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Myocyte stress 1 plays an important role in cellular hypertrophy and protection against apoptosis

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ABSTRACT

Myocyte stress 1 (MS1) is a recently described striated muscle actin-binding protein that is up-regulated in the early stages of pressure overload left ventricular hypertrophy. The aim of this study was to determine whether MS1 induces cellular hypertrophy and protects against apoptosis. Over-expressed MS1 co-localized with actin in H9c2 cells and altered expression of genes of the myocardin-related transcription factor (MRTF)/serum response factor (SRF) transcriptional pathways and in addition the apoptosis repressor with caspase recruitment domain (*Nol3*) gene. The size of cells over-expressing MS1 was significantly increased by 55% and over-expression of MS1 dramatically inhibited staurosporine-induced apoptosis by 89%. These findings suggest the involvement of MS1 in cellular hypertrophy and protection against apoptosis.

1. Introduction

Left ventricular hypertrophy (LVH) is an adaptation of the heart in response to an increased workload, stress or injury [1]. Initially this adaptation of the heart is beneficial but sustained cardiac hypertrophy is associated with decompensation and heart failure, partly due to cell death caused by apoptosis [2]. Many signaling pathways have been shown to regulate hypertrophy but the molecular controllers that sense pressure overload and initiate hypertrophy are unclear. To better understand the initial molecular mechanisms that lead to pressure-induced LVH, we have identified and characterized a novel gene, designated myocyte stress 1 [MS1; also known as STARS (striated muscle activator of Rho signaling) and ABRA (actin-binding Rho activating protein)], which is up-regulated within 1-h in the left ventricle following aortic banding in the rat with a peak expression well before any detectable increase in LV mass [3,4]. This suggests a possible role for MS1 in the initial signaling of the hypertrophic response. *Ms1* is also expressed during embryonic cardiac development, is restricted to striated muscle and is transiently up-regulated during ischemia-reperfusion in vitro [4,5], which suggests that MS1 may play a more widespread role in cardiac development and physiology.

The mouse homolog of *ms1* termed STARS was shown to be a sarcomeric actin-binding protein which stimulates serum response factor (SRF)-dependent transcription by inducing the nuclear accumulation of the SRF co-factors, myocardin-related transcription factors (MRTFs) MRTF-A and MRTF-B through a mechanism dependent on RhoA and actin polymerization [5,6]. Experiments using a cell line that expresses dominant negative MRTF-A identified genes that were MRTF-dependent where some were known SRF target genes [7]. Many of the MRTF-dependent genes have been associated with cardiac hypertrophy [8,9] and cell survival [10,11]; therefore, it is conceivable that these MRTF-dependent genes are putative targets of MS1. Here, we have investigated the role of MS1 in cellular hypertrophy and survival and report that MS1 over-expression increases cell size and protects against apoptosis in vitro.

Abbreviations: LVH, Left ventricular hypertrophy; MS1, myocyte stress 1; STARS, striated muscle activator of Rho signaling; ABRA, actin-binding Rho activating protein; SRF, serum response factor; MRTF, myocardin-related transcription factor; PBS, phosphate-buffered saline; FITC, fluorescein isothiocyanate; BSA, bovine serum albumin; NOL3, apoptosis repressor with caspase recruitment domain; LIF, leukemia inhibitory factor; IL-6, interleukin-6; fra-1, fos-related antigen-1; BNP, brain natriuretic peptide

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2. Materials and methods

2.1. Cell size and cell proliferation measurements

Following transfection for 72 h, H9c2 cells were trypsinized, washed with phosphate-buffered saline (PBS) and then cells were re-suspended in 1 mL PBSA (PBS containing 0.03% (w/v) sodium azide and 0.2% (w/v) bovine serum albumin (BSA)). Viable cells were counted and adjusted to $\sim 1 \times 10^5$ cells/100 μl PBSA. Cells were then fixed and permeabilized using a Leucoperm kit (Serotec). During permeabilization mouse anti c-Myc fluorescein isothiocyanate (FITC) antibody (Serotec) was added at 1:10 dilution in PBS and left for 30 min at room temperature in the dark. The c-Myc FITC fluorescence of individual transfected cells and the size of cells (forward scatter) were measured using a Beckman Coulter Epics XL·MCL flow cytometer and System II software.

Cell proliferation was examined 72 h after transfection by direct cell counting and flow cytometry (see Supplementary data for details).

2.2. Measurement of apoptosis (DNA fragmentation)

Initial studies looked at the effects of staurosporine on H9c2 cells. Cells were treated with various concentrations of staurosporine (5–25 nM) for 24 h and stained with Vybrant DyeCycle Violet Stain (Invitrogen) according to the manufacturer's protocol. The percentage of apoptotic cells (sub-G_1) were measured using a DakoCytomation CyAn ADP flow cytometer and analyzed using the Summit v4.3 software.

Following transfection for 24 h, H9c2 cells were treated with 5 nM staurosporine (Sigma) for 24 h. Cells were then collected, labeled with mouse anti c-Myc FITC antibody to detect transfected cells and stained with the Vybrant DyeCycle Violet stain (Invitrogen) according to the manufacturer's protocol.

Methods for Myc-MS1 expression plasmid, Cell culture and transfection, Immunofluorescence microscopy, real-time and semi-quantitative RT-PCR, and Western blotting, see Supplementary data for details.

2.3. Statistical analysis

Differences between sets of data were compared using Student's *t*-test. A *P*-value < 0.05 was considered to be statistically significant.

3. Results

3.1. Altered expression of hypertrophic and cell survival genes in H9c2 cells over-expressing MS1

The MS1 expression vector over-expressed *ms1* mRNA and MS1 protein in H9c2 cells and in cells over-expressing MS1 the MS1 signal co-localized with actin (see Supplementary data for details).

Over-expression of MS1 significantly induced the known MRTF-dependent genes leukemia inhibitory factor (*LIF*) and interleukin-6 (IL-6) and the known MRTF-SRF target genes adrenomedullin, jun-B, and *fra-1* (Fig. 1). *LIF* and IL-6 were up-regulated by 2.5-fold ($P < 0.01$) and 5.9-fold ($P < 0.001$), respectively. A significant induction of 1.6-fold was observed for adrenomedullin ($P < 0.05$). Jun-B and *fra-1* were increased 2.3-fold ($P < 0.01$) and 2.1-fold ($P < 0.01$), respectively. In addition, genes that have been associated with cardiac hypertrophy such as cardiac α-actin and *BNP* were also up-regulated in H9c2 cells over-expressing MS1 by 2.3-fold ($P < 0.01$) and 4.5-fold ($P < 0.01$), respectively (Fig. 1).

It is well established that apoptosis accounts for cardiac cell death during ischemia/reoxygenation, hypertension, and in maladaptive hypertrophy [12], disease states which induces cardiac *ms1* expression. The BCL-2 family proteins play a central role in regulating apoptosis in the heart [2,13], therefore we measured the expression of two pro-survival factors (*Bcl-2* and *Bcl-x_L*) in H9c2 cells over-expressed with MS1 but found no change in their mRNA expression (data not shown). Another factor of interest is *Nol3* (Apoptosis repressor with caspase recruitment domain), an apoptosis repressor gene expressed predominantly in striated muscle [14] and has been shown to inhibit staurosporine-induced apoptosis [15]. Over-expression of MS1 increased *Nol3* mRNA abundance in H9c2 cells by 2.2-fold ($P < 0.05$).

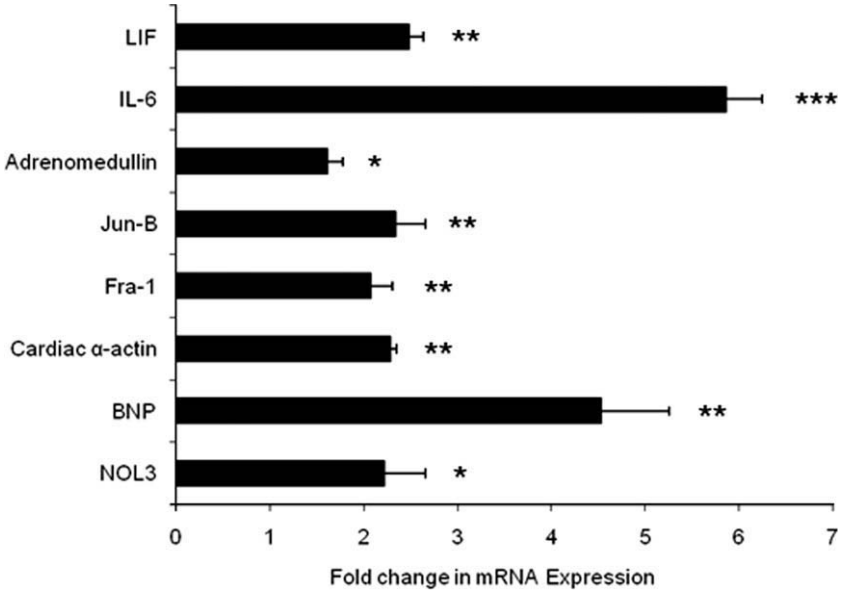

Fig. 1. Changes in gene expression following myocyte stress 1 (MS1) over-expression. Transcript levels for each gene were quantified by real-time RT-PCR following transfection for 24 h except for brain natriuretic peptide (BNP) and cardiac α-actin which were measured by semi-quantitative RT-PCR 72 h after transfection. Each gene was normalized to an internal control and the fold change in abundance is presented relative to empty vector control. Bars show mean ± S.E.M from 6 separate experiments except for BNP, cardiac α-actin and NOL3 ($n = 3$). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus empty vector control.

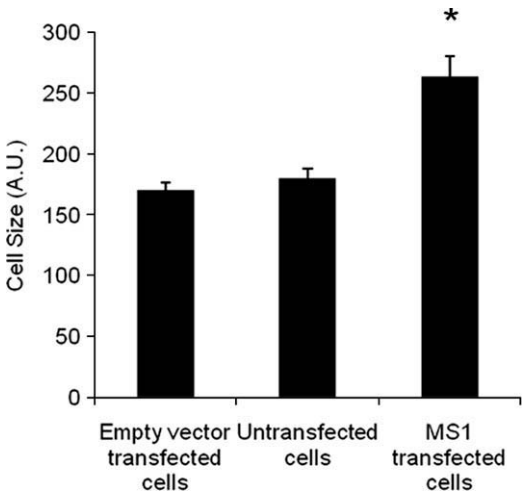

Fig. 2. Over-expression of MS1 in vitro results in cellular hypertrophy. H9c2 cells were transfected with a Myc-MS1 expression plasmid or empty vector control and stained with c-Myc FITC antibody to detect transfected cells. The median cell size (forward angle light scatter) expressed in arbitrary units of MS1 transfected cells versus empty vector transfected cells and untransfected cells (cells that remained untransfected following transfection of the Myc-MS1 expression plasmid) were quantified by flow cytometry. Bars show mean ± S.D. from 4 separate experiments. $^{*}P < 0.01$.

3.2. MS1-over-expression in vitro results in cellular hypertrophy

Within the population of cells transfected with the Myc-MS1 expression plasmid, on average 13.1 ± 0.5% of cells over-expressed MS1. In each experiment 10 000 cells were analysed by flow cytometry and therefore ~1300 quantified cells over-expressed MS1. The size of cells over-expressing MS1 was significantly increased by 46.6 ± 6.7% ($P < 0.01$) when compared to the untransfected portion of cells (Fig. 2). Similarly, they were significantly larger by 54.5 ± 8.5% ($P < 0.01$) then cells exposed to empty vector control.

Neither the transfection procedure nor the over-expression of MS1 affected cell proliferation. This was measured by direct cell counting and flow cytometry, which showed no difference in the proportion of cells over-expressing MS1 in either S phase or the G_2/M phase of the cell cycle when compared to the untransfected portion of cells or cells exposed to empty vector control (data not shown).

3.3. MS1-over-expression in vitro protects against staurosporine-induced apoptotic cell death

Within the population of cells exposed to the Myc-MS1 expression plasmid, 1.6 ± 0.5% of the untransfected portion of cells showed evidence of apoptosis under basal conditions. This proportion was similar (2.1 ± 0.7%) in cells exposed to empty vector. However, in MS1 expressing cells, the proportion of apoptotic cells was significantly lower (0.7 ± 0.3%) compared to the untransfected portion of cells or cells exposed to empty vector ($P < 0.05$) (Fig. 3). Treatment with a sub-maximal concentration of 5 nM staurosporine for 24 h (Supplementary data) markedly increased the percentage of apoptotic untransfected portion of cells exposed to the Myc-MS1 plasmid to 29.0 ± 2.9% ($P < 0.001$ compared with basal level). This level of increase was similar in cells exposed to empty vector (32.4 ± 3.8%, $P = 0.23$). However, in MS1 transfected cells, while there was a significant increase in apoptotic cells after 5 nM staurosporine for 24 h (3.5 ± 1.0%, $P < 0.05$ compared with basal level), the increase was markedly attenuated ($P < 0.001$) compared with the empty vector exposed cells or the untransfected cells. The attenuation in apoptosis compared with the empty vector exposed cells was ~89%. These results suggest that MS1 over-expression affects basal apoptosis and largely prevents staurosporine-induced apoptotic cell death.

4. Discussion

Previous studies have shown that cardiac *ms1* is over-expressed during the development of LVH in experimental animal models [4,16]. More recently, *ms1* expression has also been shown to parallel the development and regression of skeletal muscle hypertrophy in humans [17]. Consistent with these observations, our present in vitro finding that increased MS1 levels results in an increase in cell size, provides further evidence of its role in striated muscle hypertrophy. Interestingly, we found no evidence that increased MS1 levels affected cell proliferation suggesting that its effects are restricted to causing hypertrophy rather than hyperplasia.

Here, we confirmed that MS1 associates with the actin cytoskeleton and causes the over-expression of hypertrophic and cardio-

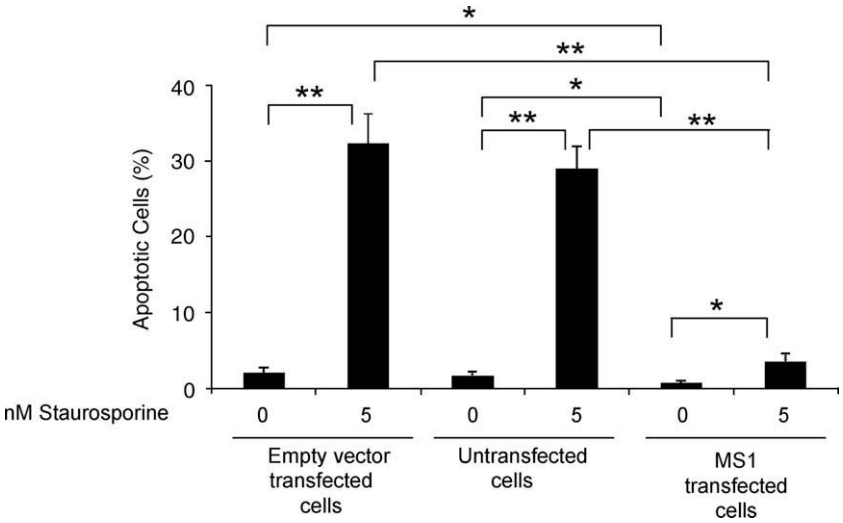

Fig. 3. MS1 over-expression inhibits apoptosis induced by staurosporine in H9c2 cells. H9c2 cells were transfected (24 h) with a Myc-MS1 expression plasmid or empty vector control and left untreated or treated with 5 nM staurosporine for 24 h, stained with c-Myc FITC antibody and Vybrant DyeCycle Violet Stain to detect transfected and apoptotic cells (sub-G_1 phase), respectively, by flow cytometry. Shown are the percentage of apoptotic MS1 transfected cells versus empty vector transfected cells and the untransfected portion of cells exposed to the Myc-MS1 plasmid. The results are the mean ± S.D. from 4 independent experiments. $^{*}P < 0.05$, $^{**}P < 0.001$.

protective genes that were identified to be MRTF-dependent genes [7]. The structural gene cardiac α-actin has also been identified as MRTF-dependent [18]. Four (cardiac α-actin, jun-B, fos-related antigen-1 (fra-1) and adrenomedullin) out of these six MRTF-dependent genes contains one or more conserved SRF binding sites which have been experimentally validated as SRF target genes [7,19–23], suggesting that MS1 causes the over-expression of several genes of the MRTF/SRF signaling pathways. In addition, MS1 increased the expression of the hypertrophic marker brain natriuretic peptide (BNP), which is known to be a direct target of SRF [24]. Of particular interest, *ms1* itself has a conserved SRF binding site, which can be bound by SRF in vivo (Ounzain and Chong, unpublished), raising the possibility that *ms1* is a SRF target, thus creating a previously unrecognised feed-forward mechanism.

Perhaps our most notable finding is the demonstration that MS1 markedly attenuated staurosporine-induced apoptosis of H9c2 cells. The specific mechanism whereby MS1 protects against staurosporine-induced apoptosis remains to be elucidated but it may involve the expression of Bcl family and related genes [14,15,25]. Specifically we have shown that MS1 up-regulated *Nol3*, a known cardioprotective gene [14,25–27]. Interestingly, apoptosis repressor with caspase recruitment domain (NOL3) has been shown to inhibit staurosporine-induced apoptosis [15] therefore, it is reasonable to suggest that MS1 may prevent staurosporine-induced apoptosis via NOL3.

The technique we employed for assessing the effect of transient MS1 over-expression on apoptosis has been widely used and validated against other assays of apoptosis [28–30]. Also, we observed a marked protective effect of MS1 against staurosporine-induced apoptosis making it highly likely that the effect is genuine. Nonetheless, further studies using different assays are necessary to confirm the current findings.

In summary, we have demonstrated for the first time the involvement of MS1 in cellular hypertrophy and protection possibly via a MRTF/SRF and/or NOL3 signaling mechanism. Although these findings require confirmation in vivo, results presented here provide support that MS1 plays a central role in the MRTF/SRF signaling pathway, with important cellular consequences. Collectively, these findings support further studies to explore the beneficial effects of the MS1-MRTF-SRF and NOL3 pathway as a therapeutic target for the treatment of cardiovascular disease.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2009.08.011.

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