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Abstract—The endoplasmic reticulum (ER) stress response (ERSR) is activated when folding of nascent proteins in the ER lumen is impeded. Myocardial ischemia was recently shown to activate the ERSR; however, the role of this complex signaling system in the heart is not well understood. ER stress activates the transcription factor ATF6, which induces expression of proteins targeted to the ER, where they restore protein folding, thus fostering cytoprotection. We previously developed a transgenic mouse line that expresses a conditionally activated form of ATF6 in the heart. In this mouse line, ATF6 activation decreased ischemic damage in an ex vivo model of myocardial ischemia/reperfusion and induced numerous genes, including mesencephalic astrocyte-derived neurotrophic factor (MANF). In the present study, MANF expression was shown to be induced in cardiac myocytes and in other cell types in the hearts of mice subjected to in vivo myocardial infarction. Additionally, simulated ischemia induced MANF in an ATF6-dependent manner in neonatal rat ventricular myocyte cultures. In contrast to many other ER-resident ERSR proteins, MANF lacks a canonical ER-retention sequence, consistent with our finding that MANF was readily secreted from cultured cardiac myocytes. Knockdown of endogenous MANF with micro-RNA increased cell death upon simulated ischemia/reperfusion, whereas addition of recombinant MANF to media protected cultured cardiac myocytes from simulated ischemia/reperfusion-mediated death. Thus, a possible function of the ERSR in the heart is the ischemia-mediated induction of secreted proteins, such as MANF, that can function in an autocrine/paracrine manner to modulate myocardial damage from ER stresses, including ischemia. **(*Circ Res*. 2008;103:1249-1258.)**

Key Words: adenovirus ■ cardiac myocytes ■ endoplasmic reticulum stress ■ hypoxia ■ ischemia ■ myocardial infarction ■ unfolded protein response

Numerous proteins that are critical to cellular function are synthesized on endoplasmic reticulum (ER) ribosomes and then folded and further posttranslationally modified in the ER lumen. Stresses that impede ER protein folding trigger the ER stress response (ERSR),[1–4] a signaling system that has not been studied extensively in the heart. ER stress activates the transcription factors X-box binding protein (XBP1) and activator of transcription-6 (ATF6), which induce numerous ERSR proteins designed to restore efficient ER protein folding, which contributes to resisting the stress.[5,6] Many ERSR genes are induced by ATF6 or XBP1, whereas others exhibit a requirement for only 1 of the 2 factors.[7]

The folding of proteins in the ER lumen requires molecular oxygen,[8] suggesting that that hypoxia-mediated induction of ERSR proteins during myocardial ischemia may enhance ER protein folding and survival of cardiac myocytes and limit ischemic damage. Consistent with this hypothesis are recent studies showing that simulated ischemia (sI) activates the ERSR in cultured rat and mouse ventricular myocytes and that ER stress is activated in the surviving myocytes adjacent to the damaged region in a mouse model of in vivo myocardial infarction.[9–12]

To examine potential functions for ER stress in the heart, we developed a transgenic (TG) mouse line that expresses a conditionally activated form of ATF6 in the heart,[9] allowing selective activation of this branch of the ERSR at any time. A microarray analysis showed that 1 of the 381 genes induced in response to ATF6 activation in cardiac myocytes in vivo was ARMET (arginine-rich mutated in early tumors).[13] The protein encoded by the ARMET had not been isolated until recent studies showed that in astrocytes, ARMET encodes a 158-aa secreted protein that enhances survival of cultured dopaminergic neurons.[14] Based on these characteristics, the authors of this study named the protein mesencephalic astrocyte-derived neurotrophic factor (MANF).

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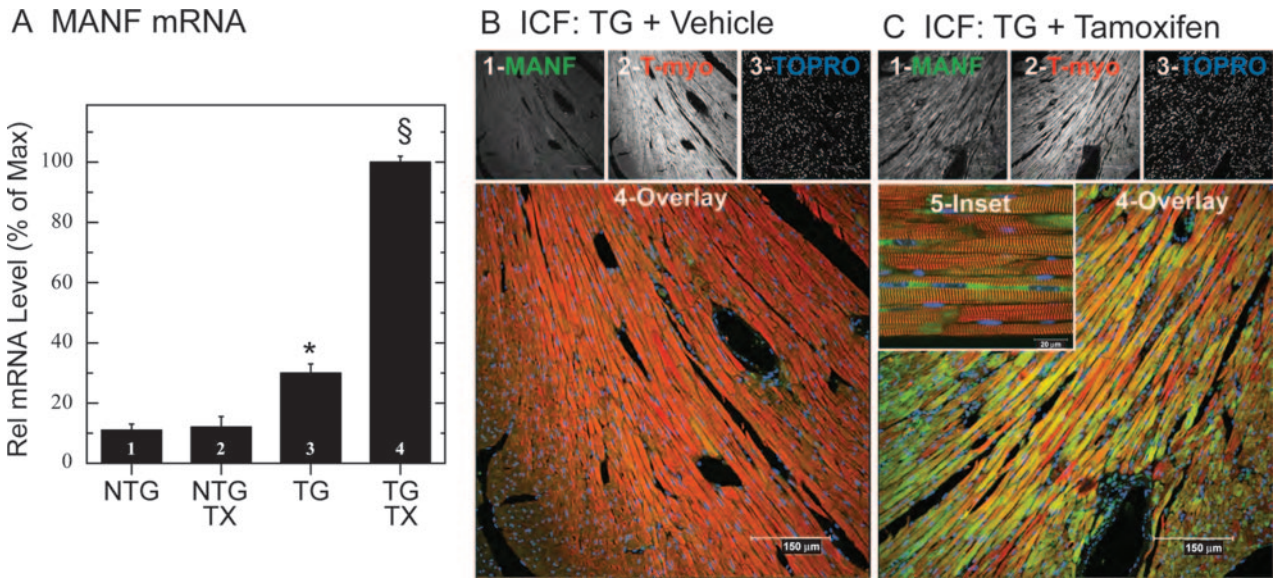

Figure 1. Effect of ATF6 on MANF expression in the mouse heart. NTG and ATF6-MER TG mice were treated with or without tamoxifen (TX), as described,[13] and euthanized, and the hearts were extracted for RNA or sectioned for immunohistochemistry. A, MANF and GAPDH mRNA levels were assessed as described in Materials and Methods. Results are the mean relative mRNA (MANF mRNA/GAPDH mRNA) normalized to the maximal value set to 100% (N=3 mouse hearts/sample). B, Sections of hearts from TG mice treated with vehicle only were examined for MANF (subpanel 1), tropomyosin (subpanel 2) or TOPRO-3 (subpanel 3) to identify nuclei and then examined by confocal fluorescent microscopy, as described.[10] C, Sections of hearts from TG mice treated with vehicle plus tamoxifen were examined as in B.

Previous microarray studies on fibroblasts showed that MANF might be an ERSR gene.[7] Moreover, the MANF gene was induced by hypoxia in HeLa cells,[15] as well as in the surviving myocardium in mouse hearts subjected to in vivo myocardial infarction.[16] The present study was undertaken to determine whether MANF is expressed as an ischemia-inducible ERSR gene in cardiac myocytes and to examine possible roles for MANF on cardiac myocyte survival during ischemic stress.

Materials and Methods

Animals

The TG mice used in this study have been described previously.[9] Approximately 100 neonatal rats and 24 adult male non-TG (NTG) and TG C57/BL6 mice were used. All procedures were in compliance with the San Diego State University Institutional Animal Care and Use Committee. In vivo myocardial infarction, immunoblotting, and sectioning for immunocytofluorescence were carried out as described.[17]

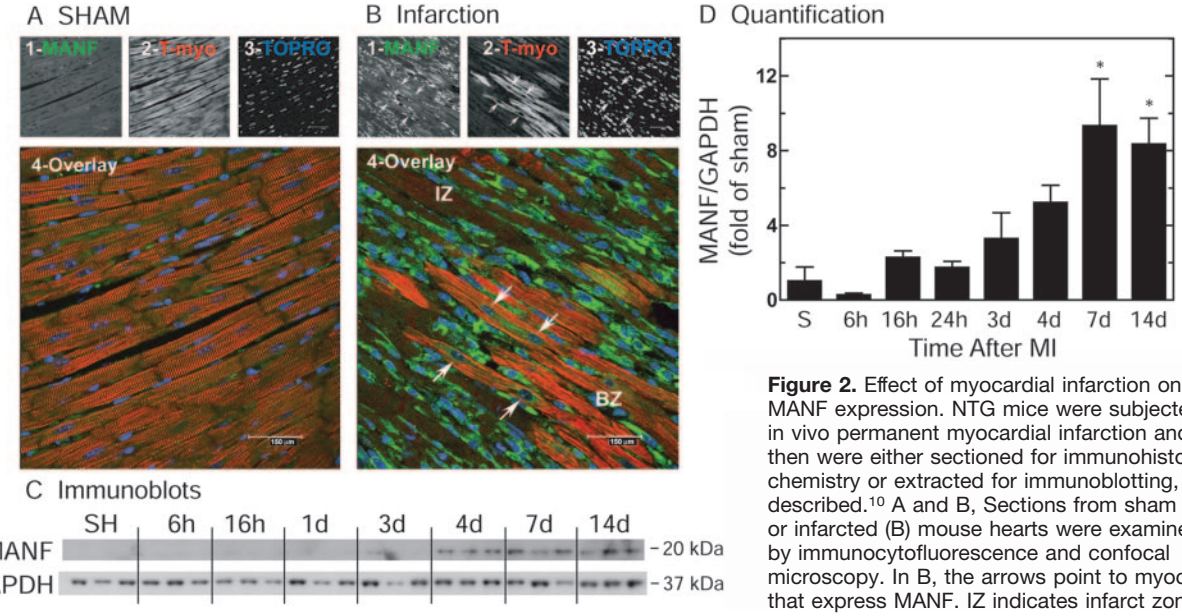

Figure 2. Effect of myocardial infarction on MANF expression. NTG mice were subjected to in vivo permanent myocardial infarction and then were either sectioned for immunohistochemistry or extracted for immunoblotting, as described.[10] A and B, Sections from sham (A) or infarcted (B) mouse hearts were examined by immunocytofluorescence and confocal microscopy. In B, the arrows point to myocytes that express MANF. IZ indicates infarct zone; BZ, border zone of surviving myocardium. This analysis was carried out on 3 different mouse hearts; a representative micrograph from 1 mouse heart is shown. C, NTG mice were subjected to sham or permanent infarction surgery. Mice were euthanized at the times shown, and regions of the infarct border zone were obtained, as described.[30] Shown are MANF and GAPDH immunoblots carried out on 3 different mouse heart samples for each postprocedure time point. D, The immunoblot shown in C was quantified by densitometry. MANF levels were normalized to GAPDH levels, and the sham sample was set to 1. All values represent N=3 mouse hearts±SE.

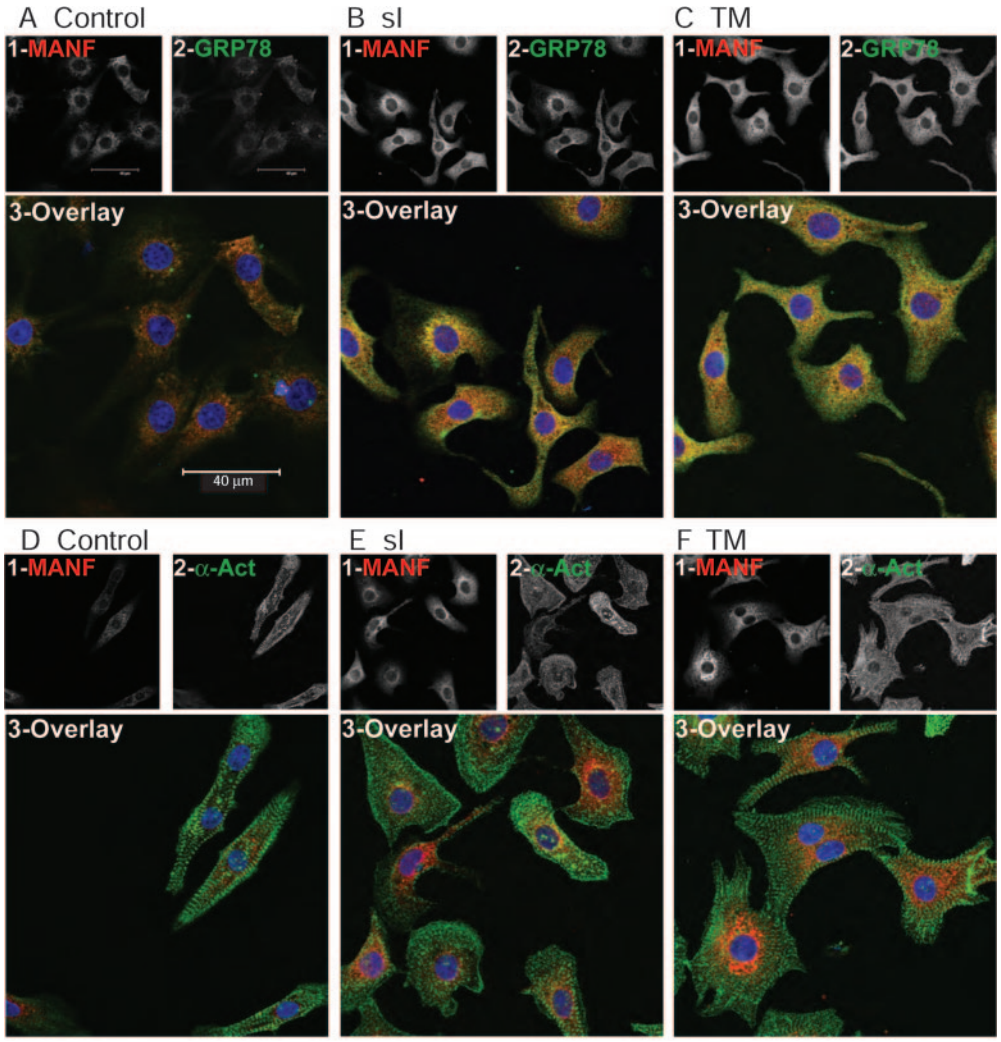

Figure 3. Microscopic examination of MANF in cultured cardiac myocytes. NRVMCs were either maintained in serum-free medium for 48 hours (Control) (A and D) or treated with sI, as previously described[10] (sI) (B and E), or treated with TM (C and F) and then analyzed by confocal immunofluorescence. Following these treatments, cultures were fixed and examined by immunocytofluorescence confocal microscopy for MANF (subpanel 1), GRP78 (A2, B2, and C2) or α-actinin (α-act) (D2, E2, and F2), as previously described.[10] Scale bar in A3, 40 μm.

Statistics

Statistical treatments were carried out by ANOVA, followed by Newman–Keuls post hoc analysis. Unless otherwise stated, * and § indicate statistical differences compared with all other values ($P<0.05$).

Cultured Cardiac Myocytes

Primary neonatal rat ventricular myocyte cultures (NRVMCs) were prepared as previously described.[10]

Real-Time Quantitative PCR

Real-time quantitative PCR was performed as previously described.[9]

The following rat primers were used as follows: MANF forward, TGCAAAGGCTGTGCAGAGAAG; MANF reverse, ATGAACTGCTGTTTCCCTCCG; GAPDH forward, CCTGGCCAAGGTCATCCAT; and GAPDH reverse, GTCATGAGCCCTTCCACGAT. The following mouse primers were used: MANF forward, TGGGTGCGTTCTTCGACAT; MANF reverse: GACGGTTGCTGGATCATTGAT.

Immunocytofluorescence and Immunoblots

Immunofluorescent confocal microscopy was carried out as previously described.[10,17] The MANF antibody (R&D Systems, catalog no. AF3748) was used at 1:25. Immunoblots were performed as previously described[10] using the MANF antibody at 1:2000.

MANF Mammalian Expression Construct and Adenovirus

The coding sequence of the mouse MANF gene (GenBank accession no. AK131997) was cloned into pcDNA 3.1 containing a C terminus 3× hemagglutinin tag using standard cloning procedures. A recombinant adenoviral strain was then prepared using the mouse MANF cDNA, as described.[10]

MANF Bacterial Expression Construct

The mouse MANF cDNA without the N-terminal signal sequence (amino acids 1 to 21) was amplified and cloned into a cleavable N-terminal His_6 containing prSET-B (Invitrogen, catalog no. V351–20) and then transformed into BL-21 cells (Stratagene, catalog no. 200131). Recombinant protein was purified from bacterial extracts using Ni-NTA agarose columns (Qiagen, catalog no. 30210). The His tag was removed as described in the manual (Invitrogen, catalog no. 45-0437). The cleaved protein was purified by size-exclusion column (Millipore Centricon, catalog no. 4225).

Caspase-3 Activity Assay

Cells were infected with various recombinant adenovirus strains (AdV) in 2% FCS-containing medium for 6 hours, after which, cells were washed and fed with the same medium but without the AdV. One day after infection (day 0), cells were maintained in this serum-starved condition for 0 to 7 days. In other experiments, recombinant MANF or equivalent molar quantities of bovine serum albumin was added to culture medium; in these experiments, all cells were infected with control AdV to maintain consistency between experimental protocols. At days 0, 3, or 7, cultures were extracted in assay buffer containing 50 mmol/L Hepes, pH 7.4, 0.1% CHAPS, 0.1 mmol/L EDTA. A total of 50 μL of the lysate and 10 μL of the assay buffer were then combined with 45 μL of reaction buffer (40

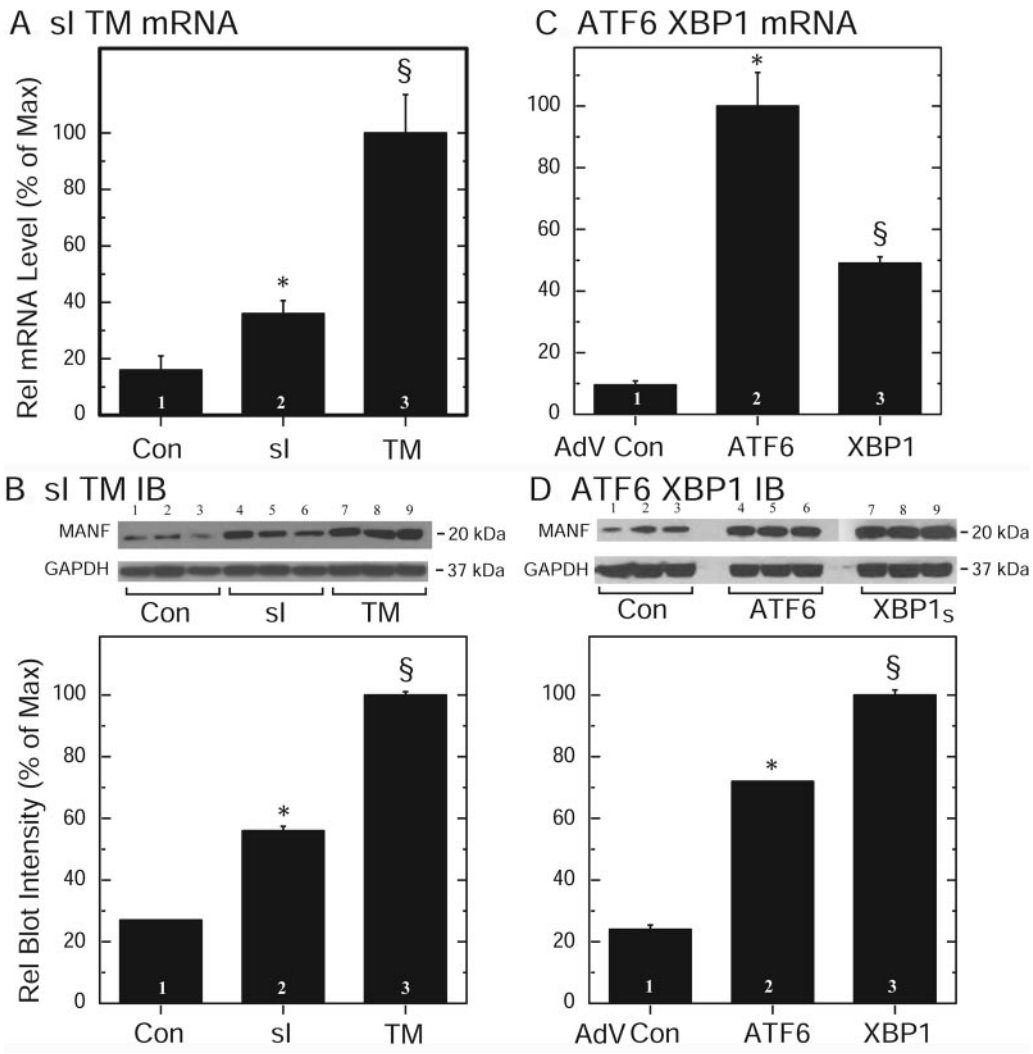

Figure 4. Effects of sI, TM, ATF6, or XBP1 on MANF expression. A and B, NRVMCs were subjected to sI or TM, as in Figure 3, and then assessed for MANF mRNA (A) or MANF protein (B) levels. C and D, NRVMCs were infected with a control adenovirus (Con) or with adenovirus encoding active forms of ATF6 or XBP1. Forty-eight hours later, cultures were extracted for mRNA (C) or MANF protein (D). N=3 cultures for each treatment. Values are mean relative mRNA or relative protein (MANF/GAPDH)±SE.

μL caspase assay buffer, 1 mmol/L DTT, 40 μmol/L DEVD-AFC in DMSO [Sigma, catalog no. A0466]). After 1 hour at 37°C, fluorescence was measured at an excitation wavelength of 400 nm and an emission wavelength of 505 nm. Caspase activity was defined as fluorescence/protein.

MANF–Luciferase and MANF-M–Luciferase Constructs

Native and mutated versions of the 5′-flanking sequence of the mouse MANF gene from −1513 to +17 were cloned into a luciferase reporter vector.

Reporter Enzyme Assay

Reporter enzyme assays for luciferase and β-galactosidase were carried out as previously described.[10]

Results

MANF mRNA was examined in the hearts of ATF6 TG mice that constitutively express a tamoxifen-activated form of ATF6 in cardiac myocytes.[9] MANF mRNA was very low in the hearts of NTG mice treated with or without tamoxifen and was increased by 2.5-fold in the hearts of untreated TG mice (Figure 1A, bars 1 to 3). However, compared to NTG, MANF mRNA was increased by 10-fold in the hearts of tamoxifen-treated TG mice (Figure 1A, bar 4). MANF protein expression was examined in mouse heart sections by confocal immunocytofluorescence microscopy. Whereas MANF was almost undetectable in the myocardium of TG mice in the absence of tamoxifen (Figure 1B, 1 and 4), it was elevated considerably in hearts from tamoxifen-treated TG mice (Figure 1C, 1, 4, and 5). These results demonstrated that MANF mRNA and protein were induced on ATF6 activation in the myocardium, in vivo, consistent with the hypothesis that ER stresses, such as ischemia, which can activate ATF6, might also induce MANF expression in the heart.

Because ER stress is activated in the mouse heart by myocardial infarction, in vivo,[10] the expression of MANF in infarcted mouse hearts was examined using confocal immunocytofluorescence microscopy and immunoblotting. MANF expression was essentially undetectable in sections of the hearts of mice subjected to sham infarct surgery (Figure 2A, 1 to 4). In contrast, 4 days after infarct surgery, MANF protein was elevated in cells in the infarct zone, which, because they did not stain for tropomyosin, may be nonmyocytes, and in tropomyosin-positive myocytes bordering the infarct (Figure 2B, 1 to 4; arrows point to myocytes). MANF expression was also examined by immunoblotting of heart tissue obtained at various times from the periinfarct zone of

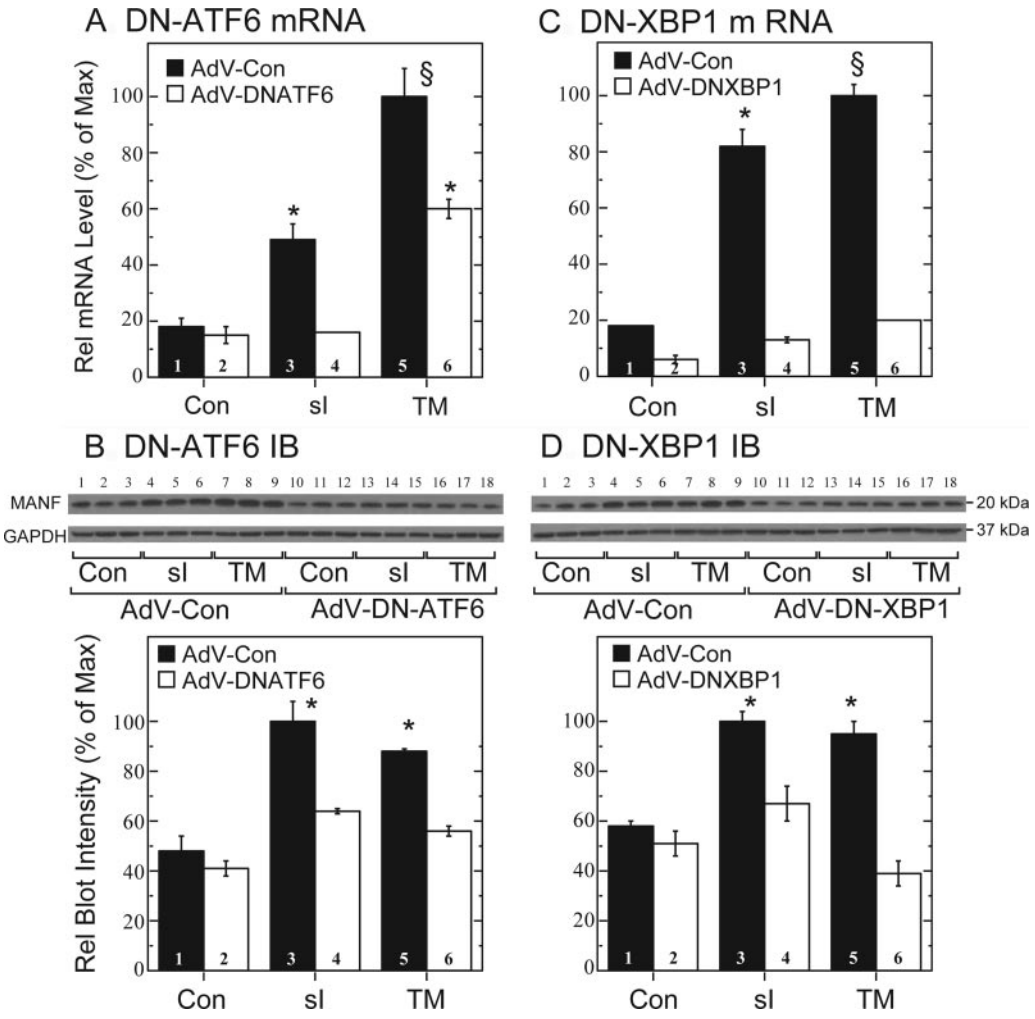

Figure 5. Effects of adenovirus-encoded DN-ATF6 or DN-XBP1 on MANF expression. NRVMCs were infected with a control adenovirus (AdV-Con) or with adenovirus encoding DN-ATF6 (A and B) or DN-XBP1 (C and D). Cultures were then subjected to either sI or TM, as in Figure 4, and then analyzed for MANF mRNA (A and C) or for MANF protein (B and D). N=3 cultures for each treatment. Values are mean relative mRNA or relative protein (MANF/GAPDH)±SE.

hearts subjected to sham or permanent occlusion infarct surgery, as described previously.[17] Whereas MANF was undetectable in the sham sample, as well as samples from hearts obtained from animals up to 3 days after infarction, by 4 days following infarction, MANF levels were clearly elevated. The level of MANF increased further at 7 and 14 days following infarction (Figure 2C and 2D). Taken together, these results demonstrate that MANF increased in myocytes and, perhaps nonmyocytes, in the border zone and infarct zone in a mouse model of in vivo myocardial infarction.

To examine MANF expression on the cellular level in cardiac myocytes, a neonatal rat ventricular myocyte culture (NRVMC) model system was used. Immunocytofluorescence showed that whereas it was expressed in very low levels in untreated cultured cardiac myocytes (Figure 3A and 3D, 1), MANF increased dramatically in cells subjected to sI (Figure 3B and 3E, 1), or in cells treated with the prototypical ER stressor, tunicamycin (TM) (Figure 3C and 3F, 1). To examine the intracellular location of MANF, the expression of GRP78, a well-characterized ER-resident ERSR protein, was assessed. As expected for an ERSR protein, GRP78 was increased by sI or TM (Figure 3B and 3C, 2). Moreover, there was significant, although incomplete, colocalization of MANF and GRP78, as indicated by the striking similarities in the staining patterns (Figure 3B and 3C, compare 1 and 2), as well as yellow fluorescence in the merged image of the red and green MANF and GRP78 images, respectively (Figure 3B and 3C, 3). Finally, costaining for the cardiac myocyte-specific protein α-actinin demonstrated that the cultures were mostly cardiac myocytes, that MANF staining was mostly perinuclear, and that it did not localize in a sarcomeric pattern (Figure 3E and 3F, 2 and 3). Thus, most of the MANF in cardiac myocytes is colocalized with the known ER-resident protein GRP78, consistent with MANF being an ER-resident protein.

To examine molecular characteristics of MANF expression, MANF mRNA and protein were measured in extracts of NRVMCs subjected to ER stress. MANF mRNA increased by 2- and 5-fold when NRVMCs were subjected to sI or treated with TM, respectively (Figure 4A, bars 2 and 3). Moreover, cellular MANF protein increased by 2- to 2.5-fold in response to sI or TM, respectively (Figure 4B, lanes 4 to 9 and bars 2 and 3). Thus, in cultured cardiac myocytes, MANF is induced by 2 different activators of ER stress, TM and sI.

To study the mechanism of MANF induction, the effects of overexpressing activated ATF6 or XBP1 were examined. Cardiac myocytes infected with recombinant adenoviral strains encoding activated forms of ATF6 or XBP1 exhibited 10- and 5-fold increases in MANF mRNA, respectively

(Figure 4C, bars 2 and 3). ATF6 and XBP1 also increased cellular MANF protein levels by 3.5- and 5-fold, respectively (Figure 4D, lanes 4 to 9 and bars 2 and 3). These results indicate that MANF gene expression in cultured cardiac myocytes can be induced by either ATF6 or XBP1, a hallmark of many ERSR genes.

To explore the roles for endogenous ATF6 and XBP1 on MANF induction, cultures were infected with recombinant adenovirus strains encoding dominant negative (DN) forms of each transcription factor, which are recombinant forms that retain their DNA-binding domains but do not have transcriptional activation domains.[10,18] Compared to cells infected with a control adenovirus, cardiac myocytes infected with DN-ATF6 adenovirus exhibited reduced levels of MANF mRNA and protein in response to sI or TM (Figure 5A, bars 3 to 6; Figure 5B, lanes 13 to 18 and bars 3 to 6). Cultures infected with DN-XBP1 adenovirus also exhibited reduced MANF mRNA and protein (Figure 5C and 5D), indicating that either endogenous ATF6 or XBP1 participate in MANF induction in response to ER stress in cultured cardiac myocytes.

To determine whether ER stress can activate the MANF promoter in cardiac myocytes, a construct encoding the 5′-flanking sequence and promoter of mouse MANF comprised of nucleotides −1513 to +17 driving firefly luciferase was generated (Figure 6A). When cultured cardiac myocytes were transfected with this construct, relative luciferase increased by 3- and 10-fold when cells were subjected to sI or TM, respectively (Figure 6B, bars 1 to 3). In contrast, induction of MANF-luciferase by either treatment was effectively blocked by DN-ATF6 (Figure 6B, bars 4 to 6) or DN-XBP1 (Figure 6B, bars 7 to 9). ATF6 and XBP1 confer ERSR gene induction through at least 3 types of ER stress response elements (ERSEs); the canonical ERSE,[19–21] the ERSE-II,[22] and the unfolded protein response element.[22] A search of the mouse or human MANF 5′-flanking sequence showed that there are 2 putative ERSEs in the promoter-proximal 1.5 kb; 1 canonical ERSE,[19] located at −492 to −473, and 1 ERSE-II,[23] located at −134 to −124 in the mouse gene. Mutation of the putative ERSE-II located at −134 to −124 (Figure 6A, M1) resulted in a 10-fold decrease of MANF-luciferase induction by sI or TM (Figure 6C, bars 1 to 3 versus 4 to 6); however, mutation of the putative ERSE located at −492 to −473 (Figure 6A, M2) resulted in much less inhibition (Figure 6D, bars 1 to 3 versus 7 to 9). The requirement of the ERSE at −134 to −124 for MANF induction by either ATF6 or XBP1 is consistent with other ERSR genes, where an ERSE-II can bind either factors and confer transcriptional induction in response to ER stress.[22] These findings are also supported by a recent study showing the need for the ERSEII at −130 bp in the mouse MANF gene for promoter induction by TM in cultured pancreatic β-cells.[24]

Because MANF possesses a C-terminal RTDL that does not conform with canonical ER-retention/retrieval sequences (ie, KDEL), it is possible that MANF is not effectively retained in the ER. When cardiac myocytes were cultured under nonstressed conditions, MANF was detectible in cell extracts, but not in culture medium (Figure 7A, lanes 1 to 3).

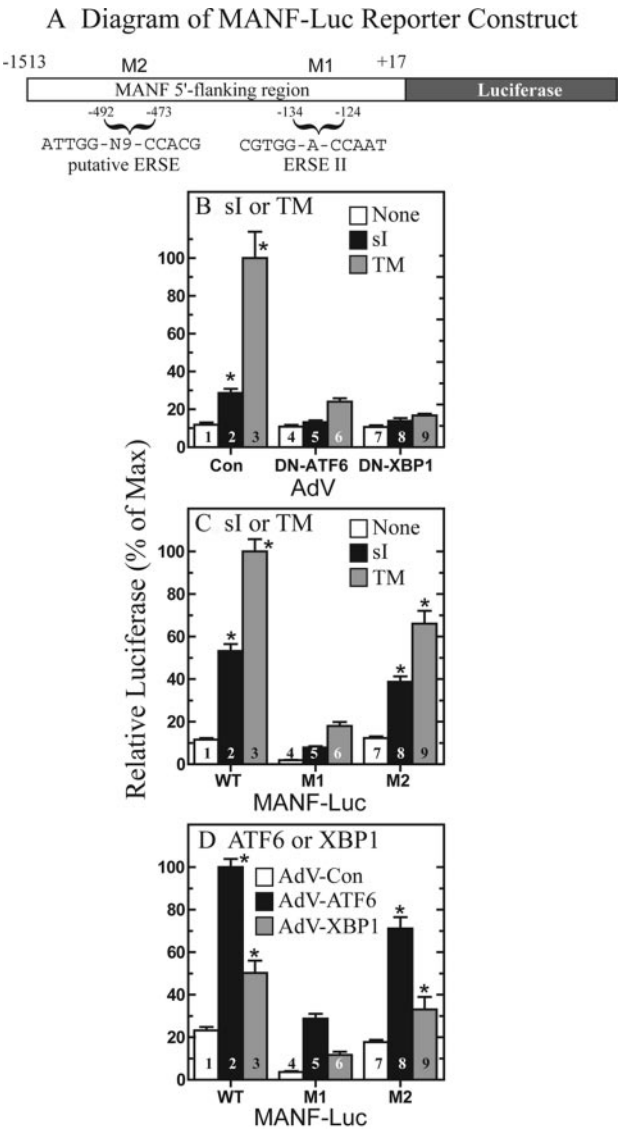

Figure 6. MANF promoter analyses. A, Approximately 1.5 kb of the mouse MANF 5′-flanking sequence (−1513 to +17) was cloned upstream of the firefly luciferase gene. The regions between −492 to −473 and −134 to −124 were identified as a putative ERSE and an ERSE-II, respectively. Mutations M2 and M1 were prepared in a manner predicted to disrupt ATF6 and/or XBP1 binding to these elements. B, NRVMCs were transfected with the wild type MANF-luciferase promoter/reporter construct, then infected with either a control adenovirus (Con), or with DN-ATF6 or DN-XBP1, as described in Figure 5. Twenty 4 hours later, cultures were subjected to either sI or TM, then analyzed for reporter activities, as described.[10] C, NRVMCs were transfected with the wild-type (WT) or mutated (M1, M2) MANF–luciferase promoter/reporter constructs, subjected to either sI or TM, and then analyzed for reporter activities. D, NRVMCs were transfected with the MANF–luciferase promoter/reporter constructs shown and then infected with either a control adenovirus (Con) or AdV-ATF6 or AdV-XBP1. Cultures were then subjected to either sI or TM and then analyzed for reporter activities.

However, when subjected to ER stress, cellular MANF expression increased, as expected, and there was a robust increase in MANF in the medium (Figure 7A, lanes 4 to 6). Immunoblots for ERSR proteins that have a canonical C-terminal KDEL demonstrated strong induction of glucose regulated proteins 94 and 78 (GRP94 and GRP78) after ER

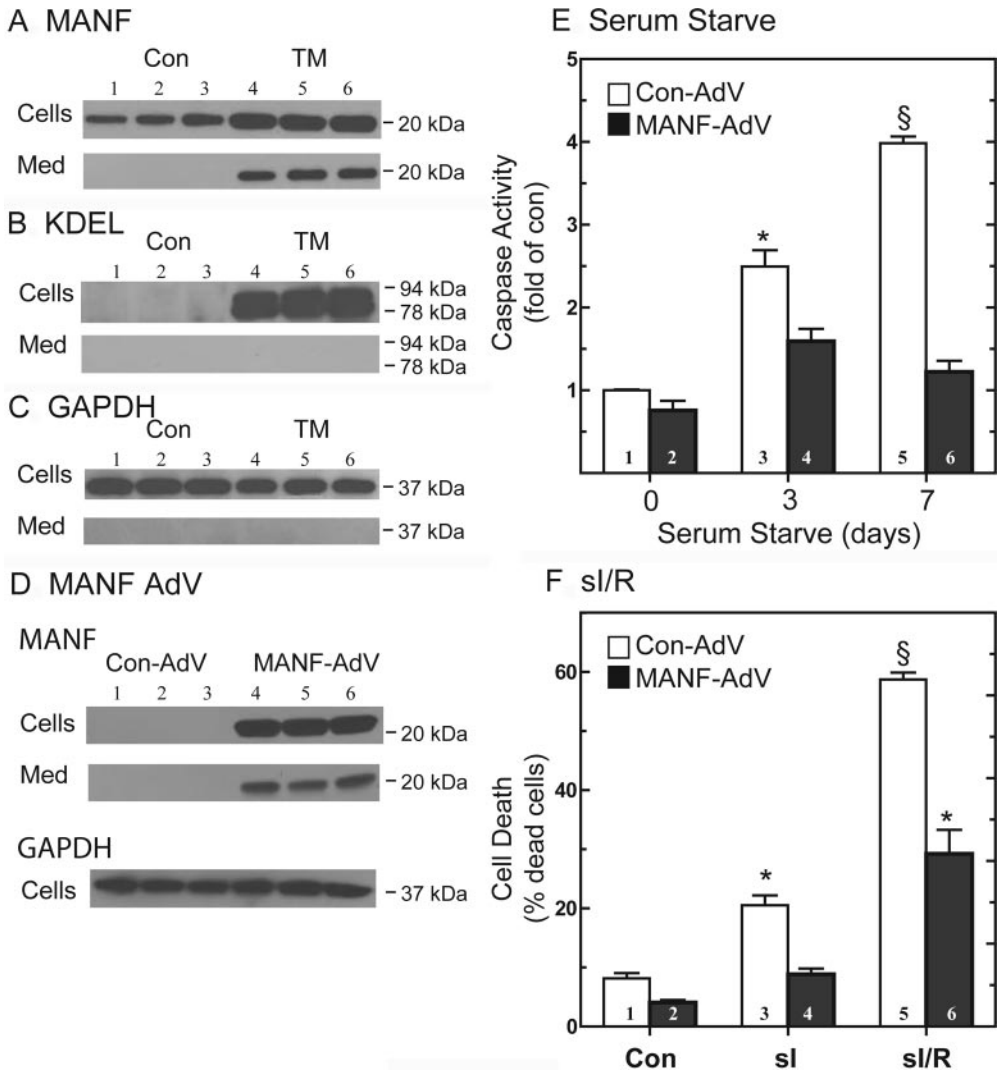

Figure 7. MANF secretion and MANF overexpression. A through C, NRVMCs were incubated with or without TM, and then medium samples and cell extracts were analyzed for MANF, KDEL, and GAPDH by immunoblotting. N=3 cultures for each treatment. D, NRVMCs were infected with either control or MANF-AdV. After 48 hours, cell and medium samples were analyzed as described above. E, NRVMCs were infected with either control or MANF-AdV, placed in serum-free medium for 0, 3, or 7 days, and then assayed for caspase-3 activity. F, NRVMCs were infected with either control or MANF-AdV and then subjected to sI or sI/R, after which cultures were examined for cell death, as described[10] (N=3 cultures/treatment). Values in E and F are means±SE. *$P<0.05$, §$P<0.05$ different from 0 days serum starve (E) or Con (F).

stress; however, even with this strong induction, neither was detected in the medium (Figure 7B, lanes 4 to 6) nor was GAPDH (Figure 7C). The lack of GRP94, GRP78, and GAPDH in the medium verified that the cells were not dying but, instead, were releasing their contents, including MANF, in response to TM treatment and that the appearance of MANF in the medium is most likely attributable to secretion. Thus, unlike proteins with a C-terminal KDEL, MANF can be released from cardiac myocytes, suggesting that it may function, in part, in an extracellular manner.

To examine possible functions of MANF in cardiac myocytes, a MANF-encoding recombinant adenoviral strain (MANF-AdV) was generated. Compared to cells infected with a control strain of adenovirus (Con-AdV), cells infected with MANF-AdV exhibited increased cellular and secreted MANF (Figure 7D, lanes 4 to 6), even in the absence of ER stress. When Con-AdV–infected cells were maintained for various times in serum-free medium, which lacks growth factors and leads to apoptosis of cultured cardiac myocytes,[25] the activity of caspase-3, an indicator of apoptosis, increased by 3- and 4-fold after maintenance for 3 and 7 days in serum-free medium, respectively (Figure 7E, bars 1, 3 and 5). In contrast, MANF-AdV–infected cells exhibited no significant increase in caspase-3 activation (Figure 7E, bars 2, 4, and 6). The effects of MANF-AdV on sI or simulated ischemia/reperfusion (sI/R)-mediated cell death were also examined. In Con-AdV–infected cultures, sI and sI/R increased cell death by approximately 2- and 3-fold, respectively (Figure 7F, lanes 1, 3, and 5). However, cell death from either sI or sI/R was decreased by ≈50% in cultures infected with MANF-AdV (Figure 7F, lanes 2, 4, and 6). These results indicate that MANF can protect cardiac myocytes from apoptotic cell death in response to a variety of stresses.

To examine whether MANF functions in an extracellular manner, recombinant mouse MANF (rMANF), purified from bacterial extracts, was added to NRVMC medium. Compared with equimolar quantities of a control protein, bovine serum albumin, rMANF decreased caspase-3 activation by serum starvation in a dose-dependent manner, with a half-maximal effect at 6.6 nmol/L (Figure 8A, bars 4 to 7). This level was lower than that of MANF found in the medium of TM-treated NRVMC, 140 nmol/L, which was estimated using immunoblotting and rMANF as a standard (Figure 8B), consistent with the hypothesis that secreted MANF is responsible, at least in part, for the protection observed in MANF-overexpressing cells. The effects of rMANF on sI or sI/R-

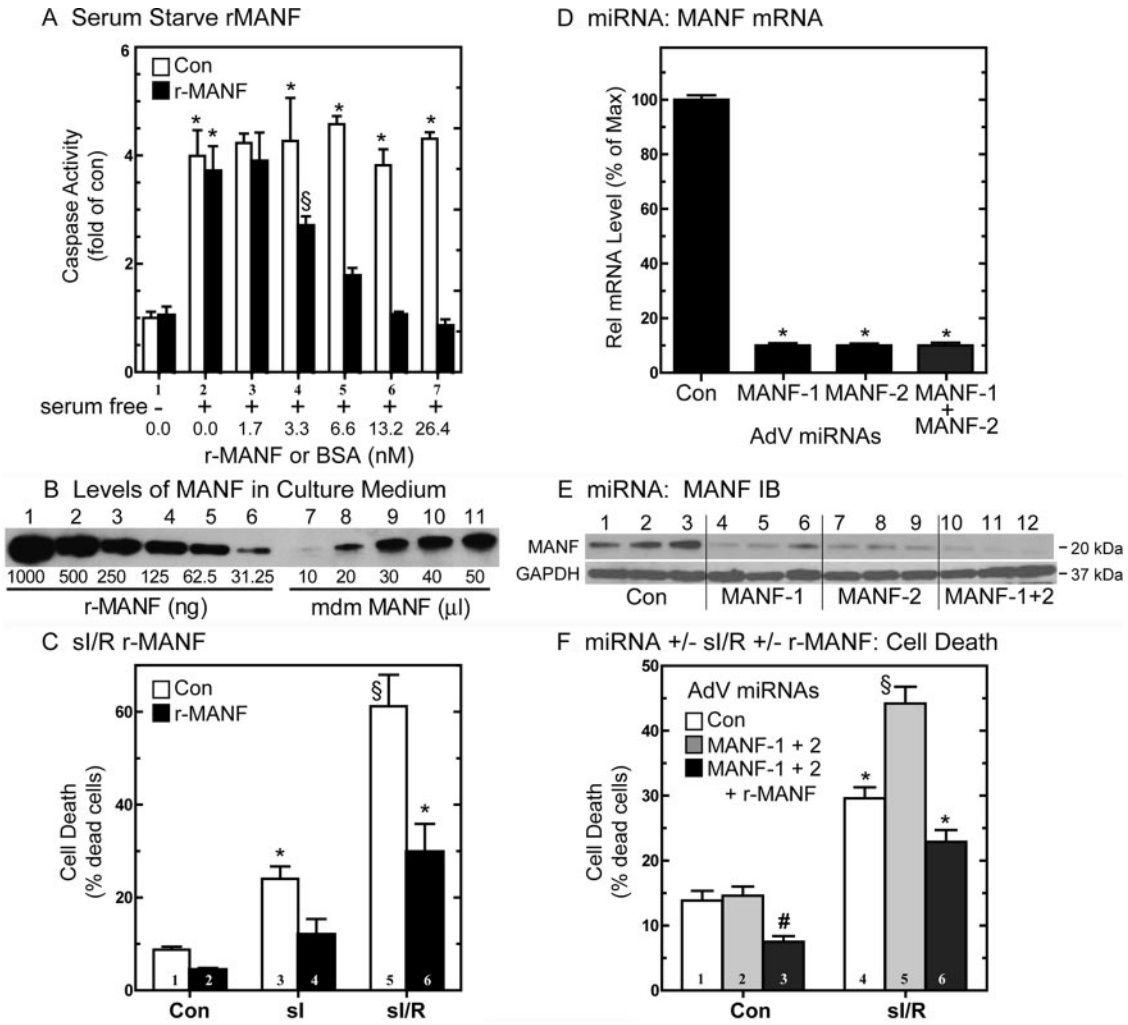

Figure 8. Effects of recombinant MANF on cardiomyocyte death. A, Purified recombinant MANF (rMANF) or bovine serum albumin (Con) was added to NRVMCs at various concentrations up to 26.4 nmol/L. Cultures were placed in serum-free medium for 7 days and then extracted and assayed for caspase-3 activity. B, MANF immunoblots were performed to compare known levels of rMANF (lanes 1 to 6) with different quantities of medium from TM-treated NRVMCs from the experiment shown in Figure 7A through 7C (lanes 7 to 11). Using this method, it was determined that the concentration of MANF in culture medium was ≈140 nmol/L. C, rMANF or bovine serum albumin (Con) was added to NRVMCs at 26.4 nmol/L. Cultures were then subjected to sI or sI/R and then examined for cell death as described.[10] D, Cultures were infected with an AdV expressing a control miRNA (Con), AdV (lanes 1 to 3), or 2 different miRNAs directed against MANF, AdV miMANF-1 (lanes 4 to 5), AdV miMANF-2 (lanes 7 to 9), or both AdV miMANF-1 and AdV miMANF-2 (lanes 10 to 12), and extracts were examined for MANF mRNA levels by real-time quantitative PCR. E, Cultures were infected as described in D, followed by extraction and immunoblotting for endogenous MANF or GAPDH. F, Cultures were infected as described in D, treated with or without 26.4 nmol/L r-MANF, subjected to sI/R, and then examined for cell death as in C. In B and C, n=3 cultures/treatment. Values are mean caspase-3 or mean cell death±SE. *$P<0.05$, §$P<0.05$, $P<0.05$ different from no rMANF or BSA. In E and F, n=3 cultures/treatment. Values are means±SE. *$P<0.05$, §$P<0.05$, #$P<0.05$ different from all other values.

induced cardiac myocyte death were also examined. sI- and sI/R-mediated cell death were significantly reduced by adding rMANF to culture medium (Figure 8C).

To examine the effects of endogenous MANF on cell survival, recombinant AdV encoding micro-RNAs (miRNAs) targeted to rat MANF were generated, as was miRNA that was not targeted to any known mRNA. Either of the 2 miRNAs, AdV MANF-1 and/or AdV MANF-2, decreased endogenous MANF mRNA and protein levels in cultured cardiac myocytes by 90% (Figure 8D and 8E). Whereas MANF miRNA had no effect on cell death under control conditions (Figure 8F, bars 1 and 2), it significantly increased sI/R-mediated cell death (Figure 8F, compare bars 4 and 5), consistent with a protective role for endogenous MANF. Interestingly, the addition of recombinant MANF to cultures that expressed MANF miRNA reduced sI/R-mediated cell death back to levels observed in cells expressing control miRNA (Figure 8F, bars 4 and 6). These results are consistent with a protective role for MANF and demonstrate that the loss of protection observed on miRNA-mediated knock down of endogenous MANF can be partially restored by addition of recombinant MANF to the culture medium.

Discussion

This study supports the hypothesis that MANF is a novel ERSR gene in the heart that can be induced and secreted in response to ER stresses, including ischemia, and that extracellular MANF may protect cardiac myocytes in an autocrine

and paracrine manner. The results that support this hypothesis include the findings that MANF was induced by ATF6 activation in the myocardium, in vivo, and in myocytes and nonmyocytes in the infarct and infarct border zones in a mouse model of in vivo myocardial infarction. Additionally, the MANF promoter, mRNA, and protein were all induced in an ATF6 and XBP1-dependent manner in cultured cardiac myocytes by TM or sI. Finally, the demonstration that MANF can be released from cardiac myocytes, and that at nanomolar levels, extracellular recombinant MANF protected myocytes from apoptosis, even in the absence of endogenous MANF, supports a paracrine and/or endocrine mechanism of function for this novel secreted protein.

This is the first study to demonstrate the secretion of an ERSR protein from cardiac myocytes and to show that an ERSR protein can function in an autocrine and/or paracrine manner to protect cardiac myocytes. The structure of MANF may reveal important details of the possible mechanism by which this ERSR protein functions in the heart. MANF is targeted to the ER in a manner that is similar to many other ER-targeted ERSR proteins. The MANF gene predicts a 179-aa protein, and, according to SignalP 3.0, the N-terminal 21 amino acids serve as a signal sequence, responsible for targeting nascent MANF to the ER lumen in a cotranslational manner.[26] Accordingly, the cotranslational removal of the 21-aa signal sequence is predicted to result in the observed 158-aa mature product in the ER lumen. However, MANF is unusual among ER lumen-targeted ERSR proteins, because, as shown in the present study, it is inefficiently retained in the ER, which may lead to its secretion during ER stress. There are several ways in which proteins are retained in the ER lumen; the major mechanism requires a C-terminal KDEL sequence, which facilitates binding of ER proteins the ER-transmembrane KDEL receptor, facilitating retention in the ER.[27] Thus, whereas most proteins retained in the ER possess an C-terminal KDEL, MANF has a C-terminal RTDL, which fosters ER retention, albeit, less effectively than KDEL.[28] In contrast to the KDEL-containing ER-resident, ERSR proteins GRP94 and GRP78, significant quantities of MANF were found in the medium during ER stress (Figure 7), suggesting a weaker retention in the ER than KDEL-containing proteins.

The results from this study indicate that MANF may exert at least a portion of its protective function extracellularly, in a paracrine and/or autocrine manner. Because it is a relatively large, hydrophilic protein that is not predicted to readily pass through the cell membrane, MANF most likely functions by binding to a cell surface receptor. In carrying out bioinformatics analyses in attempts to determine the nature of such receptors, we were unable to find any MANF-like proteins for which cell surface receptors had been identified. Thus, at the present time, we cannot predict the nature of the receptor(s) responsible for the effects of extracellular MANF on cardiac myocyte survival. However, the concentration of MANF needed to protect cardiac myocytes (≈6 to 7 nmol/L) (Figure 8), which is similar to that needed to protect dopaminergic neurons (≈3 nmol/L) in a previous study,[29] is typical for other cell surface receptor–mediated events, lending reasonable support to the hypothetical existence of MANF receptors on cardiac myocytes.

In summary, the present study has revealed many features of MANF as a novel secreted protein; however, a great deal remains to be understood about the role of MANF in the heart. For example, identification of the MANF receptor, as well as the signaling pathway by which MANF exerts its protective function, will be required to more fully appreciate the possible roles of MANF in the normal and diseased myocardium.

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Disclosures

None.

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