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Mesencephalic Astrocyte-derived Neurotrophic Factor Protects the Heart from Ischemic Damage and Is Selectively Secreted upon Sarco/endoplasmic Reticulum Calcium Depletion*

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Background: Intra- and extracellular MANF are protective; however, the conditions governing MANF secretion are unknown.
Results: Of the conditions examined, only SR/ER Ca^{2+} depletion increased MANF secretion.
Conclusion: SR/ER Ca^{2+} depletion-mediated MANF secretion was due to decreased Ca^{2+}-dependent binding of MANF to the SR/ER-resident chaperone, GRP78.
Significance: This mechanism of regulating intra- and extracellular MANF levels may contribute to survival of Ca^{2+}-stressed cells.

The endoplasmic reticulum (ER) stress protein mesencephalic astrocyte-derived neurotrophic factor (MANF) has been reported to protect cells from stress-induced cell death before and after its secretion; however, the conditions under which it is secreted are not known. Accordingly, we examined the mechanism of MANF release from cultured ventricular myocytes and HeLa cells, both of which secrete proteins via the constitutive pathway. Although the secretion of proteins via the constitutive pathway is not known to increase upon changes in intracellular calcium, MANF secretion was increased within 30 min of treating cells with compounds that deplete sarcoplasmic reticulum (SR)/ER calcium. In contrast, secretion of atrial natriuretic factor from ventricular myocytes was not increased by SR/ER calcium depletion, suggesting that not all secreted proteins exhibit the same characteristics as MANF. We postulated that SR/ER calcium depletion triggered MANF secretion by decreasing its retention. Consistent with this were co-immunoprecipitation and live cell, zero distance, photo affinity cross-linking, demonstrating that, in part, MANF was retained in the SR/ER via its calcium-dependent interaction with the SR/ER-resident protein, GRP78 (glucose-regulated protein 78 kDa). This unusual mechanism of regulating secretion from the constitutive secretory pathway provides a potentially missing link in the mechanism by which extracellular MANF protects cells from stresses that deplete SR/ER calcium. Consistent with this was our finding that administration of recombinant MANF to mice decreased tissue damage in an *in vivo* model of myocardial infarction, a condition during which ER calcium is known to be dysregulated, and MANF expression is induced.

All eukaryotic cells have an endoplasmic reticulum (ER)[2]; muscle cells also have a specialized form of the ER, called the sarcoplasmic reticulum (SR), which is important for regulating muscle contraction. Herein, we use the term sarco/endoplasmic reticulum (SR/ER) to describe the SR and/or ER in a variety of cell types. The SR/ER is the most expansive organelle, constituting as much as 50% of the total membrane (1). Most secreted and membrane proteins are synthesized in the rough ER, after which they are transported to the Golgi apparatus, where they are sorted to their final destinations (2–4). This substantial protein biosynthetic workload depends on the maintenance of an environment in the SR/ER that optimizes protein folding and transport to the Golgi. Nutrient and/or oxygen starvation, decreased SR/ER calcium, or changes in SR/ER redox status can impair protein folding, leading to the accumulation of potentially toxic, terminally misfolded proteins in the SR/ER (5). Such accumulation constitutes a stress in the SR/ER, which initiates the unfolded protein response (6). Initially, the unfolded protein response leads to a genetic reprogramming that changes the levels of numerous proteins that restore nascent SR/ER protein folding, which promotes cell survival (7, 8). However, if these aspects of the response are not sufficient to restore protein folding, subsequent events guide the cell toward

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[2] The abbreviations used are: ER, endoplasmic reticulum; SR, sarcoplasmic reticulum; MANF, mesencephalic astrocyte-derived neurotrophic factor; ANF, atrial natriuretic factor; TM, tunicamycin; TG, thapsigargin; SERCA, sarco/endoplasmic reticulum Ca^{2+}-ATPase; IP, immunoprecipitation; IB, immunoblotting; ATF6, activation of transcription factor 6; GRP78, glocose regulated protein 78 kD.

apoptosis (9). Thus, the response to proteotoxic SR/ER stress can be protective or damaging, depending on the nature of the stress (10).

Although many genes are induced in response to the accumulation of misfolded proteins in the SR/ER, few encode proteins destined for secretion. This may be because impaired protein folding in the SR/ER is predicted to decrease the processing and transport of proteins secreted by the classical secretory pathway (11). However, one recently discovered gene induced during the unfolded protein response encodes mesencephalic astroctye-derived neurotrophic factor (MANF), which is secreted (12). MANF is expressed basally in several tissue and cell types, and its levels increase further in response to accumulation of misfolded proteins in the SR/ER (13, 14).

Unlike most secreted proteins, MANF has a C-terminal sequence, RTDL, which is similar to the C-terminal KDEL motif found in many ER-resident proteins. It is by virtue of binding to the KDEL receptor that proteins with a C-terminal KDEL are retrieved from the *cis*-Golgi and transported back to the ER. KDEL receptor-mediated retrieval fosters retention in the ER, thus averting secretion (15, 16). Although the role of the C-terminal RTDL of MANF as a retention sequence is not clear, based on a survey of the effects of KDEL and various KDEL-like sequences on model secreted proteins, it has been predicted that MANF is retained in the ER (17). Consistent with this prediction are reports that MANF knockdown increased the sensitivity of HeLa cells to chemical ER stress-induced cell death (13) and increased cardiac myocyte death in response to simulated ischemia/reperfusion (14). However, MANF is also secreted. For example, MANF was discovered as a component of astrocyte-conditioned medium that protected cultured neurons from cell death, so it was concluded in that study that MANF was a secreted neurotrophic factor (12). In support of functions for secreted MANF are several studies showing that extracellular MANF protects against cell death in response to a variety of stresses, including oxygen and nutrient deprivation or ischemia (14, 18–21).

Although there is evidence that MANF is a secreted protein that is protective before, as well as after secretion, neither the mechanism of its secretion nor the conditions under which it is secreted are understood. Accordingly, we studied MANF secretion from HeLa cells and cultured ventricular myocytes, two cell types in which MANF has been shown to be induced by and to protect from stress (13, 14).

The rate of release of proteins that are secreted in a regulated manner can be increased rapidly by a variety of stimuli, whereas proteins secreted constitutively are released at rates dictated by their expression levels. Surprisingly, we found that even though neither HeLa cells nor ventricular myocytes are known to release secreted proteins in a regulated manner, MANF secretion was regulated. MANF release was increased minutes after the cells were treated with compounds that deplete SR/ER calcium. This unusual form of regulating secretion was due, at least partly, to the calcium-dependent retention of MANF by the ER-resident chaperone, GRP78 (glucose-regulated protein 78). This secretion mechanism is consistent with the ability of extracellular MANF to protect cells from ER stress, such as simulated ischemia/reperfusion of cultured cardiac myocytes (14).

EXPERIMENTAL PROCEDURES

Laboratory Animals—The research reported in this paper has been reviewed and approved by the San Diego State University institutional animal care and use committee, and it conforms to the Guide for the Care and Use of Laboratory Animals published by the National Research Council.

Osmotic Minipumps—Osmotic minipumps (ALZET, catalog number 1003D) were used to infuse 6–8 week old male FVB mice at 300 ng/h/g with recombinant MANF that had been prepared as previously described (14). Minipumps were implanted subdermally, as described previously (22).

In Vivo Ischemia/Reperfusion—Twenty-four hours after minipump implantation, *in vivo* myocardial ischemia/reperfusion was carried out. *In vivo* coronary occlusion followed by reperfusion was performed essentially as previously described (23–25). Myocardial infarction was produced in mice by a 30-min occlusion of the left ascending coronary artery, followed by 24 h of reperfusion. After reperfusion, the mice were anesthetized, the chests were reopened, and the hearts were injected with 1% Evans Blue and then harvested. Transverse 1-mm-thick slices of the ventricles were stained in 1% triphenyltetrazolium chloride. Infarct size was calculated by computerized videoplanimetry.

HeLa Cells—HeLa cells were maintained in growth medium (Dulbecco's modified Eagle's medium/F-12 containing 10% fetal calf serum). All of the experiments were carried out on cultures that were ≤70% confluent.

Cardiac Myocytes—Cells were isolated by enzymatic digestion of 1–4-day-old neonatal rat hearts, as described (26), and then cardiac myocytes were purified by Percoll density gradient centrifugation, essentially as described (27).

Media and Cell Samples—Conditioned medium samples were collected and combined with the appropriately concentrated form of Laemmli sample buffer and then boiled before SDS-PAGE and immunoblotting (see below). In one experiment, cultured cardiac myocytes were subjected to conditions that simulate ischemia/reperfusion, as previously described (28). The cells were extracted in a minimal volume of cell lysis buffer composed of 20 mM Tris (pH 7.5), 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 1× protease inhibitor mixture (Roche Applied Science; 05 892 791 001). Quantities of the cell extracts ranging from 5 to 25 μg were analyzed by SDS-PAGE, followed by immunoblotting.

Expression Plasmids—Standard molecular cloning methods were used to prepare expression plasmids that encode untagged, FLAG, and HA epitope-tagged versions of mouse MANF and GRP78. The constructs were engineered to express each of these proteins with an N-terminal ER targeting signal sequence, followed by a 3× FLAG, or 3× HA and then the full coding sequence of MANF or GRP78.

Adenovirus—Recombinant adenovirus encoding an miRNA targeted to rat MANF was prepared as previously described (14, 29).

Immunoprecipitation—Cell extracts, prepared as described in the Media and Cell Samples section above, were incubated with protein G-Sepharose beads (20 μl) for 3 h at 4 °C to remove any material that bound nonspecifically to the beads. MANF,

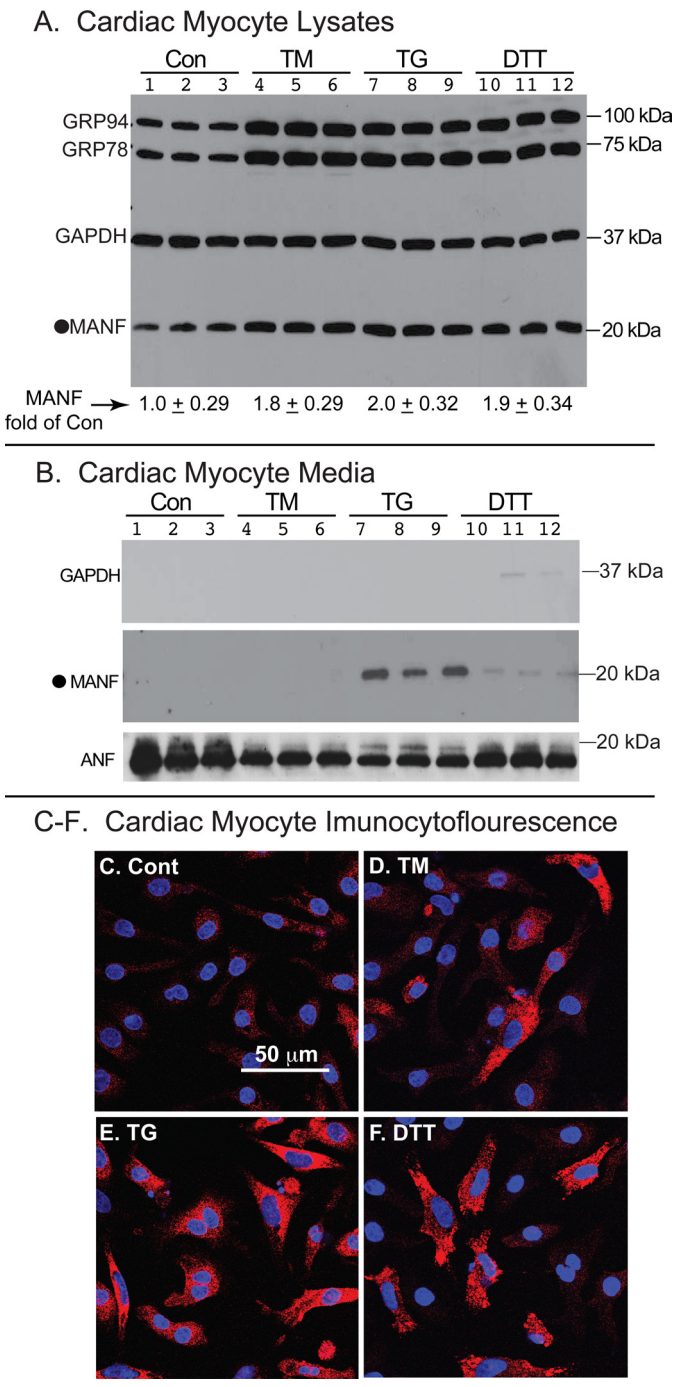


FIGURE 1. **Effects of ER stress on cardiac myocytes.** *A*, cellular MANF. Cultured cardiac myocytes were treated for 20 h with TM (10 μg/ml), TG (1 μM), or DTT (1 mM). Cell extract samples (20 μg of protein) were fractionated by SDS-PAGE, followed by immunoblotting for GRP94 and GRP78, using an anti-KDEL antibody, as well as GAPDH and MANF. Shown is an immunoblot of a representative experiment; the migration locations of molecular mass markers are shown on the *right side* of the blot, and the identities of immunoreactive proteins, are shown on *left side* of the blot. $n = 3$ cultures per treatment. Band intensities of MANF were normalized to those for GAPDH, and the MANF levels ± S.E., compared with control, are shown beneath the blot. Unless otherwise stated, the quantification of band intensities was carried out the same way for subsequent experiments. *B*, medium MANF. Medium samples (35 μl each) from the cultures described in *A* were fractionated by SDS-PAGE followed by immunoblotting for GAPDH, MANF, and ANF. *C–F*, immunocytofluorescence. Cardiac myocytes were treated for 20 h with TM, TG, and DTT, as described for *A*, and subjected to immunocytofluorescence followed by laser scanning confocal microscopy. *Red*, anti-MANF; *blue*, Sytox Blue. *Con* or *Cont*, control.

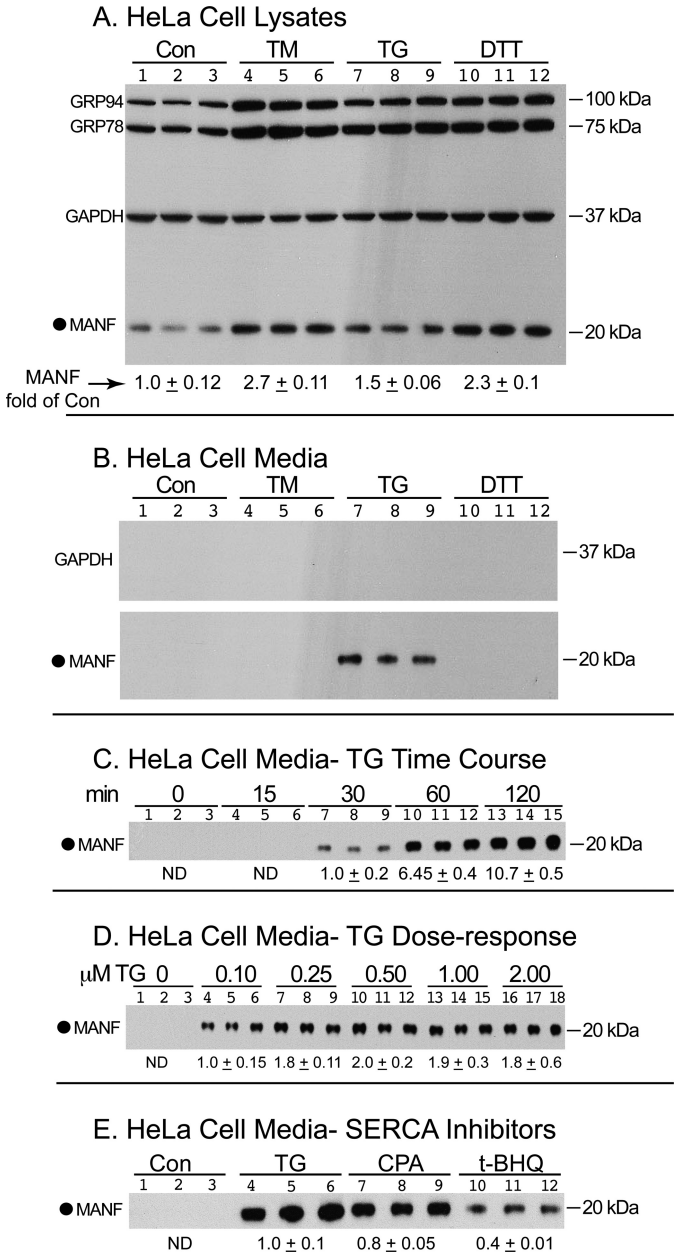


FIGURE 2. **Effects of ER stress on HeLa cells.** *A*, cellular MANF. HeLa cells were treated with TM, TG, and DTT, and then cell extracts (30 μg of protein) were subjected to SDS-PAGE and immunoblotting, as described in the legend to Fig. 1. *B*, medium MANF. Medium samples (25 μl) from the cultures described for *A* were fractionated by SDS-PAGE and immunoblotting for GAPDH and MANF. *C*, effect of different times of thapsigargin on medium MANF. HeLa cells were treated with 1 μM TG for the times shown, and then medium samples were subjected to SDS-PAGE and immunoblotted for MANF. $n = 3$ cultures per treatment. *D*, effect of thapsigargin concentration on medium MANF. HeLa cells were treated with the various concentrations of TG shown, and then medium samples were subjected to SDS-PAGE and immunoblotted for MANF. $n = 3$ cultures per treatment. Band intensities were normalized to the 0.10 μM TG value as the control (*i.e.*, *fold of Con*). *E*, effect of different SERCA inhibitors on medium MANF. HeLa cells were treated for 2 h with TG (1 μM), 2,5-di-(t-butyl)-1,4-hydroquinone (*t-BHQ*, 30 μM), or cyclopiazonic acid (*CPA*, 50 μM), and then medium samples were subjected to SDS-PAGE and immunoblotted for MANF. $n = 3$ cultures per treatment. Band intensities were normalized to the TG value as the control (*i.e.*, *fold of Con*). *ND*, not detectible; *Con*, control.

FLAG-MANF, or HA-GRP78 was then immunoprecipitated using anti-MANF antibody, anti-FLAG beads (EZview Red Anti-FLAG M2 Affinity Gel, Sigma-Aldrich F2426), or anti-

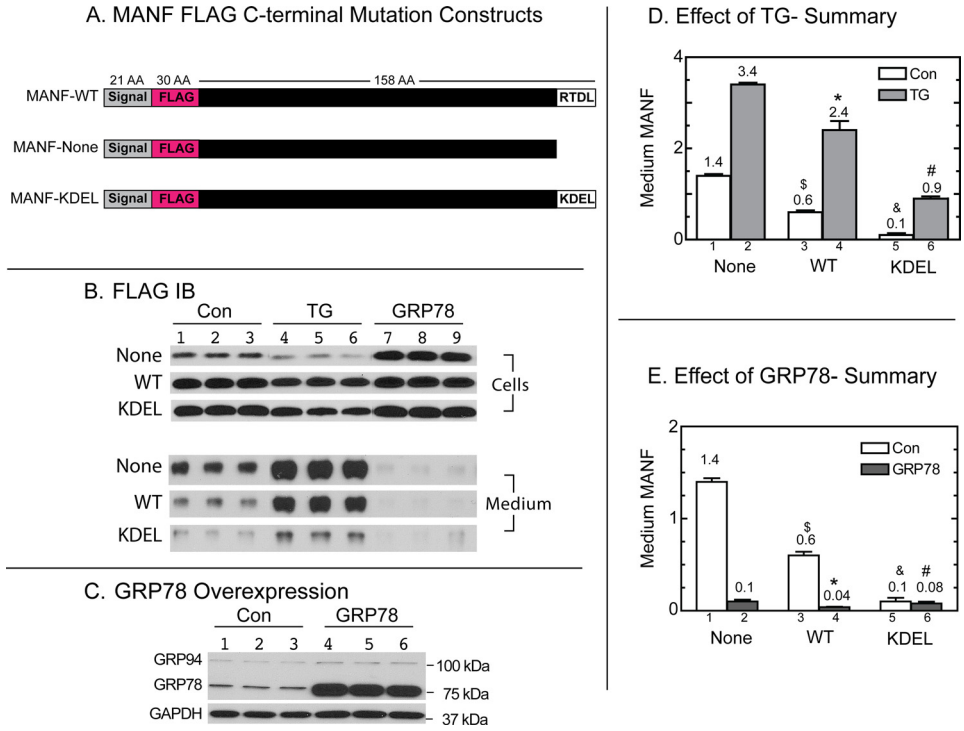


FIGURE 3. **Expression of various forms of MANF.** *A*, diagram of native and variant forms of MANF. Shown is a diagram of native and variant forms of MANF, depicting the N-terminal 21-amino acid signal sequence (−21 to 1), which is proteolytically removed in the ER lumen, co-translationally. The constructs were engineered so that the mature proteins would have the epitope tag at the N terminus, because the KDEL receptor binding sequences must be at the extreme C terminus to interact with the KDEL receptor. *B*, FLAG immunoblots of cellular and media MANF. HeLa cells were transfected with 2 μg of constructs encoding the various forms of mouse MANF and/or with 20 μg of a construct encoding mouse GRP78 or empty vector (control and TG), as shown, and then plated. The cells were treated with 1 μM thapsigargin for 2 h. Aliquots of cell extracts (~1% of the total extract) and media (~10% of the total medium sample) were then subjected to SDS-PAGE and immunoblotting. $n = 3$ cultures per construct. *C*, GRP78 immunoblots. HeLa cells transfected with or without 20 μg of a construct encoding mouse GRP78, as described in *B*, were subjected to SDS-PAGE and immunoblotting for GRP78. $n = 3$ cultures per construct. On average, GRP78 expression increased by 4-fold in cells that had been transfected with the plasmid encoding GRP78. *D*, effect of TG. Image quantification data of the medium immunoblots in *B* from control and TG-treated cells were plotted. The image quantification values are shown above each bar. Shown are the means ± S.E. $ and &, $p < 0.05$ different from all other control values; * and #, $p \leq 0.05$ different from all other TG values. *E*, effect of GRP78. The image quantification data of the medium immunoblots in *B* from Con and GRP78-transfected cells were plotted; the image quantification values are shown above each bar. Shown are the means ± S.E. $ and &, $p \leq 0.05$ different from all other control values; * and #, $p \leq 0.05$ different from all other GRP78 values. *Con*, control.

HA beads (EZview Red anti-HA affinity gel; Sigma-Aldrich, E-6779) at a ratio of 5 μl of beads/50 μl of cell extract and then incubated at 4 °C overnight. Unless otherwise indicated, the beads were washed three times with cell lysis buffer at 4 °C and then eluted with 1× Laemmli buffer.

Immunoblotting—SDS-PAGE gels were transferred to PVDF membranes. The membranes were probed with the following antibodies: ANF (Bachem; 1:2,000), FLAG (Sigma-Aldrich F1804; 1:12,000), GAPDH (RDI, TRK5G4; 1:15,000), GRP78 (C-20) (Santa Cruz Biotechnology, Inc.; SC1051; 1:1,000), KDEL (ENZO Life Sciences; ADI-SPA-827; 1:8,000), and MANF (R & D Systems, Inc.; AF3748; 1:200 for media blots, 1:500 for cell extract blots).

Cross-linking—The cells were subjected to photo Leu/photo Met cross-linking, as described (30).

Liquid Chromatography Coupled to Tandem Mass Spectrometry—HeLa cells were subjected to photo Leu/photo Met cross-linking, or not, followed by immunoprecipitation and SDS-PAGE. The gels were then subjected to silver staining, and bands migrating at 100 kDa were excised and then digested with trypsin (10 μg/ml) at 37 °C overnight. LC-MS/MS analyses of in-gel trypsin digested proteins was carried out as described (31) using a LTQ Velos Orbitrap mass spectrometer (Thermo Fisher Scientific, San Jose, CA) equipped with an Advion nanomate ESI source (Advion, Ithaca, NY). The peptides were separated by reversed phase HPLC. Data-dependent scanning was performed using Xcalibur v 2.1.0 and a survey mass scan at 30,000 resolution in the Orbitrap analyzer scanning *m/z* 400–1600, followed by collision-induced dissociation tandem mass spectrometry (MS/MS) of the 14 most intense ions in the linear ion trap analyzer (32). MS/MS spectra were searched using Thermo Proteome Discoverer 1.2 (Thermo Fisher Scientific). Proteins were identified at the 95% confidence level, as described (33). In non-cross-linked samples, no peptides corresponding to GRP78 or MANF were identified. In duplicate cross-linked culture extracts, one analysis resulted in three peptides from GRP78 and one peptide from MANF, and the second analysis resulted in seven peptides from GRP78 and one peptide from MANF. Thus, the average % coverage was 10.5% for GRP78 and 7.75% for MANF.

Statistics—The values are the means ± S.E. Statistical analyses were performed using either a Student's *t* test or using a one-way analysis of variance followed by a Student's Newman-Keuls post hoc analysis.

RESULTS

Thapsigargin Increases MANF Secretion—Because extracellular MANF has been reported to protect cultured cardiac myo-

cytes (14), in initial experiments, we examined the mechanism of MANF secretion from ventricular myocytes. Neonatal rat ventricular myocytes secrete other proteins, such as atrial natriuretic factor (ANF), constitutively, at rates dictated by expression levels (34). Accordingly, we hypothesized that MANF secretion would vary in coordination with its expression, as reported for other constitutively secreted proteins (3). To test this hypothesis, cultured cardiac myocytes were treated for 20 h with tunicamycin (TM), thapsigargin (TG), and DTT, which increase the expression of ER stress response genes by inhibiting glycosylation (35), decreasing ER calcium (36), and altering ER redox status (37), respectively. As expected, each compound increased the expression of two well known ER stress gene products, GRP94 (glucose-regulated protein 94 kDa) and GRP78 (Fig. 1*A*, *GRP94* and *GRP78*). In the absence of ER stress, MANF was expressed at moderate levels, which increased in response to each ER stressor (Fig. 1*A*, *MANF*). Immunocytofluorescence also showed that MANF was induced by these ER stressors and that it was found in a punctate, perinuclear pattern, consistent with its localization in the ER and sarcoplasmic reticulum of cardiac myocytes (Fig. 1, *C–F*). Unexpected, however, was the finding that the only compound that significantly increased MANF in the medium was thapsigargin (Fig. 1*B*, *MANF*). Moreover, in contrast to MANF, ANF was found in lower quantities in the media from cells treated with all of the ER stressors (Fig. 1*B*). In further contrast to MANF, compared with untreated cells, all of the ER stressors decreased medium and cellular levels of ANF, which is consistent with decreased export and synthesis of some proteins from the SR/ER upon accumulation of misfolded proteins (38).

The effect of ER stress on MANF secretion from HeLa cells was also examined. All three ER stressors increased cellular levels of GRP94, GRP78, and MANF (Fig. 2*A*). Consistent with the results observed in cardiac myocytes, only thapsigargin increased MANF secretion from HeLa cells (Fig. 2*B*). These results demonstrated that thapsigargin did not enhance MANF release by increasing constitutive secretion.

Because thapsigargin depletes ER calcium (36), these findings suggested that decreased ER calcium might trigger MANF secretion. Therefore, we determined whether the kinetics and the doses of MANF secretion coordinated with the abilities of thapsigargin to deplete ER calcium. Previous studies have shown that thapsigargin depletes ER calcium in cultured cells within ~30 min (39). Accordingly, HeLa cells were treated with thapsigargin for times short enough to deplete ER calcium but not long enough to induce MANF gene expression (40). MANF was detected in the media after as little as 30 min of thapsigargin treatment, and it continued to accumulate throughout the 120 min of the time course (Fig. 2*C*). These results suggest that MANF secretion was due to the ability of thapsigargin to acutely decrease ER calcium and that it did not require MANF gene induction.

Thapsigargin depletes ER calcium by inhibiting sarcoplasmic/endoplasmic reticulum calcium ATPase (SERCA) over a concentration range of ~0.1–0.5 mM thapsigargin (41). When HeLa cells were treated with various concentrations of thapsigargin ranging from 0.1 to 2 mM, MANF secretion increased in

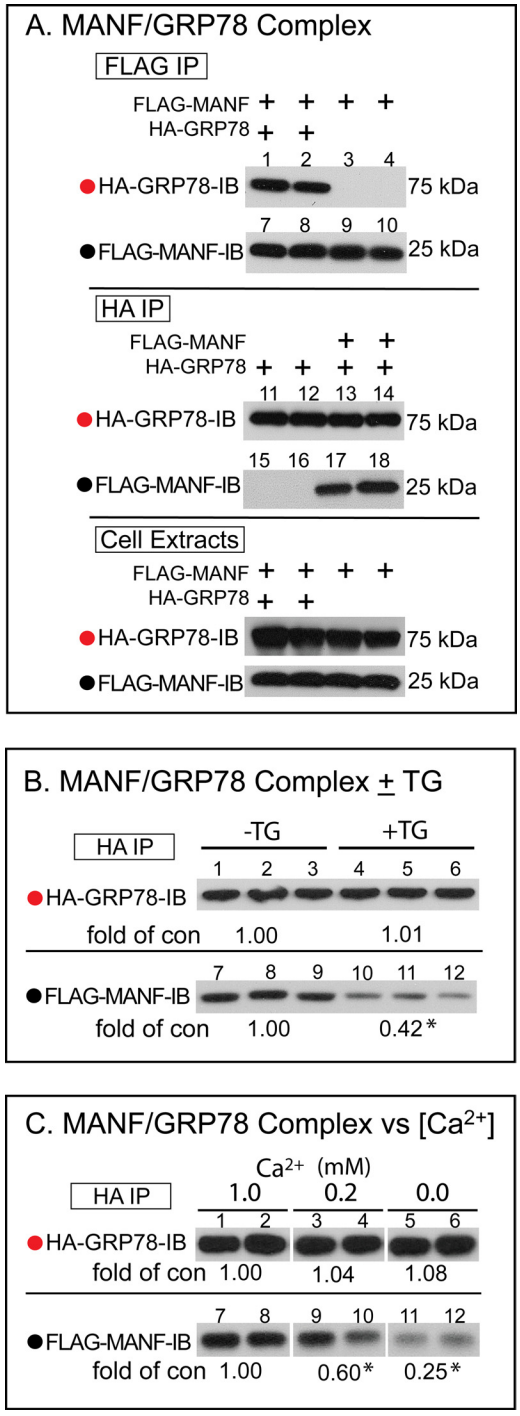


FIGURE 4. **Co-immunoprecipitation of MANF-WT and GRP78.** *A*, MANF-GRP78 complex. Duplicate HeLa cell cultures were transfected with 5 μg of FLAG-MANF-WT and/or 20 μg of HA-GRP78 expression constructs, as shown. The cell extracts were subjected to SDS-PAGE followed by immunoblotting for HA or FLAG (*Cell Extracts*). The cell extracts were also subjected to FLAG or HA IP followed by SDS-PAGE and then IB for FLAG or HA, as shown. *B*, effect of TG. Duplicate HeLa cell cultures were transfected as shown, treated for 2 h with TG (1 μM), then extracted, and subjected to IP and IB, as described for *A*. The image quantification values are shown as *fold of con*. *, $p \leq 0.05$ different from control. *C*, effect of varied calcium. Duplicate HeLa cells cultures were transfected as shown and then extracted in cell lysis buffer, to which calcium was added to final concentrations of 1, 0.2, or 0 mM, as shown. IPs were then carried out in the presence of 1, 0.2, or 0 mM calcium, and the samples were subjected to IB. Image quantification values are shown as *fold of con*. *, $p \leq 0.05$ different from control.

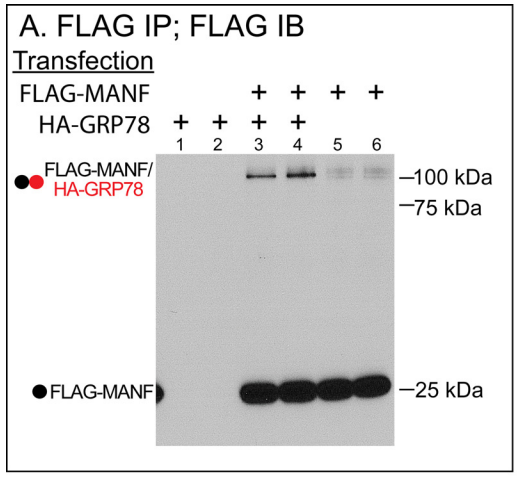
a concentration-dependent manner, reaching a maximum at 0.5 mM thapsigargin (Fig. 2*D*). Thus, the concentration range over which thapsigargin increased MANF secretion was the same as that which acutely depletes ER calcium.

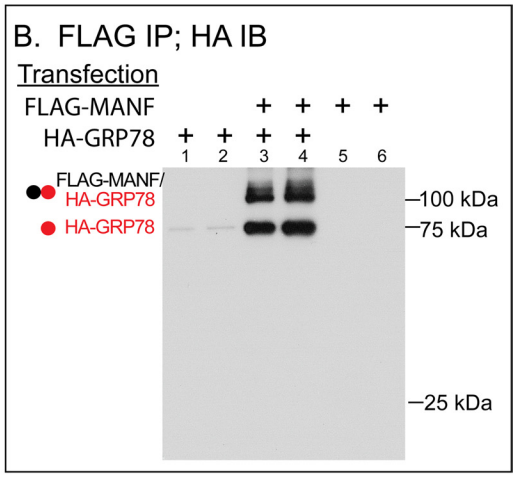
We next examined the effects of two other compounds, cyclopiazonic acid (42), and *t*-butyl hydroquinone (43), which are structurally distinct from thapsigargin, but are known to deplete ER calcium by inhibiting SERCA. When used at concentrations known to inhibit SERCA and to cause ER calcium depletion (44), both cyclopiazonic acid and *t*-butyl hydroquinone increased MANF secretion (Fig. 2*E*). Taken together, these results demonstrate that it is by acutely depleting ER calcium that thapsigargin causes MANF secretion. Moreover, because thapsigargin did not increase constitutive secretion, it must be enhancing MANF release through a different mechanism, such as decreasing its retention.

Thapsigargin-stimulated MANF Secretion Does Not Require Its C-terminal RTDL—One retention mechanism that we previously proposed (45) involves the RTDL at the C terminus of MANF, which could mimic the ER retention mediated by a C-terminal KDEL sequence (46). To explore this possibility, expression constructs encoding N-terminally FLAG epitope-tagged wild type MANF (WT) or MANF with the C-terminal four amino acids removed (None) were generated. It was critical to place the tag on the N terminus, between the signal sequence and the first amino acid of mature MANF, and not on the C terminus, because interaction of the KDEL receptor with its ligand proteins requires KDEL or a KDEL-like sequence to be at the C terminus. To compare the effects of RTDL to the canonical KDEL receptor-binding motif, a construct encoding MANF with the C-terminal RTDL converted to a KDEL (KDEL) was generated (Fig. 3*A*). The rank order of the cellular levels of each MANF variant was None < WT = KDEL (Fig. 3*B*, *lanes 1–3*, *Cells*), which contrasted with the rank order of medium levels, which was None > WT > KDEL (Fig. 3*B*, *lanes 1–3*, *Medium*). Thus, whereas the C-terminal RTDL of MANF-WT contributed to its retention and decreased secretion by ~2-fold (Fig. 3*D*, *bar 1 versus bar 3*), it was less effective than KDEL, which decreased secretion by 14-fold (Fig. 3*D*, *bar 1 versus bar 5*).

To determine whether RTDL-mediated MANF retention was involved in increased thapsigargin-mediated MANF secretion, the effects of thapsigargin on secretion of each form of MANF were determined. Thapsigargin increased the secretion of all forms of MANF (Fig. 3*B*, *lanes 4–6*, *Medium*). Thus, even in the absence of the C-terminal RTDL, the secretion of MANF-None could be increased by thapsigargin (Fig. 3*D*, *bars 1* and *2*), leading to the conclusion that there must be RTDL-independent MANF retention mechanisms that are thapsigargin-sensitive.

MANF Is Retained by GRP78—Because the SR/ER-resident chaperone, GRP78, associates with other proteins in the ER (47), we examined whether it contributes to RTDL-independent MANF retention. Accordingly, the effect of overexpressing GRP78 on the retention and secretion of each form of MANF was examined. Overexpressing GRP78 (Fig. 3*C*) increased the cellular levels of each form of MANF, although the greatest effect was on MANF-None (Fig. 3*B*, *lanes 7–9*, *Cells*). In con-





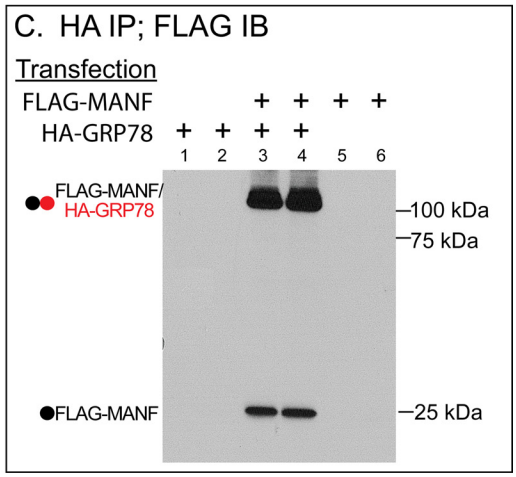


FIGURE 5. **Live cell cross-linking of FLAG-MANF-WT and HA-GRP78.** *A*, FLAG IP/FLAG IB. Duplicate HeLa cell cultures were transfected with FLAG-MANF and/or HA-GRP78, as shown and then subjected to photo methionine/photo leucine zero distance live cell cross-linking. Cell extracts were then subjected to FLAG IP followed by SDS-PAGE and FLAG IB. *B*, FLAG IB/HA IB. Duplicate HeLa cell cultures were transfected and cross-linked, as described for *A*. The cell extracts were then subjected to FLAG IP followed by SDS-PAGE and HA IB. *C*, HA IP/FLAG IB. Duplicate HeLa cell cultures were transfected and cross-linked, as described for *A*. The cell extracts were then subjected to HA IP followed by SDS-PAGE and FLAG IB.

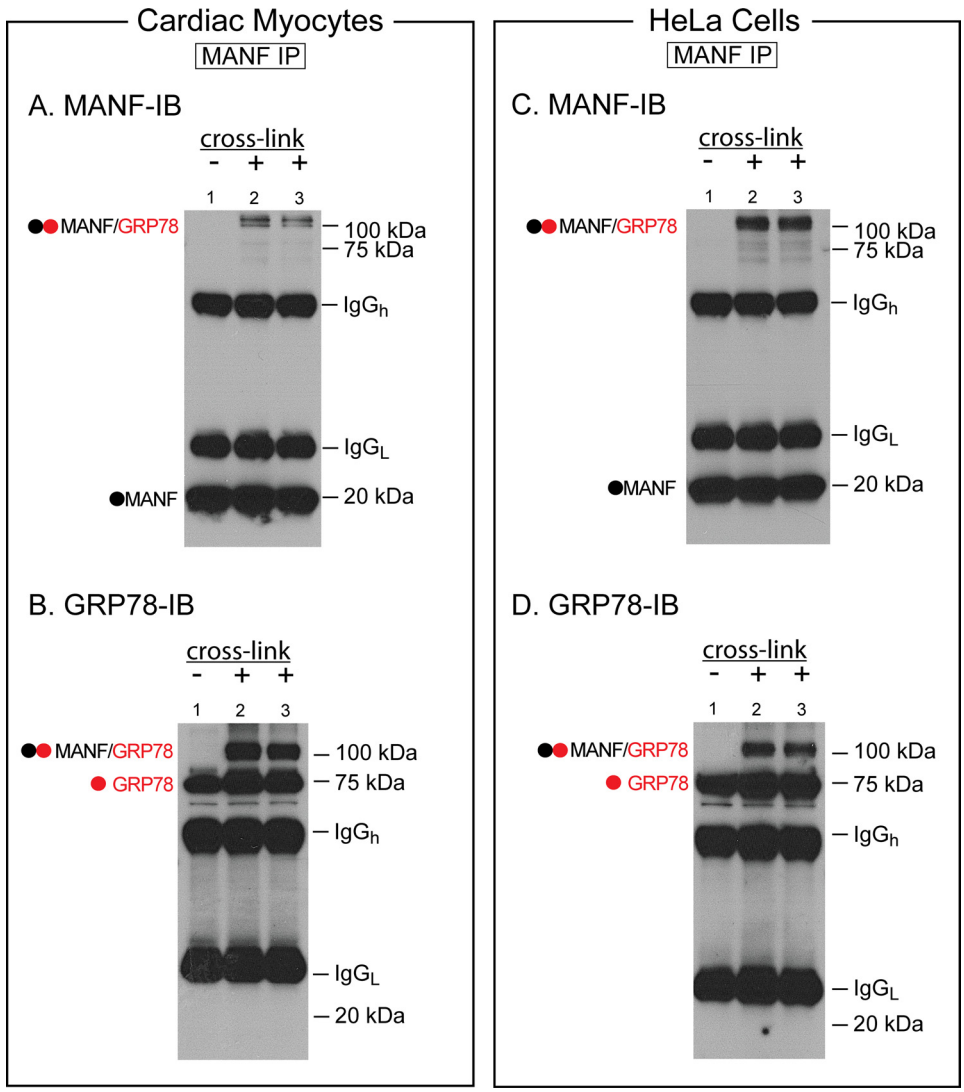


FIGURE 6. **Live cell cross-linking of endogenous MANF and GRP78.** *A* and *B*, cardiac myocytes. Cultured cardiac myocytes were maintained for 20 h in photo methionine/photo leucine media containing 2% fetal bovine serum and TM (10 μg/ml), after which they were extracted (− *cross-link*) or subjected to UV irradiation (+ *cross-link*) and then extracted. The cell extracts were then subjected to MANF IP followed by SDS-PAGE and either MANF IB (*A*) or GRP78 IB (*B*). IgG_h and IgH_L are the immunoglobulin light and heavy chains, respectively, from the MANF antibody that was used in the IP. $n = 1$ culture for − *cross-link*; $n = 2$ cultures for + *cross-link*. *C* and *D*, HeLa cells. HeLa cells were maintained for 20 h in photo methionine/photo leucine media containing 2% fetal bovine serum and TM (2 μg/ml), after which cell extracts were subjected to IP and IB, as described for *A* and *B*.

trast to its effects on cellular MANF, GRP78 overexpression dramatically decreased the medium levels of all forms of MANF (Fig. 3, *B*, *lanes 7–9*, *Medium*, and *E*, *bars 2*, *4*, and *6*). Thus, overexpression of GRP78 increased MANF retention, independently of the C-terminal RTDL.

GRP78 and MANF Reside in a Macromolecular Complex—We next examined whether GRP78-mediated MANF retention was due to complex formation between the two proteins. Accordingly, cells were transfected with constructs encoding HA-GRP78 and/or FLAG-MANF-WT and then subjected to immunoprecipitation (IP) followed by immunoblotting (IB). FLAG IP of extracts from cells transfected with FLAG-MANF-WT contained similar amounts of FLAG-MANF, as expected (Fig. 4*A*, *lanes 7–10*). However, the FLAG IP of extracts from cells that had been co-transfected with FLAG-MANF *and* HA-GRP78 contained HA-GRP78 (Fig. 4*A*, *lanes 1* and *2*), consistent with a GRP78-MANF complex. In complementary experiments, HA IPs followed by HA or FLAG IBs showed that FLAG-MANF co-immunoprecipitated with HA-GRP78 (Fig. 4*A*, *lanes 11–18*), providing additional evidence of a GRP78-MANF complex. Similar experiments demonstrated that GRP78-MANF complex formation was not dependent upon the C-terminal RTDL of MANF (not shown).

GRP78-MANF Complex Formation Requires Calcium—If retention of MANF by GRP78 contributes to thapsigargin-mediated MANF secretion, then we reasoned that the GRP78-MANF complex should be thapsigargin-sensitive. To test this possibility, the cells transfected, as described above, were treated with thapsigargin, and GRP78-MANF complex formation was assessed. Although thapsigargin had no effect on the amount of immunoprecipitated HA-GRP78 (Fig. 4*B*, *lanes 1–6*), it decreased the amount of FLAG-MANF that co-immunoprecipitated with HA-GRP78 by ~60% (Fig. 4*B*, *lanes 7–12*), consistent with the hypothesis that the GRP78-

MANF complex is sensitive to thapsigargin-mediated ER calcium depletion.

To further examine the effect of calcium on the GRP78-MANF-containing complex, cells transfected as described above were lysed in the presence or absence of calcium, and IP was performed in the presence or absence of calcium. Although removing calcium did not affect the amount of immunoprecipitable HA-GRP78 (Fig. 4*C*, *lanes 1–6*), GRP78-MANF complex levels decreased in coordination with decreases in calcium, such that 60% of the complex remained at 0.2 mM calcium and only 25% remained at 0 mM calcium (Fig. 4*C*, *lanes 7–12*). Thus, the GRP78-MANF complex was thapsigargin-sensitive and sensitive to changes in calcium concentrations that mimicked those changes that take place in the ER upon agonist-mediated ER calcium release and ER stress-mediated ER calcium depletion (48–50).

GRP78 and MANF Interact Directly—Next, we determined whether GRP78 and MANF interact, directly, in living cells. To do this, we employed zero distance photo cross-linking using incorporation of photo-activatable forms of the amino acids, methionine and leucine, into proteins in live cells (30). Accordingly, cells were co-transfected, as described above, and then subjected to UV irradiation prior to extraction and IP/IB. FLAG IP followed by FLAG-IB revealed FLAG-MANF at 25 kDa, as expected (Fig. 5*A*, *FLAG-MANF*, *25 kDa*), as well as FLAG-MANF at 100 kDa (Fig. 5*A*, *FLAG-MANF*, *100 kDa*), the latter of which was most prominent in cultures that had been co-transfected with both FLAG-MANF and HA-GRP78 (Fig. 5*A*, *lanes 3* and *4*). A mass of 100 kDa is consistent with a cross-linked complex made of MANF (~25 kDa) and GRP78 (~75 kDa). When the FLAG IPs from the same cell extracts were examined by HA immunoblotting, we detected HA-GRP78 at 75 kDa (Fig. 5*B*, *HA-GRP78*, *75 kDa*), which was due to the co-IP of non-cross-linked HA-GRP78 and FLAG-MANF. Additionally, a portion of the FLAG-immunoprecipitable HA-GRP78 migrated at 100 kDa (Fig. 5*B*, *HA-GRP78*, *lanes 3* and *4*, *100 kDa*), which was due to the co-IP of cross-linked HA-GRP78 and FLAG-MANF. Complementary experiments in which HA IPs were carried out showed the co-IP of FLAG-MANF at 25 kDa, as well as 100 kDa in HA-IPs (Fig. 5*C*). When analyzed by in-gel trypsin digestion, followed by LC-MS/MS, the 100-kDa band was shown to contain both GRP78 and MANF (see "Experimental Procedures" for details).

To determine whether endogenous MANF and GRP78 interact, cross-linking studies were carried out with cardiac myocytes or HeLa cells that had not been transfected. We found that endogenous MANF also migrated at 100 kDa, but only in cardiac myocytes or HeLa cells subjected to cross-linking (Fig. 6, *A* and *C*, *100 kDa*), indicating an interaction of endogenous MANF and GRP78. In further support of this, was our finding that endogenous GRP78 also migrated at ~100 kDa but only in cells subjected to cross-linking (Fig. 6, *C* and *D*, *100 kDa*).

MANF Is Protective in Vivo—Myocardial ischemia is the nutrient and oxygen deprivation that occurs upon coronary vessel occlusion. Reopening of the vessel results in tissue reperfusion. Ischemia/reperfusion can result in myocardial damage, or infarction. MANF expression is induced in the ischemic mouse heart (14). Moreover, myocardial ischemia/reperfusion

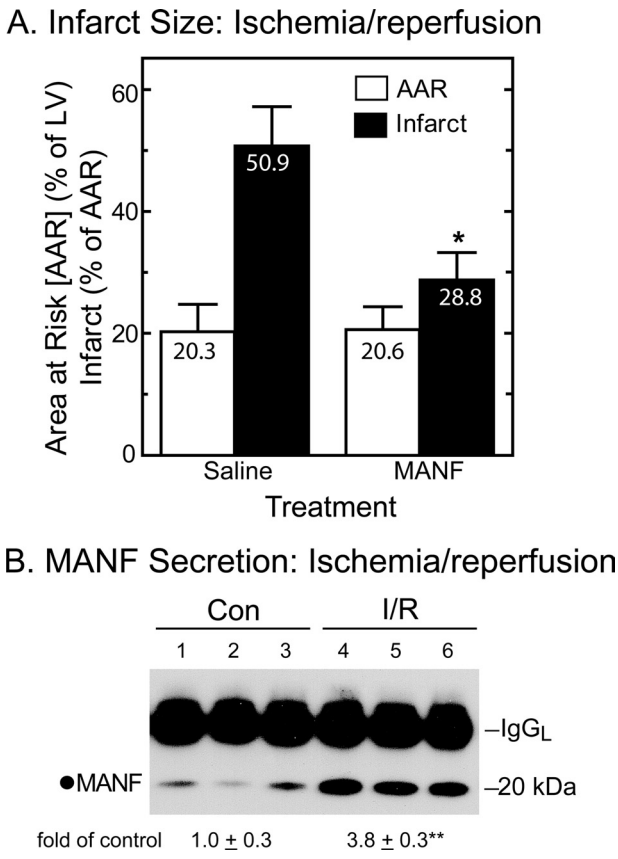


FIGURE 7. **MANF and ischemia/reperfusion.** *A*, effect of MANF on myocardial ischemia/reperfusion injury *in vivo*. Osmotic minipumps containing either saline or recombinant MANF were implanted into mice subcutaneously 24 h prior to subjecting them to 30 min of myocardial ischemia, followed by 24 h of reperfusion *in vivo*. Hearts were then examined to determine the area at risk (*AAR*) of the left ventricle (*LV*) and the infarct size, as described under "Experimental Procedures." Shown are the average infarct sizes and areas at risk ± S.E. *, $p \leq 0.05$ different from saline determined by Student's *t* test. $n = 6$ for each treatment. *B*, effect of ischemia/reperfusion on MANF secretion. Cultured cardiac myocytes were subjected to control conditions (*Con*, *lanes 1–3*) or to conditions that simulate ischemia/reperfusion (I/R; *lanes 4–6*). MANF was immunoprecipitated from medium samples, as described under "Experimental Procedures" and then subjected to SDS-PAGE followed by MANF immunoblotting. The immunoblots were quantified, and the amount of MANF secreted upon simulated ischemia/reperfusion was compared with the amount secreted under control conditions. **, $p \leq 0.01$ different from control.

can dysregulate calcium in the ER and sarcoplasmic reticulum of the heart in ways that could lead to MANF secretion (51). Accordingly, we examined the effects of extracellular MANF in an *in vivo* model of myocardial ischemia/reperfusion. Recombinant MANF was infused into mice, which were subsequently subjected to *in vivo* myocardial ischemia/reperfusion, which results in an infarction with 24 h. The size of the infarct in mice treated with recombinant MANF was decreased by ~44% compared with mice treated with PBS (Fig. 7*A*). Thus, extracellular MANF was cardioprotective, which supports roles for secreted MANF in the heart, *in vivo*. Consistent with possible protective roles for extracellular MANF was our finding that MANF secretion from cultured cardiac myocytes was increased by nearly 4-fold when cultures were subjected to conditions that simulate ischemia/reperfusion (Fig. 7*B*), the latter of which are known to mimic the abilities of thapsigargin to decrease SR/ER calcium (52–56).

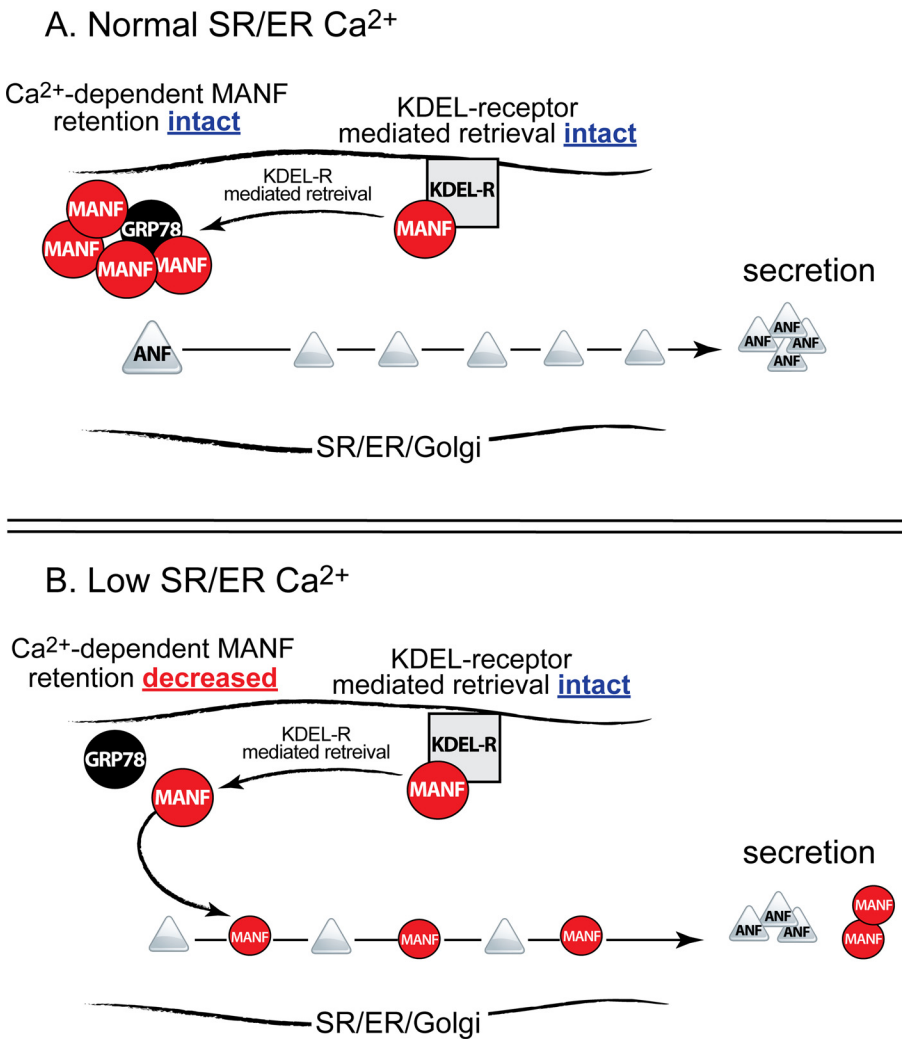


FIGURE 8. **Hypothesis of the mechanism by which SR/ER calcium affects MANF secretion.** *A*, under conditions of normal SR/ER calcium, MANF is retained in a KDEL receptor-mediated fashion, as well as by calcium-dependent binding to the ER-resident chaperone, GRP78. This results in the efficient retention and low secretion of MANF. ANF secretion is not affected by either KDEL receptor- or calcium-dependent binding to GRP78. *B*, when SR/ER calcium is decreased, MANF retention by the KDEL receptor is unaffected; however, its retention by GRP78 is decreased. This results in a decrease in MANF retention, with a corresponding increase in its secretion. ANF secretion is not increased by decreases in SR/ER calcium but is actually decreased, perhaps as a result of impaired SR/ER to Golgi to secretory vesicle trafficking upon ER stress and/or reduced cellular levels of ANF under conditions of SR/ER calcium depletion.

DISCUSSION

This study demonstrated that the KDEL receptor and GRP78 regulate MANF retention in the SR/ER of cardiac myocytes and HeLa cells (Fig. 7). To the best of our knowledge, this is the first study to describe how two different retention mechanisms collaborate to determine the conditions under which an ER luminal protein is secreted.

Previous studies led to the hypothesis that MANF protects cells after its secretion (12, 13). However, the conditions under which MANF is secreted are not well understood. In the present study, we found that under most conditions, MANF was not secreted but was retained in cells (Fig. 7*A*). Moreover, we found that the strength of retention was reduced, and thus, MANF was secreted upon ER calcium depletion (Fig. 7*B*). A retention mechanism involving the interaction of MANF with the ER-resident chaperone, GRP78, was found to be calcium-dependent. The concentration of calcium required for dissociation of the MANF-GRP78 complex fell within the range of ER calcium concentrations in cells treated with agonists that mobilize ER calcium (49, 50), in neurons and cardiac myocytes subject to hypoxia (48), and in the ischemic brain and heart (57). Thus, calcium-dependent binding of MANF to GRP78 regulates its secretion in response to changes in ER calcium over the physiological concentration range. Under these conditions, extracellular MANF could function in an autocrine and/or paracrine capacity to protect cells from death in response to ER calcium depletion, as previously reported (13).

Although this mechanism for regulating secretion is unusual, there have been a few studies on the effects of ER calcium depletion on other ER proteins (57–62). However, in contrast to its effects on MANF, for the most part, those studies showed that ER calcium depletion increased retention in the ER. Additionally, some studies showed that the increased retention was due to enhanced association of unfolded proteins with macromolecular complexes that included GRP78 (63, 64). Consistent with those results are other reports showing that GRP78 overexpression increased the retention of several proteins in the ER (65–67).

GRP78 is also known to regulate protein function by determining protein location. For example, GRP78 binds to the ER transmembrane protein, activation of transcription factor 6 (ATF6). Although GRP78 does not assist in ATF6 folding, it regulates the activity of ATF6 by conditionally retaining it in the ER (5, 6, 8). In nonstressed cells, GRP78 anchors ATF6 in the ER, a location that does not allow its activation. However, upon ER stress, GRP78 dissociates from ATF6, the latter of which moves to the Golgi where proteases in this location cleave it, liberating a soluble, active form of ATF6 that translocates to the nucleus, where it induces ER stress response genes (68–72). The ability of GRP78 to retain MANF in the ER is analogous to its ability to retain ATF6 in the ER, suggesting that, like ATF6, MANF serves signaling roles during ER stress. However, in contrast to ATF6, MANF appears to be released only by ER stresses that deplete ER calcium, indicating that it has roles specific to ER calcium dysregulation. In further contrast to ATF6, when released MANF translocates to the Golgi, because it is an ER-luminal protein, it can either be secreted or bind to the KDEL receptor and be retrieved back to the ER, as we previously postulated (45).

The KDEL receptor and GRP78 comprise two separate MANF retention mechanisms. Our results indicate that the KDEL receptor-mediated MANF retention is not calcium-dependent, whereas the GRP78-mediated retention is calcium-dependent. Accordingly, the KDEL receptor works in conjunction with GRP78 to finely tune the amount of MANF secreted upon ER calcium depletion. Our studies using MANF-WT, -None, and -KDEL demonstrate this point, as follows: Although removal of the C-terminal RTDL from MANF reduced its retention and increased its secretion, the secretion of this form of MANF was activated further by thapsigargin. This result supports the hypothesis that the KDEL receptor is not responsible for calcium-dependent MANF retention. Additionally, we found that the RTDL of MANF served as a relatively weak retention motif. This was demonstrated when we compared the relative secretion rates of MANF-None, MANF-RTDL, and MANF-KDEL. Compared with MANF-None, the secretion of MANF-RTDL (MANF-WT) was reduced by only 30% (Fig. 3*B*, *bars 2* and *4*), whereas secretion of MANF-KDEL was decreased by more than 70% (Fig. 3*B*, *bars 2* and *6*). Thus, although the relatively weak retention property of RTDL modulates the amount of MANF released upon ER calcium depletion, the release of MANF from GRP78 is the essential determinant of whether MANF will be secreted. Thus, the quantity of MANF secreted is fine-tuned by KDEL receptor-mediated retention, the latter of which is determined by the retention efficiency of the RTDL of MANF. Because the C-terminal RTDL is a conserved structural feature of MANF across species, its relatively weak retention characteristics are evidently an important aspect of its function.

In summary, the present studies of MANF, which is known to serve protective roles from within cells, as well as after its secretion, have revealed a mechanism by which its retention can be finely tuned so that its secretion, via the constitutive classical secretory pathway, can be regulated. Furthermore, our findings imply that the secretion of other proteins made in the ER might be increased during stresses that impair the movement of most other secreted proteins from the ER to the Golgi (Fig. 1*B*, compare MANF with ANF; Fig. 8). Thus, secreted proteins that are induced as a result of SR/ER calcium depletion, such as MANF, must be resistant to the impaired ER to Golgi transport that affects other secreted proteins, suggesting that they can take a detour around the protein trafficking "roadblock" in the classical secretion pathway that can be created by the accumulation of misfolded proteins in the SR/ER.

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