Antioxidant Betalains from Cactus Pear (*Opuntia ficus-indica*) Inhibit Endothelial ICAM-1 Expression

C. GENTILE,[a] L. TESORIERE,[a] M. ALLEGRA,[a] M. A. LIVREA[a] AND P. D'ALESSIO[b]

[a]*Dipartimento Farmacochimico, Tossicologico e Biologico, Università di Palermo, Italy*

[b]*INSERM U602, Université Paris XI Orsay, France*

ABSTRACT: It has been suggested that some pigments would have antioxidant properties and that their presence in dietary constituents would contribute to reduce the risk of oxidative stress–correlated diseases. Among others, inflammatory response depends on redox status and may implicate oxidative stress. Vascular endothelial cells are a direct target of oxidative stress in inflammation. We have tested the impact of the free radical scavenger and antioxidant properties of betalains from the prickle pear in an *in vitro* model of endothelial cells. Here we show the capacity of betalains to protect endothelium from cytokine-induced redox state alteration, through ICAM-1 inhibition.

KEYWORDS: endothelial cells; ICAM-1; betalains; antiinflammatory drugs

INTRODUCTION

Molecules of plant origin have been very much used in medicine, and new phytochemicals are continuously being examined for potential pharmacological applications. Contained in some families of the caryophyllales, including red beet and cactus pear, the betalain pigments are betalamic acid derivatives. We distinguish betacyanins as conjugates of betalamic acid with 3,4-dihydroxyphenilalanine (DOPA), which may or may not be glycosylated, and betaxanthins, conjugated with amino acid residues. The red betanin (the adduct contains 5-O-glucose cyclo-DOPA) and the yellow indicaxanthin (the adduct contains proline) have recently been isolated from cactus pear (*Opuntia ficus-indica*) and characterized by our group. Both compounds are able to quench the 2,2′-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid) (ABTS) cation radical.[1] More important, protective antioxidant effects of betalains have been shown in a number of *in vitro* models.[2,3] In particular, we have demonstrated that betanin and indicaxanthin may bind to human low-density lipoproteins (LDLS),[3] inhibiting their oxidation. Last, the synergistic interaction with the LDL-

Address for correspondence: P. D'Alessio, INSERM U602, Université Paris XI Orsay, France. Voice: 33-1-4550-6148; fax: 33-3-4559-5359.
dalessio@vjf.inserm.fr

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vitamin E appears to add a remarkable potential in indicaxanthin to scavenge lipoperoxyl radicals.[3]

Reactive oxygen or nitrogen species (ROS/RNS) may be toxic or display a regulatory character. The redox status of cells is continuously surveyed by antioxidant molecules and enzymes in order to avoid damage to cell components, as well as to regulate redox-mediated signaling pathways involved in cell growth, senescence, and death. This kind of regulation is also at work during the inflammatory reaction, originating in the activation of vascular endothelial cells.[4] Moreover, in acute and chronic inflammatory diseases, such as ischemia-reperfusion injury, rheumatoid arthritis, or inflammatory bowel disease, the oxidant tone is also responsible for endothelial dysfunction.[5] Moreover, ROS-generated endothelial dysfunction appears to be crucial in the pathogenesis of atherosclerosis, as well as a relevant support for cancer dissemination.[6] Hence, endothelium seems to be an ideal target for anti-inflammatory molecules. We have shown that betalains are potent radical scavengers in chemical systems and behave as efficient antioxidants in a number of biological models.[1,2]

Cell-adhesion molecules of the Ig-superfamily[7] are characteristically expressed by vascular endothelial cells in response to proinflammatory stimuli. Their membrane expression is devoted to the recruitment of activated leukocytes, finalized to surveying of tissue or defense from microorganisms. Vascular endothelium expresses the constitutive form of intercellular adhesion molecule-1 (ICAM-1), present in all cell types with the exception of red blood cells. Its expression is greatly enhanced by the stimulation of endothelial cells by tumour necrosis factor-α (TNF-α) a major cytokine that is pivotal in the inflammatory reaction as well as in a plethora of other functions.[8] The mechanism of action of TNF-α has been shown to depend on the

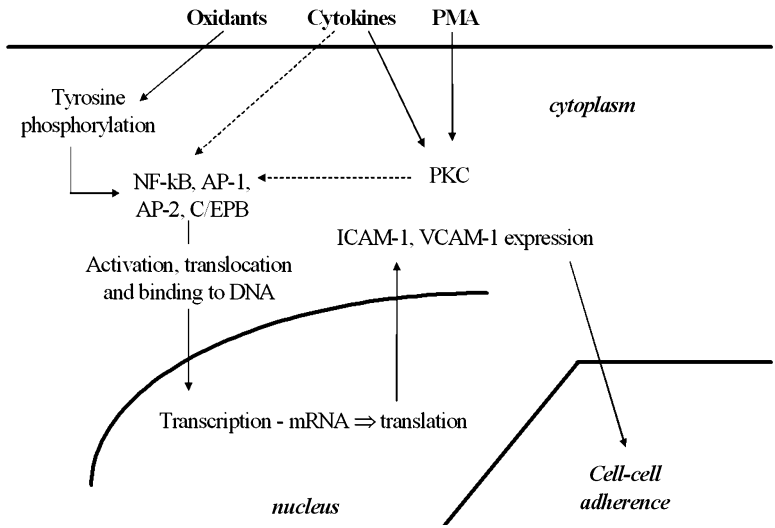

FIGURE 1. Signal transduction pathways of cell adhesion molecule expression.

production of ROS through the activation of at least two transcription factors, NF-κB, and AP-1 (FIG. 1). In this work, human primary endothelial cells, isolated from umbilical cords, are stimulated by TNF-α, and the two antioxidant molecules betanin and indicaxanthin are tested for their capacity to inhibit the expression of ICAM-1.

MATERIALS AND METHODS

Cell cultures. Human umbilical vein endothelial cells (HUVECs) were isolated after collagenase digestion of human umbilical cord veins collected from healthy, nonsmoking women. Umbilical cords were collected with the patients' informed consent. Cells were cultured in endothelial cell basal medium MV2 (ECBM MV2, PromoCell), supplemented with epidermal growth factor (50 ng/mL), hydrocortisone (0.2 μg/mL), vascular endothelial growth factor (5.0 ng/mL), basic fibroblast factor (10 ng/mL), R3IGF-1 (20 ng/mL), ascorbic acid (1 μg/mL), amphotericin B (50 ng/mL), gentamycin (50 μg/mL), and fetal calf serum (10%). HUVEC were subcultured by trypsinization (Trypsin-EDTA, Sigma) and used for the investigation up to passage 6. Cell incubations were done at 37°C in a humidified atmosphere with 5% CO^2.

Betalains, antibodies, and cytokines. Betanin and indicaxanthin were purified from fresh pulp of cactus pear (*Opuntia ficus-indica*), as previously described.[1] TNF-α was purchased from R&D Systems. Betalains and TNF-α were dissolved in PBS and kept at –20°C as stock solutions. Anti-human ICAM-1Fitc was obtained from Diaclone Research and kept at 4°C.

MTT assay. MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (Sigma) assay was used to check the viability of cells to the exposure to betanin and indicaxanthin. Briefly, HUVEC were seeded in duplicate on 96-well culture plates at 2.104 cells/cm^2. After 24-h incubation, either betanin or indicaxanthin was delivered at various concentrations, and the incubation was continued for 24 h. Then, MTT stock solution (5 mg/mL) was added to each well, and plates were incubated for 4 h. At the end of the incubation period, the medium was removed, and converted dye was solubilized with DMSO. Absorbance of converted dye was measured at 550 nm with a microplate reader.

Betanin and indicaxanthin treatments. HUVEC were seeded in duplicate in 12-well culture plates at a density of 2.104 cells/cm^2 in ECBM MV2 10% FCS. After 48 h the cells were starved in serum-free medium for 8 h and then incubated with 5 μM of either betanin or indicaxanthin. The medium was removed after 5 h of incubation, and the cells were incubated with 1 ng/mL TNF-α and/or 5mM betalains for 16 h. Incubation in the presence of betalains and relevant controls were done in ECBM MV2 without ascorbic acid and FCS.

Flow cytometry analysis. After treatments, HUVECs were harvested, and flow cytometry measurements were performed by incubation of cells with anti-human ICAM-1 Fitc diluted 1/100 in FACS buffer (0.5% BSA in PBS). After fixation with 1% phormol in PBS, the cells were analyzed for fluorescence on a Becton Dickinson FACSCalibur flow cytometer, and data were acquired and analyzed using FACS/CELLQuest software (San Jose, CA) on a Power Macintosh 7600/120 computer (Apple Computers, Cupertino, CA).

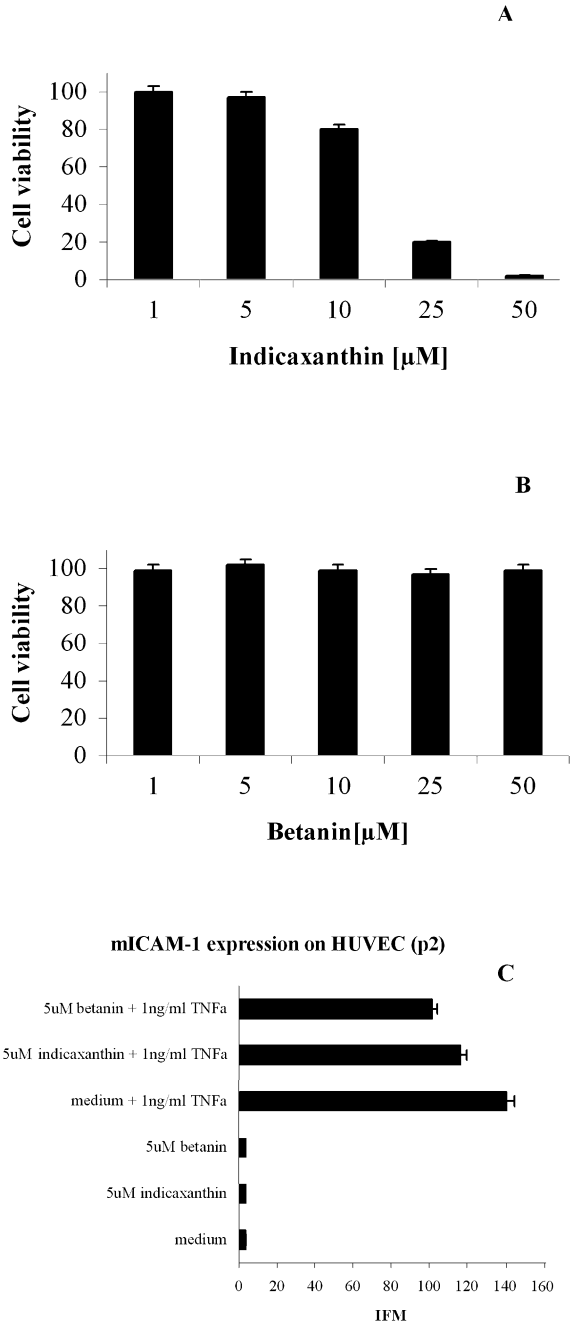

FIGURE 2. Effect of Betalains on cell viability **(A and B)** and on ICAM-1 expression **(C)**.

RESULTS

The activity of betanin and indicaxanthin on the endothelial expression of adhesive molecule ICAM-1 has been tested by incubating HUVECs with either compound in the presence or absence of TNF-α. Both betalains are nontoxic within a concentration range of 1 to 10 μM (indicaxanthin) and 1 to 50 μM (betanin) (FIG. 2 A and B). Inhibition of ICAM-1 expression is obtained at 5 μM (FIG. 2 C). Betanin (30% inhibition, $P < 0.001$ with respect to control cells incubated in the absence of betalains) was more effective than indicaxanthin (17% inhibition, $P < 0.001$).

DISCUSSION

During the last decades, one of the most challenging pharmacological approaches has focused on the identification and characterization of molecules of plant origin, integrating notions of traditional phytotherapy, biochemistry, cell biology, and synthetic chemistry. In some cases, new chemical entities have emerged, such as taxol[9] and resveratrol,[10] which have been found to be effective in the treatment of cancer and degenerative diseases. Betalains are reducing substrates and, as with many antioxidants, could affect intracellular signal transduction. This study has investigated the effect of betanin and indicaxanthin, known for their antioxidant effects in lipid peroxidation, on the expression of endothelial adhesion molecules, using an *in vitro* model of the inflammatory reaction. Both pigments were able to slightly inhibit the expression of the cell-adhesion molecule ICAM-1 at a micromolar concentration. The expression of adhesion molecules is a complex multifactorial redox-regulated process in which the first step could be represented by an intracellular increment of oxidants.[11] It is under current investigation whether betalains could modulate, by their radical scavenging activity, cellular ROS/RNS levels and/or the activity of the two main redox-sensitive transcription factors NF-κB and AP-1. Our present data demonstrate that these phytochemicals, in addition to their radical-scavenging and antioxidant effects, can also act as modulators of adhesive molecule expression in endothelial cells. The combination of these two properties could be of pharmacological interest in pathologies characterized by tissue degeneration due to endothelial dysfunction such as atherosclerosis, atherothrombosis, low limb ischemia, and stroke.[12]

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