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RESEARCH ARTICLE

The anti-inflammatory effects of resveratrol on human peripheral blood mononuclear cells are influenced by a superoxide dismutase 2 gene polymorphism

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Abstract Resveratrol is an molecule that provides both anti-inflammatory and antioxidant properties. However, it is unclear whether the basal oxidative state of the cell has any influence on the effects of this compound. In humans, a single nucleotide polymorphism (SNP) is present in the enzyme manganese superoxide dismutase (SOD2), localized in codon 16 (rs4880), which can either be an alanine (A) or valine (V). This SNP causes an imbalance in the cellular levels of SOD2, where AA- and VV-genotypes result in higher or lower enzymatic activity, respectively. Furthermore, the VV-genotype has been associated with high levels of inflammatory cytokines. Here, we examined the effects of a range of resveratrol concentrations on the in vitro activation of human peripheral blood mononuclear cells (PBMCs) carrying different Ala16Val-SOD2 genotypes. Cell proliferation, several oxidative biomarkers and cytokines (IL-1β, IL-6, TNFα, Igγ and IL-10) were analyzed. In addition, the effects of resveratrol on the expression of the *sirt1* gene were evaluated by qRT-PCR. After 24 h exposure to resveratrol, A-genotype PBMCs displayed a decrease in cell proliferation, whilst VV-cells contrasted; At 10 μM resveratrol, there was a significant decrease in the production of inflammatory cytokines in A-allele cells; however, VV-cells generally displayed a subtle decrease in these, except for TNFα, which was not affected. In all SOD2 genotypes cells exposed to resveratrol resulted in an upregulation of Sirt1 levels. Together, these results suggest that the effect of resveratrol on human PBMC activation is not universal and is dependent on the Ala16Val-SOD2 SNP.

Keywords Superoxide dismutase manganese-dependent · Sirtuin 1 · Cytokines · Oxidative stress · Resveratrol · Inflammation

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Introduction

Resveratrol (3,5,4′-trihydroxy-*trans*-stilbene); a polyphenol compound found at high concentrations in grapes that displays anticarcinogenic, cardioprotective

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and neuroprotective properties (Švajger and Jeras 2012) and effect against chronic inflammation that is strongly affected by oxidative stress (Cavagnat et al. 2012; Fulop et al. 2014).

Resveratrol has been linked to its role as a caloric restriction mimetic since increases cell lifespan where it can activate the expression of gene products such as Sirtuin 1 (silent mating type information regulation 2 homolog 1; Sirt1), a deacetylase and/or ADP-ribosyl-transferase protein that can modulate the effects of energy intake over the lifespan of an organism (Poulsen et al. 2013). The effects of resveratrol on the inhibition of T cell activation appear to involve an increase in Sirt1 activity (Wang et al. 2013; Zou et al. 2013). However, it is unclear whether the metabolic oxidative state of cells has an influence over the anti-inflammatory effects that are linked to this drug.

In humans, some people present a basal superoxide anion ($O_2^{-\bullet}$) and hydrogen peroxide (H_2O_2) imbalance, which is genetically determined by a single nucleotide polymorphism (SNP) localized in the manganese superoxide dismutase (MnSOD or SOD2) gene. SOD2 is an antioxidant enzyme that represents the major defense against reactive oxygen species (ROS) within the mitochondria, a cellular compartment where there is a continuous production of $O_2^{-\bullet}$ by the electron transport chain. (Holley et al. 2012; Kamiński et al. 2013).

The SNP that causes basal metabolic SOD2 imbalance is located at codon 16 (rs4880) and encodes for either an alanine or valine residue at the—9 position in the mitochondrial targeting sequence (MTS). The results of an Ala16Val-SOD2 SNP is that the overall $O_2^{-\bullet}$ scavenging efficiency in the cell is reduced but rather than this being related to enzymatic activity, it is due to defects in its transport into the mitochondria (Shimoda-Matsubayashi et al. 2006). The Ala variant is able to traverse both mitochondrial membranes quickly in order to enter the matrix, whilst most of the Val16 variant remain imbedded within the inner membrane (Sutton et al. 2005). This manifests itself as an imbalance in $O_2^{-\bullet}$ and H2O2 levels in mitochondria and several studies have described an association between an A-allele and AA-genotype with several types of cancer. On the other hand, other investigations have described an association between a V-allele and VV-genotype with metabolic morbidities such as hypercholesterolemia, obesity, cardiovascular dysfunction and diabetes complications (Bresciani et al. 2013a). However, these Ala16Val-SOD2 SNP derived outcomes are influenced by environmental factors such as dietary antioxidant intake and exercise (Ambrosone et al. 1999; Bresciani et al. 2013b).

Previous in vitro investigations using samples of peripheral blood mononuclear cells (PBMCs) from carriers of Ala16Val-SO2 genotypes showed that this polymorphism has an influence on the viability, antioxidant and inflammatory responses to hepatocytes cryopreservation (Martin et al. 2009) exposure to ultraviolet radiation and methylmercury (dos Santos Montagner et al. 2010; Algarve et al. 2013), elevated glucose/insulin levels (Montano et al. 2012) and a number of drugs such as clomiphene citrate (Costa et al. 2012) and methotrexate (Barbisan et al. 2014). In this study we have examined the impact of resveratrol on the activation of human PBMCs carrying different Ala16Val-SOD2 genotypes evaluating its effect on cell proliferation, oxidative stress biomarkers and on Sirt1 expression.

Materials and methods

Volunteers and Ala16Val-SOD2 SNP genotyping

The study described here is associated with a research project that was previously approved by the Ethics Committee at the Universidade Federal de Santa Maria, Brazil. 120 subjects were invited to participate of the present study and were enrolled from Santa Maria university community and presented similar health, anthropometric and lifestyle conditions including no previous history of any chronic degenerative disease or dysfunction that could influence the results. Most volunteers were undergraduate student with a similar habitual activities. Subjects that were non-smokers and not obese did not present previous non-transmissible diseases, chronic use of medication or vitamin supplements and other dysfunctions that could influence oxidative metabolism and *sirt1* gene expression. Blood samples were collected by venipuncture in EDTA tubes from this group and the Val16Ala polymorphism group. Ala16Val-SOD2 genotyping was performed as described by (Barbisan et al. 2014) using a Phusion High-Fidelity PCR kit (Thermo Scientific CO) to access genomic DNA and amplify the *SOD2* gene segment by tetra-primer ARMS-PCR

analysis: Primer F1 (forward): CACCAGCACTAG CAGCATGT; F2 (forward): GCAGGCAGCTGGCT ACGGT; R1 (reverse): ACGCCTCCTGGTACTTC TCC; R2 (reverse): CCTGGAGCCC AGATACCC TAAAG.

The PCR reaction was carried out in a total volume of 40 µl containing 20–40 ng of genomic DNA template, 0.5 µM of each primer, 100 µM dNTP mix, 1.25 mM $MgCl_2$, PCR buffer (20 mM Tris–HCl (pH 8.4), 50 mM KCl), 5 % dimethyl sulfoxide (DMSO) and 1.25 U of DNA polymerase. The PCR amplification was carried out with an initial denaturation at 94 °C for 7 min, followed by 35 cycles of 60 s at 94 °C (denaturation), 20 s at 60 °C (annealing), and 30 s at 72 °C (extension), with an additional 7 min of extension at 72 °C at the end of the final cycle. A 20 µl aliquot of the PCR product was mixed with 6 µl of loading buffer and resolved by electrophoresis in a 1.5 % agarose gel. This procedure resulted in three bands in heterozygotes (514, 366, and 189 bp) and two bands in homozygotes (Val/Val resulting in bands of 514 and 189 bp, and Ala/Ala resulting in bands of 514 and 366 bp). The SOD2 genotype frequencies were the following: AA, 26.7 %; VV, 28.2 %; and AV, 45.1 %. We performed calculations to assess any deviations from the Hardy–Weinberg equilibrium, used to assess the Chi squared goodness-of-fit, which showed that the samples were in genetic equilibrium. From the 120 genotyped subjects, we selected 6 VV, 10 AV and 6 AA carriers to obtain blood samples to perform the in vitro protocols. At least, each volunteer donated three blood samples in order to make the triplicate analysis. Volunteers were asked to avoid eat/drink antioxidant foods such as vegetables and fruits before 24 h as well as multivitamin supplements to minimize some effect on oxidative metabolism of blood samples.

PBMC cultures and resveratrol treatment

A randomized sub-sample of volunteers were selected to obtain blood samples to perform the in vitro experimentation as described previously by Algarve et al. (2013), with slight modifications. The fasting blood collections were obtained from 5–6 volunteers each with Ala16Val-SOD2 genotypes. To carry out all remaining protocols, blood samples were collected from 12 volunteers. The volunteers were asked to avoid the consumption of antioxidant-containing foods (e.g. salads, fruits and natural/manufactured juices) 24 h prior to each blood collection. Blood samples were collected by venous puncture into grey and red top Vacutainer tubes with heparin (5 ml) and PBMCs were obtained within 1 h of collection using Histopaque (Hp) density gradient medium centrifuged for 15 min at 2500×*g*. The PBMCs were counted in a Neubauer chamber using Trypan exclusion dye and 1×10^6 PBMCs were cultured in RPMI 1630 culture medium with 10 % fetal bovine serum and 1 % penicillin/streptomycin at 37 °C in a humidified 5 % CO_2 atmosphere for 24 h. After this period, the supernatant containing PBMCs was collected and transferred into the same culture medium with the addition of phytohemagglutinin (PHA) mitogen, used to trigger T-lymphocyte cell division. Concomitantly these cells (1×10^5 concentration) were distributed in a 96-well plate with and without a range of resveratrol concentrations. The effect of resveratrol on PBMC activation was determined by analysis of the cellular proliferation of PBMCs carrying different SOD2 genotypes after 24 and 72 h.

The resveratrol compound was supplied in powder form and dissolved (stock solution, 100 mm) in DMSO (Sigma Chemical Co., St. Louis, MO), and added directly to cell culture medium at a final concentration of 0.1 % DMSO. To discard possible cytotoxic effects of resveratrol on the cells, we also performed an analysis of cellular mortality and oxidative stress biomarkers after 24 h. Over the same period we also determined the levels of the following cytokines involved in inflammation pathways: interleukin β (IL-β), interleukin 6 (IL-6), tumor necrosis factor alpha (TNFα), interferon gamma (Igγ) and interleukin 10 (IL-10). The effect of resveratrol on gene (*sirt1*) and protein (Sirt1) expression was also evaluated. The protocols used to perform these analyses are described below.

Cellular proliferation assay

Cellular proliferation was evaluated using a colorimetric MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay (Mosmann 1983), confirmed by flux cytometry analysis. The MTT assay measures the level of NAD(P)-H-dependent cellular oxidoreductase enzymes, which reflects the number of viable cells. After the incubation period, cells were stained for 1 h at 37 °C with 20 µl/well MTT reagent (10 %

concentration) and 5 mg/ml in PBS. Then 100 µl of DMSO was added per well to solubilize the purple formazan crystals that were produced. The absorbance of each well was measured at 570 nm and results were expressed as the average percentage of concentration compared to the control.

Cellular viability assay

To evaluate the potential cytotoxic or cytoprotective effects of resveratrol on PBMC activation, the concentration of free double stranded DNA (dsDNA) in cell culture was measured using a specific fluorochrome dye DNA Picogreen®. This creates a highly stable complex with dsDNA in alkaline conditions but not with single stranded DNA (ssDNA), proteins, SDS or urea. This selective characteristic can be used to identify dsDNA free in culture medium and indicates cellular death. The test was performed as previously described by Parra et al. (2012). Briefly, the cell medium was centrifuged at 2500×*g* for 10 min and the supernatant was collected and used to perform the test. The dsDNA was measured using 50 µl of the sample and 50 µl of the DNA Picogreen® dissolved in TE buffer, 1× (1:1; v/v), following by incubation for 5 min in the dark. Fluorescence was measured at an excitation of 485 nm and an emission of 520 nm at room temperature (SpectraMax M2/M2e Multi-mode Plate Reader, Molecular Devices Corporation, Sunnyvale, CA, USA). Results were expressed as a percentage of dsDNA calculated for each treatment in relation to the untreated control samples. Values below 100 % indicated a decrease and values above 100 % indicate an increase in the mortality of cells when compared to the control group.

Oxidative metabolism parameters

The ROS and lipoperoxidation levels of PBMCs after 24 and 72 h of supplementation with resveratrol were determined. The ROS levels were determined by dichlorofluorescein acetate fluorimetric assay (DCFDA) (Halliwell and Whiteman 2004) with fluorescence measured at an excitation of 485 nm and an emission of 520 nm. Lipoperoxidation was estimated by the formation of thiobarbituric acid reactive substances (TBARS) measured by spectrophotometry as described by Jentzsch et al. (1983). All measurements were performed in triplicate and the results are presented as a percentage compared to the untreated control group.

Cytokines and Sirt1 protein quantification

The inflammatory cytokines IL-1β, IL-6, TNFα, Igγ, anti-inflammatory cytokine IL-10 and Sirt1 were quantified with an immunological test, using a Sirt1 Human sandwich ELISA kit (ab123457), according to the manufacturer's instructions (ABCAN, Technology, San Diego, CA). Cytokines were measure in culture supernatants. The results were expressed as a percentage compared to the untreated control group of each Ala16Val-SOD2 genotype, as previously described in Montano et al. (2012).

Analysis of *sirt1* gene and Sirt1 protein expression

RNA was extracted using Trizol reagent and the quantity and purity was analyzed using a NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, Del., USA). A ratio of $A_{260/280}$ of 1.9–2.1 signified a pure RNA sample. Single stranded cDNA was synthesized using an iScript cDNA Synthesis Kit (Bio-Rad). The *sirt1* gene expression was evaluated by real-time polymerase chain reaction (PCR) performed with a StepOne Real Time PCR system (Applied Biosystems, Foster City, Calif., USA) with the SYBR Green detection method. PCR primers for *sirt1* and *β-actin* (a housekeeping gene) were designed by Primer Express 3 software (Applied Biosystems) and purchased from Metabion. The relative expression was calculated using the comparative Ct and was expressed as the fold expression compared to the control. The specific primer pairs used in this study were: *sirt-1* Forward ACAGGTTGCGGGAATCCAA and Reverse TCGTACAGCTTCACAGTCAACTTTG and *β-actin* Forward TGTGGATCAGCAAGCAGGAGTA and Reverse TGCGCAAGT TAGGTTTTGTCA (Wang et al. 2015). The PCR reactions were performed as follows: PCR proceeded in special optical tubes in 96-well reaction plates (MicroAmp Optical, ABI, Foster City, Calif., USA) with 10 µl of Power SYBR Green PCR Master Mix (Applied Biosystems), 0.5 µl forward primer, 0.5 µl reverse primer, 2 µl cDNA template and made up to 20 µl in DEPC treated water. The wells were sealed with optical adhesive film (Applied Biosystems) and the plate was centrifuged for several seconds 250 g. Amplification was performed

using the standard two-step run protocol: 95 °C for 3 min followed by 40 cycles of 95 °C for 10 s, 60 °C for 30 s and a melt curve of 65–95 °C in 0.5 °C increments for 5 s. For each gene, mRNA expression levels were normalized to the level of *β-actin* mRNA. The fold change in gene expression was computed using the comparative Ct ($2^{-\Delta\Delta Ct}$) method.

Statistical analysis

The data were analyzed by GraphPad Prism software by two-way analysis of variance followed by post hoc Bonferroni test. A p value ≤ 0.05 was considered statistically significant.

Results

Whilst the activation of PBMCs by PHA was affected by resveratrol treatment, the results were significantly influenced by Ala16Val-SOD2 genotypes. After 24 h the proliferation rate decreased significantly in AA-cells treated with all resveratrol concentrations when compared to untreated control (Fig. 1a). Cells carrying a heterozygous genotype showed a decrease in cell proliferation just in the high resveratrol concentrations tested here (10 and 30 μM). In contrast to AA- and AV-genotypes, cells carrying the VV-genotype did not show any significant differences in proliferation rate when they were exposed to resveratrol at 2 and 5 μM, but they did present a significant increase in cell proliferation when exposed to resveratrol at 10 and 30 μM. After 72 h incubation, resveratrol caused a higher cellular proliferation in Ala16Val-SOD2 homozygous genotypes (AA and VV) at ≥5 μM. The AV-cells displayed a decrease in proliferation when exposed to 2, 5 and 10 μM resveratrol; however, higher concentrations (30 μM) did not affect cellular proliferation when compared to the untreated control group (Fig. 1b).

A complementary assay that monitors free dsDNA levels present in culture medium was performed to evaluate whether resveratrol could affect cell viability within the first 24 h of exposure (Fig. 1c). The results show a significant decrease in dsDNA levels in cells exposed to resveratrol when compared to the untreated control groups, which indicates that resveratrol has a cytoprotective effect. However, this result was independent of the Ala16Val-SOD2 SNP and was detected in all concentrations of resveratrol tested here.

The effects of resveratrol on the levels of ROS and lipoperoxidation in activated PBMCs are shown in Fig. 2. After 24 h of PBMC activation, AV-cells exposed to resveratrol at all concentrations presented lower ROS levels when compared to the untreated control group (Fig. 2a). However, when AA- and VV-cells were exposed to resveratrol concentrations of 2 and 5 μM, the observed ROS levels were similar to those in the control group. Lipoperoxidation evaluated by TBARS levels after 24 h of exposure to resveratrol also showed a dependence on the Ala16Val-SOD2 SNP (Fig. 2b). Whereas AV-cells exposed to resveratrol did not present differences in TBARS levels compared with AV-untreated cells, at 10 μM resveratrol, TBARS levels were significantly decreased for VV-cells, whereas the opposite was observed for AA-cells.

The ROS and lipoperoxidation levels were also evaluated after 72 h (Fig. 2c). Whilst AV-cells, as measured at 24 h, presented low levels of ROS when treated with 5 and 10 μM resveratrol, both homozygous Ala16Val-SOD2 cells presented an increase in ROS levels when exposed at low resveratrol concentrations (2 μM). However, whereas we observed a decrease in ROS levels in AA-cells in the presence of 5 and 10 μM resveratrol, VV-cells displayed a significant increase in ROS levels at the same concentrations. At low resveratrol concentration (2 μM), an increase of TBARS levels was observed in all PBMCs independent of the SOD2 genotype. In contrast, in A-allele cells (AA and AV) high TBARS levels were also observed in samples treated with 5 μM resveratrol, whilst VV-cells showed lower levels when compared to the VV-control group. However, at the higher resveratrol concentration tested here, TBARS levels also significantly decreased in A-allele cells when compared to the untreated control groups.

We next examined the effect of resveratrol (10 μM) on cytokine and Sirt1 expression. Resveratrol significantly decreased the inflammatory cytokines in A-allele-carrying PBMCs (Fig. 3); however, the anti-inflammatory effect of resveratrol was more evident in AA-PBMCs. In VV-cells compared to the untreated VV-control group, resveratrol did not significantly decrease the level of TNFα, although it did have a mild effect on the levels of other inflammatory cytokines.

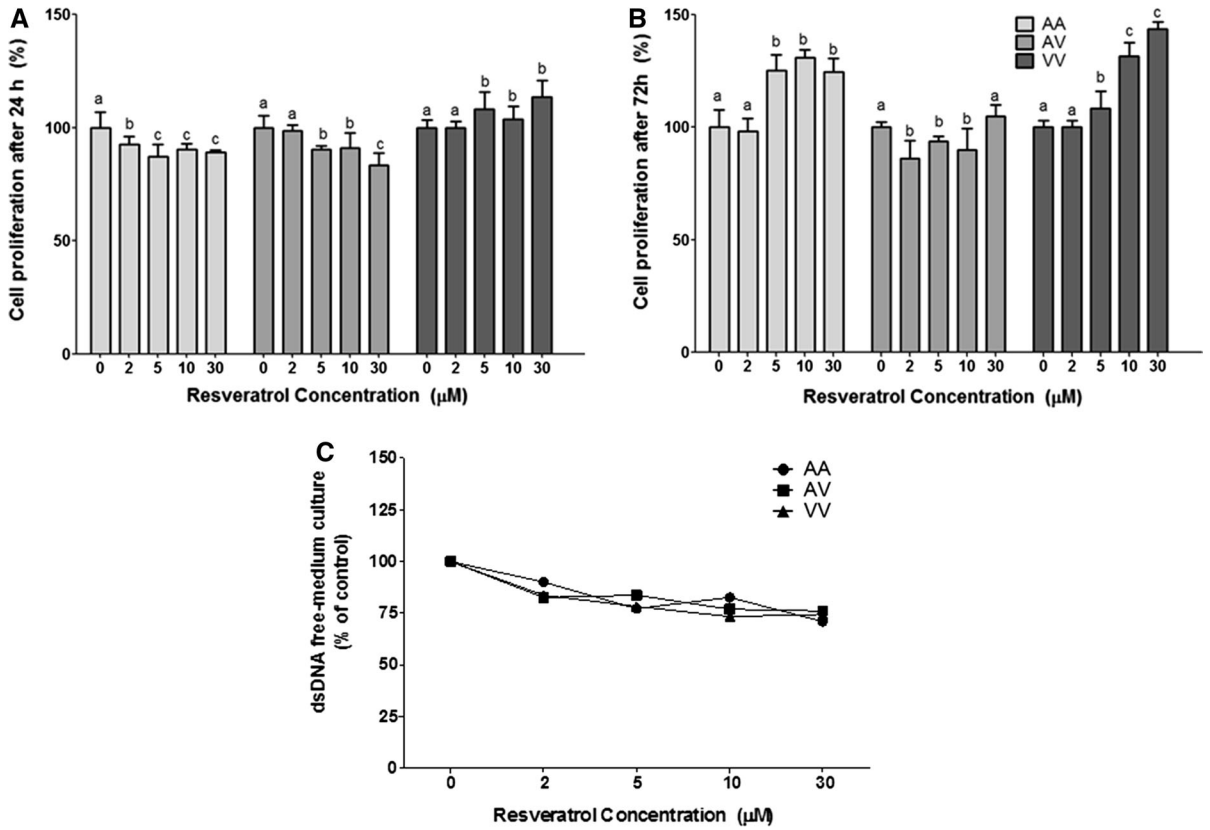

Fig. 1 Resveratrol effect on human PBMC carrier's different Ala16Val-SOD2 genotypes (AA, VV and AV). **a** Cell proliferation after 24 h and **b** 72 h exposition evaluated by MTT assay. Double-strand DNA degradation of cells exposed to different resveratrol concentrations evaluated by fluorimetry using DNA Picogreen dye. Different *letters* indicated statistical differences analyzed by One-way analysis of variance followed by Tukey post hoc test. Significance was considered when $p \leq 0.05$

Resveratrol did, however, significantly increase the levels of the anti-inflammatory cytokine IL-10 independent of the Ala16Val-SOD2 SNP.

The effect on *sirt1* gene and Sirt1 protein expression in PBMCs exposed to 10 μM resveratrol was also evaluated. As is evident in Fig. 4a, *sirt1* is upregulated by resveratrol independently of the Ala16Val-SOD2 genotype, however, this was more intense in AA-cells compared with cells carrying the V-allele. Furthermore, after 24 h exposure to resveratrol, Sirt1 protein levels (Fig. 4a) are in line with *sirt1* gene activation in all PBMCs, independent of Ala16Val-SOD2 genotypes. In relation to the untreated control group (100 %), AA-cells present Sirt1 levels at 163 %, VV-cells at 154 % and AV-cells at 139 %. This suggests that AA-cells are more responsive to resveratrol, leading to increased upregulation and higher protein levels of Sirt1 in this compared to the other Ala16Val-SOD2 genotypes.

Discussion

In general, our results corroborate previous investigations describing the inhibition of immune cell proliferation, decrease in inflammatory cytokine production (Fulop et al. 2014) and upregulation of *sirt1* gene expression (Hori et al. 2013) by resveratrol. However, to our knowledge we show for the first time that these effects on human PBMC activation are apparently not universal but influenced by the oxidative metabolic status of the cell associated with SOD2 enzymatic function. Studies performed by Kamiński et al. (2013) suggest that the SOD2 enzyme constitutes an

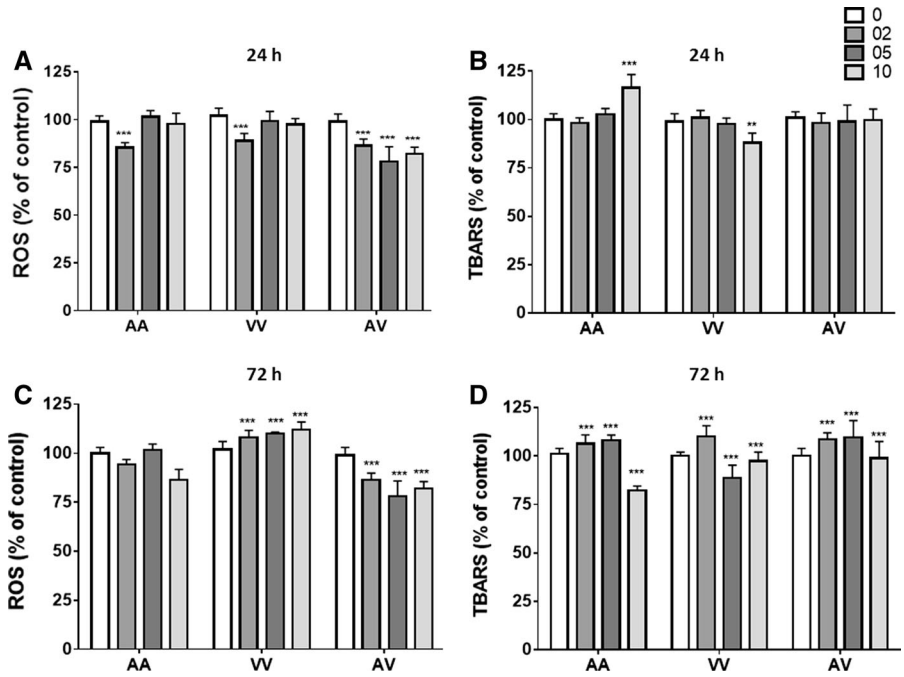

Fig. 2 Resveratrol effect on human PBMC carrier's different Ala16Val-SOD2 genotypes (AA, VV and AV) on reactive oxygen species evaluated by DCF-H fluorimetry assay and lipoperoxidation evaluated by TBARS-MDA quantification. **a** and **b** = levels after 24 h exposition; **c** and **d** = levels after72 h exposition. Different *letters* indicated statistical differences analyzed by One-way analysis of variance followed by Dunnet post hoc test. * $p = 0.05$; ** $p = 0.01$; *** $p = 0.001$

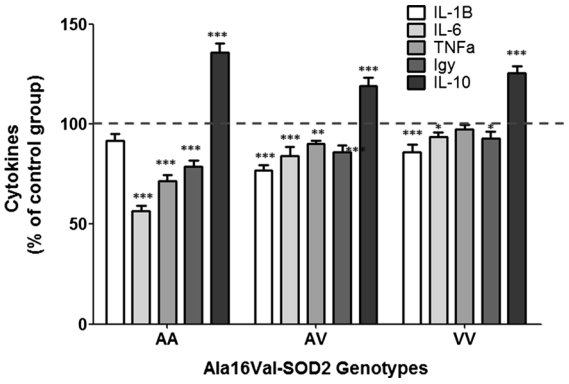

Fig. 3 Cytokine levels of PBMC carrier's different Ala16Val-SOD2 genotypes after 24 h exposition of 10 mg/mL resveratrol. *Results* are presented as percentage of untreated control group (represented by *dashed* line on the graph) evaluated by Elisa immunoenzymatic assay. Different *letters* indicated statistical differences analyzed by One-way analysis of variance followed by Dunnet post hoc test. * $p = 0.05$; ** $p = 0.01$; *** $p = 0.001$

important control switch in the process of activation-induced oxidative signal generation in T-cells. The authors observed that SOD2 overexpression triggers T-cell activation. It is therefore plausible to infer that cells with differential SOD2 efficiency may provide differential responses to molecules that act on PBMC activation.

It is generally accepted that the SOD2 enzyme is essential for the survival of cells under aerobic conditions. Here oxidative phosphorylation is used to generate energy for metabolism from ATP production and generates $O_2^{-\bullet}$ as a sub-product (Holley et al. 2012; Dröse et al. 2014). Other investigations have estimated that during normal respiration the $O_2^{-\bullet}$ concentrations in mitochondria can reach pM concentrations (Dröse et al. 2014). However, the control of $O_2^{-\bullet}$ levels by SOD2 needs to be finely regulated: low SOD2 efficiency can increase $O_2^{-\bullet}$ levels, whereas high SOD2 efficiency can increase H_2O_2 levels. Moreover, both of these situations can cause oxidative stress, potential cellular damage (Cavagnat et al. 2012) and have differential effects on the immune system. In fact, the proliferation of AV-PBMCs decreased in the presence of resveratrol treatments in a similar way as described in previous studies using immune cells as an experimental model (Holley et al. 2012; Wang et al. 2013; Zou et al. 2013). However, these results were not the same in homozygous carrier cells suggesting that there may be a pharmacogenetic effect of Ala16Val-SOD2 in PBMC activation when exposed to resveratrol.

We show here that AA-allele high efficiency SOD2 cells display greater responsiveness to T-cell

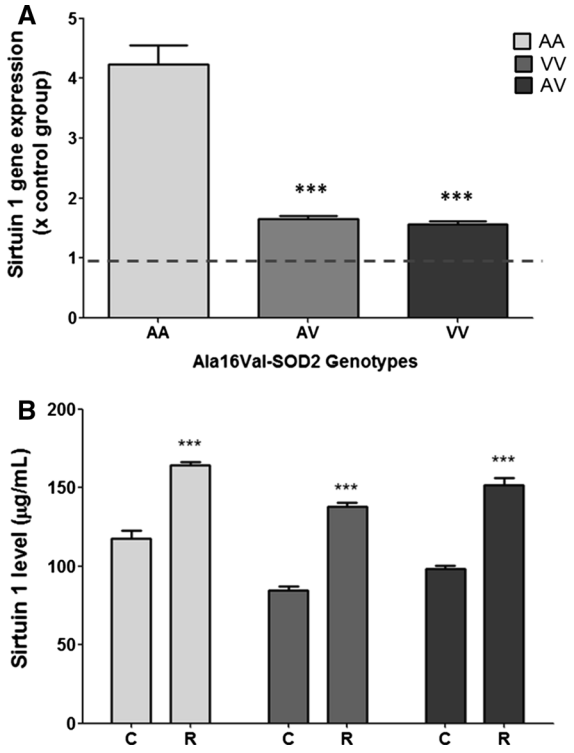

Fig. 4 Ala16Val-SOD2 gene polymorphism effect on gene expression and protein response of Sirtuin 1 to 10 mg/mL resveratrol exposition. **a** Sirtuin gene expression normalized to β-actin gene; **b** Sirtuin 1 protein level represented as determined by Elisa immunoenzymatic assay (µg/mL). *C* control, *R* resveratrol group; Results were compared by One-way analysis of variance followed by Dunnet post hoc test. * $p = 0.05$; ** $p = 0.01$; *** $p = 0.001$

lymphocyte inhibition when treated with resveratrol. These cells also show a decrease in inflammatory cytokines and ROS levels. However, after 72 h this effect is totally reverted and at ≥5 µM resveratrol concentrations cell proliferation in AA-cells increased significantly when compared to the untreated control group. In VV low-efficiency SOD2 cells, supplementation with resveratrol (≥5 µM) in culture medium stimulated cellular proliferation after 24 and 72 h at the same concentrations as was also observed in VV-cells. Although resveratrol decreased the levels of some inflammatory cytokines in VV-cells, this effect was less evident in A-allele carriers. These results corroborate previous investigations that describe high levels of inflammatory cytokines in VV-allele PBMCs when compared to A-allele PBMCs (Montano et al. 2012) indicating that VV-cells were more resistant to antioxidant and anti-inflammatory molecules such as resveratrol. In contrast, the effect of resveratrol in AA-cells appears to be transient, precipitating a reversion in T-cell activation response after 72 h of exposure.

Despite these differences, resveratrol treatment was able to activate Sirt1 in PBMCs, independent of the Ala16Val-SOD2 SNP, although this effect is more evident in AA-cells. There is evidence that Sirt1 can preserve mitochondrial function of non-immune cells, such as cardiomyocytes, attenuating myocardial oxidative damage during ischemia reperfusion involving SOD2 upregulation (Holley et al. 2012). As sirt1 expression is upregulated in all Ala16Val-SOD2 genotypes in response to resveratrol, differences in the PBMC proliferative response are likely a consequence of $O_2^{-\bullet}$ and H_2O_2 imbalance found in SOD2 homozygous genotypes. These may either be not fully reverted by *Sirt1* gene regulation or are a direct antioxidant effect of resveratrol.

Whilst a consistent number of studies have associated the Ala16Val-SOD2 SNP with chronic diseases and biological dysfunctions, there are a number of inconsistent results that may be due to environmental factors such as dietary and physical activity (Bresciani et al. 2013a). Unfortunately, epidemiological investigations do not permit the isolation and control of the contributions of environmental variables in genetic studies. For this reason, the use of Ala16Val-SOD2 SNP as a model to investigate the impact of constitutive oxidative metabolic imbalance in response to pharmacological and nutritional molecules under in vitro controlled conditions can be considered relevant. However, only recently have investigations been performed using in vitro genetic-models to analyze the effect of stressor agents such as cryopreservation, UV radiation, elevated levels of glucose/insulin and the effects of pharmacological drugs on cytotoxicity, oxidative stress and inflammatory biomarkers (Martin et al. 2009; dos Santos Montagner et al. 2010; Costa et al. 2012; Barbisan et al. 2014). In all studies presented so far, the cellular response shows some differential effect related to $O_2^{-\bullet}$ and H_2O_2 imbalance found in AA- and VV-genotypes.

In this study, we tested for the first time the effect of an antioxidant and recognized anti-aging molecule, resveratrol, on cells carrying different SOD2 genotypes. Despite the occurrence of some methodological limitations related to in vitro studies and the low number of volunteers used to perform these experiments, we conclude that the effects of resveratrol

on human PBMCs is not universal. Complementary in vivo pharmacogenetic investigations evaluating the resveratrol effect on immunological biomarkers could help us to validate the results presented here and confirm any impact this data has on human physiology.

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