doi: 10.13241/j.cnki.pmb.2014.11.005

Effect of Ursolic Acid on NQO1 Expression in HUVECs Stimulated by ox-LDL*

SU Yuan-zhen[1], WANG Chun-bo[2], XIN Hui[1△], ZHANG Rui[1], TIAN Jia-wei[3], ZHANG Jin-song[1], YAO Ru-yong[4]

(1 Department of Cardiology, Affiliated Hospital of Qingdao University Medical College, Qingdao, Shandong, 266003,China;

2 Education research of pharmacology, Qingdao University Medical College, Qingdao, Shandong, 266071, China;

3 Department of Emergency, Affiliated Hospital of Qingdao University Medical College, Qingdao, Shandong, 266003, China;

4 The central laboratory, Affiliated Hospital of Qingdao University Medical College, Qingdao 266071, China)

ABSTRACT Objective: To investigate the effects of Ursolic acid on NQO1 (Quinone Oxidoreductase1, NQO1)incultured umbilical vein endothelial cells (HUVECs) stimulated by ox-LDL, so as to approach the mechanism of UA in atherosclerosis. **Methods:** HUVECs were cultured in different concentration and divided into four groups (n=5,each): Group Ⅰ, control group without; Group Ⅱ, HUVECs stimulated with ox-LDL (20 mg/L) in endothelial basal medium for 24 hours; Group Ⅲ, HUVECs treated with ox- LDL(20m g/L) for an half hours, and treated with ox- LDL (20 mg/L) and UA (1.5 μmol/L) in endothelial basal medium for 24 hours; Group Ⅳ, HUVECs treated with ox- LDL(20 mg/L) for an half hours, and treated with ox- LDL (20 mg/L) and UA (4.5μmol/L) in endothelial basal medium for 24 hours. MTT assay was used to determine against HUVECs injury. Expression of NQO1mRNA was determined by RT-PCR. Expression of NQO1 protein was determined by Western blot. **Results:** Ursolic acid decreased the effect on reducing the cytotoxicity of ox- LDL. Expression of NQO1mRNA was significantly highter in group Ⅱ (0.624± 0.009)than that in group Ⅰ (0.521± 0.007), P<0.01. Ursolic acid dose-dependenly increased expression of NQO1 mRNA (Group Ⅲ vs Group Ⅱ: 0.722± 0.058 vs 0.624± 0.009, P<0.01; Group Ⅳ vs Group Ⅱ: 0.826± 0.059 vs 0.624± 0.009, P<0.01). Expression of NQO1 Protein was significantly highter in group Ⅱ (0.574± 0.024)than that in group Ⅰ (0.438± 0.039), P<0.01. Ursolic acid dose-dependenly increased expression of NQO1 Protein(Group Ⅲ vs Group Ⅱ: 0.710± 0.058 vs 0.574± 0.024, P<0.01; Group Ⅳ vs Group Ⅱ: 0.831± 0.034 vs 0.574± 0.024, P<0.01). **Conclusion:** NQO1 expression in ox-LDL- treated HUVECs could be increased by UA which suggests that UA may attenuate atherosclerosis by reducing ox-LDL-induced oxidative stress responses.

Key words: Ursolic acid; Ox- LDL; NQO1; Oxidative stress; Atherosclerosis

Chinese Library Classification (CLC): 282.71, R543 Document code: A

Article ID: 1673-6273(2014)11-2019-05

Introduction

Atherosclerosis is one of the most common and important of atherosclerotic Vascular disease. It is characterized by thickening of the arterial wall harden, loss of elasticity and lumen narrowing. There is appearance on the accumulation of lipid in the arterial intima yellow atherosclerosis, so called atherosclerosis. It mainly involves large and medium-sized artery and its clinical manifestations are mainly thesymptoms of the affected organs [1]. With the development of social economy and people life style changing, heart cerebrovascular accident[2,3]caused by atherosclerosis has become a major killer of human health. The incidence of the disease and mortality in the western developed countries are various diseases, and there is an upward trend in the developing countries including China. Atherosclerosis has already caused the attention of the countries of the world. Vascular endothelial cell injury and lipid deposition has become a recognized atherosclerosis dynamic factors [4]. Today, there are numerous of drugs treatment of atherosclerosis, such as lipidemicmodulating, antiplatelet drugs , thrombolytic and anticoagulants etc. These drugs brought a lot of benefits to many patients in a large number of clinical applications. Along with the application of these drugs, however, its disadvantages are gradually revealed: first of all, patients need long-term use of these drugs,then social and family to bring huge economic pressure. Secondly, more or less doses of he drug atherosclerosis in patients will cause side effect. In recent years, China has accelerated the pace of research for the treatment of atherosclerosis with Chinese herbs. Ursolic acid is a kind of triterpene compounds present in the plant, is one of the main effective ingredients of Chinese herbs [5,6], and has the pharmacological effects of resistance to atherosclerosis [7,8]. It has a wide range of sources, and less adverse reaction, and that conducive to the popularization and application[9-12].

*Foundation item : Natural Science Foundation of China (81173593)

Author introduction:SU Yuan-zhen,(1985-), male, master, Mainly engage in Cardiovascular disease research,Tel:13589242993, Email:yuanzhenhaoren@126.com

△Corresponding author: XIN Hui(1967-),Email:xhqy2002@163.com

(Received: 2013-11-19 Accepted: 2013-12-18)

In the past, there are many researchs on Ursolic acid treatment of tumor [13-16]. However, there is less research for ursolic acid resistance atherosclerosis, plays an important role in protecting cells against oxidative stress injury [17]. This study was to investigate the effect of ursolic acid on NQO1 stimulated by ox-LDL, so as to approach the mechanism of UA in atherosclerosis.

1 MATERIALS AND METHODS

1.1 Materials and reagents

HUVECs were provided by the Department of ATCC. DMEM medium was purchased from Gibco Company (USA). Fetal calf serum was purchased from Hyclone Company(USA)). Ursolic acid was obtained from Sigma-Aldrich Company(USA). Human High Ox-LDL was purchased from Yiyuan biotechnology Company (China). Primer was obtained from Sangon Biotech Company (China). PrimeScript RT-PCR Kit was purchased from-TaKaRa Bio Group. Goat Anti-Mouse IgG,HRR conjugated and Anti β-actin Mouse Monoclonal Antibody was purchased from Cowin Bioscience (China). Anti-NQO1antibody was purchased from Abacam.

1.2 Cells culture

HUVECs were maintained at 37℃ in a 5% CO_2 humid incubator and cultured in DMEM medium (Gibco,USA) and supplemented with 10% of fetal bovine serum. HUVECs were cultured in different concentration and divided into four groups(n=5,each): GroupⅠ,control group without ; GroupⅡ, HUVECs stimulated with ox-LDL (20 mg/L)in endothelial basal medium for 24 hours; GroupⅢ, HUVECs treated with ox- LDL (20 mg/L) for an half hours, then treated with ox- LDL (20 mg/L) and UA (1.5 μmlo/L) in endothelial basal medium for 24 hours ; GroupⅣ, HUVECs treated with ox- LDL(20 mg/L) for an half hours, then treated with ox- LDL (20 mg/L) and UA (4.5 μmlo/L) in endothelial basal medium for 24 hours.

1.3 MTT assay

The assay was performed by seeding HUVECs at a density of 5× 10^3 cells/well in 96-well plates. After attaching to plates, cells were treated with various concentrations for 24 h. In order to examine the effect of Ursolic acid on ox-LDL-induced cell damage, cells were treated with Ursolic acid for an half hour before the addition of ox-LDL. MTT solution (5 mg/ml, 20 μL)was added to each well at 37 ℃ for 4 h, then the supernatant was removed and discarded. The formazan crystals were dissolved in 150 μL of DMSO followed by measurement of absorbance at 490 nm using an enzyme- linked immunosorbent reader. The experiment was repeated five times.

1.4 Reverse transcriptase polymerase chain reaction (RT-PCR)

Total RNA was isolated from cells using Trizol-reagent and quantified by UV absorption at 260 and 280 nm. RT-PCR was performed according to the manufacturer,s instructions. The primer pairs of NQO1 were forward 5'-CTGATCGTACTGGCT-CACTC-3' and reverse 5'-GAACAGACTCG-GCAGGATAC -3,; GADPH, 5'-GGATTTGGTCGTATTGGG3' and reverse5'-GGAA-GATGGTGATGGGATTT-3'. PCR conditions were as follows: 32 cycles of 95 ℃ for 30 s;52 ℃ for 30 s; and 72 ℃ for 1min. Amplified products were visualized on 1.5 % agarose gel electrophoresis, stained with ethidium bromide,and photographeunder ultraviolet light. Densitometric analysis of five different observations was performed Quantity One Software. The quantity of each transcript was normalized to that of GAPDH.

1.5 Western blot analysis

After treatment,total proteins were isolated by RIPA buffer, which consisted of 50 mM Tris-Cl (pH 7.6), 150 mM NaCl, 0.2 mM PMSF, 1 % (m/v) NP-40, 0.1 mM NaF, 1.0 mM EDTA and 1.0 mM DTT. The lysates were centrifuged at 12,000 rpm for 10 min at 4 ℃, the supernatant was collected. Polyacrylamide gel was made up. Each experiment hole was full of 10 μLsample. NQO1 was separated on SDS-polyacrylamide gels and transferred into polyvinylidene difluoride (PVDF) membranes. The blotswere incubated with a blocking buffer (1× PBS and 5 % nonfat dry milk) for 1 h at room temperature and detected with primary antibodies at 4 ℃ overnight, followed by the second antibody for 1 h at room temperature. To control equal loading of total protein in all lanes, blots were stained with Antiβ-actin Mouse Monoclonal Antibody for equal loading of proteins. Protein bands were visualized using an enhanced chemiluminescence system.

1.6 Statistical analysis

All experiment were performed five times. All data were presented as mean± standard deviation (SD) unless otherwise described. Statistical analyses were performed with SPSS statistical software (version 17.0).The statistical differences were determined by analysis of variance.Values of $P<0.05$ were considered to be statistically significant.

2 RESULTS

2.1 Ursolic acid attenuates the MTT reduction of HUVECs induced by ox-LDL

Ox - LDL group cell absorbance value (0.445± 0.051)was lower than that in the control group (1.000± 0.000),$P<0.01$; Ox - LDL + UA group cell absorbance value was higher than the ox - LDL group;$P<0.01$; ox - LDL + High concentration UA group (0.760± 0.060)cell absorbance value was higher than that in the ox - LDL + Low concentration UA group (0.616± 0.048); Between the four groups were considered to be statistically significant. $F=130.727$, $P<0.01$.Fig 1, table 1.

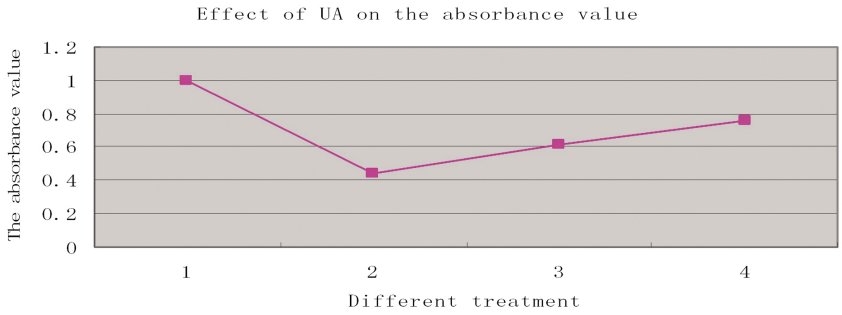

Fig. 1 Effect of UA on the absorbance value of HUVECs1.control group; 2.ox-LDL(20 mg/L)group ; 3.ox-LDL(20 mg/L)+ Low concentration UA(1.5 μmlo/L) group; 4.ox-LDL(20 mg/L)+High concentration UA(4.5 μmlo/L) group.Compared with control group, P<0.01;Compared with ox-LDL group, P<0.01(n=5)

Table 1 Effect of UA on the absorbance value of HUVECs($\bar{x}\pm s$,n=5)

group	cell absorbance value
1	1.000± 0.003
2	0.445± 0.051
3	0.616± 0.048
4	0.760± 0.060

1.control group; 2.ox-LDL(20 mg/L)group; 3.ox-LDL(20 mg/L)+ Low concentration UA(1.5 μmlo/L) group; 4.ox-LDL(20 mg/L)+ High concentration UA(4.5 μmlo/L) group.Compared with control group, P<0.01; Compared with ox-LDL group, P<0.01(n=5)

2.2 Effect of Ursolic acid on Expression of NQO1mRNA

The expression of NQO1mRNA in Ox - LDL group(0.624± 0.009) was higher than that in the control group (0.521± 0.007), P<0.01; The expression of NQO1 mRNA in the Ox - LDL + UA group was higher than that in the ox - LDL group, P<0.01;The expression of NQO1mRNA in ox - LDL + High concentration UA group (0.826± 0.059)was higher than that in ox - LDL + Low concentration UA group (0.722± 0.058); There was statistically significant btween the four groups . F=49.190,P<0.01. Fig 2, table 2.

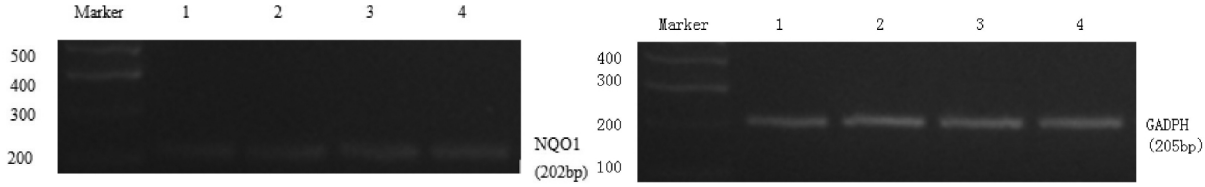

Fig. 2 Effect of Ursolic acid on Expression of NQO1mRNA

Table 2 Effect of UA on Expression of NQO1mRNA($\bar{x}\pm s$,n=5)

group	cell absorbance value
1	0.521± 0.007
2	0.624± 0.009
3	0.722± 0.058
4	0.826± 0.059

1.control group; 2.ox-LDL(20 mg/L)group; 3.ox-LDL(20 mg/L)+ Low concentration UA(1.5 μmlo/L) group; 4.ox-LDL(20 mg/L)+ High concentration UA(4.5 μmlo/L) group.Compared with control group, P<0.01;Compared with ox-LDL group, P<0.01(n=5)

2.3 Effect of Ursolic acid on Expression of NQO1 protein

The expression of NQO1 protein in Ox - LDL group(0.574± 0.024)was higher than that in the control group (0.438± 0.039), P<0.01; The expression of NQO1 protein in Ox - LDL + UA group was higher than that in the ox - LDL group, P<0.01;The expression of NQO1 protein in ox - LDL + High concentration UA group (0.831± 0.034)was higher than that in ox - LDL + Low concentration UA group (0.710± 0.584); There was statistically significant between the four groups. F=86.814,P<0.01. Fig 3, Table 3.

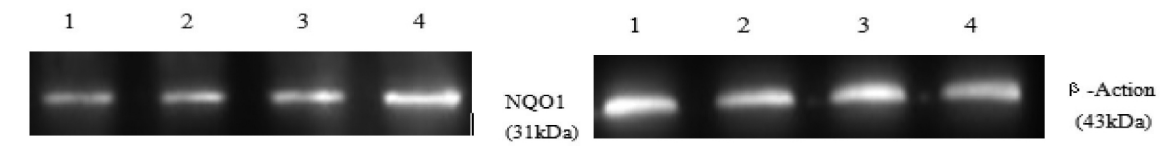

Fig. 3 Effect of Ursolic acid on Expression of NQO1 protein

Table 3 Effect of UA on Expression of NQO1 protein($\bar{x}\pm s$,n=5)

group	cell absorbance value
1	0.438± 0.039
2	0.574± 0.024
3	0.710± 0.058
4	0.831± 0.034

1.control group; 2.ox-LDL(20 mg/L)group; 3.ox-LDL(20 mg/L)+ Low concentration UA(1.5 μmlo/L) group; 4.ox-LDL(20 mg/L)+ High concentration UA(4.5 μmlo/L) group.Compared with control group, P<0.01; Compared with ox-LDL group, P<0.01(n=5)

3 DISCUSSION

NQO1(Quinone Oxidoreductase1，NQO1) is a flavin protease widespread in most eukaryotic cells. It belongs to phase Ⅱ detoxifying enzymes,and eases many toxicities of natural and synthetic [18]. It is a kind of inducible reductase. The expression of NQO1 protein can be induced by many of the external environment factors,such as Oxidative stress , Polycyclic aromatic hydrocarbons and Azo fuel etc [19]. NQO1 enzyme molecules that exists in the form of dimers in the human body. It is widely distributed in the cell cytoplasm, microsome, mitochondria, golgi apparatus [20]. There are two kinds of way reduction of quinones substances in the body. One way is that it is changed into the active half quinine by phase Ⅰ enzyme reduction, further damages cells through producing large amounts of reactive oxygen species and free radicals. Another way is that It is changed into stable hydroquinone by phase Ⅱ detoxifying enzyme, and the majority of hydroquinone can be excreted in a cell. It plays a role through the second way. MTT texts show that ox-LDL group cells was lower than the control group, P<0.01; Ursolic acid dose-dependenly increased cells, P<0.01. It can be confirmed that Ursolic acid inhibits human umbilical vein endothelial cells oxidative damage induced by ox -LDL.RT-PCR results show that ox-LDL group （0.624± 0.009） raised the expression of of NQO1mRNA than control group （0.521± 0.007）. In the intervention of ox-LDL, human umbilical vein endothelial cells can against oxidative stress damage by improving the NQO1mRNA expression. But this itself regulation mechanism still cannot completely against oxidation caused by the ox-LDL. Ursolic acid dose-dependenly increased expression of NQO1 mRNA (GroupⅢ vs GroupⅡ：0.722± 0.058 vs 0.624± 0.009, P<0.01; GroupⅣ vs GroupⅡ：0.826± 0.059 vs 0.624± 0.009, P<0.01). Western blot results also show that ox-LDL group （0.574± 0.024）raised the expression of of NQO1 protein than control group （0.438± 0.039）. Ursolic acid dose-dependenly increased expression of NQO1 Protein (GroupⅢ vs GroupⅡ：0.710± 0.584 vs 0.574± 0.024, P<0.01; GroupⅣ vs GroupⅡ：0.831± 0.034 vs 0.574± 0.024, P<0.01). In conclusion, expression of the NQO1 in ox-LDL- treated HUVECs could be further increased by UA. It suggests UA may attenuate atherosclerosis by reducing ox-LDL-induced oxidative stress responses. This experiment provides a certain theoretical basis that Ursolic acid inhibits atherosclerosis.

References

[1] Moghadasian MH.Experimental atherosclerosis:a historical overview [J]. Life Sci,2002, 70(8):855-865

[2] Williams JK, Sukhova GK, Herrington DM, et a1. Pravastatin has cholesterol lowering independent effects on the artery wall of atherosclerotic monkeys[J]. J Am Coll Cardiol, 1998, 31(3):684-691

[3] Danesh J, Wheeler JG, Hirschfield GM, et a1. C-reactive protein and other circulating markers of inflammation in the prediction of coronary heart disease[J]. N Engl J Med, 2004, 350(14):1387-1397

[4] Somova LO, Nadar A, Riunnlansn P, et a1. Cardiovascular, antihypeflipidemie and antioxidant effects of oleanolic acids and ursolic acids in experimental hypertension [J]. Phytomedieine, 2003, 10(2-3):115-121

[5] Zhu Yun, Fan Xiao-hong, Zhao Wen-bin, et al. Determination of ursolic acid in Cynomorumsongaricum Ruprby spectrophotometry[J]. ANHUI MEDICAL AND PHARMACEUTICA-LJOURNAL, 2009, 13(11):1009- 6469

[6] Li Guo-Zhang, Yu Hua-zhong, Bu Xiao-Ying, et al. Determination of Ursolic Acid in Ilex Kudingcha C. J. Tseng by Spectrophotometry[J]. CHINESE JOURNAL OF SPECTROSCOPY, SPECTROSCOPY, 2006, 23(2):10 04-8138

[7] Saraswat B, Visen PK, Agrwal DP. Ursolic acid isolatedfrom Eucalyptus tereacornis protects ethanol toxicity in isolated rat hepatoeytes[J]. phytother Res, 2000, 14(3):163-166

[8] Yu Y X, Gu Z L, Yin J L, et a1. Ursolic acid induces humanhepatoma cell line SMMC-7721 apoptosis via p53·dependentpathway [J]. Chin Med J(Ensl), 2010, 123(14):1915-1923

[9] Kowalczyk M C, Walaszek Z, Kowalczyk P, et a1. Differential effects of several phytochemicals and their deri-vativeson routine kemtinocytes invitro and in vivo: implications for skin cancer prevetion[J]. Carcinogenesis, 2009, 30(6):1008-1015

[10] Saravanan R, Pugalendi V. Impact of Ursolic acid on chronic ethanol-induced oxidativb stress in the rat heart [J]. PhannacolRep, 2006, 58(1):41-47

[11] Ali M S, IbraIlim S A, Jalil S, et a1. Ursotic acid: a potent inhibitor of mperoxides produced in the cellular system [J]. PIIytother Res, 2007, 21(6):558-61

[12] Pozo M, Castilla V, Gutierrez, et al. Ursolic acid inhibits neointima formation in the rat carotid artery injury model [J]. Atherosclerosis, 2006, 184(1):53-62

[13] Tian S, Shi Y, Yu Q, et al. Determination of oleanolic acid and ursolic acid contents in Ziziphora clinopodioides Lam by HPLC method[J]. Pharmacogaa Mug, 2010, 6(22):116-119

[14] Zhang T, He Y M, Wang J S, et a1. Ursolic acid inducesHI.60 monocyfic differentiation and upregulates C/EBPpexpression by ERK pathway activation[J]. Anticancer Drugs, 2011, 22(2):158-165

[15] Jung T Y, Pham T N, Umeyama A, et a1. Ursolic acid isolated from

Unearia rhynchophytla activates human dendriticcells via TLR2 and/or TLR4 and induces the production of IFN-gamma by CD+4; naive T cells[J]. Eur J Pharmacol, 2010, 643(3):297-303

[16] Lee J H, Lee D U, Jeong C S. Gardenia jasminoides Ellisethanol extract and its constituents reduce the risks of gastritisandr;evefse gastric lessions in rats[J]. Food ChemToxicol, 2009, 47(6):1127-1131

[17] Siegel D, Ross D. Immunodetection of NAD (P)H: quinone oxidoreductase(NQO1)in human tissues[J]. Fre Rad Biol Med, 2000, 29(3-4):246-253

[18] Dai NY, Lu Z X. NQO1 and tumor [J]. Foreign medical: genetics booklet, 2003, 26(3):141-144

[19] Smith M T, Benzene. NQOl and genetic susceptibility to cancer[J]. Proc Natl Acad Sci USA, 1999, 96(14): 7624-7626

[20] Jaiswal A K, McBride O W, Adesnik M, et al. Human dioxininducible cytosolic NAD (P)H: menadione oxidoreductase. cDNA sequence and localization of gene to chromosome 16[J]. J Biol Chem, 1988, 263(27):13572

熊果酸对 ox-LDL 诱导的人脐静脉血管内皮细胞 NQO1 表达的影响 *

苏元镇[1] 王春波[2] 辛 辉[1△] 张 蕊[1] 田嘉伟[3] 张进松[1] 姚如永[4]

（1 青岛大学医学院附属医院心内科 山东 青岛 266003；2 青岛大学医学院药理学教研室 山东 青岛 266071；
3 青岛大学医学院附属医院急诊科 山东 青岛 266003；4 青岛大学医学院附属医院中心实验室 山东 青岛 266071）

摘要 目的：研究熊果酸对经氧化性低密度脂蛋白（ox-LDL）干预后人脐静脉血管内皮细胞（human umbilical vein endothelial cells, HUVECs）醌还原氧化酶 1 表达的影响，以进一步探讨熊果酸抗动脉粥样硬化的机制。**方法：**体外培养人脐静脉内皮细胞，进行分组处理，每组 n=5。对照组，不加任何处理；ox-LDL 组，加入 ox-LDL 培养 24h，终浓度为 20mg/L；ox-LDL+ 低浓度熊果酸组，先加入 ox-LDL（浓度 20mg/L）孕育半小时，然后与熊果酸（浓度 1.5μmlo/L）共同培养 24h；ox-LDL+ 高浓度熊果酸组，先加入 ox-LDL（浓度 20mg/L）孕育半小时，然后与熊果酸（浓度 4.5μmlo/L）共同培养 24h；采用 MTT 试验测定细胞吸光度值，检测熊果酸对 ox-LDL 损伤的保护作用，采用 RT-PCR 法检测 NQO1mRNA 的表达，采用 Western blot 法检测 NQO1 蛋白的表达。**结果：**熊果酸减弱 ox-LDL 对 HUVECs 的损伤作用；ox-LDL 组 NQO1mRNA 的表达量(0.624± 0.009)明显高于对照组(0.521± 0.007)，P<0.01。熊果酸呈浓度依赖性的提高 NQO1mRNA 的表达量（ox-LDL+ 低浓度熊果酸组 vs ox-LDL 组：0.722± 0.058 vs 0.624± 0.009, P<0.01；ox-LDL+ 高浓度熊果酸组 vs ox-LDL 组：0.826± 0.059 vs 0.624± 0.009, P<0.01）。ox-LDL 组 NQO1 蛋白的表达量 (0.624± 0.009)明显高于对照组(0.521± 0.007)，P<0.01。熊果酸呈浓度依赖性的提高 NQO1 蛋白的表达量（ox-LDL+ 低浓度熊果酸组 vs ox-LDL 组：0.710± 0.058 vs 0.574± 0.024, P<0.01；ox-LDL+ 高浓度熊果酸组 vs ox-LDL 组：0.831± 0.034 vs 0.574± 0.024, P<0.01）。**结论：**熊果酸可上调 ox-LDL 诱导的人脐静脉血管内皮细胞 NQO1 的表达，表明其可能具有抗氧化应激及抗动脉粥样硬化的作用。

关键词：熊果酸；氧化性低密度脂蛋白；醌还原氧化酶 1；氧化应激；动脉粥样硬化

中图分类号：282.71，R543 **文献标识码：**A **文章编号：**1673-6273(2014)11-2019-05

* 基金项目：国家自然科学基金项目（81173593）

作者简介：苏元镇（1985-），男，硕士，主要从事心血管病方面的研究，电话：13589242993，E-mail:yuanzhenhaoren@126.com

△通讯作者：辛辉，E-mail: xhqy2002@163.com

（收稿日期：2013-11-19 接受日期：2013-12-18）