Mitofusin-2 Is a Major Determinant of Oxidative Stress-mediated Heart Muscle Cell Apoptosis*

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An inexorable loss of terminally differentiated heart muscle cells is a crucial causal factor for heart failure. Here, we have provided several lines of evidence to demonstrate that mitofusin-2 (Mfn-2; also called hyperplasia suppressor gene), a member of the mitofusin family, is a major determinant of oxidative stress-mediated cardiomyocyte apoptosis. First, oxidative stress with H2O2 led to concurrent increases in Mfn-2 expression and apoptosis in cultured neonatal rat cardiomyocytes. Second, overexpression of Mfn-2 to a level similar to that induced by H2O2 was sufficient to trigger myocyte apoptosis, which is associated with profound inhibition of Akt activation without altering ERK1/2 signaling. Third, Mfn-2 silencing inhibited oxidative stress-induced apoptosis in H9C2 cells, a cardiac muscle cell line. Furthermore, Mfn-2-induced myocyte apoptosis was abrogated by inhibition of caspase-9 (but not caspase-8) and by overexpression of Bcl-xL or enhanced activation of phosphatidylinositol 3-kinase-Akt, suggesting that inhibition of Akt signaling and activation of the mitochondrial death pathway are essentially involved in Mfn-2-induced heart muscle cell apoptosis. These results indicate that increased cardiac Mfn-2 expression is both necessary and sufficient for oxidative stress-induced heart muscle cell apoptosis, suggesting that Mfn-2 deregulation may be a crucial pathogenic element and a potential therapeutic target for heart failure.

An imbalance between cell survival and cell death signaling pathways triggers either proliferative or degenerative disorders, diseases in which apoptosis (or programmed cell death) can be either abnormally blocked or undesirably activated. In this regard, Ras plays a central role in the regulation of cell fate, including cell proliferation, differentiation, senescence, and survival or apoptosis. In particular, Ras-mediated activation of Akt has emerged as a focal point for signal transduction pathways promoting cell survival, whereas Ras-activated ERK1/24 and Akt signaling cascades drive cell cycle progression (1–3). Thus, dysfunction of Ras signaling pathways has been implicated in a multitude of degenerative and proliferative diseases.

Originally, Ras was identified as a viral oncoprotein because of its causal relationship with various cancers (3). Over the past decade, increasing evidence has, however, placed Ras signaling at the center of pathways for diverse cardiovascular diseases such as congestive heart failure, hypertensive vascular proliferative growth, and endothelial dysfunction (4). We have recently identified a powerful endogenous Ras inhibitor, mitofusin-2 (Mfn-2), also named hyperplasia suppressor gene because of its anti-proliferative effects (5). Although previous studies have shown that human Mfn-2 and its homologs localize to the mitochondrial outer membrane and play an essential role in mitochondrial fusion, thus regulating mitochondrial morphology and function (6–11), we recently demonstrated that Mfn-2 profoundly suppresses cell growth and proliferation in multiple tumor cell lines and rat vascular smooth muscle cells in vivo and in culture systems via inhibition of the Ras-ERK MAPK signaling pathway and that down-regulation of Mfn-2 contributes to various vascular proliferative disorders (5).

In addition to its anti-proliferative effects, previous studies have shown that Mfn-2 associates with Bax, a pro-apoptotic member of the Bcl-2 family (12–14), at mitochondrial scission sites during the initial stages of apoptosis (9, 11), suggesting that Mfn-2 might participate in mitochondrial apoptotic signaling. In contrast, emerging evidence suggests a protective effect of Mfn-2 in mammalian cells. For instance, a dominant-active form of Mfn-2 (promoting mitochondrial fusion) protects against Bax-mediated cytochrome c release and reduces free radical-mediated mitochondrial injury (11), whereas a dominant-negative form of Mfn-2 (inhibiting mitochondrial fusion) causes loss of mitochondrial membrane potential and fragment-

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3 The abbreviations used are: ERK, extracellular signal-regulated kinase; Mfn-2, mitofusin-2; MAPK, mitogen-activated protein kinase; FBS, fetal bovine serum; TUNEL, terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling; Adv-GP, adenovirus expressing green fluorescent protein; m.o.i., multiplicity of infection; siRNA, small interfering RNA; P13K, phosphatidylinositol 3-kinase; Adv-CA-P13K, adenovirus expressing constitutively active phosphatidylinositol 3-kinase mutant; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase.
roduction of mitochondria (15). In addition, overexpression of both Mfn-1 and Mfn-2 provides some protection against apoptotic stimuli (16). This phenomenon has been interpreted to indicate that high levels of fused mitochondria might constitute a cell defense mechanism against the accumulation of oxidative lesions. It is also noteworthy that some pro-apoptotic members of the Bcl-2 family, including Bax and Bak (17), participate in mitochondrial fusion via their interaction with Mfn-2, indicating that Bax and Bak are required for normal morphogenesis of mitochondria (18). The functionally opposing roles of Bax and Bak in normal cells versus those in apoptotic cells suggest that Bax and Bak might undergo conformational change between anti- and pro-apoptotic conformations to regulate both mitochondrial morphology and apoptosis (18). Nonetheless, it still remains highly controversial as to whether Mfn-2 promotes cell survival or apoptosis.

Of all cell types and tissues, Mfn-2 is expressed predominantly in the heart (5), but its functional role in cardiac myocytes is poorly understood. Here, we sought to determine whether Mfn-2 plays an essential role in regulating the fate of heart muscle cells (survival or death) and, if so, to explore the underlying mechanism. Our results indicate that increased cardiac Mfn-2 expression is both necessary and sufficient for oxidative stress-induced heart muscle cell apoptosis, suggesting that Mfn-2 deregulation may be a crucial pathogenic element and a potential therapeutic target for heart failure.

**EXPERIMENTAL PROCEDURES**

**Isolation, Culture, and Adenoviral Infection of Cardiac Myocytes**—Neonatal rat ventricular myocytes were isolated from 1-day-old Sprague-Dawley rats by the method described previously (19). Cardiac myocytes were plated at a density of 6.6 × 10^4 cells/cm^2 in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (FBS) in the presence of 0.1 mM 5-bromo-2-deoxyuridine. The purity of these cultures was >98% cardiac myocytes as indexed by positive staining of smooth muscle α-actin. Adenovirus-mediated gene transfer was implemented after 24 h of quiescence in serum-free Dulbecco’s modified Eagle’s medium following 48 h of culture in Dulbecco’s modified Eagle’s medium containing 10% FBS.

**Real-time PCR**—Quantitative real-time PCR was performed using the DNA Engine Opticon system (MJ Research) in combination with SYBR Green (Roche Applied Science, Mannheim, Germany) as described previously (5). Briefly, total RNA was extracted using Trizol Reagent (Sigma). 1.5 μg of RNA was then reverse-transcribed to first-strand cDNA using oligo(dT) primer and Moloney murine leukemia virus reverse transcriptase (Promega Corp.) following the manufacturer’s protocol. The primers used were as follows: Mfn-2, 5′-CTCACGGACGCGGTTATTTTCTTCT-3′ (forward) and 5′-TGTGAGGACCGAAGCTATCTCT-3′ (reverse), producing a 412-bp fragment; and 18S RNA, 5′-GGAGGGCAACCAGGACT-3′ (forward) and 5′-TGCAGCCCGGACAATCGAAG-3′ (reverse). The PCR profile was as follows: 95°C for 30 s and 35 cycles at 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s. The amount of SYBR Green was measured at the end of each cycle. The cycle number at which the emission intensity of the sample rose above the base line is referred to as the threshold cycle and was proportional to the target concentration. The data presented are the average of four independent experiments.

**Western Blotting**—Western blotting to assess protein abundance of Mfn-2 was performed with an affinity-purified anti-chicken Mfn-2 primary antibody (GenWay Biotech, Inc., San Diego, California) as described previously (5) in samples from cardiomyocytes in the presence and absence of H_2O_2 treatment or adenoviral infection. In subset experiments, phosphorylation of ERK1/2 and Akt was measured with anti-phospho-p44/42 MAPK Thr202/Tyr204 antibody or anti-phospho-Akt Ser473 antibody (Cell Signaling Biotechnology, Inc.) as described previously (19–21).

**Terminal Deoxynucleotidyltransferase-mediated dUTP Nick End Labeling (TUNEL) and Hoechst Staining in Cultured Cardiac Myocytes**—Nuclear fragmentation was detected by TUNEL staining with an apoptosis detection kit (R&D Systems) or by incubation in 10 mM Hoechst 33342 in fixed cells (70% alcohol and 30% acetone) as described previously (19, 21). 500–700 cells in 10 randomly chosen fields from each dish were counted for the percentage of apoptotic nuclei; each data point shows the results from 5000–7000 cells in four to eight independent experiments.

**MitoTracker Red Labeling and Confocal Imaging**—Neonatal rat cardiac myocytes were infected with adenovirus expressing green fluorescent protein (Adv-GFP; a control virus) or Adv-Mfn-2 at a multiplicity of infection (m.o.i.) of 100. After 24–48 h of infection, cells were incubated in serum-free medium containing 0.2 μM MitoTracker Red CMXRos (Molecular Probes) at 37°C for 20 min and then washed twice. The intact (unfixed) cardiomyocytes were imaged with a Zeiss 510 inverted confocal microscope with a ×40 numerical aperture 1.3 oil immersion lens.

**DNA Laddering**—Cells were lysed in lysis buffer (10 mM Tris-Cl (pH 8.0), 150 mM NaCl, 10 mM EDTA, 0.4% SDS, and 100 μg/ml protease K) after adenoviral infection for 48 h; incubated at 50°C for 5 h with gentle agitation; and then extracted with phenol/CHCl3/isoamyl alcohol, followed by CHCl3/isoamyl alcohol. DNA fragmentation was detected by loading 10 μg of total DNA onto 2% agarose gel in Tris acetate/EDTA buffer and visualized by ethidium bromide staining as described previously (21).

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide Cell Viability Assay—Cells were infected with Adv-GFP or Adv-Mfn-2 at m.o.i. 100 and cultured for various times. 0.5 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide was added to the cells for 4 h, and the absorbances at 490 nm were measured as described previously (5). A 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide kit was purchased from Roche Applied Science.

Small Interfering RNA (siRNA)—For siRNA assay, 21-nucleotide siRNAs comprising 19 nucleotides and a dTdT overhang at each 3′ terminus corresponding to coding region 1973–1991 of Mfn-2, as described by previously (22), were chemically synthesized: RNAi-1, 5′-AGGAGGCCUCUAAAGGCACATT-3′; and RNAi-2, 5′-UGGCGCUUGAAGGCCUCUTT-3′. H9C2
cells were transfected with Oligofectamine (Lifetechn) according to the manufacturer’s protocol.

Cytochrome c in Cytosolic and Mitochondrial Fractions—Release of cytochrome c was measured using a cytochrome c release Apoptosis Assay kit (Oncogene) following the manufacturer’s instructions. Briefly, cells were lysed in cytosol extract buffer and homogenized 40 times. After centrifugation, the supernatant was collected as a cytosolic fraction, and the pellet was resuspended as a mitochondrial fraction (see Fig. 4A). Cytochrome c was detected by Western blotting with an anti-cytochrome c antibody.

Materials—Unless indicated otherwise, all chemicals were purchased from Sigma. The caspase-9 inhibitor Z-LEHD-FMK and the caspase-8 inhibitor Z-IE(OMe)TD(OMe)-FMK were purchased from Calbiochem.

Statistical Analysis—Data are expressed as the mean ± S.E. Statistical comparisons used one-way analysis of variance, followed by Bonferroni’s procedure for multiple-group comparisons. *p < 0.05 was considered statistically significant.

RESULTS

Oxidative Stress Elevates Endogenous Mfn-2 Gene Expression and Triggers Apoptosis in Cultured Cardiomyocytes—Mounting evidence has demonstrated that reactive oxygen species-mediated oxidative stress plays a major role in apoptotic cell death (23) and that reactive oxygen species formation is greatly increased in heart failure and initiates myocyte apoptosis, thus contributing to the development of heart failure (24–26). To investigate the potential functional role of Mfn-2 in regulating the fate of cardiac myocytes, we first adopted an oxidative stress-mediated myocyte apoptotic model using cultured neonatal rat cardiomyocytes subjected to a widely used oxidative insult, H2O2 (19), which is a major endogenous source of reactive oxygen species produced in mitochondria and greatly augmented in heart failure (25). Fig. 1A illustrates the time course of endogenous Mfn-2 gene expression (quantified by real-time PCR) in response to H2O2 (200 μM). The increase in Mfn-2 mRNA abundance occurred after 4 h of treatment, reached its maximum at 8 h, and then declined to a sustained 2-fold increase for at least 24 h. Fig. 1B shows a typical example and the average dose response of H2O2-induced augmentation of Mfn-2 protein abundance in cells after 24 h of treatment. It is noteworthy that H2O2 (200 μM) profoundly promoted myocyte apoptosis, as evidenced by a 6.2-fold increase in TUNEL staining-positive cells (Fig. 1C) and severe DNA fragmentation revealed by DNA laddering assay (Fig. 1D). The apoptotic nuclei appeared blue by TUNEL staining, with a varying degree of chromatin condensation and fragmentation (Fig. 1C). Similarly, serum starvation, hypoxia, or simulated ischemia also concurrently augmented Mfn-2 gene expression and myocyte apoptosis (data not shown). These results indicate that Mfn-2 expression is markedly induced by various death-inducing stimuli, particularly oxidative stress in cardiomyocytes, and that severe heart muscle cell apoptosis is accompanied by elevated Mfn-2 expression.

Up-regulation of Mfn-2 Is Sufficient to Trigger Cardiac Myocyte Apoptosis—To determine whether up-regulation of Mfn-2 alone is sufficient to trigger heart muscle cell apoptotic death, we overexpressed Mfn-2 to a level (2.35 ± 0.47-fold over the base line; n = 4) similar to that induced by oxidative stress (Fig. 1B) in cultured neonatal rat cardiomyocytes using adeno viral gene transfer (Fig. 2A). Infection of cardiomyocytes with Adv-Mfn-2 (m.o.i. = 100) displayed a time-dependent decrease in cell viability compared with uninfected cells or those infected with a control virus, Adv-GFP (m.o.i. = 100) (Fig. 2B). Furthermore, DNA fragmentation (assayed by DNA laddering) was detected in myocytes infected with Adv-Mfn-2, but not in uninfected or Adv-GFP-infected cells (Fig. 2C). The apoptotic effect of Mfn-2 was further manifested by Mfn-2-induced increases in chromatin condensation and fragmentation revealed by both Hoechst (Fig. 2D) and TUNEL staining (Fig. 2E). On average, the percentage of apoptotic cardiomyocytes was increased by 2–3-fold in cells infected with Adv-Mfn-2 relative to those infected with Adv-GFP (both at m.o.i. = 100 for 48 h) (Fig. 2, D
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Overexpression of Mfn-2 triggers rat neonatal cardiomyocyte apoptosis. Rat neonatal cardiomyocytes were transfected with Adv-Mfn-2 or Adv-GFP (both at m.o.i. = 100 for 24 h). A, shown is a typical Western blot for Mfn-2 in uninfected cells or those infected with Adv-Mfn-2 or Adv-GFP. Similar results were obtained in four independent experiments. B, shown is the time course of cell viability assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) measurement (n = six independent experiments for each data point). *, p < 0.01 versus uninfected and Adv-GFP groups. C–E, overexpression of Mfn-2 markedly increased rat neonatal cardiomyocyte apoptosis assayed by DNA laddering (C) (n = three independent experiments), Hoechst staining (D), and TUNEL staining (E) (n = six and eight independent experiments, respectively). The total number of cells in each group was 5000 – 7000. *p < 0.01 versus Adv-GFP.

Mfn-2 Silencing Blocks Oxidative Stress-induced Apoptosis in a Heart Muscle Cell Line (H9C2 Cells)—Because our recent studies have shown that Mfn-2 binds to Ras and inhibits Ras-activated ERK1/2) in Cardiomyocytes

Mfn-2 Silencing Blocks Oxidative Stress-induced Apoptosis in a Cardiac Cell Line (H9C2 Cells)—Next, we sought to determine whether Mfn-2 is necessary for oxidative stress-induced apoptosis using siRNA-mediated silencing of Mfn-2 in H9C2 cells, a rat cardiac muscle cell line, because siRNA transfection efficiency is greater in H9C2 cells than in primary cultured rat neonatal cardiomyocytes. Fig. 3 (A and B) illustrates that Mfn-2 siRNA (siRNA+), but not the scrambled siRNA (siRNA−; used as a negative control), substantially reduced basal Mfn-2 protein abundance and prevented H2O2-induced up-regulation of Mfn-2. More important, Mfn-2 siRNA fully protected H9C2 cells against H2O2-induced apoptosis, as assayed by DNA laddering (Fig. 3C) and Hoechst staining (data not shown), indicating that Mfn-2 up-regulation is required for oxidative stress-induced heart muscle cell apoptosis.

Mfn-2 Increases Mitochondrial Cytochrome c Release and Activates Caspase-9 and Caspase-3—As is the case for most cells, upstream apoptotic signaling is often classified into two general pathways: the death ligand receptor-mediated pathway involving activation of caspase-8 and its downstream executioner caspases and the mitochondrial pathway (or the intrinsic pathway) involving sequentially the release of cytochrome c, the recruitment of Apaf-1 (apoptotic protease-activating factor-1), and the activation of caspase-9 and downstream executioner caspases (27, 28). We sought to determine the signaling pathways involved in Mfn-2-mediated cardiomyocyte apoptosis. First, we found that cytosolic cytochrome c was markedly elevated in cells infected with Adv-Mfn-2 relative to cells infected with Adv-GFP (m.o.i. = 100 for 36 h for both groups) (Fig. 4, A and B). Moreover, we treated cardiomyocytes with either caspase-8 or caspase-9 inhibitor for 48 h to distinguish between the mitochondrion-dependent and -independent pathways. The caspase-9 inhibitor Z-LEHD-FMK effectively abrogated Mfn-2-induced DNA fragmentation (Fig. 4C) and the increase in Hoechst staining-positive cells (Fig. 4D), indicating that caspase-9 plays a crucial role in Mfn-2-evoked apoptotic signaling. In contrast, inhibition of caspase-8 with Z-IE(OMe)TD(OMe)-FMK had no detectable effect in either assay (Fig. 4, C and D). Furthermore, both caspase-9 and caspase-3 were overtly activated in cells overexpressing Mfn-2, as evidenced by increases in cleaved procaspase-9 and procaspase-3 (Fig. 4E). In addition, adenoviral gene transfer of a mitochondrial anti-apoptotic protein, Bcl-xL (12–14, 29, 30), protected myocytes from Mfn-2-induced apoptosis (Fig. 4F). These results indicate that elevated Mfn-2 triggers myocyte apoptosis by the primary mitochondriald apoptotic pathway.

Overexpression of Mfn-2 Inhibits Activation of Akt (but Not ERK1/2) in Cardiomyocytes—Because our recent studies have shown that Mfn-2 binds to Ras and inhibits Ras-activated
MAPK signaling in vascular smooth muscle cells and cancer cell lines (5), here we examined the potential effect of Mfn-2 on the Ras-ERK1/2 MAPK pathway in cultured cardiac myocytes. Surprisingly, overexpression of Mfn-2 had no detectable effect on either basal or 10% FBS-induced activation of ERK1/2 in cultured rat neonatal cardiac myocytes (Fig. 5A). Thus, Mfn-2-mediated cardiomyocyte apoptosis is unlikely attributable to inhibition of ERK1/2 MAPK signaling despite its essential role in Mfn-2-induced anti-proliferation in vascular smooth muscle cells (5). Next, we examined the possibility that Mfn-2 triggers cell apoptosis by suppressing other Ras-dependent cell survival signals. To this end, we defined the possible effect of Mfn-2 on the Ras-phosphatidylinositol 3-kinase (PI3K)-Akt pathway, which is crucially involved in the regulation of cell survival as well as cell proliferation (31, 32). Unlike ERK1/2, both basal and mitogenic stimulus-induced activation of Akt was markedly attenuated by overexpression of Mfn-2 in cultured neonatal rat cardiomyocytes. Fig. 5B illustrates that Mfn-2 overexpression profoundly suppressed basal phosphorylation of Akt in a time-dependent manner without altering total Akt protein abundance. Moreover, serum-mediated Akt phosphorylation was also largely abrogated in cells infected with Adv-Mfn-2 (m.o.i. = 100) (Fig. 5, C and D). Furthermore, coexpression of a constitutively active PI3K mutant (Adv-CA-PI3K) increased PI3K-mediated Akt activation (Fig. 5E) and abolished Mfn-2-induced DNA fragmentation in the co-infected cells (Fig. 5F). Used as a negative control, coexpression of a control gene, β-galactosidase, did not affect Mfn-2-mediated cardiomyocyte apoptosis (Fig. 5F). These results suggest that inhibition of the Ras-evoked PI3K-Akt cell survival signal constitutes a primary mechanism responsible for Mfn-2-mediated heart muscle cell apoptosis in the context of myocardial oxidative stress.

Mfn-2 Apoptotic Effect Is Independent of Mfn-2-induced Alterations in Mitochondrial Morphology—To address whether Mfn-2-induced cell death is causally related to mitochondrial dynamics, we visualized living cell mitochondrial morphology under various experimental conditions by confocal microscopic imaging of cells incubated with 0.2 µM MitoTracker Red CMXRos at 37 °C for 20 min. Interestingly, adenovirus-mediated overexpression of Mfn-2 led to mitochondrial perinuclear clustering (24-h infection) and elongation and networks (36- and 48-h infections) (Fig. 6, B and C), instead of fragmentation, although under the same experimental conditions, overexpression of Mfn-2 triggered myocyte apoptosis (Figs. 2 and 4). To further determine whether the morphological changes in mitochondria contribute to Mfn-2-mediated apoptosis, Adv-Mfn-2-infected cells were compared with those treated with H2O2 or co-infected with Adv-CA-PI3K to induce or abolish the Mfn-2 apoptotic effect, respectively. H2O2 (200 µM for 36 h) treatment-mediated mitochondrial morphological changes were similar to those induced by Mfn-2 overexpression. Notably, coexpression of CA-PI3K did not alter Mfn-2-induced morphological changes in mitochondria even though it protected cells against apoptosis (Fig. 6), suggesting that the Mfn-2 apoptotic effect is not causally related to altered mitochondrial dynamics in cultured neonatal rat cardiomyocytes.

**DISCUSSION**

Mfn-2 (also named hyperplasia suppressor gene) is a ubiquitously expressed and phylogenetically well conserved gene. In addition to its well established functional role in mitochondrial fusion (6–11, 15, 16), our recent studies have demonstrated that Mfn-2 exhibits a profound anti-proliferative effect on vascular smooth muscle cells in vitro and in vivo via inhibition of the Ras-Raf-MEK-ERK1/2 MAPK signaling pathway (5). Here, we have revealed another important functional role of Mfn-2 in...
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FIGURE 5. Overexpression of Mfn-2 inhibits activation of Akt without affecting ERK1/2 signaling. A, overexpression of Mfn-2 had no effect on phosphorylation of ERK1/2 in response to 10% FBS stimulation. Upper panel, phosphorylated ERK1/2 (p-ERK); lower panel, total ERK1/2 protein. B, infection of rat neonatal cardiomyocytes with Adv-Mfn-2 for 48 h inhibited basal Akt phosphorylation in a titer-dependent manner without altering total Akt protein abundance. Con, control. C, overexpression of Mfn-2 suppressed both basal and 10% FBS-induced Akt phosphorylation without changing total Akt protein abundance. D, shown are the average data for Akt phosphorylation in myocytes infected with Adv-Mfn-2 or Adv-GFP (n = 6). *, p < 0.01 versus all of other groups; †, p < 0.05 versus GFP in the absence of FBS. E, infection of cells with Adv-CA-P13K (but not Adv-GFP or Adv-β-galactosidase (β-gal)) elevated Akt phosphorylation. F, co-infection of cardiomyocytes with Adv-CA-P13K (but not Adv-β-galactosidase) prevented Mfn-2-mediated DNA fragmentation. In A–D, cardiomyocytes were infected with Adv-GFP or Adv-Mfn-2 for 48 h and then stimulated by 10% FBS for 15 min, whereas in E and F, cells were infected with the indicated adenovirus at m.o.i. = 100 for 48 h. For all panels, similar results were obtained in cells at an earlier time point of 24-h adenoviral infection (data not shown).

FIGURE 6. Mfn-2-mediated myocyte apoptosis is not causally related to altered mitochondrial dynamics. The mitochondrial morphology of intact cultured neonatal rat cardiac myocytes infected with Adv-GFP or Adv-Mfn-2 in the presence or absence of H$_2$O$_2$ or upon co-infection with Adv-β-galactosidase or Adv-CA-P13K was visualized by confocal imaging in conjunction with living cell MitoTracker Red staining. A, shown is the normal dispersed distribution pattern of mitochondria in neonatal rat cardiac myocytes infected with Adv-GFP for 36 h. B, shown is the slight perinuclear clustering of mitochondria in cells infected with Adv-Mfn-2 for 24 h. C and D, in addition to perinuclear clustering, mitochondrial elongation and networks were formed in cells infected with Adv-Mfn-2 or treated with H$_2$O$_2$ (200 μM), respectively, for 36 h. E and F, co-infection of cardiomyocytes with Adv-β-galactosidase (β-gal; a control virus) or Adv-CA-P13K, respectively, did not prevent Mfn-2-induced mitochondrial perinuclear aggregation. In all panels, results are representative of at least three experiments; the titer of each adenovirus used was m.o.i. = 100.
heart muscle cell apoptosis, marking Mfn-2 deregulation as a crucial pathogenic element and a promising therapeutic target for heart failure.

**Mfn-2 Promotes Cardiomyocyte Apoptosis via the Mitochondrial Death Pathway**—In this study, we have shown that Mfn-2-induced heart muscle cell apoptosis is associated with increased cytochrome c release from mitochondria into the cytosol and activation of caspase-9 and caspase-3. Furthermore, we have utilized caspase-8 and caspase-9 inhibitors to distinguish between the mitochondrion-dependent and -independent pathways and found that inhibition of only caspase-9 (but not caspase-8) effectively blocks Mfn-2-induced apoptosis, suggesting that Mfn-2 promotes myocyte apoptosis mainly via the mitochondrial death pathway. This conclusion is substantiated by the fact that adenoviral gene transfer-mediated overexpression of a member of the Bcl-2 anti-apoptotic protein family, Bcl-xL (12–14, 29, 30), protects myocytes against Mfn-2-induced apoptosis. This is also supported by the previous notion that Bax, a crucial pro-apoptotic member of the Bcl-2 family (12–14), associates with Mfn-2 at mitochondrial scission sites during the initial stages of apoptosis (9, 11). Altogether, these findings support the perception that Mfn-2-evoked cardiomyocyte apoptosis is primarily through the mitochondrion-dependent apoptotic pathway.

**Mfn-2-mediated Myocyte Apoptosis Is Not Causally Related to Altered Mitochondrial Dynamics**—We have examined steady-state mitochondrial morphology under various experimental conditions. Our results indicate that Mfn-2-induced cell death is unlikely dependent on Mfn-2-mediated mitochondrial morphological changes such as mitochondrial perinuclear clustering, elongation, and network formation (Fig. 6). Notably, the CA-PI3K mutant-mediated blockade of the Mfn-2 apoptotic effect does not apparently affect Mfn-2-induced mitochondrial morphological alterations (Fig. 6), indicating that the pro-apoptotic competency of Mfn-2 is independent of its role in mitochondrial fusion. Furthermore, disruption of Mfn-2 mitochondrial targeting via deletion of the transmembrane domain did not prevent Mfn-2-mediated apoptosis in vascular smooth muscle cells, although we and others have previously demonstrated that the transmembrane domain of Mfn-2 protein is essential for its role in mitochondrial fusion (5, 6–11).

**Distinct Signaling Modes Underlying Mfn-2-mediated Apoptosis Versus Anti-proliferation or Myogenesis**—Because our previous study demonstrated that Mfn-2 elicits a profound anti-proliferative effect in cultured vascular smooth muscle cells and cancer cell lines via binding to Ras and subsequently suppressing the Ras-Raf-ERK1/2 MAPK signaling pathway (5), we have examined the potential effect of Mfn-2 on ERK1/2 activation in heart muscle cells. To our surprise, overexpression of Mfn-2 has little or no effect on either basal or serum-stimulated activation of ERK1/2 in cultured rat neonatal cardiac myocytes. In sharp contrast, Mfn-2 overexpression profoundly suppresses basal as well as serum-stimulated Akt activation. Moreover, enforced expression of a CA-PI3K mutant fully protects cardiac myocytes from Mfn-2-induced apoptosis. These findings strongly suggest that suppression of the PI3K-Akt cell survival signaling pathway constitutes a primary mechanism underlying Mfn-2-evoked apoptotic cell death.

In this regard, a recent study reported that an essential role of Mfn-2 in regulating insulin-mediated myogenesis is dependent on its inhibitory effect on the Ras-MEK-MAPK signaling pathway in clonal lines of muscle cells (40). Specifically, insulin-induced Mfn-2 up-regulation is mediated by a PI3K-dependent mechanism, but blocked by activation of MEK-MAPK signaling. In turn, the increased Mfn-2 expression enhances insulin-mediated myogenesis likely through inhibition of the Ras-MEK-MAPK signaling pathway (40), the same mechanism underlying Mfn-2-mediated anti-proliferation in vascular smooth muscle cells (5). Thus, the signaling pathway responsible for Mfn-2-mediated apoptosis distinctly differs from that underlying Mfn-2-induced anti-proliferation or myogenesis.

**Distinct Functional Roles of Mfn-2 Versus Mfn-1**—There are multiple lines of evidence suggesting that the pro-apoptotic Mfn phenotype might be a unique function of Mfn-2 and not shared by Mfn-1 and that, whereas Mfn-1 constitutes a molecular means specifically involved in mitochondrial fusion, Mfn-2 acts as a versatile signaling molecule involved in various vital cellular processes in addition to mitochondrial fusion. First, a recent study of Detmer and Chan (41) demonstrated that wild-type Mfn-1 is able to complement mutant Mfn-2 in Charcot-Marie-Tooth disease type 2A through their heterodimerization; in contrast, wild-type Mfn-2 cannot rescue mutant Mfn-2 in terms of mitochondrial fusion, indicating that Mfn-1 plays a predominant role in the mitochondrial fusion pathway. Second, Mihara and co-workers (42) have shown that Mfn-1-harboring mitochondria are efficiently tethered in a GTP-dependent manner, whereas Mfn-2-harboring mitochondria are tethered with much lower efficiency, suggesting that Mfn-1 is mainly responsible for GTP-dependent membrane tethering. Similarly, Scorrano and co-workers (43) reported that OPA1-mediated mitochondrial tabulation and fusion require the presence of Mfn-1, but not Mfn-2. Thus, whereas Mfn-1 plays a predominant role in mitochondrial fusion, Mfn-2 participates in various cell signaling cascades, including the fusion pathway.

In summary, we have demonstrated, for the first time, that Mfn-2 is markedly induced by oxidative stress in primary cultured mammalian cardiomyocytes and cultured H9C2 cells, a cardiac cell line. The up-regulation of Mfn-2 is both necessary and sufficient to trigger apoptotic heart muscle cell death. These findings have defined an important genetic modifier of cardiac myocyte apoptosis in response to ischemia or oxidative injury. Because cardiomyocyte apoptosis is a key cellular process involved in the development of heart failure, our findings may reveal new drug targets and therapeutic strategies for hitherto lethal heart diseases, including myocardial infarction, dilated cardiomyopathy, and congestive heart failure.

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