bandpass filter was used to measure the red fluorescence due to the PI-bound DNA. The data was analyzed as a single parameter frequency histogram in an SFIT model.

**Northern Blot Analysis**—The cells were cultured in DMEM with 10% FBS until they reached 70% confluence. They were then incubated under hypoxic conditions or treated with the indicated agents. The total RNA was extracted from the treated or untreated cells using a phenol and guanidine thiocyanate solution (Tri Reagent; Molecular Research Center, Inc. Cincinnati, OH). The RNA was then fractionated by electrophoresis on 1.0% agarose gels containing formaldehyde, and transferred to membranes. The blots were hybridized overnight with a $2 \times 10^6$ cpm/ml cDNA probe, labeled with $[P^{32}]$dCTP (PerkinElmer, Boston, MA) by random-priming, washed, and exposed to X-ray film at -70°C, as described previously (28). As a loading control, glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) mRNA was detected using a *GAPDH* cDNA probe.

**Western Immunoblotting**—The cells were washed twice with cold PBS on ice and harvested by scraping with a rubber policeman. The cells were pelleted by centrifugation at 4°C and resuspended directly into a Laemmli sample buffer containing 62.5 mM Tris-HCl, pH 6.8, 2% SDS (w/v), 10% glycerol (w/v) and 5% mercaptoethanol (v/v). The extracted proteins were resolved by 10% SDS-PAGE and transferred to nylon membranes, as described previously (29). The GADD153 polyclonal antibody (R-20) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA).
Stable or Inducible Overexpression of GADD153—The transfection of the GADD153 gene into the H9c2 cells was carried out using an expression plasmid vector encoding human GADD153 cDNA or the control pcDNA3. The construction of the GADD153 expression vector was achieved by ligating the human GADD153 (a gift from Dr. Nikki J. Holbrook) with pcDNA3 in the BamHI and XhoI sites in either the sense or in the antisense orientation, as described elsewhere (29). The transfections were carried out by lipofectin (Invitrogen, Gaithersburg, MD) according to the manufacturer’s protocol. The GADD153-transfected or neo-transfected cells were selected in the presence of 600 mg/ml G418 for 2-3 weeks. Finally, the individual colonies were isolated using cloning rings, expanded and assayed for the expression of the transfected gene using Northern and Western blotting analysis. For the pTRE2-GADD153 construct, a fragment of GADD153 DNA from pcDNA3-GADD153 was ligated to the BamHI and SalI sites of pTRE2hyg (BD Biosciences Clontech, Palo Alto, CA). The H9c2 cells were transfected with the pTet-Off vector (BD Biosciences Clontech) and selected with 600 µg/ml G418 for 2-3 weeks. The surviving cells (Tet-Off cell lines) were expanded and screened for tTA regulatory protein expression by a transient transfection with TRE2hyg-Luc (BD Biosciences Clontech) for the clones with a low background and a high level of luciferase induction in response to tetracycline. An H9c2 Tet-Off clone was transfected with the pTRE2hyg-GADD153 expression construct. After selection with 50 µg/ml hygromycin for 2-3 weeks, the surviving cells were expanded and Northern blotting was used to determine the presence of GADD153 gene expression. The effect of CARP regulation on cell survival was determined by generating an antisense CARP expression plasmid by ligating an open reading frame PCR amplified to pcDNA in the XbaI and KpnI sites in an antisense orientation using a set of primers containing
either XbaI (sense primer, 5´GCTCTAGACCTTCAGCCAACATGATGG) or KpnI (antisense, 5´GGGGTACCCCTCAGAATGTAGCTATGC). The sense CARP expression plasmid was generated by ligating an open reading frame PCR amplified to pcDNA3.1/HisA in the KpnI and XhoI sites in a sense orientation using a set of primers containing KpnI (sense primer, 5´GGGGTACAGCCAACATGATG) or XhoI (antisense, 5´CCCTCGAGGCTCAGAATGTAGC).

**Cellular Localization of the Functional GADD153 Protein and Immunofluorescence**—The H9c2 cells were transfected with the expression vector, pEGFP-GADD153 deletion mutants, which were kindly provided by Dr. Joel F. Habener (30). After 48 hours, the cells were stained with DAPI and the transfected cells were examined using laser-scanning microscopy (LCM510, Carl Zeiss, Jena, Germany). For immunofluorescence, the cells were washed twice with cold PBS on ice and fixed in 4% PBS-paraformaldehyde for 30 min, which was followed by membrane permeabilization in 0.1% Triton X-100/PBS buffer for 1 min. The nonspecific antibody binding was blocked using PBS/bovine serum albumin 1% w/v for 1 h followed by an overnight incubation with the primary antibody against 5 mg/ml CARP at 4°C. The primary antibody binding was detected with the tetramethylrhodamine isothiocyanate isomer R (TRITC)-conjugated swine anti-rabbit immunoglobulin (1:100, Dako, Glostrup, Denmark). The polyclonal antibody to CARP was affinity-purified from the sera of the immunized rabbits with the recombinant GST-CARP protein produced in E. coli.

**Luciferase assay**—PCR amplification was performed from the human liver genomic DNA to generate the luciferase reporter gene CARP–206Lucas described elsewhere (31). The
PCR product was subcloned into the promoterless luciferase reporter gene vector, pGL3–Basic (Promega, Madison, WI). Cells were plated at $2 \times 10^4$ cells per well in 24-well plates, and 18 hr later the cells were then cotransfected at 37°C for 16 hr with 500 ng CARP–206Luc plasmid along with various concentration of the GADD153 expression plasmid and lipofectin (Invitrogen, Gaithersburg, MD). The cells were lysed in 120 µl of a lysis buffer at the indicated time intervals and stored at –20°C until assayed. The luciferase activity was measured using a Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer’s instructions, and was normalized using a Renilla luciferase activity.

Statistical Analysis—All the data was entered into the Microsoft Excel 5.0 package and GraphPad Software was used to perform the two-tailed $t$ tests. $P$ values < 0.05 were considered significant.

RESULTS

Induction of GADD153 during Hypoxia–Induced Apoptosis—Hypoxia effectively induced apoptotic cell death in the cultured cells stained with the DNA-binding dye DAPI in a time dependent manner. This revealed the condensed chromatin and fragmented nuclear morphologies that are characteristic of apoptosis in H9c2 cells (Fig. 1A and B). An overnight culture under hypoxic conditions caused apoptosis in approximately 35% of the H9c2 cells. The level of DNA fragmentation was analyzed by gel electrophoresis. The H9c2 cells cultured under hypoxia showed increased fragmentation of the lower molecular weight DNAs in a time dependent manner, which supported the above findings (Fig. 1C). The percentage of apoptotic
cells measured using DAPI was similar to the apoptotic population measured by FCM, i.e. the percentage of cells in the sub-G1 fraction stained with PI showed similar results (data not shown). During apoptotic cell death, it was found that the GADD153 gene is expressed in proportion to the level of hypoxia induced-apoptotic cell death in H9c2 cells in a time dependent manner (Fig. 1D). Therefore, this study examined whether or not the hypoxic induction by chemicals including divalent cations (CoCl2) and iron chelators (DFO and DP) causes apoptotic cell death and GADD153 mRNA overexpression. As expected, these “hypoxia imitators” effectively induced GADD153 expression (Fig. 1E), which was in proportion to the level of apoptotic cell death observed in the H9c2 cells (Fig. 1F). The iron chelators induced higher GADD153 mRNA overexpression and apoptotic cell death than the divalent cations.

Effect of Ectopic GADD153 Over-expression on Apoptosis—In order to determine the role of GADD153 mRNA over-expression, the GADD153 expression system was introduced to the H9c2 cells because these cells expressed a low level of constitutive GADD153. Two types of H9c2 cells (HG-10 and HG-19) that stably express different levels of human GADD153 mRNA were isolated (Fig. 2A). The cells were then maintained under hypoxic conditions at 37°C. Compared with the control cells transfected with the empty vector alone (VC-4 and VC-5), the HG-10 and the HG-19 cells became apoptotic within 12 h of being exposed to the hypoxic conditions (P < 0.01), i.e. up to 80% of the HG-10 and HG-19 cells were apoptotic while only 18% of the VC-4 and VC-5 cells were apoptotic. The proportion of apoptotic cells appears to be related to the GADD153 protein expression level. FCM analysis found that the apoptotic sub-G1 fraction of the cells also increased in response to hypoxia (Fig. 2B). A GADD153 expression construct was produced by placing GADD153 cDNA under the control of a tetracycline (tet)-
repressible promoter (32). The H9c2 Tet-Off clone was then transfected with the pTRE2hyg-GADD153 expression construct described in EXPERIMENTAL PROCEDURES. After selection with hygromycin for a 2-3 week period, the surviving cells were expanded and screened for GADD153 gene expression using Northern blotting. Two clones, TREG8 and TREG20, which showed efficient tet-repressible expression of the GADD153 transgene, were selected for the cell growth experiments. In both clones, GADD153 over-expression was induced by the depletion of tetracycline in a time-dependent manner (Fig. 2C). The level of apoptotic cell death was increased as a result of tetracycline depletion in the TREG8 and TREG20 cells in response to hypoxia for 16 h (85.6% and 87.5% versus 37.4% and 36.6%, respectively) (Fig. 2D), which correlates with the observed GADD153 mRNA over-expression.

The transient co-transfection of an antisense expression plasmid of GADD153 (3 µg) with pEGFP (1 µg) was performed to determine if the prevention of GADD153 over-expression might lead to the inhibition of hypoxia-induced apoptosis. The level of apoptotic cell death was determined by gating the GFP-positive cells and calculating the sub-G₁ fraction of cells transfected with the antisense GADD153 cDNA. The antisense transfectants were significantly resistant to hypoxia e.g. approximately 15.8% of the transfectants were apoptotic ($P < 0.01$), compared with 35.7% of the empty vector control cells (Fig. 2E). The accumulation of GADD153 mRNA in the H9c2 cells transfected with the antisense GADD153 cDNA was effectively decreased to less than 50% of the control controls (right panel). This suggests that the inhibition of GADD153 expression correlates positively with cell survival in the H9c2 cells.

Proapoptotic Ability and Cellular Localization of Deletion GADD153 Mutants—In order to determine what regions of the GADD153 molecule play a role in hypoxia-induced
apoptosis, various GADD153 deletion mutants (30) were examined for their ability to stimulate apoptosis upon transfection (Fig. 3A). The fluorescence-activated cells were gated and analyzed for a hypodiploid fraction in the cells transfected with the GFP fusion deletion mutants of GADD153. The level of apoptosis (sub-G1 fraction) increased 4-fold in the wild-type GFP-GADD153 (Fig. 3B). The mutant where the entire transactivation (TA) region was missing (pEGFP-bZIP) did not enhance the level of apoptotic cell death to almost the same degrees as the GFP vector control. In contrast, the other deletion mutants containing the TA elicited apoptotic cell death in response to hypoxia, irrespective of the partial deletion of the first third of the TA region (36 amino acids) or point mutations at Ser78 and Ser81 of the TA region (Fig. 3C). This suggests that the proapoptotic ability of GADD153 resides in the TA region of the protein and is independent of a partial deletion or point mutation in the TA region.

The proapoptotic ability of these deletion mutants was then examined to determine if they were relevant to their cellular localization. Interestingly, the wild-type GFP-GADD153 was located in the nucleoplasm, which is devoid of nucleoli. pEGFP-bZIP is mainly located in the nucleoli. The deletion mutant, GFP-LZ(-) (missing ZIP), is located in both the nucleoplasm and the nucleoli. The GFP-GADD153Ala78,81 mutant is located at the nucleoplasm, whereas the GFP-TA(-) (missing partial deletion of TA) is initially located at the nucleoplasm but migrates to the nucleoli (Fig. 3D). Therefore, the nucleoplasmic localization resulting from the TA region appears to be related to apoptotic cell death. In the process of apoptotic cell death, the nuclear translocation of the GADD153 protein was partially impaired and was retained in the cell cytoplasm until the late stage. This was also observed with the nuclear localization results (Fig. 3E).

The stress-activated phosphorylation of GADD153 by the p38 mitogen-activated protein kinase (p38 MAPK) was examined.
protein kinases (p38 MAPK) at serines, 78 and 81, is responsible for cell apoptosis, and a pretreatment with a p38-kinase inhibitor, SB203580, blocks both the phosphorylation of GADD153 and stress-mediated apoptosis (33). However, the data shows that an Ala78,81 mutation of GADD153 has no impact on the hypoxia-mediated apoptosis. Therefore, this study next examined whether or not p38 MAPK activation still functions during apoptosis. SB203580 (20 µM or 40 µM) effectively enhanced cell survival (3.1 or 4.0 fold) (Fig. 4A) and did not alter the GADD153 protein levels (Fig. 4B), which suggests that alternative pathway to p38 MAPK activation or a p38 MAPK-independent pathway is involved. Furthermore, NAC (2 mM or 4 mM) effectively inhibited the hypoxia-induced apoptosis and enhanced the level of cell survival by approximately 3.1 or 3.8 times compared with the vehicle controls (P < 0.01) (Fig. 4A). Similarly, NAC did not inhibit the over-expression of the GADD153 protein, rather the higher concentration (4 mM) of NAC significantly stimulated GADD153 protein expression (Fig. 4B), which indicates that NAC functions downstream of GADD153 expression.

**GADD153 Down-regulate the Expression of CARP**—It was observed that a treatment with adriamycin induces apoptotic cell death and the concomitant overexpression of GADD153 mRNA in H9c2 cells. It should be noted that adriamycin (2 µg/ml) effectively induced apoptotic cell death (66%) and the concomitant GADD153 overexpression in a time dependent manner over a 48-h period (Fig. 5A). In addition, the expression of the CARP gene during adriamycin-induced apoptosis was examined because adriamycin-mediated cardiac toxicity is related to the down-regulation of CARP gene expression via oxidative stress (34). The results showed that adriamycin consistently down-regulated CARP mRNA expression. However, CARP down-regulation fluctuated with GADD153 mRNA expression during the 36-h adriamycin treatment.
The relationship between \textit{GADD153} and \textit{CARP} mRNA expression in response to hypoxia was examined. Both genes were inversely expressed in a time dependant manner during the hypoxia-induced apoptosis (Fig. 5C). Therefore, oxidative stress might result in \textit{GADD153} over-expression, which subsequently down-regulates the \textit{CARP} expression level. In order to examine this hypothesis, the expression of \textit{CARP} mRNA was tested in the cells stably expressing \textit{GADD153}. As expected, the \textit{CARP} mRNA expression level was reduced in the stable transfectants. The \textit{CARP} mRNA expression level was inversely proportional to the \textit{GADD153} mRNA expression level (Fig. 5D). Another approach to support this hypothesis was to demonstrate the promoter activation by \textit{GADD153}. The reporter plasmid, pGL3B-CARP (-206), which consists of 206 bp fragment of the 5′-flanking sequence and a 170 bp fragment of the 5′-untranslated region of the human \textit{CARP} gene, was transiently cotransfected into the H9c2 cells using either the empty vector or the pcDNA3-GADD153 expression vector. Cotransfection with the pcDNA3-GADD153 expression plasmid decreased the luciferase activity driven by the \textit{CARP} promoter in a dose dependent manner (Fig. 6A). Four µg of \textit{GADD153} reduced the promoter activity to 25% of the control. In addition, the immunofluorescence assay revealed that the expression of the \textit{CARP} protein was localized in the cytoplasm or the nucleus of the cells, which perfectly overlaps with the ectopic expression of the GFP-CARP fusion protein (Fig. 6B, upper panels). Moreover, the results showed that the over-expression of GADD153 down-regulated the \textit{CARP} expression level (Fig. 6B, middle panels). This suggests that GADD153 induction by oxidative stress might mediate the transcriptional downregulation of \textit{CARP}.

\textit{Ectopic Expression of CARP Increases Resistance to Hypoxia-Induced Apoptosis in}
H9c2 Cells—In order to determine the role of CARP in hypoxia-induced apoptotic cell death in H9c2 cells, the stable transfectants expressing CARP mRNA (CARP-6 and CARP-7) were isolated and the level of apoptotic cell death caused by hypoxic stress for 16 h was measured (Fig. 7). CARP effectively decreased the susceptibility of the cells to hypoxia-induced apoptotic cell death, compared with the vector controls (17% and 16.7% versus 35% and 33.6%, P < 0.01). This suggests that the down-regulation of CARP might contribute at least in part to apoptotic cell death in the H9c2 cells.

**DISCUSSION**

The heart is subjected to oxidative stress in many clinical situations including ischemia-reperfusion injury and anthracycline chemotherapy (35). The loss of cardiac myocytes through cardiac myocyte apoptosis is a major problem in heart failure. In addition, the molecular mechanism of cardiac myocyte apoptosis is not fully understood. This study examined the role of the proapoptotic transcriptional factor, GADD153, in cardiac myocytes apoptosis as a response to hypoxia. Furthermore, GADD153 was found to be involved in anthracycline doxorubicin-induced cardiac myocytes apoptosis.

GADD153 is a transcriptional factor that is induced by cellular stress, and has been suggested to play a role in the signal transduction from the stressed endoplasmic reticulum (ER) to apoptosis. The blocking N-linked glycosylation (a post-translational event specific to the proteins entering the ER) with tunicamycin resulted in the strong induction of GADD153 along with the up-regulation of BiP and GRP94, which are specific proteins in the unfolded protein
Other chemicals that interfere with ER related processes. These include thapsigargin (inhibition of the ER Ca\(^{2+}\)-ATPase), brefeldin A (an inhibitor of vesicle transport between ER and Golgi), and AIF4- (inhibition of trimeric G-proteins), also induce GADD153 (37). Glucose-regulated proteins (GRPs) are induced by chemical agents such as tunicamycin, A23187 and hypoxia, which disrupt protein trafficking in the endoplasmic reticulum. The treatment of the NIH-3T3 cells with chemical inducers of GRPs increases the levels of GADD153 mRNA as well as GRP78 mRNA. In addition, hypoxia can also increase the GADD153 and GRP78 mRNA level. Therefore, the GRP and GADD genes can be activated by similar stimuli (e.g., hypoxia and chemical inducers). However, the mechanisms leading to the increased GRP78 and GADD gene mRNA levels are different, and might involve distinct protein kinases (38). Based on this hypothesis, rat cardiomyocytes were maintained under hypoxic conditions and treated with adriamycin. Both situations clearly up-regulated GADD153. Therefore, the hypoxia-mediated apoptosis of cardiac myocytes appears to be related to ER stress. Recently, the over-expression of GADD153 sensitized cells to ER stress via the down regulation of Bcl-2 expression. This down-regulation of Bcl-2 expression increased the level of oxidant injury, e.g. the depletion of cellular glutathione and the exaggerated production of reactive oxygen species (39). However, altered Bcl-2 expression was not observed in this experimental system (data not shown). Although, few studies have addressed the mechanistic link between GADD153 expression and cell death, the induction of GADD153 has been observed in the apoptotic pathways of FAS-regulated apoptosis and C6-ceramides-mediated cell death (40).

This study found that either hypoxia or hypoxia-mimicking chemicals in vitro induced GADD153 over-expression in cardiomyocytes. The hypoxia-regulated genes involved in
controlling the cell cycle or apoptosis are either HIF-1α-dependent (those encoding the proteins p53, p21, Bel-2) or HIF-1α-independent (p27, GADD153) (41). The GADD153 gene promoter activity is stimulated by the binding of the AP-1 family of proteins to an AP-1 site in a GADD153 gene promoter region as a result of oxidative stress such as exposure to reactive oxygen species (17). This shows that the stable expression, i.e. tet-inducible expression, and the transient expression of GADD153 increase the level of apoptotic cell death. The GADD153 molecule contains a N-terminal transactivation domain, a basic region, and a leucin zipper. This study determined which region contains the important proapoptotic domains of GADD153 in relation to their subcellular localization. The data suggests that the TA domain plays a key role in apoptotic cell death, and that this region is responsible for the nucleoplasmic localization of GADD153. The partial deletion of the N-terminal of the TA domain failed to reduce the level apoptotic cell death. These results are somewhat different from those reported elsewhere (34). This disparity appears to be derived from the different quantitative method used. However, a mutation of serine 78 and 81 of the TA domain to alanine does not alter the pro-apoptotic activity, which is consistent with a previous report (30) suggesting that the p38 MAPK mediated phosphorylation site is not essential for the proapoptotic function of GADD153. However, it was observed that SB203580, a specific p38 MAPK inhibitor, effectively inhibited the hypoxia-mediated apoptosis in cardiomyocytes without altering the GADD153 expression level. Therefore, it is proposed that hypoxia-mediated GADD153 induces the apoptosis of cardiomyocytes via a p38 MAPK dependent or independent mechanism, as reported elsewhere (30).

The CARP expression level increases during human heart failure (42) and in animal models of cardiac hypertrophy (43), and decreases in cardiomyocytes exposed to adriamycin
CARP has been suggested to act as a nuclear transcription co-factor that negatively regulates the cardiac gene expression and might play a key role in the pathophysiology of heart failure. The β-adrenoceptor agonist, isoprenaline, induces hypertrophy and increases the CARP expression level. Furthermore, CARP is up-regulated in response to shear stress in vitro. This suggest that CARP expression might be associated with pathological stress in cardiomyocytes. This study found that hypoxic stress leads to the up-regulation of GADD153 expression and the down-regulation of CARP expression. Moreover, the results suggest that GADD153 expression is involved in the transcriptional down-regulation of CARP. The adriamycin–mediated down-regulation of CARP may in part be derived from oxidative stress. Furthermore, it was observed that an oxidative stress-inducing agent, 4HPR, similarly down-regulated CARP expression (data not shown). It is unclear if the down-regulation of CARP is directly or indirectly involved in the GADD153-mediated sensitization of the cardiomyocytes to apoptotic cell death. However, these results show that the ectopic overexpression of CARP decreased the level of apoptotic cell death induced by oxidative stress. Therefore, CARP induction may at least in part play a role in the GADD153-mediated sensitization of apoptotic cell death.

In summary, this study demonstrated that hypoxia-induced GADD153 induction is partly responsible for the apoptotic cell death in cardiomyocytes. The deletion mutant experiments showed that the wild-type GADD153 located at nucleoplasm, which is devoid of nucleoli, and the nucleoplasmic localization of the TA region is related to apoptotic cell death. The antioxidant and the p38 MAPK inhibitor effectively decreased the hypoxia-induced apoptotic cell death but had little effect on GADD153 expression. Hypoxic stress also down-regulates CARP expression, which might be mediated by GADD153. This understanding of the mechanisms through which
hypoxic stress damages the cardiac myocytes might assist further studies aimed at reducing the
effect of hypoxia or ischemia/reperfusion injury.

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**FIGURE LEGENDS**

**Fig. 1.** Hypoxia induced apoptosis in H9c2 cells and concomitant \textit{GADD153} mRNA expression. \(A\), The H9c2 cells were cultured under hypoxic conditions for 16 h and the apoptotic cells were counted by DAPI staining at the indicated time intervals. The values are represented as the mean ± S.E. of three independent experiments carried out in duplicate. \(B\), the nuclei of the cells in the hypoxic culture showing the typical apoptotic morphology of fragmentation and condensation after staining with DAPI. \(C\), induction of internucleosomal DNA fragmentation by hypoxic incubation. The DNA was extracted and analyzed by 2% agarose gel electrophoresis in the presence of ethidium bromide at the indicated times. \(D\), induction of \textit{GADD153} mRNA in hypoxic cultured cells as a function of time. The total RNA was extracted at the indicated times and fractionated by electrophoresis on 1% agarose gel. The blot was hybridized overnight with \textit{GADD153} cDNA and reprobed with \textit{GAPDH} cDNA as a loading control. The experiments were performed at least three times, and the result of one representative experiment is shown. \(E\), The \textit{GADD153} mRNA levels increase in H9c2 cells in response to the hypoxia mimics. The cells were incubated in the presence of 100 \(\mu\)M CoCl$_2$, 100 \(\mu\)M DFO, or 100 \(\mu\)M DP for 48 h. The total RNA was extracted at the indicated times and fractionated by electrophoresis on 1% agarose gel. The blot was hybridized overnight with \textit{GADD153} cDNA and reprobed with \textit{18S} cDNA as a loading control. The experiments were performed at least three times, and the result of one representative experiment is shown. \(F\), quantification of the apoptotic fractions was performed using a FACScan as described in “EXPERIMENTAL PROCEDURES.” Each value represents the mean ± S.E. of triplicate experiments. **, \(p < 0.01\) compared with the control.
**Fig. 2. Effect of ectopic GADD153 over-expression on apoptosis induced by hypoxia.**

A, Northern blot analysis of *GADD153* in H9c2 cells transfected with the GADD153 expression plasmid (HG-10 and HG-19) or the vector control (VC-4 and VC-5). The effect of the hypoxic culture for 12 h on apoptotic cell death in the cells stably over-expressing GADD153. Apoptotic cell death was determined by DAPI (lower). The vertical bars represent the means ± S.E. of two experiments carried out in duplicate. **, significantly different from the vector controls at *p* < 0.01.

B, FCM analysis of the sub-G1 fraction from the HG-10 and HG-19 cells stably over-expressing GADD153 compared with the VC-4 and VC-5 cells in response to hypoxia (M1, apoptotic fraction). The values represent the means ± S.E. of two experiments carried out in duplicate. **, significantly different from vector controls at *p* < 0.01.

C, the accumulation of *GADD153* mRNA was induced in tet-regulated transfectants (TREG8 and TREG20) in a time dependent manner. The transfectants were cultured in the absence of tetracycline for the indicated times. The total RNA was extracted at the indicated time periods and fractionated by electrophoresis on 1% agarose gel. The blot was hybridized overnight with *GADD153* cDNA and reprobed with *18S* cDNA as a loading control.

D, apoptotic cell death during the induction of *GADD153* mRNA in the tet-regulated transfectants (TREG8 and TREG20). The cells were cultured in the presence (+) or absence (-) of 1 µg/ml tetracycline for 48 h and then incubated under hypoxic conditions for 16 h. The level of apoptotic cell death in response to hypoxia was determined by DAPI staining. The values are the means ± S.E. of three experiments carried out in duplicate. **, *p* < 0.01.

E, inhibition of apoptotic cell death in cells co-transfected with the antisense *GADD153* and pEGFP were incubated under hypoxic conditions for 16 h. The fluorescence-activated cells were gated and analyzed for the
hypodiploid fraction. **, \( p < 0.01 \) compared with the vector control. The total RNA was extracted and fractionated by electrophoresis on 1% agarose gel. The blot was hybridized overnight with GADD153 cDNA and reprobed with 18S cDNA as a loading control (right panel).

**Fig. 3.** Proapoptotic activity and cellular localization of the domain of GADD153. A, a scheme to show the constructs of the deletion mutants of GFP-GADD153. Each number represents an amino acid within GADD153. TA, transactivation domain; b, basic region; ZIP, leucin zipper. B, GADD153-induced apoptosis demonstrated in gated population of cells by fluorescence-activated cell scanning. The H9c2 cells were transfected with either GFP or GFP-GADD153 in 60-mm dishes and incubated for 24 h. They were then cultured under normoxic or hypoxic conditions for 8 h. The cells were harvested and stained with PI. Fluorescence-activated cells were gated (upper panels) and analyzed for the hypodiploid fraction (M₁). *, \( p < 0.01 \) compared with the vector control. C, the indicated deletion mutants of GFP-GADD153 were evaluated for their ability to induce apoptosis in H9c2 cells under hypoxic conditions for 8 h. The bars represent means ± S.E. of three experiments carried out in duplicate. **, \( p < 0.01 \) compared with GFP-GADD153. D, cellular localization of the deletion mutants. H9c2 cells grown on coverslips were transiently transfected with 2 µg of the plasmid encoding either the GFP-GADD153 or the deletion constructs of the GFP-GADD153, or with a vector control (pEGFP). The GFP fluorescence was then analyzed using confocal microscopy. Bar, 20 µm. E, cellular localization of GFP-GADD153 fusion protein in the cells cultured under hypoxic conditions for 8 h and 16 h, respectively. Bar, 20 µm.

**Fig. 4.** Increased cell survival by p38 MAPK-inhibitor SB203580 or by NAC. A, The
H9c2 cells were incubated under hypoxic conditions in the presence of the p38 MAPK inhibitor, SB203580 (20 and 40 µM, respectively), or NAC (2 and 4 mM, respectively). The H9c2 cells were cultured under hypoxic conditions for 20 h and the level of cell survival was determined by trypan blue dye exclusion. The bars indicate the mean ± S.E. of three experiments carried out in duplicate. **, \( p < 0.01 \).

**B**, GADD153 expression during inhibited cell death in H9c2 cells treated with SB203580 (20 and 40 µM, respectively) or NAC (2 and 4 mM, respectively). The proteins were extracted and underwent immunoblot analysis using a polyclonal antibody against GADD153 (R-20). The GADD153 levels were quantified using densitometric scanning and are normalized to the actin levels. The bars are the means ± S.E. of three experiments. **, \( p < 0.01 \).

**Fig. 5.** Adriamycin or hypoxia-mediated reciprocal regulation between *GADD153* and *CARP* mRNA expression. **A**, adriamycin-induced apoptotic cell death in a time dependent manner. The H9c2 cells were treated with 2 µg/ml adriamycin for 48 h. The level of apoptotic cell death was determined by DAPI staining. The values represent a mean ± S.E. of two experiments carried out in duplicate. **B**, adriamycin-mediated down-regulation of *CARP* mRNA or up-regulation of *GADD153* mRNA during apoptotic cell death. The total RNA was extracted at the indicated times and fractionated by electrophoresis on 1% agarose gel. The blot was hybridized overnight with *GADD153* cDNA and reprobed with *GAPDH* cDNA as a loading control. The experiments were performed at least three times, and the result of one representative experiment is shown. **C**, the expression of *GADD153* mRNA inversely related to the expression of *CARP* mRNA in response to hypoxia. The H9c2 cells were incubated under hypoxic conditions for 16 h. The total RNA was extracted at the indicated times and fractionated by electrophoresis on 1% agarose gel. The blot was hybridized overnight with *GADD153* or *CARP* cDNA.
cDNA and reprobed with GAPDH cDNA as a loading control. The experiments were performed at least three times, and the result of one representative experiment is shown. D, the expression of CARP mRNA in the H9c2 cells stably expressing GADD153 mRNA (HG-10 and HG-19) compared with the control cells stably transfected with the empty vector (VC-4 and VC-5).

FIG. 6. Regulation of the CARP promoter activity by GADD153. A, The H9c2 cells were co-transfected with the CARP (-206/+170) luciferase reporter gene and various concentrations of the GADD153 expression plasmid (pcDNA3-GADD153) for 48 h. The vertical bars represent the mean ± S.E. of three experiments carried out in duplicate. B, The H9c2 cells were transfected with 2 µg of the expression vectors for GFP-CARP, GFP-GADD153, or GFP. The cells were fixed with 4% paraformaldehyde and permeabilized in 0.4% Triton-X for 10 min at room temperature. The cells were stained 15 min with 1 µg/ml DAPI to visualize the nucleus (blue) and for indirect immunofluorescence staining against CARP (red). The cells were then stored in 50% glycerol in PBS at 4°C. GFP fluorescence (green) was analyzed using confocal microscopy. Bar, 20 µm.

FIG. 7. Inhibition of apoptotic cell death by the ectopic overexpression of CARP in H9c2 cells. The cells stably expressing CARP (CARP-6 and CARP-7) were isolated and analyzed for CARP mRNA expression (upper panels). The stable transfectants were incubated under hypoxic conditions for 16 h. The percentage of apoptotic cells was evaluated by DAPI staining. Each value represents the mean ± S.E. of triplicate experiments. **, p < 0.01 compared with the vector controls (VC-1 and VC-3).
Fig. 1

A) Apoptotic cells (%) over time (h).

B) Control vs. Hypoxia images.

C) DNA content analysis.

D) GADD153 and GAPDH mRNA expression over time (h).

E) GADD153 and 18S mRNA expression under different treatments.

F) Relative cell number and DNA content under control, CoCl₂, DFO, and DP treatments.

G) Statistical summary of DNA content.

** indicates significance at p < 0.01.
Fig. 2
Fig. 3

A

B

C

D

E

Fig. 3
Fig. 5
A

![Graph showing relative luciferase activity](attachment:image.png)

B

![Immunofluorescence images](attachment:image.png)

Fig. 6
Cell death (%)
Involvement of GADD153 and cardiac ankyrin repeat protein in hypoxia-induced apoptosis of H9c2 cells

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