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Protection of vascular smooth muscle cells by over-expressed methionine sulphoxide reductase A: Role of intracellular localization and substrate availability

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Abstract
Methionine sulphoxide reductase A (MSRA) that reduces methionine-S-sulphoxide back to methionine constitutes a catalytic antioxidant mechanism to prevent oxidative damage at multiple sub-cellular loci. This study examined the relative importance of protection of the cytoplasm and mitochondria by MSRA using A-10 vascular smooth muscle cells, a cell type that requires a low level of reactive oxygen species (ROS) for normal function but is readily damaged by higher concentrations of ROS. Adenoviral over-expression of human MSRA variants, targeted to either mitochondria or the cytoplasm, did not change basal viability of non-stressed cells. Oxidative stress caused by treatment with the methionine-preferring oxidizing reagent chloramine-T decreased cell viability in a concentration-dependent manner. Cytoplasmic MSRA preserved cell viability more effectively than mitochondrial MSRA and co-application of S-methyl-L-cysteine, an amino acid that acts as a substrate for MSRA when oxidized, further increased the extent of protection. This suggests an important role for an MSRA catalytic antioxidant cycle for protection of the cytoplasmic compartment against oxidative damage.

Keywords: *Vascular smooth muscle cells, methionine sulphoxide reductases, ROS, protein oxidation, oxidative stress, vascular diseases*

Abbreviations: *VSMCs, vascular smooth muscle cells; Met-S-O, methionine-S-sulphoxide; ROS, reactive oxygen species; MSRA, methionine sulphoxide reductase A; ChT, chloramine-T.*

Introduction

Oxidative stress to vascular cells is a major contributing factor for the pathogenesis of cardiovascular diseases including hypertension and atherosclerosis. In the atherogenic vessel, endothelial cells and vascular smooth muscle cells (VSMCs) are easily damaged by excessive reactive species such as reactive oxygen species (ROS), often leading to their functional impairment and apoptosis [1–3]. However, because numerous signalling cascades involved in the regulation of VSMC differentiation, proliferation and migration require intracellular ROS as vital second messengers [4–8], exuberant elimination of ROS

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inhibits VSMC proliferation and causes apoptotic death [9–13]. Thus, unintentional and deleterious effects on the VSMC function may result from antioxidant-based therapies designed to protect VSMCs by diminishing the ROS level.

Methionine sulphoxide reductase A (MSRA), which catalyses the reduction of methionine-S-sulphoxide (Met-S-O) back to methionine [14,15], participates in the cellular antioxidant mechanism [16] by restoring functionally critical methionine residues in proteins, such as in calcium/calmodulin (Ca^{2+}/CaM)-dependent protein kinase II (CaMKII) [17]. Additionally, MSRA may promote a more reduced cellular redox-state by cyclic reduction of free and/or protein-bound oxidized methionine that is not essential for protein function. According to this MSR-mediated catalytic antioxidant mechanism [18,19], methionine acts as a 'sink' for ROS and thereby protects other cellular molecules against oxidative damage.

The *msrA* transcript is alternatively spliced to produce multiple variants that differ in their N-terminus, resulting in differential sub-cellular localization patterns [20–24]. The presence of such MSRA forms in heart and aortic tissue extracts indicates that the enzyme has an important function in the cardiovascular system [24,25]. Recently we have reported that the enzyme MSRA is endogenously expressed in mitochondria of A-10 rat VSMCs [24]. In this study, we tested the hypothesis that over-expression of human MSRA protects VSMCs against oxidative stress without interfering with ROS-dependent signalling essential for normal VSMC survival. To address the role of intracellular localization, we over-expressed MSRAs specifically targeted to either mitochondria or the cytoplasm. The results show that over-expression of MSRA protects VSMCs against oxidative stress and that cytoplasmic MSRA is surprisingly more effective than the mitochondrial form. Concurrent treatment with cytoplasmic MSRA and a substrate S-methyl-L-cysteine (SMLC) [19] further increases the extent of cell protection, consistent with the importance of the MSRA-catalytic antioxidant mechanism.

Materials and methods

Cell culture

The vascular smooth muscle cell line A-10 (rat thoracic aorta) [26] was obtained from American Type Culture Collection (ATCC, Manassas, VA) and cultured in DMEM medium supplemented with 10% foetal bovine serum at 37°C and 5% CO_2. Unless indicated, cells between passage 10 and 30 were used for cell viability experiments.

Over-expression of human MSRA enzymes

Constructs. The coding sequences of human MSRA and $MSRA^{\Delta N}$ (deletion of Met1-Arg22) were cloned into the viral expression vector pacAd5CMV using the restriction sites *Xho*I and *Not*I [27]. Control Ad5CMV plasmids encoded for EGFP. Coding sequences were verified by sequencing (University of Pennsylvania, School of Medicine, DNA Sequencing Facility) and recombinant virus particles were generated at the Gene Transfer Vector Core of the University of Iowa.

Transient over-expression. VSMCs were plated into 96-microwell plates at a density of 5000 cells per well. After 12 h, cells were infected with virus particles applied at a multiplicity of infection (MOI) rate of 400 in medium containing 2% serum and infection medium was replaced by medium containing full serum 12 h later. Uniform infection rates were confirmed by comparing the infection rates after immunocytochemical detection of the over-expressed proteins.

Confirmation of MSRA over-expression in VSMCs. Expression was confirmed for each construct by semi-quantitative RT-PCR amplification of the complete open reading frame (Titanium One-Step RT-PCR kit, Clontech Laboratories, Inc., Mountain View, CA) and Western blot detection using an antibody directed against human MSRA [28].

For immunocytochemical detection, cells were plated on glass coverslips and infected with MSRA virus particles. Mitochondria were stained with CM-H_2Xros MitoTracker Red (Molecular Probes, Eugene, OR) followed by immunostaining with the aforementioned MSRA antibody at 1:2000 dilution. For staining of nuclei, specimens were mounted in UltraCruz™ mounting medium (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) containing 4′,6-diamidino-2-phenylindole (DAPI). Photographs were taken with a Nikon Eclipse fluorescence microscope at the Biomedical Imaging Core of the University of Pennsylvania.

Apoptosis assay. A-10 cells, plated on glass coverslips and infected with MSR virus particles at MOI 400, were assayed after 48 h for apoptosis using terminal deoxynucleotidyl transferase-mediated dUTP biotin nick-end labelling (TUNEL) assay and Annexin-V-FLUOS staining kit (Roche Diagnostics Corp., Indianapolis, IN), followed by immunocytochemical detection of individual MSR over-expressing cells as described above. For controls, cells were incubated for 15 h in medium supplemented with 5 mM vitamin C, adjusted to pH 7.4.

S-methyl-L-cysteine (SMLC) treatment. Control and $MSRA^{\Delta N}$ expressing cells were incubated with

medium containing sterile filtered SMLC (Sigma-Aldrich, Inc., St. Louis, MO) at a concentration of 50 mM. After 24 h, cells were thoroughly washed and exposed to oxidative stress.

Oxidant treatment and cell viability assay

To induce oxidative stress, cells were incubated in a freshly prepared medium containing the oxidant chloramine-T (ChT; Sigma-Aldrich). The indicated concentrations refer to the initial concentrations applied for 15 h. For a time course study, ChT was applied for 15, 30 and 45 h. MTT cell proliferation assay (ATCC, Manassas, VA) was used to monitor cell viability according to the manufacturer's protocol and absorbance was measured at 540 nm using a microplate reader (Thermo Electron Corp., Vantaa, Finland). Background was determined by measuring absorbance of cell-free medium containing the appropriate oxidant concentrations.

Statistical analysis

In each cell viability experiment, six microwells were analysed and all experiments were repeated three times to confirm reproducibility. The results presented (mean $\pm$ SEM) are those from one representative experiment. One-way analysis of variance (ANOVA) followed by Bonferroni post-hoc test was used to determine statistical differences between group means. Differences were considered statistically significant when $p \leq 0.05$.

Results

Oxidative stress induces apoptosis of VSMCs

To induce oxidative stress, we applied the oxidant chloramine-T (ChT). Chloramines are physiologically generated through the reactions involving the enzyme myeloperoxidase and implicated in cardiovascular and inflammatory diseases [29,30]. ChT damages proteins by preferentially oxidizing methionine to Met-S-O and Met-R-O [31,32]. In order to assess the biological activity of ChT when supplemented to the cell culture medium, we measured how quickly ChT removes inactivation of a ROS-sensitive voltage-gated sodium channel (Supplementary Methods). Based on such experiments we determined that a concentration of 2 mM ChT in culture medium for 1 h has a potency to modify the ROS-sensor equivalent to $\sim$12 μM ChT; after 24 h incubation in medium at 37°C the apparent activity has even dropped to below 1 μM (supplementary Figure 1). Thus, in the following experiments we supplemented the medium with up to 2 mM ChT to yield physiologically relevant low levels of ChT activity. Treatment with ChT decreased cell viability of A-10 VSMCs in a concentration-dependent manner (Figure 1A). The cell-killing effect of ChT also depended on the treatment duration (Figure 1B); extended exposures to 1.4 mM ChT for 30 and 45 h led to significantly lower viability values ($45.5 \pm 2.7\%$, $18.1 \pm 1.3\%$) compared to a 15-h treatment ($58.6 \pm 1.5\%$) ($p < 0.05$).

In mouse aortic SMCs oxidative damage accumulates during ageing, caused by an increase in generation of ROS and/or a decline in the cellular antioxidant mechanism [33], suggesting that aged VSMCs may be more vulnerable to ROS-induced damage than younger cells. We tested this possibility using early (25–35) and late (75–85) A-10 passage cell populations, as performed in other studies of cellular ageing [34,35]. We observed a significantly lower viability for the aged A-10 cell population ($41.1 \pm 2.8\%$), compared to the younger cell group ($79.0 \pm 1.1\%$; $p < 0.05$) (Figure 1C).

Treatment of A-10 cells with ChT caused a dramatic change in cell morphology. After exposure to ChT at concentrations higher than 1.0 mM, the cells lost their normal adherent and flattened appearance and developed a spherical shape (Figure 1D). Furthermore, coeval treatment with ChT had a more pronounced effect on the cell morphology in the aged than in the younger cell population (Figure 1D). ChT-induced cytotoxicity was caused by apoptosis, as confirmed by Annexin V and TUNEL labelling (Figure 1E).

Over-expression of MSRA variants does not affect normal VSMC viability

To study the effect of enhanced MSRA enzymatic activity on VSMC viability, we over-expressed the enzyme in a sub-cellular location-specific manner. As shown previously in multiple studies [20,24,36], full-length MSRA was preferentially localized in mitochondria by incorporating the MSRA amino-terminal mitochondrial targeting sequence. The absence of the targeting sequence in $MSRA^{\Delta N}$ directed the enzyme primarily to the cytoplasm [28].

As confirmed by an enzymatic activity assay [37], MSRA (15.4 pmol/μg) and $MSRA^{\Delta N}$ (15.2 pmol/μg) showed comparable reductase activities after plasmid transfection into HEK tsA cells (EGFP control: 0.2 pmol/μg) (Figure 2A). Adenoviral over-expression of *msrA* and $msrA^{\Delta N}$ transcripts in A-10 cells was confirmed by RT-PCR amplification (Figure 2B) and robust protein expression was detected by Western blotting: single bands for MSRA and $MSRA^{\Delta N}$, respectively, were detected in lysates of the cells treated with the virus particles (Figure 2C), but no signal was detected in EGFP expressing control samples (Figure 2C, left lane). Comparable infection rates for MSRA and $MSRA^{\Delta N}$ were verified by immunocytochemical detection, confirming almost confluent over-expression of the two constructs in A-10 VSMCs (Figure 2D). Mitochondrial and cytosolic

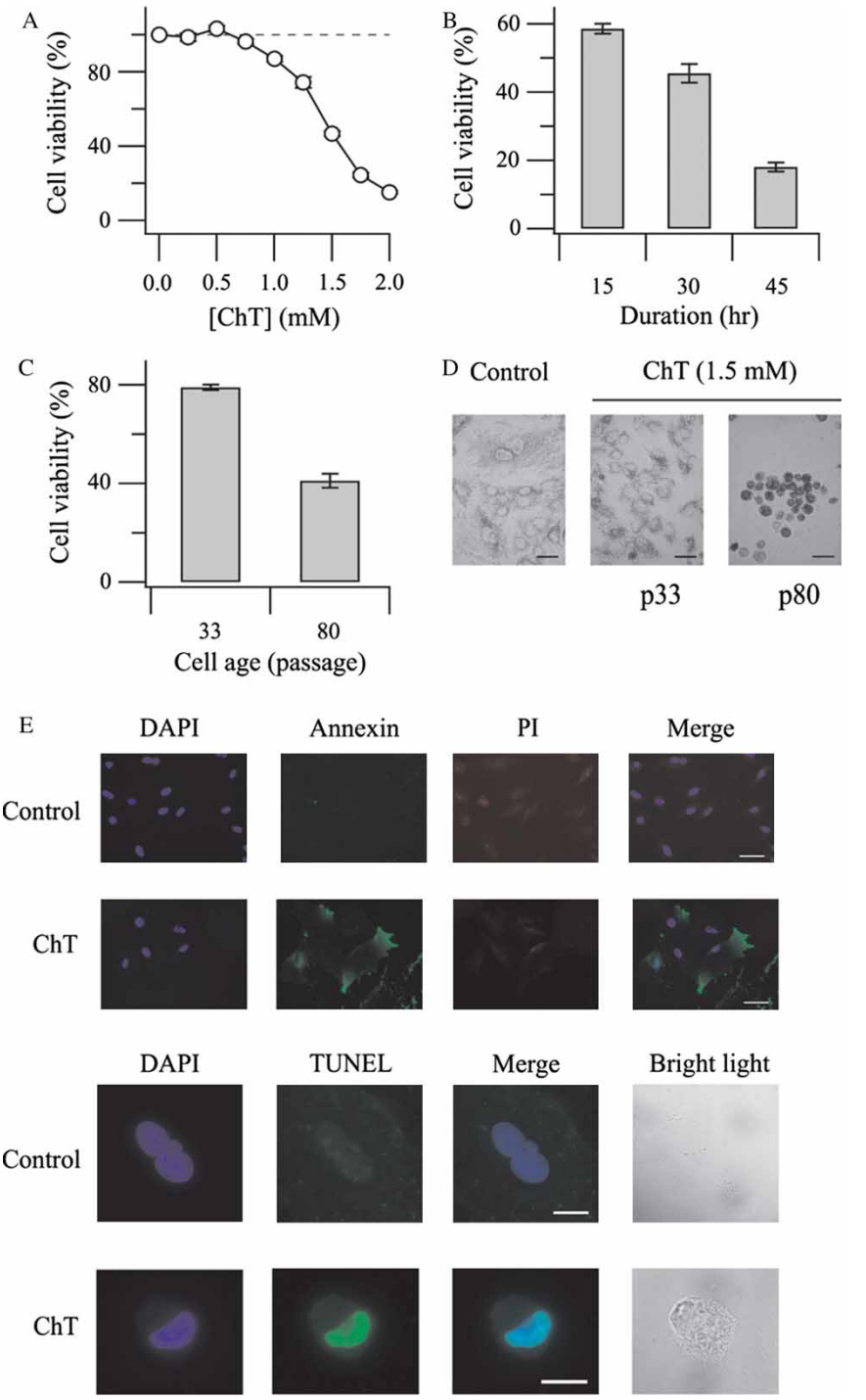

Figure 1. Effect of ChT on A-10 VSMC viability. (A) Treatment with the oxidant ChT for 15 h diminishes A-10 cell viability, measured by MTT assay, in a concentration-dependent manner. Error bars are in part smaller than symbol sizes. (B) Time course of decline in the cell viability caused by treatment with ChT (1.4 mM) for 15, 30 and 45 h. (C) Decline in viability of A-10 cells cultivated for 33 and 80 passages after treatment with ChT (1.5 mM). (D) Representative images of control A-10 cells and cells treated with ChT (1.5 mM), which causes round shaped cell phenotype. Morphological transformation is much less noticeable in the younger cell population (passage 33) than in the aged cells (passage 80). Cells were photographed after uptake of 3-(4,5-dimethylthiazolyl-2)-2,5-diphenlytetrazolium bromide (tetrazolium MTT; Sigma); scale: 50 μm. (E) Detection of apoptosis by Annexin V (top panel; scale: 100 μm) and TUNEL (bottom panel; scale: 25 μm) labelling in control (top row) and ChT-treated (1.5 mM; bottom row) A-10 VSMCs; blue, DAPI stained nuclei.

targeting of the over-expressed constructs was verified by immunocytochemical localization, where MSRA co-localized well with the mitochondrial dye MitoTracker red (Figure 2E, supplementary Figure 2). The results confirm that adenoviral infection robustly increased the level of functional MSRA proteins in an organelle-specific fashion.

VSMCs require intracellular ROS as a second messenger for cellular survival and proliferation [4–8], such that treatment with antioxidants often

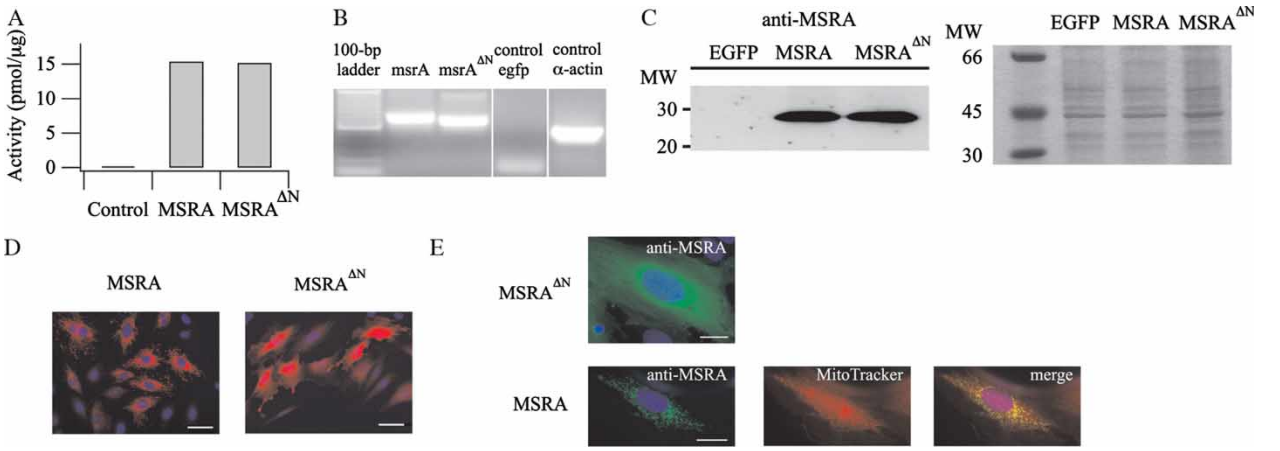

Figure 2. Over-expression of human *msrA* variants in A-10 VSMCs. (A) Reductase assay confirming similar enzymatic activities for MSRA (15.4 pmol/µg) and MSRA$^{\Delta N}$ (15.2 pmol/µg) constructs. (B) RT-PCR amplification of the coding sequences of transiently expressed *msrA* (708-bp) and *msrA*$^{\Delta N}$ (640-bp) shows a formidable transcription of both constructs in infected A-10 cells; *egfp*-infected control cells show no RT-PCR product for *hmsrA*. (C) Western blot signals of over-expressed MSRA and MSRA$^{\Delta N}$ are detected in the appropriate extracts of msrA and msrA$^{\Delta N}$-infected cells, but not in egfp-infected controls. Staining of the protein gel (bottom) served as internal loading control. (D) Comparison of MSRA and MSRA$^{\Delta N}$ expression rates in A-10 cells demonstrated by immunocytochemical detection; scale: 100 µm. (E) Intracellular localization of expressed human MSRA variants in A-10 cells, examined by immunofluorescence staining (green signals). Full-length MSRA (bottom panel) is targeted to mitochondria (red; mitochondrial marker MitoTracker Red). Deletion of the first 22 amino acids leads to cytosolic localization of MSRA$^{\Delta N}$ (top); blue, DAPI-stained nuclei; scale: 25 µm.

induces cell death [9–13]. We asked whether enhanced MSRA enzymatic activity caused by over-expression of the enzyme affects the A-10 cell survival under normal non-stressed conditions. We found no significant differences in cell viability (Figure 3A) as measured by the MTT assay among the cells that over-expressed either EGFP or both, mitochondrial and cytoplasmic, MSRA forms (MSRA: $98.1 \pm 1.0\%$; MSRA$^{\Delta N}$: $103.5 \pm 1.2\%$) ($p = 0.11$). To further confirm that MSRA over-expression is not detrimental in A-10 cells, we identified individual cells that robustly over-express MSRA and MSRA$^{\Delta N}$ by immunocytochemical detection and assayed for apoptosis using Annexin V and TUNEL staining. Fluorescence microscopy analysis of 15 cells for each construct showed that MSRA over-expressing cells did not show signs of apoptosis under a non-stressed condition (Figure 3B). Treatment with ascorbic acid, which is known to induce apoptosis of arterial SMCs [11], validated the suitability of both assays labelling for apoptosis (Figure 3B, right). These results together show that MSRA over-expression in A-10 cells is not detrimental for cell survival under normal conditions without exogenous oxidative stress.

MSRA variants confer protection against oxidative stress

Recent evidence suggests that over-expression of MSRA confers greater oxidative stress resistance in a variety of systems [38–42]; however, it is not known which form of MSRA, mitochondrial or cytoplasmic, is most effective in providing oxidative stress protection. To address this issue, we treated VSMCs with the oxidant ChT, which shows biochemical similarities to chloramines generated by leukocytes at atherosclerotic vessel sites [30]. Cells over-expressing MSRA and MSRA$^{\Delta N}$ were exposed to increasing concentrations of the oxidant for 15 h and cell viability was assayed using the MTT assay that already has been validated in other oxidant involved cell viability studies [40,43]. Compared with control cells that expressed EGFP, cells over-expressing the MSRA enzymes were more resistant to oxidative stress induced by ChT up to 1.4 mM ($p \leq 0.2$) (Figure 4). While mitochondrial MSRA enhanced cell viability maximal by 13% (1.0 mM ChT), cytoplasmic MSRA$^{\Delta N}$ was clearly more effective than mitochondrial MSRA ($p < 0.01$): cells expressing MSRA$^{\Delta N}$ showed no change in cell viability up to 1.2 mM ChT (Figure 4A) and at 1.4 mM ChT showed a 23% greater viability ($p < 0.01$; Figure 4B). Increasing the amount of applied Ad*msr* virus particles per cell to boost MSRA and MSRA$^{\Delta N}$ over-expression had no further rescue effect on cell viability (supplementary Figures 3 and 4).

Cytosolic MSRA$^{\Delta N}$ *delays loss of cell viability*

To further quantify the robust protective effect of cytoplasmic MSRA$^{\Delta N}$, we examined the cell viability as a function of ChT treatment durations (Figure 5). Cells heterologously expressing MSRA$^{\Delta N}$ or EGFP were incubated for 15, 30 and 45 h in medium containing 1.2 mM ChT. Viability of the control cells progressively decreased by $21.1 \pm 2.0\%$, $43.8 \pm 2.1\%$ and $58.4 \pm 2.1\%$. Over-expression of MSRA$^{\Delta N}$ markedly delayed this decline in cell viability; there was no significant change in cell viability after 15 h ($+1.9 \pm 1.7\%$; $p = 0.3$) and, compared with the EGFP group, a remarkably lesser decline after 30 h ($16.6 \pm 4.7\%$) and

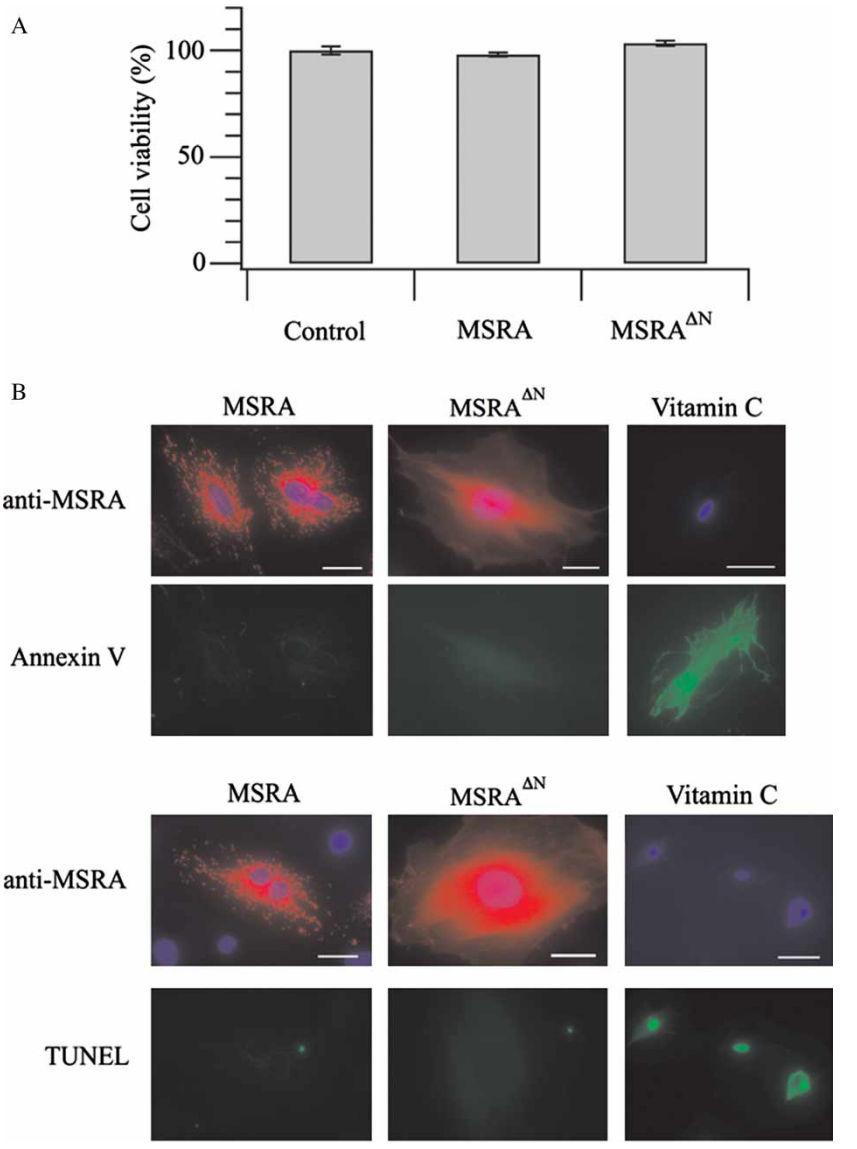

Figure 3. Effect of MSRA over-expression on A-10 VSMC survival. (A) Over-expression of mitochondrial MSRA and cytoplasmic $MSRA^{\Delta N}$ for 48 h statistically has no effect on A-10 cell viability ($p = 0.11$), measured by MTT assay. The results were standardized to EGFP-over-expressing controls. (B) Single-cell analysis of apoptotic cell death in A-10 cells over-expressing mitochondrial MSRA (left) and cytoplasmic $MSRA^{\Delta N}$ (centre) by MSRA immunocytochemical detection and Annexin V (top panel) and TUNEL (bottom panel) labelling in corresponding cells. Control cells were treated with vitamin C (5 mM) to induce apoptosis (right panel); blue, DAPI-stained nuclei; scale: 50 μm.

45 h ($27.4 \pm 3.2\%$) exposure to ChT ($p < 0.05$). Thus, exposure of $MSRA^{\Delta N}$ over-expressing cells to the oxidant for at least 45 h caused a similar decrease in cell viability as measured for control cells after 15 h.

Enhancing the MSRA catalytic antioxidant cycle

The 'sink hypothesis' of the MSRA action [18] postulates that the catalytic antioxidant cycle mediated by MSRA prevents oxidative damage by providing more methionine residues to scavenge excessive ROS. According to this hypothesis, increasing the substrate availability could enhance the efficacy of the MSRA antioxidant mechanism. Our previous study using *Drosophila* showed that MSRA reduces the methionine analogue S-methyl-L-cysteine (SMLC) when oxidized and that dietary supplementation of SMLC confers greater oxidative stress resistance and prevents the locomotor defect caused by ectopic expression of human alpha-synuclein [19]. Therefore, we hypothesized that expression of $MSRA^{\Delta N}$ contemporaneous with application of SMLC may offer even greater protection against oxidative stress caused by ChT. Expression of $MSRA^{\Delta N}$ ($28.0 \pm 1.7\%$; control: $21.9 \pm 1.6\%$; $p < 0.05$) or SMLC pre-treatment ($25.0 \pm 0.9\%$; $p = 0.2$) alone failed or only moderately protected A-10 cell viability in response to treatment with ChT (1.75 mM; Figure 6A). However, combined treatment with $MSRA^{\Delta N}$ and SMLC together efficiently protected

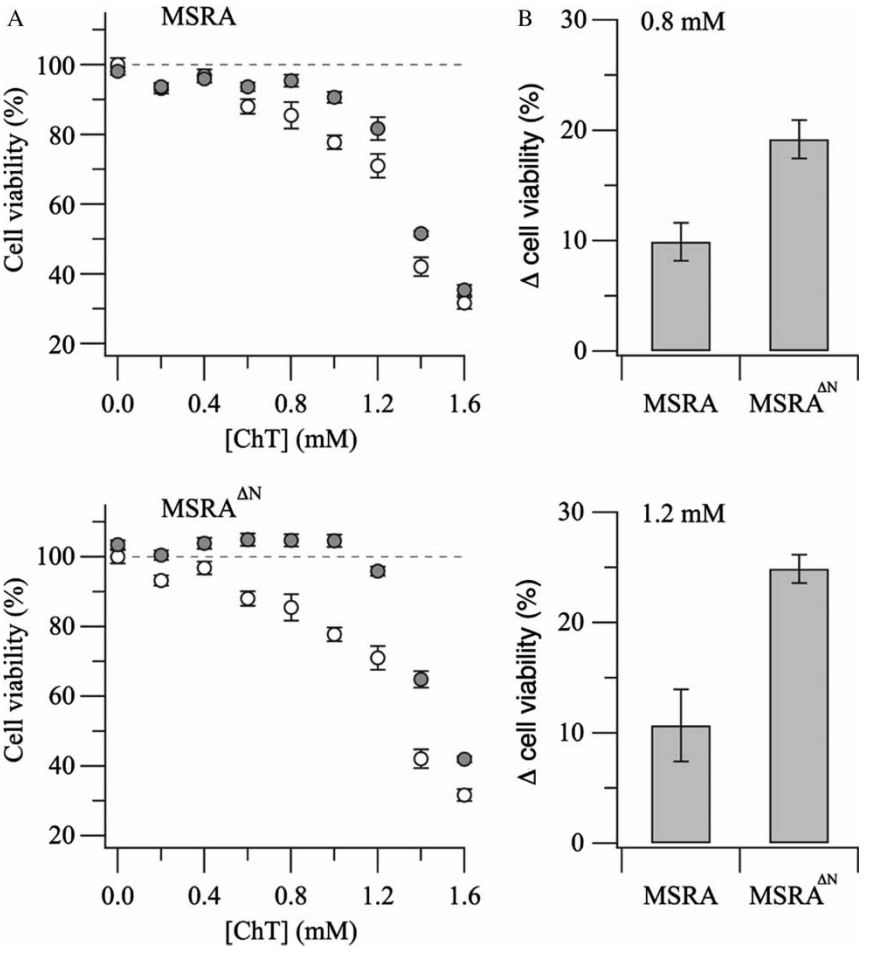

Figure 4. MTT cell viability assay of MSRA variants over-expressing A-10 cells after 15 h of ChT treatment. (A) Representative graphs depicting protective capabilities against increasing concentrations of the oxidant for MSRA and MSRA$^{\Delta N}$ (filled circles), relatively to EGFP over-expressing controls (open circles). (B) For the indicated ChT concentrations mitochondrial localized MSRA increased resistance, on average, by 10%. Cytoplasmic targeted MSRA$^{\Delta N}$ completely prevented loss of cell viability for up to 1.2 mM ChT ($p \geq 0.05$) (A) with an average increase in viability of 27%.

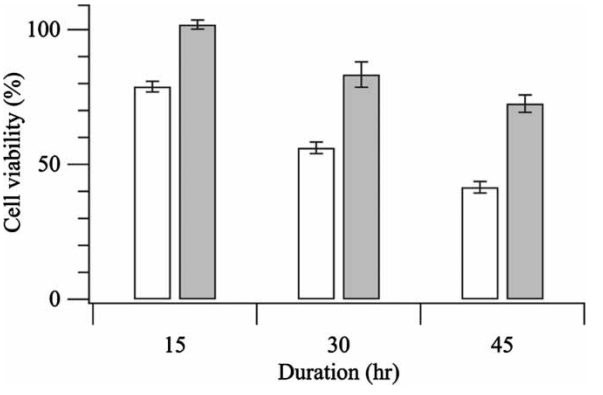

Figure 5. Protective effect of cytoplasmic MSRA$^{\Delta N}$ analysed for increasing ChT exposure times. Over-expression (solid bars) of MSRA$^{\Delta N}$ significantly delays decline in A-10 cell viability caused by extended ChT treatment, relative to EGFP infected controls (open bars) ($p < 0.05$). Cells were incubated in serum-supplemented medium containing 1.2 mM ChT for 15, 30 and 45 h and cellular viability was measured using MTT assay.

the cells, increasing the cell viability by 3-fold ($61.7 \pm 2.3\%$; $p < 0.05$) (Figure 6A).

As found using the cell viability assay, expression of MSRA$^{\Delta N}$ or treatment with SMLC alone failed to protect normal A-10 appearance with 1.5 mM ChT. However, treatment of MSRA$^{\Delta N}$ and SMLC together effectively prevented the morphological change under such a condition, keeping the appearance of oxidant treated cells indistinguishable from control cells (Figure 6B). It has to be stressed that in such experiments the cells incubated with SMLC were washed before ChT treatment to prevent inactivation of the oxidant by SMLC in the medium.

Discussion

The progression of atherogenesis frequently culminates in plaque rupture caused by apoptotic cell death of VSMCs, leading to severe cardiovascular outcomes, including thrombosis and cardiac infarction. Because VSMC apoptosis within the atherosclerotic

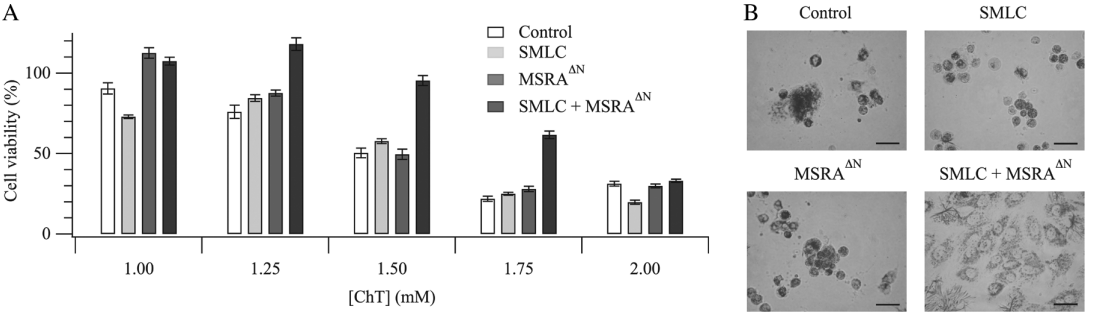

Figure 6. Enhanced protection against oxidative stress by co-treatment of cytoplasmic MSRA$^{\Delta N}$ and the MSRA substrate S-methyl-L-cysteine (SMLC). (A) MTT cell viability assay depicting diminished viability of either MSRA$^{\Delta N}$ or SMLC (50 mM) pre-treated cells after exposure to ChT concentrations higher than 1.0 mM and enhanced protective effect of combined treatment with MSRA$^{\Delta N}$ and SMLC. (B) Representative images of oxidant treated A-10 VSMCs, photographed after uptake of tetrazolium MTT; scale: 50 μm.

lesion probably involves oxidative stress [3], protection against ROS-induced oxidative modifications has to be considered an important therapeutic approach to prevent plaque destabilization [44] and the sensitivity of VSMCs to oxidative stress has significant physiological and pathophysiological implications [3]. Our study demonstrates that preferential oxidation of methionine by the oxidant ChT [31], structurally similar to chloramines produced physiologically by the reactions involving myeloperoxidase [30], diminishes cell viability in a concentration- and time-dependent manner by promoting apoptotic cell death in A-10 VSMCs. The sensitivity of A-10 cells to oxidative stress increases with the age of the culture, suggesting that an altered redox-state in aged VSMCs may contribute, in part, to the functional impairment of the VSMC layer in the ageing vasculature [33].

The damaging effects of reactive species are widely known, but increasing evidence suggests that some ROS are also required for normal VSMC function as essential second messengers. Consistent with this possibility, exogenous application of general ROS scavengers causes apoptosis in VSMCs [9–13]. This dependence of VSMCs on ROS thus likely precludes the use of general ROS scavengers to limit the extent of oxidative damage as a strategy to slow atherogenesis. In contrast, our results here show that over-expression of MSRA, which participates in the catalytic antioxidant cycle [18,19], either in mitochondria or in the cytosol, does not alter the cell viability under non-stressed conditions. Noteworthy, transient over-expression of mitochondrial MSRB2 (CBS-1), which reduces Met-R-O back to methionine [45], likewise does not affect normal A-10 viability (data not shown), indicating that enhanced reduction of both enantiomers of oxidized methionine does not interfere with ROS-dependent VSMC signalling.

Reactive species are generated at many sub-cellular loci [46]. Among them, mitochondria produce superoxide anions, which in turn lead to formation of other damaging molecules [47]. Conversion of superoxide and peroxide in mitochondria by manganese superoxide dismutase (SOD2) and phospholipid hydroperoxide glutathione peroxidase (PHGPx) is pivotal for protecting against oxidative damage [48,49]. Consequently, attempts to prevent and limit cellular oxidative damage as an intervention strategy in cardiovascular diseases, neurodegenerative diseases and ageing have focused on protection of mitochondria [50]. For example, mitochondrial over-expression of the peroxide-converting enzyme catalase in transgenic mice led to extension of median and maximum lifespan along with a delayed development of cardiac pathologies [51].

Our study demonstrates that over-expression of MSRA in the cytoplasm is more effective in protecting A-10 cells against the oxidative stress induced by ChT than MSRA over-expression in mitochondria. MSRA repairs Met-S-O back to methionine and is a component of the methionine-based catalytic antioxidant mechanism [16,18]. The superior effectiveness of over-expression of MSRA in the cytoplasm in protecting cells against the oxidative insult by ChT suggests that, for normal cellular viability, protection of the cytoplasm may become a limiting factor. The importance of protection of the cytoplasm against oxidative stress in normal cell function and survival is in contrast with the widely held view that protection of mitochondria against oxidative stress is important. We speculate that one critical variable is the locus of generation of reactive species. In VSMCs, considerable amounts of damaging reactive species may be generated in the cytoplasm itself by the activity of NOXes and other redox enzymes [52,53]. Some reactive species may be derived from extracellular sources, especially with inflammation [30]. It is plausible that in the presence of reactive molecules from the cytoplasm [52] and/or extracellular sources such as activated macrophages and leukocytes or after uptake of oxidized low-density lipoproteins from the blood [54,55], protection of the cytoplasmic compartment, for example by the MSRA catalytic antioxidant system, becomes a limiting factor in cell survival. Endogenous antioxidant systems, such as

the robustly expressed MSRB1 (Sel-X), may protect the A-10 cytoplasm under normal conditions [24]. However, application of ChT, which is similar to chloramines produced physiologically by the reactions involving myeloperoxidase [30], leads to an imbalance between oxidant generation and antioxidant capacity, causing oxidative damage and ultimately cell death. The over-expressed $MSRA^{\Delta N}$ may constitute, together with the endogenous MSRB, an efficient shield against oxidative stress and prevents oxidative damage particularly in the cytoplasmic compartment. It should be noted that $MSRA^{\Delta N}$ showed a superior protection against another oxidant, t-butyl-hydroperoxide (supplementary Figure 5), suggesting the importance of cytoplasmic protection against oxidative stress induced by many different agents.

The significance of cytoplasmic protection may be cell-type specific. For example, in lung epithelial cells that were exposed to 250 μM H_2O_2, adenoviral-mediated over-expression of catalase provided better protection when the enzyme was targeted to mitochondria, compared to cytoplasmic localization [56]. In contrast, catalase in either mitochondria or cytosol was equally beneficial for HepG2 cells exposed to 100 μM H_2O_2 [57], whereas in the insulin-producing RINm5F cell line cytoplasmic, rather than mitochondrial, catalase provided distinctly better protection against peroxide toxicity [58].

Oxidation of methionine by oxidants such as ChT used in our study should produce both Met-S-O and Met-R-O, which are reduced back to methionine by MSRA and MSRB, respectively [21]. It is then expected that over-expression of MSRB, which reduces Met-R-O [45], is similarly effective to MSRA. Indeed, we found that over-expressed MSRB2 (CBS-1), when localized in mitochondria, confers comparable protection to mitochondrial MSRA (supplementary Figure 6). We speculate that over-expression of MSRB in the cytosol, in spite of its ample endogenous presence there [24], may further enhance this protection.

The MSRA-mediated protection of A-10 cell viability can be further enhanced by supplementation with SMLC, an amino acid that acts as a substrate for MSRA when oxidized [19]. The effectiveness of SMLC only in the presence of over-expressed cytoplasmic MSRA (Figure 6) is consistent with the idea that the compound serves as a recyclable ROS scavenger and enhances the efficacy of the MSRA catalytic antioxidant system by increasing the number of available scavenging sites [19]. Although further analysis of the underlying molecular mechanism is needed, the beneficial effect of SMLC is further supported by a significant delay in morphological transformation of co-treated cells under an oxidatively stressed condition.

In summary, we have demonstrated that over-expression of MSRA in the cytoplasm confers significantly greater protection against extracellularly applied oxidative stress without compromising the cell viability under normal conditions. The protective efficacy of cytoplasmic MSRA can be further enhanced by contemporaneous treatment with SMLC, an amino acid that serves as a substrate for MSRA. Thus, protection of the cytoplasm by the catalytic antioxidant system involving MSRA may hold promise as a therapeutic strategy for many forms of cardiovascular diseases in which oxidative stress is implicated.

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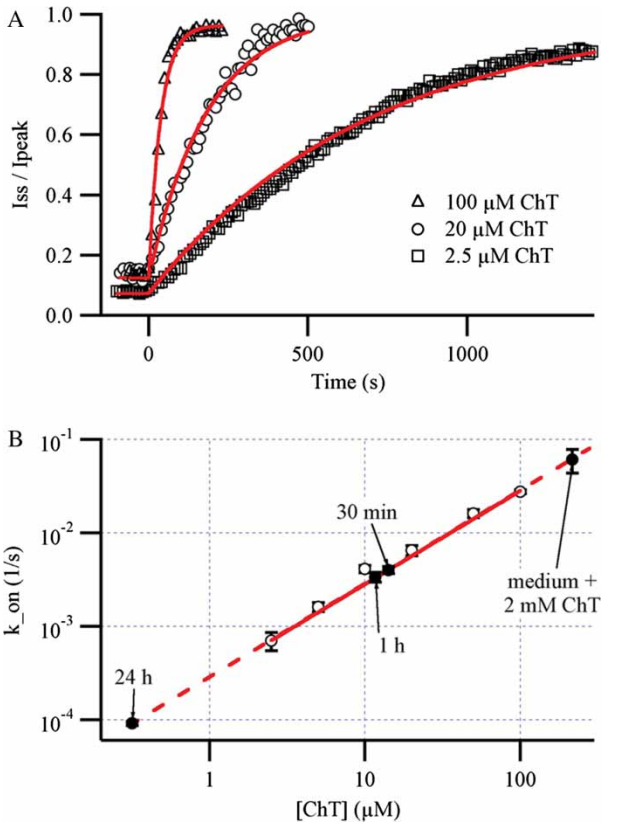
Supplementary Methods

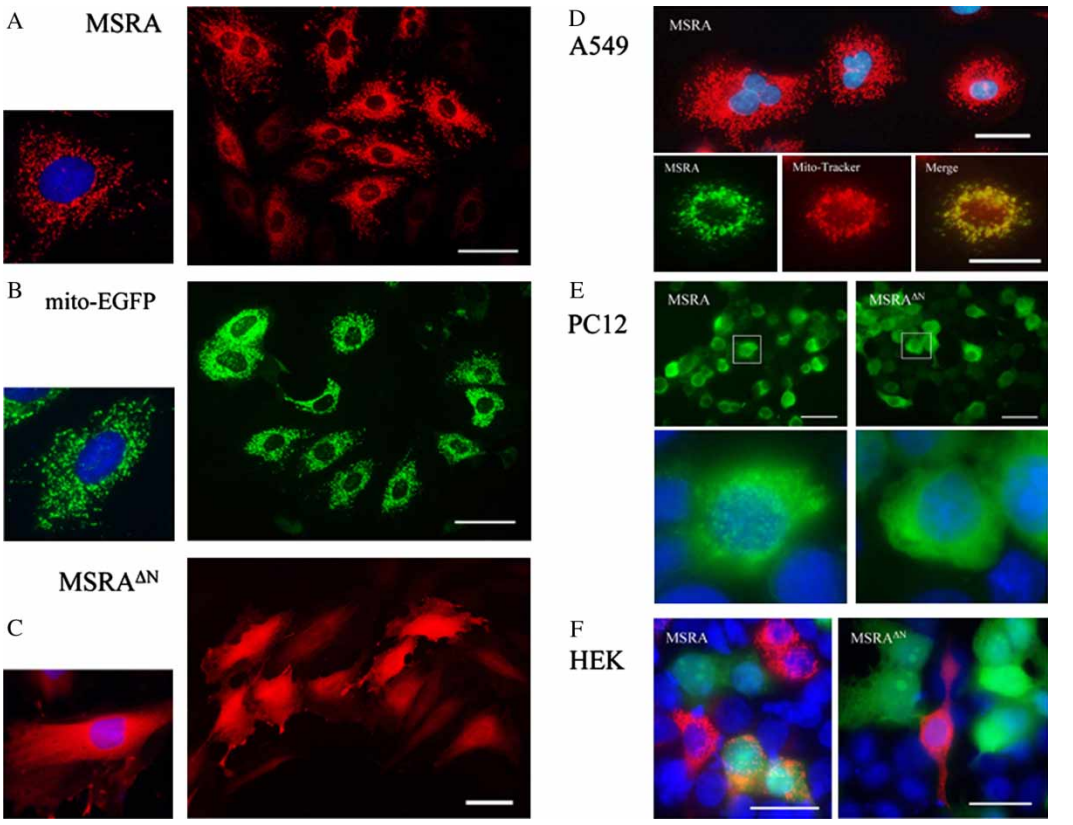
The inactivation motif 'IFM' of rat skeletal muscle sodium channel ($rNa_V1.4$) was altered to 'ICM' by PCR-based mutagenesis to yield mutant $rNa_V1.4\_F1304C$, here termed $rNa_V1.4\_ICM$. The mutant was transiently expressed in HEK 293 cells and currents were measured in the whole-cell configuration of the patch-clamp method as described earlier (Kassmann *et al.* 2008). ChT was added to the cells under voltage-clamp control by replacing the entire volume of the recording bath chamber.

Supplementary References

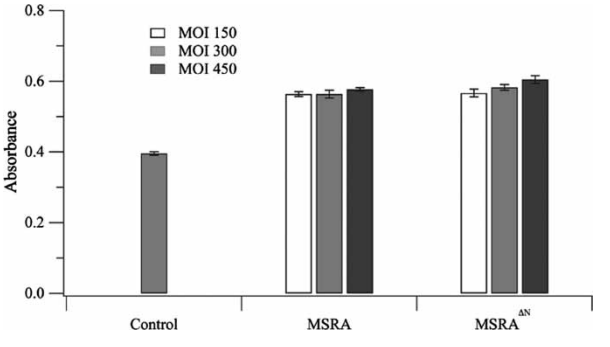
Kassmann M, Hansel A, Leipold E, Birkenbeil J, Lu SQ, Hoshi T, Heinemann SH. Oxidation of multiple methionine residues impairs rapid sodium channel inactivation. Pflügers Archiv 2008; 456:1085–1095.



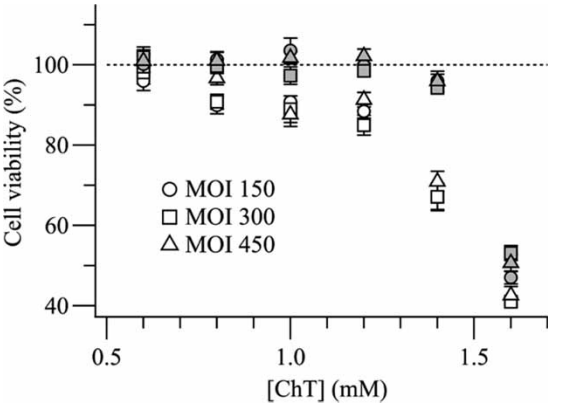
Supplementary Figure 1. Assay for the activity of ChT in cell culture medium. (A) The loss of rapid channel inactivation was assayed for the sodium channel mutant $rNa_V1.4\_ICM$ by measuring the ratio of steady-state current vs the peak current (Iss/Ipeak) elicited by repetitive depolarizations to –20 mV in the whole-cell patch-clamp configuration. The time course describing the loss of inactivation upon application of ChT was described with single-exponential functions (superimposed red curves). (B) The rate of the loss of inactivation (k_{on}) as a function of the ChT concentration (open symbols) is well described by a linear fit (red line). Filled symbols indicate measurements in which 2 mM ChT incubated in cell culture medium at 37°C for the given durations were applied to the cells. The estimated remaining ChT activities to remove inactivation of $rNa_V1.4\_ICM$ were 216 µM (directly applied after mixing), 14.2 µM (30 min incubation), 11.7 µM (1 h incubation) and 0.32 µM (24 h incubation). Error bars indicate SEM values.



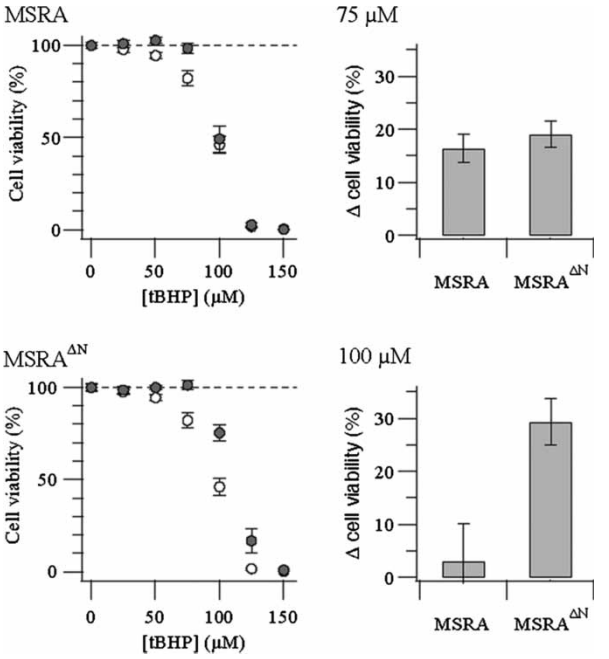
Supplementary Figure 2. Sub-cellular localization of over-expressed human MSRA variants in A-10 VSMCs (A–C), A549 cells (D), PC12 cells (E) and HEK tsA cells (F). In all four cell lines, full-length MSRA is localized predominantly in mitochondria, indicated by the granular distribution pattern comparable to EGFP containing the mitochondrial localization sequence of pyruvate dehydrogenase alpha 1 (B) and co-localization with a Mito-Tracker (D, bottom). MSRA lacking the amino-terminal signalling sequence ($MSRA^{\Delta N}$) resides in the cytosol (C; E, right; F, right). HEK tsA cells were co-transfected with cytoplasmic EGFP (green). Scale: 50 μm.



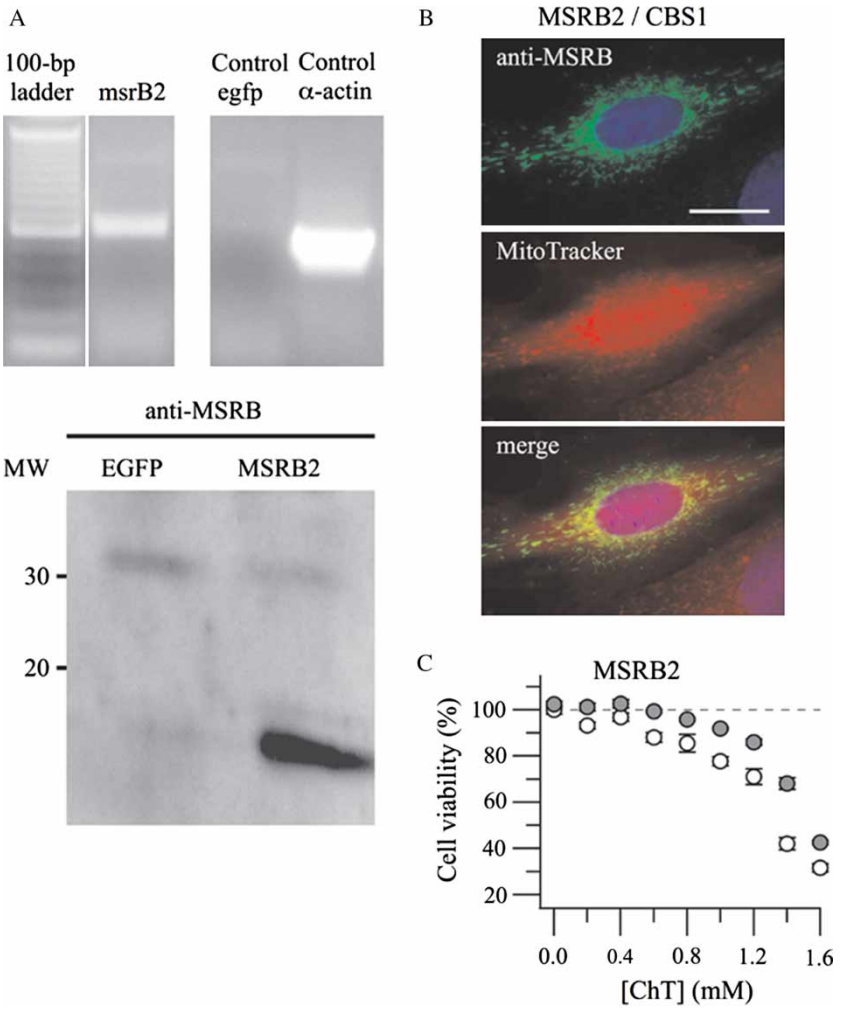
Supplementary Figure 3. Effect of Ad*msr* virus particle quantity on A-10 cellular viability. Cells were infected with MSRA and $MSRA^{\Delta N}$ encoding virus particles, respectively, applied at different multiplicity of infection rates (MOI 150, 300, 450). After treatment with the oxidant ChT (1.0 mM), absorption after MTT reduction was determined. Increased application of Ad*msr* virus particles per cell had no further rescue effect on cell viability.



Supplementary Figure 4. Decrease in $MSRA^{\Delta N}$ protection (filled symbols) under high oxidative stress conditions (1.6 mM ChT). Enhanced over-expression of $MSRA^{\Delta N}$ (circle, MOI 150; square, MOI 300; triangle, MOI 450) has no further protective effect on cell viability. Control, corresponding MOI values for over-expression of Ad*egfp* (open symbols).



Supplementary Figure 5. MTT cell viability assay of MSRA variants (filled circles) over-expressing A-10 cells treated with the oxidant *t*-butyl-hydroperoxide (tBHP) for 15 h. While mitochondrial MSRA (top) modestly protected cell viability against the oxidant by maximal 17% (75 µM tBHP), cells over-expressing the cytoplasmic localized $MSRA^{\Delta N}$ (bottom) were more resistant against up to 125 µM tBHP with a maximal increase in viability by 30% (100 µM tBHP). Open circles: EGFP control.



Supplementary Figure 6. Effect of MSRB over-expression on viability of oxidatively stressed A-10 cells. (A) RT-PCR amplification and Western blot detection of over-expressed *msrB2/CBS-1* shows robust transcript (549-bp) and protein (19-kDa) levels of the construct after adenoviral infection of A-10 cells; *egfp*-infected control cells show no appropriate signals. (B) MSRB2 is targeted to mitochondria and co-localized well with the mitochondrial marker MitoTraker red. Blue, DAPI-stained nuclei; scale: 25 μm. (C) MTT cell viability assay of MSRB2 over-expressing A-10 cells after 15 h of ChT treatment. Representative graph depicting protective capabilities against increasing concentrations of the oxidant for MSRB2 (filled circles), relatively to EGFP over-expressing controls (open circles). For the indicated ChT concentrations mitochondrial localized MSRB2 increased resistance, on average, by 13%.