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Delphinidin inhibits VEGF induced-mitochondrial biogenesis and Akt activation in endothelial cells

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ABSTRACT

Delphinidin, an anthocyanin present in red wine, has been reported to exert vasculoprotective properties on endothelial cells, including vasorelaxing and anti-apoptotic effects. Moreover, delphinidin treatment in a rat model of post-ischemic neovascularization has been described to exert anti-angiogenic property. Angiogenesis is an energetic process and VEGF-induced angiogenesis is associated with mitochondrial biogenesis. However, whether delphinidin induces changes in mitochondrial biogenesis has never been addressed.

Effects of delphinidin were investigated in human endothelial cells at a concentration described to be anti-angiogenic *in vitro* (10^{-2} g/l). mRNA expression of mitochondrial biogenesis factors, mitochondrial respiration, DNA content and enzyme activities were assessed after 48 h of stimulation.

Delphinidin increased mRNA expression of several mitochondrial biogenesis factors, including NRF1, ERRα, Tfam, Tfb2m and PolG but did not affect neither mitochondrial respiration, DNA content nor enzyme activities. In presence of delphinidin, VEGF failed to increase mitochondrial respiration, DNA content, complex IV activity and Akt activation in endothelial cells.

These results suggest a possible association between inhibition of VEGF-induced mitochondrial biogenesis through Akt pathway by delphinidin and its anti-angiogenic effect, providing a novel mechanism sustaining the beneficial effect of delphinidin against pathologies associated with excessive angiogenesis such as cancers.

1. Introduction

Epidemiological studies reported a reduction in cardiovascular risk and vascular protection associated with diet rich in polyphenols, including those from red wine (Renaud and de Lorgeril, 1992). It has been previously shown that the anthocyanin delphinidin possesses the same pharmacological profile as a total extract of red wine polyphenolic compounds (RWPC) to promote endothelial nitric oxide (NO) production leading to endothelium-dependent relaxation (Andriambeloson et al., 1998). NO production induced by delphinidin involves activation of estrogen receptor alpha (ERα) (Chalopin et al., 2010), increase of intracellular calcium concentration and activation of tyrosine kinases (Martin et al., 2002). Moreover, delphinidin has demonstrated anti-angiogenic properties in both *in vitro* and *in vivo* angiogenesis models (Favot et al., 2003; Martin et al., 2003a; Baron-Menguy et al., 2007). Its anti-angiogenic effect is associated with inhibition of endothelial cell migration and proliferation induced by vascular endothelial growth

Abbreviations: ER, estrogen receptor; ERRα, estrogen-related receptor α; FCCP, carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone; Delph, delphinidin; HUVECs, human umbilical vein endothelial cells; mtDNA, mitochondrial DNA; ND5, NADH dehydrogenase subunit 5; NO, nitric oxide; NOS, nitric oxide synthase; NRF-1, nuclear respiratory factor 1; PGC1α, peroxisome proliferative activated receptor-γ co-activator 1 α; PolG, mitochondrial DNA polymerase G; PRC, PGC1-related co-activator; RWPC, red wine polyphenolic compounds; Tfam, mitochondrial transcription factor A; Tfb2m, mitochondrial dimethyladenosine transferase 2; VEGF, vascular endothelial growth factor.

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Table 1
Sequences of primers used for real-time PCR.

Gene name	Forward primer	Reverse primer	Tm (°C)
Actin (qRT-PCR)	5′ CGACATGGAGAAAATCTGGC 3′	5′ AGGTCCAGACGCAGGATG 3′	58
ERRα (qRT-PCR)	5′ GTGGCATCCTGTGAGGCCTG 3′	5′ GCCGCCGCTTGTACTTCTG 3′	62
NRF-1 (qRT-PCR)	5′ CTCGCCTTCTTCTCCCGAGG 3′	5′ GGACAATAGCTTGCTGTCCC 3′	62
PolG (qRT-PCR)	5′ GTGAGAAGACTGAAGCTTCG 3′	5′ GTTCGTGATCTCCTAGGTTC 3′	60
PRC (qRT-PCR)	5′ GTGGTTGGGGAAGTCGAAG 3′	5′ AGATAAGGGAGGCATCCATG 3′	62
Tfam (qRT-PCR)	5′ CCGAGGTGGTTTTCATCTGT 3′	5′ CAGGAAGTTCCCTCCAACGC 3′	58
Tfb2m (qRT-PCR)	5′ CTATGTCTTCTCGAGGGCTC 3′	5′ CTGGATTTCCGGGATCTGC 3′	62
Actin (qPCR)	5′ ATCATGTTTGAGACCTTCAAC 3′	5′ AGGTCCAGACGCAGGATGG 3′	64
ND5 (qPCR)	5′ CCTCCTAGACCTAACCTGAC 3′	5′ CTTTGTATGATTATGGGCGTTG 3′	64

factor (VEGF) *in vitro* (Favot et al., 2003; Martin et al., 2003a). Mechanisms sustaining the anti-angiogenic effect of delphinidin are not completely elucidated and imply transient activation of ERK1/2, cyclin dependent-pathway (Favot et al., 2003) and inhibition of VEGF receptor 2 (VEGFR2) transactivation (Lamy et al., 2006).

Very recently, we reported a key role of mitochondria in regulation of angiogenesis by RWPC. Indeed, angiogenesis is an energetic process and the pro-angiogenic effect of RWPC is associated with stimulation of mitochondrial energetic capacity through a mechanism sensitive to ERs and NO synthase inhibitors (Duluc et al., 2013). Moreover, the pro-angiogenic factor VEGF is also described to increase mitochondrial biogenesis in endothelial cells through an Akt3-dependent pathway (Wright et al., 2008). However, the involvement of mitochondria in the anti-angiogenic effect of delphinidin has never been assessed yet.

Therefore, the present study was conducted to determine the role of mitochondria in the anti-angiogenic effect of delphinidin. For this purpose, effects of delphinidin were studied on both basal and VEGF-stimulated mitochondrial capacity. The mitochondrial capacity was assessed by mitochondrial respiration measurement, mitochondrial biogenesis factors mRNA expression, mitochondrial complexes activities and mitochondrial DNA (mtDNA) content in endothelial cells.

2. Material and methods

2.1. Reagents

The anthocyanin delphinidin chloride was purchased from Extrasynthèse (Genay, France) and used at 10^{-2} g/l. This concentration was described to induce the maximal relaxing effect on rat aorta *ex vivo* (Andriambeloson et al., 1998), to prevent angiogenesis and to inhibit migration, proliferation (Favot et al., 2003; Martin et al., 2003a) and apoptosis (Martin et al., 2003b) of endothelial cells *in vitro*. Delphinidin was diluted in dimethylsulfoxide (DMSO, Sigma Aldrich, St Quentin Fallavier, France). The final concentration of DMSO in experiments never exceeded 0.1%. Compounds interfering with respiration, including oligomycin, carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP), and antimycin A were purchased from Sigma Aldrich (St Quentin Fallavier, France). Vascular endothelial growth factor (VEGF) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

2.2. Cell culture

The human umbilical vein endothelial cells (HUVECs) cell line EA.hy926 were maintained at 37 °C in a humidified incubator gased with 5% CO_2 in air and was cultured in growth medium (Dulbecco's modified Eagle's medium: Ham's F-12,1:1; Lonza) supplemented with 1% L-glutamine, 1% aminopterin/thymidin/hypoxanthin, 1% non-essential amino acids, 1% Na-pyruvate, 1% streptomycin/penicillin and 10% of heat-inactivated fetal bovine serum (Lonza). Cells were stimulated with DMSO (final concentration 0.1%, used as control), delphinidin, VEGF or both during 48 h.

2.3. RNA extraction and quantitative PCR analysis (qPCR)

Total RNA was isolated from cells using the RNA extraction kit Macherey-Nagel (Hoerdt, France). Quantification, degradation and contamination of the samples were assessed using the Agilent 2100 Bioanalyzer (6000 Nano Assays – Agilent Technologies, Waldbronn, Germany) following manufacturer's procedure. Samples were stored at −80 °C until quantification step. For total RNA samples, retrotranscriptions were performed using Advantage® RT-for-PCR Kit (Clontech, Mountain View, USA) following manufacturer's recommendations. Quantification reactions were performed starting with 40 ng cDNA for a 20 μl final reaction volume using the iQ™ SYBR® Green Supermix following manufacturer's instructions (Bio-Rad, Hercules, CA) and the CFX96™ Real-Time PCR Detection System (Bio-Rad). Standards were obtained by PCR performed on total cDNA of control cells as described previously (Jacques et al., 2006). We explored gene expression of peroxisome proliferative activated receptor-γ co-activator 1 (PGC1)-related co-activator (PRC), estrogen-related receptor alpha (ERRα), nuclear respiratory factor-1 (NRF-1), mitochondrial transcription factor A (Tfam), mitochondrial dimethyladenosine transferase 2 (Tfb2m), and mitochondrial DNA polymerase G (PolG). Forward and reverse primers used and corresponding annealing/elongation temperature are summarized in Table 1. Gene expression was normalized by reference to actin gene expression for each sample. Fidelity of qPCR reaction was determined by melting temperature analysis and visualization of standard product on a 2% agarose gel. Quantifications were performed using the efficiency-adjusted ΔΔCT method.

2.4. Measurement of cellular respiration

Respiration rates were measured in endothelial cells stimulated with delphinidin, VEGF or both during 48 h. The cells were collected by trypsinization, washed once in culture medium and centrifuged ($500 \times g$ x 5 min). The pellet was resuspended in respiratory medium, corresponding to normal culture medium without fetal bovine serum and antibiotics, both reagents known to interfere with mitochondrial respiration. Mitochondrial oxygen consumption was measured at 37 °C using a high-resolution Oxygraph-2K respirometer (Oroboros, Innsbruck, Austria). Oxygraphy experiments were performed on cells cultured in T75 flasks, corresponding to an average of 6 millions cells per flask. Basal respiration rate of cells was determined by measuring the linear rate of oxygen consumption, which reflects the aerobic metabolic activity of cells with physiological substrates in culture medium. Oligomycin (1 μg/ml) was then added to determine the non-phosphorylating respiration rate. The uncoupling respiration rate was also recorded by stepwise addition of FCCP ($(2–10) \times 10^{-7}$ M) up to the optimal concentration representing the maximal capacity of the respiratory chain