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Antioxidants increase number of progenitor endothelial cells through multiple gene expression pathways

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Abstract

To date, there is no report on the effect of antioxidants on endothelial progenitor cells (EPCs). This study shows that *in vitro* incubation of EPCs with vitamin C and E reverted the already well documented lowering effect of TNF-α on EPC number and increased p-p38 expression levels. In order to document major changes of gene expression levels and gain insight into signalling pathways, microarray analysis was performed and a significant variation of the expression of 5389 genes in EPCs following antioxidant treatment was detected. Also *in vivo* evidence is provided about the positive effect of antioxidant vitamins on EPCs, since vitamin C and E supplementation potentiated the physical training-induced increase of EPC number and VEGF levels. Together, these data indicate that antioxidant treatment ameliorates EPC number and causes major changes of gene expression within these cells *in vitro*. Furthermore, concomitant antioxidant supplementation and physical training *in vivo* raised the levels of circulating EPCs and serum VEGF more than physical training alone.

Keywords: *Antioxidants, vitamins, endothelial progenitor cells, VEGF, microarray*

Introduction

Bone marrow-derived endothelial progenitor cell (EPC) is a circulating immature precursor that is capable of differentiating into mature endothelial cells and homing to site of vascular damage or neovascularization to promote vascular repair or neoangiogenesis [1]. These beneficial effects are impaired when the number and/or functional activities of EPCs are reduced, as it happens in the presence of severe endothelial dysfunction and in patients with diabetes, coronary heart disease (CHD) or multiple coronary risk factors [2–10]. Additionally, levels of circulating EPCs predict cardiovascular events in patients with CHD and represent a biological marker of vascular function [11–15]. EPCs are mobilized into peripheral blood and recruited to the foci of pathophysiological neovascularization and

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reendothelialization, thereby contributing to vascular regeneration [16,17]. The oxidative-modification hypothesis of atherosclerosis [18] has prompted the study of antioxidant vitamins in the prevention of cardiovascular disease. Putative beneficial effects of antioxidant may be attributed to their ability to scavenge free radicals, regulate nitric oxide (NO) synthesis [1,2,15,19–23] or release, regulate antioxidant enzyme activities, thus preventing or reducing extensive molecular and cellular damage. Current treatments of cardiovascular diseases, including pharmacological therapies, antioxidant supplementation, and promotion of regular and moderate physical activity only incompletely ameliorate endothelial dysfunction.

Here, we specifically investigated the effect of vitamin C and E on EPC number in the presence/absence of the detrimental activity of tumour necrosis factor-α (TNF-α). We also analysed bioactivity and gene expression of EPCs following administration of the antioxidant vitamins. Finally we tested *in vivo* the response of EPC number to supplementation with the antioxidants alone or in combination with physical exercise.

Materials and methods

EPC isolation

EPCs were isolated from total peripheral blood mononuclear cells (PBMCs) of healthy human donors that were obtained by density gradient centrifugation and cultured as previously described in detail [24]. Briefly, after 3 days of culture, non-adherent cells were removed by thorough washing with PBS and adherent cells ($\sim$10% of the total plated PBMCs) were used for further analysis.

EPC characterization and counting

Isolated EPCs were characterized after staining with 1,1'-dioctadecyl-3,3,3',3' tetramethylindocarbocyanine-labelled acetylated LDL (Dil-Ac-LDL) (Biomedical Technologies Inc., Massachusetts, USA) and FITC-labelled lectin from *Ulex europaeus* (Sigma-Aldrich, Milan, Italy) as previously described in detail [24]. Cells were subsequently visualized by fluorescence microscopy and analysed by FACS. Dual positive cells for both Dil-Ac-LDL and lectin were considered and EPCs fields were randomly counted using a computer-based program (Leica FW4000) [24]. EPC number was calculated as percentage of positive cells for Dil-Ac-LDL/lectin dual staining and was expressed as mean $\pm$ SE, each analysis included 5000 events. The EPC number was expressed as a percentage of positive cells.

Cell treatments

EPCs were treated with the antioxidant vitamin E (50 μM) and C (10 μM) [25], in the presence or absence of TNF-α (10 ng/ml), for 3 days (day 0–3) without changing the medium as described [24]. TNF-α was added after a pre-incubation of 30 min with antioxidants.

Western blot analysis

Total cell extracts (20–50 μg/lane) were loaded onto SDS-polyacrylamide gels and blotted onto polyvinylidene difluoride membranes. Western blots were performed by use of antibodies against phospho-p38 and γ-tubulin as described [24].

Detection of apoptosis by ELISA analysis

Adherent cells were washed with PBS and incubated with Caspase 3 & 7 FLICA kit according to the manufacturer's protocol (Immunochemistry Technologies, LLC) and analysed with a Tecan Infinite M200.

Proliferation assay

Adherent cells were incubated with bromodeoxyuridine (BrdU) labelling reagent for 3 days at 37°C. Cells were washed with PBS, fixed and incubated for 90 min with an anti-BrdU-POD antibody according to the manufacturer's protocol (Roche, Milan, Italy). The analysis was carried out with a Tecan Infinite M200.

RNA extraction, micro-arrays analysis and RT-PCR

After 3 days of culture, total RNA was extracted by using TrizolTM (Invitrogen, Milan, Italy). The first and second strand cDNA synthesis was performed using the GeneChip One-Cycle cDNA Synthesis Kit (Affymetrix, UK). Labelled cRNA was prepared using the GeneChip IVT Labelling Kit (Affymetrix, UK). Following the IVT reaction, the unincorporated nucleotides were removed using RNeasy columns (Qiagen, Milan, Italy) [26,27]. cRNA was transferred for hybridization onto the Affymetrix U133 Plus 2.0 GeneChips. The washing and staining procedure was performed in the Affymetrix Fluidics Station 450. The probe arrays were scanned at 560 nm using a confocal laser-scanning microscope (GeneChip® Scanner 3000). The readings from the quantitative scanning were analysed by the Affymetrix Gene Expression Analysis Software [26]. RNA was reverse transcribed with Superscript III (Invitrogen) using 1 μg total RNA and 100 ng random primers. Results were normalized to glyceraldehyde-3-phophate deydrogenase (GAPDH), used as a housekeeping gene. We used a GAPDH-specific fragment to verify the

integrity of the RNA preparation. The intensity of the amplified bands was quantified by densitometry and normalized to that obtained with GAPDH (Quantity One, Bio-Rad).

Ontology assessment

We subjected the list of differentially expressed transcripts to gene ontology using DAVID (Database for Annotation, Visualization and Integrated Discovery), web-based applications (http://david.abcc.ncifcrf.gov) that allow access to a relational database of functional annotations [28].

Animal protocols

C57BL/6J mice (Charles River, Sulzefer, Germany) were used for graduated physical exercise studies. Animals were treated in compliance with the Guide for the Care and Use of Laboratory Animals and the Guidelines of the American Heart Association. Graduated physical exercise consisted of a progressive swimming programme, as described previously in detail [19,29,30]. The study protocol lasted 28 days; vitamins E and C were added to the drinking water at an appropriate dilution (6%), as described [19,29,30]. At the end of treatment, mice were killed by CO_2 asphyxia. Blood was drawn from the inferior vena cava into heparinized tubes [19,29,30].

Evaluation of mouse EPC number and VEGF levels

FACS analysis was performed for anti-SCA1-FITC and anti-VEGFR2 as described [31]. Quantitative fluorescence analysis was performed with a FACSCANTO instrument (BD Biosciences, San Sose, USA). Each analysis included 10 000 events. Serum levels of VEGF were determined with a mouse VEGF ELISA kit (Oncogene Research, Calbiochem, Darmstadt, Germany).

Statistical analysis

Data are given as mean ± SE. Differences were assessed by *t*-test and a *p*-value < 0.05 was considered to be significant.

Results

EPC number and intracellular signalling

As shown in Figure 1, incubation of EPCs with TNF-α decreased their number, accordingly to previous results [31]. This effect was clearly reverted by pre-treatment with the antioxidant vitamins E and C ($p < 0.05$). Moreover, after only 3 days of culture these cells rarely formed clusters. These data were further confirmed by FACS analysis of double positive cells (Dil-Ac-LDL and lectin) (Figure 2).

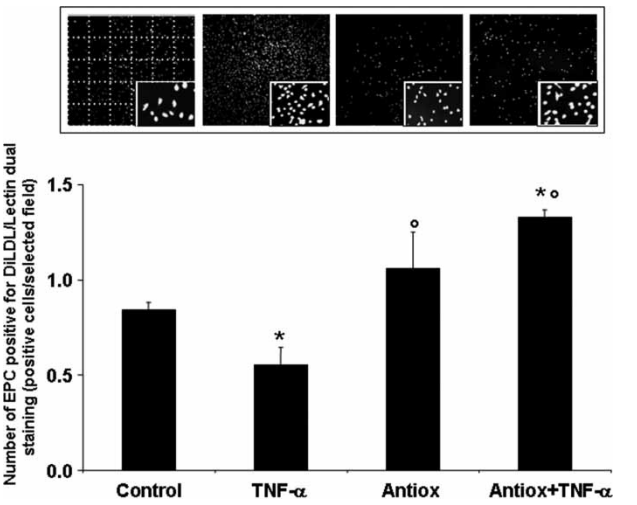

Figure 1. Effects of antioxidants in presence or absence of TNF-α on EPC number. Dil-Ac-LDL uptake and lectin binding staining of isolated EPCs were determined by fluorescence microscopy, Hoechst positive nuclei from PBMCs. Upper panel, Micrographs show EPCs double positive for Dil-Ac-LDL and lectin staining (arrows) are representative of five different experiments in duplicate. Lower panel, Bar graph shows EPC number calculated dividing for the number of sub-fields, excluding empty sub-fields. Results are expressed as mean ± SE and are representative of five different experiments in duplicate. $^{*}p < 0.05$ vs untreated; $^{\circ}p < 0.05$ vs treated with TNF-α.

To investigate the response of some signalling pathways to the same stimuli we analysed the activation of p38-kinase. This kinase has been involved in the altered proliferation of EPCs isolated from individuals with cardiovascular risk factors [24,31]. In the present study, incubation of EPCs with TNF-α determined an increase of the level of phosphorylated p38 (Figure 3). Interestingly, this effect was abolished by the concomitant incubation with antioxidants and TNF-α.

Microarray analysis of gene expression in EPCs after treatments with antioxidants

We next studied the regulation of gene expression in the presence of the antioxidant vitamins. The cRNA was generated in duplicate from a pool of three different mRNA extractions (a mean of two different experiments, each in duplicate) from EPCs for each experimental condition (control, antioxidants, TNF-α, antioxidants + TNF-α). Arrays were generated and contained 14 500 well-characterized human genes (out of 22 215 total genes comprehensive of EST sequences); arrays were used to evaluate the effect of the different treatments on EPC gene expression. Only genes with a threshold of at least 50 arbitrary detection units and a Signal Log ratio value ≥ 1 and ≤ -1, corresponding to an absolute fold change (FC) value of 2 ($FC = 2^{[\text{Signal Log Ratio (SLR)}]}$) have been included in the list of the analysed genes. The SLR algorithm measures the magnitude and direction of change between transcript levels of the

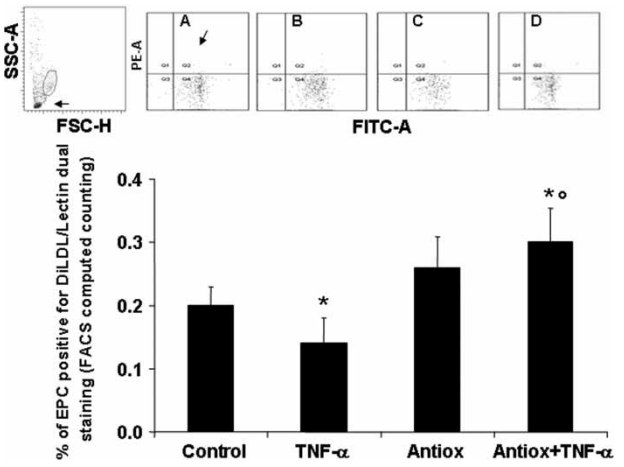

Figure 2. Effect of TNF-α and antioxidants on human EPC number. DiLDL uptake and lectin binding staining of control EPCs (A) or treated with TNF-α (10 ng/ml) (B) for 3 days, antioxidants alone (C), or TNF-α in the presence of antioxidants (D) were determined by FACS-computed counting. Upper panel: Forward (FSC) and side scatter (SSC) analysis show the selected population. Lower panel, Bar graph of corresponding EPCs number. $^{\star}p < 0.05$ vs untreated; $^{\circ}p < 0.05$ vs treated with TNF-α. Results are expressed as mean $\pm$ SE and are representative of five different experiments in duplicate.

experimental and baseline chips. The use of logs in the analysis between the probe sets eliminates difficulties caused by one very high data point in the set masking information from lower valued data points. Base 2 is used as the log scale, therefore a SLR of 1 represents a 2-fold increase in abundance of an mRNA and a value of -1 represents a 2-fold reduction in transcript expression. Overall, antioxidant treatment decreased transcription of 3643 genes and increased transcription of 1746 genes compared with untreated cells; TNF-α treatment decreased transcription of 296 genes and increased transcription of 557 genes compared with untreated cells; antioxidants and TNF-α treatment decreased transcription of 2716 genes and increased transcription of 1414 genes compared with untreated cells. Important changes were observed in transcription of genes coding for different proteins with known biological functions such as: (1) protein folding (heat shock protein 90 kDa, 1 beta, tumour rejection antigen (gp96) -1); (2) angiogenesis (Neuropilin 2, Neuropilin 1); (3) chemotaxis (interleukin 8 receptor, alpha; chemokine (C-X-C motif) ligand 3); (4) transcription (early growth response 1, early growth response 3); (5) cell adhesion (selectin P ligand); (6) phospholipids scramblase 1 (phospholipids scrambling); and (7) immune response (SAM domain and HD domain 1). Representative genes are listed in Tables I–III. The biological process ontology and KEGG pathway terms associated with the differentially expressed genes were examined using the online available DAVID bioinformatics database (Figure 4A and B). To validate data obtained by microarray analysis, we performed RT-PCR on differentially expressed genes (Figure 4C). The levels of induction detected by RT-PCR were similar to those observed by microarrays, although there were significant differences in the quantification of the gene expression level by the two analytical methods.

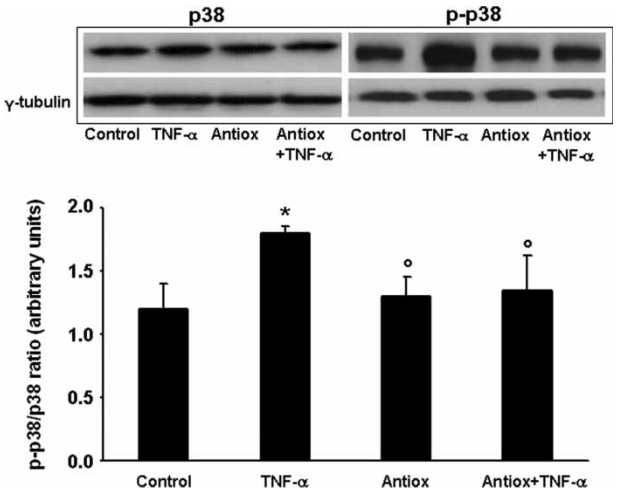

Figure 3. Detection of p38 and p-p38 expression levels. Western blot analysis of total p38 (A) and p-p38 were performed on cell lysates prepared from EPCs treated with media alone (control, lane 1), 10 ng/ml TNF-α (TNF-α, lane 2), 50 μM vitamin E, 10 μM vitamin C (antiox, lane 3), or 10 ng/ml TNF-α + 50 μM vitamin E, 10 μM vitamin C (TNF-α + antiox, lane 4) as described in Materials and methods. γ-tubulin served as loading control; total p38 has been analysed to express the level of the phosphorylated form (p-p38) as a ratio p-p38/total p38. Data are mean $\pm$ SE and are representative of three different experiments, $^{\star}p < 0.05$ vs untreated, $^{\circ}p < 0.05$ vs TNF-α.

Antioxidants prevent TNF-α-induced apoptosis

TNF-α-treated EPCs showed a significant increase in apoptosis by caspase assay vs untreated cells ($p < 0.05$) and antioxidants counteracted TNF-α-induced apoptosis, even though not significantly. Moreover, treatment with the sole antioxidants determined a lower level of apoptosis compared with TNF-α-treated EPCs (Figure 5, $p < 0.05$). On the other hand, antioxidants did not increase EPC proliferation, as assessed by BrdU assay (data not shown).

Table I. Modulation of gene expression by antioxidant vitamins E and C in EPCs detected by microarray experiments.

Up-regulated genes	Down-regulated genes
Phospholipid scramblase 1	Enolase 1, (alpha)
Neuropilin 1	Jumonji domain containing 2B
Interleukin 8 receptor, alpha	Calcium activated nucleotidase 1
SAM domain and HD domain 1	Heat shock 90 kDa protein 1, beta
Centaurin, delta 1	Apolipoprotein E

Table II. Modulation of gene expression by TNF-α in EPCs detected by microarray experiments.

Up-regulated genes	Down-regulated genes
Early growth response 1	Jumonji domain containing 2B
Selectin P ligand	Calcium activated nucleotidase 1
Phospholipid scramblase 1	Tumor rejection antigen (gp96) 1
Rho/rac guanine nucleotide exchange factor (GEF) 2	Rhophilin, Rho GTPase binding protein 1
Sorting nexin family member 27	Immunoglobulin superfamily, member 10

Effects of antioxidants and physical training on EPCs in mice

To analyse the *in vivo* effect of the antioxidant treatment, vitamins C and E were administered to mice either alone or in combination with moderate physical training. Results indicated that moderate physical training determined a significant increase in EPC number ($p < 0.05$ vs respective group of sedentary) and this effect was further amplified by concomitant antioxidant supplementation ($p < 0.001$ vs respective group of sedentary) (Figure 6A). However, antioxidant treatment by itself did not modify the number of circulating EPCs. The same was true for the VEGF serum concentration, which was significantly increased by physical training alone ($p < 0.05$ vs respective group of sedentary) and, more intensely, by physical training combined with antioxidant supplementation ($p < 0.001$ vs respective group of sedentary) (Figure 6B).

Discussion

The major findings of the present study are: (1) a beneficial *in vitro* effect of antioxidant vitamins C and E on TNF-α-treated EPC number and p-p38 expression levels; (2) regulation of gene expression in cultured EPC following treatment with vitamins C and E as detected by microarray analysis; and (3) an *in vivo* beneficial effect of vitamins C and E supplementation and physical exercise on EPC number.

Impaired EPC proliferation is induced by TNF-α [31] and is associated with decreased levels of VEGF [32]. Our recent *in vitro* and *in vivo* studies show that EPC number and functional activities can be ameliorated by resveratrol, vitamin E and L-arginine [29,31,33,34].

Table III. Modulation of gene expression by antioxidants and TNF-α in EPCs detected by microarray experiments.

Up-regulated genes	Down-regulated genes
Growth arrest and DNA-damage-inducible, beta	Cleft lip and palate associated transmembrane protein 1
Neuropilin 2	Rhophilin, Rho GTPase binding protein 1
Early growth response 1	Phospholipase D family, member 3
Phospholipid scramblase 1	Rap guanine nucleotide exchange factor (GEF) 1
Neuropilin 1	Talin 1

Vitamins C and E are known to exert protective potentials in many disease states associated with enhanced oxidative stress [35]; we observed that administration of these vitamins was able to revert the reduction of cell number induced by the pro-inflammatory cytokine TNF-α. This effect could be explained by an anti-apoptotic mechanism rather than an increased proliferation induced by the antioxidant vitamins. Indeed, our data show that TNF-α-treated EPCs undergo a higher level of apoptosis, which is reduced by antioxidant vitamin treatment, and is not different from the basal condition when cells are co-incubated with vitamins and TNF-α. On the contrary, vitamin treatment of EPCs did not increase their proliferation. Moreover, the *in vitro* beneficial effect of antioxidant treatment on EPC number is associated with a reduction of the increased levels of phophorylated p38 as it occurs following TNF-α exposure. This cytokine is known to induce p38 phosphorylation and phosphorylated p38 has been reported to be a negative regulator of EPC number [24,31,36]. Therefore, it is likely that the decreased level of phosphorylated p38 might play a role in the response of EPC to the antioxidant vitamins. Considering that the disease-associated reduction of circulating EPC number may be secondary to a variety of mechanisms (exhaustion of the pool of progenitor cells in the bone marrow, reduced mobilization or reduced survival and/or differentiation) these results could have interesting pathophysiological implications.

We have also tested the effect of the oral supplementation of vitamins E and C *in vivo*, by using C57BL/6J mice with or without concomitant moderate physical training [29,30]. It is noteworthy that our results indicate that moderate physical training led to a significant increase in EPC number ($p < 0.05$ vs the control group of sedentary) [30], while the sole dietary supplementation did not. Also interesting was the trend toward an amplification of the effect of physical activity by the concomitant oral administration of the antioxidant vitamins ($p < 0.001$ vs the respective group of sedentary, Figure 6A). Furthermore, these latter results were parallelled by the response of VEGF serum concentration that did not vary after the antioxidant supplementation, but rose significantly after physical training alone ($p < 0.05$ vs respective group of sedentary) [29,30] and/or in combination with the antioxidants oral supplementation ($p < 0.001$ vs respective group of sedentary, Figure 6B). This is the first indication that short-term supplementation with vitamins C and E could ameliorate the effects of moderate physical exercise on EPC number and VEGF serum levels *in vivo*. The coincident changes of EPCs and VEGF lead one to

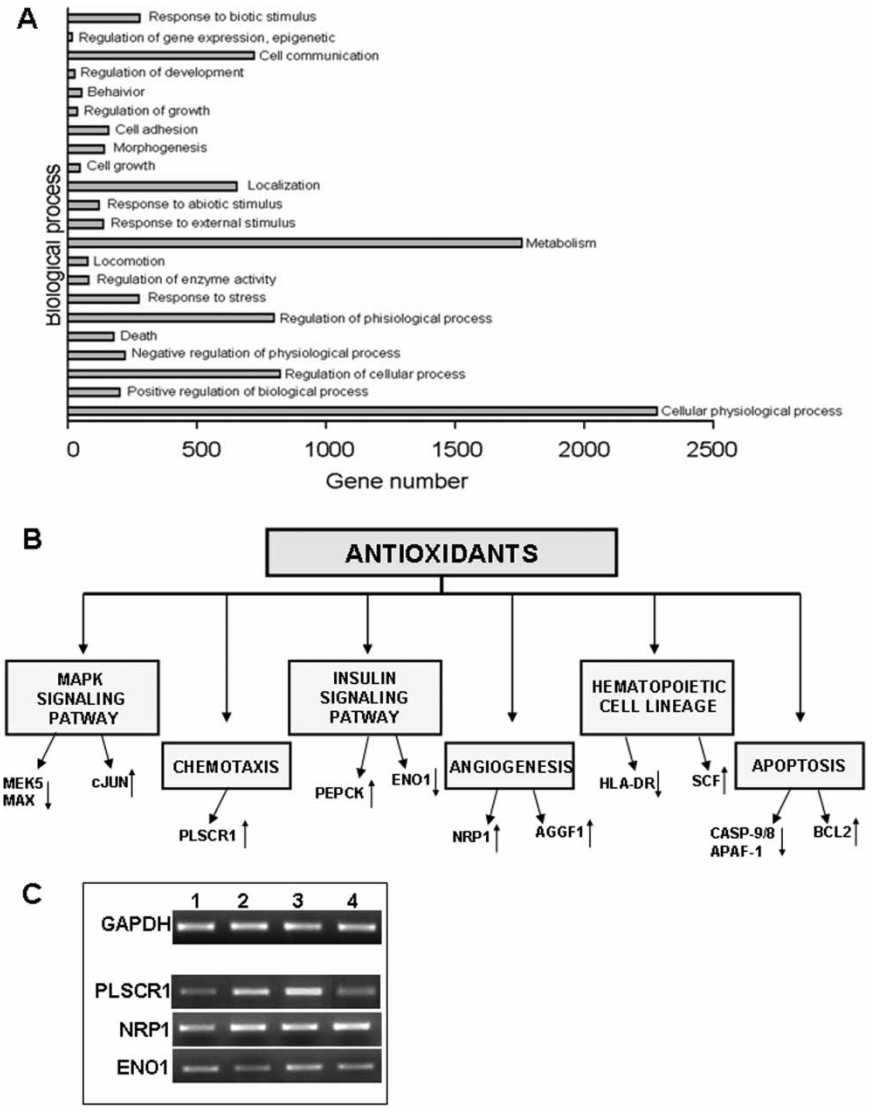

Figure 4. (A) Different pathways influenced by antioxidants treatments on EPC detected by using DAVID (http://david.abcc.ncifcrf.gov). Abbreviations: MEK5, mitogen-activated protein kinase kinase 5; MAX, MAX interactor 1; cJUN, jun oncogene; PTK2, protein tyrosine kinase 2; RRAS, related RAS viral (r-ras) oncogene homologue; PEPCK, phosphoenolpyruvate carboxykinase 2 (mitochondrial); BCL2, BCL2-antagonist of cell death; CASP8, caspase 8, apoptosis-related cysteine peptidase; APAF-1, apoptotic peptidase activating factor 1; CASP9, caspase 9, apoptosis-related cysteine peptidase; HLA-DR; major histocompatibility complex, class II, DR beta 4; SCF, KIT ligand; RAPL; Ras association (RalGDS/AF-6) domain family member 5; ITGB2, integrin, beta 2 (complement component 3 receptor 3 and 4 sub-unit); ITGA4; integrin, alpha 4 (antigen CD49D, alpha 4 sub-unit of VLA-4 receptor). ↓, decrease; ↑, increase. (B) Representation of the biological process of the 5389 selected genes regulated by antioxidants treatment by using DAVID (http://david.abcc.ncifcrf.gov). (C) RT-PCR analysis of some genes differentially expressed by microarray data. EPCs untreated lane 1, antiox treatment lane 2, TNF-α treatment lane 3, antiox and TNF-α treatment lane 4.

hypothesize a causal relationship between the growth factor and the number of EPCs. Recently we have observed similar *in vivo* effects elicited by administering either the nitric oxide precursor, L-arginine, or the antioxidants present in red wine [29,30]. Another study investigating the role of antioxidants on EPC number and function looked at beta-carotene, which has been shown to influence homing of EPCs [37]. The observation of the synergistic effect of physical training and antioxidant supplementation is coherent with the known increase of the production of reactive oxygen species in the presence of enhanced activity of the mitochondrial electron transport chain in skeletal muscle cells during physical exercise.

The capture of a gene expression signature characteristic of EPCs could be one of the first steps to unveiling the effects of such treatments on different molecular mechanisms [38]. Microarrays were designed to evaluate the gene expression profiles after antioxidants and TNF-α treatments. This report has identified a series of candidate genes involved in different biological processes that are regulated by antioxidants and/or physical training, such as 'regulation of physiological process', 'cellular physiological

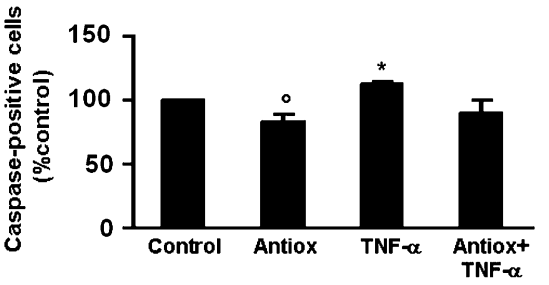

Figure 5. Antioxidants prevent TNF-α-induced apoptosis of EPC. EPCs were incubated with media alone (control, lane 1), 50 μM vitamin E, 10 μM vitamin C (antiox lane 2), 10 ng/ml TNF-α (TNF-α, lane 3), or 10 ng/ml TNF-α+50 μM vitamin E, 10 μM vitamin C (TNF-α+antiox, lane 4) and apoptosis was measured by caspase assay with a Tecan Infinite M200. Data are expressed as mean±SD, $*p<0.05$ vs control, $^{\circ}p<0.05$ vs TNF-α.

process', 'regulation of cellular process', 'death', and 'metabolism'. One of the genes that we found up-regulated in EPCs treated with antioxidants and in treatment with TNF-α is Neuropilin 1 (NRP1). This is a membrane-bound co-receptor that plays versatile roles in angiogenesis, axon guidance, cell survival,

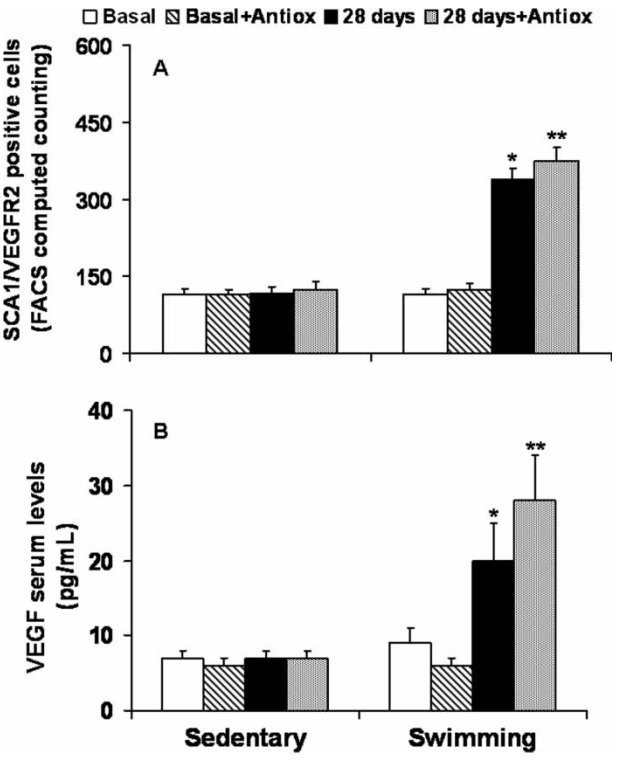

Figure 6. Effects of antioxidants on EPCs *in vivo*. EPC number and VEGF serum levels in C57BL/6J mice, in basal condition, after antioxidant supplementation, after physical exercise and after the combination of physical exercise and antioxidant supplementation. (A) FACS computed counting was used to determine the EPCs number based on the co-expression of mouse haematopoietic stem cell marker SCA1 and VEGF-receptor-2 (VEGFR-2). $*p<0.05$ vs respective group of sedentary; $**p<0.001$ vs respective group of sedentary mice. (B) Serum levels of VEGF were determined by VEGF ELISA kit mouse. $*p<0.05$ vs respective group of sedentary; $**p<0.001$ vs respective group of sedentary mice. Results are expressed as mean±SD.

migration and invasion [39]. In addition, we report the up-regulation of phospholipids scramblase 1 (PLSCR1) [40–42], a multiply palmitoylated endofacial plasma membrane protein. Our data indicated that PLSCR1 is up-regulated in cells with both TNF-α alone and associated with antioxidants.

Instead, antioxidants induced a down-regulation of alpha-enolase 1 ENO1, which is a receptor for plasminogen on several cell types, serving to localize and promote plasminogen activation pericellularly [43]. Localization of plasmin activity on the cell surface plays a critical role in fibrinolysis and in physiopathological processes involving extracellular matrix remodelling [44]. Another example of down-regulated gene is apolipoprotein E (ApoE). ApoE is a main apoprotein of the chylomicron and binds to a specific receptor on liver cells and peripheral cells. Studies of the ApoE deficient (ApoE−/−) mouse confirm genetic involvement and hypercholesterolemia as primary factors leading to atherosclerosis [45]. A gene up-regulated by TNF-α treatment, but down-regulated by antioxidants, is Selectin P ligand (SELPLG). SELPLG is the high affinity counter-receptor for P-selectin on myeloid cells and stimulated T lymphocytes. As such, it plays a critical role in the tethering of these cells to activated platelets or endothelia expressing P-selectin [46].

The biological significance of these genes in the growth, survival, differentiation and function of EPCs should be determined by further investigation; however, this gene list may help to navigate the search for the potential biomarkers and therapeutic targets to modulate vasculogenesis during pathological conditions of adult life. Vitamins E and C are widely distributed in plant-derived food. High consumption of vegetables and fruit is recommended in any healthy dietary style and together with moderate physical activity makes up an integral part of a healthy lifestyle. The present results provide additional support, at a molecular and cellular level, to the health-promoting effect of the beneficial duo of healthy diet and physical activity.

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***Declaration of interest*:** The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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