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Functions for the cardiomyokine, MANF, in cardioprotection, hypertrophy and heart failure

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Abstract

We define cardiomyokines as heart-derived secreted proteins that affect cardiovascular function via autocrine, paracrine and/or endocrine mechanisms. The subject of this review is the cardiomyokine, mesencephalic astrocyte-derived neurotrophic factor (MANF). The expression of MANF is increased in the ischemic heart, in part, through activation of ER stress, a condition that drastically impairs the expression and secretion of most cardiomyokines. This novel function of MANF suggests that it may have important roles in the ER stressed, ischemic heart. Consistent with this are recent findings showing that MANF protects against ischemic damage, and that it is anti-hypertrophic. Accordingly, in light of its function as a potentially secreted cardiomyokine, MANF has translational potential as a novel therapy for ischemic heart disease. This article is part of a special issue entitled “Key Signaling Molecules in Hypertrophy and Heart Failure.

Keywords

Cardiomyokine; MANF; ER stress; Cardioprotection; Hypertrophy

1. Introduction

1.1. Cardiomyokines and ER stress

Secreted proteins, including hormones and cytokines, are critical for the development, growth and maintenance of all tissues and cells. Numerous proteins are secreted from various tissues, such as skeletal muscle; these proteins, such as the cardioprotective peptide, Fstl1 [1,2], are called myokines [3,4]. By analogy, we propose the term cardiomyokine (CMK) to describe heart-derived myokines that exert paracrine, autocrine and/or endocrine effects.

The rough endoplasmic reticulum (ER) is the location of the biosynthesis of proteins secreted via the classical or conventional secretory pathway [5,6], as are many CMKs. Conventionally-secreted proteins are inserted into the ER lumen co-translationally, and then, upon completion of their synthesis and folding in the lumen, they are transported to the Golgi on their way to secretory vesicles, from which they are released into the extracellular space [7]. Accordingly, a robust protein synthesis and folding environment in the rough ER [8], and perhaps the sarcoplasmic reticulum (SR) of myocardial cells, is crucial for efficient biosynthesis and secretion of most CMKs.

Certain myocardial injuries, such as infarction or ischemia, impair protein folding in the ER [9]. Although not yet shown in the heart, studies in other cell and tissue types have demonstrated that impaired protein folding in the ER during hypoxic or ischemic stress reduces secretion of adiponectin [10], apolipoprotein B100 [11] and procollagen [12,13]. Additionally, impaired ER-protein folding in hypoxic tumor cells inhibits Wnt secretion [14], and in a model of renal epithelial ischemia, mis-folding of secreted proteins decreases their secretion [15]. Thus, since myocardial ischemia impairs ER-protein folding, it is likely that ischemia also impacts the secretion of many CMKs. However, there is the potential that there is a group of CMKs whose secretion may not be reduced under such conditions, but may actually be preserved, or even enhanced by virtue of their induction during the ER stress response [16,17].

There are several features of the ER stress response, which is sometimes called the unfolded protein response, that are cell-survival oriented. One feature involves the degradation of mis-folded ER-proteins by ER-associated degradation, or ERAD [18]. If ERAD is not sufficient to remove mis-folded ER-proteins, the continued accumulation of mis-folded proteins in the ER leads to activation of a conserved signal transduction program, which at the outset is oriented toward resolving the stress, usually by restoring ER-protein folding capacity. However, if the stress is severe enough, later events in ER stress signaling trigger apoptosis [19].

In the healthy heart, ER-protein folding is efficient (Fig. 1A). However, upon ischemia, mis-folded proteins accumulate in the ER (Fig. 1B). These mis-folded proteins are detected by several ER-transmembrane proteins that serve central roles in sensing and initiating UPR signaling; activation of transcription factor 6, ATF6 is one such ER stress sensor that may be the most important ER stress sensor in mammals [20,21]. In all mammalian cells, ER stress stimulates ATF6 translocation from the ER to the Golgi, where it is cleaved by site 1 and site 2 proteases, which liberates a soluble N-terminal fragment called N-ATF6. N-ATF6, which is generated on the cytoplasmic face of the Golgi, translocates to the nucleus where it binds to several forms of ER stress response elements (ERSEs), and induces transcription of ATF6-dependent ER stress response genes (Fig. 1C).

In the cardiac context, ischemia induces ER stress, as well as ATF6 translocation from the ER to the nucleus, where it serves as a transcription factor, upregulating genes that protect cardiac myocytes from ischemic damage [22]. Using a novel line of transgenic mice that express activated ATF6 in the heart, it was shown that ATF6 reduces myocardial damage and enhances left ventricular function after I/R [23]. It is presumably through ATF6-dependent genes, some of which may encode known or novel CMKs, that this protective aspect of ER stress is mediated in the heart. However, until recently, there had been no identification of ATF6-dependent genes in the heart.

1.2. MANF, an ATF6-regulated gene in the heart

Microarray analyses of ATF6 transgenic mouse hearts identified numerous ATF6-regulated genes, including a group of genes that encode putative ATF6-dependent CMKs [24]. The proteins encoded by this group of genes are potentially unique, since they are induced, synthesized and folded in the ER during adverse conditions that actually inhibit the expression of other CMKs. Accordingly, since the synthesis and folding of ATF6-dependent CMKs are increased, even in the suboptimal environment of the stressed ER, we postulate that these ER stress-inducible CMKs may serve previously unappreciated roles under conditions that induce ER/SR stress, such as myocardial ischemia. One ER stress-inducible CMK gene that was identified in the ATF6 transgenic mouse hearts is arginine-rich mutated in early tumors, or ARMET, also named mesencephalic astrocyte-derived neurotrophic factor, or MANF (Fig. 1D).

When it was identified as an ATF6-inducible gene in the heart, the ARMET gene sequence was known, but the protein encoded by this gene had not been characterized [25]. In unrelated studies that were designed to identify neuronal growth factors in astrocyte-conditioned medium, a new dopaminergic neurotrophic factor was discovered. Based on its origin and function, it was named mesencephalic astrocyte-derived neurotrophic factor, or MANF. Sequence analysis of MANF suggested that it was encoded in an open reading frame in the ARMET gene. Cloning and sequencing of the MANF cDNA verified that the MANF mRNA is encoded on a 4.3 kb region of human chromosome 3 previously identified as the ARMET gene [26].

1.3. MANF structure

The MANF primary transcript encompasses 1109 bp, which codes for a predicted 179 amino acid protein (Fig. 2A). The N-terminal 21 amino acids serve as the signal sequence that targets the nascent protein to the ER/SR in cardiac myocytes. Sequencing of the mature MANF protein isolated from astrocyte-conditioned culture medium verified the absence of the putative signal sequence from the mature protein [26], consistent with its removal by signal peptidase while the nascent MANF protein is co-translationally imported through the ER membrane and into the ER lumen (Fig. 2B). Although many other secreted proteins undergo post-translational proteolytic modification before they are secreted, it is believed that the secreted form of MANF is the full length protein without the signal sequence, i.e. 158 amino acids, and that it has no transmembrane domains and four intra-molecular disulfide bonds [27] (Fig. 2B).

The MANF sequence is highly conserved across species. For example, human and fruit fly (*D. melanogaster*) MANF exhibit 54% identity. Although this conservation exists across a broad range of species, other than the recently-described, ciliary-derived neurotrophic factor (CDNF) [28–30], which exhibits a 59% amino acid identity with MANF, when comparing the full length protein to others, MANF shows little similarity with any other protein. This uniqueness makes it difficult to predict the mechanism of MANF action by analogy to previously reported examples. Accordingly, to date, most hypotheses concerning the mechanism of MANF function are based on domains that MANF shares with other proteins.

Crystallographic analyses have shown that the N-terminus of MANF adopts a configuration similar to saposin-like proteins [27], which are known to bind membrane and free lipids. In this regard, while the putative receptor for MANF has not yet been identified, it has been proposed that extracellular MANF may exert its function by binding to cell-surface membrane lipids [27]. The C-terminus of MANF has a C-X-X-C motif, which is common in thiol/disulfide oxidoreductases, also called protein disulfide isomerases (PDIs). ER-targeted PDIs catalyze the formation of intramolecular protein disulfide bonds in this organelle. ER-targeted PDIs are often induced during ER stress, where they restore protein folding and, in so doing, exert protective functions [31,32]. Thus, it has been proposed that in the ER lumen, intracellular MANF may serve a PDI-like function to facilitate disulfide bond formation of proteins folding in this organelle [30]. However, in refutation of that hypothesis is a study which showed that MANF does not exhibit oxidoreductase activity [33]. It has been proposed that extracellular MANF may, in part, exert its action by altering disulfide bond status of cell-surface proteins; via this post-translational modification, MANF could affect the function of intracellular signaling processes [30].

2. Animal and cell models of MANF expression and secretion

2.1. MANF expression in tissues and cells

Soon after its discovery in cultured astrocyte-conditioned medium, it was shown that MANF is expressed in several other cell and tissue types, and in some cases, expression was shown

to be relatively high, even in the absence of ER stress. For example, in the absence of any ER stress-inducing maneuvers, MANF expression was low in the brain and heart, but relatively high in the stomach, and skeletal muscle [33]. Additionally, in several cultured cell lines, including mouse 3T3 fibroblasts, HeLa cells and pancreatic β cells, basal expression of MANF was shown to be low, however, the expression increased considerably upon chemically-induced ER stress [28,33]. In part, this induction is achieved via an ER stress response element located in the 5'-flanking sequence of the MANF gene [33,34].

2.2. MANF function

Recent studies of MANF in the cardiac context suggest that it may play important roles in facilitating myocardial survival from ischemic injury, as well as modulating cardiac hypertrophy and, thus, heart failure. In addition to being induced in response to ATF6 activation in mouse hearts, *in vivo* [23,24], MANF is upregulated upon ER stress or ischemia in cultured cardiac myocytes, as well as in the infarcted mouse heart, *in vivo* [34]. These findings are consistent with studies showing that MANF was upregulated by hypoxia in cultured mouse embryonic fibroblasts [35], as well as in *in vivo* models of cerebral ischemia in rats [28,36].

In terms of function, it was shown that overexpressing MANF in cultured cardiac myocytes reduced death in response to ischemia, while knocking down endogenous MANF increased myocyte death. Moreover, when added to the medium of cultured cardiac myocytes in which MANF had been knocked down, recombinant MANF replicated the protective effects of endogenous MANF against ischemia/reperfusion-mediated cell death [34], implying protective roles for MANF as an ER stress-inducible secreted CMK. These findings are consistent with studies in other tissues where, for example, the infusion of MANF protected against cerebral ischemia in rats [28,37] and protected in an *in vivo* model of Parkinson's disease in rats [38]. In addition to exerting a cytoprotective effect, it is possible that MANF may affect cell growth. In one study it was shown that MANF inhibited cell proliferation [28]. In the post-natal heart, most cardiac myocytes are post-mitotic, and in response to growth stimuli, they exhibit increases in cell size, or hypertrophic growth. In preliminary studies from our lab, it was shown that MANF decreased hypertrophy induced by α_1 -adrenergic receptor agonist, phenylephrine (unpublished results). The mechanisms by which MANF exerts its cytoprotective and anti-hypertrophic functions remain to be elucidated. However, since MANF is protective when it is added to cultured cell medium, it is possible that it exerts its effects, at least partly through a cell-surface receptor, which is yet to be characterized.

2.3. Mechanism of ER stress-enhanced MANF secretion

Several reports have shown that under non-stressed conditions, MANF secretion is minimal, and a significant proportion is retained in the ER. For example, in unstressed U2OS cells, HEK293 cells and mouse 3T3 fibroblasts, MANF was localized to the ER and Golgi [28,33], while in unstressed cultured neonatal cardiac myocytes, immunostaining studies demonstrated MANF co-localization to the ER along with the well characterized ER-protein, glucose-regulated protein, 78 (GRP78) [34], which is also induced by ER stress. However, in all of these studies, ER stress stimulated MANF secretion, but not the secretion of other ER stress response gene products that reside in the ER. These findings suggest that in comparison to many other ER stress response genes, MANF expression is induced by ER stress, but in contrast, the secretion of MANF is also enhanced by ER stress.

ER stress-enhanced secretion of MANF could depend, at least partly, on the four amino acids at its C-terminus, RTDL. Many ER-resident proteins have a C-terminal KDEL motif that facilitates high affinity binding to the KDEL-receptor (KDEL-R) [39], which is

localized primarily in the *cis*-Golgi localized [40] (Fig. 3A, Step 1). Proteins, like GRP78, with C-terminal KDEL motifs are retrieved from the Golgi and relocated back to the ER, and via this cycling, they are efficiently retained in the ER and not secreted (Fig. 3A, Step 2). Recent studies have shown that in addition to the canonical KDEL sequence, proteins with variations of this sequence can also bind to the KDEL-R, but in some cases, binding is weaker than that of KDEL [41]; one of the sequences shown in that study to bind more weakly than KDEL is RTDL (Fig 3A, Step 3). Thus, it is possible that under basal, unstressed conditions, low expression of MANF, and other KDEL-R-binding proteins fosters complete retrieval of all of them to the ER (Fig. 3A, Steps 2 and 4). However, upon ER stress, levels of ER stress response gene products that have C-terminal KDEL (e.g. GRP78) and RTDL (e.g. MANF) motifs increase, while KDEL-R levels do not change [42]. Due to their different affinities for the KDEL-R, proteins with KDEL motifs compete better for binding to the KDEL-R than proteins with RTDL motifs (Fig. 3B, Step 1). Thus, when the KDEL-R recycles ligands back to the ER, KDEL proteins are efficiently retained in the ER (Fig. 3B, Step 2), while proteins with RTDL are not efficiently retained (Fig. 3B, Step 3). In the case of MANF, inefficient retention in the ER during ER stress fosters its secretion (Fig. 3B, Step 4), allowing it to bind to a putative, cell-surface receptor on cardiac myocytes, to exert autocrine effects, as well as other cell types, to exert paracrine or endocrine effects (Fig. 3B, Step 5).

3. Translational potential

Although there are many studies yet to be carried out, based on our current understanding of MANF structure and function, one would predict it to have therapeutic potential. Due to its potentially broad spectrum of action, a MANF-based therapeutic is predicted to be of high impact. All studies carried out to date that have examined the effects of MANF have demonstrated that it is protective in culture and *in vivo*. Moreover, this protection has been seen in multiple cell and tissue types, including the ischemic heart and brain, as well as in animal models of neurodegenerative disorders. Additionally, since MANF overexpression decreases proliferation of cultured cancer cells, and decreases cardiomyocyte hypertrophy, a MANF-based cancer therapy is conceivable. However, tempering these exciting possibilities is the fact that it is not known whether the antigrowth effects of MANF are exerted through intra- or extracellular mechanisms.

Finally, MANF is only one of the many putative ER stress-inducible CMKs in the heart [24]. Accordingly, if the whole CMK family is considered, the likelihood of finding additional proteins that exhibit potential as therapeutics for heart failure increases substantially. Future studies that assess the identities, functions and mechanisms of other ER stress-inducible CMKs will be necessary to reveal the full therapeutic potential of this category of cytokines that are induced and secreted from the heart during harsh stresses, such as ischemia, that greatly impair the synthesis of almost every other secreted protein.

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Fig. 1.

Ischemia-mediated activation of ER stress in the heart. Panel A — Protein synthesis and folding are efficient in the healthy heart. Panel B — In response to injuries, e.g. ischemia, protein synthesis and folding in the ER/SR of cardiomyocytes are impaired, causing ER stress. Panel C — ER stress leads to the generation of active, N-ATF6, which translocates to the nucleus and activates ER stress response genes. Panel D — MANF is an ATF6-inducible ER stress response gene in the heart that exerts protective, as well as anti-hypertrophic effects.

A. MANF Gene, mRNA and Protein Sequence

MANF Gene (4.3 kb)
 ↓
 MANF mRNA (1109 bp); cDNA (705 bp)
 ↓
 Pre-MANF 179AA (21AA signal sequence)
 Mature MANF 158AA

-21 -1 1
 MWATQGLAVA LALSVLPGSR ALRPGDCEVC
 ISYLGRFYQD LKDRDVTFSF ATIENELIKF
 CREARGKENR LCYYIGATDD AATKIINEVS
 KPLAHHIPVE KICEKLKKKD SQICELKYDK
 QIDLSTVDLK KLRVKELKKI LDDWGETCKG
 CAEKSDYIRK INELMPKYAP KASSARTDL¹⁵⁸

B. Diagram of MANF Structure

Fig. 2.

Structure of the MANF gene, mRNA and protein. Panel A — Shown are the MANF gene, mRNA and 179 amino acid pre-MANF. The N-terminal 21 amino acid signal sequence (red) targets nascent MANF to the rough ER. Conserved cysteines are highlighted in yellow and the C-terminal KDEL-like sequence, RTDL, is shown in blue. Panel B — The locations of the conserved cysteines and hypothetical intramolecular disulfide bonds are shown, as well as the saposin-like and C-terminal domains of MANF.

A. Non-stressed: No MANF Secretion

B. ER Stress: MANF Secretion

Fig. 3. Hypothetical mechanism for the ER stress-enhanced secretion. Panel A — Under non-stressed conditions, ER-resident proteins with canonical and, in some cases, non-canonical KDEL sequences, such as MANF's RTDL, can bind to KDEL-receptors, facilitating efficient retention in the ER. Panel B — During ER stress, the differential affinities of canonical and non-canonical KDEL sequences for the KDEL-receptor lead to secretion of those proteins that bind to the KDEL-receptor more weakly than KDEL.