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Circ. Res. 2006;99;275-282; originally published online Jun 22, 2006;
DOI: 10.1161/01.RES.0000233317.70421.03
Circulation Research is published by the American Heart Association. 7272 Greenville Avenue, Dallas, TX 72514
 Print ISSN: 0009-7330. Online ISSN: 1524-4571

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Abstract—Endoplasmic reticulum (ER) stresses that reduce ER protein folding activate the unfolded protein response (UPR). One effector of the UPR is the transcription factor X-box binding protein-1 (XBP1), which is expressed on ER stress–mediated splicing of the XBP1 mRNA. XBP1 induces certain ER-targeted proteins, eg, glucose-regulated protein 78 (GRP78), that help resolve the ER stress and foster cell survival. In this study, we determined whether hypoxia can activate the UPR in the cardiac context. Neonatal rat ventricular myocyte cultures subjected to hypoxia (16 hours) exhibited increased XBP1 mRNA splicing, XBP1 protein expression, GRP78 promoter activation, and GRP78 protein levels; however, the levels of these UPR markers declined during reoxygenation, suggesting that the UPR is activated during hypoxia but not during reoxygenation. When cells were infected with a recombinant adenovirus (AdV) encoding dominant-negative XBP1 (AdV-XBP1dn), UPR markers were reduced; however, hypoxia/reoxygenation-induced apoptosis increased. Confocal immunocytofluorescence demonstrated that hypoxia induced GRP78 in neonatal rat and isolated adult mouse ventricular myocytes. Moreover, mouse hearts subjected to in vivo myocardial infarction exhibited increased GRP78 expression in cardiac myocytes near the infarct, but not in healthy cells distal to the infarct. These results indicate that hypoxia activates the UPR in cardiac myocytes and that XBP1-inducible proteins may contribute to protecting the myocardium during hypoxic stress. **(*Circ Res*. 2006;99:275-282.)**

Key Words: cardiac myocytes ■ cardiac transcription factors ■ chaperones ■ ER stress ■ hypoxia ■ ischemia ■ mouse ■ myocardial infarction ■ unfolded protein response

In the myocardium, ischemia activates stress-response pathways that can protect against tissue damage[1]; the unfolded protein response (UPR) may be such a pathway. The UPR is activated by stresses that alter protein folding in the rough endoplasmic reticulum (ER), including those that decrease ATP, ER Ca, or UDP-glucose, all of which take place during ischemia.[2–5] The initial objective of the UPR is to resolve ER stress via induction of protective ER-targeted proteins, including chaperones (eg, GRP94, GRP78, calreticulin) and protein disulfide isomerases (PDIs) (eg, ERp72) that reestablish ER Ca and redox homeostasis and restore ER protein folding. This induction is mediated in part by the UPR-activated transcription factors, ATF6 (activation of transcription factor 6),[6,7] and X-box binding protein-1 (XBP1),[8] which bind to regulatory elements of ER stress–responsive genes.[9] If the ER stress is resolved, the UPR wanes, but if unresolved, the UPR activates the apoptotic cell death program.[10–12]

The few studies on ER stress in the heart suggest that the UPR may be activated in cardiac myocytes, where it may enhance myocyte survival. For example, reduction of ER Ca increases GRP78 in H9c2 cardiac myocytes[13] and activates ATF6 and the GRP78 promoter in cardiac myocyte cultures.[14] GRP78 is also elevated in the embryonic mouse myocardium,[15,16] and XBP1 knockout mice die in utero from cardiac myocyte apoptosis,[17] suggesting a requirement for the UPR during cardiac development. GRP78 is induced in mouse hearts on transverse aortic constriction,[18] suggesting UPR activation by pressure overload. Preinducing GRP78 protects H9c2 cells from oxidative stress–induced death,[19] and overexpression of GRP94 in H9c2 cells reduces stress-induced death.[20] Finally, transgenic mice expressing a dysfunctional KDEL receptor, which reduces the levels of ER-targeted chaperones, exhibit increased ER stress in the heart and dilated cardiomyopathy.[21]

The goals of this study were to determine whether hypoxia activates the UPR in cardiac myocytes and, if so, to examine the functional consequences of UPR activation. Accordingly, we examined cultured cardiac myocytes subjected to hypoxia, as well as a mouse model of in vivo myocardial infarction. Hypoxia and ischemia were found to mediate UPR activation in cardiac myocytes in vitro and in vivo. Moreover, dominant-negative XBP1 inhibited the XBP1 branch of the UPR and increased cardiac myocyte apoptosis during hypoxia/reoxygenation. To our knowledge, this is the first study to investigate the functional role of XBP1-mediated transcriptional induction on cultured cardiac myocyte survival and the

Original received December 16, 2005; revision received June 8, 2006; accepted June 12, 2006.
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Circulation Research **is available at http://circres.ahajournals.org** **DOI: 10.1161/01.RES.0000233317.70421.03**

first to show increased expression of UPR markers in cardiac myocytes near an infarct.

Materials and Methods

Animals

Approximately 100 neonatal rats and 8 mice were used in this study. All procedures involving animals were in accordance with the San Diego State University Institutional Animal Care and Use Committee.

Mouse Myocardial Infarction

The hearts of five 10-week old male FDB mice were subjected to permanent myocardial infarction by occlusion of the left anterior descending coronary artery, as previously described.[22] Mice were euthanized 4 days after infarction, at which time the hearts were processed for tissue embedding.

Immunofluorescence and Confocal Microscopy of Mouse Heart Sections, Adult Mouse Cardiac Myocytes, and Neonatal Rat Ventricular Myocytes

Adult mouse hearts were embedded and sectioned as previously described.[23] Briefly, hearts were removed and perfused via the aorta with PBS followed by relaxation buffer containing $CdCl_2$ and KCl and then neutral buffered formalin. After 24 hours in formalin, hearts were cut by cross-section into thirds and processed for standard paraffin embedding. All portions of each heart were included in each block. Cells were isolated and cultured, as previously described.[14,24] Mouse heart sections or cultured cells were incubated with a goat antiserum to GRP78, at 1:30 (catalog no. SC-1051; Santa Cruz Biotechnology, Santa Cruz, Calif), and either a mouse monoclonal antibody to tropomyosin, at 1:100 (catalog no. T9283; Sigma-Aldrich), or a mouse monoclonal antibody to sarcomeric α-actinin, at 1:200 (catalog no. A7811; Sigma-Aldrich), at 4°C for 16 to 18 hours. Appropriate secondary antibodies were applied for 90 minutes at room temperature. Nuclei were labeled with TOPRO-3 iodide. Samples were then visualized on a Leica TCS SP2 confocal laser scanning microscope.

Cell Culture and Hypoxia/Reoxygenation

After maintaining neonatal or adult heart cell cultures for 48 hours in serum-free medium (neonatal) or 16 hours in 2% FBS-supplemented medium, hypoxia, or hypoxia followed by reoxygenation was performed essentially as described previously,[25] and as described in the legends of the figures.

XBP-1 Splicing

XBP1 splicing was assessed using RT-PCR and the following primers that spanned the splice site in the XBP1 mRNA: forward, ACGAGAGAAAACTCATGGC; reverse, ACAGGGTCCAACTTGTCCAG. PCR products were resolved on a 2% agarose gel and viewed by UV illumination. PCR products were sequenced to confirm their identities.

Immunoblots

Cells were extracted, fractionated by SDS-PAGE, and subjected to immunoblotting as described,[26] using an anti-KDEL mouse monoclonal antibody, at 1:2000 (catalog no. SPA-827; Stressgen Biotechnologies Inc, San Diego, Calif), a goat antiserum to GRP78, at 1:1000 (catalog no. SC-1050; Santa Cruz Biotechnology), a rabbit antibody to the C-terminal of XBP1, at 1:500 (catalog no. 619502; BioLegend, San Diego, Calif), rat monoclonal antibody to caspase-12, at 1:200 (catalog no. C7611; Sigma-Aldrich), a mouse monoclonal antibody to CHOP/GADD 153, at 1:500 (catalog no. SC-7351; Santa Cruz Biotechnology), a mouse monoclonal antibody to GAPDH, at 1:15,000 (catalog no. RDI-TRK 5G4–65C; Research Diagnostics Inc, Flanders NJ), or a mouse anti-FLAG M2 monoclonal antibody, at 1:15,000 (catalog no. F-3165; Sigma-Aldrich). Bands on membranes were visualized with horseradish peroxidase–conjugated anti-IgG (Santa Cruz Biotechnology) and enhanced chemiluminescence and quantified as previously described.[27]

Transfection and Reporter Assays

Neonatal rat ventricular myocytes were cotransfected with plasmids encoding SV40-β-galactosidase (pCH110, Amersham) and either pGL2p (control-luciferase) or glucose-regulated protein 78 (GRP78)-luciferase composed of the human GRP78-promoter (−284 to +221) driving luciferase. Cultures were then plated and extracts were assayed for β-galactosidase and luciferase, as previously described.[28]

Adenovirus Preparation and Infection

A recombinant adenovirus strain expressing green fluorescence protein (GFP) and XBP1dn, driven from 2 different CMV promoters, was created using the AdEasy system as previously described.[29,30] Briefly, a construct encoding 3X FLAG-XBP1 spliced (s) encompassing amino acids 1 to 188 of rat XBP1, a known dominant-negative form of XBP1, XBP1dn,[31] was generated. This fragment was cloned into the adenovirus shuttle vector, pAdTrack-CMV, and used to create a new AdV strain, as previously described.[27]

TUNEL Staining and Cell Death Assays

TUNEL staining and was performed as previously described.[26] Assessment of cell death was performed using ethidium homodimer-1 (catalog no. E1169; Molecular Probes) and Calcein Blue AM (catalog no. C1429; Molecular Probes).

Statistics

Each experiment was performed on a minimum of n=3 different cultures or animals and was repeated at least 3 times; the immunoblots and immunocytofluorescence results shown are from 1 experiment that is representative of the results from 3 replicate experiments, and the other results are the compilation of the data derived from at least 3 different experiments. Statistical analyses were performed using a 1-way ANOVA followed by a Student's Newman–Keuls post hoc analysis. The probability values are described in the legends of the figures.

Results

To characterize UPR activation in cardiac myocytes, neonatal rat ventricular myocytes were treated with the prototypical UPR activators, tunicamycin (TM), or dithiothrietol (DTT), which inhibit ER protein glycosylation and alter the ER redox environment, respectively. The levels of several UPR-inducible proteins were assessed by immunoblotting with an antiserum specific for the C-terminal ER-targeting sequence KDEL. Three KDEL-positive proteins were detected, and the quantities of each increased on treatment with TM or DTT (Figure 1A). The 100- and 75-kDa proteins are GRP94 and GRP78, respectively, well-known ER stress–response proteins[32–34]; however, the identity of the 50-kDa protein is not known. The TM- and DTT-mediated increases in GRP78 were confirmed using a GRP78-specific antibody (Figure 1B). GRP78 promoter activity was also increased by ≈4-fold on treatment of cardiac myocytes with TM or DTT (Figure 1C). These results demonstrate that in cultured cardiac myocytes, the prototypical ER stressors, TM and DTT, increase promoter activity and protein levels of at least 2 well-characterized UPR-inducible proteins.

Because hypoxia has recently been shown to activate XBP1 in human tumor cells,[35] we investigated the effects of hypoxia on XBP1 mRNA splicing in neonatal rat ventricular myocytes using a previously published PCR-based assay[9,36] (Figure 2A). When cardiac myocytes were subjected to

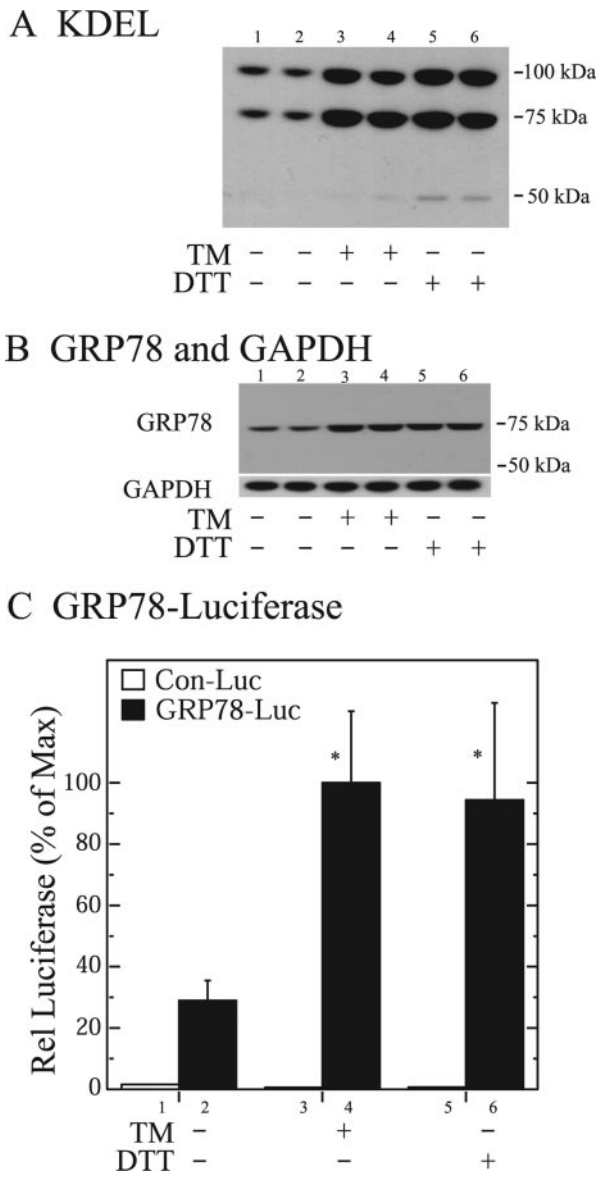

Figure 1. Effect of TM or DTT on UPR-inducible protein levels and promoter activity in cardiac myocytes. A and B, Neonatal rat ventricular myocytes were maintained for 48 hours in serum-free medium and treated with or without TM (10 μg/mL) or DTT (1 mmol/L) for 16 or 8 hours, respectively, and extracted. Equal amounts of protein were analyzed by SDS-PAGE and immunoblotting for KDEL (A) or GRP78 or GAPDH (B). Locations of molecular-weight marker proteins are shown (n=2 cultures per treatment). C, Myocytes were transfected with a GRP78-luciferase construct, as described in Materials and Methods, and then treated as in A. Extracts were assayed for reporter enzymes, which are shown as the mean relative luciferase (ie, luciferase/β-galactosidase) compared with untreated cells transfected with Con-Luc (n=mean of 3 experiments±SE). *P<0.05 different from all other groups.

hypoxia, levels of the spliced form of the XBP1 mRNA increased by ≈5-fold (Figure 2B, XBP1 splicing lanes 4 to 6; Table I in the online data supplement, available at http://circres.ahajournals.org), similar to that seen on TM treatment (Figure 2B, lanes 16 to 18; supplemental Table I). Time course experiments showed that the greatest splicing occurred on 12 to 16 hours of hypoxia. We also examined the levels of other UPR markers, including XBP1, GRP94, GRP78,

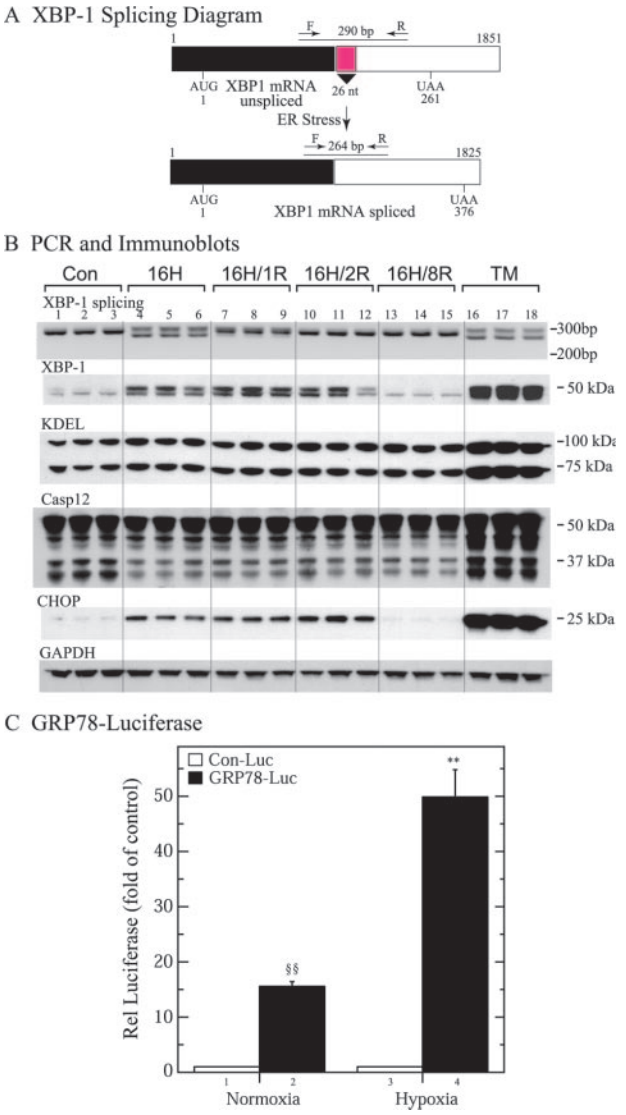

Figure 2. Effects of hypoxia and reoxygenation on XBP1 splicing, UPR-inducible protein levels, and promoter activity in neonatal rat ventricular myocytes. A, XBP1 splicing diagram. In the absence of ER stress, the XBP1 mRNA encodes a 261 amino acid protein of unknown function.[9] However, on ER stress, XBP1 mRNA splicing results in removal of a 26-nucleotide intron, resulting in a frameshift that alters the open-reading frame to generate the 376 amino acid protein, XBP1, that is an active transcription factor, XBP1.[50] B, Splicing and immunoblots. Neonatal rat ventricular myocytes, maintained in 2% FBS, were subjected to hypoxia (H) alone or hypoxia followed by reoxygenation (R) for the times shown or to TM for 16 hours. XBP1 splicing: RT-PCR was used to assess the approximate relative levels of XBP1 unspliced (290 bp) and XBP1 spliced (264 bp), as described in Materials and Methods. Immunoblots: cultures treated as described above were extracted and analyzed by immunoblotting, as described in Materials and Methods (n=3 cultures per treatment). This experiment was repeated 3 times and quantified; the results are shown in supplemental Table I. C, Promoter assay. Neonatal rat ventricular myocytes were transfected as described for Figure 1C, treated±hypoxia for 16 hours, and then extracted and assayed for reporter levels; hypoxia time courses showed 16 hours to be the optimal time for reporter induction. Shown are relative luciferase values (ie, luciferase/β-galactosidase) expressed as the fold of control, ie, normoxia-treated Con-Luc–transfected cells, which was set to 1.0 (n=mean of 3 different experiments±SE). §§,**P<0.01 different from all other groups.

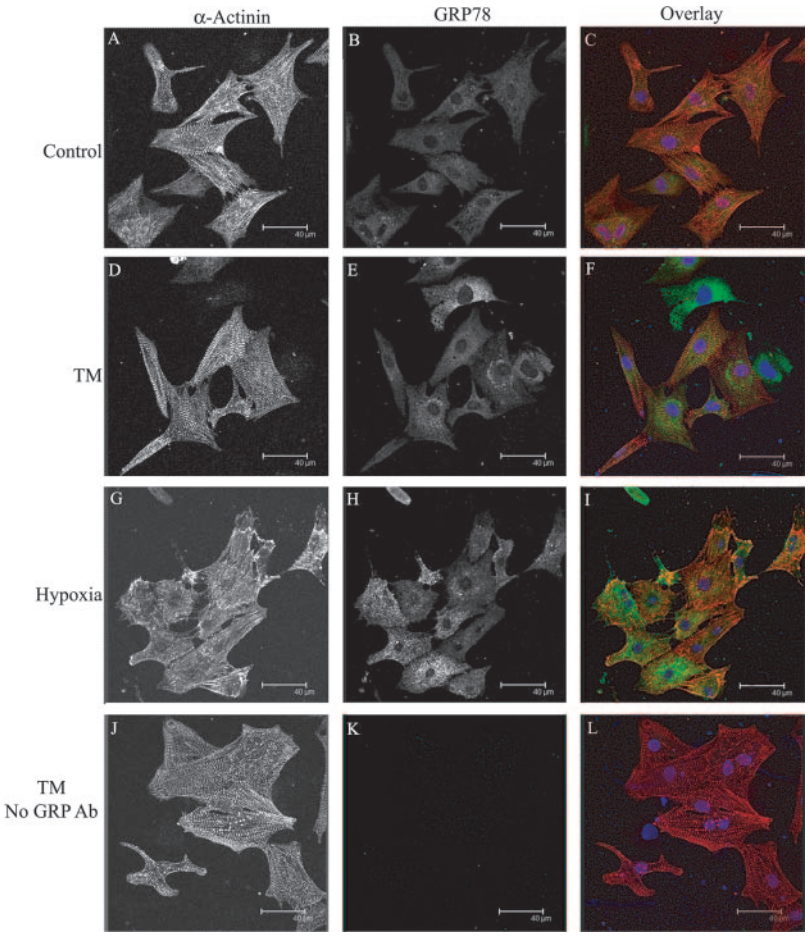

Figure 3. Confocal immunocytofluorescence of neonatal rat ventricular myocytes. Neonatal rat ventricular myocyte cultures, maintained in 2% FBS, were subjected to 16 hours of no treatment (Control) or treatment with hypoxia or TM. Cultures were then fixed and analyzed for sarcomeric α-actinin (A, D, G, and J) and GRP78 (B, E, and H) by confocal fluorescence microscopy, as described in Materials and Methods. Nuclei were also stained using TOPRO-3 (not shown as an individual micrograph). Overlays of the α-actinin (red), GRP78 (green), and nuclei (blue) are shown (C, F, I, and L). A GRP78 control was performed by leaving the GRP78 antibody out of the staining procedure that was applied to a TM-treated culture (J, K, and L).

caspase-12 fragments (indicators of casapse-12 activation), and CHOP. Following hypoxia, we observed an approximate 3.5-fold increase in XBP1, an approximate 1.5-fold increase in GRP94 and GRP78, 5- to 7-fold increase in CHOP, but a slight decrease of caspase-12 fragments (Figure 2B; supplemental Table I). These results indicated that in neonatal rat ventricular myocytes, with the exception of caspase-12, all UPR markers examined were increased by hypoxia. We also assessed the effects of reoxygenation on UPR markers. XBP1 mRNA splicing was reduced by ≈50% after 1 hour of reoxygenation, whereas the levels of XBP1, GRP94, GRP78, and CHOP remained elevated through 2 hours of reoxygenation (Figure 2B; supplemental Table I). XBP1 and CHOP were reduced after 8 hours of reoxygenation; however, GRP94 and GRP78 remained elevated. The levels of caspase-12 fragments remained low throughout all times of reoxygenation examined.

To determine whether a prototypical ER stress–inducible promoter is activated by hypoxia, cells were transfected with GRP78-luciferase and then subjected to normoxia or hypoxia. Although luciferase activity in cells transfected with the control reporter was not changed by hypoxia, the luciferase activity in GRP78-luciferase–transfected cells was increased ≈3.5-fold by hypoxia (Figure 2C), consistent with an effect of hypoxia on GRP78 promoter activation.

Confocal immunocytofluorescence was performed to determine the effects of hypoxia on GRP78 induction on a cellular level in neonatal rat ventricular myocytes. Whereas the levels of GRP78 in control cultures of neonatal rat ventricular myocytes were relatively low (Figure 3A through 3C), following TM or hypoxia, perinuclear GRP78 staining was increased in α-actinin–positive myocytes, as well as the few α-actinin–negative nonmyocytes in the cultures (Figure 3D through 3F [TM]; Figure 3G through 3I [hypoxia]). The perinuclear staining pattern of GRP78 is consistent with localization to the RER. Control experiments demonstrated that the GRP78 staining was specific, because omission of the GRP78 antibody resulted in essentially no detectible staining in TM-treated cardiac myocytes (Figure 3J through 3L). These results demonstrated that hypoxia increased GRP78 expression in neonatal rat ventricular myocytes in a manner similar to the prototypical ER stressor TM.

We next examined the effects of inhibiting XBP1-mediated gene induction with adenoviral-encoded XBP1 (1-188) (AdV-XBP1dn), a previously characterized dominant-negative form of XBP1.[31] XBP1dn is believed to heterodimerize with endogenous XBP1 and to facilitate its degradation,[37] which reduces the levels of endogenous XBP1 and inhibits XBP1-mediated gene induction. Immunoblot analysis demonstrated that cardiac myocytes infected with AdV-XBP1dn expressed a FLAG-tagged protein of the correct size (Figure 4A, lanes 4 to 6). As expected, AdV-XBP1dn inhibited TM-stimulated GRP78 promoter activation (Figure 4B, bar 2 versus bar 4). Compared with AdV-

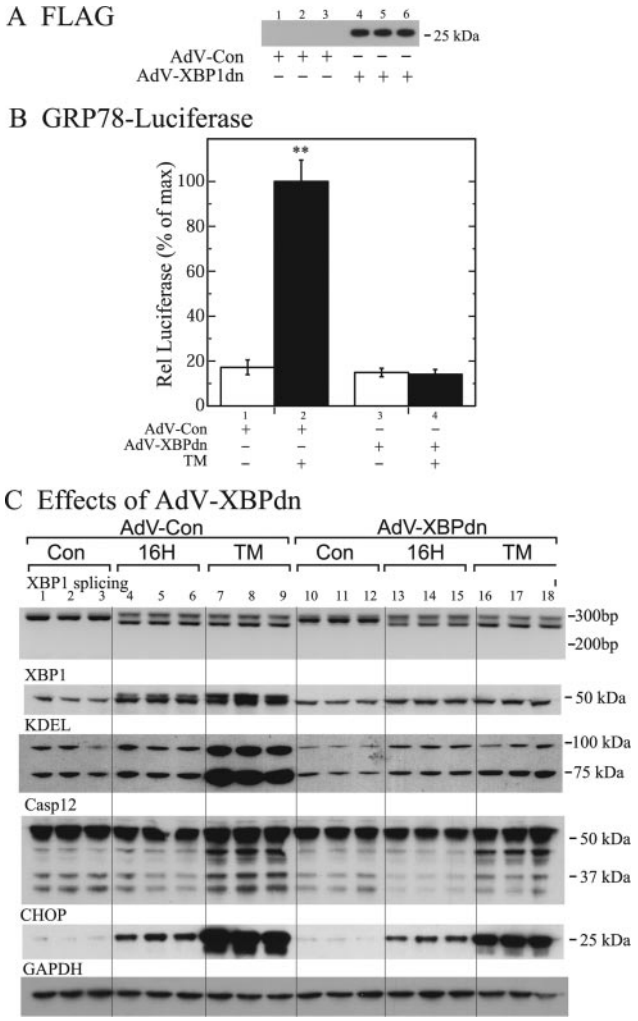

Figure 4. Effect of XBP1dn on the UPR in neonatal rat ventricular myocytes. A, AdV-XBP1dn protein expression. Neonatal rat ventricular myocytes were infected with AdV-Con or AdV-XBP1dn (n=3 cultures for each strain), maintained for 48 hours in 2% FBS and extracted, and then the level of Flag-XBP1dn was estimated by SDS-PAGE followed by immunoblotting with anti-FLAG, as described in Materials and Methods. B, Effect of AdV-XBP1dn on TM-mediated GRP78 promoter activation. Neonatal rat ventricular myocytes were transfected as in Figure 1, infected with the AdV strains shown, and then maintained for 48 hours in 2% FBS. Cultures were then treated±TM for 16 hours, extracted, and analyzed for reporter activities. Shown are relative luciferase values (ie, luciferase/β-galactosidase) as a percentage of the maximal value, which was set to 100% (n=mean of 3 different experiments±SE). **$P<0.01$ different from all other groups. C, Splicing and immunoblots. Neonatal rat ventricular myocytes were subjected to hypoxia (H) or TM for 16 hours. The UPR markers shown were assessed as described in Materials and Methods and for Figure 2B. This experiment was repeated 3 times and quantified; the results are shown in supplemental Table II.

Con, AdV-XBP1dn decreased the UPR markers in control cultures, as well as those subjected to hypoxia or TM (Figure 4C, lanes 1 to 9 versus 10 to 18; supplemental Table II).

Like GRP94 and GRP78, many other proteins induced by XBP1 are protective. Accordingly, the effects of AdV-XBP1dn on cardiac myocyte apoptosis and cell death in response to hypoxia/reoxygenation were examined. Under control conditions, AdV-XBP1dn exerted a slight increase in

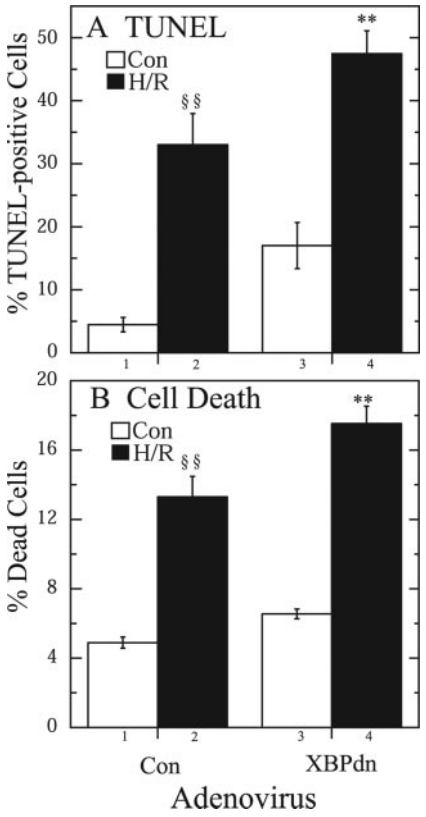

Figure 5. Effect of XBP1dn on apoptosis markers and cell death in neonatal rat ventricular myocytes. Neonatal rat ventricular myocytes were infected with AdV-Con or AdV-XBP1dn, maintained in serum-free medium, treated±hypoxia (16 hours)/reoxygenation (24 hours), and then examined for DNA fragmentation by TUNEL analysis (A) or cell death (B), as described in Materials and Methods. Shown is the mean±SE (n=3 different experiments). §§ and **$P<0.01$ different from all other groups.

TUNEL formation and cell death (Figure 5A and 5B, white bars 1 versus 3). However, when treated with hypoxia/reoxygenation, compared with AdV-Con, cultures infected with AdV-XBP1dn exhibited statistically significant increases in TUNEL formation and cell death (Figure 5, black bars 2 versus 4). These results indicate that under these conditions, the XBP1 branch of the UPR is protective.

Confocal immunocytofluorescence was performed to determine the effects of hypoxia on GRP78 induction on a cellular level in isolated adult mouse ventricular myocytes. Relatively low levels of perinuclear GRP78 staining were apparent in untreated adult mouse ventricular myocytes (Figure 6A through 6C), consistent with the known localization of this protein to the RER. We also observed small amounts of GRP78 staining in more distal regions of the cells, some of which was near α-actinin, consistent with a possible sarcomeric localization. Treatment with either TM or hypoxia increased both perinuclear and sarcomeric GRP78 staining (Figure 6D through 6I), indicating that in comparison with the neonatal rat ventricular myocytes, the UPR can be activated by hypoxia or TM in adult mouse ventricular myocytes. Control experiments demonstrated that the GRP78 staining was specific, because omission of the GRP78 antibody resulted in essentially no detectible staining (Figure 6J through 6L).

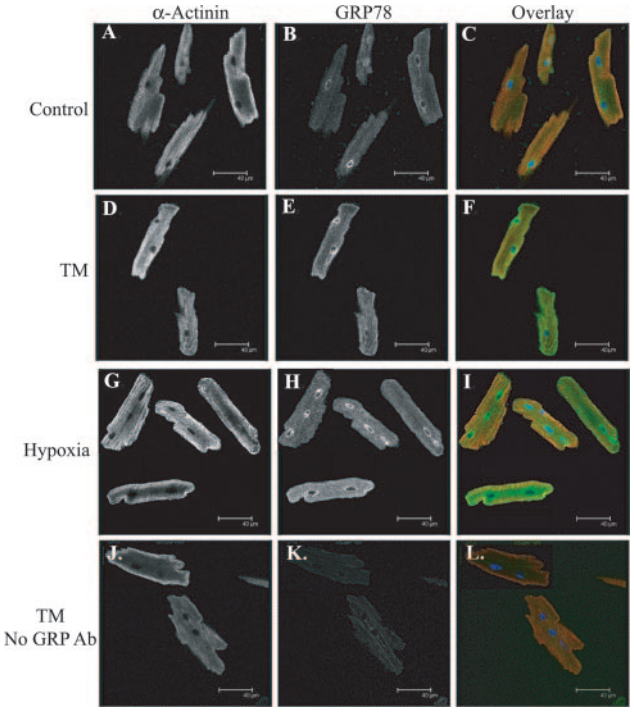

Figure 6. Confocal immunocytofluorescence of adult mouse ventricular myocytes. Adult mouse ventricular myocytes were maintained in serum-free medium for 16 hours, then subjected to 8 hours of no treatment (control), TM, or hypoxia. Cultures were then fixed and analyzed for sarcomeric α-actinin, (A, D, G, and J) and GRP78 (B, E, and H) by confocal fluorescence microscopy, as described in Materials and Methods. Nuclei were also stained using TOPRO-3 (not shown as an individual micrograph). Overlays of the α-actinin (red), GRP78 (green), and nuclei (blue) are shown (C, F, I, and L). A GRP78 control was performed by leaving the GRP78 antibody out of the staining procedure that was applied to a TM-treated culture (J, K, and L).

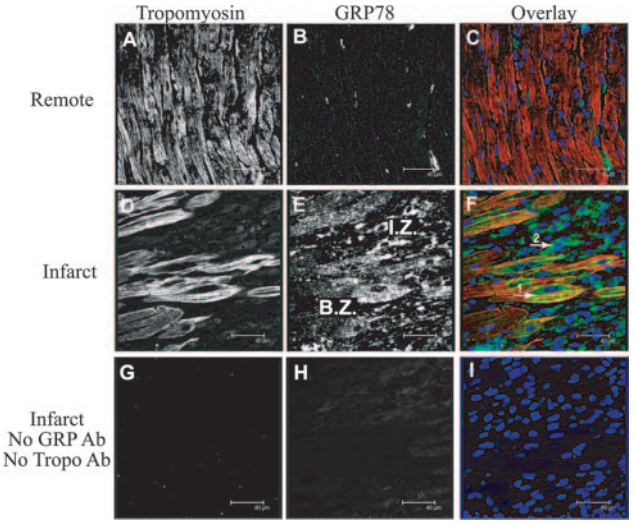

Figure 7. Effect of myocardial infarction in vivo on GRP78 levels in the mouse heart. In vivo myocardial infarction was performed in mice, as described in Materials and Methods. Four days after infarction, paraffin sections were subjected to immunocytofluorescence for tropomyosin (A and D) and GRP78 (B and E) and to staining for nuclei with TOPRO-3 (not shown). Overlays of the tropomyosin (red), GRP78 (green), and TOPRO-3 (blue) are shown (C and F). Regions of the hearts that were distant from the site of infarction (Remote) and those that were near the infarction (Infarct) were viewed by confocal fluorescence microscopy. A tropomyosin and GRP78 control was performed by leaving the tropomyosin and GRP78 antibodies out of the staining procedure that was applied to a section from the infarct zone (G, H, and I). The infarct and border zones are shown as I.Z. and B.Z., respectively, in E. In F, an example of a GRP78-positive cardiac myocyte is shown by arrow 1, and a GRP78-positive noncardiac myocyte is shown by arrow 2. This experiment was performed in 5 different mice, and a representative result is shown here.

To assess the effects of hypoxia in vivo, sections of mouse hearts subjected to permanent myocardial infarction were stained for GRP78 and then examined by confocal immunofluorescence microscopy. When healthy sections of the heart remote to the infarction were examined, very little GRP78 staining was observed, and what little was seen was mostly between myocytes, suggesting nonmyocyte cell types (Figure 7A through 7C). In contrast, when sections near the infarct were examined, GRP78 staining was increased in tropomyosin-positive cells, ie, myocytes (Figure 7F, cell 1) nearest the infarct, as well as tropomyosin-negative cells, ie, nonmyocytes (Figure 7F, cell 2) in the infarct zone. The tropomyosin-negative/GRP78-positive cells may represent cardiomyocytes that have entered necrotic or apoptotic cell death pathways and, therefore, lost cross-reactive sarcomeric proteins, or they may be noncardiomyocytes, such as macrophages and other cell types known to infiltrate the infarct after several days. Taken together with the results shown in Figure 6, these findings suggest that the UPR is activated in adult mouse myocytes in culture, and in vivo it is activated in nonmyocytes and myocytes in the infarct zone, as well as regions bordering the infarct zone, consistent with the hypothesis that hypoxia activates the UPR in the heart.

Discussion

The UPR has been studied extensively in tumor cells, where hypoxia-mediated activation of XBP1 and induction of GRP94 and GRP78[11,38–40] are necessary for survival.[35,41,42] Results in the present study suggest that hypoxia may also activate XBP1 in the heart, where it might contribute to protection. For example, hypoxia led to XBP1 mRNA splicing and increases in XBP1, GRP94, and GRP78, the latter 2 of which are known to be induced by XBP1 and to be protective in other cell types. Moreover, although we found that hypoxia increased CHOP, an XBP1-inducible transcription factor that fosters cell death, we did not find evidence of caspase-12 activation, indicating that under these conditions, not all aspects of the UPR were activated. We also found that blocking XBP1-mediated events increased myocyte death in culture, suggesting that on balance, the XBP1-dependent aspects of the UPR activated by hypoxia may be protective in the cardiac context.

In addition to the UPR, hypoxia activates other signaling pathways, such as hypoxia-inducible factor-1 (HIF-1), which is known to induce GRP94 expression.[6,43,44] However, because XBPdn conferred such a strong decrease in the UPR markers examined in this study, as well as an increase in cardiac myocyte apoptosis, a major contribution by UPR-independent pathways, such as HIF-1, seems unlikely, albeit

formally possible. Addressing this possibility might involve inhibition or activation of the HIF-1 system by manipulating levels of HIF-1α. For example, it would be of interest to determine whether UPR markers are induced in myocytes transfected with a constitutively stable hybrid form of HIF-1α[45] or with small interfering RNA (siRNA) directed against HIF-1α-prolyl-4 hydroxylase-2,[46] both of which have been shown to induce HIF-1–dependent pathways and to protect cardiac myocytes. It would also be of interest to examine the effects of HIF-1 inhibition using HIF-1α siRNA[47] on UPR marker induction and on myocyte survival during hypoxia. Such studies would begin to address whether there exists overlap or crosstalk between the HIF-1 and UPR signaling systems in cardiac myocytes.

In addition to XBP1, ATF6 and PKR-like endoplasmic reticulum kinase (PERK) are also effectors of the UPR.[39] Although the current study focused on XBP1, it is of interest to address whether the ATF6 and/or PERK are also activated by hypoxia in cardiac myocytes. To the best of our knowledge, there has been only 1 other investigation of the UPR in hypoxic cultured cardiac myocytes, published while the current study was in progress.[48] That study showed that hypoxia can lead to PERK activation in cultured cardiac myocytes, but the status of ATF6 activation by hypoxia was not examined. However, a recent study from our laboratory has shown that hypoxia activates features of the UPR in mouse heart ex vivo and that mimicking ATF6 activation in transgenic mouse hearts protects from ischemia/reperfusion injury and decreases apoptosis and necrosis, suggesting a potentially protective role for ATF6 in the heart.[49]

In summary, the present study demonstrated that hypoxia, but not reoxygenation increased the levels of the protective, UPR-inducible proteins, XBP1, GRP94, and GRP78 in cultured cardiac myocytes and that GRP78 was increased in cardiac myocytes, as well as nonmyocytes in an in vivo mouse model of myocardial infarction. Combined with evidence that inhibiting XBP1 signaling increased cardiac myocyte apoptosis and cell death, these findings support the hypothesis that hypoxia can activate certain aspects of the UPR in cardiac myocytes in vivo and in vitro, underscoring the potential importance of this signaling system as a regulator of myocardial survival during ischemia.

Sources of Funding

This work was supported by NIH grants NS/HL-25037 and HL-75573 (to C.C.G.). N.G. is a fellow of the Rees-Stealy Research foundation and the San Diego State University Heart Institute.

Disclosures

None.

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Online Table 1: Quantification of PCR products and immunoblots from Figure 2B

	Treatment					
	Control	16H	16H/1R	16/2R	16H/8R	TM
XBP1 splicing	1.00 ± 0.02	5.29 ± 1.18	2.16 ± 0.26	1.01 ± 0.04	0.08 ± 0.07	7.20 ± 0.59
XBP1	1.00 ± 0.09	2.88 ± 0.40	3.50 ± 0.61	2.43 ± 0.65	0.85 ± 0.25	6.44 ± 1.25
KDEL 94 k	1.00 ± 0.06	1.61 ± 0.15	1.65 ± 0.12	1.67 ± 0.17	1.67 ± 0.08	2.88 ± 0.13
KDEL 78 k	1.00 ± 0.05	1.45 ± 0.09	1.65 ± 0.09	1.69 ± 0.08	1.63 ± 0.08	3.31 ± 0.23
Casp12	1.00 ± 0.07	1.20 ± 0.40	0.44 ± 0.05	0.51 ± 0.07	0.39 ± 0.05	1.87 ± 0.23
CHOP	1.00 ± 0.08	5.25 ± 1.08	5.47 ± 1.11	6.80 ± 1.75	0.38 ± 0.1	53.55 ± 18.18
GAPDH	1.00 ± 0.02	0.86 ± 0.06	0.83 ± 0.03	0.84 ± 0.04	0.88 ± 0.04	0.99 ± 0.04

The immunoblots derived from 3 different experiments like that shown in Figure 2B were quantified by densitometry. The PCR product from spliced XBP1, and the fastest migrating caspase-12 fragment were quantified. For the other blots, all bands shown were quantified. Shown is the mean ± SE for each band compared to control, which was set to 1.00.

Online Table 2: Quantification of PCR products and immunoblots from Figure 4C

	Treatment					
	AdV-Con			AdV-XBP1dn		
	Control	16h	TM	Con	16H	TM
XBP1 splicing	1.00 ± 0.08	5.53 ± 0.57	10.06 ± 0.92	2.86 ± 0.47	5.16 ± 0.37	8.19 ± 0.54
XBP1	1.00 ± 0.05	2.47 ± 0.34	3.57 ± 0.36	1.07 ± 0.07	1.42 ± 0.21	1.43 ± 0.14
KDEL 94 k	1.00 ± 0.10	1.51 ± 0.08	4.43 ± 0.49	0.60 ± 0.11	0.92 ± 0.08	0.93 ± 0.10
KDEL 78 k	1.00 ± 0.06	1.78 ± 0.15	6.00 ± 0.76	0.79 ± 0.15	1.01 ± 0.17	2.06 ± 0.36
Casp12	1.00 ± 0.09	0.36 ± 0.09	1.51 ± 0.14	0.70 ± 0.10	0.23 ± 0.06	0.64 ± 0.11
CHOP	1.00 ± 0.07	9.63 ± 2.12	65.80 ± 8.16	1.23 ± 0.23	5.99 ± 1.35	33.98 ± 8.51
GAPDH	1.00 ± 0.04	0.89 ± 0.03	0.87 ± 0.04	0.94 ± 0.07	0.97 ± 0.03	0.80 ± 0.09

The immunoblots derived from 3 different experiments like that shown in Figure 4C were quantified by densitometry. The PCR product from spliced XBP1, and the fastest migrating caspase-12 fragment were quantified. For the other blots, all bands shown were quantified. Shown is the mean ± SE for each band compared to control, which was set to 1.00.