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Lycopene inhibits endothelial cells migration induced by vascular endothelial growth factor A increasing nitric oxide bioavailability

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ABSTRACT

Lycopene is a carotenoid found in tomatoes previously shown bioactive in endothelial cells. We studied *in vitro* cell migration, key step in angiogenesis using the scratch wound healing assay to test the effects of lycopene (2 μmol/L) on Human Umbilical Vein Endothelial Cells. To explore the cellular mechanism, we measured nitric oxide (NO) release and protein kinase B (Akt) phosphorylation. Lycopene inhibited cell migration induced by vascular endothelial growth factor-A (VEGFA) and reduced Akt phosphorylation. NO generation in endothelial cells, assessed by using difluorofluorescein diacetate, was increased by lycopene alone or in combination with VEGFA and blunted by NO synthase inhibition. Increased nitrite-nitrate concentration in culture medium indicates effects of lycopene on NO bioavailability, potentially implicated in the reduction in VEGFA-induced cell migration, as observed with a NO-donor. In conclusion, lycopene inhibits endothelial cells migration modulating VEGFA signalling system. Increased NO generation partly explains lycopene bioactivity.

1. Introduction

Lycopene, the most abundant carotenoid in tomato fruit (Lenucci, Cadinu, Taurino, Piro, & Dalessandro, 2006), has been investigated as a putative major contributor to the health benefits of tomato and tomato based products (Clinton, 1998; Martin, Wu, & Meydani, 2000). Observational studies suggest that a diet rich in lycopene might be associated with a reduced incidence of cardiovascular diseases, as recognized in the Framingham Heart Offspring Study (Jacques, Cassidy, Rogers, Peterson, & Dwyer, 2015) and cancer (Giovannucci, Rimm, Liu, Stampfer, & Willett, 2002; Milani, Basirnejad, Shahbazi, & Bolhassani, 2017). Mechanistic studies represent the background information to support these evidences, while clinical intervention studies are lacking.

Lycopene ($C_{40}H_{56}$) is a carotenoid with antioxidant properties acting as a quencher of oxygen free radicals thus protecting lipids, proteins and nucleic acids from oxidative modification. Not only protection of cells from oxidative injury, but also a variety of biological effects of lycopene have been observed *in vitro*, including improvement of endothelium-dependent vascular relaxation, anti-inflammatory and anti-platelet activities (Di Tomo et al., 2012; Feng, Ling, & Duan, 2010; Fuhrman, Elis, & Aviram, 1997; Karppi, Nurmi, Kurl, Rissanen, & Nyyssönen, 2010; Palozza et al., 2010; Story, Kopec, Schwartz, & Harris, 2010). Proapoptotic, antiproliferative effects on cancer cells were observed, that might substantiate potential beneficial effects of lycopene on human health (Nahum et al., 2001; Takeshima et al., 2014). Inhibition of angiogenesis was also observed to be determined by lycopene, although the underlying mechanisms are poorly defined (Chen, Lin, Yang, & Hu, 2012; Elgass, Cooper, & Chopra, 2012; Huang, Chuang, Lo, & Hu, 2013; Sahin, Sahin, & Gumuslu, 2012). Angiogenesis, the process of new blood vessel growth, consists in a sequence of events that include migration, proliferation of endothelial cells and formation of the vasculature (Folkman, 1995). Vascular endothelial growth factors (VEGFs) are crucial regulators of blood vessel formation. VEGFA has a primary role in stimulating vascular endothelial cell migration (Olsson, Dimberg, Kreuger, & Claesson-Welsh, 2006).

The binding of VEGFA to the VEGF tyrosine kinase receptor-2 (VEGFR-2), leads to the activation of the proangiogenic signalling systems. Downstream, Akt phosphorylation is a crucial step in VEGFA pathway, promoting the activation of a variety of target proteins implicated in cell proliferation, migration, survival, and permeability.

Abbreviations: DAF-FM DA, 4-amino-5-methylamino-2′, 7′-difluorofluorescein diacetate; DAN, 2,3-diaminonaphthalene; DETA-NO, diethylenetriamine NONOate; HUVEC, human umbilical vein endothelial cell; NO, nitric oxide; ROS, reactive oxygen species; VEGFA, vascular endothelial growth factor A

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Nitric oxide (NO) was reported to modulate angiogenesis by direct and indirect mechanisms exerting anti- and pro-angiogenic effects depending on concentration and duration of NO exposure (Fukumura, Kashiwagi, & Jain, 2006; Burke, Sullivan, Giles, & Glynn, 2013). NO released after VEGFR-2 activation is a critical mediator of endothelial cell migration (Bouloumie, Schini-Kerth, & Busse, 1999; Murohara et al., 1998; Palmer, Ashton, & Moncada, 1988; Papapetropoulos, García-Cardeña, Madri, & Sessa, 1997).

Particularly under clinical conditions characterized by increased oxidative stress, NO may react with superoxide anion (O_2^-) to form peroxynitrite ($ONOO^-$), a strong oxidant agent quenching NO signalling. This reaction can be limited by reactive oxygen species (ROS) scavengers.

Microvascular disease in diabetes mellitus represents a clinical condition in which increased inactivation of NO by ROS and impaired endothelial cells migration are implicated. In this setting, lycopene-rich food might exert preventive or dampening effects (Murillo & Fernandez, 2016).

In the present study, we investigated the effects of lycopene on VEGFA-induced endothelial cell migration, VEGFA signalling and NO release. Our results indicate that lycopene inhibits *in vitro* VEGF-induced endothelial cells migration, and that this anti-migratory activity implicates the downregulation of VEGF-activated signalling system and the increased bioavailability of NO.

2. Materials and methods

2.1. Cell culture and chemicals

Human umbilical vein endothelial cells (HUVECs) and endothelial cell basal medium-2 (EBM-2) with EGM-2 Single Quots were purchased from Lonza Srl. (Basel, Switzerland). In all experiments, HUVECs were used between passages three and eight. Recombinant human vascular endothelial growth factor A (VEGFA) was purchased from PreProTech Ltd. (London, UK). Diethylenetriamine NONOate (DETA-NO), was purchased from Cayman Chemical Co. (Ann Arbor, MI). Nω-Nitro-L-arginine methyl ester hydrochloride (L-NAME) and L-arginine were purchased from Sigma-Aldrich (Milan, Italy). TO-PRO-3 iodide was purchased from Thermo Fisher (Italy). Anti-phospho-Akt (Ser473), anti-Akt, were from Cell Signalling Technology (Beverly, MA, USA). Lycopene was purchased from Sigma-Aldrich (Milan, Italy), dissolved in a dimethyl sulfoxide (DMSO) to obtain a 1.86 mM stock solution with a minimal exposure to air and light and aliquoted and stored at −80 °C until used. The stock solution was diluted with EGM-2 medium and added to the culture medium at a final concentration of 2 µM. The final concentrations of the DMSO in the medium was 0.1% (v/v).

2.2. Cell migration assay

Migration of HUVECs were determined *in vitro* by scratch wound healing assay (Treggiari et al., 2015). Cells were seeded in 6-well plates pre-coated with fibronectin and grown in EGM-2 to confluence. After overnight starvation in EBM-2 with 1% foetal bovine serum (FBS), the cell were wounded by scratching with a pipette plastic tip. Cells were washed twice with phosphate buffered saline (PBS) to remove non-adherent cells and then placed in EBM-2 with 1% FBS and treated with 10 ng/mL VEGFA in the presence or absence of 2 µmol/L of lycopene and incubated for 20 h. Cells treated with DMSO (0.1%) only served as the vehicle control. Images were taken at 0 h and at 20 h. The migrated HUVEC cells were manually counted. The images were taken from three randomly selected field.

2.3. Cell viability

We performed the viability test using 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium Bromide (MTT, Sigma Aldrich) to evaluate the potential cytotoxic effects of lycopene (0.5–5 µmol/L) and DMSO (0.03–0.27% of the final volume). HUVECs grown to confluence in EGM-2 were trypsinized and seeded in 96-wells plate at concentration of 150,000/100 µl; HUVECs were incubated for 20 h in the presence or absence of lycopene dissolved in DMSO, or DMSO alone. MTT (50 µg/well) was added and after 3 h at 37 °C, the reaction was stopped with 100 µl of isopropanol/0.04 M HCl. Absorbance at 540 nm was measured in Victor™ X4 microplate reader (Perkin Elmer).

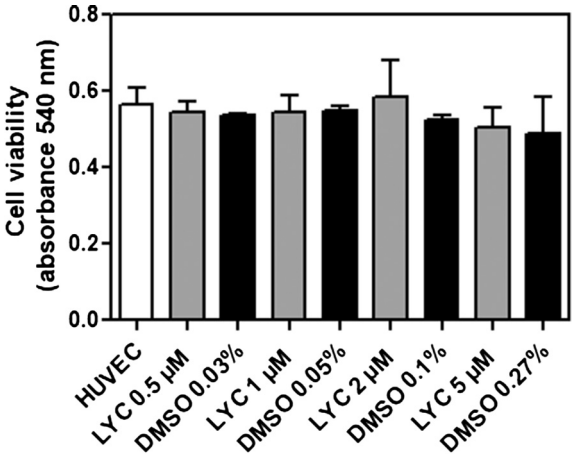

Fig. 1. Effects of lycopene and DMSO on endothelial cells viability. The MTT assay was performed using adherent HUVECs incubated for 20 h in the presence of different concentrations of lycopene, which was dissolved in DMSO, DMSO alone or in the absence of any added compound (control conditions). Values are expressed as mean ± SE (n = 3). One-way ANOVA was applied to the whole set of data.

2.4. Nitric oxide measurement

NO generation in HUVECs was measured by using 4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate (DAF-FM DA) (Merk Millipore, Italy). DAF-FM DA stock solution (5 mmol/L) was aliquoted and stored at −20 °C in darkness and working dilution was prepared just before use. HUVECs were plated onto 13 mm glass cover-slide in the 6-well plate. The cells were grown in EGM-2 medium for 24 h and serum starved overnight in medium containing EBM-2 and 1% FBS. Medium was replaced with Krebs Ringer-Phosphate (KRP) buffer (154 mmol/L NaCl, 5.6 mmol/L KCl, 2.2 mmol/L $CaCl_2$, 1.1 mmol/L $MgSO_4$, 10 mmol/L glucose, 2.15 mmol/L Na_2HPO_4, 0.85 mmol/L NaH_2PO_4, pH 7.20). Then, 1 µmol/L DAF-FM DA was added to cells preparations that were incubated for 30 min at 37 °C. Afterwards, the excess probe was removed and the cells were incubated for an additional 10 min to allow for complete de-esterification. Cells were then washed three times with KRP and then incubated with 10 ng/mL VEGFA and 2 µmol/L of lycopene in KRP with 1% FBS, at 37 °C for 30 min. L-NAME 300 µmol/L was used as negative control. Cells treated with DMSO (0.1%) only served as the vehicle control. Cells were then washed three times with KRP, fixed with 4% formaldehyde for 10 min at room temperature and loaded with TO-PRO-3 (1:2000) for 10 min in PBS for nucleus stain. Cells were placed on a confocal microscopic stage after washing the coverslip in water. NO fluorescence was measured using excitation and emission wavelengths of 488 and 515 nm, respectively. For each data point, images from 4 randomly chosen fields were taken. The total fluorescence intensity and the number of cells in each field were noted, and the mean fluorescence intensity per cell (MFI/cell) was determined by using ImageJ software.

2.5. Nitrate/Nitrite assay

The nitrate/nitrite concentration was measured by using 2,3-diaminonaphthalene (DAN) fluorometric assay kit (Cayman Chemical

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