RESEARCH ARTICLE

β-Carotene and lycopene affect endothelial response to TNF-α reducing nitro-oxidative stress and interaction with monocytes

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Scope: Cardiovascular disease (CVD) is associated with vascular oxidative imbalance and inflammation. Increased reactive oxygen species (ROS) generation is associated with a functional inactivation of nitric oxide (NO) due to the reaction with O_2^-, leading to peroxynitrite ($ONOO^-$) formation and subsequent reduction in the beneficial effect of vascular NO bioavailability. Carotenoids'-rich diets have been associated with decreased risk of CVD, but the underlying mechanism is still unknown.

Methods and results: In human umbilical vein endothelial cells (HUVECs), both β-carotene (BC) or lycopene (Lyc) significantly affected tumor necrosis factor-α (TNF-α)-induced inflammation, being associated with a significant decrease in the generation of ROS (spectrofluorometry) and nitrotyrosine (an index of $ONOO^-$ formation, cytofluorimetry), an increased NO/cGMP (cyclic guanosine monophosphate) levels (EIA), and a down-regulation of NF-κB-dependent adhesion molecule expression (Western blot and EMSA) and monocyte–HUVEC interaction (adhesion assay). Our results indicate that BC or Lyc treatment reduce the inflammatory response in TNF-α-treated HUVECs. This is due to the redox balance protection and to the maintenance of NO bioavailability.

Conclusion: Our observations provide background for a novel mechanism for carotenoids' anti-inflammatory activity in the vasculature and may contribute to a better understanding of the protective effects of carotenoid-rich diets against CVD risk.

Keywords:
Carotenoids / Endothelial dysfunction / Inflammation / Nitric oxide / Reactive oxygen species

1 Introduction

More than 700 carotenoids have been identified, but few of them are considered of nutritional relevance and circulate at micromolar levels, therefore receiving most attention by health researchers [1, 2]. Among them, α-carotene, β-carotene (BC) and β-cryptoxanthin are the major carotenoids having a significant pro-vitamin A activity [3], while lutein, lycopene (Lyc) and zeaxanthin are not converted into active retinoids by

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Abbreviations: BC, β-carotene; **CVD,** cardiovascular disease; **HUVECs,** human umbilical vein endothelial cells; **L-NAME,** L-nitro-arginine-methyl ester; **Lyc,** lycopene; **NO,** nitric oxide; **NOS,** nitric oxide synthase; **ROS,** reactive oxygen species; **TNF-α,** tumor necrosis factor-α

humans [4]. Their biological activity is therefore independent from retinoids-associated pathways. Given their chemical structure, carotenoids have been proposed to act as free-radical scavengers, even though a number of reports claimed that carotenoids can act as the pro-oxidant molecules, at least at high oxygen concentration [5]. However, more recently, a large number of biological beneficial activities related to their ability to regulate different cellular pathways and functions have been proposed [5]. Of note, a number of epidemiological studies have shown a correlation between elevated dietary carotenoids intake and circulating levels and decreased risk of cardiovascular disease (CVD) [6, 7].

In fact, recently, it has been demonstrated that circulating serum carotenoids were associated, in an apparently beneficial direction, with markers of inflammation, oxidative stress and endothelial dysfunction [8], which are known to be associated with CVD [9–11].

At present, it is widely accepted that one of the earliest detectable pathogenic events in both human and experimental atherosclerosis [12] is represented by the over-expression of a wide spectrum of cell surface adhesion molecules, which is a pivotal and critical event in the binding of normally non-thrombogenic circulating cells, such as monocytes, to the endothelial surface [13]. One of the major features of this event is the activation of NF-κB pathway [14, 15] triggering, in turn, the up-regulation of the expression of the vascular cell adhesion molecules (VCAM-1), intercellular cell adhesion molecules (ICAM-1) and E-Selectin in response to various inflammatory cytokines [16].

Nitric oxide (NO), constitutively generated by endothelial cells, plays an important role in the maintenance of vascular homeostasis and in the pro-inflammatory response that characterizes the early stages of atherosclerosis [17]. It is known that NO inhibits the vascular inflammatory response by quenching NF-κB nuclear transfer thanks to its regulatory activity on IκBα synthesis [18] and also by directly inhibiting NF-κB binding to DNA [19]. These events, in turn, down-regulate NF-κB-dependent expression of adhesion molecules [20]. The maintenance of endothelial NO bioavailability is therefore considered beneficial to endothelial functions and more in general to vascular health. However, in tumor necrosis factor-α (TNF-α)-stimulated endothelium, NO rapidly reacts with superoxide anion (O_2^-) to form a stable potent oxidant peroxynitrite ($ONOO^-$) resulting in decreased vascular relaxation, and contributing to the up-regulation of NF-κB-dependent cellular response. Thus, the general effect of anti-oxidant molecules on the biological function of NO is likely to be due, at least in part, to a direct removal of O_2^- [21]. Within this scenario, carotenoids may be considered potential anti-oxidant modulators of endothelial response to pro-oxidant/inflammatory stimuli.

According to this hypothesis, Martin et al. reported that BC, lutein and Lyc significantly reduced both VCAM-1 and ICAM-1 expression, BC and lutein inhibited E-selectin expression in IL-1β-stimulated human aortic endothelial cells, while Lyc only attenuated both IL-1β-stimulated and spontaneous monocyte–endothelial interactions [22]. Even though it has been recently demonstrated that Lyc is able to inhibit the activation of NF-κB induced by TNF-α [21], the mechanism underlying carotenoids anti-inflammatory activity in endothelial cells, is still largely unknown.

In the present study, we report that Lyc and BC affect NF-κB-dependent expression of adhesion molecule and monocyte–human umbilical vein endothelial cell (HUVEC) interaction induced by TNF-α and protect NO bioavailability therefore reducing TNF-α-induced nitro-oxidative stress.

2 Materials and methods

All methods are described briefly, for details see Supporting Information.

2.1 Cell cultures and experimental procedures

Umbilical cords were obtained from randomly selected healthy mothers delivering at the Chieti and Pescara University Hospital. All procedures were in agreement with the ethical standards of the Institutional Committee on Human Experimentation (Reference Number: 1879/09COET) and with the Declaration of Helsinki Principles. After approval of the protocol by the Institutional Review Board, signed informed consent form was obtained from each participating subject. Primary HUVECs were obtained, cultured and used between the third and fifth passages in vitro, as described previously [23].

Our experimental aim was to evaluate the ability of carotenoids either in the reverting or preventing TNF-α-triggered inflammatory response in endothelial cells.

To evaluate the potential carotenoids' reverting effects on TNF-α-activated inflammatory response, serum-starved HUVECs were incubated for 16 h with TNF-α (1 ng/mL, Sigma-Aldrich, Taufkirchen, Germany) and then treated with carotenoids. In detail, the effect of a 2-h treatment with BC and Lyc (2.5 μmol/L) on free-radical production stimulated by TNF-α was studied by measuring the levels of reactive oxygen species (ROS), nitrotyrosine (NT), cyclic guanosine monophosphate (cGMP) and NO release, as described in the Supporting Information. In particular, time-dependent NO release was evaluated by incubating BC or Lyc (2.5 μmol/L) for different time periods (1–90 min) and assessed by 4,5-diaminofluorescein diacetate (DAF-2DA, Calbiochem, Germany), as described in the Supporting Information. Total nitric oxide synthase (NOS) activity was evaluated following 15 min BC or Lyc (2.5 μmol/L) incubation by measuring the conversion of L-[^{3}H]-arginine into L-[^{3}H]-citrulline, as described in the Supporting Information. In the same experimental conditions, basal- and carotenoid- (2.5 μmol/L) stimulated endothelial NOS (eNOS) protein levels were evaluated by Western blot analysis, as described in the Supporting Information.

To evaluate the effects of carotenoids in preventing TNF-α-induced inflammatory response, serum-starved HUVECs were incubated in the presence of carotenoids and then stimulated with TNF-α (1 ng/mL) at different time points. In detail, to study NF-κB signaling pathway and adhesion molecule protein levels, HUVECs were incubated with BC or Lyc (24 h) and then stimulated with TNF-α (1 ng/mL) for 2 and 16 h, respectively. At the time point, HUVECs were collected and EMSA and Western blot analysis were performed, as described in the Supporting Information. Finally, to test the consequences of the modulation of adhesion molecules expression by carotenoids at a functional level, cell adhesion was assayed utilizing U937 monocytes and HUVECs. BC or Lyc were added to the incubation medium either before or after TNF-α stimulation.

2.2 Preparation and stability study of carotenoid stock solutions

BC (Fluka, Shnelldorf, Germany) and Lyc (Sigma-Aldrich, Steinheim, Germany) were dissolved in tetrahydrofuran (THF, Sigma-Aldrich) to give a final concentration of 1 mmol/L (at this concentration THF did not affect any evaluated cellular parameters, thus the data were not shown). Stock solutions were stored at −80°C under argon in sealed pyrex tubes and used without any dilution and further purification. The stability of stock solutions was checked over 3 months by high-performance liquid chromatography with UV detection (HPLC-UV) as previously described [24].

2.3 Carotenoids determination

Cellular association of test carotenoids was determined by HUVECs and concentrations in media were measured by HPLC-UV [25].

2.4 ROS determination

ROS levels were measured using the fluorogenic reagent dichlorofluorescein diacetate (DCF-DA, Sigma-Aldrich) using a spectrofluorimeter (LS45 Luminescence Spectrometer, PerkinElmer Instruments, Waltham, MA, USA) with excitation at 485 nm and emission at 530.

2.5 NT determination

NT levels were evaluated by cytofluorimetric analysis using a fluorescent-activated cell sorting (FACS) Calibur flow cytometer (BD Biosciences, California, USA) equipped with 488 nm argon ion laser. Data were analyzed using the CELLQuest 3.2.1.f1 (BD Biosciences) software.

2.6 NO production by DAF-2DA cytometric evaluation

NO production was studied by using DAF-2DA in the serum-starved culture conditions. 10 000 events for each sample were analyzed [26] using an FACS Calibur flow cytometer (BD Biosciences). Data were analyzed using the CELLQuest 3.2.1.f1 software (BD Biosciences) and expressed as MFI or ΔMFI Ratio.

2.7 cGMP determination

Intracellular cGMP levels were evaluated by using a commercially available Enzyme Immunoassay (EIA) kit (GE Healthcare, Little Chalfont, Buckinghamshire, UK).

2.8 NOS activity by conversion of L-[^{3}H]-arginine into L-[^{3}H]-citrulline

NOS activity was determined by measuring the conversion of L-[^{3}H]-arginine into L-[^{3}H]-citrulline as previously described [27].

2.9 NF-κB activation by electrophoretic mobility shift assay (EMSA)

Nuclear protein extraction and EMSA were carried out as described by Cimino et al. [28].

2.10 Western blot analysis

Adhesion molecules, p65, phospho-IkBα, total IkBα, eNOS protein levels and β-actin were evaluated by Western blot analysis as previously described [26, 29].

2.11 MTT Assay

The effect of carotenoids on HUVECs viability was assessed by the 3-(4,5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide (MTT, Sigma-Aldrich) method. The spectrometric absorbance at 540 nm was read using a microplate reader (SpectraMAX 190, Molecular Devices, Sunnyvale, California, USA).

2.12 [^{3}H]-thymidine incorporation assay

The HUVEC-incorporated radioactivity was measured in a scintillation counter (TopCount NXT, Scintillation and Luminescence Plate Counter, PerkinElmer).

2.13 Cell cycle progression

Samples of 10 000 cells were analyzed for DNA content by flow cytometer, using an FACS Calibur instrument (BD Biosciences) and cell cycle phase distributions were analyzed by the ModFit LT™ software (Verity Software House, Toshan, ME, USA).

2.14 Preparation of U937 cells and adhesion assays

For adhesion assays, HUVECs were grown to confluence in six-well tissue culture plates and U937 cell adhesion was evaluated as described [26].

2.15 Statistical analysis

The results were presented as mean ± standard deviation (SD) or standard error of the mean (SEM). Significance of differences between treatments for each treatment time was evaluated by one-way Kolmogorov–Smirnov Test (KS test) and ANOVA. Post hoc analysis (Bonferroni test) was used to determine the significance between each treatment time. Data were considered to be significant if $p < 0.05$. Statistical analysis was performed using the XLStat2007.1 (Microsoft, USA) software.

3 Results

3.1 BC and Lyc stock solutions stability

Without any synthetic anti-oxidants preventing carotenoid degradation, check of stock concentrations is mandatory [30]. Then, the levels of carotenoids were determined by HPLC-UV prior to each use over 3 months. The levels of Lyc decreased of 16 ± 1% (mean ± SD, $n = 3$), while a 28 ± 1% decrease was calculated for BC stored under the same conditions. Then, BC solutions were prepared every 2 months, so that degradation was reduced to an acceptable level 13 ± 1%.

3.2 Carotenoids determination

Incubation of HUVECs for 24 h with medium containing ~2.5 μmol/L carotenoids increased cellular levels as shown in Table 1. Different solubilities, due in part to intrinsic chemical characteristics of the carotenoids, did not allow to obtain equivalent final concentrations of the two carotenoids in culture medium [22]. However, we were able to incorporate each carotenoid into HUVECs to a measurable level although there was at most 23-fold difference in medium concentrations (Table 1). Cellular association varied over tenfold range, suggesting that there was a concentration-dependent association that possibly was also carotenoid- and tissue-specific. In control medium and cell monolayers incubated in unsupplemented media, carotenoids were not detectable.

Table 1. Cellular association of β-carotene and lycopene by HUVEC

	Medium (μmol/L)	Cells (pmol/L/10^6 cells)
Control	n.d.	n.d.
β-Carotene	0.017 ± 0.002	22 ± 8
Lycopene	0.47 ± 0.28	253 ± 38

n.d., not detected.

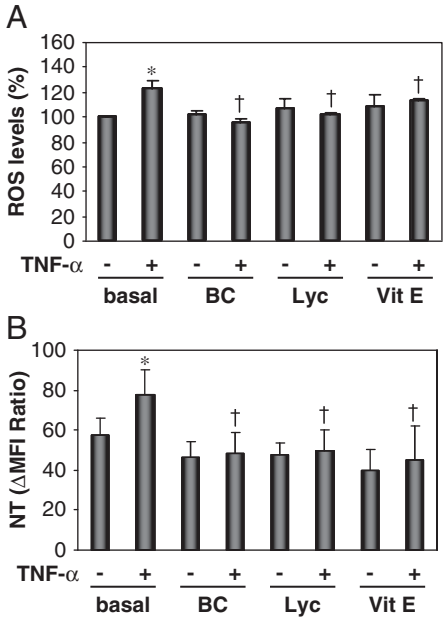

Figure 1. Effects of carotenoids on TNF-α-induced ROS and NT generation. (A) Intracellular ROS levels in HUVECs treated for 16 h with TNF-α (1 ng/mL) and then for 2 h with 2.5 μmol/L carotenoids and for 1 h with 20 μmol/L vitamin E (Vit E) (*$p < 0.05$ versus basal, †$p < 0.05$ versus TNF-α). (B) In the same experimental conditions, intracellular NT levels (index of $ONOO^-$ formation) was evaluated in 10 000 events for each sample by cytofluorimetric analysis and expressed as Δmean fluorescence intensity (MFI) ratio. ΔMFI ratio was calculated subtracting the value of the MFI of negative events (MFI of secondary antibody) from that of positive events then dividing by the MFI of negative events (*$p < 0.05$ versus basal, †$p < 0.05$ versus TNF-α). Data refer to at least three separate experiments.

3.3 Effects of carotenoids on TNF-α increased nitro-oxidative stress

Figure 1A shows that both BC and Lyc (2.5 μmol/L, 2 h) totally abolished TNF-α-induced ROS levels (1 ng/mL, 16 h, $p < 0.05$). In parallel, cytofluorimetric evaluation of

intracellular NT levels (index of $ONOO^-$ formation), indicated that the addition of carotenoids completely reverted the increase in NT levels induced by TNF-α in HUVECs (Fig. 1B, $p<0.05$). A known anti-oxidant, such as vitamin E (Vit E) (20 μmol/L, 1 h), totally abolished either TNF-α-induced ROS and NT levels ($p<0.05$). The incubation with the vehicle alone [0.05–0.25% v/v THF or veh] was not associated with any significant variation in ROS or NT levels (data not shown).

3.4 Effects of carotenoids on NO bioavailability

In comparison to unsupplemented cells, Fig. 2A and B shows that incubation with BC or Lyc (2.5 μmol/L) subsequent to TNF-α treatment was associated with a significant increase in NO levels in a time-dependent manner. As expected, at each time point, basal NO levels observed in the presence of TNF-α were significantly lower than values at the baseline ($p<0.01$), indicating that a decrease in basal NO bioavailability occurs after TNF-α treatment. Of note, the increase in NO production in the presence of either BC or Lyc was not affected by the preincubation with the eNOS inhibitor L-nitro-arginine-methyl ester (L-NAME). Thus, indicating that both carotenoids affect NO levels through decreasing oxidative unbalance (leading to an increased NO bioavailability) rather than by increasing eNOS activity or levels. In addition, in the absence of TNF-α preincubation, both BC and Lyc (2.5 μmol/L) slightly but significantly increased NO production in a time-dependent manner ($p<0.01$) and this effect was not affected by the preincubation with the eNOS inhibitor L-NAME (data not shown).

In order to confirm this hypothesis, we also evaluated the potential effect of carotenoids in modulating NOS enzymatic activity. The L-[^{3}H]-arginine/L-[^{3}H] to citrulline conversion assay demonstrated that neither BC nor Lyc affect eNOS activity (data not shown). As expected, a significant increase in enzymatic NO production was observed in HUVECs treated with the positive control ionomycin, which was significantly inhibited by preincubation with L-NAME, demonstrating that increased NO production was due to eNOS activation (0.054 ± 0.0096 versus 0.022 ± 0.0056 pmol NO/min/mg protein, ionomycin versus control, $p<0.001$; 0.0044 ± 0.001 versus 0.054 ± 0.0096 pmol NO/min/mg protein, ionomycin+L-NAME versus ionomycin, $p<0.0001$).

We also measured ionomycin-stimulated NO production by DAF-2DA cytometric evaluation confirming data obtained by L-[^{3}H]-arginine/L-[^{3}H] to citrulline conversion assay (ΔMFI ratio: 16.8 ± 1.3 and 11.0 ± 1.2, ionomycin versus control, respectively; $p<0.001$; 9.8 ± 2.7 and 16.8 ± 1.3, ionomycin+L-NAME versus ionomycin, respectively, $p<0.001$).

Finally, in order to exclude that the observed NO release associated with carotenoids treatment was not due to an increased eNOS expression, we also assessed eNOS protein

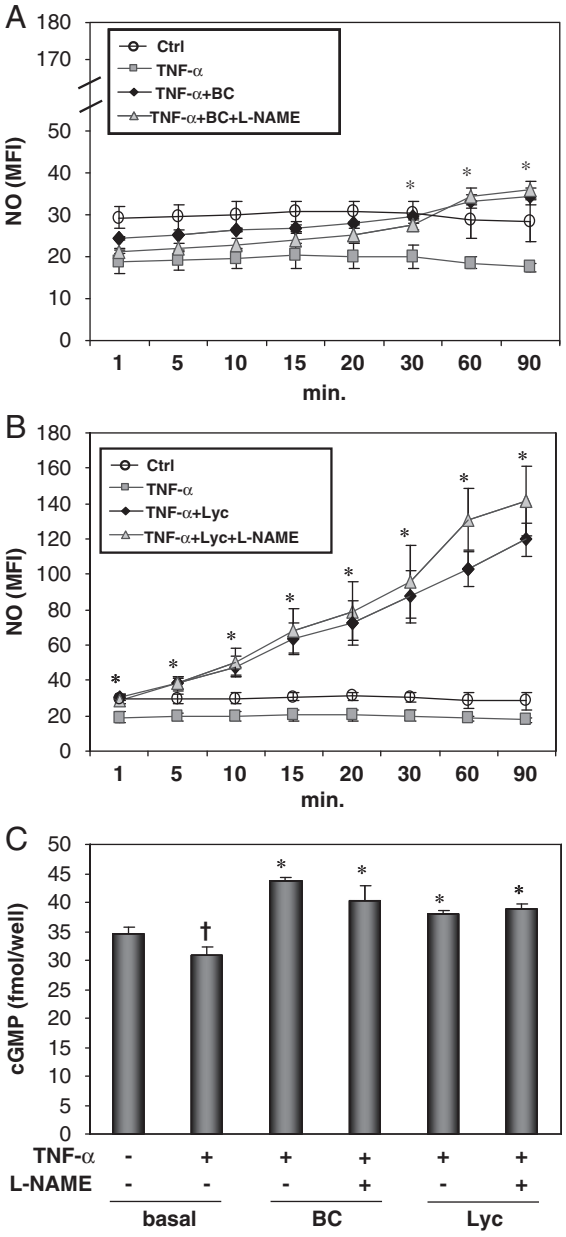

Figure 2. Effects of carotenoids on NO production and intracellular cGMP levels. Time-dependent NO generation measured by DAF-2DA cytometric analysis in HUVECs stimulated with TNF-α (1 ng/mL) for 16 h and then incubated (1–90 min) with 2.5 μmol/L BC (A) or Lyc (B) in the presence or absence of L-NAME (1 mmol/L). Data are expressed as mean fluorescence intensity (MFI) and are shown as mean ± SEM from three independent experiments. Statistical analysis showed significant differences between the untreated cells (Ctrl) and TNF-α-treated cells (TNF-α) at all time points ($p<0.01$). In the presence or absence of L-NAME pretreatment, statistical analysis showed significant differences between TNF-α+BC or +Lyc versus TNF-α (*$p<0.01$). (C) cGMP levels measured by EIA in HUVECs stimulated with TNF-α (1 ng/mL) for 16 h and then incubated for 2 h with BC or Lyc (2.5 μmol/L) and/or L-NAME (1 mmol/L) (*$p<0.05$).

levels in basal conditions and after stimulation with TNF-α. In all conditions, the presence of carotenoids at the tested concentrations (0.5–2.5 μmol/L) did not induce any detectable change in eNOS protein expression (data not shown).

To confirm the role of carotenoids in maintaining NO bioavailability, we also measured in the same experimental conditions the intracellular levels of cGMP, a biological target of NO activity [31]. Figure 2C shows that the stimulation with TNF-α induced a significant decrease in cGMP levels ($p<0.05$) and that the subsequent treatment with carotenoids (2.5 μmol/L, 2 h), either in the presence or absence of L-NAME was associated with a significant increase in cGMP levels ($p<0.05$), demonstrating that an increase in NO production and bioavailability occur in association with the treatment with carotenoids. The incubation with the vehicle, THF, alone did not induce any significant variation in NO bioavailability (data not shown).

3.5 Effects of carotenoids on TNF-α modulated NF-κB pathway

Since NO can inhibit vascular inflammatory NF-κB activity by inducing IκBα synthesis and then inhibiting NF-κB nuclear transfer [18], the potential ability of carotenoids in affecting NF-κB translocation induced by TNF-α was studied by EMSA, utilizing the binding to a canonical NF-κB oligonucleotide-binding sequence of a nuclear protein extract. As expected, TNF-α treatment was associated with NF-κB nuclear translocation. Twenty-four preincubation with 2.5 μmol/L BC and Lyc significantly decreased the amount of nuclear NF-κB following TNF-α treatment (Fig. 3A and B). In addition, in order to better understand the potential role of carotenoids in inhibition of NF-κB pathway, NF-κB p65 protein expression in nucleic fractions was also determined. Figure 3C shows that NF-κB p65 subunit was expressed at very low levels in the nuclei of non-stimulated cells (lanes 1 and 2, basal and vehicle, respectively). However, a marked translocation of p65 from cytosol to nucleus was observed after TNF-α stimulation (lane 3). BC and Lyc pre-treatment (2.5 μmol/L) was associated with a significant decrease in the amount of p65 subunit translocation into the nucleus (lanes 4 and 5, respectively). These observations suggest that BC and Lyc affect the formation of NF-κB-DNA complexes by inhibiting NF-κB nuclear translocation.

The increase in NF-κB activity results from the phosphorylation and rapid inactivation of the inhibitory subunit IκB through proteolysis [32]. Thus, we next determined whether BC and Lyc (2.5 μmol/L) affected IκB phosphorylation. The stimulation by TNF-α induced a rapid IκBα phosphorylation with a simultaneous decrease in cytosolic IκBα protein levels (lane 3). Twenty-four hours pre-treatment with BC and Lyc resulted in a slight though significant inhibition of TNF-α-induced IκBα expression and phosphorylation (Fig. 3D, lanes 4 and 5, respectively).

3.6 Effects of carotenoids on TNF-α increased adhesion molecules protein levels and U937 adhesion on HUVECs

As known, TNF-α stimulation increased VCAM-1, ICAM-1 and E-Selectin expression in HUVECs. Figure 4 shows that this increase was significantly inhibited by BC (1–2.5 μmol/L, panel 4A) and Lyc (2.5 μmol/L, panel 4B), further supporting the possible preventive effect of carotenoids on inflammation. Consequently, in order to test the functional consequences of the inhibition of adhesion molecules expression by BC and Lyc induced by TNF-α, a cell adhesion assay was performed utilizing the human monocyte line U937. Figure 4C shows that few U937 cells were adherent on HUVECs monolayer in the absence of TNF-α (panel a), whereas the number of adherent cells dramatically increased following the stimulation of HUVECs with TNF-α (panel g). Twenty-four hours pre-treatment with either 2.5 μmol/L BC or Lyc resulted in a significant reduction in monocyte adhesion to HUVECs (panels h and i, respectively). The incubation of HUVECs with carotenoids (2.5 μmol/L) in the absence of TNF-α was not associated with any significant change in monocyte adhesion (panels e and f).

In addition, to further support also the potential effects of carotenoids in reverting TNF-α-activated inflammatory response, HUVECs were stimulated with TNF-α (1 ng/mL for 16 h) before the treatment with 2.5 μmol/L carotenoids for 24 h, in the presence or absence of L-NAME. Also in this experimental condition, carotenoids significantly reduced monocyte–HUVEC interaction (Fig 5A and B). Of note, the pre-treatment with L-NAME did not affect the inhibitory activity of carotenoids on U937 adhesion on HUVECs induced by TNF-α, confirming that their effects are likely to be independent on the stimulation of eNOS enzymatic activity (Fig. 5A and B).

As shown in Fig. 4C (panels b–d) and in inset Fig. 5B, the treatment with saturating concentrations of anti-VCAM-1 or anti-ICAM-1 or anti-E-Selectin antibodies also resulted in the inhibition of U937 adhesion to HUVECs. This observation suggests that the hyper-expression of these molecules on the cell surface was among the major mechanisms responsible for the increased adhesion of U937 monocytes to HUVECs.

The inhibition of adhesion molecule expression and monocytes–HUVECs interaction was evidently not due to carotenoids' cytotoxicity. In fact, both BC and Lyc, at the doses considered in the present study, did not affect HUVECs viability, proliferation and cell cycle progression, as determined by MTT assay, [^{3}H]-thymidine incorporation assay and FACS analysis, respectively (data not shown, see Supporting Information).

4 Discussion

The major scope of this study was to provide further insights on the mechanism/s underlying the effect of some

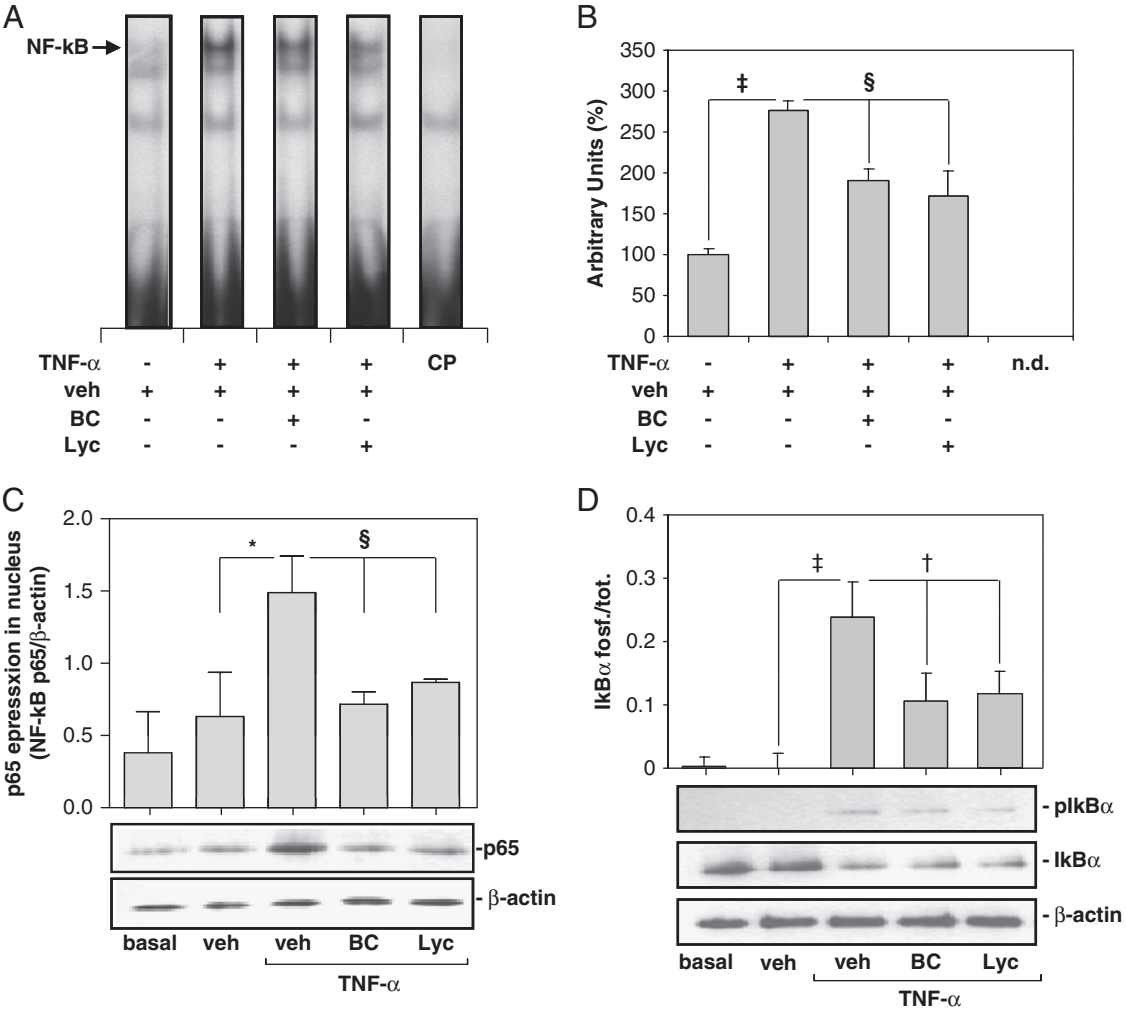

Figure 3. Effects of carotenoids on TNF-α-induced NF-kB activation. (A) A representative EMSA experiment out of at least three separate experiments. HUVECs were treated for 24 h with 2.5 μmol/L of carotenoids and vehicle alone (TNF, veh) followed by 2 h TNF-α (1 ng/mL) stimulation. CP is Cold Probe (non-labeled). The results are reported in histograms (B) and are expressed as percentage of relative intensity (Arbitrary Units) relative to vehicle alone. (C) and (D) Representative protein blotting for NF-κB p65 subunit translocation to nucleus and IκBα phosphorylation, respectively, in HUVECs treated in the same experimental conditions. Histograms show anti-NF-κB p65 and anti-phospho- and total-IkBα Western blot densitometric analysis, respectively. The ratios of NF-κB p65, phosphorylated or total IkBα and β-actin are expressed as the relative intensity. Data are shown as mean ± SD from three independent experiments. n.d., not detectable. $^{*}p<0.05$ versus vehicle alone, $^{\dagger}p<0.05$ versus TNF-α, $^{\ddagger}p<0.01$ versus vehicle alone, $^{\S}p<0.01$ versus TNF-α.

carotenoids of nutritional importance on TNF-α-induced endothelial inflammatory response and to obtain a better understanding of their possible implication in the in vivo evidences indicating a protective role of carotenoids in preventing CVD. We considered two molecules: BC, which is characterized by pro-vitamin A activity and Lyc which is a molecule having no pro-vitamin A activity. Both are largely represented in the majority of human dietary patterns [1, 2].

We demonstrated that both BC and Lyc significantly reduced the expression of vascular adhesion molecules levels and monocyte–endothelial adhesion triggered by TNF-α (Figs. 4 and 5), in both the conditions studied with carotenoids incubated before and after TNF-α stimulation. Both carotenoids, in fact, were able to quench the inflammatory response induced by TNF-α either when administered to HUVECs before the induction or after the pro-inflammatory stimulus.

This observation strongly suggests that their activity is likely to be "directly" targeted toward the reactive oxygen and nitrogen species generated during inflammation than to a signaling effect even though the simultaneous contribution of alternative mechanisms, possibly due to the activation of carotenoids-specific cellular signaling, is not to be excluded.

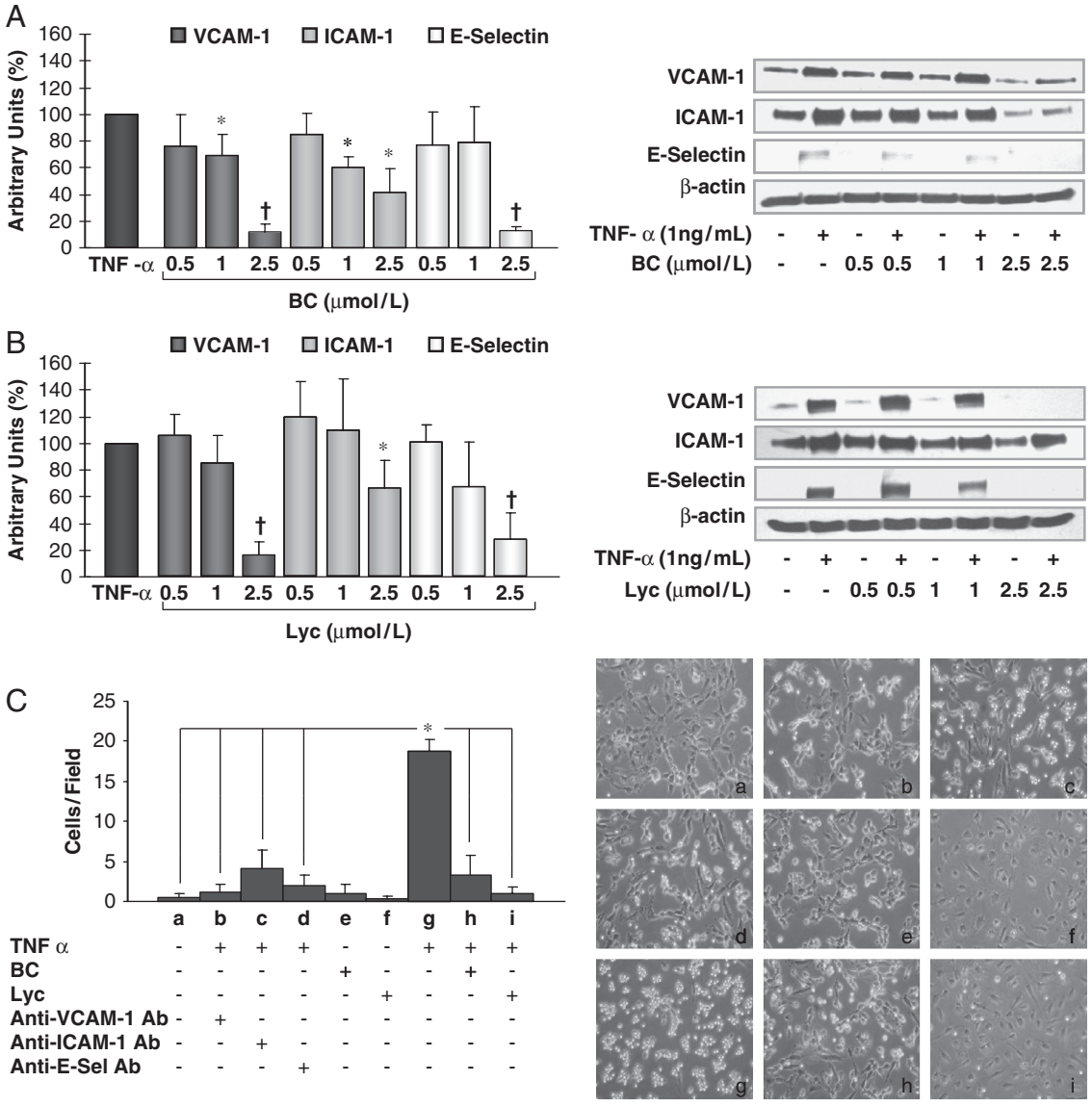

Figure 4. Effects of carotenoids on adhesion molecules expression and monocytes–HUVECs interaction induced by TNF-α. Right: Representative Western blot for VCAM-1, ICAM-1 and E-Selectin in HUVECs incubated for 24 h with 0.5–2.5 μmol/L of BC (A) and Lyc (B) before stimulation with or without 1 ng/mL TNF-α for 16 h. (C) In the same experimental conditions, HUVECs were incubated with the highest carotenoid concentration (2.5 μmol/L) to evaluate monocytes–HUVECs interaction by U937 cell adhesion assay. Left: (A) and (B) Histograms show VCAM-1 (dark-gray bars), ICAM-1 (light-gray bars) and E-selectin (white bars) Western blot densitometric analysis. VCAM-1, ICAM-1 or E-Selectin and β-actin ratio are expressed as relative intensity (Arbitrary Units). Data are shown as a percentage change (relative to TNF-α stimulation, black bar) in band densities and shown as mean ± SD from three independent experiments. $^{*}p<0.05$, $^{\dagger}p<0.0001$ versus TNF-α-treated HUVEC. (C) Quantitative data express number of U937 cells adhering within a high-power field (3.5 mm^2). Each measurement is the mean ± SD of adhering cells from three experiments, each consisting of eight counts for condition. HUVECs are also incubated with anti-VCAM-1, ICAM-1 and E-Selectin antibody for 1 h before the assay. $^{*}p<0.001$ versus TNF-α.

One of the effects responsible for the observed activity of carotenoids was the inhibition of the TNF-α-induced activation of NF-κB pathway (Fig. 3). Interestingly, in our cellular model these effects were associated with an ability to protect HUVECs from TNF-α-induced nitro-oxidative stress (Fig. 1), suggesting that the observed anti-inflammatory activity of BC and Lyc may be due, at least in part, to the maintenance of NO bioavailability (Fig. 2), which plays a fundamental role in vascular functions and homeostasis [21].

The present study stems from several previous observations, based on both in vivo and in vitro experiments, suggesting that carotenoids play an important role in preventing vascular inflammation. It is known that chronic

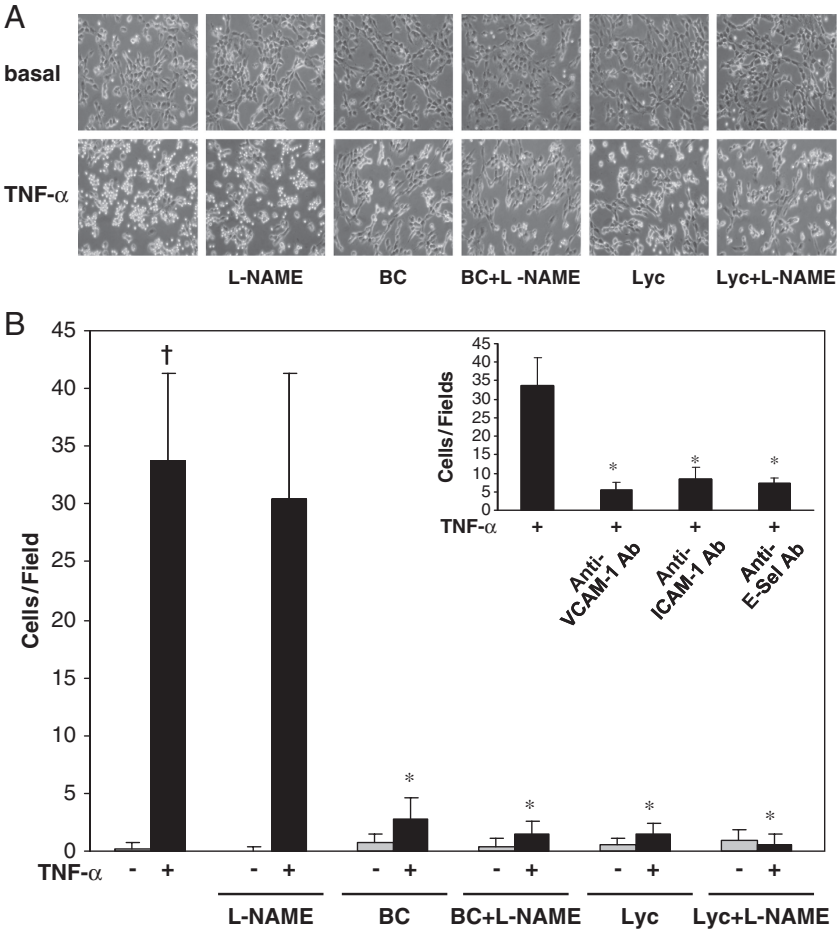

Figure 5. Effects of carotenoids, in the presence or absence of L-NAME, on TNF-α-induced monocytes–HUVECs interaction. (A) Untreated HUVECs (basal) and preincubated cells with 1 ng/mL TNF-α for 16 h (TNF-α) were stimulated with 2.5 μmol/L carotenoids in the presence or absence of L-NAME (1 mmol/L). (B) Quantitative data express number of U937 cells adhering within a high-power field (3.5 mm^2). Each measurement is expressed as mean ± SD of adhering cells from three experiments, each consisting of eight counts for each condition. HUVECs were also incubated with anti-VCAM-1, ICAM-1 and E-Selectin antibody for 1 h before the assay. $^{*}p<0.01$ versus TNF-α, $^{\dagger}p<0.001$ versus basal.

inflammatory conditions and the excess of free radicals, generated within an inflammatory milieu, are likely to play an important role in the development of vascular dysfunction and CVD [33, 34]. Dietary anti-oxidants, such as those present in fruits and vegetables, have been frequently reported to play a beneficial effect in vitro by reducing the levels of inflammation and free radicals [3]. In spite of a consolidated agreement of in vitro studies, the effect of anti-oxidants in atherosclerosis and CVD in vivo is still subjected to constant debate. However, several studies reported that dietary profiles providing high intakes of carotenoids, are associated with lower risk of CVD [6, 7, 35, 36]. In cultured aortic endothelial cells, Lyc has been reported to inhibit cytokines-induced ICAM-1 and VCAM-1 expression [22]. Recently and in agreement with the present study, Hung et al. [21] found that Lyc is able to inhibits TNF-α-induced NF-κB activation, ICAM-1 expression and monocyte–endothelial interaction. However, the mechanism/s of action of carotenoids on TNF-α-primed HUVECs has/have not been fully elucidated.

In our study, we incubated HUVECs with two of the most prevalent carotenoids in human plasma. Due to their different solubilities in aqueous medium, we could not reach the same final concentration of the two molecules in the culture medium, which was much higher for Lyc than for BC (0.5 ± 0.28 and 0.02 ± 0.002 μmol/L, respectively). In spite of this difference, we could achieve a significant enrichment of the culture medium up to levels similar to those usually found in the plasma of subjects consuming a normal diet, rich in fruits and vegetables [37]. This evidence might be of relevance since several in vivo observations suggest a possible biphasic response of carotenoids in promoting health when taken at dietary levels, but adverse effects have been observed at higher amounts [3]. Remarkably, our preliminary results on HUVECs treated with high carotenoids concentrations (2.5 μmol/L to 1 mmol/L), strongly indicate that in these experimental conditions all tested carotenoids are active in suppressing cell proliferation and decreasing cell viability (data not shown). These evidences also corroborate the hypothesis that in vivo supplementation of carotenoids at pharmacological levels may have adverse effects, possibly through their pro-oxidant activity or, in the case of pro-vitamin A molecules, by over-activating retinoid acid related signaling [3, 38]. However, at

lower doses (below 2.5 μmol/L) we found that both carotenoids reduced U937–endothelium interaction, confirming potential beneficial effects in reducing vascular inflammation.

In order to elucidate the mechanism/s potentially underlying the effects of carotenoids, we considered their role in modulating NO bioavailability, according to the evidence that an increased release of this molecule lead to a down-regulation of the expression of NF-κB-dependent adhesion molecules in endothelial cells [20, 39]. In the present study, we have demonstrated that in a model of vascular inflammation, the presence of ''physiological'' concentrations of BC and Lyc is associated with a significant increase in NO level and bioavailability (as indicated by the increase in cGMP levels, Fig. 2). As expected, TNF-α treatment led to a fall of NO availability and release due to the reduction of eNOS phosphorylation and to an increase in ROS generation inducing a situation of endothelial nitro-oxidative stress [40]. In fact, the inactivation of NO by ROS, in particular O_2^-, is recognized to be a crucial factor in reducing NO bioavailability [41]. In this respect, we demonstrated that in cultured HUVECs, either BC or Lyc (2.5 μmol/L, 2 h) suppressed the increase in ROS generation and the intracellular levels of NT (an index of $ONOO^-$ formation) due to TNF-α treatment. This activity significantly quenched the oxidative stress generated by an inflammatory condition, allowing NO to exert its biological activities, as documented by cGMP increased levels (see Figs. 1 and 2).

Of note, the increase in NO levels associated with BC and Lyc treatment was not affected by the presence of the eNOS inhibitor L-NAME, indicating that their effect was not due to a specific enzymatic activation of eNOS. In order to confirm this hypothesis, we also evaluated the direct effect of both carotenoids in modulating NOS enzymatic activity. Neither BC nor Lyc affected enzymatic NO production, while HUVECs exposed to positive control, ionomycin, exhibited a significant increase in eNOS-dependent NO production, confirming both the integrity and the reactivity of our cultured cells model.

We propose that the candidate mechanism/s potentially responsible for the positive modulation of NO bioavailability by BC and Lyc is associated with their reducing potential. Carotenoids are known to be able to directly interact with several free-radical species in vitro [3], and this may account for their radical quenching or scavenging properties. In prooxidant conditions, such as in the presence of TNF-α, reducing molecules might contribute to maintain NO availability by directly interacting with O_2^- and therefore minimizing its reaction with intracellular NO and the formation of the potent oxidant-nitrosylating agent, peroxinitrite.

In conclusion, our results, obtained in human endothelial cells exposed to physiological concentration of carotenoids, similar to those occurring in the vessels of subjects consuming a ''normal'' diet, provide a robust evidence that these molecules may act on vascular inflammatory state by increasing vascular NO bioavailability thanks to their reducing activity. This may represent another interesting mechanism to further elucidate why carotenoids can prevent and/or delay cardiovascular disease.

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