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Biochemical and Biophysical Research Communications 342 (2006) 145–152
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www.elsevier.com/locate/ybbrc

Functioning methionine sulfoxide reductases A and B are present in human epidermal melanocytes in the cytosol and in the nucleus

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Received 4 January 2006
Available online 3 February 2006

Abstract

Oxidation of methionine residues by reactive oxygen (ROS) in protein structures leads to the formation of methionine sulfoxide which can consequently lead to a plethora of impaired functionality. The generation of methionine sulfoxide yields ultimately a diastereomeric mixture of the *S* and *R* sulfoxides. So far two distinct enzyme families have been identified. MSRA reduces methionine *S*-sulfoxide, while MSRB reduces the *R*-diastereomer. It has been shown that these enzymes are involved in regulation of protein function and in elimination of ROS via reversible methionine formation besides protein repair. Importantly, both enzymes require coupling to the NADPH/thioredoxin reductase/thioredoxin electron donor system. In this report, we show for the first time the expression and function of both sulfoxide reductases together with thioredoxin reductase in the cytosol as well as in the nucleus of epidermal melanocytes which are especially sensitive to ROS. Since this cell resides in the basal layer of the epidermis and its numbers and functions are reduced upon ageing and for instance also in depigmentation processes, we believe that this discovery adds an intricate repair mechanism to melanocyte homeostasis and survival.

Keywords: Reactive oxygen species; Methionine sulfoxide reductases; Thioredoxin reductase; Epidermal melanocytes

The human skin with its outer layer constantly combats as a first line of defence against oxidative stress due to exogenous and endogenous generation of reactive oxygen species (ROS) from oxygen produced as superoxide anion, hydrogen peroxide, hydroxyl radicals and singlet oxygen, peroxynitrite anion, nitric oxide as well as organic peroxides and hydroperoxides by physical, chemical, and biological reactions. Proteins, membrane lipids, carbohydrates, and nucleic acids are all subject to cellular damage [1,2]. In this context, it has been shown that several amino acids such as histidine, proline, arginine, cysteine, methionine and tryptophan are especially susceptible to hydroxyl radicals which are produced from hydrogen peroxide via the Haber–Weiss as well as the Fenton reaction, yielding oxidation of those residues and consequently fragmentation of the protein structure, cross-linking, and proteolytic digestion [2]. ROS are a major source for nuclear and mitochondrial DNA strand breaks/damage. Moreover, ROS have been implied in mutagenesis and carcinogenesis as well as in ageing [3].

However, it is also important to notice that oxidative stress only occurs if the rate of ROS production exceeds its removal. Consequently there is a need for powerful defence mechanisms to keep the redox status and cellular homeostasis under fine control. Defence strategies include enzymes such as the superoxide dismutases, catalase, glutathione reductase/glutathione glutathione peroxidases, thioredoxin/thioredoxin reductase, and the thioredoxin peroxidases. Moreover, small molecules such as thiols, vitamins C and E, the amino acids methionine and

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0006-291X/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2006.01.124

tryptophan, 6 and 7 tetrahydrobiopterin and lipoic acid as well as the metalloid selenium play an important role [4]. Besides the absorbing effects of skin pigment, the biopolymer melanin functions as a significant radical trap. In this context, it is well recognised that a correlation between skin colour and individual free radical defence exists because darker skin photo types (Fitzpatrick classification) express significantly more effective trapping mechanisms [4,5].

It has been shown that hydrogen peroxide (H_2O_2) can oxidise the sulfur containing methionine and cysteine residues in protein sequences leading to disruption of protein and peptide structures and in turn to dysfunction [6]. Examples for deactivation of enzymes due to structural alteration of their active sites include amongst others catalase, dihydropteridine reductase, and acetylcholinesterase [7–10]. Within the protein structure, methionine and cysteine are the most sensitive residues for oxidation by H_2O_2 and this chemical oxidation yields roughly equal concentrations of the two diastereomers (*R*) and (*S*) of methionine sulfoxide and the formation of intra- and intercellular disulphide bridges.

MSRA specifically reduces methionine sulfoxide (*S*), whereas MSRB reduces methionine sulfoxide (*R*) [11,12]. Recently MRSB proteins were identified in various organisms including mammals [13–15] and to date three MSRB proteins were found and designated. MSRB1 is a selenocysteine (Sec)-containing enzyme and is designated as SelR. It has been found in mammals in the cytosol as well as in the nucleus [16,17]. Because of the presence of Sec in its active site, it exhibits the highest enzyme activity amongst all of its isoenzymes [11]. MSRB2 is designated as CBS-1 because cys is present in place of Sec and it resides in mitochondria. It also has only been described in mammals [13]. Two splice variants have been discovered in MSRB3 (MSRB3A and B3B) [11]. All MSRB isoenzymes are zinc-containing proteins [11].

The recent discovery of epidermal methionine-*S*-sulfoxide reductase A (MSRA) and methionine-*R*-sulfoxide B (MSRB) added a major repair mechanism for methionine sulfoxide reduction to the pool of defence mechanisms against ROS induced damage in human skin [18,19].

Interestingly, both sulfoxide reductases utilise the same NADPH driven thioredoxin reductase/thioredoxin pathway as electron donor [1].

Since epidermal melanocytes express only low catalase levels [20] which would imply a special sensitivity to H_2O_2 generation and accumulation, we here asked the question whether these cells possess the thioredoxin reductase/thioredoxin/methionine-*S*–*R* sulfoxide reductases cascade as repair mechanisms for methionine sulfoxide formation.

In this report, we present evidence that human epidermal melanocytes do indeed transcribe and translate both methionine sulfoxide reductases as well as thioredoxin reductase in their cytosol and in the nucleus. Moreover, we present MSR enzyme activities in cytosolic as well as in nuclear extracts of these cells. In conclusion epidermal melanocytes have the capacity to protect their methionine proteins against ROS induced stress.

Materials and methods

Cell cultures. In order to establish human epidermal melanocyte cell cultures full thickness skin received as surgical waste from routine cosmetic face-lifts and breast reduction was utilised. Briefly, after removal of fat, the skin was washed in a solution containing 5% penicillin/streptomycin (P/S) and 5% fungisone followed by dispase treatment (Roche Applied Science, Roche Diagnostics Corporation, Indianapolis, USA) for 12 h at 4 °C. The epidermis was peeled off and placed into sterile phosphate buffered saline (PBS) and then trypsinised (1× trypsin/EDTA (TE), Sigma) for 10 min at 37 °C. The cell suspension was centrifuged at 1000*g* and re-suspended in MCDB154 medium (Cascade Biologics, Mansfield, Nottinghamshire, UK) containing 1% P/S and 0.03 M calcium and finally seeded into T_{225} flasks (Scientific Laboratory Supplies, Nottingham, UK).

In order to establish pure melanocyte cultures from mixed cell population, cells were grown to 70–80% confluence, followed by sequential trypsination and finally seeded in MCBD154 medium containing melanocyte supplements (Cascade Biolobics, Mansfield, Nottinghamshire, UK). For immunofluorescence studies, melanocytes were seeded into chamber slides (Nalge Nunc International, Naperville, Illinois) and cultured for 48 h, followed by removal of the medium and a three times wash in sterile PBS, pH 7.4. Finally cells were fixed in ice cold methanol for 5 min prior to the staining procedure. For the experiments reported herein we established three primary melanocyte cultures from three different donors.

Preparation of cytosolic and nuclear extract from melanocyte cell cultures. Cytosolic and nuclear extracts were prepared from primary melanocyte cell culture (passage 3) using a nuclear extraction kit (Active Motif, Rixensart, Belgium) following the manufacture's instructions. Briefly, medium was aspirated from the flasks and washed with ice cold PBS containing phosphatase inhibitors. Cells were gently detached and centrifuged at 1000*g* for 5 min. The cell pellet was resuspended in hypotonic buffer and incubated for 15 min on ice, followed by thorough mixing and finally centrifuged at 1400*g* for 30 s. The supernatant contained the cytosolic cell extract. The protein content was determined at the $OD_{280\ nm}$ using the method of Kalb and Bernlohr [21].

From the pellet the nuclear extract was obtained following the manufacturer's instructions. The purity of the nuclear extract was tested by utilising the method of Howell et al. [22]. The protein content was determined as described above.

Human skin biopsies. After signed consent, full thickness skin 3 mm punch biopsies were taken under local anaesthesia from the inner proximal arm from healthy human volunteers ($n = 3$; skin phototype III, Fitzpatrick classification) [5] followed by embedding in optimal cutting temperature compound (OCT) (Sakura, RA Lamb, Eastbourne, UK) and cut in 4–5 μm sections using a Leica CM1800 or Leica CM3050 S cryostat. Sections were placed onto poly-L-lysine coated slides and stored at −80 °C until further use.

This study was approved by the local Ethics Committees and was in agreement with the principles of the Helsinki declaration.

In situ double immunofluorescence studies. Frozen slides were air dried for 60 min at room temperature (RT), fixed in ice cold methanol for 6 min, and blocked in 10% normal donkey serum (NDS, Jackson Immuno Research Laboratories, Cambridgeshire, UK) for 90 min at RT followed by a 5-min wash in PBS. To demonstrate the presence of MSRB protein expression we used a monoclonal mouse anti-human antibody (Autogen Bioclear UK, Calne, Wiltshire, UK) diluted 1:50 in 1% NDS and incubated overnight at 4 °C. In order to detect MSRA protein we utilised a polyclonal rabbit anti-human antibody (Autogen Bioclear UK, Calne, UK) diluted 1:50 in 1% NDS and incubated for 3 h at RT. For detection of thioredoxin reductase on human epidermal melanocytes and keratinocytes a rabbit anti-human thioredoxin reductase antibody (Upstate, Dundee, UK) at the dilution 1:20 and incubation overnight at 4 °C was utilised. In addition we used for the detection of melanocytes either the

melanocyte specific mouse anti-gp100 protein antibody (NKI beteb, Monosan Antibodies, Buckinghamshire, UK) in a dilution 1:20 and incubation overnight at 4 °C or a polyclonal goat anti-tyrosinase antibody (Santa Cruz Biotechnology, Inc. Santa Cruz, CA) in a dilution of 1:20 and incubation overnight at 4 °C. Briefly, sections were blocked for 60 min with 10% normal donkey serum (NDS) in phosphate buffered saline (PBS, pH 7.4). Subsequently, specimens were incubated with the above primary antibodies. The slides were washed in PBS 4× for 5 min. Sections were air dried followed by incubation at RT for 1hr with a fluorescent secondary antibody (Jackson Immuno Research Laboratories, Cambridgeshire, UK) in a dilution of 1:100 fluorescein isothiocyanate conjugated donkey anti-rabbit (FITC) for MSRB and NKI beteb. Tetramethyl rhodamine isothiocyanate conjugated donkey anti-mouse (TRITC) was utilised for detection of MSRA, thioredoxin reductase, and tyrosinase. After incubation with the secondary antibody slides were three times washed in PBS. Finally the slides were dried and mounted in Vectashield Mounting Medium containing 4,6-diamino-2-phenylindole (DAPI) (Vector Laboratories, CA, USA) and viewed under a Leica DMIRB/E fluorescence microscope (Wetzlar, Germany). Images were captured using a colour video camera coupled to a computer using Image Grabber PCI graphics program (Optivision, Osset, West Yorkshire, UK). In addition, some pictures were captured using a Nikon Eclipse 80i microscope with a DS-U101 Nikon camera coupled to a Nikon ACT-2U capture program (Nikon, Europe).

Paint Shop Pro™ 7 was utilised to merge the two different fluorochromes in order to follow possible co-localisation.

In vitro immunofluorescence labelling of melanocytes. In order to detect immunoreactivity in melanocytes we used the same procedure as described above but melanocytes were incubated overnight at 4 °C using a dilution 1:500 of both methionine reductase antibodies. Thioredoxin reductase was used in the dilution 1:20 and cells were incubated overnight at 4 °C. The expression of MSRA and thioredoxin reductase was visualised after incubation with TRITC labelled secondary antibody, while MSRB was labelled with FITC labelled antibody using the same conditions as described above.

RNA isolation and reverse transcription polymerase chain reaction (RT PCR). Total RNA was isolated from primary melanoctye cell culture (passage one). Cells were harvested with trypsin and pelleted by centrifugation at 100*g* for 5 min. The total RNA was isolated using the Qiagen RNeasy Mini Kit (Qiagen Ltd., Crawley, West Sussex, UK) following the manufacture's instruction. The yield of isolated RNA was determined by measuring the at $OD_{260\ nm}$. An aliquot of 1 μg total RNA was incubated at 70 °C for 10 min. For reverse transcription of total RNA the Promega Reverse Transcription System (Promega, Southampton, UK) was utilised. The primers for all enzymes were designed based on the published primer sequences by Marchetti et al. [15]. The primers for thioredoxin reductase were based on the Gene Accession No. G29720. All primers are listed in Table 1.

The basic reaction mixture contained 10× PCR buffer (Life Technologies, Paisley, UK) 1×, dNTP (Promega, Southampton, UK) 0.2 mM, melanocyte cDNA 2 μl, *Taq* Polymerase (Promega, Southampton, UK) 0.05 U/μl and nuclease free water (Promega, Southampton, UK) in a final volume of 50 μl. The specific conditions were for

MSRA: $MgCl_2$ (Life Technologies, Paisley, UK) 0.5 mM, PCR conditions were 5 min at 95 °C, 1 min at 95 °C, 1 min at 54.8 °C, 30 s at 72 °C for 35 cycles, and10 min at 72 °C, 4 °C.

MSRB1: $MgCl_2$ of 1 mM, PCR conditions were 5 min at 95 °C, 1 min at 95 °C, 1 min at 49.5 °C, 30 s at 72 °C for 35 cycles, and 10 min at 72 °C, 4 °C.

MsrB2: $MgCl_2$ 1.5 mM, PCR conditions were 5 min at 95 °C, 1 min at 95 °C, 1 min at 51.9 °C, 30 s 72 °C for 35 cycles, and 10 min at 72 °C, 4 °C.

MsrB3: $MgCl_2$ 1.5 mM, PCR conditions were 5 min at 95 °C, 1 min at 95 °C, 1 min at 54.4 °C, 30 s at 72 °C for 35 cycles, and 10 min at 72 °C, 4 °C.

Thioredoxin reductase: $MgCl_2$ of 2 mM, PCR conditions were 5 min at 92 °C, 1 min at 92 °C, 1 min at 60 °C, and 1 min at 72 °C for 35 cycles.

PCR products were analysed in an agarose gel electrophoresis in Tris-acetate–EDTA (TAE) buffer (0.04 M Tris, 0.001 M EDTA). Fifteen microlitres sample was mixed in 3 μl of 6× loading dye (Promega, Southampton, UK) and loaded into a gel. A 100-bp DNA ladder (Promega, Southampton, UK) served as standard. Addition of ethidium bromide (Sigma–Aldrich Co., Poole, Dorset UK) to the gel allowed visualisation of PCR products by UV transillumination.

Western blotting. Cellular cell and nuclear extracts from human primary melanocyte cell cultures were separated in 10% SDS–PAGE and proteins transferred to a PVDF membrane (Immobilon™-P Millipore, Bedford, UK). The membrane was blocked with 0.5% gelatine in TBS-T buffer (150 mM NaCl, 20 mM Tris, and 0.047% Tween, pH 7.4) and incubated overnight with anti-human MSRB or anti-human MSRA antibody (both at the dilution 1:2000). For thioredoxin reductase the membrane was blocked with 5% non-fat dried milk for 2 h at RT and incubated with anti-thioredoxin reductase overnight at 4 °C (at the dilution 1.5 μg/ml). The blot was washed and incubated for 1 h at RT with an anti-rabbit immunoglobulin G (IgG) peroxidase-conjugated antibody (Fc specific, dilution 1:5000, Cell Signalling Technology Inc., Beverley, MA). Visualisation of the MSR bands was performed using a modified enhanced chemiluminescence (ECL) fixed on a film sheet (X-OMAT™ Kodak, USA).

Determination of MSRA and MSRB enzyme activities in cytosolic and nuclear extracts from primary epidermal melanocyte cell cultures. In order to determine enzyme activities, we utilised methyl ^{3}H-labelled *S*-adenosylmethionine (SAM, 250 μCi, 81 Ci/mmol, Life Sciences Amersham, Bucks, UK) as starting material. SAM was oxidised for 2 h with a 10-fold excess of H_2O_2 and the product formation was subjected to Silica gel thin layer chromatography (TLC, GF 1000 μM, Merck SG Darmstadt, Germany) using a mixture of isopropanol:formic acid:water as solvent (V/V 20:1:5) at RT. Analysis of 5 μl product yielded four spots after detection with ninhydrin at 110 °C (Sigma–Aldrich, Poole, Dorset, UK). The R_f values were for the unreacted SAM ($R_f = 0$), methionine sulfone ($R_f = 0.31$), methionine sulfoxide ($R_f = 0.52$), and methionine ($R_f = 0.77$). Based on these results the formed ^{3}H labelled methionine sulfoxide and [^{3}H]methionine sulfone were used as substrates for the determination of methionine sulfoxide reductase activities by measuring the formation of ^{3}H labelled methionine in cytosolic and nuclear extracts from epidermal melanocytes (protein content 4.0 mg/ml in Tris/HCl buffer, pH 7.5).

Table 1
PCR-Primers for MSRA, MSRB1, MSRB2, MSRB3, and thioredoxin reductase (TR)

PCR primer	Primer sequence	Length (bp)	Product size (bp)
MsrA forward	5′-AGTACCTGAGCAAGAACCCCA-3′	21	329
MsrA reverse	5′-TCACTCAGACCCCAGAAGACA-3′	21	
MsrB1 forward	5′-GACGTTACACCCTCACCTT-3′	19	328
MsrB1 reverse	5′-AGCTACTTCCGCACAGATT-3′	19	
MsrB2 forward	5′-CAAGGAAGCAGGAATGTATCA-3′	21	308
MsrB2 reverse	5′-ATGGTCAGTGTTTCCTTGGTTT-3′	22	
MsrB3 forward	5′-CCGGGTCGTGTAGGGATAAA-3′	20	328
MsrB3 reverse	5′-TGAGCACCACACTGAGAGCA-3′	20	
TR forward	5′-ACATGGAAGAACATGGCA-3′	18	479
TR reverse	5′-CTCCTCAGAAAGGCCACAAG-3′	20	

The reaction mixture contained 100 μl cell extract (0.4 mg), 10 μl NADPH (1 mM), 10 μl thioredoxin reductase from *Escherichia coli* (0.07 U) (Sigma–Aldrich, Poole, Dorset, UK) 50 μl thioredoxin from *E. coli* (0.35 U) (Sigma–Aldrich, Poole, Dorset, UK), 10 μl cold methionine sulfoxide (Sigma–Aldrich, Poole, Dorset, UK) containing either 700 μmoles for MSRA or 200 μmol for MSRB and 10 μl of ^{3}H labelled methionine sulfoxide/methionine sulfone in a 5:1 ratio. Reaction rates were measured after incubation at 10, 20, 30,40, and 60 min in 5 μl of product at each time point using the above TLC system. Products were detected by ninhydrin/110 °C development. The corresponding methionine and methionine sulfoxide spots were scraped and counted in scintillation fluid (Ready Safe, Sigma Aldrich, Poole, Dorset, UK) in the ^{3}H channel in a Tri Carb 2100 TR Packard scintillation counter.

Calculation of the formed [^{3}H]methionine and the remaining [^{3}H]methionine sulfoxide in μmol was based on cpm/μmol in the reaction mixture allowing to distinguish between the formed [^{3}H]methionine and the unreacted [^{3}H]methionine sulfoxide in the examined extracts.

Results

Human epidermal melanocytes express both MSRA and MSRB and their electron donor thioredoxin reductase in situ

The expression of MSRA is shown in Fig. 1A, a confirming the result of Ogawa et al. [18]. The enzyme is expressed throughout the entire epidermis as well as in the stratum corneum. In order to detect MSRA in melanocytes in situ we employed the melanocyte specific gp100 protein to follow the expression of MSRA in these cells (Fig. 1A, b). The overlay of both images reveals the presence of this enzyme in some but not all melanocytes in situ. (Fig. 1A, c). MSRB is also distributed throughout the epidermis. (Fig. 1B, a) In order to demonstrate the presence of this enzyme in melanocytes, we used a tyrosinase antibody to overcome the host incompatibility (Fig. 1B, b). The overlay of both protein immunoreactivities indicates a strong co-localisation of MSRB and tyrosinase supporting the presence of this enzyme in melanocytes in situ (Fig. 1B, c). Since thioredoxin reductase/thioredoxin is the electron donor for both enzymes, we wanted to show the presence of this enzyme in these cells. Thioredoxin reductase is also expressed throughout the epidermis (Fig. 1C, a). Co-localisation with NKIbeteb shows the presence of the enzyme in melanocytes (Fig. 1C, b/c). For negative control the antibodies were omitted (data not shown).

Human melanocytes express both MSRA/MSRB and thioredoxin reductase under in vitro conditions in the cytosol and in the nucleus

The expression of both methionine sulfoxide reductases is demonstrated in Fig. 2a/b. Both enzymes are expressed in the cytosol with much stronger granular expression in the nucleus. Co-expression of both proteins is very pronounced in the nucleus (Fig. 2c). Thioredoxin reductase is expressed in the cytosol and in the nucleus. The expression is also much more pronounced in the nucleus (Fig. 2d). To prove specificity of the immunoreactivity the antibody was omitted(data not shown).

RT-PCR confirms the presence of mRNA for both reductases, their isoenzymes, and thioredoxin reductase in melanocytes

The presence of mRNA for MSRA, MSRB1, MSRB2, and MSRB3 in melanocytes is presented in Fig. 3, lanes 2–5. The presence of thioredoxin reductase is shown in Fig. 3, lane 6. The size of all products was in agreement with the expected targets.

Western blot analysis confirms the presence of MSRA, MSRB, and thioredoxin reductase in the cytosol and in the nucleus of melanocytes

Western blot analysis of cytosolic and nuclear extract originating from human primary melanocytes demonstrated the presence of both MSRA and MSRB as well as thioredoxin reductase in the cytosol and in the nucleus (Fig. 4). Since the commercially available MSRB antibody does not distinguish between the isoforms B1, B2, and B3 we cannot prove the presence of any specific MSRB isoform by this methodology.

Cytosolic and nuclear extracts from epidermal melanocytes exhibit methionione sulfoxide reductase enzyme activity

The result of the enzyme assay clearly showed the reduction of ^{3}H labelled methionine sulfoxide back to ^{3}H labelled methionine in the cytosol as well as in the nucleus of epidermal melanocytes. Since the optimal enzyme activity of MSRA requires 700 μmol–1 mmol of substrate, while MSRB activities have their optimum at 200 μmol substrate concentrations and the latter activities are inhibited with 700 μmol substrate, we decided to follow enzyme activities under these two different substrate conditions [11]. The result in the presence of 700 μmol substrate yielded indeed high enzyme activity (Fig. 5A). Moreover, the result showed that only 50% of the substrate was turned over after 30 min which would be in agreement with reduction of only the *S*-diastereomer. These data suggest the presence of a functioning MSRA in the cytosol and in the nucleus of melanocytes (Fig. 5A). In the presence of 200 μmol substrate (the optimum for MSRB) we found significantly slower enzyme activities in the cytosol (Fig. 5B). Moreover, the reaction was not complete after 1 hour. In fact only 30% of the substrate was turned over. This result would favour the presence of active MSRB in the cytosol (Fig. 5B). Unfortunately at this point we cannot distinguish between the contributions of the different MSRB isoforms.

Discussion

The epidermis is especially vulnerable to oxidative stress caused by multiple exogenous stimuli as well as by a plethora of endogenous metabolic events. Over the past many mechanisms have been identified in control of keeping the redox-balance in phase [1,4]. As pointed out by Ogawa

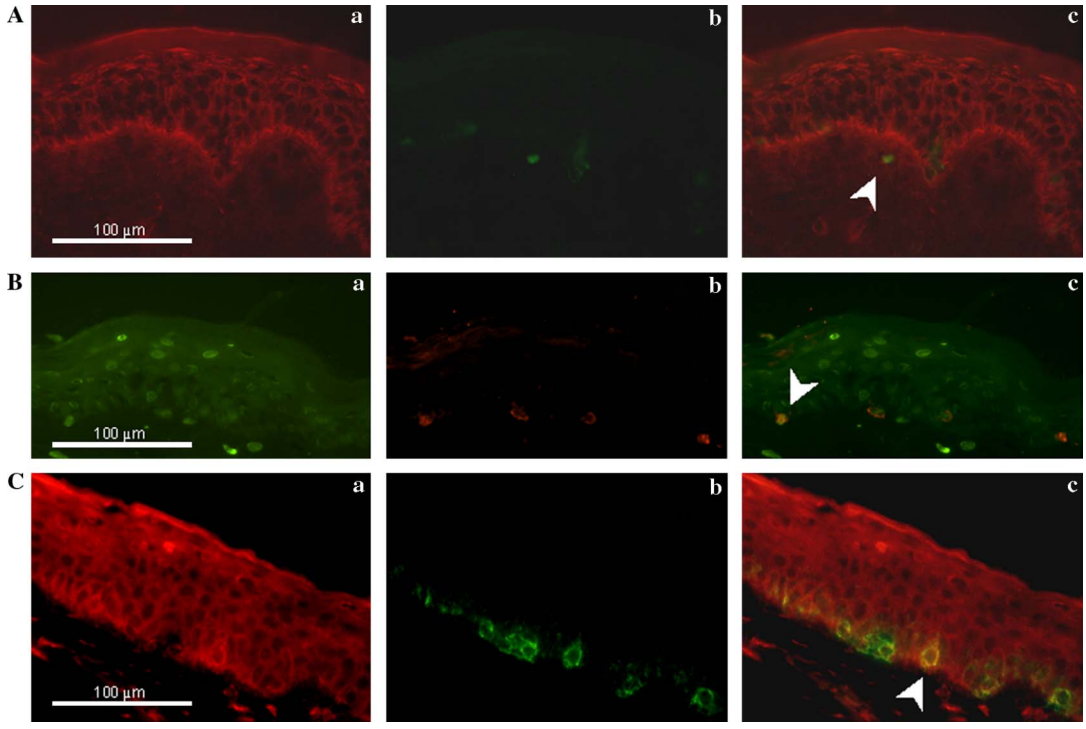

Fig. 1. MSRA, MSRB, and thioredoxin reductase protein are expressed in situ. (A) MSRA: (a) MSRA (TRITC labelling), (b) NKI/beteb (FITC labelling), (c) overlay of a/b showing the expression of MSRA in melanocytes which is indicated by arrow (scale bar: 100 µm). (B) MSRB: (a) MSRB expression (FITC-labelling), (b) tyrosinase expression (TRITC-labelling), (c) overlay of a/b proving the presence of MSRB in melanocytes, arrow (scale bar: 100 µm). (C) Thioredoxin reductase: (a) thioredoxin reductase expression (TRITC-labelling), (b) NKI/beteb expression (FITC-labelling), (c) overlay of a/b demonstrating presence of thioredoxin reductase in epidermal melanocytes, arrow (scale bar: 100 µm).

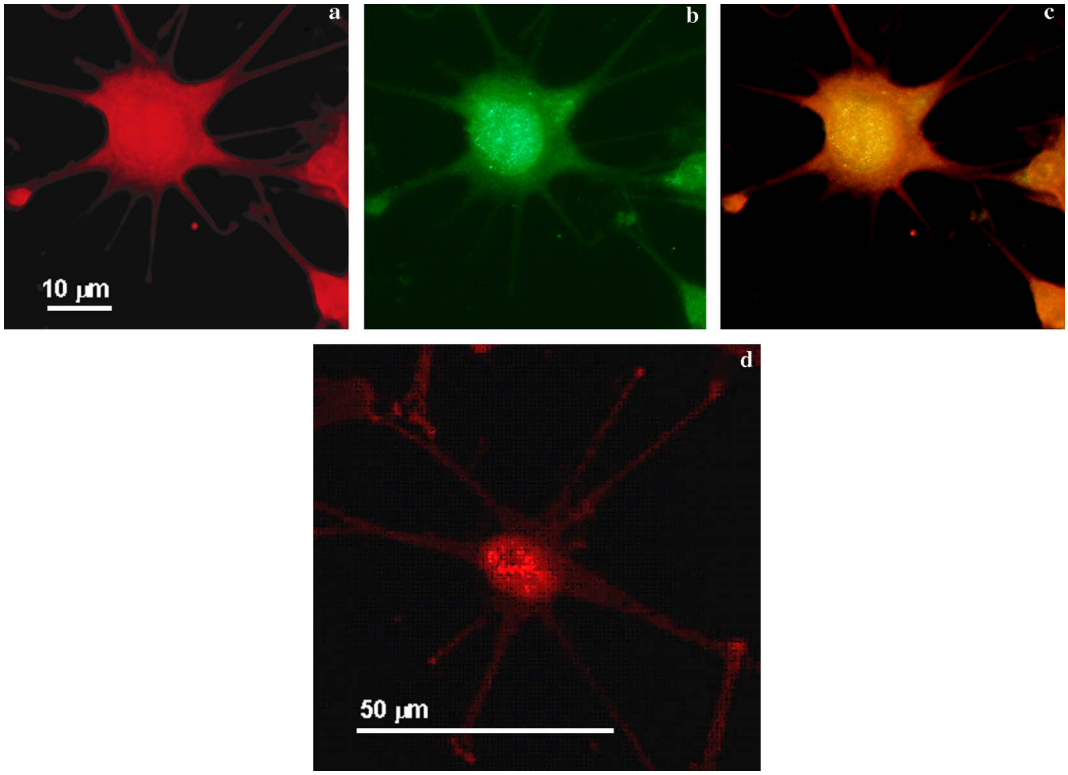

Fig. 2. In vitro expression of MSRA, MSRB, and thioredoxin reductase in human epidermal melanocytes. (a) MSRA (TRITC-labelling), (b) MSRB (FITC-labelling), (c) overlay of a/b. Both MSRA and MSRB are expressed throughout the entire cell with strong staining in the nucleus (scale bar: 10 µm), (d) thioredoxin reductase is expressed mainly in the nucleus in a granular pattern. Much weaker expression of the enzyme is present in the cytosol (scale bar: 50 µm).

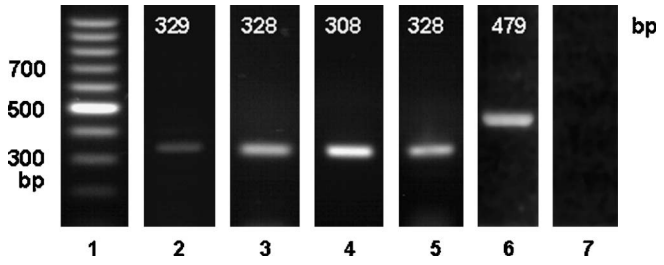

Fig. 3. RT-PCR demonstrates mRNA expression of MSRA, MSRB and its isoenzymes as well as thioredoxin reductase in human epidermal melanocytes. Lane 1, ladder; lane 2, MSRA; lane 3, MSRB1; lane 4, MSRB2; lane 5, MSRB3; lane 6, thioredoxin reductase, and lane 7, negative control. All bands are in agreement with the expected size (indicated on each lane).

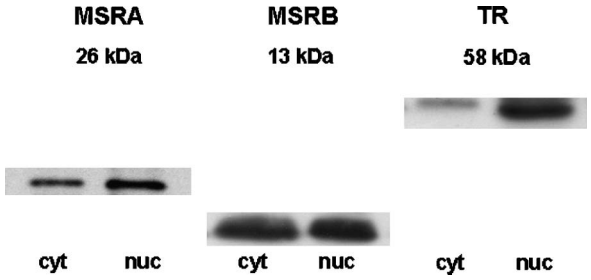

Fig. 4. Western blot analysis confirms the presence of MSRA, MSRB, and thioredoxin reductase in melanocytes in the cytosol and in the nucleus. MSRA was found at the size of 26 kDa in the cytosol and in the nucleus. MSRB was found at the expected molecular weight of 13 kDa in the cytosol and in the nucleus. Thioredoxin reductase was present in the nucleus and in the cytosol and the molecular weight was in agreement with 58 kDa. The protein expression is much weaker in the cytosol compared to the nuclear extract which is in agreement with the in vitro immunoreactivity (Fig. 2D).

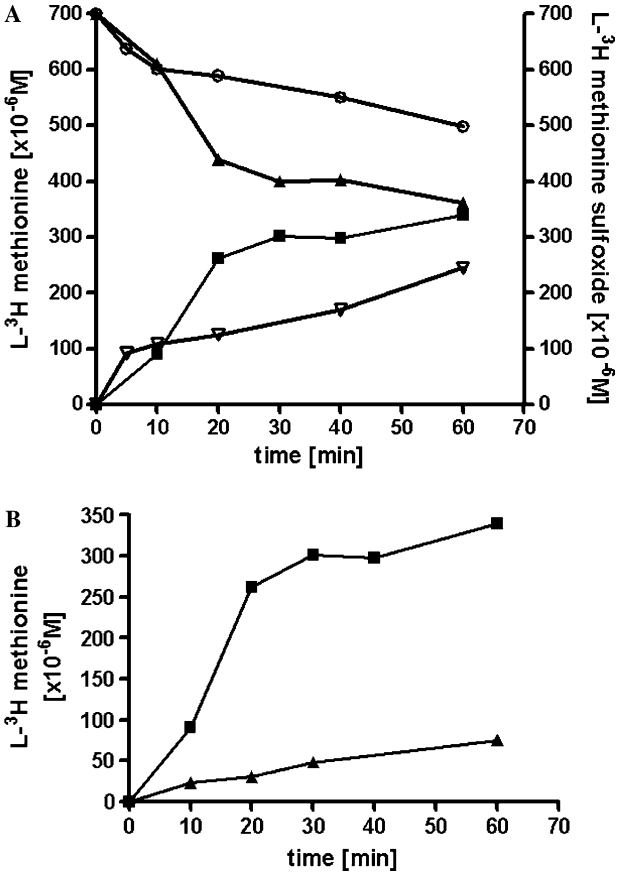

Fig. 5. Methionine sulfoxide reductase activities in the cytosol and in the nucleus of human epidermal melanocytes. (A) Enzyme activities were measured over time in the presence of 700 μmol ^{3}H labelled L-methionine sulfoxide by following the formation of ^{3}H labelled methionine in nuclear (∇) and cytosolic extracts (■) from ^{3}H labelled L-methionine sulfoxide in the same extracts (nuclear ○/cytosol ▲) as outlined in methods. The substrate concentration is optimal for MSRA activity [11]. The conversion of L-methionine sulfoxide to L-methionine shows for the cytosolic enzyme 50% of stoichiometry (■, ▲). For nuclear extracts (∇, ○) the reaction is slower and is not completed after 60 min. (B) Comparative study of cytosolic MSR activities in the presence of different substrate concentrations The formation of the product [L-^{3}H]methionine is much slower in the presence of 200 μmol (▲) compared to 700 μmol (■) substrate. Since the concentration of 200 μmol is the optimum for MSRB activity, this result indicates MSRB activities in the cytosol [11].

et al. [18] and by earlier work from the same group, skin ageing is associated with a decreased capacity to neutralise ROS and repair damaged proteins as well as DNA [6]. In this context, Shinenaga and colleagues showed already in 1994 that mitochondrial stability decreases with age due to lipid peroxidation of mitochondrial membranes by ROS causing the release of cytochrome *c* from the electron transport chain providing a signal for caspase-mediated apoptosis [3]. Very recently, the scenario vis a vis the role of ROS in the mitochondrion has received a great deal of attention in the context of ageing in mammals and it has been shown that overexpression of mitochondrial catalase prolongs life in mice [23]. Moreover, a lower expression of MRSA decreased the lifespan by 40% in these animals, while its overexpression in *Drosophila melanogaster* leads to a 70% increase. Importantly, thioredoxin reductase/thioredoxin (TR/T) functions as the electron donor for both MSRA and MSRB [11,12] and overexpression of this system increased the lifespan by 70% in the murine model [24]. Taking into consideration that the number of epidermal melanocytes, which are neural crest derived cells, are decreasing with age and that their functionality greatly varies from younger skin, we were interested to investigate this specific cell and its possible methionine sulfoxide repair capacity in the light of low catalase levels in these cells as documented earlier [20]. Moreover, since these cells also have autocrine $6BH_4$ synthesis/recycling and since it was shown that dihydropteridine reductase, the last step in $6BH_4$ recycling is subjected to methionine oxidation in its sequence by H_2O_2, it was tempting to investigate whether these cells have functioning methionine sulfoxide reductases [9]. A great deal of information on oxidative stress in the human epidermis has been learned from the depigmentation disorder vitiligo because the epidermis of affected individuals generates continuously H_2O_2 in the 10^{-3} M range by various pathways leading to deactivation of catalase, the enzyme which degrades this ROS to H_2O and O_2 [25]. Only very recently it was shown that not only the porphyrin ring of this enzyme is affected by oxidation, it

was realised that also oxidation of methionine residues affects seriously the assembly of the tetramer of this enzyme [7,8]. Hence, under oxidising conditions and in the absence of efficient repair mechanisms, melanocytes would be a good target for oxidative damage. However, in this context it is fascinating that protein alteration caused by H_2O_2-mediated oxidative stress depends on the concentration of this ROS. At low concentrations it can have a very beneficial effect because it upregulates both enzyme protein expression and activities (for Review see Schallreuter 2005 [26]). Interestingly, the increased transcription/activity of MSRA by UVA-light or by H_2O_2 as reported by Ogawa et al. [18] mimics the transcriptional activation of thioredoxin reductase which together with its electron acceptor thioredoxin provides the electron source for enzyme activities of both MRSA&B [1]. Whether induction/activity of MSRB is also stimulated by this ROS or UV needs to be shown. Clearly to recover oxidised methionines due to the production of both diastereomers (*R*) and (*S*) of methionine sulfoxide by H_2O_2 needs both enzymes.

Since we have found MSRB expression and activity in the cytosol and in the nucleus and taking into consideration that the presence of MSRB1 (Sel 1) has been documented in these compartments, while MSRB2 has been found in the miochondrion and MSRB3 in the endoplasmic reticulum [11], it is tempting to speculate that our results show MSRB1 activities. Further research is needed to clarify the contribution from the different isoforms especially in the light that we detected the mRNA for all three isoforms.

In summary, the discovery of the MSR's in epidermal melanocytes adds yet another intricate repair mechanism for survival of these cells. Whether the reductases themselves are possibly also targets for oxidative stress needs to be shown. However, the presence of methionine sulfoxide repair in melanocytes may shed some more light on melanocyte survival, apoptosis, and cell death under multiple physiological and pathological conditions reaching from aging per se to greying of the hair.

Acknowledgments

This research was kindly supported by Deutsche Vitiligo-Verein Hamburg, Germany and by Stiefel International.

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