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Identification of a new functional splice variant of the enzyme methionine sulphoxide reductase A (MSRA) expressed in rat vascular smooth muscle cells

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Abstract
Reactive oxygen species contribute to ageing of the vascular system and development of cardiovascular disease. Methionine-S-sulphoxide, an oxidized form of methionine, is repaired by the enzyme methionine sulphoxide reductase A (MSRA). The enzyme, targeted to mitochondria or the cytosol by alternative splicing, is vital for oxidative stress resistance. This study was designed to examine the endogenous expression and intracellular localization of MSRA in rat aortic vascular smooth muscle cells (VSMCs). We detected robust MSRA immunoreactivity exclusively in mitochondria. Sequence analysis of *msrA* transcripts revealed the presence of a novel mitochondrial splice variant, *msrA2a*, in cultured rat VSMCs as well as in aortic tissue preparations. The enzymatic activity of a recombinant MSRA2a protein was confirmed by the reduction of methionine sulphoxide in a model substrate peptide. We conclude that multiple MSRA variants participate in the repair of oxidized proteins in VSMC mitochondria, but that other protective mechanisms may exist in the cytoplasmic compartment.

Keywords: *Oxidative stress, vasculature, MSRA, protein oxidation, alternative splicing*

Abbreviations: *met-O, methionine sulphoxide; MSRA/B, methionine sulphoxide reductase A and B; ROS, reactive oxygen species; VSMC, vascular smooth muscle cell.*

Introduction

Regulation of blood pressure requires proper functioning of the vascular smooth muscle cells (VSMCs) lining the blood vessels. However, VSMCs are easily damaged by excess reactive oxygen species (ROS) [1–4], which may be generated by their mitochondria, cytoplasmic NADPH oxidases (NOXes) (see [5] for review) or from extracellular sources including oxidized low-density lipoprotein (oxLDL) and macrophages [1,6,7]. Oxidatively damaged molecules accumulate with age and contribute to accelerated ageing and development of age-associated cardiovascular diseases such as hypertension and atherosclerosis [8–11].

Enhanced oxidative stress associated with ageing and many pathophysiological conditions induces oxidation of various cell constituents, including amino acid residues in proteins. The amino acid methionine with its reactive sulphur atom is particularly susceptible to oxidation, leading to formation of

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two diastereomers of methionine sulphoxide: methionine-S-sulphoxide (met-S-O) and methionine-R-sulphoxide (met-R-O). Unlike other oxidized amino acids, met-S-O and met-R-O are stereo-specifically reduced by the enzymes methionine sulphoxide reductase (MSR) A (MSRA, also referred to as methionine-S-oxide reductase (MSOR)) and methionine sulphoxide reductase B (MSRB, also referred to as methionine-R-oxide reductase (MROR)), respectively. While both MSRA and MSRB reduce oxidized methionine using a similar chemical reaction mechanism to participate in the redox cycle of methionine, the two enzyme systems have distinct characteristics. For example, three *msrB* genes (*msrB1* [*selenoprotein R*], *msrB2* [*CBS-1*], *msrB3* [*CBS-2*]) are known in mammals [12–14], but only one *msrA* gene exists [15,16]. Analysis of the *msrA* genomic organization shows that the gene contains six exons and that the first exon is alternatively spliced to create two *msrA* splice variants [17,18]. The resulting two proteins differ in the distal amino-terminal sequence, which represents either a mitochondrial signal sequence or a shorter, four (mouse) or five (human) residues encompassing sequence. Whereas the MSRA protein without the mitochondrial signal sequence is localized in the cytoplasmic compartment (cytoplasmic MSRA), the protein with the mitochondrial signal sequence (mitochondrial MSRA) is present in mitochondria [17–20]. However, MSRA with the signal sequence can be detected in cytosol and nucleus when over-expressed in different eukaryotic cells [21]. The mitochondrial and cytoplasmic versions of MSRA share a highly conserved catalytic active-site motif, GCFWG [22], which, in mammals, is coded in the third exon of the gene. High-resolution structural studies indicate that MSRA consists of a core domain harbouring the active centre. The peripheral N- and C-termini are highly variable in different species and are folded into long coiled structures [23].

Multiple lines of evidence have underscored the importance of methionine oxidation and MSR enzymes, especially MSRA, in the cellular response to oxidative stress. Over-expression of MSRA confers greater cell viability following treatment with exogenous oxidants in a variety of cell types, such as yeast, PC12 cells, lens epithelial cells and fibroblasts [24–28]. *In vivo*, over-expression of the enzyme in *Drosophila* leads to greater resistance to oxidative stress and also to lifespan extension [29]. Conversely, functional impairment of the MSRA system increases vulnerability of cultured cells to oxidative stress [27,30] and shortens lifespan in yeast and mouse [31,32].

Despite the importance of MSRA as a critical component of the cellular anti-oxidant system and potentially as a tool to manage age-associated diseases [27,29,33], little is known about its expression and function in VSMCs. Such information is indispensable for understanding normal ageing of the vascular system as well as in devising therapeutic strategies against numerous oxidative stress-associated vascular diseases. Therefore, we examined the expression of MSRA in rat aortic VSMCs and identified different mitochondrial *msrA* splice forms. We show here that mitochondria in VSMCs posses two distinct MSRA variants to reduce oxidized methionine residues, indicating additional levels of complexity in met-S-O reduction. In contrast, our results show that the vascular A-10 cell cytoplasm is largely free of MSRA and other protective mechanisms, like the reduction of met-R-O by MSRB, must contribute to the oxidative stress resistance.

Materials and methods

Cell line and culture

The rat aortic vascular smooth muscle cell line A-10 [34] 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.

Immunocytochemical and Western blot detection of MSRA

A-10 cells, plated on glass cover slides, were rinsed with PBS and fixed in 4% formaldehyde at 37°C for 10 min. Cells were washed in 50 mM Tris buffer (pH 7.4) and incubated with primary antibody directed against rat MSRA (kindly provided by B. Friguet, Université Denis Diderot, Paris), followed by secondary Cy2-conjugated anti-rabbit antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA). Mitochondria were stained with CM-H_2Xros Mito-Tracker Red (Molecular Probes, Eugene, OR). Specimens were mounted in UltraCruz™ mounting medium (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) containing 4′,6-diamidino-2-phenylindole (DAPI) for staining of nuclei. The fluorescence images were obtained using a Nikon Eclipse E 800 fluorescence microscope at the Biomedical Imaging Core of the University of Pennsylvania.

For Western blot detection, dissected rat thoracic aorta was homogenized in cell fractionation buffer (Clontech Laboratories, Inc., Mountain View, CA) using a Dounce tissue grinder and confluent growing A-10 cells were harvested. The samples were mixed with loading buffer containing 5% (v/v) 2-mercaptoethanol and 20 mM DTT and separated on a 10% (w/v) SDS/PAGE protein gel. After blotting on a nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA), MSRA was detected using primary anti-rat MSRA antibodies at a dilution of 1:50.000. Blots were developed using ECL™ Western Blotting

Detection Reagents (GE Healthcare, Buckinghamshire, UK).

RT-PCR amplification of rat msrA

For amplification of endogenous rat *msrA*, A-10 cells were harvested and total RNA was isolated (RNeasy mini kit, Qiagen Inc., Valencia, CA) for RT-PCR amplification (Titanium One-Step RT-PCR kit, Clontech Laboratories, Inc.). Based on the published sequence of rat *msrA* (Genbank: NM_053307) forward primer mito-F1 and backward primer *msrA*-B1 were used to amplify a 330-bp fragment of mitochondrial *msrA* (for primer sequences see Table I). To obtain a fragment of rat cytoplasmic *msrA*, splice form specific forward primers cyto-F1 and cyto-F2 were used together with the backward primer *msrA*-B1 (332-bp fragment and 310-bp fragment) as well as the forward primer cyto-F3 in combination with the backward primer *msrA*-B2 (198-bp fragment). The thermal cycling conditions were 50°C for 60 min; 94°C for 5 min; 30 cycles of 94°C for 30 s, 53°C for 30 s, 68°C for 50 s; 68°C for 5 min.

For selective amplification of *msrA2a*, the splice version specific forward primer ASF-F1 and the backward primer *msrA*-B3 (153-bp fragment) were used in a nested PCR (Advantage-HF2 PCR kit, Clontech Laboratories, Inc.). The thermal cycling conditions were 94°C for 1 min; 30 cycles of 94°C for 30 s, 54°C for 30 s, 68°C for 50 s; 68°C for 5 min. PCR products were separated on a DNA gel, sub-cloned into pGEM-T vector (Promega Corporation, Madison, WI) and prepared for sequencing at the Sequence Core of the University of Pennsylvania.

Table I. (RT-) PCR primer sequences for amplification of rat *msrA* splice forms.

(RT-) PCR primer	Primer sequence
msrA splice form amplification	
mito-F1	5′-ATGATGGGCGACTCATCTTC-3′
cyto-F1	5′-CTGTGGTTGGTAGCCTGAGC-3′
cyto-F2	5′-GGGGGAGAAGACAGGTCGTA-3′
cyto-F3	5′-AGGCCTGTGTATGGATGGAG-3′
msrA-B1	5′-CTCAAAGCTGACGTGCTCTG-3′
msrA-B2	5′-TACACAACCCGGACGACTTC-3′
ASF-F1	5′-AGCCTCTCTCCCAACCAGA-3′
msrA-B3	5′-GAAGCAGCCCATTCCAAATA-3′
5′-end amplification	
mito-F2	5′-TCCATCCCGGTACGGATGAT-3′
cyto-F3	5′-AGGCCTGTGTATGGATGGAG-3′
ASF-B	5′-GCCAGCTATGTTTTCCAGGT-3′
3′-RACE PCR	
mito-F1	5′-ATGATGGGCGACTCATCTTC-3′
ASF-F1	5′-AGCCTCTCTCCCAACCAGA-3′
ASF-F2	5′-ACCTGGAAAACATAGCTGGC-3′

Amplification of 5′- and 3′-ends of msrA2a splice form

To determine the 5′-end of the *msrA2a* mRNA the transcript was amplified by RT-PCR using forward primers specific for mitochondrial (mito-F2) or cytoplasmic (cyto-F3) *msrA* and the splice form specific backward primer ASF-B. For amplification of 3′-ends a 3′-RACE PCR (Roche Diagnostics, Indianapolis, IN) was performed using forward primer mito-F1, followed by the splice form specific primers ASF-F1 and ASF-F2.

Amplification of msrA2a from various rat tissues

A male Fisher 344 rat (5 weeks of age) was obtained from Charles River Laboratories (Wilmington, MA) and sacrificed by cervical dislocation. Thoracic aorta, brain, liver and kidney were dissected, rinsed in PBS and immediately frozen in liquid nitrogen for storage. Total RNA was isolated (RNeasy mini kit, Qiagen) and mitochondrial *msrA* was obtained by RT-PCR amplification using the described primers mito-F1 and *msrA*-B1. To amplify a fragment of the splice form *msrA2a*, we performed a nested PCR using 5 μl of the PCR-product and the *msrA2a* specific forward primer ASF-F1 and primer *msrA*-B3, using the mentioned PCR parameters. All obtained PCR products were analysed by sequencing.

The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

Recombinant protein expression

The published rat *msrA* coding sequence (GenBank: NM_053307) was amplified from total RNA of A-10 cells using the primers 5′-ATGCTCTCCGCCTC CAGAAGGACTCT-3′ and 5′-TTACTTTTTAAT GGCCGTGGGACAGG-3′. To obtain a full-length form of *msrA2a*, a PCR fragment that contained the alternatively spliced sequence was amplified using primers 5′-ATGATGGGCGACTCATCTTC-3′ and 5′-CTCAAAGCTGACGTGCTCTG-3′ and inserted into the *msrA* ORF using the endogenous restriction sites *Hind*III and *Age*I.

For recombinant over-expression in *E. coli* we used *msrA* lacking the amino-terminal targeting sequence ($MSRA^{\Delta 1-19}$). For this, the coding sequences of *msrA* and *msrA2a* were amplified using primers 5′- GCAGGATCC*ATG*ATGGGCGACTCATCTT CGAAGGT-3′ and 5′- AGCCTGCAGTTACTT TTTAATGGCCGTGGGACAGG-3′ (*Bam*HI and *Pst*I restriction sites underlined, initial ATG italic), respectively, for sub-cloning of the PCR products into the pQE-30 expression vector (Qiagen). All plasmids were verified by sequencing. Over-expressed proteins were purified using Ni-NTA material as described by the manufacturer (Qiagen). The correct size of the

proteins was verified by SDS-polyacrylamide gel electrophoresis, followed by Western blot detection using polyclonal antibodies directed against human MSRA [35].

Enzyme activity assay

A synthetic peptide (KIFM(O)K, M(O) is met-O) derivatized with a dinitrophenyl (DNP) group (Peptide Specialty Laboratories, Heidelberg, Germany) was used to determine the enzymatic activity of MSRA2a. Each reaction mixture contained 3 nM of substrate, diluted in 50 mM Tris buffer (pH 7.3), 20 mM DTT and 1 ng of recombinant enzyme. After incubation at 37°C for 45 min, the reaction mixtures were analysed.

For analysis the reaction mixes were passed through a Source 15RPC-ST4.6/100 column (GE Healthcare, Buckinghamshire, UK) using an ÄKTA purifier-10 system (GE Healthcare). The DNP group allowed the detection of the substrate peptide in the reaction mixture at 365 nm, without any background absorption of other components. In a linear gradient from H_2O/0.1% trifluoroacetic acid (TFA) to 84% acetonitrile/0.1% TFA the oxidized peptide eluted as a single peak at 57% acetonitrile. After incubation with MSR enzyme a second peak at 61% acetonitrile/0.1% TFA appeared and the ratio between the peak areas of reduced peptide and reduced plus oxidized peptide provided a measure for enzyme activity.

Over-expression of MSRA2a-EGFP fusion protein in A-10 cells

The coding sequences of rat *msrA* and *msrA2a* splice forms were sub-cloned into EGFP-N1 vector (Clontech Laboratories, Inc.) using the restriction sites *Xho*I and *Kpn*I. The correct reading frame was confirmed by sequencing. A-10 cells, plated on glass cover slides, were transfected with 2 μg plasmid DNA (FuGENE 6 Transfection Reagent, Roche) and 48 h later analysed with a fluorescence microscope. Mitochondria and nuclei were stained as described earlier.

Results

Expression of MSRA in A-10 VSMCs

Western blot detection revealed the presence of MSRA peptide in rat thoracic aorta as well as in the vascular smooth muscle cell line A-10, derived from rat thoracic aorta [34] (Figure 1A). Consistent with a previous report [16], the apparent molecular weight of the protein was ~26-kDa, which is predicted by the cDNA sequence for mitochondrial *msrA*. In contrast, a control sample derived from rat liver extract contained MSRA that has a slightly lower molecular weight than the protein in A-10 cell and aortic tissue extracts (Figure 1A). In blotted samples of mitochondrial and cytosolic A-10 cell fractions, MSRA was detectable in the mitochondrial fraction only, where it predominantly appeared as a dimer at ~52-kDa (supplementary Figure 1).

An immunocytochemical assay confirmed the robust expression of MSRA in the A-10 cell line. MSRA immunoreactivity (MSRA-IR) co-localized well with the mitochondrial dye MitoTracker Red in every cell, confirming the consistency of the assay system and the preferential localization of MSRA in mitochondria surrounding the nucleus (Figure 1B). Both mitochondrial and cytoplasmic variants of MSRA arise from a single gene and share identical sequences downstream of the N-terminal sub-cellular targeting motifs [18]. Thus, polyclonal antibodies raised against MSRA recognize both forms as previously demonstrated by Western blot detection of mitochondrial and cytoplasmic MSRA in rat liver cell extracts [20]. Likewise, we detected MSRA immunoreactivity in the cytoplasm of a rat liver cell line (*clone 9*, ATCC) (data not shown). However, using the same experimental protocols, we failed to detect any MSRA-IR in the cytoplasm of every A-10 cell (15 cells analysed). The apparent lack of MSRA-IR outside of mitochondria in the cytoplasmic compartment suggests that A-10 cells have no or only a very low amount of functional cytoplasmic MSRA. In contrast, the whole cytoplasm revealed an intensive immunoreactivity against methionine-R-sulphoxide reductase MSRB1 (Figure 2A), whose expression was also confirmed by RT-PCR amplification (Figure 2B).

To complement the immunological assays, we analysed the expression of both mitochondrial and cytoplasmic *msrA* variants in A-10 cells by detecting the appropriate mRNA transcripts using RT-PCR. The mitochondrial *msrA* transcript was detected using a primer specific for the 5′ mitochondrial targeting sequence encoded by the first exon of the *msrA* gene. Our results indeed confirmed the presence of a transcript for mito-MSRA (Figure 1C, lane 1). Because the sequence of a cytoplasmic *msrA* splice version was not previously described in rat, we searched the expressed sequence tag (*est*) database for putative rat cyto-MSRA sequences and identified one cDNA clone (Genbank: CB757497) that encodes an MSRA protein with an alternative N-terminus. In this clone, translation at the initial start codon generates the sequence MEQQPQ that corresponds to the N-terminal starting sequence (MCSEP) found in human (GenBank: AY690665) [17] and monkey (GenBank: AY958431) [19] cytoplasmic MSRA. Using three different primers that bind to this alternative rat *msrA* first exon, we failed to obtain RT-PCR products, suggesting the absence of an *msrA* transcript encoding the cytoplasmic variant of the enzyme in A-10 cells (Figure 1C, lane 2). Therefore, the results obtained by the RT-PCR experiments are consistent with the

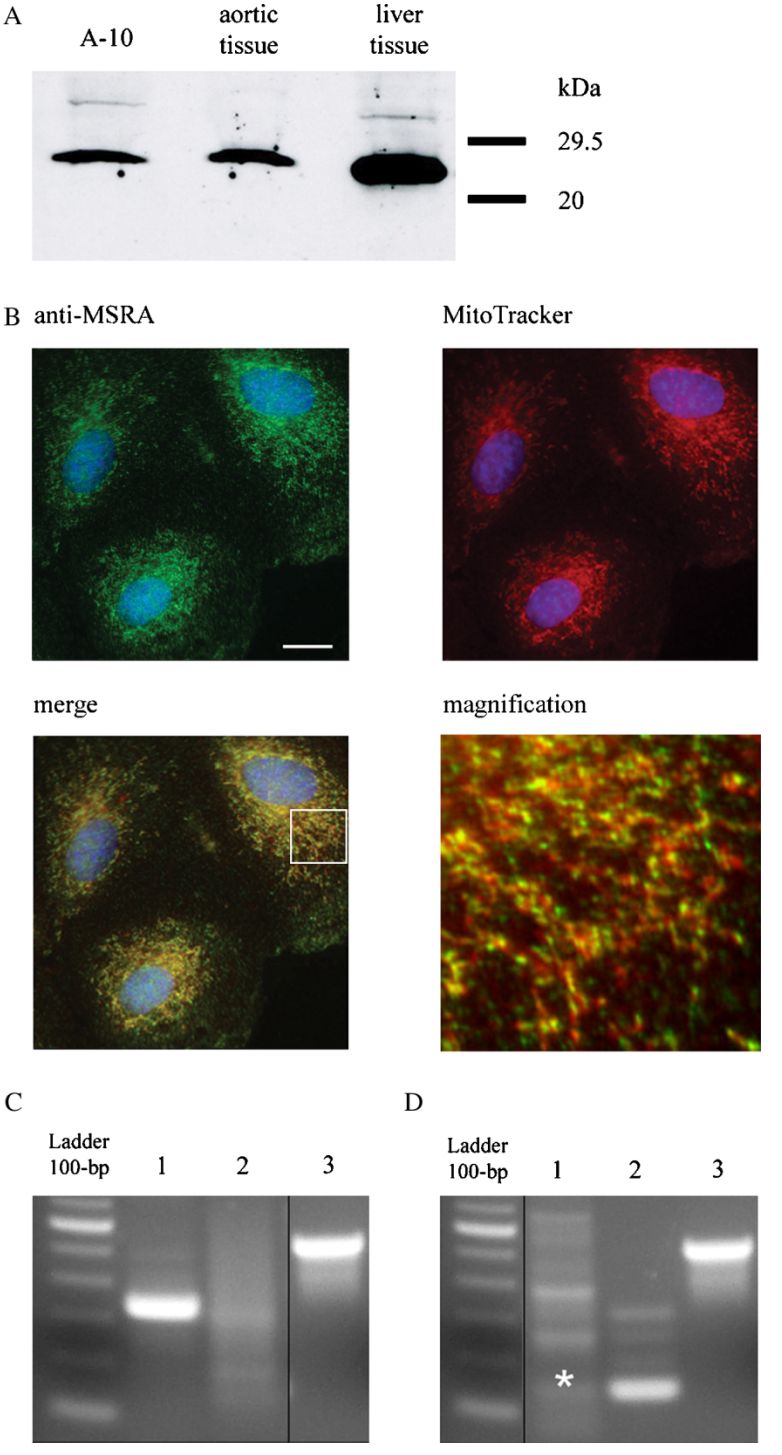


Figure 1. Endogenous expression of MSRA in A-10 VSMCs. (A) Western blot analysis demonstrates the presence of MSRA in lysates of A-10 VSMCs, rat aortic extract and liver tissue extract. The MSRA found in the VSMC line and thoracic aorta has a molecular weight of ~ 26-kDa and MSRA expressed in rat liver is slightly smaller. (B) Immunocytochemical detection of MSRA shows localization specifically in mitochondria: top left, anti-MSRA antibody; top right, mitochondrial marker (MitoTracker Red); bottom left, merged image; bottom right, high resolution of selected area in merged image. DAPI-stained nuclei appear blue; scale bar: 25 μm. (C) RT-PCR amplification of mitochondrial (1) and cytoplasmic (2) *msrA* splice form in A-10 cells. Use of a specific primer directed against the mitochondrial targeting sequence generated a 330-bp PCR product, but no product was detected with primers specific for an alternative sequence that encodes for cytosolic MSRA (representative shown for cyto-F3 *msrA*-B2 primer pair); (3) control, β-actin. (D) Selective amplification of *msrA2a* splice form in A-10 cells. The expected 160-bp fragment (*) is not identified after a first RT-PCR (1), a 153-bp fragment, sequenced as *msrA2a*, can be detected after nested PCR (2); (3) control, β-actin.

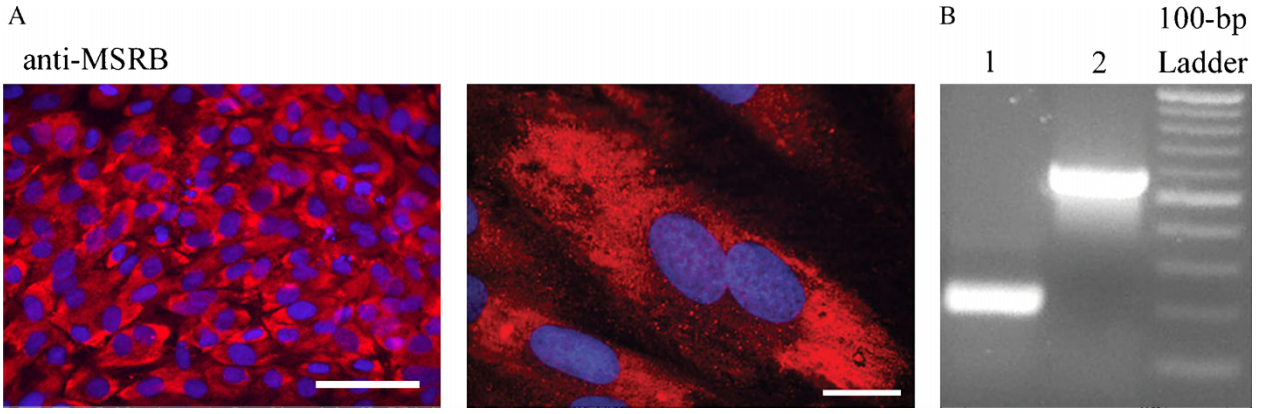


Figure 2. Endogenous expression of MSRB1 (SelR) in A-10 VSMCs. (A) Immunocytochemical detection of MSRB1 using a primary antibody directed against human MSRB1 (Abcam Inc., Cambridge, MA) and Cy3-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories) reveals its abundant expression in A-10 cells (left). MSRB1 immunoreactivity is present in the cytosol (right). Scale bar: left, 100 μm; right, 25 μm. (B) RT-PCR amplification of the predicted rat *msrB1 (SelR)* transcript (GenBank: XM_001058492) in A-10 cells. Primers for amplification of a 195-bp fragment (1) were FW 5′-GCGAGGTCTTCCAGAATCAC-3′ and BW 5′-ACTTGCCACAGGACACCTTT-3′; (2) control, *β*-actin.

immunocytochemical results; A-10 cells have no detectable cytoplasmic MSRA and contain mitochondrial MSRA only.

Identification of various msrA splice forms

Mapping of the amplified mitochondrial *msrA* nucleotide sequences to the genomic sequence led to the identification of three different *msrA* transcripts in A-10 VSMCs. In addition to the published rat *msrA* sequence (GenBank: NM_053307), we detected two novel splice forms: *msrA2a* and *msrA2b*. The alternative splice event occurred at the level of the second exon and led to the insertion of one additional exon (2a and 2b, respectively; Figure 3). Their sequences correspond to position 88.721-88.804 and 24.997-25.098, respectively, within the rat genomic DNA on chromosome 15.

In the *msrA2a* splice version (GenBank: DQ989019), exon 2a encodes additional 28 amino acids, five of which are proline (Figure 4). Analysis of this sequence by ProtScale (http://us.expasy.org/tools/protscale.html) [36] showed no notable pattern of polar or hydrophobic amino acids, but the sequence may form a self-contained domain, as indicated by a relatively high value for bulkiness. A database search by BLAST showed that the sequence ^{4}TPSLSPNQKLN^{14} in the splice insertion has a high degree of sequence similarity to the N-terminus of a bacterial oxidoreductase of *Solibacter usitatus* (GenBank: ZP_00523405). Besides the insertion, the *msrA2a* transcript itself contained all other exons present in *msrA*, as revealed by amplification of its 3′-end by RACE-PCR. On the genomic level, a detailed analysis of the chromosomal DNA at the site of the *msrA2a* exon revealed that the sequences at the exon-intron boundaries comply with the GT-AG rule, which is a prerequisite for splicing of the exon. Taking these results together, this transcript most likely is translated into a full-length MSRA protein.

Unlike exon 2a, exon 2b in *msrA2b* contains two consecutive stop-codons, which may prevent its translation into a functional MSRA protein (Figure 4). Alternative splicing of exon 2b was found to generate two splice forms, in which 2b either is inserted between exon 1 and 2 (*msrA2b1*; GenBank: DQ989020) or completely replaces the original exon 2 (*msrA2b2*; GenBank: DQ989021) (Figure 3). The latter generates an additional, third TGA stop codon at the junction between exon 2 to 3.

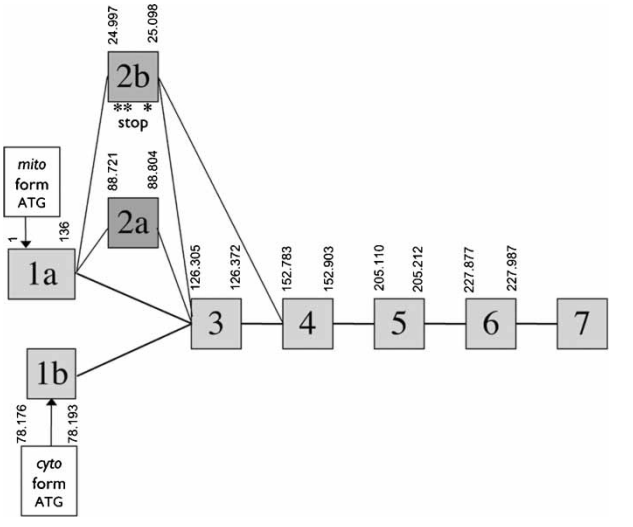


Figure 3. Genomic organization of the rat *msrA* gene. The corresponding positions of the six, or alternatively seven, exons were identified by mapping their sequences on the genomic DNA. Two splice variants differ in the first exon, which either translates a mitochondrial targeting sequence (1a) or directs the protein into the cytoplasm (1b). Two newly identified splice forms contain an additional exon 2; either with a continuous ORF that shows homology to the N-terminus of a bacterial oxidoreductase (2a) or with consecutive stop codons, which may prevent the translation into a functional MSRA protein (2b).

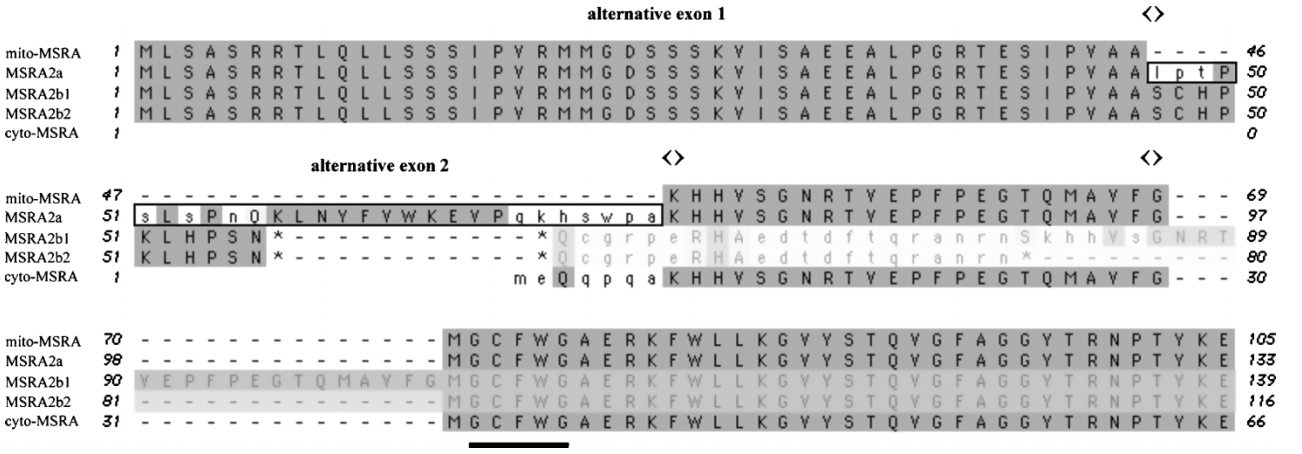


Figure 4. Alignment of amino-termini of rat MSRA proteins up to the catalytic domain (underlined) generated by alternative splicing. The top four splice forms are mitochondrial proteins that include an amino-terminal targeting sequence (first 21 amino acids), encoded by an alternative exon 1. A 28 amino acid insertion (framed), located on an alternative exon 2, is specific for the new identified splice form MSRA2a (second sequence). For MSRA2b1 and MSRA2b2 splice forms, a different alternative exon 2 contains stop codons, which result in the truncation of the protein; the non-translated sequences are shadowed. The cytosolic splice form (bottom) has an alternative amino-terminus contributed by an alternative exon 1.

Mitochondrial localization of MSRA2a splice form

Mitochondrial localization of human and mouse MSRA depends on an N-terminal motif that contains hydrophobic amino acid residues and is encoded by the alternatively spliced first exon of MSRA [18,35]. To study the intracellular localization of the new identified splice form MSRA2a, we transiently over-expressed rat MSRA-EGFP and rat MSRA2a-EGFP fusion proteins, where fusion of EGFP to the MSRA C-terminus ensures a free N-terminus of the enzyme, in A-10 cells. As previously described, rat MSRA containing the mitochondrial targeting sequence indeed is localized in mitochondria (Figure 5), albeit cytosolic and nuclear MSRA-EGFP was found in some cells (supplementary Figure 2). For the new variant MSRA2a, the alternative splicing event occurred at the level of the second exon and the presence of the mitochondrial targeting sequence indicated that an MSRA2a-EGFP fusion protein also can be targeted to mitochondria. After over-expression of

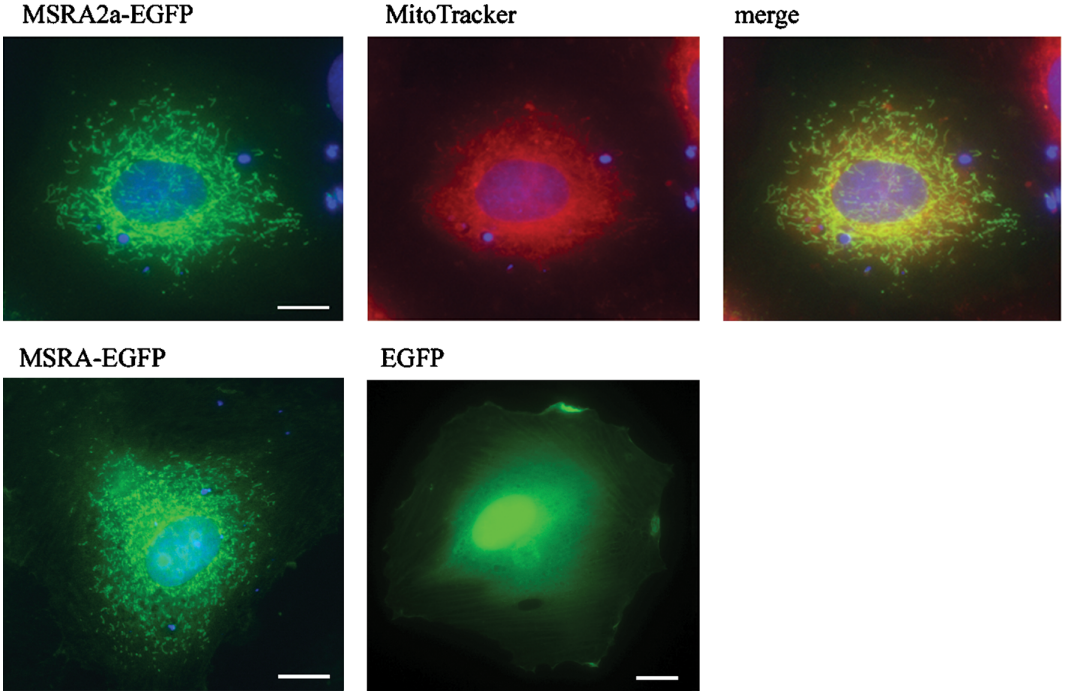


Figure 5. Mitochondrial localization of rat MSRA2a splice form in A-10 cells (top panel). The EGFP signal of a transiently over-expressed MSRA2a-EGFP fusion protein (top, left) co-localizes with the mitochondrial marker MitoTracker red (top, middle). As previously described for human MSRA [35], the rat MSRA-EGFP fusion protein is localized to mitochondria, too (bottom, left). Control, EGFP (bottom, right); scale bar: 25 μm.

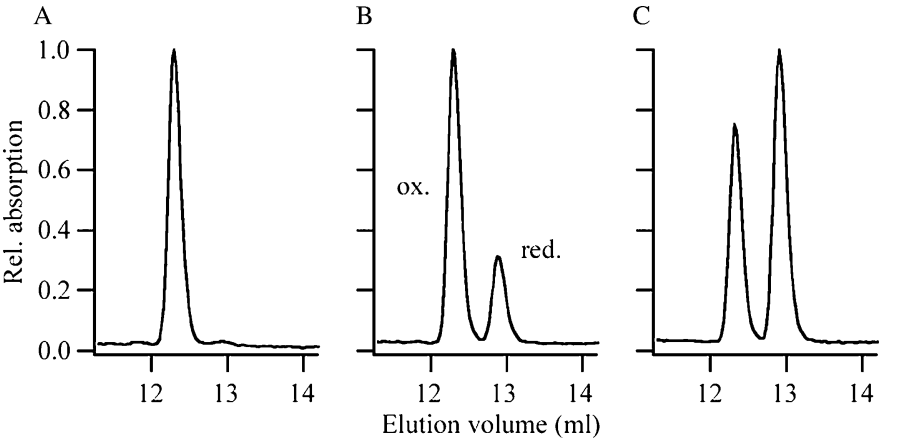


Figure 6. Activity assay of MSRA2a and MSRA splice forms, determined by measuring changes in the elution of a model substrate peptide by reverse-phase HPLC. (A) Oxidized peptide incubated without MSRA is measured as a single peak. The newly identified splice form MSRA2a has reductase activity (B), although the amount of reduced peptide (right peak) is smaller compared to the MSRA first identified (C; right peak).

MSRA2a-EGFP in A-10 cells, the fusion protein indeed was predominantly localized in mitochondria around the nucleus, confirmed by co-localization with the mitochondrial dye MitoTracker red (Figure 5). Likewise, MSRA2a-EGFP can be found in mitochondria when over-expressed in the rat liver cell line *clone 9*, a cell type that possesses endogenous MSRA in the cytoplasm (supplementary Figure 3).

Activity of MSRA2a splice form

We prepared the recombinant MSRA2a form and compared its catalytic activity with recombinant rat MSRA. The activity of the recombinant MSRAs was determined by measuring changes in the elution of a model substrate peptide by reverse-phase HPLC. The results showed that MSRA2a is enzymatically active (Figure 6B). However, the amount of peptide reduced by MSRA2a during a fixed incubation period was significantly smaller than the amount of peptide reduced by MSRA (Figure 6C).

Identification of msrA2a transcripts in rat tissues

In addition to its presence in the A-10 cell line, the *msrA2a* transcript was expressed in other rat tissues. We detected strong expression of *msrA* transcripts in the isolated rat aorta and, as described by Petropoulos et al. [16], in brain, liver and kidney by RT-PCR amplification (Figure 7). The amplified PCR product contained a mixture of *msrA* and the new splice form *msrA2a*. Even though *msrA* is the predominant variant, *msrA2a* was detectable in a nested PCR step using a primer for selective amplification of this splice form (Figure 7). The 153-bp PCR product obtained, covering the 5′-end of the insertion and partially the fourth exon, was ubiquitously present in all the tissue samples examined.

Discussion

Normal vascular function critically depends on a proper redox status of VSMCs. Low levels of ROS are probably essential for VSMC survival but in excess, ROS are clearly damaging [1–4,37]. In fact, many cardiovascular diseases are associated with enhanced oxidative stress caused by excess ROS [11,38]. To prevent oxidative damage, all cells have developed effective systems such as catalase and superoxide dismutase that eliminate excessive reactive species. Enzymatic repair of oxidized proteins by MSRs is another important component in cellular protection against oxidative stress. The results of various studies show that over-expression of the enzyme promotes cellular survival under oxidative stress [24–28].

Here we analysed the endogenous expression of MSR enzymes in VSMCs using the thoracic aorta derived A-10 cell line [34]. This cell line is frequently used as a model to study ROS-dependent signalling in VSMC proliferation [39] as well as mechanisms of oxidative stress-induced apoptosis and antioxidant protection [40–42] in the vasculature. Our study shows a robust expression level of MSRA. Depending on the N-terminal sequence specified by alternatively splicing, the enzyme may be targeted to the cytoplasm or mitochondria when expressed as EGFP fusion in human embryonic kidney HEK293 or monkey kidney CV-1 cells [17,18]. Immunological studies on rat liver cells [20] and human epidermal melanocytes [43] identified the presence of endogenous MSRA in the cytoplasm in addition to mitochondria and nucleus. In rat heart extracts, MSRA activities are detected in both mitochondrial and cytoplasmic fractions [44]. In contrast, our results clearly show that A-10 VSMCs express MSRA preferentially in mitochondria. An immunocytochemical analysis of rat PC12 cells showed a similar

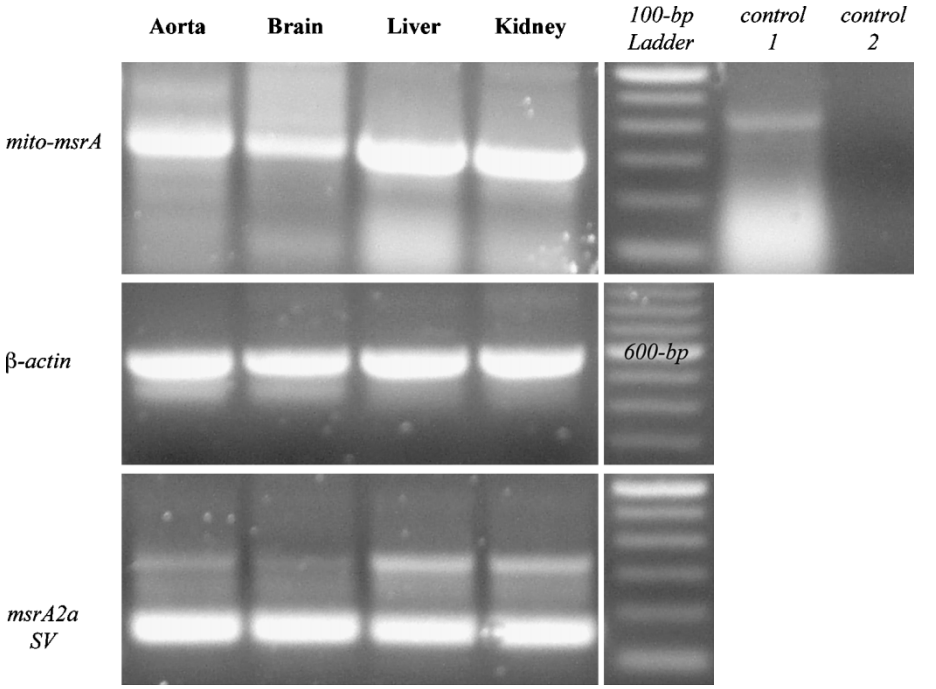
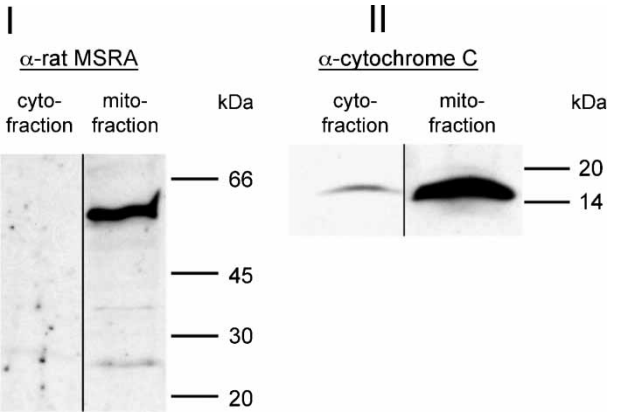


Figure 7. Detection of the *msrA2a* splice form in rat aorta, brain, liver and kidney tissue. In each tissue a 330-bp RT-PCR fragment of *msrA*, covering exon 1 to exon 4 of mitochondrial *msrA* (mito-*msrA*), was amplified (top). Control 1, no reverse transcriptase enzyme; control 2, no RNA. *β*-actin amplification served as internal RT-PCR control (middle). For each sample a 153-bp fragment of the *msrA2a* splice form (*msrA2a* SV) can be amplified in a nested PCR with a splice form-specific primer (bottom). The larger band at ~330-bp was identified as the *msrA* template DNA.

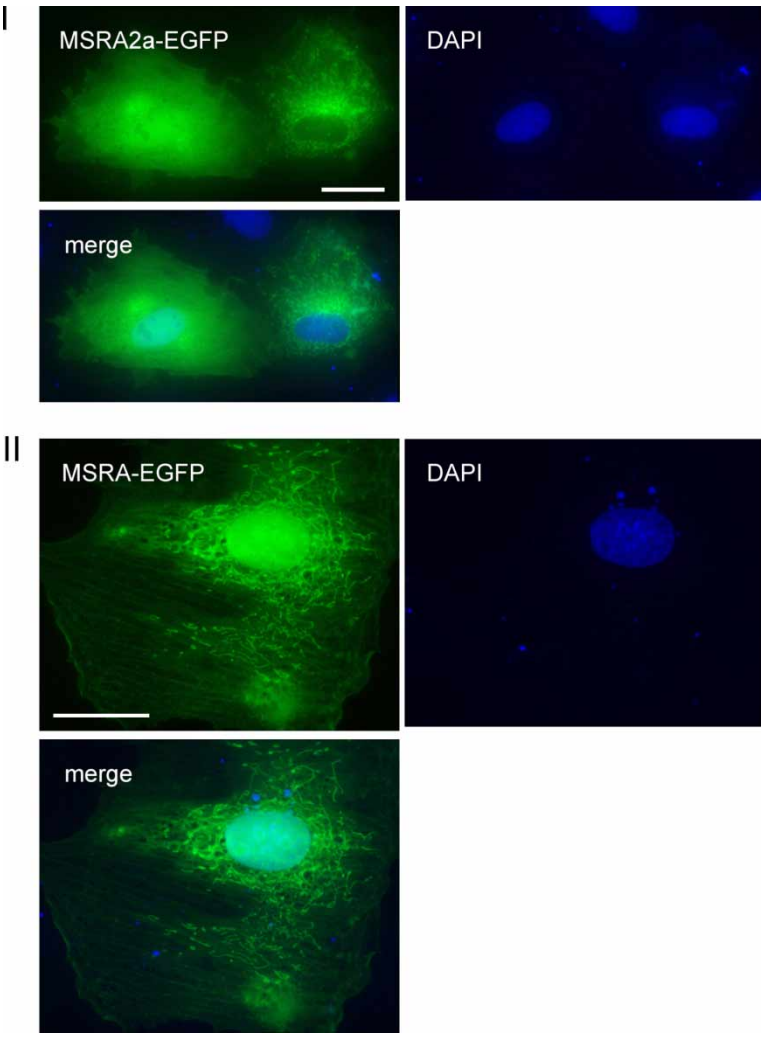
expression of the enzyme specifically in the mitochondrial compartment (unpublished), but a more comprehensive survey on the sub-cellular localization of MSRA among different cell types will be of interest.

Mitochondria represent one of the primary sources of cellular ROS and the exclusive presence of MSRA in A-10 mitochondria is probably related to MSRA-mediated repair/protection of mitochondrial proteins. In comparison, the apparent lack of MSRA in the cytoplasm raises the important question of how cytoplasmic proteins are repaired/protected. An alternative mechanism must exist. One such possibility is that MSRB1 (selenoprotein R) [45,46], which reduces met-R-O, is present in the cytoplasm of A-10 cells in a large quantity (Figure 2) and may compensate for the apparent lack of MSRA. This MSRB compensation possibility does not, however, address how met-S-O is repaired. Possibly, the cytoplasm may somehow generate met-R-O exclusively and MSRA is not required. Alternatively, there may be a mechanism to convert met-S-O to met-R-O.

The 28-residue insertion close to the targeting sequence in the newly identified MSRA2a variant does not disrupt the mitochondrial targeting of the enzyme in A-10 cells, indicating that the overall structure of the N-terminus remains intact. In some cells, over-expressed MSRA-EGFP and MSRA2a-EGFP were prominently found in the cytosol and nucleus, as previously reported for mouse MSRA [21]. An interaction of the flexible N-terminal domain [22] with the central core may be responsible for the extra-mitochondrial localization [21]. In comparison, our immunological analysis of endogenously expressed MSRA did not detect comparable levels of the enzyme in the A-10 cytoplasm. Thus, there may exist additional factors, such as alternative folding mechanisms, which differentially control the



Supplementary Figure 1. Detection of MSRA in fractionated A-10 cell lysates. (I) Western blotting showed the appearance of MSRA dimer (52-kDa) and to a lesser extend MSRA monomer (26-kDa) in the mitochondrial fraction only, but the protein was not detectable in the cytosolic fraction. Fractionation of lysates (ApoAlert Cell Fractionation Kit, Clontech) was verified by the detection of cytochrome C, a mitochondrial protein in non-apoptotic cells (II).

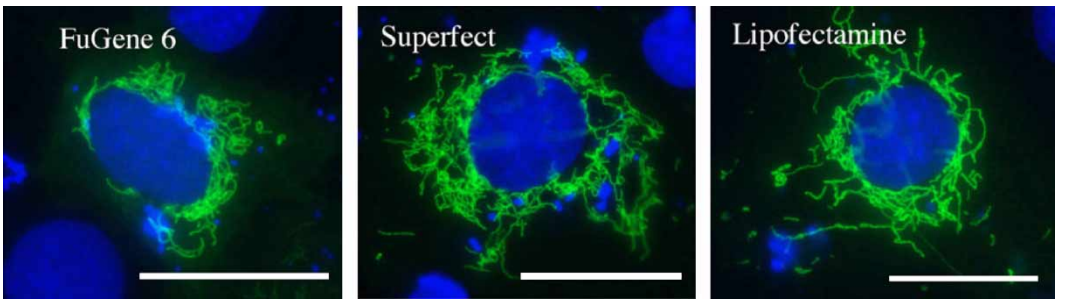


Supplementary Figure 2. Localization of over-expressed rat MSRA2a-EGFP and MSRA-EGFP in A-10 cells showing multiple localizations of the EGFP fusion protein. In (I) two cells that were transfected with MSRA2a-EGFP are shown and the fusion protein is localized either in the cytosol and nucleus (left cell), or exclusively restricted to mitochondria (right cell). (II) Nuclear and mitochondrial distribution of over-expressed MSRA-EGFP in a single A-10 cell. Cytosolic-nuclear as well as selectively mitochondrial localization patterns were found for this construct in subsequent analyzed cells, too (data not shown). Scale bar: 50 μm.

intracellular targeting of endogenous and over-expressed MSRA.

The presence of the *msrA2a* transcript in rat aorta as well as in brain, liver and kidney tissue suggests that this new variant may have a physiological role in a wide variety of tissues. Sequence comparison with published MSRA from a variety of organisms, including *N. meningitidis*, *E. coli*, *Arabidopsis*, yeast, *Drosophila* and human, revealed that the described splice form has not been identified in other organisms. On the genomic level, we identified a corresponding sequence with homology to the alternative exon 2a within the mammalian *msrA* genes of mouse (90.9% identity), Canis lupus (66.7% identity), Pan troglodytes (71.1% identity) and human (70% identity) (chromosomal positions are listed in supplementary materials), indicating that this sequence is conserved among distantly related mammalian species. It would be interesting to examine, e.g. by RT-PCR amplification, if this sequence becomes alternatively spliced in various species.

The splice insertion described here is unlikely to alter the overall structure of the MSRA protein [23]. A main feature of the MSRA structure are the N- and C-terminal ends that do not comprise secondary-structural elements and form coils that interact with the catalytic centre-containing core [23]. It is assumed that the coiled structure of the N-terminus is caused by frequently occurring proline residues [23] and the insertion in MSRA2a also contains numerous prolines, representing the most abundant amino acid (18%) encoded by the alternatively spliced exon. Based on the structure of *E. coli* MSRA, we hypothesized that an insertion at the N-terminus is unlikely to disrupt the catalytic activity of the enzyme and compared MSRA and MSRA2a enzymatic activity. Indeed, MSRA2a reduced met-S-O in a model substrate, although the substrate turnover rate was



Supplementary Figure 3. Mitochondrial localization of over-expressed MSRA2a in rat liver cells (clone 9). Transfection of liver cells using three different transfection reagents results in mitochondrial localization of the MSRA2a-EGFP fusion protein. Scale bar: 25 μm.

lower than for MSRA. This finding is consistent with those of previous studies using human and *E. coli* MSRA that demonstrated that MSRA with a deleted N-terminus remains enzymatically active [35,47].

The inserted sequence shows some similarity to the N-terminus of a bacterial oxidoreductase. Although the function of this motif is yet to be discovered, it may act as a regulatory domain. Alternatively, the N-terminal domain is known to be important for substrate affinity of the enzyme [21] and the insertion may affect the substrate specificity of MSRA2a. However, experimental evidence confirming a substrate specificity of MSRA2a is not yet available and further studies of the three-dimensional structure, determining its N-terminal folding and putative effects on substrate binding, are necessary.

Two of the newly identified transcripts contain stop codons within the alternatively spliced exon. They would be translated into a truncated MSRA peptide without reductase activity. Other, likely enzymatically inactive, MSRA splice forms were also identified by Kim and Gladyshev [18]. Recent reports identified a decline in MSRA activity in tissues of aged rats [16]. Transcriptional regulations that promote splicing of truncated MSRA forms may contribute to this decline. Since different cardiovascular diseases, like atherosclerosis or hypertension, are caused by oxidative damage to VSMCs and the cells may rely on a continuous anti-oxidative protection by MSRA, it is of interest to explore the regulatory mechanisms that control splicing of *msrA* transcripts.

Supplemental material

The nucleotide sequences of the reported splice forms have been submitted to GenBank under accession numbers DQ989019 (rat *msrA2a* mRNA, partial fragment), DQ989020 (rat *msrA2b1*, partial fragment) and DQ989021 (rat *msrA2b2*, partial fragment).

Chromosomal positions of exon 2a homologue sequences in inspected mammalian *msrA* genes: mouse, GenBank: NC_000080 (chromosome 14, nucleotides 103.118 to 103.202); Canis lupus, GenBank: NC_006607 (chromosome 25, nucleotides 116.260 to 116.355); Pan troglodytes, GenBank: NW_001240294 (chromosome 8, nucleotides 113.300 to 113.386); human, GenBank: NC_000008 (chromosome 8, nucleotides 112.804 to 112.890).

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