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Polymorphism in the manganese superoxide dismutase gene

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Abstract
Oxidative stress and mitochondrial damage occur in sepsis. Manganese superoxide dismutase (MnSOD) provides the main defence against oxidative stress within mitochondria. Ala9Val is a single nucleotide polymorphism (SNP) in the *MnSOD* gene, predicted to affect intra-mitochondrial transport of the enzyme. We found a significant difference in the genotype frequency between healthy subjects ($n = 100$) and patients with sepsis ($n = 40$, $p = 0.009$). For assessment of functionality ten healthy subjects of each homozygous genotype (A/A or V/V) were studied. Peripheral blood mononuclear cells were separated and incubated for 18 h with lipopolysaccharide (LPS), followed by analysis of mitochondrial and cytosolic fractions. There was no difference between genotypes in MnSOD activity and cytochrome *c* concentration, and minor differences in total antioxidant capacity (TAC) and mitochondrial membrane potential, which did not affect response to LPS. Despite predictions from structural enzyme studies that mitochondrial trafficking would be affected by the Ala9Val polymorphism of the *MnSOD* gene had little functional effect.

Keywords: *Manganese superoxide dismutase, antioxidants, oxidative stress, genetics, polymorphism, sepsis*

Introduction

Mitochondria are the major source of reactive oxygen species in a resting cell. If antioxidant protection is inadequate this will result in oxidative stress and cause mitochondrial dysfunction. We have previously shown oxidative stress in patients with sepsis, as shown by increased levels of lipid peroxides and direct detection of circulating radicals; decreased antioxidant capacity associated with non-survival; decreased concentrations of individual protective antioxidants; detectable circulating redox-reactive iron; ischemia–reperfusion leading to xanthine oxidase activation, and abnormal handling of exogenous antioxidants [1–6].

Manganese superoxide dismutase (MnSOD, SOD2, EC 1.15.1.1) is the only known enzyme that scavenges superoxide radical within mitochondria, providing the main defence against oxidative stress [7,8]. MnSOD is localised in the mitochondrial matrix, which is an optimum location for the removal of superoxide generated within the mitochondria [9]. MnSOD expression is induced by oxidative stress, hypoxia, tumor necrosis factor α (TNFα), interleukin-1 (IL-1) and lipopolysaccharide (LPS) [10,11].

MnSOD is encoded by a nuclear gene located on the long arm of chromosome 6 (6q25). It is synthesized as a precursor protein in the cytoplasm with a cleavable N-terminal mitochondrial targeting sequence (MTS). The MTS drives the mitochondrial importation of MnSOD precursor through the translocase of the outer membrane and the translocase of the inner membrane. Inside the mitochondrial matrix, the MTS is cleaved by a mitochondrial processing peptidase, and four monomers of the mature MnSOD protein assemble into the active MnSOD homotetramer [12,13].

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Ala9Val (C1183T) is a diallelic single nucleotide polymorphism (SNP) in the MTS of the MnSOD enzyme [14,15]. The polymorphism changes the amino acid codon at position-9 of the signal peptide, from alanine (GCT) to valine (GTT). Protein secondary structure analysis has revealed that the presence of alanine results in an amphiphilic α helical structure, which is crucial for intra-mitochondrial transport, while the presence of valine is predicted to form a β-pleated sheet conformation [14,16]. This alteration may be expected to affect the cellular allocation of the enzyme and transport of MnSOD into the mitochondrion thus reducing biological availability. The presence of valine is predicted to lead to lower mitochondrial MnSOD activity than the alanine form.

Inefficient targeting of MnSOD could leave mitochondria without their full defence against oxidative stress and reduced MnSOD expression results in mitochondrial dysfunction. Inadequate antioxidant protection through low MnSOD activity is likely to result in mitochondrial dysfunction, cytochrome *c* release and increased apoptosis, which have been observed in sepsis [17,18]. We hypothesised that the MnSOD Ala9Val polymorphism might affect the functional activity of MnSOD enzyme, and hence mitochondrial function. This could affect the mitochondrial response to sepsis. The aim of the study was to determine the genotype frequency in patients with sepsis compared to healthy subjects and the functionality of the Ala9Val polymorphism using different markers of mitochondrial function.

Materials and methods

MnSOD genotyping

A power calculation was performed to detect difference in the frequency of any of the possible genotypes (Trialaid, version 1.02). It was found that in order to detect a 30% difference in V/V genotype frequency between patients and controls at a power of 80% and $p < 0.05$, 40 subjects were required in each group. After local research ethics committee approval and written informed consent, 100 healthy volunteer subjects were recruited; 64 females and 36 males, median (range) age 29 (21–61) years, all Caucasian. In addition 40 consecutive patients (18 female, 22 male, age 67 (19–91) years) admitted to the intensive care unit of Aberdeen Royal Infirmary, who fulfilled the consensus criteria for sepsis [19,20], all Caucasian, were recruited.

Twenty millilitre venous blood was withdrawn from each subject for DNA extraction and genotype analysis was performed by polymerase chain reaction (PCR) amplification and restriction fragment length polymorphism (RFLP) analysis. DNA extraction was performed using the BACC 3 genomic DNA extraction kit (Nucleon, Amersham Biosciences, Buckinghamshire, UK). According to the DNA sequence, two primers were designed to flank a 246 bp area of DNA, which includes the SNP position (C1183T), with the following sequence:

Upstream primer: 5′ AGC CCA GCC TGC GTA GAC GGT C 3′.
Downstream primer: 5′ TAC TTC TCC TCG GTG ACG TTC AG 3′.

Following PCR amplification of the desired segment of DNA including the polymorphism site, an RFLP reaction was carried out. The polymorphism creates a restriction site for the digestion enzyme *BsaWI* (15,21) such that the enzyme cuts the DNA segment only when there is T at position 1183, giving two DNA fragments of 87 and 159 bp size. In the presence of C in this position the enzyme does not cut the DNA leaving it as one single segment of 246 bp length. Subjects were assigned to one of three possible genotypes, homozygous to alanine (A/A: one band of 246 bp), homozygous to valine (V/V: two bands of 87 and 159 bp) or heterozygous (A/V: three bands 87, 159 and 246 bp, Figure 1).

Ala9Val functionality

Ten healthy subjects of each homozygous genotype (A/A and V/V) were recruited. Functionality of

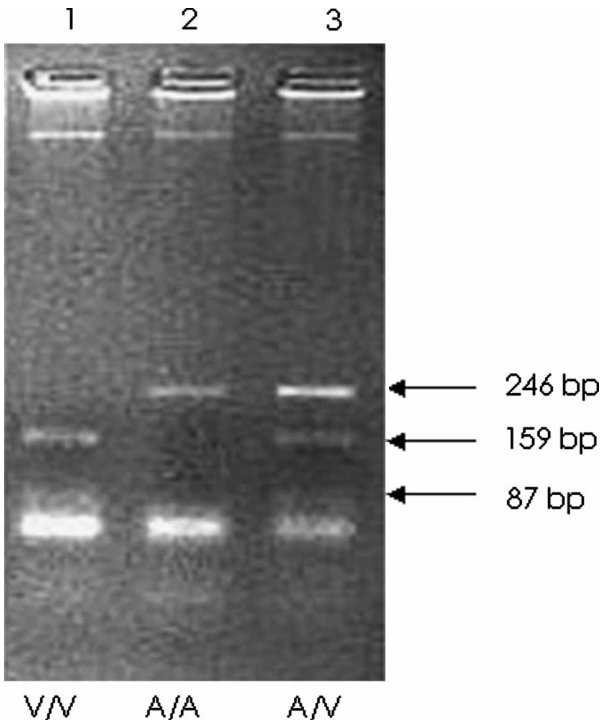

Figure 1. Electrophoresis gel demonstrating the different genotypes after PCR–RFLP analysis. Lane 1: V/V homozygous subject showing two bands (159 and 87 bp). Lane 2: A/A homozygous subject with one band of uncut DNA (246 bp). Lane 3: heterozygous genotype, where three bands (246, 159 and 87 bp).

the polymorphism was determined in terms of MnSOD enzyme activity, cytochrome *c* concentration, total antioxidant capacity (TAC) in the presence and absence of 2 μg/ml LPS.

Cell preparation. Mononuclear cells were separated by density gradient centrifugation using Ficoll-paque (Amersham Biosciences, Buckinghamshire, UK). Isolated cells were incubated for 18 h at 37°C, in a humid atmosphere of 95% air/5% CO_2 with 2 μg/ml LPS from *Escherichia coli* 026:B6 (Sigma, Poole, Dorset, UK) or phosphate buffered saline (PBS), in RPMI 1640 medium with Glutamax-l (Gibco BRL), containing 5000 U/ml penicillin G sodium, 5000 μg/ml streptomycin sulphate and 10% heat-inactivated foetal calf serum. Purity was 94–98% and viability, assessed by trypan blue exclusion, was in excess of 94%.

Following incubation, cells were washed twice at 400 g for 10 min with ice cold PBS (pH 7.4), then resuspended in buffer A containing 20 mM/l HEPES, 1.5 mM/l $MgCl_2$, 10 mM/l KCl and 1 mM/l each ethylene diamine tetra-acetic acid (EDTA), ethylene glycol tetra-acetic acid and dithiothreitol. The buffer was prepared and autoclaved before the addition of 250 mM sucrose (sterilised by filtration) and 1x protease inhibitor cocktail. Cells were then left to swell on ice for 30 min. Igepal (10% v/v in buffer A) was added for further 20 min followed by homogenisation. Cell lysis was confirmed by light microscopy. Homogenates were vortexed for 30 s, and centrifuged at 1000*g* for 10 min to pellet nuclei and cell debris. The supernatant was then centrifuged at 10,000*g* for 15 min, to pellet the mitochondria. Mitochondria were resuspended in buffer containing 100 mM/l KCl, 1 mM/l EDTA, 10 mM/l TrisCl and 340 mM sucrose. The supernatant was then centrifuged at 1,00,000*g* for 60 min to pellet the microsomal fraction, leaving a pure cytosolic fraction. Both fractions were stored at −80°C. Bradford reagent (Sigma) was used to determine the protein concentration in all samples.

MnSOD activity. MnSOD activity was assayed using a commercial kit using the water soluble tetrazolium dye (Dojindo Molecular Technologies Inc., Japan), in the presence of 1 mM potassium cyanide.

Cytochrome c. Cytochrome *c* was measured using a quantitative sandwich enzyme immunoassay technique (R&D Systems Europe, Abingdon, Oxon, UK).

Total antioxidant capacity. TAC was measured as previously described [22]. Briefly, the exposure of metmyoglobin to hydrogen peroxide leads to the formation of ferrylmyoglobin. Reducing agents can reduce the ferrylmyoglobin back to myoglobin. Interaction of the phenothiazine compound ABTS with ferrylmyoglobin leads to the production of the radical cation $ABTS^{\cdot+}$, a product with absorption maxima at 650, 734 and 820 nm. In the presence of antioxidants, the absorbance of this radical cation is quenched to an extent related to the antioxidant capacity added [2,22,23]. The assay was standardized using Trolox, a water soluble analogue of vitamin E, to produce Trolox equivalent antioxidant capacity (TEAC) defined as the concentration (mmol/l) of Trolox having the equivalent antioxidant capacity to a 1 mmol/l solution of the substance under investigation.

Mitochondrial membrane potential. Ten healthy subjects of each homozygous genotype (A/A and V/V) were recruited and 40 ml blood was obtained. Mononuclear cells were separated and the whole cells were then incubated for 18 h in culture medium as described before, with or without 20 μg/ml LPS, or PBS as control. Mitochondrial membrane potential was measured in intact mononuclear cells, i.e. without sub-cellular fractionation. Following incubation, analysis was performed using flow cytometry.

JC-1 (Molecular Probes, Invitrogen Ltd, Paisley, UK) is a fluorescent lipophilic cationic dye, which accumulates in mitochondria in a potential-dependent manner, causing a shift in its fluorescence emission. In healthy cells, JC-1 accumulates inside the mitochondrial matrix and stains the mitochondria bright red, due to the negative electrochemical gradient that allows the positively charged dye to enter, and when excited at 490 nm emits light at 590 nm, giving red fluorescence. In cells where the mitochondrial membrane potential is collapsed, JC-1 cannot accumulate inside the mitochondria, and the dye remains in the cytoplasm in a monomeric form, emitting green fluorescence.

Following incubation, cells were washed twice with PBS and incubated with JC-1 (10 μg/ml final concentration) for 30 min. Flow cytometry and data analysis were carried out on a BD LSR System (Becton Dickinson, San Jose, CA, USA), with an argon laser exciting at 488 nm. Green fluorescence was detected at 530 nm (FL-1), and red fluorescence was detected at 610 nm (FL-3). Typically 15,000 events per sample were collected in list mode. Data were analysed by the CellQuest Pro™ Software (Becton Dickinson). Valinomycin (5 μM) was used as a positive control.

Statistical analysis

Data were not normally distributed and so are presented as median (range). Statistical differences in enzyme activity between genotypes (A/A vs. V/V)

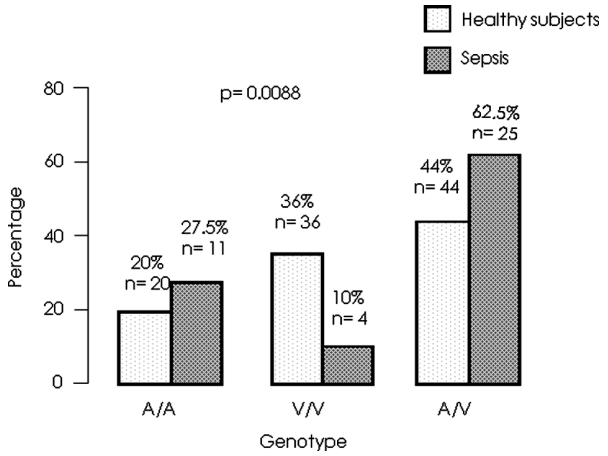

Figure 2. Genotype frequency of the Ala9Val MnSOD polymorphisms in healthy volunteers ($n = 100$) and patients with sepsis ($n = 40$). Genotype frequency was significantly different between groups (Chi-squared, $p = 0.0088$).

were analysed using the Mann–Whitney U-test. The differences in frequencies of genotypes and alleles in patients and controls and assessment of deviation from Hardy–Weinberg equilibrium was analysed by Chi-square testing. Statistical analysis was performed using Analyse-it software for Excel (version 1.63) and a p value of < 0.05 was considered significant.

Results

MnSOD genotyping

The genotype frequency of the Ala9Val polymorphism in a healthy local population and in patients with sepsis is given in Figure 2. Statistical analysis revealed a significant difference in the genotype frequency between healthy subjects and sepsis patients ($p = 0.009$ Chi-square test).

Allele frequencies were calculated as the percentage of the number of alleles, to the numbers of chromosomes:

$$\text{Allele frequency} = \frac{\text{Number of the alleles}}{\text{Number of chromosomes}} \times 100\%$$

The allele frequencies were $A = 0.42$, $V = 0.58$ in healthy controls and $A = 0.59$, $V = 0.41$ in patients. Statistical analysis revealed a significant difference in the allele frequency between both groups ($p = 0.024$ Chi-square test). Expected genotype frequencies were calculated from the allele frequencies and compared to observed genotype frequencies to test for deviation from Hardy–Weinberg equilibrium. Chi-square values were 0.9 (NS) and 3.3 (NS) for controls and patients, respectively, showing that there was no deviation from equilibrium.

Ala9Val functionality

There was no difference between genotypes in terms of MnSOD enzyme activity, cytochrome *c* level or TAC in mitochondrial or cytosolic cell fractions after LPS stimulation (Table I). In control (unstimulated) cells TAC was significantly higher in mitochondria from V/V homozygotes that in those with A/A genotype ($p = 0.05$, Table I). No other differences between genotypes were observed in unstimulated cells.

Cells treated with LPS had significantly higher MnSOD activity in mitochondrial fractions from subjects of both A/A genotype ($p = 0.027$) and in V/V genotype ($p = 0.037$). In addition, LPS caused a significant increase in cytochrome *c* concentration of mitochondrial fractions both in A/A genotype ($p = 0.027$) and in V/V genotype ($p = 0.002$). On the other hand, TAC was significantly lower in the cytosolic fraction of LPS stimulated compared to unstimulated cells from A/A homozygous subjects ($p = 0.027$) and V/V homozygous subjects ($p = 0.037$) subjects (Table I).

Table I. MnSOD activity, cytochrome *c* concentration and TAC in sub-cellular fractions of mononuclear cells in the presence and absence of lipopolysaccharide in relation to genotype.

Sub-cellular fraction	Cytosolic + LPS		Cytosolic control		Mitochondrial + LPS		Mitochondrial control	
Genotype	A/A	V/V	A/A	V/V	A/A	V/V	A/A	V/V
MnSOD activity units/mg protein	35.3 24.9–62.0	27.8 20.9–47.8	42.7 22.8–84.8	34.9 11.1–97.4	63.5* 3.0–126.4	47.1* 4.1–117.7	48.7 3.3–112.7	39.5 2.8–116.0
Cytochrome *c* ng/mg protein	33.9 18.6–42.3	27.2 20.7–92.7	45.5 15.8–108.3	33.1 15.9–95.4	100.5* 29.8–139.5	78.9* 43.6–131.3	74.2 12.5–160.5	41.9 10.9-95.4
Total antioxidant capacity μmol/mg protein	0.6† 0.1–1.8	0.7† 0.4–2.5	0.97 0.3–3.5	1.2 0.6–6.3	2.1 0.1–10.9	5.6 0.1–28.6	1.7 0.5–3.4	3.6‡ 0.7-11.3

Median (range); * significantly higher than in the absence of lipopolysaccharide ($p < 0.05$); † significantly lower than in the absence of lipopolysaccharide ($p < 0.05$); ‡ significantly higher than the A/A genotype ($p = 0.05$).

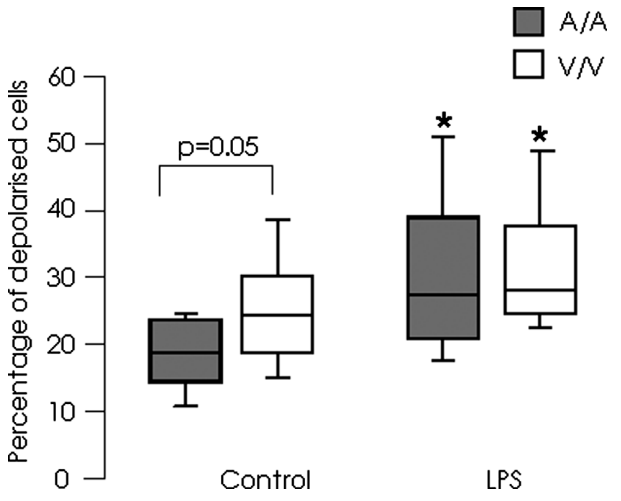

Figure 3. Percentage of mononuclear cells with depolarised mitochondria identified using JC-1 staining in the presence and absence of LPS in relation to Ala9Val genotype. Box and whisker plots show median, 25th/75th percentiles and full range and $n = 10$ for each genotype. *, significantly higher than in the absence of LPS $p < 0.05$.

Mitochondrial membrane potential. Mononuclear cells were first stimulated with or without 2 μg/ml LPS and incubated for 18 h in culture medium, before being stained with JC-1 for mitochondrial membrane potential assay. At this level of cell stimulation, changes in mitochondrial membrane potential observed were much the same as that observed in PBS controls. A dose-finding study was conducted to establish the optimum concentration of LPS stimulation for mitochondrial membrane potential (data not shown), based on the results of which, cells were stimulated with 20 μg/ml LPS for mitochondrial membrane potential assay.

In unstimulated control cells there was a significant difference in terms of the percentage of cells with depolarised mitochondria between genotype groups ($p = 0.05$, Figure 3). When cells were stimulated with LPS there was an increase in the percentage of cells with depolarised mitochondria compared to control cells ($p = 0.02$ in the A/A group and $p = 0.06$ in V/V the group), but there was no difference between genotype groups ($p = 0.57$, Figure 3).

Discussion

We investigated the frequency of a polymorphism in the MTS of the *MnSOD* gene in healthy controls compared to patients with sepsis. We found a significant difference in the distribution of the polymorphism between controls and patients, with a reduced frequency of the valine (V/V) allele in patients with sepsis compared to healthy controls (10 vs. 36%, respectively). We also undertook a pilot study to measure the *in vitro* functionality of the polymorphism in homozygous subjects. We found minor differences in some of the parameters studied between A/A and V/V homozygotes.

Genetic epidemiological studies suggest a strong genetic influence on the outcome from sepsis, and genetics may explain the wide variation in the individual response to infection that has long been a dilemma for physicians. It was found that the heterozygous genotype (A/V) was most frequent in the local population (44%), while A/A homozygote was least common (20%). The predominance of the A/V heterozygous genotype group is in agreement with other studies of other Caucasian populations (Germans, Swedes, Finns, Saamis and Russians) [15,24,25]. In contrast, in Asian ethnic groups the V/V genotype is the most common (Taiwanese and Japanese) [26,27].

Several studies have suggested a role of the Ala9Val polymorphism of MnSOD in human disease but there is still inconsistency. The alanine allele was reported to be a risk factor for Parkinson's disease [14] colorectal cancer [28] sporadic motor neurone disease [29] and severe alcoholic liver disease [30] while the V/V genotype was reported as a risk factor for lung carcinoma or cardiomyopathy [27]. Also, while an association between the alanine allele and increased risk of breast cancer had been reported [31] a more recent case-control study did not support any association of the Ala-9Val MnSOD polymorphism in the development of breast cancer [32].

However, disease associations in one population have not been confirmed in other populations. For example the association of Parkinson's disease with the alanine allele in a Japanese population [14] was not confirmed in a German population [15] and the association of the A/A genotype with alcoholic liver disease in France [30] was not confirmed in a UK study [33]. The allele frequency of alanine (expected to be associated with higher intra-mitochondrial MnSOD activity) was higher in sepsis patients than the valine allele in our study. MnSOD is the major antioxidant protecting mitochondria, which are a particular target for oxidative stress in sepsis. Altered frequency of the MnSOD polymorphism may have been associated with altered protection against oxidative damage and the *MnSOD* gene may represent a plausible candidate gene for mitochondrial damage in sepsis. We therefore undertook an *in vitro* pilot study to investigate possible functional effects of the Ala9Val polymorphism in selected homozygous subjects. The advantage of an *in vitro* study is that it provides standardised and uniform conditions for all studied samples, leaving genotype differences as the only variable. We chose not to use patients for the functional study since biochemical variations could have resulted from differences in infective organisms, site of infection, duration of infection, LPS load, cytokine levels, drugs used, management protocols,

differences in co-morbidities and exposure to other oxidative stresses.

Increased intra-mitochondrial trafficking of newly synthesised MnSOD may lead to a better protection from oxidative damage. Structural analysis of Ala9Val certainly suggested that the polymorphism would affect the cellular allocation of the enzyme [14,16]. The V/V genotype was expected to be associated with lower mitochondrial MnSOD activity and less efficient mitochondrial defence against oxidative stress, thus affecting mitochondrial function and apoptosis. If this hypothesis was correct the V/V genotype would be associated with decreased MnSOD activity, higher cytosolic cytochrome *c* levels and lower antioxidant capacity, which may be more pronounced following an oxidative insult. It could also be speculated that decreased mitochondrial antioxidant protection or sub-optimal responses to oxidative stress could result in greater consumption of antioxidants after oxidative insult. It has been shown that importation of the alanine variant into rat mitochondria resulted in mitochondrial MnSOD activity 30% higher than when valine variant was imported, which suggested that A/A homozygotes might have higher MnSOD activity than V/V subjects [34]. Although we found that MnSOD activity was higher in mitochondria of cells exposed to LPS, suggesting upregulation of the enzyme, there was no difference between A/A and V/V genotypes, despite previous predictions that this would be the case.

In our study, we used an *in vitro* model of sepsis to study the different biochemical responses to endotoxin stimulation in relation to MnSOD genotype. The dose and duration of LPS incubation were selected to provide optimal cell stimulation for identifying different responses to endotoxin stimulation. This model is widely used as an *in vitro* model of sepsis [35,36] The findings of a significant increase in mitochondrial MnSOD and mitochondrial cytochrome *c*, and the drop in cytosolic TAC demonstrates the stimulation effect of LPS on the cells.

The choice of 18 h LPS exposure was based on the finding of previous studies. Tsan et al. showed that while induction of MnSOD mRNA was evident 3 h after LPS treatment in human peritoneal macrophages, the increase in MnSOD activity was not observed until 18–20 h after LPS treatment [35]. In another study, there was a marked increase in monocyte MnSOD activity after incubation with LPS for 18 h, but not at 1 or 4 h [37].

We observed a significant increase in MnSOD activity and cytochrome *c* concentration following LPS stimulation in mitochondrial fractions from subjects of both genotypes. MnSOD and cytochrome *c* were also detected at low concentrations in both mitochondrial and cytosolic fractions in unstimulated cells [38,39]. However, LPS exposure resulted in increased MnSOD activity only in the mitochondria, the site where it is biologically active. LPS is a potent stimulus for mitochondrial biogenesis, causing up-regulation of genes (e.g. cytochrome *c*, and cytochrome *c* oxidase) involved in oxidative phosphorylation [40]. We found an increase in cytochrome *c* following LPS stimulation consistent with LPS stimulation of mitochondrial biogenesis. Suliman et al. in an *in vivo* study in rats, also demonstrated up-regulation of cytochrome *c* expression following LPS stimulation, and provided evidence that ROS regulate oxidant sensitive nuclear genes, indicating stimulation of mitochondrial biogenesis by LPS [40]. Increased activity of mitochondrial MnSOD possibly prevented the leak of cytochrome *c* to the cytosol after LPS stimulation.

LPS induces oxidative stress and promotes apoptosis in some cells through both the release of pro-apoptotic cytochrome *c* in the cytosol and up-regulation of the expression of pro-apoptotic Bcl-2 proteins [41]. Agents that inhibit the formation of reactive oxygen species offer protection against LPS-induced apoptosis [41,42]. Cytochrome *c* release from the mitochondria involves the formation of the mitochondrial transition pore (MTP) [43]. MnSOD activity may prevent cytochrome *c* release by blocking the formation of the MTP and several studies have shown that both MnSOD and cytochrome *c* are specific mitochondrial targets of oxidative damage [44]. We found that mitochondrial cytochrome *c* levels were higher in cells exposed to LPS, but there was no difference in cytochrome *c* between genotype groups in either the mitochondrial or the cytosolic fractions.

Conversely, reduced MnSOD activity is related to an increase in cytosolic release of cytochrome *c*. In a study on cerebral focal ischaemia in mice [45] cytosolic cytochrome *c* expression in heterozygous MnSOD (SOD2 −/+) knock out mice was higher compared to wild type mice, accompanied by apoptosis. Equally, over-expression of MnSOD has been shown to protect from apoptosis [46].

Zhang et al. measured plasma MnSOD enzyme activity in patients with tardive dyskinesia vs. controls, in relation to Ala9Val genotype. Although MnSOD activity was higher in patients, they found no difference in the enzyme activity between genotype A/A and V/V [21]. These findings are clearly in agreement with our study. In contrast, Shimoda-Matsubayashi et al. reported higher MnSOD enzyme activity in the mitochondrial fraction of patients with Parkinson's disease who were A/A genotype compared to both A/V and V/V genotypes [16]. However, it should be noted that only a single patient with the A/A genotype was studied.

TAC represents the ability of the entire antioxidant repertoire to resist oxidant reactions [47] and may give more biologically-relevant information than that obtained from measuring concentrations of individual antioxidants. Several studies have shown that in sepsis,

impairment of cellular TAC plays a central role in oxidative damage to the cell, and that the measurement of TAC in such conditions gives an indication of the overall ability to withstand oxidant damage [23]. Reduced-plasma TAC in patients with sepsis who develop organ dysfunction has been described, and failure to achieve a normal plasma antioxidant potential was strongly associated with an unfavourable outcome [2]. We found that TAC was lower in the cytosol of cells exposed to LPS, suggesting consumption of antioxidants. In mitochondria from control (unstimulated) cells, we found that TAC was higher in subjects of the V/V genotype. This does not concur with the prediction of decreased MnSOD activity associated with the valine form of MnSOD. However, it is possible that sub-optimal MnSOD activity is compensated for by other antioxidants which contribute to overall TAC.

Energy released during oxidative reactions in the mitochondrial respiratory chain is stored as an electrochemical gradient across the membrane. Thus mitochondrial membrane potential is considered a sensitive indicator of the energy state of the mitochondria. Following LPS stimulation, the increased exposure of the mitochondrial inner membrane to oxidative stress results in opening of the permeability transition pores in the inner mitochondrial membrane which collapses mitochondrial membrane potential leading to loss of the outer mitochondrial membrane integrity and release of inter-membrane proteins into the cytosol [48,49]. Several studies have demonstrated a direct effect of MnSOD on the stabilization of the mitochondrial membrane and, in SOD2 + /− mice, membrane potential was found to be lower than in wild type mice [49]. This suggests that a functional polymorphism which affects mitochondrial enzyme activity is likely to affect mitochondrial membrane potential and this may be more pronounced following exposure to oxidative challenge. We found that the percentage of cells with depolarised mitochondria was higher in those cells exposed to LPS but that there is no relationship with genotype after LPS stimulation. However, the percentage of cells with depolarised mitochondria was higher in V/V homozygous subjects compared to A/A homozygotes. The relevance of this finding is unclear, since cells did not appear to be more susceptible to LPS-induced mitochondrial damage.

The implications of the findings of our pilot study are either that the Ala9Val polymorphism has little functional effect on MnSOD activity, or that it has a functional effect, but factors other than MnSOD are more important in modifying the response to oxidative stress and, therefore, disease risk in sepsis. These factors may include other inflammatory mediators and other antioxidant defence systems. In addition any functional effect of the polymorphism may be confined to certain populations.

Conclusion

In conclusion, we showed that the Ala9Val polymorphism in the *MnSOD* gene was associated with only minor differences in TAC and mitochondrial membrane potential despite predictions from prior structural enzyme studies that mitochondrial trafficking would be affected. These findings suggest that the finding of a different allele frequency in patients with sepsis is likely to be unrelated to the functional effects on cellular responses to oxidative stress.

Acknowledgements

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