Methionine Sulfoxide Reductases A and B Are Deactivated by Hydrogen Peroxide (H_2O_2) in the Epidermis of Patients with Vitiligo

Karin U. Schallreuter[1,2], Katharina Rübsam[2], Nicholas CJ. Gibbons[1,2], Derek J. Maitland[3], Bhaven Chavan[1], Carsten Zothner[1,2], Hartmut Rokos[1,2] and John M. Wood[1]

Patients with the depigmentation disorder vitiligo have low catalase expression/activities and constantly accumulate 10^{-3} M hydrogen peroxide (H_2O_2) in their skin. Such high concentrations of H_2O_2 oxidize L-methionine residues in proteins and peptides to (*R* and *S*)-methionine sulfoxide diasteriomers. *In vivo* FT-Raman Spectroscopy revealed the presence of methionine sulfoxide in the depigmented skin of patients with active vitiligo. In normal healthy human skin, methionine sulfoxide reductases A and B specifically reduce methionine sulfoxides (*S*) and (*R*), respectively, back to L-methionine consequently repairing oxidatively damaged proteins and peptides. In this report, we show that the expression/activities of MSRA and MSRB are significantly decreased in the epidermis of patients with vitiligo compared to healthy controls. Also, we used recombinant human MSRA and MSRB1 to show that both enzymes are deactivated by 10^{-3} M H_2O_2 by 85 and 40%, respectively. Structural modelling based on the crystal structure of human MSRA revealed that the active site of this enzyme is significantly altered after H_2O_2-mediated oxidation of L-methionine, L-tryptophan, and L-cysteine residues in its active site. Taken together, our results confirm that very important anti-oxidant enzymes are seriously affected in acute vitiligo.

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INTRODUCTION

Vitiligo has been recognized as a model disease for hydrogen peroxide (H_2O_2)-mediated oxidative stress (Schallreuter *et al.*, 1999, 2005). Both the epidermis and to a lesser extent the vascular system of patients with active vitiligo have been shown to be affected by the constant accumulation of H_2O_2 (Schallreuter *et al.*, 1991, 1994, 1999, 2006a, 2007b; Maresca *et al.*, 1997; Hasse *et al.*, 2004; Schallreuter, 2005; Grando *et al.*, 2006; Dell'Anna *et al.*, 2007; Spencer *et al.*, 2007a). In proteins and peptides, the sulfur-containing amino acids L-methionine and L-cysteine are especially sensitive to oxidation by H_2O_2 consequently leading to a disturbed redox-balance in the skin of affected individuals (Thiele *et al.*, 1999; Schallreuter and Wood, 2001; Gibbons *et al.*, 2006; Ogawa *et al.*, 2006; Schallreuter, 2006). This oxidation reaction leads to the production of the two diasteriomers of methionine sulfoxide (*R* and *S*) and the formation of inter- and intramolecular disulfide bridges from L-cysteine residues. Under normal healthy conditions, the skin expresses high levels of MSRA and MSRB (B1, B2, B3) and its electron donor system thioredoxin reductase/thioredoxin (TR/T) (Thiele *et al.*, 1999; Schallreuter and Wood, 2001; Ogawa *et al.*, 2006; Schallreuter, 2006, 2006c). This antioxidant electron transfer chain (NADPH/TR/T/MSRA/MSRB) reduces the two diastereomers of methionine sulfoxide back to L-methionine, while NADPH/TR/T also reduces disulfide bridges back to L-cysteine (Nordberg and Arner, 2001). Therefore, these systems are critical for the repair of proteins as well as peptide hormones in the epidermis (Gibbons *et al.*, 2006; Schallreuter, 2006; Spencer *et al.*, 2007a).

The constant accumulation of H_2O_2 in the epidermis in patients with vitiligo is responsible for the oxidation/deactivation of many enzymes, proteins, peptides, and cofactors (Table 1). As MSRA and MSRB are deactivated by the oxidation of methionine-to-methionine sulfoxide (*R* and *S*), the above enzymes, proteins, peptides, and cofactors cannot be repaired. Certainly H_2O_2 leads to severe problems in vitiligo and many causes for its generation in the epidermis have been documented (Schallreuter, 2005). Whether H_2O_2 is the primary cause or the consequence needs yet to be shown.

[1]*Clinical and Experimental Dermatology, Department of Biomedical Sciences, University of Bradford, Bradford, UK;* [2]*Institute for Pigmentary Disorders in Association with EM Arndt University of Greifswald, Germany and University of Bradford, Bradford, UK and* [3]*Department of Forensic Sciences, University of Bradford, Bradford, UK*

Correspondence: Professor Karin U. Schallreuter, Clinical and Experimental Dermatology, Department of Biomedical Sciences, University of Bradford, Bradford, West Yorkshire BD7 1DP, UK.
E-mail: K.Schallreuter@bradford.ac.uk

Abbreviations: HMBC, heteronuclear multiple bond correlation; H_2O_2, hydrogen peroxide; PC-KUS, pseudocatalase Karin U Schallreuter; ROS, reactive oxygen species; TLC, thin layer chromatography; TR/T, thioredoxin reductase/thioredoxin

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Table 1. Enzymes, proteins, peptides, and cofactors oxidized/deactivated by H_2O_2 in vitiligo (Current state of the art)

A	Anti-oxidant
	Catalase (Aronoff, 1965; Schallreuter *et al.*, 1991, 1999; Maresca *et al.*, 2006; Wood and Schallreuter, 2006)
	Thioredoxin Reductase (Schallreuter and Wood, 2001)
	Glutathione Peroxidase (Beazley *et al.*, 1999)
	MSRA/B (Schallreuter *et al.*, 2006c)
	Albumin (Rokos *et al.*, 2004)
B	Pigmentation
	Phenylalanine hydroxylase (Schallreuter *et al.*, 2005)
	Tyrosinase (Wood *et al.*, 2005)
	Tyrosinase related protein (TRP1) (Jimbow *et al.*, 2001)
	Calmodulin-dependent calcium ATPase (Schallreuter *et al.* 2007b)
C	Prohormone convertases and POMC-derived peptides
	Prohormone Convertase 1 (PC-1) (Spencer, 2007)
	Prohormone Convertase 2 (PC-2) (Spencer, 2007)
	PACE-4 (Spencer, 2007)
	Furin (Spencer, 2007)
	α-MSH (Spencer *et al.*, 2007b)
	ACTH (Spencer *et al.*, 2007b)
	β-Endorphin (Spencer *et al.*, 2007b)
D	Enzymes of the neuroendocrine system
	Acetylcholinesterase (Schallreuter *et al.*, 2004)
	Butyrylcholinesterase (Schallreuter *et al.*, 2006b)
E	6-Tetrahydrobiopterin biosynthesis/recycling
	GTP CHI (Chavan and Schallreuter (2007))
	PCD (Schallreuter *et al.*, 2001)
	DHPR (Hasse *et al.*, 2004)
	6-BH_4 (Rokos *et al.*, 2002; Schallreuter *et al.*, 1994)
F	EF-hand calcium binding sites on calmodulin and butyrylcholinesterase (Schallreuter *et al.* (2007a))

MSRA specifically reduces methionine sulfoxide (*S*) meanwhile MSRB reduces methionine sulfoxide (*R*) (Kryukov *et al.*, 1999, 2002; Jung *et al.*, 2002; Kim and Gladyshev, 2004; Neiers *et al.*, 2004). Previously it was shown that MSRA and MSRB (B1, B2, B3) are present in human epidermal keratinocytes and melanocytes *in vitro* with melanocytes > undifferentiated keratinocytes > differentiated keratinocytes (Ogawa *et al.*, 2006; Schallreuter, 2006; Schallreuter *et al.*, 2006c). In this report, we show by NMR Spectroscopy that L-methionine sulfoxide contains a (49:51) mixture of (*R* and *S*) diastereomers. Patients with active vitiligo fail to repair/reduce methionine sulfoxide in their epidermis, where *in vivo* FT-Raman Spectroscopy has been used to identify the S = O stretch at 1,026 cm^{-1} as an indicator for methionine oxidation. However, we found that the loss of expression/activity of MSRA/MSRB can be partially recovered by treating these patients with a narrowband UVB-activated topical pseudocatalase Karin U Schallreuter (PC-KUS), which in turn reduces epidermal H_2O_2 from 10^{-3} to 10^{-6} M (Schallreuter *et al.*, 1999). A comparative study of enzyme activities of the combined methionine sulfoxide reductases in epidermal suction blister extracts from patients with active vitiligo revealed a significant decrease in ^{14}C methionine sulfoxide reduction compared to healthy controls confirming the loss of enzyme activity. This result was confirmed by studies with recombinant human MSRA and MSRB1 yielding loss of enzyme activities reaching from 85 to 40%, respectively, in the presence of 10^{-3} M H_2O_2. Computer simulation of MSRA confirmed disruption of the enzyme active site due to oxidation of Met, Trp, and Cys residues by H_2O_2 in its catalytic domain.

RESULTS

Decreased epidermal MSRA and MSRB expression of patients with vitiligo is caused by H_2O_2-mediated oxidative stress

The expression of MSRA and MSRB in the lesional (vit, $n = 13$) and non-lesional skin (nl, $n = 11$) of patients with vitiligo was studied *in situ* and compared to healthy control subjects ($n = 4$) of the same skin phototype (Fitzpatrick *et al.*, 1971). Levels of both MSRA ($P_{vit} < 0.001$; $P_{nl} < 0.001$) and MSRB ($P_{vit} < 0.001$; $P_{nl} < 0.001$) are significantly decreased. Protein expression improves after reduction of H_2O_2 in the epidermis with a topical application of pseudocatalase PC-KUS (Figure 1a and b). Statistics is based on the 95% confidence interval.

^{1}H NMR – Spectroscopy of the (*R* and *S*) diastereomer mixture of L-methionine sulfoxide

In the ^{1}H NMR spectrum of L-methionine sulfoxide, a *pseudo*-pentuplet at δ 3.70 due to the presence of two partially overlapping triplets for the methine protons on C-2 confirmed the presence of two diastereomers. A set of three multiplets δ 2.74–2.98 (Figure 2a) due to the methylene protons on C-4 with at proton ratio of 1:2:1 clearly confirms the presence of two diastereomers. ^{1}H-^{1}H COSY and ^{1}H-^{13}C HMQC spectra confirmed that the multiplets centered at δ 2.76 and 2.95 were coupled and on the same carbon of a diastereomer. Integration of this region gave a 96:100 ratio of the diastereomers consistent with the slight intensity variation observed within each pair of signals in the ^{13}C NMR spectra. This ability to distinguish in the ^{1}H NMR spectra between the two different diastereomers in the δ 2.74–2.98 region opens up the possibility to confirm enzyme substate specificity for each diastereomer.

The ^{13}C NMR spectra of L-methionine sulfoxide showed the presence of four sets of pairs of signals consistent with the presence of two diastereomers. The peak heights within each pair were not quite equal, suggesting that the diastereomers were not present in exactly the same concentrations.

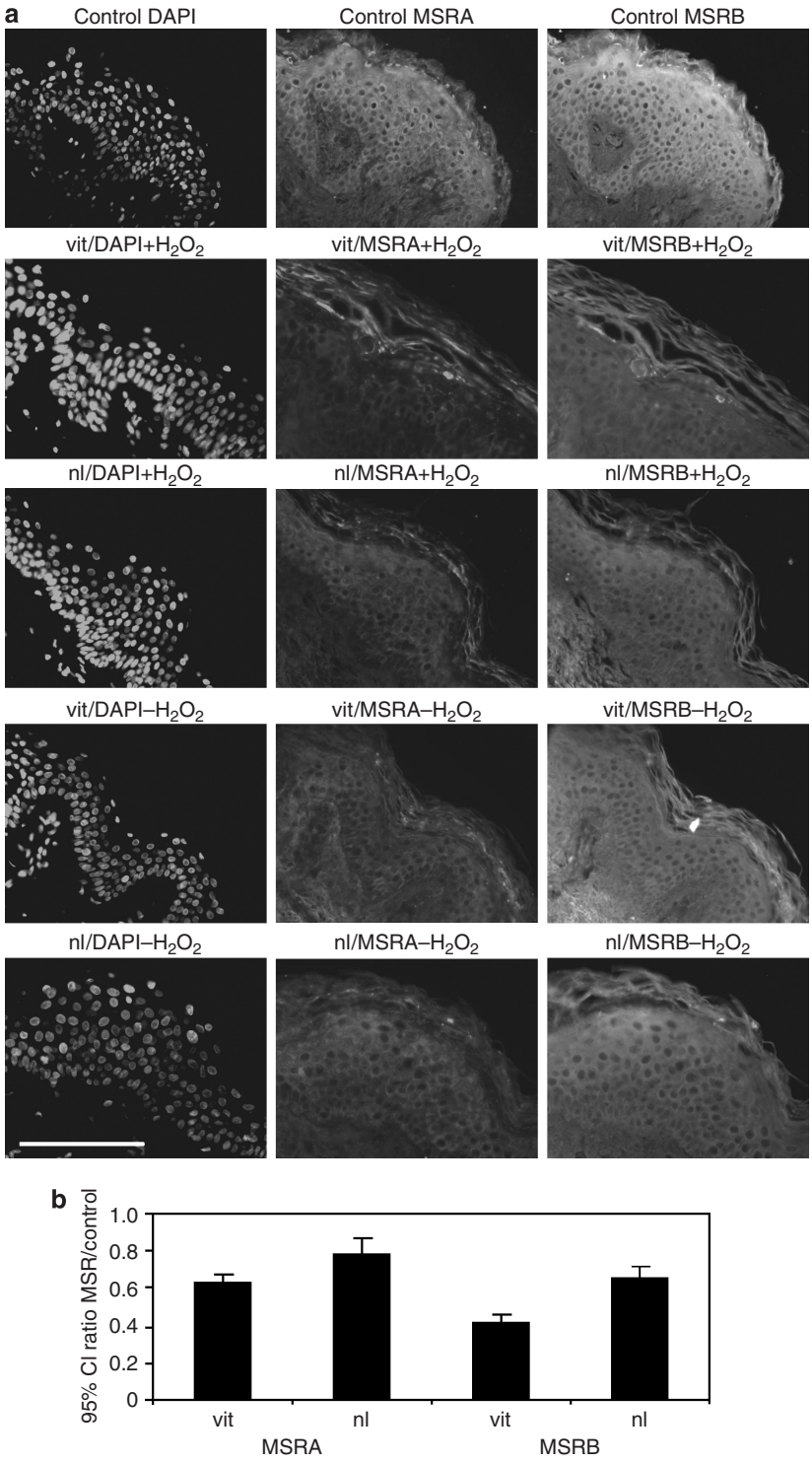

Figure 1. *In situ* expression of epidermal MSRA and MSRB in lesional and non-lesional skin of patients with vitiligo before and after treatment with pseudocatalase PC-KUS. (**a**) One example of MSRA/MSRB expression in healthy control skin phototype III compared to enzyme expression in lesional (vit) and non-lesional (nl) skin of a patient with active vitiligo ($+H_2O_2$) and after reduction/removal of epidermal H_2O_2 by a pseudocatalase PC-KUS ($-H_2O_2$). Bar = 100 μm. (**b**) Statistical analysis based on the 95% confidence interval. Both MSRA and MSRB protein expression was significantly lower compared to controls using the quotient of MSR over controls. Levels of both MSRA ($p_{vit}<0.001$; $p_{nl}<0.001$) and MSRB ($p_{vit}P<0.001$; $p_{nl}<0.001$) are significantly decreased, but they return to almost normal values after the removal of H_2O_2 from the epidermis with a topical application of pseudocatalase PC-KUS. There was no significant difference in protein MSRA expression between lesional (vit) and non-lesional (nl) skin, whereas the difference in MSRB was significant ($P<0.001$).

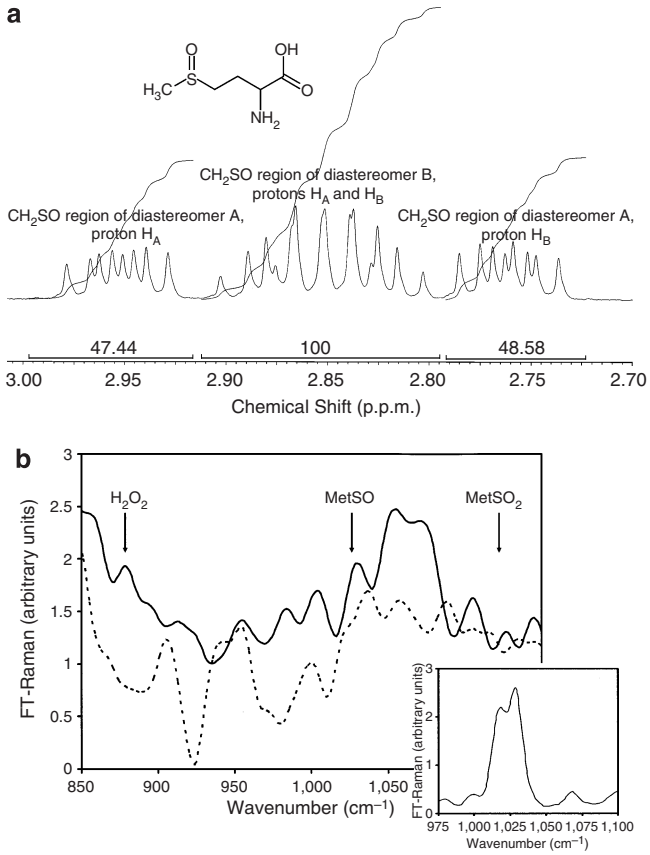

Figure 2. *In vitro* and *in vivo* identification of methionine sulfoxide by ^{1}H NMR spectroscopy and FT Raman spectroscopy. (**a**) ^{1}H NMR spectrum of (*R* and *S*)-L-methionine sulfoxide. Region of 600 MHz ^{1}H NMR spectrum of L-methionine sulfoxide shows signals from protons on C-4 due to two diastereomers. Assignments: δ_H (D_2O 600 MHz) 2.10–2.17 (4H, m, H-3), 3.58 (6H, s, CH_3SO), 2.74–2.78 (^{1}H, m, H-4, isomer A), 2.80–2.90 (2H, m, H-4, isomer B), 2.93–2.98 (^{1}H, m, H-4, isomer A), 3.69 (^{1}H, t, *J* 6.3 Hz, H-2), and 3.72 (^{1}H, t, *J* 6.4 Hz, H-2). ^{13}C NMR: δ_C (D_2O 150 MHz); 23.79 and 23.82 (C-3), 36.40 and 36.54 (CH_3SO), 48.21 and 48.26 (C-4), 53.32 and 53.52 (C-2), 173.26 and 173.29 (C-1). (**b**) *In vivo* FT-Raman Spectrum of a patient with active vitiligo. FT-Raman spectrum of the lesional skin of a patient with active vitiligo showing the O–O stretch for H_2O_2 at 875 cm^{-1} and the S = O stretch at 1,026 cm^{-1} for Met S = O (- - - control, —— patient). Inset shows the FT-Raman spectrum of methionine sulfoxide (S = O stretch).

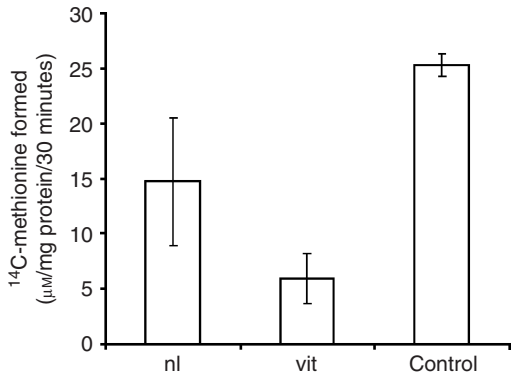

Figure 3. Decreased MSRA/MSRB activities in vitiligo. A comparative study of MSRA/MSRB activities in cytosolic extracts from the epidermis of patients with vitiligo ($n = 4$) compared to skin phototype III ($n = 2$). $P < 0.001$.

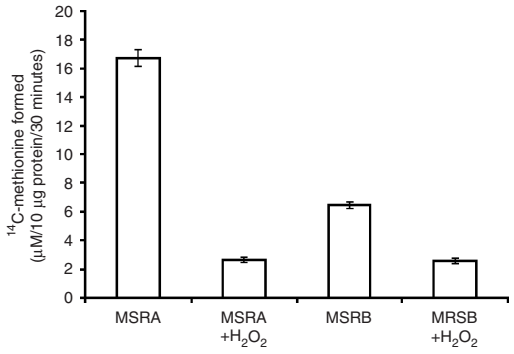

Figure 4. Decreased enzyme activities of recombinant human MSRA/MSRB1 in the presence of H_2O_2. Human recombinant MSRA and MSRB1 activities are decreased after exposure to H_2O_2 (10^{-3} M). MSRA is more sensitive (85%) to H_2O_2 than MSRB (40%). (MSRA 1 nmol of enzyme reduced 7 nmol of MetS = O per minute at 37°C, and MSRB 1 nmol of enzyme reduced 3 nmol of MetS = O per minute at 37°C).

FT-Raman Spectroscopy identifies methionine sulfoxide in the lesional skin of patients with active vitiligo

Patients with active vitiligo accumulate 10^{-3} M H_2O_2 in their epidermis *in vivo* as previously shown by FT-Raman Spectroscopy. H_2O_2 is detected as the O–O stretch at 875 cm^{-1}. Figure 2b presents a characteristic FT-Raman spectrum of the lesional skin of a patient with active vitiligo compared to healthy control skin identifying an S = O stretch assigned as methionine sulfoxide at 1,026 cm^{-1} (inset shows the methionine sulfoxide standard spectrum) (Gibbons *et al.*, 2006). This result suggests that the oxidation product methionine sulfoxide in epidermal proteins cannot be reduced back to L-methionine by MSRA and MSRB.

MSRA and MSRB enzyme activities are significantly reduced in the epidermis of patients with vitiligo compared to healthy controls

The combined activities of MSRA/MSRB were determined in suction blister extracts in both the lesional and non-lesional skin of four patients with active vitiligo compared to four healthy controls of the same skin phototype. The results are presented in Figure 3 showing that in patients with vitiligo, both the lesional and non-lesional epidermis have significantly lower enzyme activities compared to controls.

Deactivation of human recombinant MSRA and MSRB1 by H_2O_2

To test the stability of MSRA and MSRB1 against the influence of 10^{-3} M H_2O_2, activities of pure human recombinant enzymes were determined in the presence and absence of H_2O_2. The results showed that both enzyme activities were lower after oxidation. However, MSRA was significantly less stable than MSRB1 (Figure 4).

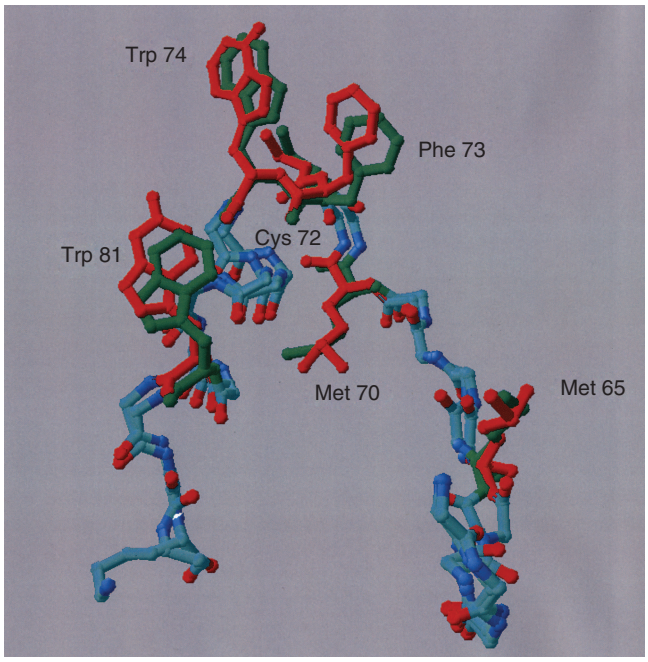

Figure 5. Structural modelling of MSRA indicates H_2O_2-mediated deactivation of the enzyme active site. Overlay of native MSRA (green) compared to its H_2O_2-mediated oxidation of the enzyme active site (red). After oxidation of MSRA by H_2O_2 the active site Cys^{72} and adjacent residues Trp^{74}, Trp^{81}, Met^{65}, Met^{70}, and Phe^{73} (red) is disorientated compared to native enzyme (green) supporting deactivation of the enzyme.

H_2O_2-mediated oxidation affects the active site of MSRA

Based on the X-ray crystal structure of human MSRA, several residues in the active site could be target to H_2O_2-mediated oxidation (Lowther *et al.*, 2000). The catalytic site for MSRA contains Cys^{72} and the methyl group-binding site for methionine sulfoxide Phe^{73} and Trp^{74}. Glu^{115}, Asp^{150}, and Tyr^{155} are believed to bind the S=O group, whereas Glu^{115} has been implicated in proton transfer (Lowther *et al.*, 2000). After oxidation with H_2O_2, Trp^{74}, Trp^{81}, Met^{65}, and Met^{70} yield 5-OH-Trp and MetS=O residues, respectively. The net result shows that both the binding site for the substrate and the active site residue Cys^{72} are disoriented. In addition, the oxidation of Trp^{136} and Met^{145} alters the position of important catalytic residues Asp^{150} and Tyr^{155}. Taken together, the structural modelling supports significant deactivation of MSRA by H_2O_2 (Figure 5).

DISCUSSION

The human epidermis expresses high levels of anti-oxidant enzymes such as catalase, glutathione reductase/glutathione peroxidase, TR/T/thioredoxin peroxidase, and methionine sulfoxide reductases A and B (B1, B2, B3) together with a battery of other antioxidants to keep the redox homeostasis under control (Schallreuter *et al.*, 1991, 2006c; Schallreuter and Wood, 2001; Ogawa *et al.*, 2006; Schallreuter, 2006). In normal healthy human epidermis, these enzymes cope with the generation of H_2O_2/$OH^{\bullet}$ from UVB-irradiation and the production of singlet oxygen/$OH^{\bullet}$ from UVA light (Schallreuter *et al.*, 1999; Maresca *et al.*, 2006; Wood and Schallreuter, 2006). Hydroxyl radicals react with proteins, lipids, and DNA (Marks *et al.*, 1996). In the depigmentation disorder vitiligo, the redox balance is seriously compromised primarily due to the loss of functioning catalase and thioredoxin reductase activities (Schallreuter, 2005). This severe oxidative stress is apparent upon examination of the depigmented epidermis of these patients where there is extensive vacuolization (lipid peroxidation) and the loss of functioning melanocytes (Moellmann *et al.*, 1982; Bhawan and Bhutani, 1983; Tobin *et al.*, 2000). This pathology has been shown to be due to the continuous accumulation of H_2O_2 (10^{-3} M) in the epidermis of patients with active disease (Schallreuter *et al.*, 1991, 1999). The removal of H_2O_2 from the epidermis of these patients with a topical application of narrow band UVB-activated pseudocatalase PC-KUS results in the re-establishment of the redox balance in the skin of patients with vitiligo followed by repigmentation (Schallreuter, 2005; Schallreuter and Elwary, 2007). In this report, we show for the first time that the protein repair enzymes MSRA and MSRB also partially recover in the epidermis of patients with vitiligo after treatment with PC-KUS (Figure 1). Experiments with recombinant MSRA and MSRB1 revealed that both enzymes are subject to deactivation by 10^{-3} M H_2O_2, although MSRA was more sensitive than the seleno-enzyme MSRB (Figure 4). This could be due to the low potential for the selenohydryl group being able to reduce H_2O_2, as it does for the seleno-enzymes thioredoxin reductase and glutathione peroxidise (Nordberg and Arner, 2001). The inactivation of MSRA by H_2O_2 was supported by computer-assisted molecular modelling of this enzyme indicating disruption of the active site after oxidation (Figure 5). Previous studies revealed that the electron donor system (TR/T) for MSRA and MSRB is also deactivated by H_2O_2-mediated oxidative stress (Gibbons *et al.*, 2006). The lesional skin of patients with vitiligo revealed significantly lower activities of MSRA, MSRB compared to control subjects, and even the non-lesional skin of these patients is significantly affected by this reactive oxygen species (ROS) (Figure 3). Earlier, this scenario has also been shown for TR/T and catalase (Schallreuter *et al.*, 1991; Schallreuter and Wood, 2001). Interestingly, both MSRA/MSRB as well as TR/T increase with increasing skin color providing a rationale for extra photoprotection with increasing skin phototypes (I–VI) (Schallreuter and Wood, 2001) (data not shown). In conclusion, both MSRA and MSRB are deactivated by H_2O_2 (10^{-3} M) as shown with the model disease vitiligo. Based on our results presented herein, we propose that MSRA as well as MSRB are targets to H_2O_2-mediated oxidative stress in a concentration-dependent manner despite both enzymes play a critical role in protein repair.

MATERIALS AND METHODS

^{14}C L-methionine (50 mCi mmol^{-1}) and all other reagents used in this study were obtained from Sigma (Poole, Dorset, UK). Silica gel thin-layer chromatography (TLC) plates (GF 1,000 μm) were obtained from Merck (Darmstadt, Germany).

Synthesis and purification of ^{14}C methionine sulfoxide

^{14}C methionine sulfoxide (5×10^{-4} M) (14,907 c.p.m.) was synthesized by the oxidation of ^{14}C L-methionine in the presence of 10^{-3} M H_2O_2 for 1 hour. The products from the oxidation reaction were separated by TLC, using L-methionine and methionine sulfoxide standards, which were detected by ninhydrin after heating the plate to100°C. L-methionine yielded a purple spot (Rf 0.77) and methionine sulfoxide gave an orange spot (Rf 0.52) after chromatography in isopropanol: formic acid:water (20:1:5). ^{14}C methionine sulfoxide (95% yield) was scraped from the silica gel plate and dissolved in 2.0 ml of distilled water.

Human skin biopsies

Full thickness skin (3 mm punch biopsies) was taken under local anesthesia from the inner proximal arm from the lesional skin of patients with acute vitiligo ($n = 13$) before and after treatment with pseudocatalase (PC-KUS) ($n = 11$) (Schallreuter *et al.*, 1995, 1999) and from four healthy controls (skin phototype III, Fitzpatrick classification; Fitzpatrick *et al.*, 1971). Epidermal suction blisters were obtained from both the lesional and non-lesional skin of four patients with acute vitiligo and from four healthy controls using the method of Kiistala *et al.* (Kiistala and Mustakallio, 1967). The study was approved by the local ethics committee and was in agreement with the Declaration of the Helsinki principles. Written and signed consent was obtained in all cases.

In situ immunofluorescence studies

Frozen slides were air dried for 60 minutes at room temperature before fixing in ice-cold methanol for 6 minutes and blocked in 10% normal donkey serum (Jackson Immunoresearch Laboratories, Cambridge, UK) for 90 minutes following a 5-minute wash in phosphate-buffered saline. MSRA was detected by using a polyclonal rabbit anti-human antibody (Autogen Bioclear, Calne, Wiltshire, UK) diluted 1:50 in 1% normal donkey serum followed by incubation at room temperature for 3 hours. For MSRB, we used a monoclonal mouse antihuman antibody (Autogen Bioclear, Calne, Wiltshire, UK) diluted 1:50 in 1% normal donkey serum incubated overnight at 4°C. Then, the slides were washed 4 × with phosphate-buffered saline, air dried, and incubated for 1 hour with a fluorescent secondary antibody (FITC-conjugated donkey anti-rabbit at a dilution of 1:100 (Jackson Immunoresearch Laboratories, Cambridge, UK). Slides were washed 3 × with phosphate-buffered saline air dried and mounted on Vectashield Mounting Medium (Vector Laboratories, Peterborough, UK). Slides were viewed under a Leica DRMIB/E fluorescence microscope (Wetzlar, Germany). Images were captured using a digital camera (C8484-05G, Hamamatsu Photonics UK, Welwyn Garden City, Herts, UK) coupled to a computer and evaluated with the imaging software IPLab version 3.7.4 (Scanalytics, Fairfax, VA).

Statistical analysis

Statistical analysis is based on one sample *t*-test using SPSS version 12.0. (SPSS Inc., Chicago, IL).

NMR Spectroscopy of L-methionine sulfoxide

NMR spectra of L-methionine sulfoxide (7.5 mg), dissolved in D_2O (99.9%, 1 ml), were acquired at 20°C on a JEOL ECA600 Multi-nuclear FT-NMR Spectrometer equipped with an X-H 5 mm auto-tune field gradient probe operating at 150.9 MHz for ^{13}C and 600.1 MHz for ^{1}H. ^{1}H and ^{13}C assignments were validated using a combination of ^{1}H-^{1}H DQF-COSY, ^{13}C-{^{1}H} DEPT135, ^{1}H-^{13}C HMQC, and ^{1}H-^{13}C heteronuclear multiple bond correlation (HMBC) experiments in addition to the standard ^{1}H and ^{13}C-{^{1}H} single pulse experiments.

Detection of methionine sulfoxide in the lesional skin of patients with active vitiligo using non-invasive FT-Raman Spectroscopy

FT-Raman spectra were measured using a Bruker RFS 100/S spectrometer (Bruker Karlsruhe, Germany) with a liquid nitrogen-cooled germanium detector equipped with a fiber optic cable. Near infrared excitation was produced with an Nd^{3+}:YAG laser operating at 1,064 nm. Each spectrum of the lesional skin of patients with vitiligo was accumulated over 400 scans (12 minutes) at 1,000 mW with a resolution of 4 cm^{-1}.

Determination of MSRA/MSRB enzyme activities in cell extracts prepared from epidermal suction blister tissue

Epidermal suction blister extracts were prepared using a pestle and mortar chilled to −80°C and fine sand. The soluble fraction was taken up in 1.0 ml of 0.1 M Tris/HCl buffer pH 7.4, followed by centrifugation. Protein concentrations were determined by the method of Kalb and Bernlohr (1977). Standard assays were carried out using cell extracts (50 µl), dithiothreitol as electron donor (10 µl) 5×10^{-3} M, and ^{14}C methionine sulfoxide (10 µl) 5×10^{-3} M. Reactions were incubated for 30 minutes at room temperature then 5.0 µl of the reaction product was applied to a TLC silica gel plate (GF 1,000 µm, Merck, Darmstadt, Germany) and chromatographed in isopropanol:formic acid:water (20:1:5). L-Methionine and methionine sulfoxide were detected by ninhydrin. Radiolabelled ^{14}C L-methionine spots were scraped from the TLC plates and added to 3.0 ml of scintillation fluid (Ready Safe, Beckman Coulter, Fullerton, CA) and counted on the ^{14}C channel in a Packard Tricarb Liquid Scintillation Counter (2001 TR, Packard Instruments, Meriden, CT). The ^{14}C L-methionine formed was standardized to µmoles per mg of protein per 30 minutes.

Oxidation of recombinant MSRA and MSRB1 with H_2O_2

Recombinant MSRA and MSRB1 were obtained from Jena Bioscience (Jena, Germany). Each enzyme (10 µg) was incubated with 10^{-3} M H_2O_2 for 1 hour followed by the removal of excess H_2O_2 with 2 µl of catalase (5.0 mg ml^{-1}) for 30 minutes. Using the method described above, enzyme activities (µM ^{14}C L-methionine formed per 30 minutes) were compared for both MSRA and MSRB1 before and after H_2O_2-mediated oxidation. Experiments were performed in triplicate.

Molecular structural modelling of the MSRA active site before and after H_2O_2-mediated oxidation

The active site of MSRA taken from the X-ray crystallographic analysis was studied before and after the oxidation of Trp, Met, and Cys residues in the catalytic center (Lowther *et al.*, 2000). Native and oxidized MSRA were modelled using Hyperchem™ software (Hypercube, Gainsville, FL) followed by Minimisation and Deep View analysis (Swiss Institute for Bioinformatics, Lausanne, Switzerland). Unfortunately, MSRB cannot be modelled at the current time due to the absence of X-ray crystallographic data for the human enzyme.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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