ELSEVIER

International Journal of Cardiology 127 (2008) 33–39

International Journal of Cardiology

www.elsevier.com/locate/ijcard

# Effects of different statins on endothelial nitric oxide synthase and AKT phosphorylation in endothelial cells ☆

Juyong Wang [a,*], Zhenye Xu [a], Isao Kitajima [b], Zhongqi Wang [a]

[a] *Tumor Institute of Traditional Chinese Medicine, Longhua Hospital; Shanghai University of Traditional Chinese Medicine, Shanghai 200032, China*
[b] *Department of Clinical Laboratory and Molecular Pathology, University of Toyama, Toyama 930-0194, Japan*

Received 15 March 2007; received in revised form 16 August 2007; accepted 27 October 2007
Available online 16 January 2008

**Abstract**

*Background:* In the present study, we examined effects of pitavastatin and cerivastatin on NO production and their mechanisms in EC.
*Methods:* HUVEC cells ($1 \times 10^4$ cells/well) were seeded into 96-well plates in 100 μl of culture medium for overnight, and then treated with various concentrations of pitavastatin or cerivastatin for 48 h. The cytotoxicity was evaluated using a WST-8 assay; The cells were cultured for 6 h in 200 μl of fresh medium containing increasing doses of pitavastatin or cerivastatin at 37 °C for 6 h, the NO production was detected by diaminofluoresceins (DAFs) assay; Simultaneously, The cells ($1 \times 10^5$ cells/well) were seeded into 96-well plates in medium for overnight, and then treated with reagents at 37 °C for 30 min, cGMP level was measured by enzyme-immunoassay. The cells were cultured in 2 ml of fresh medium containing increasing doses of pitavastatin or cerivastatin at 37 °C for 30 min, the phosphorylations of eNOS and Akt were detected by Western blotting.
*Results:* We found that pitavastatin not only induced NO production, but also increased cGMP level in HUVECs. Furthermore, EC were incubated with pitavastatin or cerivastatin for 30 min, Western blot analysis showed that pitavastatin (0.1 μM) significantly upregulated the phosphorylation of eNOS and Akt about 1.4-fold or 1.3-fold compared with control, however, cerivastatin (0.1 μM) did not have any effects on them.
*Conclusion:* Low dose of pitavastatin (0.1 μM) involves Akt pathway, activates eNOS activity, increases cGMP level and produces NO in EC, which is higher than that of cerivastatin.


*Keywords:* Nitric oxide synthase; Vascular endothelial cell; Akt; Statin

## 1. Introduction

3-Hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, commonly referred to as statins, are widely prescribed for the treatment of hypercholesterolemia. Interestingly, studies performed in the last few years have revealed that statins protect against stroke and myocardial ischemia/reperfusion injury possibly through NO-dependent mechanism [1,2]. These findings are consistent with the notion that the cardioprotective effects of statins are partly independent of their serum lipid-lowering effects and may be due to the activation of endothelial nitric oxide synthase (eNOS) through NO-dependent pathways in the vascular endothelium [1,2]. Nitric oxide (NO) is an important component of vascular homeostasis as it acts to regulate vascular tone, arterial pressure, platelet and leukocyte adhesion to the endothelial surface, and vascular smooth muscle cell proliferation [3]. An increased NO bioavailability

☆ Supported by Shanghai Leading Academic Discipline Project, Project number: Y0302.
* Corresponding author. Tumor Institute of Traditional Chinese Medicine, Longhua Hospital, Shanghai University of Traditional Chinese Medicine, 725 Southern of Wan Ping Road, 69# box, Shanghai, China. Tel.: +86 21 64385700 2320, +86 13761470296; fax: +86 21 64398310.
*E-mail address:* wangjuyong1@126.com (J. Wang).

0167-5273/$ - see front matter © 2007 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.ijcard.2007.10.034

is observed within the first few weeks of statin treatment and is essential for improving vascular function in clinical trial [4].

Pitavastatin, also known as NK-104, is the first totally synthetic inhibitor of HMG-CoA reductase, the rate-limiting enzyme in cholesterol biosynthesis [5]. Pharmacokinetic studies have suggested that pitavastatin is only slightly metabolized by the cytochrome *P450* (CYP) system [6]. In the clinical setting, pitavastatin lowered total cholesterol and low-density lipoprotein (LDL) cholesterol levels by 28% and 40%, respectively [7]. Recently, we have reported that pitavastatin induces NO production, which is dependent on post-transcriptional regulation [8]. However, the protective effect of pitavastatin in EC compared with other statins remains unclear. Therefore, the purpose of the present study was to examine the effects of pitavastatin or cerivastatin treatment of EC on NO production, cGMP level, the phosphorylations of eNOS and Akt and eNOS activity. Here we show that low dose of pitavastatin (0.1 μM) involves Akt pathway, activates eNOS activity and produces NO in EC, which is higher than that of cerivastatin.

## 2. Materials and methods

### 2.1. Reagents

Pitavastatin (trade name: LIVALO®, code name: NK-104) was kindly provided by Kowa Co., Ltd. (Nagoya, Japan) and Nissan Chemical Industries, Ltd. (Tokyo, Japan). Dimethyl sulfoxide (DMSO) and cerivastatin were purchased from Wako Pure Chemical Industries (Osaka, Japan). Diaminofluorescein-2 (DAF-2) was purchased from Daiichi Pure Chemicals Co., Ltd. (Tokyo, Japan). Mevalonic acid (MEV) and geranylgeranyl pyrophosphate (GGPP) were purchased from Sigma (St. Louis, MO, USA). Krebs-Ringer phosphate buffer (KRP buffer) consisted of NaCl (120 mM), KCl (4.8 mM), $CaCl_2$ (0.54 mM), $MgSO_4$ (1.2 mM), glucose (11 mM), and sodium phosphate (15.9 mM), pH 7.2.

### 2.2. Cell culture

Human umbilical vein endothelial cells (HUVECs) were purchased from Takara Bio Inc. (Otsu, Japan). HUVECs were maintained in endothelial growth medium (EBM-2) containing 2% fetal bovine serum (FBS), 0.4% hFGF-B, 0.1% hEGF, 0.1% heparin, 0.1% ascorbic acid, 0.1% VEGF, 0.1% R3-IGF, 0.04% hydrocortisone, 50 mg/ml gentamicin, and 50 μg/ml amphotericin B. Cells were maintained at 37 °C and 5% $CO_2$. For experiments, HUVECs between passages 3 and 6 were used. The cells were seeded in 60-mm tissue culture plates and grown in complete EBM-2 to 90% confluence. Then, the cells were washed with phosphate-buffered saline (PBS) and incubated for the indicated times at 37 °C in 2 ml of medium containing pitavastatin, cerivastatin or vehicle.

### 2.3. WST-8 assay

The cytotoxicity was evaluated using a WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium,monosodium salt] colorimetric assay. HUVEC cells ($1\times10^4$ cells/well) were seeded into 96-well plates in 100 μl of culture medium for overnight, and then treated with various concentrations of pitavastatin or cerivastatin for 48 h, respectively. Next, 10 μl of WST-8 reagent solution (Cell Counting Kit, Dojindo Laboratories, Japan) was added and incubated for 2 h. Cell viability was determined according to manufacturer's instructions.

### 2.4. Determination of NO from HUVECs

Diaminofluoresceins (DAFs) are fluorescent NO indicators. The reaction of NO with DAFs yields the corresponding bright green fluorescent triazolofluoresceins [9]. It is feasible to detect the generation of NO from HUVECs with these fluorescent NO indicators and a simple protocol [10]. Briefly, the cells were seeded onto 96-well flat-bottomed culture plates and incubated for 6 h. Subsequently, the cells were cultured for 6 h in 200 μl of fresh medium containing increasing doses of pitavastatin or cerivastatin at 37 °C for 6 h and washed twice with KRP buffer. Thereafter, a solution of DAF-2 (10 μM), L-arginine (1 mM), and L-NAME (10 mM) dissolved in 200 μl of KRP buffer was added. After incubation for another 2 h, the supernatants were transferred to black microplates and fluorescence was measured with a fluorescence microplate reader calibrated for excitation at 485 nm and emission at 538 nm.

### 2.5. Measurement of cGMP content

Intracellular cGMP content was measured with the cGMP enzyme-immunoassay system (Amersham Pharmacia). Briefly, HUVEC cells were seeded in 96-well plates at a density of approximately $1\times10^5$ cells/well in medium for overnight, and then replaced with fresh medium contain reagents for 30 min at 37 °C. The cell lysate was centrifuged, and transferred into the appropriate well of the immunoassay microtiter plate. cGMP was measured as described by the manufacturer's protocol.

### 2.6. Western blotting of cell lysates and conditioned medium

Cell lysates were prepared by adding 500 μl of ice cold lysis buffer (20 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40,) containing 1% aprotinin (Bayer, Leverkusen, Germany) and 1 mM phenylmethylsulfonyl fluoride. The lysates were cleared by centrifugation and protein concentration in the supernatant was determined by the DC Protein assay (Bio-Rad, Hercules, CA, USA). Aliquots of cell lysates (20 μg of protein each) were separated by 8.5% SDS-PAGE, electrotransferred to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad), blocked with 5% nonfat milk in TBS-