Transcriptional Profiling of Angiogenic Activity of Calycosin in Zebrafish

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Approved by

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澳門大學碩士學位論文

毛蕊異黃酮促斑馬魚血管新生的轉錄組學研究

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Abstract

Transcriptional Profiling of Angiogenic Effect of Calycosin in Zebrafish

by LI SHANG

Thesis Supervisor: Prof. Ming Yuen LEE

Chinese Medicinal Science

Angiogenesis plays an important role in a wide range of physiological processes and many diseases are associated with dysregulation of angiogenesis. *Radix Astragali*, a commonly used traditional Chinese medicine, also known as Huangqi in Chinese, is a potential candidate for treating angiogenesis dysregulation diseases. Calycosin, a major isoflavonoid isolated from *Radix Astragali* has been shown to possess angiogenic effect in previous studies but its underlying mechanism remains unclear. The present study investigates the angiogenic effects of calycosin *in* zebrafish embryo in vivo. *Tg(fli1:EGFP)* and *Tg(fli1:nEGFP)*transgenic zebrafish embryos were treated with different concentrations of calycosin (10, 30, 100 μM) from 72 hpf to 96 hpf prior morphological observation and angiogenesis phenotypes assessment, Calycosin was demonstrated to induce phenotypic changes and stimulate endothelial cells growth at subintestinal vessels (SIVs) in zebrafish embryo. Meanwhile, transcriptome profiling by deep sequencing suggested that calycosin modulated expression of genes involved in VEGF, FGF, Delta-Notch and Erbb signalling pathways, and these results were confirmed by qPCR and whole mount in situ hybridization. Importantly, the involvement of VEGF(R) and FGF(R) signaling pathways in calycosin induced angiogenic phenotype *in vivo* was further confirmed because the effects could be specifically blocked by a VEGF receptor kinase inhibitor and a FGF receptor kinase inhibitor (SU5402), individually.

Previous findings suggested that vascular endothelial growth factor and fibroblast growth factor are angiogenic molecules whose combined mitogenic activity and capability to activate PI3K-Akt signaling pathway is more potently synergistic. Interestingly, Mitogen-activated protein (MAP) kinases inhibitor (MEK1/2 Inhibitor) and blockers of PI 3-kinase catalytic activity (Wortmannin or LY294002) also could completely abrogate calycosin-induced angiogenesis actions. Taken together, this study revealed, for the first time, that calycosin is an active compound which can elicit angiogenic effect, involving the interaction between VEGF and FGF signaling pathways. The result should provide scientific clues to underlying mechanism of action of traditional use of Radix Astragali on promotion of "qi", a Chinese medicine philosophical concept.

Key words

calycosin, angiogenesis, zebrafish, VEGF/VEGFR, FGF/FGFR, EGF/EGFR, Notch, MAPK, Raf, MEK1/2, PI3-kinase, Src, transcriptome.

摘要

血管新生在很多生理過程中扮演著重要的角色，並且很多疾病發生與血管新生的失調有關。黃芪，作為一劑傳統中藥，在治療血管新生失調性疾病有著潛在的效果。毛蕊異黃酮，作為從黃芪中提取的主要的異黃酮成分，在我們以前的研究中發現其具有血管新生的效果，但是其中的分子機制依然不清楚。而我們當前研究既是探索毛蕊異黃酮在體內斑馬魚模型中的血管新生的效果。將轉基因斑馬魚胚胎 Tg(fli1:EGFP)和 Tg(fli1:nEGFP)在受精 72 小時後，用 10, 30, 100 μM 濃度的毛蕊異黃酮處理 24 小時後，對其形態的觀察以及血管新生的形態的評價中發現，毛蕊異黃酮能夠誘導斑馬魚胚胎腸下血管(SIV)的形態的變化及刺激內皮細胞生長。與此同時，通過深度測序的轉錄學分析發現，毛蕊異黃酮能夠調節 VEGF, FGF, Delta-Notch 和 Erbb 信號通路中一些基因的表達,而該一結果，被即時定量 PCR 和原位雜交技術再次證實。而關鍵的是，血管內皮細胞生長因數(VEGF)受體激酶的抑制劑以及成纖維細胞生長因數(FGF)受體激酶抑制劑都能特異性的抑制毛蕊異黃酮誘導的體內血管新生形態的發生，從而進一步證實了毛蕊異黃酮的促進血管新生的作用是通過 VEGF 和 FGF 信號通路而實現的。以前的研究發現，VEGF 和 FGF 作為血管新生分子都能夠通過有絲分裂活性和啟動 PI3K-AKT 信號通道的能力而發揮作用。有趣的是，有絲分裂啟動蛋白激酶抑制劑(MEK1/2 抑制劑)PI3K 酶活性抑制劑(Wortmannin 或者 LY294002) 都能抑制住毛蕊異黃酮誘導的血管新生的活動。所以，這個研究第一次發現了毛蕊異黃酮是一個促進血管新生的化合物，並且其促血管新生原理是通過啟動 VEGF 和 FGF 兩條信號通路實現的。這個結果也能提供一個科學的理解線索，關於中醫哲學上的黃芪“補氣”的概念。

關鍵字: 毛蕊異黃酮，血管新生，斑馬魚，血管內皮生長因數/受體，成纖維細胞生長因數/受體，表皮細胞生長因數/受體，有絲分裂啟動蛋白激酶

(MAPK)，Notch, MAPK, Raf, MEK1/2, PI3-kinase, Src, 轉錄組.

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LIST OF ABBREVIATIONS

%	Percent
℃	degree Celsius
Ang	Angiopoietins
AP	Alkaline phosphatase
bFGF	basic fibroblast growth factor
BrdU	bromodeoxyuridine
CAM	chorioallantoic membrane
CV	cranial vessel
DA	dorsal aorta
DAG	diacylglycerol
DBT	danggui buxue tang
DMSO	dimethyl sulfoxide
dpf	days post fertilization
ECGS	endothelial cell growth supplement
ECM	extracellular matrix
ECM	extracellular matrix
ECs	endothelial cells
ECs	endothelial cells
EDTA	Ethylenediaminetetraacetic acid
EGFP	enhanced green fluorescent protein
eNOS	endothelial nitric oxide synthase
ERKs	Extracellular signal-regulated kinases
FGF	fibroblast growth factor
FIGF	C-fos induced growth factor
FITC	fluorescein islthiocyanate filter
Flk	fetal liver kinase
Flk-1	fetal liver kinase-1

Flt-1	fms-like tyrosine kinase-1
Flt-1	fms-like tyrosine kinase
GFP	green fluorescent protein
HIF-1α	hypoxia-inducible factor 1α
HUVEC	Human umbilical vein endothelial cells
HUVECs	human umbilical vein endothelial cells
IGF-I	insulin-like growth factor-I
ISVs	intersomitic vessels
JNKs	c-Jun amino-terminal kinases
KDR	kinase domain region
KDR	kinase-domain region
MAPK	Mitogen-activated protein kinases
MEK	mitogen-activated protein kinase kinase
min	Minute
ml	Milliliter
mM	Millimole
MMPs	matrix metalloproteinases
MMPs	metalloproteinases
MTT	3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltrtrazolium bromide
MTT	3-(4, 5-dimethylthiazol-2-yl)-2, 5 -diphenyltetrazolium bromide
nM	Nanomole
NO	nitric oxide
OD	Optical density
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PCV	posterior cardinal vein
PDGF	platelet-derived growth factor
PI	propidium iodide
PI	propidium iodide

PI3K	phosphatidylinositol-3 kinase
PlGF	Placenta growth factor
PS	penicillin-streptomycin
PTU	N-Phenylthiourea
RA	Radix Astragali
RAE	Radix Astragali Extrat
Res	Resveratrol
RT	reverse transcription
RTKs	receptors tyrosine kinases
S1P	sphingosine-1-phosphate
SIV	subintestinal vessel
TCM	traditional Chinese medicine
UEA-I	Ulex europeus agglutinin I
UV	Ultraviolet
VEGF	vascular endothelial growth factor
VEGF	vascular endothelial growth factor
VSMC	vascular smooth muscle cell
β-ME	2-mercaptoethano
μg	Microgram
μM	Micromole