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Regulation of protein expression by L-arginine in endothelial cells

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1. ABSTRACT

L-Arginine is a conditionally essential amino acid for humans and plays an important role in the regulation of cardiovascular function and antioxidative defense. Previous studies have focused on the important role of L-arginine as a physiological precursor in the generation of nitric oxide and polyamines in endothelial cells (cells that line the interior surface of blood vessels). Because of the rapid development of high-throughput proteomics technology, there is now growing interest in studying roles for L-arginine in modulating endothelial-cell protein expression. Of particular interest, recent proteomics analysis has shown that treatment of coronary venular endothelial cells with a physiological level of L-arginine (e.g., 0.1 mM) increases expression of structural proteins (vimentin and tropomyosin) and cytochrome bc_1 complex iii-chain A, while decreasing expression of stress-related proteins (PDZ domain containing-3), in these cells. These findings aid in elucidating the mechanisms responsible for the beneficial effect of physiological levels of L-arginine on the circulatory system.

2. INTRODUCTION

Most mammals (including humans, pigs, sheep, and rats) can synthesize L-arginine from glutamine, glutamate, and proline via pyrroline-5-carboxylate as an essential intermediate (1). However, compelling evidence shows that L-arginine is a conditionally essential amino acid for humans and pigs, depending on developmental stages and disease states (1). This nutrient serves as the nitrogenous precursor for synthesis of nitric oxide (NO; a major vasodilator), polyamines (key regulators of DNA and protein synthesis), proline (a major constituent of extracellular matrix protein), creatine (an antioxidant and a crucial element of energy metabolism in muscle and the central nervous system), and proteins (2-4). L-Arginine plays an important role in the regulation of angiogenesis (growth of new vessels from the existing ones) and cardiovascular function (5). This beneficial effect of arginine holds great promise in enhancing wound healing, improving microcirculatory function, promoting maternal and fetal development, and treating various vascular disorders (including hypertension and atherosclerosis) (6-10).

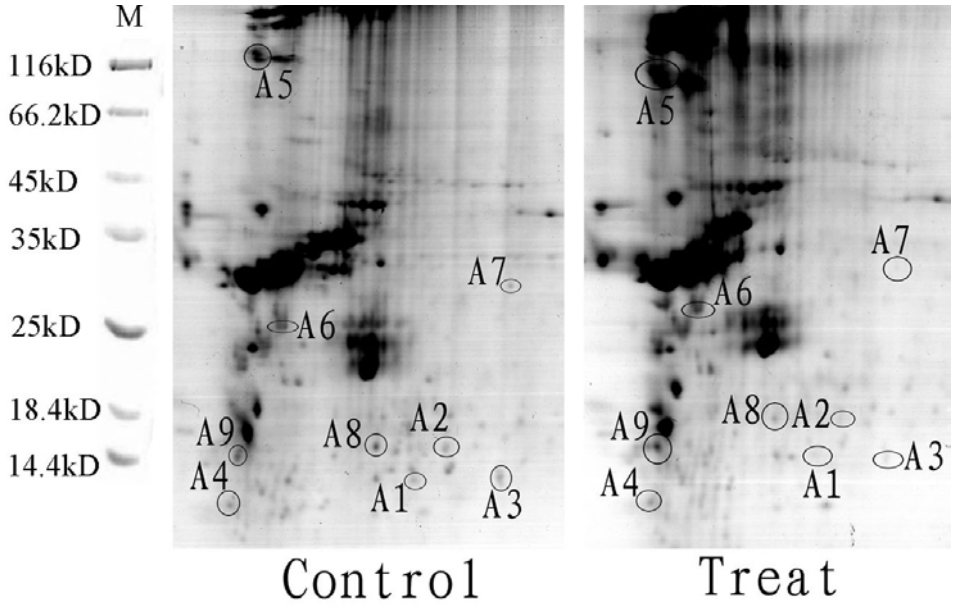

Figure 1. Locations and abundances of nine differentially expressed protein spots in CVEC supplemented with arginine. A1-A6: vimentin; A7: PDZ domain containing 3; A8: cytochrome bc_1 complex iii (EC 1.10.2.2), chain A; A9: tropomyosin, cytoskeletal type. CVEC were isolated from coronary venules 15 μm in diameter (44) and were cultured in Dulbecco's modified Eagle's medium (45). To study the effect of arginine on the proteome in CVEC, cells were cultured at 37°C in an arginine-free basal DMEM supplemented with 10% FBS, which provided 15 μM arginine in the medium (control). To study the effect of arginine on the proteome of EC, this basal culture medium was supplemented with L-arginine to achieve a final concentration of 0.1 mM (treatment [treat]), which is a physiological level in human plasma (46). At the end of 48-h culture, cells were collected for the extraction and analysis of proteins and RNA. Cell protein was extracted (47) and samples were analyzed in triplicate using 2D-PAGE (47, 48). Peptide mass fingerprinting (PMF) was generated on a Bruker ReflexTM III MALDI-TOF mass spectrometer (Bruker Daltonik GmbH, Bremen, Germany) working in the reflectron mode with 20 kV of accelerating voltage and 23 kV of reflecting voltage. Mass accuracy for PMF analysis was 0.1-0.2 Da with external calibration, and internal calibration was carried out using enzyme autolysis peaks; resolution was 12,000. The PeakClean software (www.proteomics.com.cn/tools/PkClean/) was used to remove contaminant peaks, including matrix peaks, solvent peaks, and enzyme autolysis peaks. PMF matching was performed using the MASCOT search engine (http://mascot.proteomics.com.cn/search_form_PMF.html) (37).

Physiological levels of NO have potent antioxidant and anti-atherosclerotic actions (5). Previous biochemical studies have focused on the role of L-arginine in the generation of NO and polyamines (11, 12), as well as oxidative defense (13) in endothelial cells. Available evidence shows that increasing extracellular levels of arginine drives endothelial production of NO (14), which then relaxes smooth muscle cells and stimulates blood flow. In humans and experimental animal models, an increase in blood flow causes fluid shear stress, which is a major determinant of arterial tone and vascular remodeling (5, 15). Thus, it is possible that arginine coordinately modulates both metabolic pathways and structure in the vascular system through alterations in protein profiles. However, at present, little is known about the physiological effect of arginine on changes in the proteome of endothelial cells.

The rapid development of high-throughput proteomics technologies in recent years has facilitated the simultaneous analysis of thousands of proteins in cells or tissues, therefore providing a useful tool for discovery research in cardiovascular research (5, 16-19). The major objective of this article is to highlight recent advances in effects of L-arginine on the proteome of endothelial cells. The new knowledge will help define the molecular and cellular mechanisms whereby L-arginine exerts beneficial effects on the circulatory system.

3. COMPARATIVE PROTEOME ANALYSIS OF ENDOTHELIAL CELLS EXPOSED TO ARGININE

Coronary venular endothelial cells (CVEC) have been used to study mechanisms that regulate angiogenesis (20). A representative of the proteome in these cells, which is based on the 2-dimentional polyacrylamide gel electrophoresis (2D-PAGE) technique and matrix-assisted laser desorption ionization (MALDI) mass spectrometry, is shown in Figure 1. Addition of 0.1 mM L-arginine (a physiological level of arginine in human plasma) to culture medium containing 0.015 mM L-arginine differentially affects the levels of nine protein spots in 2D gels (Figure 1). Note that an arginine concentration of 0.015 mM was present in plasma of neonates or adults with vascular dysfunction (21) and is generally considered to be deficient for mammals (22, 23).

Biochemical properties of the proteins which exhibited changes in response to arginine treatment are

Table 1. Biochemical properties of differentially expressed proteins in arginine-treated CVEC

No.	Accession	Name	MW (KD)	pI	Observed MW (KD)	Observed pI	Relative change in expression in response to 0.1 mmol/L arginine vs. Control	Sequence coverage (%)
A1	Q17QM7	Vimentin	53.695	5.06	13.500	6.00	-1.50	35
A2	Q17QM7	Vimentin	53.695	5.06	14.500	6.50	-3.13	20
A3	Q17QM7	Vimentin	53.695	5.06	13.000	9.00	-1.60	30
A4	Q17QM7	Vimentin	53.695	5.06	11.000	4.00	+2.71	38
A5	Q17QM7	Vimentin	53.695	5.06	110.000	4.90	+2.05	64
A6	Q17QM7	Vimentin	53.695	5.06	25.000	5.00	+3.13	54
A7	Q3MHE0	PDZ domain containing 3	54.548	6.10	30.000	9.00	-1.87	19
A8	1BE3A	Cytochrome bc1 complex iii, chain A	49.181	5.46	15.000	5.50	+1.80	27
A9	Q29219	Tropomyosin, cytoskeletal type	12.763	4.79	14.300	4.50	+1.21	54

CVEC were cultured for 48 h in DMEM containing 0.015 or 0.1 mM L-arginine. At the end of the culture period, cells were collected for analysis of the proteome. The ratios of protein levels in cells treated with 0.1 mM arginine to those in cells cultured with 0.015 mM arginine were calculated. The four proteins indicated in the table were differentially expressed ($P < 0.05$). The signs (-) and (+) indicate a decrease and increase, respectively, compared with the value for the control.

summarized in Table 1. Interestingly, vimentin and tropomyosin are up-regulated in response to treatment with 0.1 mM L-arginine, whereas the opposite result is obtained for PDZ domain-containing protein-3. Furthermore, concentrations of cytochrome bc1 complex 3-chain A are increased in cells cultured containing 0.1 mM L-arginine, in comparison with cell cultures with 0.015 mM L-arginine (Figure 1). It is necessary that results of proteomic analysis be verified using both quantitative RT-PCR and western blot. This can be achieved by randomly selecting one or more of the differentially expressed proteins. The example shown is mRNA and protein levels for vimentin in CVEC treated with 0.015 or 0.1 mM L-arginine (Figure 2).

Although much attention has been focused on arginine metabolism and the regulatory role of NO in cell metabolism (17, 24, 25), little is known about the effect of this amino acid on expression of proteins in vascular endothelial cells. Thus, the finding that physiological levels of arginine affect the abundance of four proteins that are related to cell structure and modulation of oxidative response is novel and important. In particular, concentrations of 2 structural proteins (vimentin and tropomyosin) and cytochrome bc_1 complex iii- chain A related to oxidative phosphorylation are increased but the concentration of a signaling protein (PDZ domain containing-protein 3) is decreased in CVEC cultured with 0.1 mM L-arginine. These results provide the first description of changes in the proteome of endothelial cells in response to supplementation of arginine to an arginine-deficient medium and also help elucidate the mechanisms for the beneficial effect of arginine on improving cardiovascular function.

Vimentin is a member of the intermediate filament family of proteins, which plays an important structural role in eukaryotic cells (26). These proteins, along with actin microfilaments and microtubules, constitute the cytoskeleton. Vimentin is expressed abundantly in mesenchyme-derived cells, including endothelial and vascular smooth muscle cells, where this filament primarily functions as an intracellular scaffold to support cell strength and tissue integrity (27). There is evidence that vimentin regulates the migration and proliferation of endothelial cells (28, 29). In addition, vimentin plays a key role in the modulation of vascular tone, possibly by modulating NO synthesis in endothelial cells (30). However, the underlying mechanism is unknown. It is possible that vimentin interacts with caveolin-bound eNOS on the plasma membrane, thereby affecting eNOS phosphorylation and activity. In support of this view, vimentin is readily phosphorylated by different protein kinases (31). Interestingly, six protein spots corresponding to vimentin were differentially expressed in CVEC in response to a physiological concentration of arginine (Table 1 and Figure 1). Also, mRNA levels for vimentin were enhanced in arginine-treated cells (Figure 2). Therefore, we suggest that physiological levels of arginine are necessary for optimal expression of the vimentin gene and protein in endothelial cells. This may be associated with augmented production of NO [an angiogenic factor (32)] and enhanced CVEC proliferation (11).

Tropomyosin is another structural protein whose levels were increased in CVEC in response to supplementation of arginine to an arginine-deficient medium. Tropomyosin is a major microfilament-associated and actin-binding protein in endothelial cells (33). This protein is crucial for maintaining hemodynamic shear stress responses and thus for regulating vessel wall function (34). Interestingly, the synthesis of tropomyosin is stimulated at the post-transcriptional level in proliferating endothelial cells (33). Conversely, reduced concentration of tropomyosin is associated with impaired angiogenesis in blood vessels (35). These results indicate an important role for arginine in regulating the synthesis of cytoskeleton-associated proteins in endothelial cells, as recently reported for global proteins in skeletal muscle cells (36, 37) and mammary gland (38).

L-Arginine is known to improve mitochondrial function in many cell types (39). Interestingly, supplementing arginine to an arginine-deficient culture

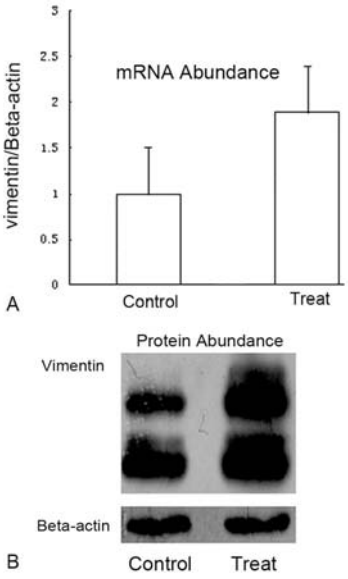

Figure 2. Real-time PCR (Figure 2A) and western blotting (Figure 2B) analysis of vimentin in endothelial cells. Data are means ± SEM, n = 6. mRNA or protein levels for vimentin differ ($P < 0.05$) between control (0.015 mM L-arginine) and arginine (treat; 0.1 mM L-arginine) groups, as analyzed by t-test. Coronary venular endothelial cells were cultured for 48 h in DMEM containing 0.015 or 0.1 mM L-arginine. At the end of the culture period, cells were collected for mRNA and protein expression analysis of vimentin. Values were normalized on the basis of beta-actin levels [used as an internal standard]. For RT-PCR analysis, total RNA was isolated from the cultured cells and its integrity was verified by electrophoresis on a 1% agarose gel stained with ethidium bromide. Then 1 μg of total RNA was reversed-transcribed in a 25-μl reaction mixture using random primer Oligo-dT18 (Sangon, Shanghai, China) and Moloney murine leukemia virus reverse transcriptase (49). Each sample was reverse-transcribed to cDNA (50) for real-time PCR analysis (51). Beta-actin was chosen as a housekeeping gene. The primers used for the beta-actin gene were: forward sequence, GTCCGTGACATCAAGGAGAAGC; reverse sequence, CAGCACCGTGTTGGCGTAG. The primers used for the vimentin gene were: forward sequence, CGCCAGATGCGTGAAATG; reverse sequence, GCTGGAAGAAATAGTCCTCC. Fragment sizes for beta-actin and vimentin were 251bp and 290bp, respectively. The annealing temperature for RT-PCR was 65°C. For western blot analysis of vimentin (a representative protein) and beta-actin (an internal reference protein), the experimental conditions (52) included the use of the primary antibody (1:20000 dilution; 2 h at room temperature and washing four times in PBST for 8 min) and the horseradish peroxidase-conjugated secondary antibody (1:10000; 1 h at room temperature and washing in PBST for 32 min). Protein bands were visualized with a chemiluminescence substrate using a gel-imaging system (Tanon Science and Technology, Shanghai, China) with the NIH Image Analysis Software (42, 53).

medium enhances the abundance of cytochrome bc_1 in CVEC (Table 1). Cytochrome bc_1 (also known as ubiquinol:ferricytochrome c oxidoreductase) resides in the inner mitochondrial membrane and is the complex III of the respiratory chain (40). This complex transfers electrons from ubihydroquinone to cytochrome c, generating a proton gradient across the mitochondrial membrane and, therefore, the oxidation of substrates to yield water. When cytochrome bc_1 activity is reduced, superoxide anion (O_2^-) production is enhanced, resulting in oxidative stress in cells (41). Conversely, when expression of the cytochrome bc_1 protein complex is stimulated in response to L-arginine, the generation of superoxide anion and related other reactive oxygen species (e.g., H_2O_2 and peroxides) is reduced (23). This outcome would minimize cellular concentrations of oxidants. Thus, our new observation offers an explanation for the previous finding that arginine plays a critical role in preventing oxidative stress in cultured endothelial cells (13). Additionally, in response to an improved redox state, expression of key antioxidant proteins is generally attenuated (22, 42). In support of this view, supplementing arginine to culture medium reduces the concentrations of stress-related proteins [PDZ domain containing-protein 3] (43) in CVEC.

4. CONCLUSION AND PERSPECTIVES

In conclusion, proteomic analysis reveals for the first time that a physiological level of arginine alters expression of key proteins related to microfilament function and oxidative defense in CVEC. The changes in these structural and regulatory proteins are associated with improvement of the cellular redox state and enhanced NO production in endothelial cells. These novel findings aid in elucidating the mechanisms responsible for the beneficial action of arginine on the endothelium. At present, it is not known whether L-arginine directly or indirectly regulates protein expression in endothelial cells. We must also recognize that the chemical structure of L-arginine is unique in that it contains a guanidino group and is a basic substance at intracellular physiological pH. We do not rule out a possibility that L-arginine modulates gene expression through one of its metabolites. Thus, we present Figure 3 to propose a mechanism whereby L-arginine increases the expression of vimentin, tropomysin, and cytochrome bc_1 in endothelial cells. According to this model, both L-arginine and NO may activate mammalian target of rapamycin (mTOR, a protein kinase) by stimulating its phosphorylation (37,54). Polyamines (products of arginine catabolism via arginase) enhance the transcription of specific genes to form respective mRNAs (21). The translation of mRNAs into proteins requires both polyamines and mTOR for initiating the formation of polypeptides (55-58), including vimentin, tropomysin, and cytochrome bc_1. Additionally, mTOR and NO may inhibit degradation of these proteins by proteases and peptidases (59,60). Such changes in the synthesis and degradation of vimentin, tropomysin, and cytochrome bc_1 could lead to an increase in their abundance in endothelial cells. Future studies are

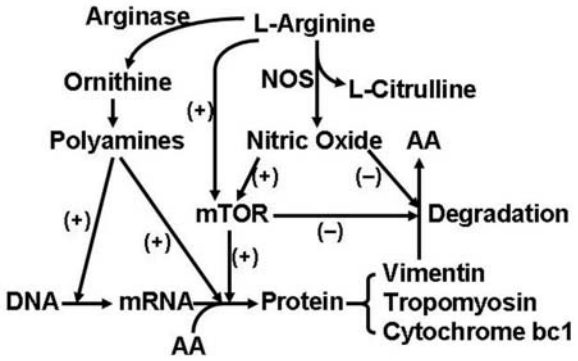

Figure 3. Proposed mechanisms for L-arginine to increase the expression of vimentin, tropomysin, and cytochrome bc1 in endothelial cells. Both L-arginine and nitric oxide (synthesized from L-arginine by nitric oxide synthase) may activate mammalian target of rapamycin (mTOR, a protein kinase) by stimulating its phosphorylation. Polyamines (products of arginine catabolism via arginase) enhance the transcription of specific genes to form respective mRNAs. The translation of mRNAs into proteins requires both polyamines and mTOR to initiate the formation of polypeptides, including vimentin, tropomysin, and cytochrome bc_1. Additionally, mTOR and nitric oxide may inhibit degradation of these proteins by proteases and peptidases. Such changes in the synthesis and degradation of vimentin, tropomysin, and cytochrome bc_1 could lead to an increase in their abundance in endothelial cells.

warranted to test these novel and important hypotheses.

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Abbreviations: CVEC: coronary venular endothelial cells; DMEM: Dulbecco's modified Eagle's medium; 2D-PAGE: 2-dimentional polyacrylamide gel electrophoresis; DPBS: Dulbecco's phosphate-buffered saline; FBS: fetal bovine serum; MALDI: matrix-assisted laser desorption ionization; NO: nitric oxide; NOS: nitric oxide synthase; PBST: PBS with 0.2% Tween 20; PCR: polymerase chair reaction; PMF: peptide mass fingerprinting; RT-PCR: real-time polymerase chair reaction.

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