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Participation of 47C>T SNP (Ala-9Val polymorphism) of the SOD2 gene in the intracellular environment of human peripheral blood mononuclear cells with and without lipopolysaccharides

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Abstract The outcome of sepsis occurs due to influence of environmental and genetic factors besides genes variants whose expression support its outcome or not. Oxidative stress is associated to the pathogenicity of sepsis, occurring when there is a reactive species overproduction associated with inflammation. The aim of this study was to characterize the cellular redox status of human peripheral blood mononuclear cells (PBMCs) with either −9Ala (AA) or −9Val (VV) SOD2 genotypes and evaluate their response to oxidative stress induced by lipopolysaccharide (LPS). The PBMCs were isolated from the blood of 30 healthy human volunteers (15 volunteers for each allele) and the following assays were performed: antioxidant enzyme activities (superoxide dismutase; catalase; glutathione peroxidase), total radical-trapping antioxidant parameter, non-enzymatic antioxidant capacity (total antioxidant reactivity), and quantification of conjugated dienes (lipid peroxidation). At basal conditions (i.e., not stimulated by LPS), cells from 47C allele carriers showed higher activities of CAT and SOD, as well as higher TAR compared to 47T allele. However, when 47CC cells were challenged with LPS, we observed a higher shift toward a pro-oxidant state compared to 47TT cells. The CAT activity and lipid peroxidation were increased in cells with both alleles, but SOD activity increased significantly only in 47TT cells. These results demonstrate that SOD2 polymorphisms are associated with different cellular redox environments at both basal and LPS-stimulated states, and identification of this polymorphism may be important for a better understanding of pro-inflammatory conditions.

Keywords Sepsis · Lipopolysaccharides · Peripheral blood mononuclear cells · SOD2 Ala-9Val polymorphism · Cellular redox environment

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Introduction

Sepsis is an important cause of admission and one of the leading causes of mortality in intensive care units (ICU), being characterized by the presence of both infection and the systemic inflammatory response syndrome (SIRS) [1, 2]. In the course of sepsis, a deficient immunologic defense may allow infection to become established; however, an excessive or poorly regulated response may harm the host through a maladaptive release of endogenously generated inflammatory compounds [3]. Septic shock represents the end of the spectrum (sepsis, several sepsis, and septic shock) of increasing inflammation and host response to an infection [3]. The release of the endotoxin lipopolysaccharide (LPS) from the cell wall of Gram-negative bacteria is generally regarded as the initiating event in the development of sepsis [4], and is well known to activate monocytes and macrophages, leading to the production of pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) [5, 6]. After activation, these cells can produce reactive oxygen species (ROS) which is addressed to kill microorganisms; however, the excess of ROS may attack cellular components causing cell

damage [7]. Antioxidant enzymes like superoxide dismutase (SOD) protect the cell from oxidative damage, presenting a potential therapeutic target for critically ill patients [8]. SOD is a family of ubiquitous metalloproteins that catalyzes the reaction of two superoxide (O_2^-) molecules into hydrogen peroxide (H_2O_2) and molecular oxygen (O_2) [9]. Manganese-dependent superoxide dismutase (MnSOD) (EC 1.15.1.1) exists as a tetramer and is initially synthesized containing a leader peptide, which targets this manganese-containing enzyme exclusively to the mitochondrial spaces. This enzyme is coded by a nuclear gene (SOD2) and is found in its active form within the mitochondrial matrix [10]. The protein is composed of 222 amino acids [11] and the first 24 amino acids of the primarily translated polypeptide might constitute the leader peptide for transport of the precursors to the mitochondria [12]. After synthesis in the cytosol, the inactive enzyme is transported posttranslationally into mitochondrial matrix, where it is activated upon loss of the second signal peptide [13]. The human gene encoding MnSOD is located in the long arm of human chromosome 6 (6q25) [14] and presents five exons and four introns [15]. This gene may present a change in the nucleotide 47 (C>T) sequence of DNA that will result in the incorporation of an alanine (GCT) or a valine (GTT) in the area of the signal peptide [16]. This polymorphism is predicted to generate an alteration in the conformational structure of MnSOD: the allele that encodes an alanine (Ala) added in the −9 position (residue 16) of the signal peptide-containing protein is predicted to induce an α-helical structure, while the allele encoding a valine (Val) is predicted to induce a β-sheet structure [17, 18]. This alteration (alanine to valine) is expected to affect the efficiency of the transport of MnSOD into the mitochondria, i.e., −9Ala can be more easily transported to the mitochondria when compared with −9Val [18]. The study of processing of these two types of leader signals suggests that the basal level activity of MnSOD decreases with the presence of the V allele (i.e., activity decreases from AA to AV and from AV to VV genotypes) [19], and that this dimorphism, besides controlling the efficiency of protein transport to mitochondria, also regulates the stability of the MnSOD mRNA [20].

Genomic medicine aims to identify inheritable genetic patterns in the different susceptibilities of individuals to develop some disease or characteristic. Epidemic genetic studies suggest a marked influence of genetic factors in the outcome of sepsis patients [21, 22]. The development of sepsis depends on environmental and genetic factors; expression of some gene variants was associated with different outcomes. Oxidative stress is related to the pathogenicity of sepsis, occurring when there is a reactive species overproduction associated with inflammation [23]. The aim of this study was to characterize the cellular redox status of human peripheral blood mononuclear cells (PBMCs) with either −9Ala (AA) or −9Val (VV) SOD2 genotypes and evaluate their response to oxidative stress induced by LPS.

Materials and methods

Subjects, design, and approval

Volunteer's participants are residents from the city of Porto Alegre, RS, Brazil (southern Brazil) which is composed of a singular genetic background: majority of inhabitants with European origin (Portuguese, Italians, Spanish, and Germans ancestry) and a small amount of individuals with African traits contributing to their genetic pool [24, 25]. To obtain the number of 30 samples, it was necessary to evaluate the genotype of 80 individuals: 47CC = 0.2, 47CT = 0.5, 47TT = 0.3 and 47C = 0.45, 47T = 0.55; Chi-square test Hardy–Weinberg equilibrium $P = 0.99$. Samples were also evaluated for another polymorphism (Ile58Thr, 173T>C) of the SOD2 gene, the allele 173C possesses smaller activity when compared to the allele 173T [26, 27] with interest in just verifying the effect of the polymorphism Ala-9Val; the heterozygote will be discarded and will just be fastened the homozygote 58Ile (173T). All human subjects signed informed consent form and the experimental protocol was approved by the Ethics Research Committee of Federal University of Rio Grande do Sul (UFRGS) protocols number 18435.

Preparation of PBMCs

The PBMCs were isolated from the blood of healthy humans by gradient centrifugation on Ficoll-Paque™ PLUS (GE Healthcare Bio-Sciences, Uppsala, Sweden) and resuspended in RPMI 1640 (GIBCO™, Invitrogen, Grand Island, NY) supplemented with 0.28 mg/ml of gentamycin sulfate and 20 % human serum (of the own donor) at final cell density of 1.9×10^6/ml as previously described [28]. Platelet contamination of these preparations was <1 %. The viability as measured by trypan blue dye exclusion was uniformly greater than or equal to 90 %. The cells were maintained in a 5 % CO_2 humidified incubator at 37 °C for 18 h.

Genotyping

Genomic DNA was extracted from leukocytes by a standard method [29]. Polymerase chain reaction (PCR) was performed at a total volume of 25 µl with about 10–100 ng of genomic DNA, 1.6 U *Taq* DNA polymerase in *Taq* buffer (Life Technologies—Brazil Ltda. INVITROGEN

Inv. São Paulo, SP, Brazil), final concentration of each dNTP 0.2 mM, and 2 mM $MgCl_2$, 10 % DMSO. The exon 2 segment of the SOD2 gene was amplified using primers: sense 5′-GCC CAG CCT GCG TAG ACG GTC CC-3′ and anti-sense 5′-TGC CTG GAG CCC AGA TAC CCC AAG-3′ (Life Technologies—Brazil Ltda. INVITROGEN Inv. São Paulo, SP, Brazil), where the underlined nucleotide represents the deliberate primer mismatches designed to introduce artificial restriction site [30] for the determination of 47C>T SOD2 SNP, as well as the exon 3 segment was amplified using primers: sense 5′-AGC TGG TCC CAT TAT CTA ATA G-3′ and anti-sense 5′- TCA GTG CAG GCT GAA GAG AT-3′ (Life Technologies—Brazil Ltda. INVITROGEN Inv. São Paulo, SP, Brazil) for the determination of 173T>C SOD2 SNP [31]. The PCR was performed on an PTC-100 thermocycler (MJ Research, Inc. Watertown, MA, USA) as follows: an initial denaturation at 95 °C for 6 min, followed by 35 cycles at 95 °C for 1 min, at 60 °C for 1 min, and at 72 °C for 1 min and 30 s. The final extension step was prolonged to 7 min. The PCR products were digested by the *Hae*III and *Eco*RV restriction endonucleases for the Val-9Ala and Ile58Thr polymorphisms, respectively. At least 15 % of the samples were subjected to a second, independent PCR restriction fragment length-polymorphism analysis in order to confirm their genotypes.

Experimental groups

All the 30 samples were organized in two groups: control (without LPS stimuli) and with LPS stimulation (100 ng/ml by 18 h) (*Escherichia coli* serotype 055:B5, Sigma, St. Louis, MO, USA). Fifteen samples were from 47C allele carriers, and 15 samples were from 47T allele carriers in each group. The selected healthy subjects have an average age of 26.1 years (19.8–32.4 years) and body mass index (BMI) (as stipulated by World Health Organization) of 22.3 (19.2–25.4) (Table 1), and subjects with a history of diabetes, alcohol abuse, cancer, or vitamin supplements were excluded. The cells from homozygote carriers of the 47C allele (the allele encoding alanine) were called 47CC cells; the cells from homozygote carriers of the 47T allele (the allele encoding valine) were called 47TT cells.

Total radical-trapping antioxidant parameter (TRAP assay)

The non-enzymatic antioxidant cellular defenses were estimated by the total radical-trapping antioxidant parameter, which determines the non-enzymatic antioxidant potential of the sample, as previously described [32]. In brief, the reaction was initiated by injecting luminol and 2,2-azobis [2-methylpropionamidine]dihydrochloride (AAPH)—a free radical source that produces peroxyl radical at a constant rate—in glycine buffer (0.1 M, pH 8.6), resulting in a steady luminescent emission. PBMCs samples (10 μg of protein) were mixed in glycine buffer in their action vial and the decrease in luminescence monitored in a liquid scintillation counter for 60 min after the addition of the sample homogenates. The area under the curve obtained of the chemiluminescence values were transformed to percentage values and compared against the control values.

Conjugated dienes

For quantification of conjugated dienes, test samples (tissue/membrane fractions) subjected to oxidative stress were treated with chloroform:methanol mixture (2:1) followed by vigorous vortexing and centrifugation at 8,000 × *g* for 10 min. The upper layer obtained was discarded

Table 1 Demographic, clinical, and genotypic profile of donors

Variables	All	With 47CC	With 47TT	*p*
Donors [n (%)]	30 (100)	15 (50)	15 (50)	
Female [*n* (%)]	21 (70.0)	11 (52.4)	10 (47.6)	0.690^{X^2}
Age [years; mean (SD)]	26.1 (6.3)	25.9 (7.3)	26.3 (5.3)	0.954^{ST}
Weight [mean (SD)]	65.2 (10.3)	65.7 (10.0)	64.7 (10.9)	0.861^{ST}
Height [mean (SD)]	1.71 (0.9)	1.71 (0.06)	1.71 (0.11)	0.057^{ST}
BMI [mean (SD)]	22.3 (3.1)	22.4 (3.1)	22.1 (3.2)	0.915^{ST}
Smokers [n (%)]	3 (10)	2 (66.7)	1 (33.3)	0.543^{X^2}
Antidepressant [n (%)]	5 (16.7)	3 (60)	2 (40)	0.624^{X^2}
Diabetes [n (%)]	0 (0)	0 (0)	0 (0)	–
Vitamin Supplements [n (%)]	0 (0)	0 (0)	0 (0)	–

p value describes a comparison between 47CC and 47TT genotype

47CC 47CC homozygotes, *47TT* 47TT homozygotes to 47C>T SOD2 SNP, *BMI* body mass index, *n* number, *SD* standard deviation of the mean, *ST* Student's *t* test, X^2 Pearson chi-square test

along with the proteins, while the lower chloroform layer was dried under a stream of nitrogen at 45 °C. The residue obtained was dissolved in cyclohexane and absorbance was taken at 233 nm against a cyclohexane. The sensitivity of this assay is up to a few nanomoles (2–3 nmol) [33].

ELISA analyses and nitrite assay

At the end of the incubation period, the medium (supernatants) were collected, centrifuged, and frozen at −70 °C until further analysis for tumor necrosis factor-alpha (TNF-α) and nitrite. All samples were assayed in triplicate.

TNF-α protein was assessed using indirect ELISA method with a minimum level of detection ranging from 0.1 to 1,000 ηg/ml using standard curve purified protein (abcam—ab9642). In brief, the samples and standards were added and the plate was incubated overnight at 4 °C. The plate was washed four times with PBS and 0.05 % Tween 20 (Sigma, St. Louis, MO, USA). The plate was blocked with 1 % bovine serum albumin and incubated for 1 h at room temperature before washing four times with PBS and 0.05 % Tween 20. Polyclonal antibody specific for TNF-α (Sigma, St. Louis, MO, USA) was added to the wells incubated for 2 h at room temperature. After washing away, a peroxidase-conjugated secondary antibody (Cell Signaling Technology, Danvers, MA, USA) was added to the wells and incubated for 1 h at room temperature. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution was added to the wells. The enzyme reaction yields a blue color product that turns yellow when the stop solution is added. The color developed in proportion to the amount of TNF-α bound in the initial step. Absorbances were measured at 450 nm.

Nitrite concentrations were determined using a microplate assay based on method of Green et al. [34]. Samples supernatant (100 µl) were added to saturated solution of vanadium chloride (VCl_3) (400 mg), prepared in 1 M hydrogen chloride (HCl 50 ml) for reduction of nitrate to nitrite, and sodium nitrite standards in complete culture medium were mixed with 100 µl Griess reagent (1:1 0.1 % naphthyl-ethylenediamine and 1 % sulfanilamide in 5 % phosphoric acid), and then incubated for 10 min at room temperature and the nitrite content was measured by absorbance at 540 nm. Nitrite concentration in the samples was calculated with a standard curve prepared using $NaNO_2$.

Antioxidant enzyme activities

Catalase (CAT) activity was assayed by measuring the rate of decrease of H_2O_2 absorbance in a spectrophotometer at 240 nm [35], and the results are expressed as U/mg protein. Superoxide dismutase enzyme activity was assessed by quantifying the inhibition of superoxide-dependent adrenaline auto-oxidation in a spectrophotometer at 480 nm, as previously described [36]. Total SOD activity was assayed without KCN, and enzymatic activity of MnSOD was determined in cellular extracts by the cytochrome *c* reduction method using 1 mM KCN as previously described [37]. The difference of total and MnSOD activity was considered to represent CuZn SOD activity. To exclude the presence of nonenzymatic SOD-like activity in the homogenate, SOD activities were assayed using the boiled homogenate, and it was confirmed that the nonenzymatic activity was under the detection limit. The results are expressed as U/mg protein. To determine glutathione peroxidase (GPx) activity, the rate of NAD(P)H oxidation was measured in a spectrophotometer at 340 nm in the presence of reduced glutathione, *tert*-butyl hydroperoxide, and glutathione reductase, as previously described [38]. A ratio between SOD activity and CAT activity (SOD/CAT ratio) was applied to better understand the effect of SOD2 Ala-9Val polymorphism upon these two oxidant-detoxifying enzymes that work sequentially converting the superoxide anion to water [39]. An imbalance between their activities is thought to facilitate oxidative-dependent alterations in the cellular environment, which may culminate in oxidative stress.

Protein determination

All the results were normalized by the protein content using the Lowry method [40].

Statistical analysis

Results were expressed as the mean ± SEM of at least three independent experiments and each sample was performed in triplicate. Data were analyzed by Student's *t* test. When appropriate, a one-way analysis of variance (ANOVA) and individual group means compared using Tukey's multiple group comparison test was performed. To test Hardy–Weinberg equilibrium, the Chi-squared test was used, as well as to evaluate the influence of individual genotype with demographic and clinical data. Differences were considered to be significant when $p < 0.05$.

Results

We first compared the antioxidant enzyme activities and redox parameters between 47CC and 47TT not stimulated by LPS, in order to establish if the different genotypes influence oxidative parameters under basal conditions. In untreated cells, basal SOD activity of 47CC cells was found to be higher than in 47TT cells (23.31 ± 8.48 U/mg protein

vs 19.15 ± 4.72 in 47TT, $p < 0.05$) (Fig. 1a). In this condition also, 47CC cells presented a higher basal activity for CAT (8.07 ± 2.45 U/mg protein vs 5.24 ± 1.26, $p < 0.01$) (Fig. 2a). However, we did not observe any differences in the SOD/GPx ratio, SOD/GPx + CAT ratio (data not shown) and SOD/CAT ratio between the two phenotypes (Fig. 2b). We also observed by TRAP/TAR analysis that 47CC cells possess a higher capacity of basal nonenzymatic antioxidant defenses as compared to the 47TT cells (12.7 ± 3.2 % chemiluminescence vs 20.1 ± 6.1; $p < 0.05$, respectively) (Fig. 3a, b). Besides, levels of conjugated dienes were not different between 47CC and 47TT cells, indicating that basal lipoperoxidation is not different between cells with different alleles (Fig. 3c).

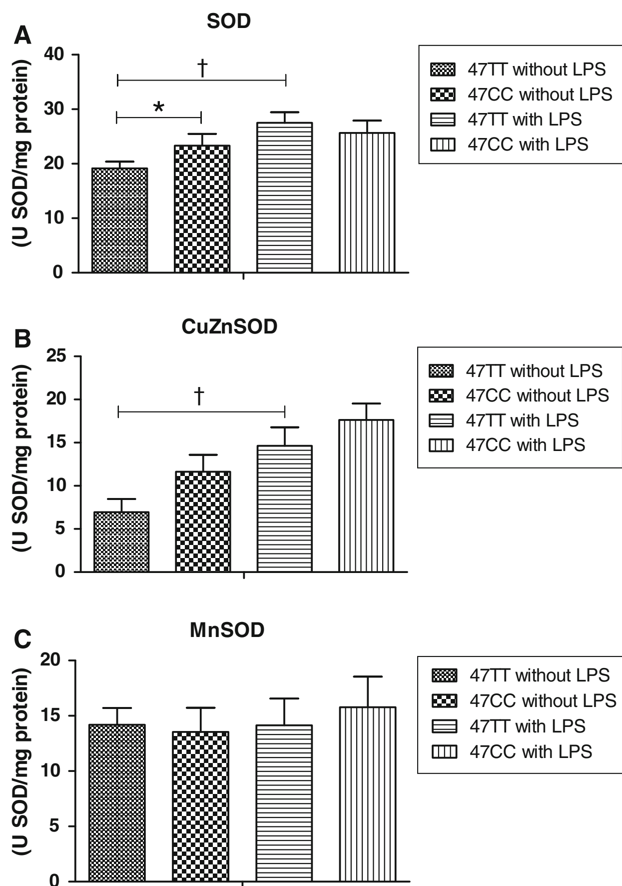

Fig. 1 SOD activity in PBMCs separate by 47C>T SOD2 SNP challenged or not with LPS 100 ng/ml by 18 h. **a** Total SOD activity was assessed by quantifying the inhibition of superoxide-dependent adrenaline auto-oxidation in a spectrophotometer at 480 nm. The difference of total and MnSOD activity was considered to represent CuZnSOD activity (**b**). **c** MnSOD activity was determined in cellular extracts by the cytochrome *c* reduction method using 1 mM KCN. $^{*}p < 0.05$ value describes a comparison between 47CC and 47TT genotype without LPS; $^{\dagger}p < 0.05$ value describes a comparison between 47TT cells without LPS and with LPS (Student's *t* test was performed)

There was no statistical difference in GPx activity among the two cell types (43.91 ± 9.91 NADPH mM/min/mg protein vs 35.27 ± 10.98, $p = 0.660$; 47CC and 47TT cells, respectively) (Fig. 4a), as well as in the TNF-α production (0.42 ± 0.05 ηg/ml vs 0.40 ± 0.03, $p = 0.091$; 47CC and 47TT cells, respectively) (Fig. 4a, c). On the other hand, nitrite production is different one among the alleles (1.21 ± 0.54 μM vs 1.04 ± 0.30, $p < 0.01$; 47CC and 47TT cells, respectively) (Fig. 4b).

We next subjected both 47CC and 47TT to LPS stimulation as described in "Materials and methods" section and evaluated antioxidant enzyme activities and redox

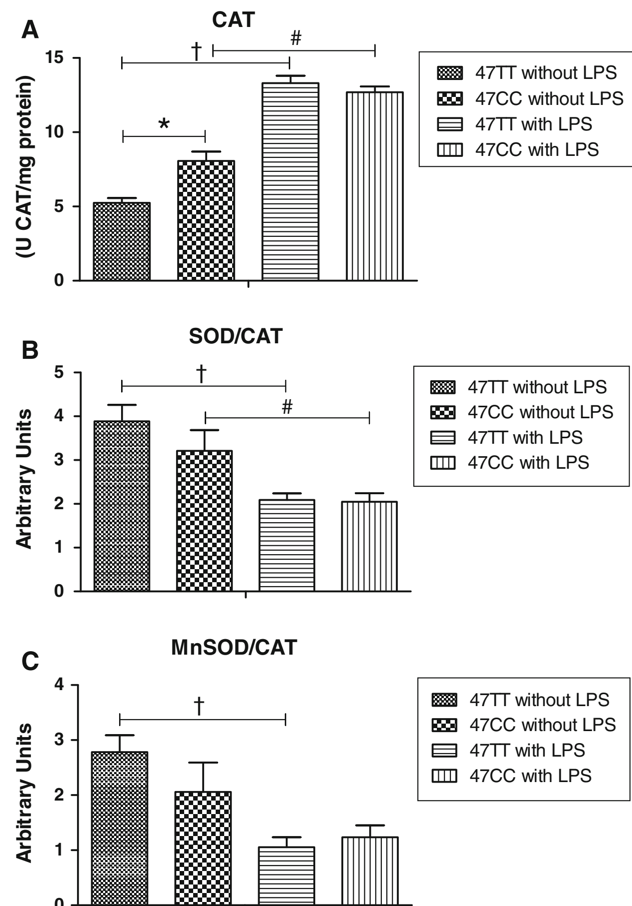

Fig. 2 Catalase activity and ratio between SOD activity and CAT activity in PBMCs separate by 47C>T SOD2 SNP challenged or not with LPS 100 ng/ml by 18 h. **a** Catalase activity was assayed by measuring the rate of decrease in H_2O_2 absorbance in a spectrophotometer at 240 nm; **b** Ratio between SOD activity and CAT activity, and **c** ratio between MnSOD activity and CAT activity are applied to better understand the effect of SOD2 Ala-9Val polymorphism upon these two oxidant-detoxifying enzymes that work sequentially converting the superoxide anion to water. $^{*}p < 0.05$ value describes a comparison between 47CC and 47TT genotype without LPS; $^{\dagger}p < 0.05$ value describes a comparison between 47TT cells without LPS and with LPS; $^{\#}p < 0.05$ value describes a comparison between 47CC cells without LPS and with LPS (Student's *t* test was performed)

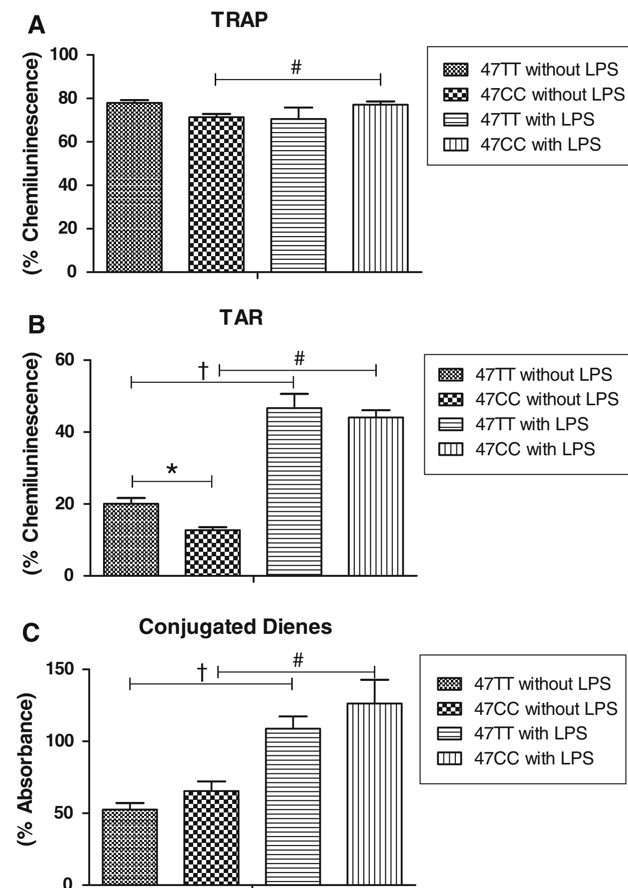

Fig. 3 Total radical-trapping antioxidant parameter, total antioxidant reactivity, and lipid peroxidation in PBMCs separate by 47C>T SOD2 SNP challenged or not with LPS 100 ng/ml by 18 h. **a** TRAP index was measured by luminol-enhanced chemiluminescence as described in "Materials and methods" section. **b** TAR is calculations through the chemiluminescence emitted from samples at 1 min of experiment. **c** The detection of conjugated dienes is done by absorption at 233 nm. $*p < 0.05$ value describes a comparison between 47CC and 47TT genotype without LPS; $^{\dagger}p < 0.05$ value describes a comparison between 47TT cells without LPS and with LPS; $^{\#}p < 0.05$ value describes a comparison between 47CC cells without LPS and with LPS (Student's *t* test was performed)

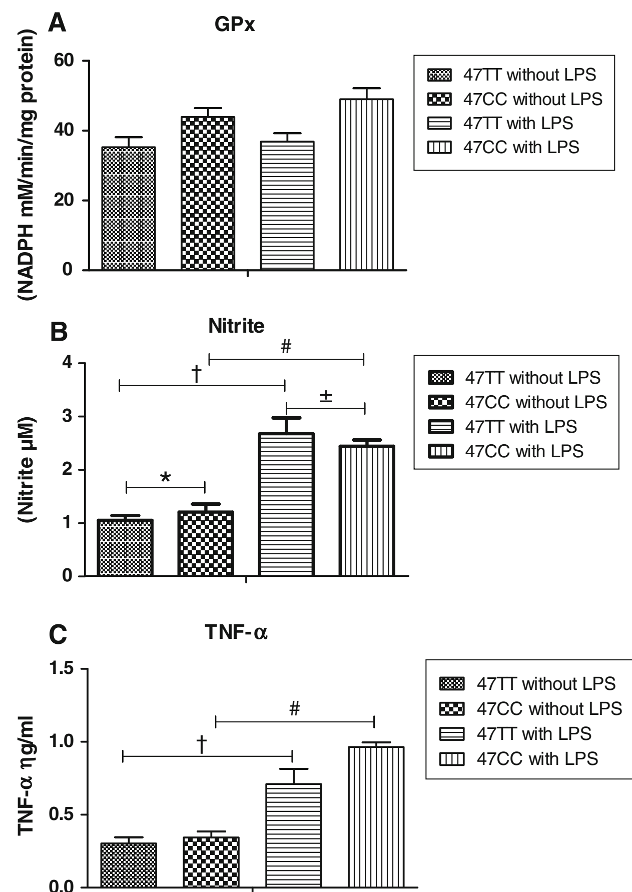

Fig. 4 Glutathione peroxidase activity, nitrite production, and TNF-α production in PBMCs separate by 47C>T SOD2 SNP challenged or not with LPS 100 ng/ml by 18 h, at the end of the incubation period, supernatants were collected for the nitrite and TNF-α quantification. **a** GPx activity the rate of NAD(P)H oxidation was measured in a spectrophotometer at 340 nm in the presence of reduced glutathione. **b** Nitrite production (nM) was measured by the Greiss assay. **c** TNF-α release was measured by ELISA. $*p < 0.05$ value describes a comparison between 47CC and 47TT genotype without LPS; $^{\dagger}p < 0.01$ value describes a comparison between 47TT cells without LPS and with LPS; $^{\#}p < 0.01$ value describes a comparison between 47CC cells without LPS and with LPS; $^{\pm}p < 0.05$ value describes a comparison between 47CC and 47TT genotype with LPS (Student's *t* test was performed)

parameters. SOD activity increased in the 47TT cells only (23.31 ± 8.48 U/mg protein vs 25.65 ± 8.81 in 47CC, $p = 0.470$; 19.15 ± 4.72 U/mg protein vs 27.52 ± 7.47 in 47TT, $p < 0.01$) (Fig. 1a) and CAT activity increased in both cell types when stimulated by LPS (8.07 ± 2.45 U/mg protein vs 12.69 ± 1.51 in 47CC, $p < 0.01$; 5.24 ± 1.26 U/mg protein vs 12.69 ± 1.51 in 47TT, $p < 0.01$) (Fig. 2a), resulting in a decrease in the SOD/CAT activity ratio (Fig. 2b). A decrease in the MnSOD/CAT ratio was observed in 47TT cells only (Fig. 2c). SOD/GPx ration and SOD/GPx + CAT ratio were not altered in both cell types (data not shown). TRAP/TAR analysis indicated that 47CC cells stimulated by LPS had a higher increase in the pro-oxidant cellular status than the 47TT cells (71.4 ± 5.9 % chemiluminescence vs 78.6 ± 4.2, and 77.1 ± 5.9 % chemiluminescence vs 75.6 ± 4.9; $p < 0.01$, respectively) (Fig. 3a, b). Moreover, both cells had equally increased lipoperoxidation induced by LPS stimulation as indicated by the increase in levels of conjugated dienes as shown in Fig. 3c. GPx activity in both 47CC and 47TT cells was not significantly altered by LPS stimulation (43.91 ± 9.91 NADPH mM/min/mg protein vs 49.02 ± 12.14 in 47CC, $p = 0.140$; 35.27 ± 10.98 NADPH mM/min/mg protein vs 36.88 ± 9.33 in 47TT, $p = 0.701$) (Fig. 4a). In nitrite production as well as difference it was

seen among the alleles in the control, it was seen in the cells challenged with LPS (2.44 ± 0.44 µM vs 2.68 ± 1.06, $p < 0.05$; 47CC and 47TT cells, respectively) (Fig. 4b), for nitrite and TNF-α production in both 47CC and 47TT cells was significant altered by LPS stimulation compared with control ($p < 0.01$) (Fig. 4b, c). But there is no difference in the TNF-α production among the challenged alleles (0.96 ± 0.12 ηg/ml vs 0.93 ± 0.15, $p = 0.233$; 47CC and 47TT cells, respectively) (Fig. 4c).

Discussion

The 47C>T SNP may have a functional effect in the activity of MnSOD [19], but its phenotypical significances and consequences on sepsis, septic shock, organ dysfunction, or mortality are still unknown. Leukocytes, particularly the macrophages, are sensitive to oxidative stress and may have their function highly impaired under conditions of severe inflammation combined with enhanced reactive species production, such as in sepsis [41]. Reactive species, in turn, are frequently associated to the intracellular signal cascade elicited by LPS that activates the nuclear factor-κB (NF-κB) and causes the release of proinflammatory cytokines, thus establishing a chronic state of proinflammatory signaling [42].

All these signaling events are observed at several degrees in the different clinical stages of sepsis. However, sepsis outcome presents a high degree of variation which is believed to be associated to multiple environmental and genetic factors [43]. SOD2 is one of the main enzymes responsible for the so-called primary antioxidant defense response, acting directly on excess superoxide radicals derived from increased mitochondrial activity [44]. In macrophages, mitochondrial electron transport chain is greatly enhanced during the respiratory burst induced by proinflammatory stimuli [45]. Here, we compared blood mononuclear cells of 30 healthy humans volunteers separated by SOD2 47C>T SNP into two different experimental groups (15 volunteer for each allele) with the objective to investigate if there is any significative difference in the redox environment before and after macrophages presenting different SOD2 polymorphisms were challenged by LPS. Data regarding BMI, tobacco smoking, and antidepressant use were analyzed along with the results of oxidative parameters (enzymatic activity, lipid peroxidation, TAR/TRAP assay) and we found significant correlation between BMI and GPx activity after LPS stimulation and also between smokers and GPx activity in cells not treated with LPS (data not shown). It is very likely that these data reflect an influence of sample number (Table 1). However, this relationship had no influence on the results of oxidative stress parameters in PBMCs with the different alleles with or without LPS stimulation.

We observed here that although the Ala-9Val polymorphism did not significantly affect MnSOD activity (Fig. 1c), it induced a change in total SOD activity and catalase activity (Figs. 1a, 2a). Elsakka et al. [46] also reported that cells with different alleles had no significant differences among MsSOD activity; however, differently from our data, they observed a decrease in enzyme activity after the challenge with LPS. Our results suggest that the 47CC cells present a lower pro-oxidant state than the 47TT cells under basal conditions. This conclusion can be drafted from the following observations: (i) higher SOD and CAT activities; (ii) more pronounced nonenzymatic antioxidant defenses in 47CC cells, as observed with lower values of chemiluminescence (Figs. 1a, 2a, 3b); (iii) increased nitrite accumulation in allele C (Fig. 4b). However, when PBMCs are challenged by LPS, the profile of oxidant status is inverted in relationship to the basal conditions, as SOD activity was increased and the Mn-SOD/CAT ratio was decreased only in 47TT cells (Figs. 1a, 2c); the TRAP assays indicated a higher pro-oxidant status in 47CC cells (Fig. 3a). Besides, the nitrite profile also is inverted (Fig. 4b).

In some conditions of biologic stress, such as ischemia or sepsis, uncoupling of electron transport may occur due to a variety of reasons, and as a consequence, O_2^- production increases [47–49]. Our data suggest that, under pro-inflammatory conditions, carriers of the 47C allele will present a higher pro-oxidant state compared to 47T allele carriers, most probably as consequence of increased H_2O_2 production. Hydrogen peroxide results from SOD2 detoxification activity on superoxide, being less reactive and potentially damaging to biomolecules. However, H_2O_2 may also favor intracellular hydroxyl radical (OH^-) formation through iron/copper-mediated Fenton reaction [45]. Hydroxyl radicals are highly toxic, powerfully reactive, and potent oxidizing agents, promoting oxidative stress to cells at several degrees. Thus, it is possible that an association of the 47C allele with a less favorable clinical outcome in sepsis is related to a more pronounced pro-oxidant state resulting from increased H_2O_2 production, especially if GPx and CAT activities are not coupled to SOD activity.

Mitochondrion is the main O_2 consuming and ROS-generating organelle of the cell. Suliman et al. [50] postulated that cell activation by LPS, which stimulates cytokine and ROS production, would damage mitochondria by oxidation of mtDNA. To test their hypothesis, they injected LPS intraperitoneally and evaluated several oxidative parameters in liver mitochondria. LPS injection caused a significant decrease in mtDNA copy number, and also depleted glutathione (GSH) and increased

mitochondrial lipid peroxidation. Besides, MnSOD gene expression was enhanced, thus indicating LPS treatment caused an increase in mitochondrial superoxide production, probably due to enhancement of both electron transport chain activity and uncoupling. These results reinforce the significance of SOD2 in the regulation of the redox state during pro-inflammatory stimulation.

The evaluation of critically ill patients admitted to the ICU is mainly accomplished through instruments that analyze the dysfunction of organs and systems through monitoring of their physiologic state. This approach, however, does not consider the genetic history of each patient. The genomic medicine aims to identify genetic patterns that turn the individual to be more or less susceptible to diseases [51]. A wide range of alterations (mutations) related to the susceptibilities to sepsis have been identified [22, 52]. The expression of such variants influences disease manifestation or outcome. The identification of SOD alleles in sepsis patients, in this regard, may be helpful to identify patients susceptibilities and to indicate clinical procedures more adequate for each case.

In conclusion, our results demonstrated that the Ala-9 val polymorphism of SOD2 actively participates in the regulation of cellular redox environment ,and the identification of the genotype/phenotype of patients may be a relevant step in understanding the clinical evolution of diseases with a pro-inflammatory component. Our group intends to perform further investigations in order to understand the influence of this polymorphism in other critical diseases, such as breast/prostate and lung cancer, motor neuron diseases.

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