JOURNAL OF PHYSIOLOGY AND PHARMACOLOGY 2011, 62, 3, 287-293
www.jpp.krakow.pl

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CELLFOOD™ IMPROVES RESPIRATORY METABOLISM OF ENDOTHELIAL CELLS AND INHIBITS HYPOXIA-INDUCED RECTIVE OXYGEN SPECIES (ROS) GENERATION

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Endothelial mitochondria, the major site of ATP generation, modulate the intracellular dynamics of reactive oxygen species (ROS), which, in turn, control endothelial function. Adequate oxygen (O_2) supply is required by endothelial cells (EC). Both hypoxia and hyperoxia may favor the overproduction of ROS leading to oxidative stress, mitochondrial damage and endothelial dysfunction. We investigated the capability and mechanisms of Cellfood™ (CF), an antioxidant compound, to modulate O_2 availability and mitochondrial respiratory metabolism and to regulate ROS generated by hypoxia in EC *in vitro*. Human umbilical vein endothelial cells (HUVEC) and ECV-304 were evaluated for the O_2 consumption using a Clark's electrode. The O_2 consumption rate rose, during the first minutes after CF addition and was associated with increase in mitochondrial oxidative capacity and good cell viability. Similar behaviours were observed when EC were exposed to CF for up to 8 days. The O_2 consumption increased and was accompanied by both intracellular rise of ATP and maintainment of LDH concentration. Hypoxia-induced ROS generation was significantly inhibited by CF, through the up-regulated expression of MnSOD, an anti-oxidant responsible for mitochondrial function preservation. The EC hypoxic response is mediated by the hypoxia master regulator HIF-1alpha whose activation was attenuated by CF, in concomitance with MnSOD up-regulation. Our results suggest a role for CF in improoving respiratory metabolism and in activating anti-oxidant mechanisms in EC, thus preserving endothelial function.

Key words: *Cellfood™, human umbilical vein endothelial cells, hypoxia inducible factor-1alpha, mitochondrial activity, reactive oxygen species generation, MnSOD, superoxide dismutase*

INTRODUCTION

The endothelium participates in many physiological functions and human diseases, and endothelial cells (EC) play an important role in mediating the focal nature of disease states (1). In particular, EC are able to regulate the homeostasis of vascular function. Thus it seems to be crucial to maintain proper EC functions, shape and viability. EC are exquisite sensors of O_2 and are equipped with mechanisms that adjust metabolism to O_2 supply changes (2). In particular, the O_2 sensing machinery in EC is well sensitive and in response to low O_2 availability EC build up a response, through the up-regulation of the hypoxia inducible factor-1 alpha (HIF-1alpha) (3). Mitochondria play a crucial role in cell energy homeostasis and function as main energy source. The endothelium is considered not to be a major energy-requiring organ, being the vascular endothelium in a quiescent state; nevertheless EC have an extensive mitochondrial network. This suggests that mitochondrial function may be important in maintaining the physiological O_2 metabolism and the bio-energetic status of EC (4). Moreover, EC mitochondria could contribute to endothelial patho-physiology, in relation to the development and progression of cardiovascular diseases (5, 6). A major initiator of endothelial injury is oxidative stress which results from an imbalanced state of increased reactive oxygen species (ROS) generation and insufficient intracellular antioxidant activity. Both oxidative stress due to excessive ROS formation and a defective capacity to detoxify intracellular oxidants are common features of several cardiovascular diseases, although the cause-effect relationship between oxidative damage and cardiovascular dysfunction is not completely understood (7).

In EC the mitochondrial respiratory chain represents a minor source but an important target of ROS, which ultimately lead to mitochondrial irreversible damage (8). Despite both mitochondrial metabolism and glycolysis are crucial for energy generation ATP-related in EC (9, 10), the endothelial dysfunction seems to be primarily associated with impaired mitochondria (11). Strategies for the targeted delivery of antioxidants to mitochondria are being developed. Cellfood™ (CF) is a food integrator containing D_2SO_4 numerous numerous minerals, aminoacids and enzymes, which could confer antioxidant properties to the compound. Despite the complexity of the formulation hampering the possibility to identify the component mostly responsible for the beneficial clinical effects, it is likely that the whole formula is effective. In this study we investigate the effect and mechanisms of action of CF on EC spontaneously immortalized (ECV 304) and on primary human umbilical vein EC (HUVEC) mitochondrial function, respiratory metabolism *in vitro*, ROS generation, MnSOD induction and HIF-1alpha pathway.

MATERIALS AND METHODS

Cell cultures

HUVEC were isolated from human cord by collagenase as described (12) and cultured in 1% gelatin-coated flasks (Falcon, BectonDickinson, Bedford, MA, USA) using endotoxin-free Medium 199 (BioWhittaker, Cambrex BioScience, Verviers, Belgium), containing 20% heat-inactivated foetal bovine serum (FBS, Hyclone, Logan, UT, USA), 1% bovine retinal-derived growth factor, 90 µg/ml heparin, 100 I.U./ml penicillin, and 100 µg/ml streptomycin (Biochrom, Berlin, Germany) (Complete Medium, CM). All experiments were carried out with HUVEC at passage 1-4 (12). ECV-304 endothelial cell line (ATCC) was cultured in Medium 199 supplemented with 10% FBS.

Hypoxia treatment

HUVEC were incubated in a temperature and humidity controlled C-chamber (BioSpherix, Redfield, NY, USA), in an atmosphere containing 1% O_2, 5% CO_2, 90% N_2.

Endothelial cells treatment with Cellfood™

Commercially available Cellfood™ (Eurodream S.r.l., La Spezia, Italy) is composed by D2SO4 in acqueous solution and by a mixture of 17 aminoacids, 34 enzymes and 78 minerals. In a set of experiments (short time-course treatment) a single dose of CF was administered to EC at a concentration of 1 µl/ml and O_2 consumption and mitochondrial potential evaluated for a time course of about 15-20 min. In another set of experiments (long time course treatment), CF was administered daily to EC at growing concentration for 8 days according to the following scheme: the first, the second and the third day 0.3 µl/ml, the fourth, the fifth and the sixth day 0.7 µl/ml, the seventh and the eighth day 1µl/ml. Untreated EC grown for 8 days served as controls.

Endothelial cells viability

Viability of EC treated for 24 hours with different concentrations of CF was evaluated with Propidium Iodide (PI) and Annexin V and then analyzed with a cytofluorimeter (FC500-Beckman Coulter).

Bright field microscopy

Microphotographs were taken with Eclipse TS100 microscope (Nikon, Melville, NY) (10X original magnification).

Measurement of O_2 consumption

O_2 consumption was measured using the Clark-type-polarographic oxygen electrode, modified to allow adherent EC monitoring. Briefly, in the cylinder was inserted a rack (R) containing six shelves, that allowed to increase total surface, each covered with glass round coverslips (13 mm), on which ECV (150,000 cells/coverslip) were seeded (*Fig. 1C*). The data were recovered with WinWedge software (TAL Technologies, Philadelphia, PA, USA). The instrument was setup at 24% of O_2(= µl of O_2 dissolved in 1 ml of aqueous medium at 1 atmosphere and at 37°C) as starting level (13). O_2 consumption was measured every 5 sec, for at least 12 minutes. Data are expressed as percentage of the basal O_2 consumption rate.

Confocal microscopy

EC plated on glass slides, either treated or not with CF, were incubated with acridine orange (AO, 0,67 µM, 10 min 37°C, Sigma-Aldrich, St. Louis, MI, USA) to assess cell viability and with MitoTracker CMXRos derivative (MT, 100 nM, 45 min, 37°C, Molecular Probes, Eugene, OR, USA) to stain mitochondria, depending on their membrane potential. MT reduced probe fluoresce in live cells, where it is oxidized. Slides were analyzed at confocal microscope (Leica TCS SP5, AOBS Microsystems GmbH, Wetzlar, Germany), 2D free projection max were obtained from z-series of images. The experiments have been repeated three times.

Measure of ATP and LDH levels in human umbilical vein endothelial cells

Intracellular ATP and LDH levels were determined using a commercial ATP luminescence assay kit (Invitrogen S.r.l., San Giuliano Milanese, Milan, Italy) and a LDH Cytotoxicity Colorimetric assay kit (LK100, Oxford Biochemical Research INC, Oxford, MI, USA), respectively. For intracellular ATP determination, HUVEC were cultured in 6 wells plates and then lysed using the boiling water method (14). ATP and LDH standards were used each time to generate a calibration curve. The protein content was determined using the Bradford method (15).

Reactive oxygen species measurement

We assessed ROS production by incubating HUVEC with the cell-permeable fluorescent probe hydroethidine (HE, Invitrogen) as previously described (16). Briefly, cells were incubated under hypoxic conditions for 24 hours, then stained with 2 µmol/l HE for 1 hour at 37°C, washed and analyzed by flow cytometry (FC500, BeckmanCoulter). Only living cells, gated according to scatter parameters, were used for the analysis.

Fluorescence microscopy

The expression of MnSOD was evaluated on HUVEC either exposed or not to hypoxia (1%O_2) in the presence/absence of CF at the indicated intervals, using Rabbit-anti-MnSOD (1:50, 4°C, overnight; Upstate-Millipore, Milan, Italy) revealed by Donkey-anti-Rabbit AlexaFluor488 (1:500, 45 min, Molecular Probes Invitrogen, Eugene, OR, USA). The expression of HIF-1alpha and of Glut-1 was evaluated using anti-human HIF-1alpha mAb (clone OZ15) from Novus Biologicals and rabbit anti-GLUT-1 from DAKO, followed by rabbit anti-mouse IgG Alexa Fluor 594 . Nuclei were assessed by 4,6- diaminidino-2-phenylindole (Sigma-Aldrich) stain.

Statistical analysis

The results are expressed as means S.E.M. Comparisons among groups were performed by one-way ANOVA. Values of $p<0.05$ were considered statistically significant.

RESULTS

Cellfood™ improves endothelial cells respiratory metabolism preserving optimal mitochondrial activity

Preliminary experiments were performed to assess CF toxicity on HUVEC (*Fig. 1A*) with increasing concentrations of the compound for 24 hours (see materials and methods). CF resulted moderately toxic for EC (15.67% PI positive cells) only at very high concentrations (≤15 µl/ml); accordingly, EC displayed optimal cellular viability and reached the confluence with concentrations ≤1 µl/ml (hereafter the concentration used), similarly to the controls. Moreover, at this concentration both

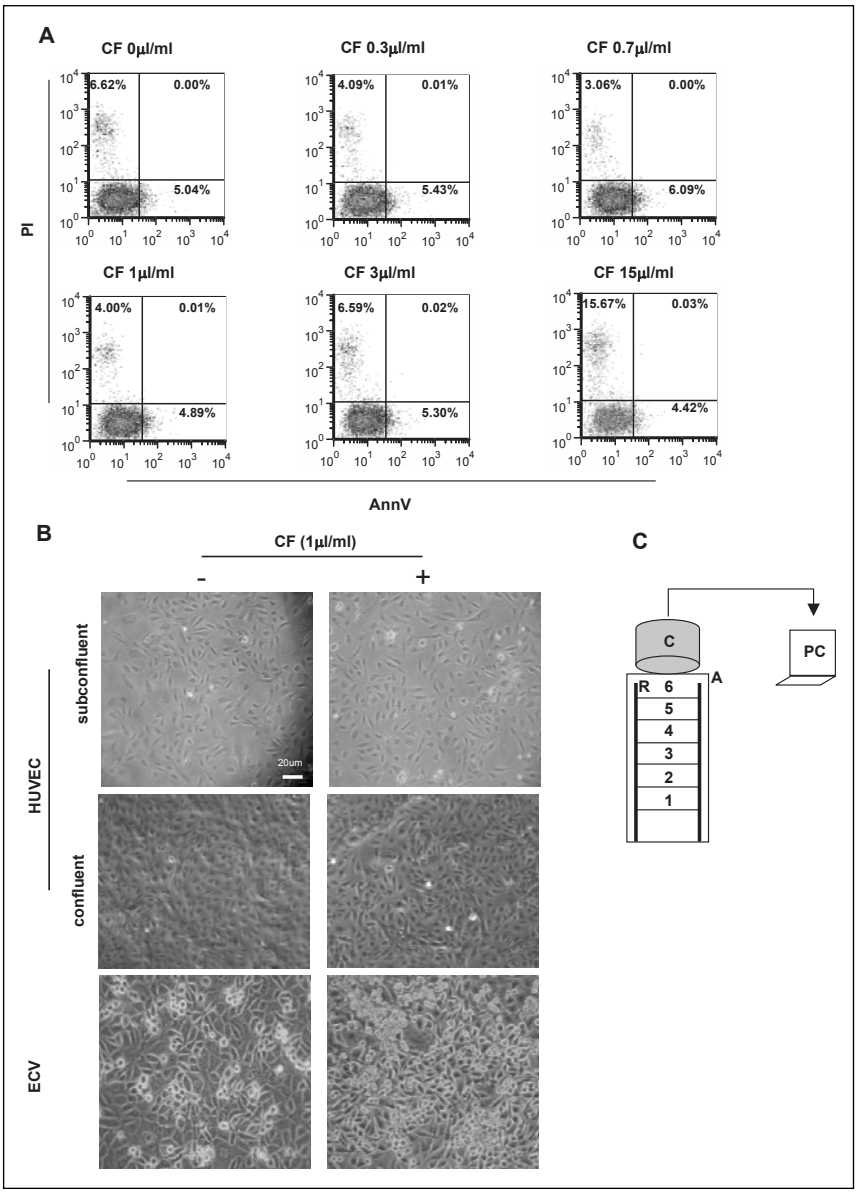

Fig. 1. CF does not affect EC viability and morphology. A: HUVEC exposed to 24 hours increasing doses of CF were stained with PI/AnnV to assess apoptosis and submitted to cytofluorimetric analysis. B: both subconfluent and confluent HUVEC and ECV were cultured in the absence/presence of CF (1 µl/ml) for 24 hours; pictures were acquired at the end of the culture. C: schematic representation of Clark's electrode, modified to assess O2 consumption of adherent cells (See Materials and Methods and results section).

HUVEC and ECV retained their morphology, shape, size and adherence (a hallmark of viability) in a manner similar to the untreated counterpart (NT) (*Fig. 1B*). Even though EC seem to generate most of their energy anaerobically, they consume O_2. It has been found that mitochondrial function decreases with passage number in EC (4); therefore, we used HUVEC between passage 4. O_2 consumption was measured by means of a modified Clark's electrode device, in house-developed, to avoid EC anoikis due to loss of adherence (*Fig. 1C*). To assess the effect of CF on O_2 consumption, ECV were kept in culture for a few min; values monitored were assumed as basal O_2 consumption (*Fig. 2A*). The addition of CF shifted toward a fast increase of O_2 consumption, as also indicated by 6% decrease of O_2, overall suggesting that the compound is available and effective. Notably, under these culture settings, pH values were stable (not shown), indicating absence of acidosis.

To support this behaviour, we determined ECV mitochondrial activity and cell viability in brief time-course experiments (up to 15 min), through the use of MT, an indicator of active mitochondria, and AO, a dye intercalating DNA and a marker of cell viability. The addition of CF resulted in brighter AO-MT fluorescence than that of untreated ECV, compatible with a good mitochondrial activity and a well preserved viability (*Fig. 2B*). To assess whether this observation was restricted to ECV, we investigated the mitochondrial activity of primary HUVEC, that indeed retained their activity for up to 15 min (*Fig. 2B*, middle panels). These findings suggest that CF is able to sustain optimal mitochondrial activity without affecting EC viability.

The O_2 consumption was then evaluated in EC for a long-time period, culturing EC with increasing CF concentrations for up to 8 days. The O_2 metabolism of treated cultures was slightly higher than that of untreated one, peaking at day 3 and 8 of culture (*Fig. 2C*). In parallel, we assessed the effect of CF on mitochondrial activity on both ECV and HUVEC (*Fig. 2D* upper and lower panel). The mitochondrial function was sustained throughout the period, both in ECV and HUVEC, without signs of suffering or death or EC de-adherence. We can conclude that CF could improve respiratory metabolism without affecting EC viability also for an extended time period.

Cellfood™ sustains ATP production

Concentration of intracellular ATP was measured at serial time points and expressed as increase/decrease over untreated

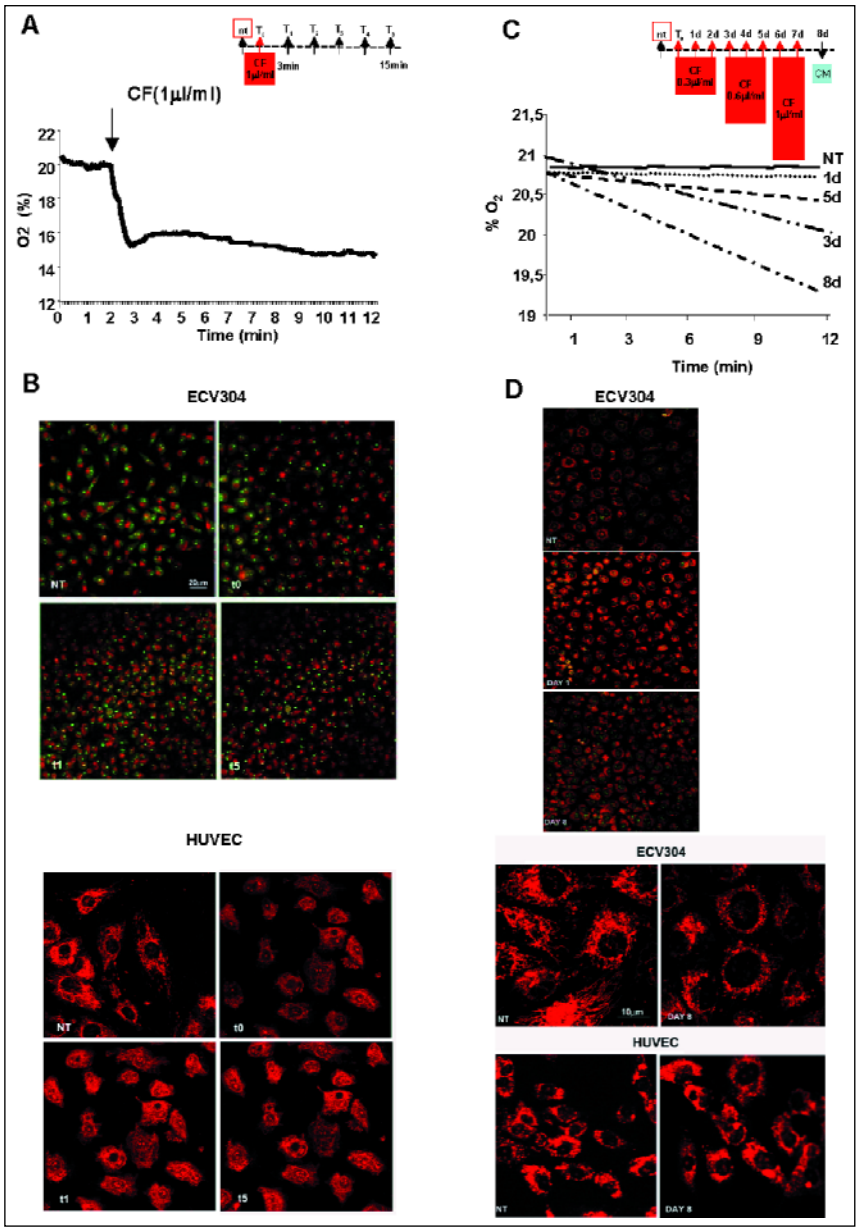

Fig. 2. Brief and long exposure to CF increases O_2 consumption preserving mitochondrial activity. A: O_2 consumption by ECV upon CF brief exposure (min) is expressed in percentage. Arrow indicates the addition of 1 µl/ml CF, that we considered the start point (T_0, see schedule schematic representation). Representative of three experiments. B: in upper panel, ECV mitochondrial activity and viability, assessed in confocal analysis with MitoTracker CMXRos derivative (red) and with acridine orange (green) respectively; in lower panel, mitochondrial activity of HUVEC. C: O_2 consumption by ECV upon CF long exposure (days, d) is expressed in percentage (see schedule schematic representation). Representative of three experiments. D: in upper panel, ECV mitochondrial activity and viability assessed in confocal analysis as mentioned before; in lower panel, ECV and HUVEC mitochondrial activity is shown.

HUVEC (76±19 pmol/µg protein); its trend showed a bell-shaped curve, with significant peaks at a short time point (3 hours) and at 8 days of CF administration (*Fig. 3A*). Conversely, LDH production, performed at same intervals, decreased significantly at 3 hours and remained at levels similar to the control ones (NT, 1.6±0.3 U/ml) throughout the period (*Fig. 3B*), consistently with the absence of pH lowering mentioned above. Cumulatively, these data suggest a shift from glycolysis to mitochondrial, respiratory pathway, determined by CF.

Cellfood™ affects reactive oxygen species generation and inhibits hypoxia-driven HIF-1alpha activation, via MnSOD regulated expression

EC are fine sensors of O_2 variations; ROS are produced continuously as natural by-products of the normal metabolism of O_2 (17, 18), and in hypoxia mitochondria stimulate the production of cellular ROS (19, 20). Indeed, HUVEC expressed constitutively ROS, and, as expected, their expression increased after a 24 hours exposure to severe hypoxia (1%O_2); notably, the addition of CF inhibited in a significant manner their up-regulation (*Fig. 4A*). To identify a mechanism responsible for CF activity, we assessed for the presence of an endogenous antioxidant enzyme, MnSOD, that balances ROS production, thereby preventing oxidant damage. The exposure of HUVEC to both 1 hours and 24 hours of hypoxia was sufficient to slightly increase the expression of MnSOD, through an intrinsic defense mechanism. Interestingly, the addition of CF in the same experimental conditions, strongly increased MnSOD expression (*Fig. 4B*), convincingly suggesting that CF, *via* upregulation of antioxidants, may exert a substantial protective mechanism against oxidative stress generated by hypoxia. The EC hypoxic response is mediated by the activation of the hypoxia inducible factor HIF-1alpha, that translocates to the nucleus leading to the transcription of down stream genes (21). Under basal culture conditions, low expression of HIF-1alpha was detectable, mainly localized in the cytoplasm (*Fig. 4C*). One hr-exposure to 1% hypoxia allowed for almost instantaneous induction of HIF-1alpha expression and translocation to the nucleus, that returned to basal levels within 24 hours. Notably, the addition of CF inhibited this pattern of expression, likely indicating that CF

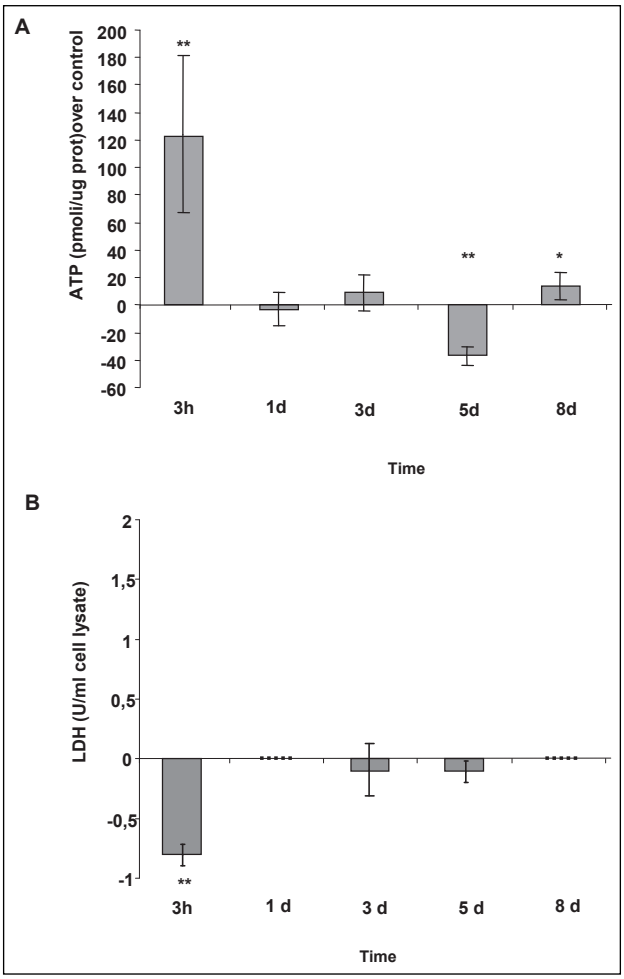

Fig. 3. ATP and LDH intracellular levels in HUVEC. Intracellular ATP and LDH contents are expressed as decrease/increase over control. The results represent the mean±S.E.M. of 3 experiments. A: * p<0.05 *vs*. 3 hours, 1d, 5d; **p<0.05 *vs*. 1d, 3d, 5d, 8d. B: *p<0.05 *vs*. 1d, 3d, 5d, 8d.

interferes with the HIF-1alpha pathway (*Fig. 4C*). Accordingly, the up-regulated expression of the glucose transporter Glut-1, as an adaptive response mechanism to hypoxia, is also inhibited by CF (*Fig. 4D*).

DISCUSSION

The endothelium participates in many physiological functions and human diseases, and endothelial dysfunction represents a risk factor for the development of clinical events, such as vascular diseases and stroke (22). As a result, the entire cardiovascular system critically depends on a structurally and functionally intact vascular endothelium. Thus, maintenance of proper EC homeostasis is a obvious requirement (23) and the need for anti-oxidant and for compounds that tightly regulate endothelial activation, is of significant biological relevance for the clinical features associated with endothelial damage and dysfunction. In this context, the crucial role of propionate has been recently described, contained in the short chain fatty acids (SCFAs) present in cellulose fibers, in inhibiting the TNF-induced endothelial both mRNA and protein expression of ICAM-1 and VCAM-1, *via* the inhibition of NF-kB p65, another master regulator of EC activation (24).

A major initiator of endothelial injury is oxidative stress, which results from an imbalanced state of increased reactive oxygen species (ROS) generation, responsible for vascular endothelial cells apoptosis (25), and insufficient intracellular antioxidants (26), including enzymes such as MnSOD.

In this regard, the vascular protective effects of resveratrol against oxidative injury have been elucidated due likely to the induction of endogenous anti-oxidative enzymes in HUVEC and EA.hy 926 (27).

Herein, we have investigated the effects and mechanisms of action of CF, a food integrator, on the EC oxidative metabolism *in vitro*. We have shown the following: 1) a single administration of CF to EC determines a significant increase in O_2 consumption with respect to NT cells, *via* the increase of the mitochondrial activity, without affecting EC viability. 2) a few days repeated administration (up to 8d) of CF to HUVEC, allows optimal O_2 consumption, mitochondrial activity and down-stream ATP production, without accumulation of undesidered LDH. Overall, these findings indicate a metabolic shift from glycolytic to mitochondrial pathway. Overall, our study clearly demonstrates the efficacy of CF in preserving mitochondrial activity of EC. The relationship between O_2 consumption and reactive oxygen species (ROS) generation has been investigated. Indeed, high dose and/or inadequate removal of ROS, especially superoxide anion, results in "oxidative stress", which has been implicated in the pathogenesis of many cardiovascular diseases, including hypercholesterolemia, atherosclerosis, hypertension, diabetes, and heart failure (28). One of the major antioxidant defense systems against $O_2^{\circ-}$ is superoxide dismutases (SODs). SOD2 is a mitochondrial manganese (Mn) containing enzyme (MnSOD) and is localized in the mitochondrial matrix; for this reason we selected this isoform to elucidate the mitochondrial involvement. The essential role of SOD2 in maintaining mitochondrial function has been recently shown by MnSOD-deficient mice with heart failure, affected by markedly impaired endothelial function (29). Herein we show that ROS expression, induced by exposure to hypoxia is significantly reverted by CF, through the important increase of MnSOD, whose expression over time is fluctuating (*Fig. 5*), as also recently well shown (30). This finding indicates that CF supports resistance of EC against ROS toxicity, thereby preserving their viability and functional activity also under conditions of oxidative stress driven by hypoxia. The EC hypoxic response is tightly regulated by the master hypoxia regulator HIF-1alpha whose activation leads to the transcription of potent pro-angiogenic cytokines and down-stream neo-acquired functions (3). Here we show that CF inhibits the nuclear translocation of HIF-1alpha and the Glut-1 up-regulated expression, suggesting that CF interferes with the hypoxic pathway and can be considered a new regulator of the endothelial hypoxic response. We argue that HIF-1alpha inhibition is mediated by MnSOD activation, being their expression on the same HUVEC inversely correlated. Moreover, the link between excessive ROS generation, loss MnSOD and HIF-1alpha activation has been recently well documented in pulmonary hypertensive EC (31).

Cumulatively, we propose that CF is able to preserve EC mitochondrial activity and optimal respiration, *via* a double mechanism, the generation of MnSOD and the inhibition of HIF-1alpha expression, thus avoiding endothelial dysfunction. CF could be a candidate in supporting the treatment of diseases characterized by vascular complications.

Conflict of interests: None declared.

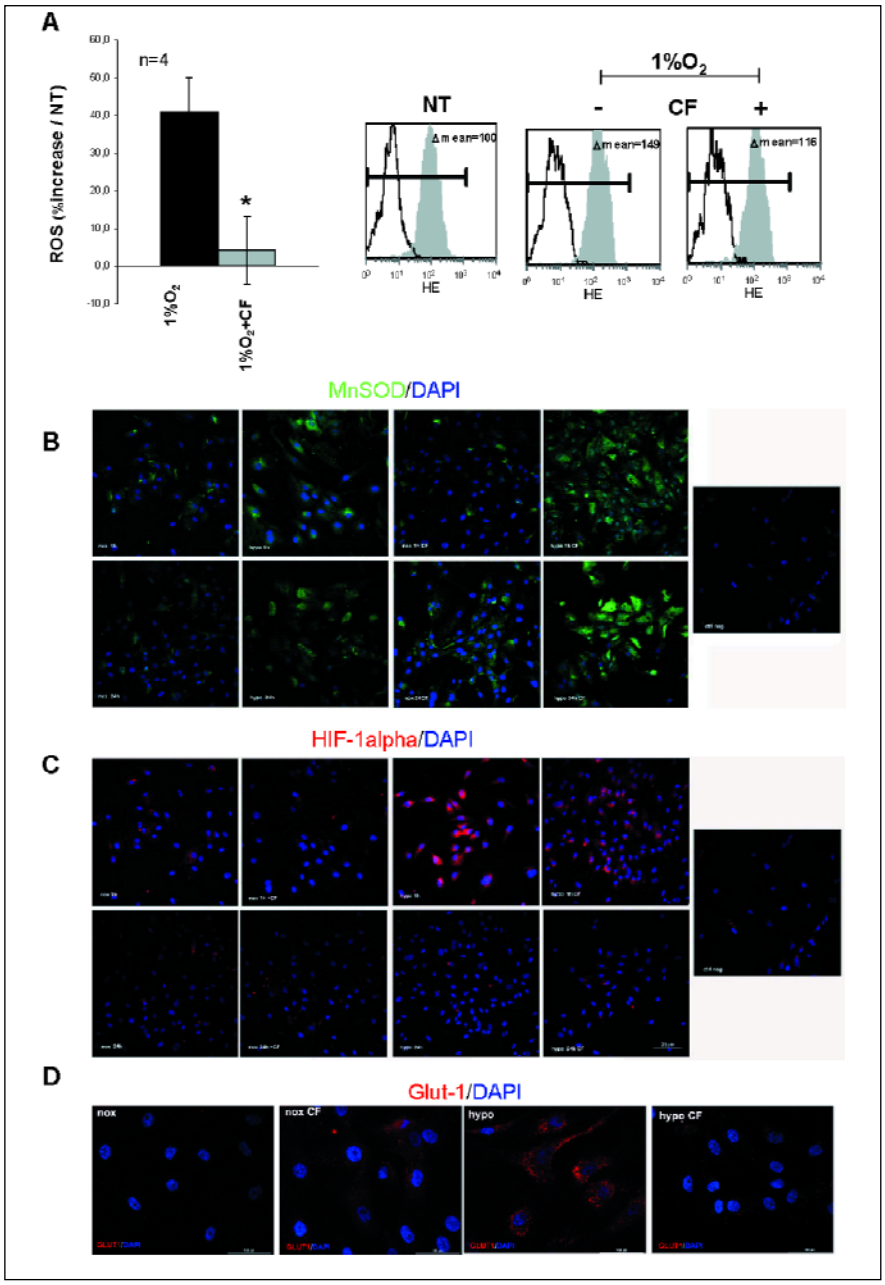

Fig. 4. CF inhibits hypoxia induced ROS generation, up-regulates MnSOD expression and interferes with HIF-1alpha pathway. A: HUVEC were exposed to 1% hypoxia for 24 hours and evaluated for the induction of ROS, in respect with normoxic counterpart, through cytofluorimetric analysis (left panel). One representative experiment out of four is shown (right panel); black line is referred to cells only (negative control); grey histogram is referred to cells stained with HE. B: MnSOD expression in HUVEC treated or not with CF (24 hours) is obtained by confocal analysis (green). C: HIF-1alpha (red) expression in HUVEC is acquired with confocal analysis. Nuclei are stained with DAPI (blue) (right panel). D: HUVEC expression of Glut-1 (red) in confocal analysis. Nuclei are stained with DAPI.

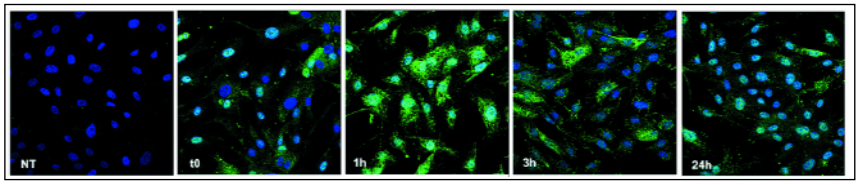

Fig. 5. Time-course of MnSOD expression in HUVEC treated with CF. MnSOD expression (green) is evaluated in confocal analysis at the indicated times. Nuclei are stained with DAPI.

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Received: July 19, 2010
Accepted: June 12, 2011

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