Fluvastatin inhibits AGE-induced cell proliferation and migration via an ERK5-dependent Nrf2 pathway in vascular smooth muscle cells

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Abstract

Advanced glycation endproduct (AGE)-induced vascular smooth muscle cell (VSMC) proliferation and reactive oxygen species (ROS) production are emerging as important mechanisms of diabetic vasculopathy, but little is known about the molecular mechanism responsible for the antioxidative effects of statins on AGES. It has been reported that statins exert pleiotropic effects on the cardiovascular system due to decreases in AGE-induced cell proliferation, migration, and vascular inflammation. Thus, in the present study, the authors investigated the molecular mechanism by which statins decrease AGE-induced cell proliferation and VSMC migration. In cultured VSMCs, statins upregulated Nrf2-related antioxidant gene, NQO1 and HO-1, via an ERK5-dependent Nrf2 pathway. Inhibition of ERK5 by siRNA or BIX02189 (a specific ERK5 inhibitor) reduced the statin-induced upregulations of Nrf2, NQO1, and HO-1. Furthermore, fluvastatin was found to significantly increase ARE promoter activity through ERK5 signaling, and to inhibit AGE-induced VSMC proliferation and migration as determined by MTT assay, cell counting, FACS analysis, a wound scratch assay, and a migration chamber assay. In addition, AGE-induced proliferation was diminished in the presence of Ad-CA-MEK5α encoding a constitutively active mutant form of MEK5α (an upstream kinase of ERK5), whereas depletion of Nrf2 restored statin-mediated reduction of AGE-induced cell proliferation. Moreover, fluvastatin suppressed the protein expressions of cyclin D1 and Cdk4, but induced p27, and blocked VSMC proliferation by regulating cell cycle. These results suggest statin-induced activation of an ERK5-dependent Nrf2 pathway reduces VSMC proliferation and migration induced by AGES, and that the ERK5-Nrf2 signal module be viewed as a potential therapeutic target of vasculopathy in patients with diabetes and complications of the disease.
Introduction

Hydroxy-3-methylglutaryl coenzyme A reductase inhibitors (statins) are potent inhibitors of cholesterol biosynthesis and are widely used to reduce serum cholesterol levels in hyperlipidemic patients [1]. However, recent reports have shown statins also ameliorate cardiovascular disorders, and remarkably, have preventative effects on cardiovascular diseases [2]. In addition, statins have been reported to improve endothelial dysfunction by increasing nitric oxide availability, to inhibit proliferation and inflammatory responses, and to stabilize atherosclerotic plaque [3, 4].

Extracellular signal-regulated kinase 5 (ERK5) is an atypical member of MAPK family, and reportedly, regulates endothelial integrity and protects against vascular dysfunction and cardiovascular diseases in rodent models. On the other hand, MEK5, an upstream kinase of ERK5, plays critical roles in cell proliferation, migration, and differentiation [5]. Transcription factor nuclear factor-erythroid 2-related factor 2 (Nrf2) is an important regulator of cellular oxidative stress [6, 7], and under homeostatic conditions, binds to Kelch-like ECH-associated protein 1, and is subsequently degraded via the proteasome system or stored in cytoplasm [8]. In the presence of oxidative stress, Nrf2 translocates to the nucleus, where it forms Nrf2/small Maf heterodimer, which binds specifically to antioxidant response elements (AREs), and activates the gene expressions of antioxidant proteins, such as, NAD(P)H:quinone oxidoreductase-1 (NQO1), and heme oxygenase-1 (HO-1) [9–15]. Nrf2 has also been reported to play an atheroprotective roles by regulating antioxidant genes in the cardiovascular system [16]. Cellular redox balance is tightly controlled by various antioxidant systems, and in VSMCs, ROS activates the Nrf2 signaling pathway, which in turn, induces anti-atherosclerotic gene expression [17], and it has been reported Nrf2-activating drugs decrease VSMC proliferation and migration by antioxidant gene expression [18, 19]. Furthermore, studies have demonstrated ERK5 is a molecular target for regulating laminar blood flow-mediating Nrf2-dependent gene expression and suggested it may have significant therapeutic potential for the treatment of atherosclerosis [20].

It was recently shown advanced glycation endproducts (AGEs) and their receptor-ligand interactions play key roles in neointimal formation after vascular injury [21, 22]. AGEs are known to be formed in diabetes and to promote inflammation via specific receptors on endothelial cells [23, 24], and to mediate pro-inflammatory responses and cell proliferation through the NFκB signaling pathway [25, 26]. We hypothesized that statin might decrease AGE-induced proliferation and migration via ERK5-Nrf2-dependent gene regulation in VSMCs, and thus, we sought to identify the molecular mechanism responsible for the reductions in AGE-induced cell proliferation and VSMC migration by statins.

Materials and methods

Reagents and antibodies

Fluvastatin, pitavastatin, and BIX02189 (a specific inhibitor of ERK5) were purchased from Selleck Chemicals (Houston, TX) [27]. AGE-BSA was obtained from Calbiochem (Darmstadt, Germany), and MTT reagents were purchased from Amresco (Solon, Ohio). Antibodies were purchased from the following vendors: ERK1/2 (#9102, anti-rabbit), ERK5 (#3372, anti-rabbit), phosphor-ERK1/2 (#9106, anti-rabbit) and phospho-ERK5 (#3371, anti-rabbit) from Cell Signaling Technology (Danvers, MA), Nrf2 (sc-13032, 200 μg/ml, anti-rabbit), NQO1 (sc-32739, μg/ml, anti-mouse), CDK4 (sc-260 C-22, 100 μg/ml, anti-rabbit), p27 (sc-528 C-19, 100 μg/ml, anti-rabbit) and HA (anti-rabbit) from Santa Cruz Biotechnology (Santa Cruz, CA), HO-1 (ADI-SPA-895, 1 mg/ml, anti-rabbit) from Enzo lifesciences, cyclin D (06–137, 1 mg/ml, anti-rabbit) from Millipore and tubulin (anti-mouse) from Sigma (St. Louis, MO).
Cell culture and treatment conditions

Sprague-Dawley rats were anesthetized with ketamine and xylazine. Primary rat vascular smooth muscle cells (VSMCs) were isolated from rat thoracic aorta. The cells were processed using a 1 mm chop setting in a 10 cm culture dish, and cultured with 50% FBS-DMEM with 1% antibiotics-antimycotics in a CO2 incubator. VSMCs were maintained in DMEM supplemented with 10% fetal bovine serum (FBS), 50 U/mL penicillin, and 50 μg/mL streptomycin at 37°C in a 95% air-5% CO2 atmosphere. VSMCs from passages 4–7 were cultured for 9–24 h in the presence of 5μM fluvastatin with or without 10 μg/ml AGEs. Cells were harvested at indicated time points. All animal experiments were handled in accordance with the protocol approved by the Institutional Animal Care and Use Committee at Yeungnam University College of Medicine, Daegu, Republic of Korea.

Western blotting analysis

Cells were lysed with radioimmunoprecipitation assay (RIPA) lysis buffer supplemented with 1 mM phenylmethylsulfonyl fluoride (PMSF) and 0.01 mM protease inhibitor cocktail (PIC), and lysates were incubated on ice for 15 min and centrifuged at 15,000 × g for 10 min at 4°C. Protein concentrations were determined using a Bradford assay. Proteins were separated by SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes, which were then immunoblotted with primary antibodies (1:1,000 dilution) followed by corresponding secondary antibodies (1:4,000 dilution). Signals were visualized using electrochemiluminescence (ECL) detection reagents (Millipore, Temecula, CA), according to the manufacturer’s instructions.

Quantitative real time RT-PCR (qRT-PCR)

The mRNA expressions of Nrf2 targeted genes were determined by qRT-PCR as described previously [7]. Briefly, total RNA was isolated with TRizol, and reverse transcription was conducted using TaqMan reverse transcription reagents, according to the manufacturer’s instructions. qRT-PCR was conducted using 1 μL of template cDNA and Power SYBR Green in an ABI PRISM 7500. Quantification was performed using the efficiency-corrected ΔΔCq method. The primers used to amplify DNA sequences were as follows: NQO1, forward 5’-TTACTATGGGATGGGGTCCA-3’ and reverse 5’-TGCCAAAAACTTTCAACCAA-3’, Nrf2, forward 5’-AAAACCCACCTGAAAGCACAG-3’ and reverse 5’-AGTGTTCTGGTGATGCCACA-3’, and GAPDH, forward 5’-GGAGCCAAAAGGGTCATCAT-3’ and reverse 5’-GTGATGGCATGGACTGTGGT-3’. PCR conditions were as follows: preliminary denaturation at 50°C for 2 min, followed by 95°C for 10 min, 95°C for 15 s, and 60°C for 1 min.

Small interfering RNA (siRNA)

VSMCs were transiently transfected with 50 or 100 pM of control or specific siRNAs against Nrf2 or ERK5 using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer’s instructions. The targeting sequences of siRNAs were as follows: rat Nrf2 siRNA (5’–CAAACAGAAGUGGACCUAAdTdT–3’); rat/mouse ERK5 siRNA(5’–GAAAGGGTGCGAGCTTATAU–3’). Non-specific control siRNA was purchased from Bioneer and used as a negative control. Cells were harvested 48–72 h after siRNA transfection, and mRNA expression levels were measured.

MTT assay

AGE-induced proliferation was quantified using a MTT assay. Briefly, VSMCs were cultured on 24-well plates and when ~80% confluent, medium was replaced with serum free DMEM.
Cells were then pretreated with BIX02189 (2 μM) and stimulated with fluvastatin (5 μM) for 24 h. MTT reagents were added for 4 h at 37˚C the removed by washing with PBS, and eluted with DMSO. Proliferation was measured using a microplate reader (Biorad) at 570 nm.

**Reporter gene assay**

A luciferase assay was used to determine ARE promoter activity. The resultant construct was co-transfected with pRL-tk vector containing Renilla luciferase reporter gene into cultured VSMCs using the lipofectamine 2000 method and Plus transfection reagent. Cells were lysed at 24 h post-transfection, and Firefly-to-Renilla luciferase activity ratios in lysates were measured with dual-luciferase assay kit (Promega) to evaluate ARE promoter activity.

**Flow cytometric analysis**

Cells (1×10^5) were trypsinized, fixed in 95% ethanol, and stained with propidium iodide (PI) (50 μg/ml) for 30 min at 37˚C. PI stained cells were filtered using a 5 ml polystyrene round bottom tube fitted with a cell-strainer cap prior to flow cytometry. All flow cytometry measurements were obtained using a FACSCalibur (Becton Dickinson, San Jose, CA). Cell cycle analysis was performed using Cell-Quest pro-software.

**Wound scratch assays and migration chamber assays**

VSMC cells were cultured until >90% confluent in 6 well dishes, and then medium was replaced with serum free DMEM overnight. Wounds were made with a sterile 200 μL pipet tip by drawing a line through cells perpendicular to the line above. Cells were then pretreated with BIX02189 (2 μM) and stimulated with fluvastatin (5 μM) for 24 h. Finally, images were taken using a phase contrast at 40×. For selective migration assay, transwell system was used. Cells were seeded in the inner chamber and pretreated with BIX02189 (2 μM) in the presence or absence of fluvastatin (5 μM). AGE (10 μg/ml) were added to the lower for 12 h. After fixing, cells were visualized by crystal violet staining. Unmigrated cells were scraped off and then migrated cells were counted under a light microscope.

**Statistics**

Results shown in the bar graph are means ± SDs. The significances of differences were determined using ANOVA and Student’s t test. P values of < 0.05 were considered significant.

**Results**

**Inhibition of ERK5 reduced statin-induced expressions of Nrf2 and NQO1 in VSMCs**

To investigate the effect of statins on the Nrf2 antioxidant system, we assessed Nrf2, NQO1, and HO-1 protein levels in VSMCs. As shown in Fig 1, fluvastatin and pitavastatin strongly increased Nrf2 and NQO1 expressions in dose- and time-dependent manner (Fig 1A and 1B). To examine the role of ERK5 in Nrf2 antioxidant system induction by statins, we used an ERK5 inhibitor and ERK5 specific siRNA. Inhibition of ERK5 was found to markedly attenuate fluvastatin-induced NQO1 and HO-1 expression (Fig 1C and 1D). Interestingly, statin-induced phosphorylation levels of ERK1/2 were not affected by ERK5 inhibitor and siRNA (Fig 1C and 1D), suggesting the specific role of ERK5 in statin-induced Nrf2 signaling in vascular smooth muscle cells.

Next, we studied the effect of fluvastatin on the mRNA expressions of Nrf2, NQO1, and HO-1 in VSMCs. Consistent with protein levels, fluvastatin significantly induced the mRNA
Fig 1. The involvement of ERK5 in statin-induced Nrf2 signaling in VSMCs. (A-B) Western blot analysis of Nrf2, NQO1, and HO-1 in statin-treated VSMCs. Cells were exposed to fluvastatin and pitavastatin for 24 h at the indicated dosages. In addition, protein samples were refined from cultured VSMCs treated with fluvastatin (5 μM) or pitavastatin (5 μM) for the indicated times. (C) Western blot analysis of ERK5, NQO1, HO-1, phospho-ERK1/2, and phospho-ERK5 in BIX02189 treated VSMCs. Protein samples were obtained from cultured VSMCs treated with 5 μM fluvastatin or 5 μM pitavastatin for 24 h. (D) VSMCs were transfected with control or ERK5 siRNA (50 pM) for 30 h and then treated with 5 μM fluvastatin for 24 h. Protein levels of NQO1, HO-1, ERK5 and tubulin were determined by Western blotting with specific antibodies. In addition, phosphorylation levels of ERK1/2 and ERK5 were determined by immunoblotting with specific antibodies. Bar graphs present the densitometric quantification of Western blot bands. Results are representative of three independent experiments. *, #, †, p<0.05; **, ##, ††, p<0.01 compared with control. NS indicates not significant. 

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expressions of Nrf2, NQO1, and HO-1 (Fig 2), and ERK5 inhibition suppressed the fluvastatin-induced expressions Nrf2, NQO1, and HO-1 mRNA (Fig 2). These results indicated that ERK5 contributed to the fluvastatin-induced Nrf2 antioxidant genes in VSMCs.

**Inhibition of ERK5 attenuated fluvastatin-induced ARE promoter activity in VSMCs**

Activated Nrf2 translocates to the nucleus and then binds specifically to ARE in the promoters of target genes, such as, NQO1 and HO-1 [8]. Thus, we investigated whether fluvastatin could promote Nrf2 to ARE promoter binding. VSMCs were transfected with the reporter ARE, and ARE promoter activity was measured using a reporter gene assay. ARE promoter activity was found to be enhanced in response to fluvastatin, and to be significantly inhibited by BIX02189 (Fig 2D). These observations suggest that ERK5 is an important regulator of fluvastatin-induced Nrf2 activation and that it does so by regulating transcription.

**Fluvastatin regulated AGE-induced cell proliferation through ERK5-Nrf2 signal module**

It has been reported that fluvastatin reduces AGE-induced VSMC proliferation [28]. To confirm this effect, VSMCs were treated with AGEs in the presence or absence of fluvastatin and then subject to MTT assay. AGEs were found to dose-dependently induce cell proliferation (Fig 3A), and this was significantly suppressed by fluvastatin (Fig 3B). In addition to MTT assay, we got the similar results with cell counting (Fig 3C). Interestingly, this suppressive effect of fluvastatin was prevented when VSMCs were pretreated with BIX02189, which suggested the involvement of ERK5 in the fluvastatin-mediated inhibition of AGE-induced cell proliferation (Fig 3B and 3C). We also examined whether ERK5 activation could reduce proliferation by using Ad-CA-MEK5α encoding a constitutively active mutant form of MEK5α (an upstream kinase of ERK5). As shown in Fig 4, AGE-induced proliferation determination by both MTT assay and cell counting was significantly diminished in the presence of Ad-CA-MEK5α, and Nrf2 depletion using siRNA restored AGE-induced proliferation. These results indicate fluvastatin inhibited AGE-induced VSMC proliferation through ERK5-Nrf2 signal module.

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**Fig 2. The involvement of ERK5 in fluvastatin-induced Nrf2 signaling in VSMCs.** (A-C) Quantitative RT-PCR analysis of the mRNA expressions of Nrf2, NQO1, and HO-1 in VSMCs treated with fluvastatin. VSMCs were treated with BIX02189 (2 μM) for 1 h and then incubated with 5 μM fluvastatin for 6 h. qRT-PCR analysis was performed in triplicate. Results are representative of three independent experiments. *p < 0.05. (D) VSMCs were co-transfected with pARE and pRL-tk and then stimulated with 5 μM fluvastatin for 24 h in the presence or absence of BIX02189 (2 μM). Promoter activity was measured by using a Dual-Luciferase reporter assay kit and a GloMax 20/20 luminometer. Transfection efficiency was normalized versus Renilla luciferase activity derived from pRL-tk construct. Reporter assay was performed in triplicate. Results are presented as the means ± SDs of three independent experiments. **p < 0.01.

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Fluvastatin regulated AGEs-induced cell cycle progression through the ERK5-Nrf2 pathway

The cell cycle is controlled by activators (cyclins) and inhibitors (Rb, p16, p21, p27). Among them, cyclin D is a major cell cycle associated cyclin and interacts with four cyclin-dependent kinases (Cdks; Cdk2, 4, 5 and 6). Cyclin D-Cdk4/6 complex accumulation is required for cell cycle progression [29, 30]. On the other hand, p27 inhibits Cdk by binding directly to Cdk-cyclin complex and blocking its protein kinase activity. Statins have been shown to induce cell cycle arrest in various cancer cells, including those of prostate [31] and breast cancer [32], and p53, p21, and p27 proteins are known to regulate VSMC proliferation [33]. Furthermore, it has been demonstrated that statins cause G1 arrest in VSMCs by up-regulating p27 and down-regulating cyclin E [34, 35].

In order to examine the effects of fluvastatin on cell cycle progression at the cell cycle regulatory gene level. We first checked that AGEs increased cyclin D1 and Cdk4 protein levels, but reduced p27 level. Fluvastatin was found to suppress cyclin D1 and Cdk4 protein levels, but to enhance p27 level in VSMCs (Fig 5A), and these effects were prevented by BIX02189 pretreatment. As compared with cells treated with fluvastatin (5 μM) in the presence of AGEs (10 μg/ml), cells in G0/G1 phase was significantly reduced by BIX02189 pretreatment (Fig 5B). To investigate whether ERK5 activation induced cell cycle arrest, we pretreated cells with Ad-CA-MEK5α. It was found that the AGE-induced protein expressions of cyclin D1 and Cdk4 were markedly reduced in the presence of Ad-CA-MEK5α, whereas depletion of Nrf2 prevented these reductions (Fig 6). These results show fluvastatin blocks VSMC proliferation via an ERK5-Nrf2 pathway and that this leads to cell cycle arrest.

Fluvastatin regulated AGE-induced cell migration through an ERK5-dependent pathway

To determine the functional significance of the fluvastatin-mediated ERK5 activation pathway, we examined the effect of fluvastatin on AGE-induced VSMC migration using both a scratch assay (Fig 7A) and a migration chamber assay (Fig 7B). Fluvastatin significantly suppressed AGE-induced cell migration in response to wound injury, and BIX02189 blocked this inhibitory effect of fluvastatin (Fig 7). Taken together, our results suggest that fluvastatin suppresses
AGE-induced cell proliferation and migration by inducing antioxidant genes through the ERK5-dependent activation of the Nrf2 pathway.

**Discussion**

Antioxidant defense mechanisms protect cells from excessive oxidative stress [16], and the transcription factor Nrf2 contributes to these processes by regulating the expressions of antioxidant genes. The present study provides evidence that in VSMCs, ERK5 activation increases

Fig 4. The effect of Nrf2 siRNA on the ERK5 activation-mediated inhibition of AGE-induced VSMC proliferation. (A) VSMC cells were infected with adenovirus encoding LacZ or CA-MEK5α for 48 h, and then exposed to AGEs for 24 h. Cell viability was determined using a MTT assay. Results are presented as the means±SEs of three independent experiments. *, p < 0.05; **, p < 0.01. (B) Cell proliferation was determined by using cell counting with same condition. Results are representative of three independent experiments. **, p < 0.01. Amounts of protein expression were determined by immunoblotting with specific antibodies for HA and Nrf2.

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AGE-induced cell proliferation and migration by inducing antioxidant genes through the ERK5-dependent activation of the Nrf2 pathway.
A number of recent reports have shown that statins may also have important anti-inflammatory effects, in addition to their effects on lowering plasma lipids [37, 38]. Since inflammation is closely linked to ROS production, the molecular basis of their observed anti-inflammatory effects might be related to their abilities to block ROS production [18, 37, 39]. It has been well established that ROS are related to the development of cardiovascular disease, and many studies support the notion that ROS released from a dysfunctional mitochondrial respiratory chain other sources plays a role in the development of atherosclerosis and its complications [40–42], which have been attributed to ROS-mediated vascular signaling pathways.
In the present study, fluvastatin activated Nrf2 in VSMCs and this markedly increased ARE-driven promoter activity (Fig 2C) and the upregulation of ARE-targeted antioxidant genes (NQO1 and HO-1). These findings provide first evidence fluvastatin exerts its antioxidant effects via the ERK5-dependent activation of the Nrf2 pathway in vascular cells [17–19, 43].

It has been recently demonstrated AGEs and their receptor-ligand interactions play key roles in neointimal formation after vascular injury [28]. In fact, AGE-induced VSMC proliferation and ROS production are emerging as important mechanisms of atherosclerosis. Before commencing the present study, we hypothesized fluvastatin might reduce AGE-induced proliferation, and thus, we sought to identify the mechanism underlying the involvement of fluvastatin in AGE-induced cellular signaling. Treatment with AGEs increased the protein levels of the proliferation markers, cyclin D1 and CDK4, and markedly decreased p27 level, and these effects of AGEs were lost when ERK5 expression was depleted or inhibited by BIX02189 (Fig 5A). Our MTT assay and cell counting results show the antioxidant effects of fluvastatin are largely dependent on the ERK5-dependent Nrf2 pathway, because Nrf2 siRNA abolished their antioxidant cytoprotective responses. In addition, the overexpression of CA-MEK5α using an adenoviral system inhibited AGE-induced cell proliferation and cell cycle progression, and these effects were prevented by Nrf2 depletion (Fig 4 and Fig 6), which indicates ERK5 activation regulates cell cycle arrest via a Nrf2-dependent pathway.

NQO1 and HO-1 have been suggested as a downstream target of Nrf2 in inhibition of vascular smooth muscle cell proliferation through cell cycle arrest [44–46]. In this study, our data showed that statin induced Nrf2 and its target genes including NQO1 and HO-1 in an ERK5-dependent manner. In addition, inhibition of ERK5 and Nrf2 failed to inhibit fluvastatin-induced protein regulation of cell cycle-related genes including cyclin D1, CDK4, and p27 suggesting that ERK5-Nrf2 signal nodule regulates G1 cell cycle arrest in vascular smooth muscle cells.

A growing body of evidence suggested that ERK5 has cytoprotective effects via multiple pathways. Based on the genetic approach with gene knock out, it has been established that ERK5 is essential for endothelial viability and protects vascular leakage [47].
laminar shear stress protects endothelial cells from oxidative stress and inflammatory responses via ERK5 activation. However, laminar shear stress reduces endothelial cell proliferation in an ERK5-dependent manner. On the other hand, ERK5 can be activated by many growth factors that are important in vascular smooth muscle cell proliferation. It is not clear whether growth factor-mediated ERK5 activation could regulate Nrf2 or not. ERK5 is not only a kinase but also a transcriptional coactivator with C-terminal transactivation domain, which is a unique characteristic compared to ERK1/2. It has been reported that statin induces both phosphorylation and transcriptional activation of ERK5 [48]. Therefore it is reasonable to pursue...
the molecular mechanism of ERK5-Nrf2 signal nodule in aspect of transcriptional regulation regarding the ambiguity of ERK5.

This study provides a molecular basis for the protective effects for statins. In particular, our finding regarding their induction of the ERK5-dependent regulation of cell proliferation and cell cycle arrest in VSMCs could eventually contribute to the development of a treatment for cardiovascular diseases. This study shows fluvastatin exerts induces antioxidant enzymes via an ERK5-dependent Nrf2 pathway.

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References
1. Maron DJ. The epidemiology of low levels of high-density lipoprotein cholesterol in patients with and without coronary artery disease. Am J Cardiol. 2000; 86(12A):11L–4L. PMID: 11374848
2. Pedersen TR, Wilhelmsen L, Faerge man O, Strandberg TE, Thorgeirsson G, Troedsson L, et al. Follow-up study of patients randomized in the Scandinavian simvastatin survival study (4S) of cholesterol lowering. Am J Cardiol. 2000; 86(3):257–62. PMID: 10922429
3. Zhou Z, Wang K, Penn MS, Marso SP, Lauer MA, Forudi F, et al. Receptor for AGE (RAGE) mediates neointimal formation in response to arterial injury. Circulation. 2003; 107(17):2238–43. https://doi.org/10.1161/01.CIR.0000063577.32819.23 PMID: 12719284
4. Duckers HJ, Boehm M, True AL, Yet SF, San H, Park JL, et al. Heme oxygenase-1 protects against vascular constriction and proliferation. Nat Med. 2001; 7(6):693–8. https://doi.org/10.1038/89068 PMID: 11385506
5. Wang X, Tournier C. Regulation of cellular functions by the ERK5 signalling pathway. Cell Signal. 2006; 18(6):753–60. https://doi.org/10.1016/j.cellsig.2005.11.003 PMID: 16376520
6. Motohashi H, Yamamoto M. Nrf2-Keap1 defines a physiologically important stress response mechanism. Trends Mol Med. 2004; 10(11):549–57. https://doi.org/10.1016/j.tmm.2004.09.003 PMID: 15519281
7. Negro P, Abe J, Woo CH, Satoh K, McClain C, O'Dell MR, et al. PKCzeta decreases eNOS protein stability via inhibitory phosphorylation of ERK5. Blood. 2010; 116(11):1971–9. https://doi.org/10.1182/blood-2010-02-269134 PMID: 20536799
8. Itoh K, Wakabayashi N, Katoh Y, Ishii T, Igarashi K, Engel JD, et al. Keap1 represses nuclear activation of antioxidant responsive elements by Nrf2 through binding to the amino-terminal Neh2 domain. Genes Dev. 1999; 13(1):76–86. PMID: 9887101
9. Forman HJ, Ruden D. Introduction to serial reviews on EpRE and its signaling pathway. Free Radic Biol Med. 2004; 36(10):1197–8. https://doi.org/10.1016/j.freeradicbiomed.2004.03.004 PMID: 15110383

10. Itoh K, Tong KI, Yamamoto M. Molecular mechanism activating Nrf2-Keap1 pathway in regulation of adaptive response to electrophiles. Free Radic Biol Med. 2004; 36(10):1208–13. https://doi.org/10.1016/j.freeradicbiomed.2004.02.075 PMID: 15110385

11. Chan K, Lu R, Chang JC, Kan YW. NRF2, a member of the NFE2 family of transcription factors, is not essential for murine erythropoiesis, growth, and development. Proc Natl Acad Sci U S A. 1996; 93(24):13943–8. PMID: 8943040

12. Ishii T, Itoh K, Takahashi S, Sato H, Yanagawa T, Katoh Y, et al. Transcription factor Nrf2 coordinately regulates a group of oxidative stress-inducible genes in macrophages. J Biol Chem. 2000; 275(21):16023–9. PMID: 10821856

13. Li J, Ichikawa T, Janicki JS, Cui T. Targeting the Nrf2 pathway against cardiovascular disease. Expert Opin Ther Targets. 2009; 13(7):785–94. https://doi.org/10.1517/14728220903025762 PMID: 19530984

14. Mann GE, Bonacasa B, Ishitani T, Siow RC. Targeting the redox sensitive Nrf2-Keap1 defense pathway in cardiovascular disease: protection afforded by dietary isoflavones. Curr Opin Pharmacol. 2009; 9(2):139–45. https://doi.org/10.1016/j.coph.2008.12.012 PMID: 19157984

15. Itoh K, Chiba T, Takahashi S, Ishii T, Igarashi K, Katoh Y, et al. An Nrf2/small Maf heterodimer mediates the induction of phase II detoxifying enzyme genes through antioxidant response elements. Biochem Biophys Res Commun. 1997; 236(2):313–22. PMID: 9240452

16. Makabe S, Takahashi Y, Watanabe H, Murakami M, Ohba T, Ito H. Fluvastatin protects vascular smooth muscle cells against oxidative stress through the Nrf2-dependent antioxidant pathway. Atherosclerosis. 2010; 213(2):377–84. https://doi.org/10.1016/j.atherosclerosis.2010.07.059 PMID: 20864107

17. Wassmann S, Laufs U, Muller K, Konkol C, Ahlbory K, Baumer AT, et al. Cellular antioxidant effects of atorvastatin in vitro and in vivo. Arterioscler Thromb Vasc Biol. 2002; 22(2):300–5. PMID: 11834352

18. Haendeler J, Hoffmann J, Zeiher AM, Dimmeler S. Antioxidant effects of statins via S-nitrosylation and activation of thioredoxin in endothelial cells: a novel vasculoprotective function of statins. Circulation. 2004; 110(7):856–61. https://doi.org/10.1161/01.CIR.0000138743.09012.93 PMID: 15289372

19. Takemoto M, Liao JK. Pleiotropic effects of 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors. Arterioscler Thromb Vasc Biol. 2001; 21(11):1712–9. PMID: 11701455

20. Kim M, Kim S, Lim JH, Lee C, Choi HC, Woo CH. Laminar flow activation of ERK5 protein in vascular endothelial cells of diabetic vasculopathy. Exp Mol Med. 2009; 41(11):802–11. https://doi.org/10.3858/emm.2009.41.11.086 PMID: 19641377

21. Hofmann MA, Drury S, Fu C, Qu W, Taguchi A, Lu Y, et al. RAGE mediates a novel proinflammatory axis: a central cell surface receptor for S100/calgranulin polypeptides. Cell. 1999; 97(7):889–901. PMID: 10399917

22. Brownlee M, Cerami A, Vlassara H. Advanced glycosylation end products in tissue and the biochemical basis of diabetic complications. N Engl J Med. 1988; 318(20):1315–21. https://doi.org/10.1056/NEJM19880519318007 PMID: 3283558

23. Hofmann MA, Drury S, Fu C, Qu W, Taguchi A, Lu Y, et al. RAGE mediates a novel proinflammatory axis: a central cell surface receptor for S100/calgranulin polypeptides. Cell. 1999; 97(7):889–901. PMID: 10399917

24. Surapisitchat J, Hoefen RJ, Pi X, Yoshizumi M, Yan C, Berk BC. Fluid shear stress inhibits TNF-alpha activation of JNK but not ERK1/2 or p38 in human umbilical vein endothelial cells: Inhibitory crosstalk among MAPK family members. Proc Natl Acad Sci U S A. 2001; 98(11):6476–81. https://doi.org/10.1073/pnas.101134098 PMID: 11358329

25. Bierhaus A, Chevion S, Chevion M, Hofmann M, Quehenberger P, Illner T, et al. Advanced glycation end product-induced activation of NF-kappabB is suppressed by alpha-lipoic acid in cultured endothelial cells. Diabetes. 1997: 46(9):1481–90. PMID: 9287050

26. Stephenson K, Tunstead J, Tsai A, Gordon R, Henderson S, Dansky HM. Neointimal formation after endovascular arterial injury is markedly attenuated in db/db mice. Arterioscler Thromb Vasc Biol. 2003; 23(11):2027–33. https://doi.org/10.1161/01.ATV.0000096394.32433.59 PMID: 14500292

27. Tatake RJ, O’Neill MM, Kennedy CA, Wayne AL, Jakes S, Wu D, et al. Identification of pharmacological inhibitors of the MEK5/ERK5 pathway. Biochem Biophys Res Commun. 2008; 377(1):120–5. https://doi.org/10.1016/j.bbrc.2008.09.087 PMID: 18834865

28. Yoon SJ, Yoon YW, Lee BK, Kwon HM, Hwang KC, Kim M, et al. Potential role of HMG CoA reductase inhibitor on oxidative stress induced by advanced glycation endproducts in vascular smooth muscle cells of diabetic vasculopathy. Exp Mol Med. 2009; 41(11):802–11. https://doi.org/10.3858/erm.2009.41.11.086 PMID: 19641377
29. Hall C, Nelson DM, Ye X, Baker K, DeCaprio JA, Seetholscher S, et al. HIRA, the human homologue of yeast Hir1p and Hir2p, is a novel cyclin-cdk2 substrate whose expression blocks S-phase progression. Mol Cell Biol. 2001; 21(5):1854–65. https://doi.org/10.1128/MCB.21.5.1854-1865.2001 PMID:11238922

30. Adams PD. Regulation of the retinoblastoma tumor suppressor protein by cyclin/cdks. Biochim Biophys Acta. 2001; 21(3):123–33.

31. Hoque A, Chen H, Xu XC. Statin induces apoptosis and cell growth arrest in prostate cancer cells. Cancer Epidemiol Biomarkers Prev. 2008; 17(1):88–94. https://doi.org/10.1158/1055-9965.EPI-07-0531 PMID:18199714

32. Sanchez CA, Rodriguez E, Varela E, Zapata E, Paez A, Masso FA, et al. Statin-induced inhibition of MCF-7 breast cancer cell proliferation is related to cell cycle arrest and apoptotic and necrotic cell death mediated by an enhanced oxidative stress. Cancer Invest. 2008; 26(7):698–707. https://doi.org/10.1080/07357900701874658 PMID:18608208

33. Zhu P, Chen JM, Chen SZ, Zhang C, Zheng SY, Long G, et al. Matrine inhibits vascular smooth muscle cell proliferation by modulating the expression of cell cycle regulatory genes. Acta Pharmacol Sin. 2010; 31(10):1329–35. https://doi.org/10.1038/aps.2010.145 PMID:20835268

34. Fouty BW, Rodman DM. Mevastatin can cause G1 arrest and induce apoptosis in pulmonary artery smooth muscle cells through a p27Kip1-independent pathway. Circ Res. 2003; 92(5):501–9. https://doi.org/10.1161/01.RES.0000091880.53735.BF PMID:12600884

35. Rao S, Gray-Bablin J, Herliczek TW, Keyomarsi K. The biphasic induction of p21 and p27 in breast cancer cells by modulators of cAMP is posttranscriptionally regulated and independent of the PKA pathway. Exp Cell Res. 1999; 252(1):211–23. https://doi.org/10.1006/excr.1999.4620 PMID:10502413

36. Kim SE, Thanh Thuy TT, Lee JH, Ro JY, Bae YA, Kong Y, et al. Simvastatin inhibits induction of matrix metalloproteinase-9 in rat alveolar macrophages exposed to cigarette smoke extract. Exp Mol Med. 2009; 41(4):277–87. https://doi.org/10.3858/emm.2009.41.4.031 PMID:19299917

37. Antonopoulos AS, Margaritis M, Lee R, Channnon K, Antoniades C. Statins as anti-inflammatory agents in atherogenesis: molecular mechanisms and lessons from the recent clinical trials. Curr Pharm Des. 2012; 18(11):1519–30. https://doi.org/10.2174/138161212799504803 PMID:22364136

38. Jain MK, Ridker PM. Anti-inflammatory effects of statins: clinical evidence and basic mechanisms. Nat Rev Drug Discov. 2005; 4(12):977–87. https://doi.org/10.1038/nrd1901 PMID:16341063

39. Alegret M, Silvestre JS. Pleiotropic effects of statins and related pharmacological experimental approaches. Timely Top Med Cardiovasc Dis. 2007; 18(11).

40. De Rosa S, Cirillo P, Paglia A, Sasso L, Di Palma V, Chiariello M. Reactive oxygen species and antioxidants in the pathophysiology of cardiovascular disease: does the actual knowledge justify a clinical approach? Curr Vasc Pharmacol. 2010; 8(2):259–75. PMID:19758111

41. Madamanchi NR, Runge MS. Mitochondrial dysfunction in atherosclerosis. Circ Res. 2007; 100(4):460–73. https://doi.org/10.1161/01.RES.0000258450.44413.96 PMID:17332437

42. Goncalves DO, Calou IB, Siqueira RP, Lopes AA, Leal LK, Brito GA, et al. In vivo and in vitro anti-inflammatory and anti-nociceptive activities of lovastatin in rodents. Braz J Med Biol Res. 2011; 44(2):173–81. PMID:21243316

43. Lee HH, Park SA, Almazari I, Kim EH, Na HK, Surh YJ. Piceatannol induces heme oxygenase-1 expression in human mammary epithelial cells through activation of ARE-driven Nrf2 signaling. Arch Biochem Biophys. 2010; 501(1):142–50. https://doi.org/10.1016/j.abb.2010.06.011 PMID:20558128

44. Choi HC, Lee KY, Lee DH, Kang YJ. Heme oxygenase-1 induced by aprotinin inhibits vascular smooth muscle cell proliferation through cell cycle arrest in hypertensive rats. Korean J Physiol Pharmacol. 2009; 13(4):309–13. https://doi.org/10.4196/kjp.2009.13.4.309 PMID:19885015

45. Kim JE, Kang YJ, Lee KY, Choi HC. Isoproterenol inhibits angiogenesis II-stimulated proliferation and reactive oxygen species production in vascular smooth muscle cells through heme oxygenase-1. Biol Pharm Bull. 2009; 32(6):1047–52. PMID:19483313

46. Oh CJ, Park S, Kim JY, Kim HJ, Jeoung NH, Choi YK, et al. Dimethylfumarate attenuates restenosis after acute vascular injury by cell-specific and Nrf2-dependent mechanisms. Redox Biol. 2014; 2:855–64. https://doi.org/10.1016/j.redox.2014.06.003 PMID:25097877

47. Hayashi M, Kim SW, Imanaka-Yoshida K, Yoshida T, Abel ED, Eliceiri B, et al. Targeted deletion of BMK1/ERK5 in adult mice perturbs vascular integrity and leads to endothelial failure. J Clin Invest. 2004; 113(8):1138–48. https://doi.org/10.1172/JC119890 PMID:15085193

48. Akaike M, Che W, Marmarosh NL, Ohta S, Osawa M, Ding B, et al. The hinge-helix 1 region of peroxisome proliferator-activated receptor gamma1 (PPARgamma1) mediates interaction with extracellular signal-regulated kinase 5 and PPARgamma1 transcriptional activation: involvement in flow-induced PPARgamma activation in endothelial cells. Mol Cell Biol. 2004; 24(19):8691–704. https://doi.org/10.1128/MCB.24.19.8691-8704.2004 PMID:15367687