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Chronic Nicotine Exposure Reduces Antioxidant Function of Simvastatin in Renal Proximal Tubule Cells

ISTVAN ARANY[1], TIBOR FÜLÖP[2,3] and MEHUL DIXIT[1]

[1]*Division of Pediatric Nephrology, Department of Pediatrics, University of Mississippi Medical Center, Jackson, MS, U.S.A.;*
[2]*Division of Nephrology, Department of Medicine, Medical University of South Carolina, Charleston, SC, U.S.A.;*
[3]*Medical Services, Ralph H. Johnson VA Medical Center, Charleston, SC, U.S.A.*

Abstract. *Background/Aim: We have previously reported that simvastatin exhibits antioxidant properties via extracellular signal-regulated kinase (ERK)/cAMP-response element binding (CREB) protein-dependent induction of heme oxygenase-1 (HO1) and chronic nicotine exposure inhibits ERK/CREB signaling in renal proximal tubule cells (through p66shc). Herein, whether nicotine dampens simvastatin-dependent HO1 induction was determined. Materials and Methods: Renal proximal tubule (NRK52E) cells were pre-treated with 200 μM nicotine for 24 h followed by 10 μM simvastatin. Promoter activity of HO1 and manganese superoxide dismutase (MnSOD) and activation of CREB and ERK (via ELK1) were determined in luciferase reporter assays. CREB and p66shc were modulated via genetic means. Results: Nicotine suppressed simvastatin-dependent activation of HO1 and MnSOD promoters and activity of CREB and ELK1 via p66shc. Overexpression of CREB or knockdown of p66shc restored simvastatin-dependent induction of HO1 and MnSOD in the presence of nicotine. Conclusion: Antioxidant efficiency of simvastatin might be significantly lessened in smokers/E-cigarette users.*

Statins not only lower dyslipidemia, but also elicit pleotropic responses (1) which, independently from their lipid-lowering effect (2), help protect the kidney from adverse effects of oxidative stress in patients with chronic kidney disease (3). Previously, we reported that simvastatin elicits protective antioxidant responses by transcriptionally inducing the promoter of the antioxidant gene heme oxygenase-1 (*HO1*) *via* the cAMP response element binding (*CREB*) protein in cultured renal proximal tubule cells (4). *CREB* is a transcription factor that regulates promoter activity of several genes including *HO1* (4, 5) and manganese superoxide dismutase (*MnSOD*) (6, 7).

It has been recognized that smoking/chronic exposure to nicotine accelerates development and progression of kidney disease in animal models and in humans (8-12) by interfering in several pivotal pathways. We showed that nicotine not only augments production of reactive oxygen species (ROS) (11, 13), but also inhibits antioxidant responses in the kidney (14) and in cultured renal proximal tubule cells (15). Interestingly, prolonged nicotine exposure reduces *CREB* activation in the brain (16). By analogy, chronic nicotine exposure may also interfere with *CREB* activation and hence with the antioxidant function of simvastatin in the kidneys of smokers. We reported that nicotine activates the *p66shc* gene (13), which in turn shuts down extracellular signal-regulated kinase (*ERK*) activation (17, 18) and consequently *CREB* activation, which is pivotal for inducing *HO1* or *MnSOD*.

Accordingly, our aim was to determine whether nicotine pretreatment attenuates simvastatin-mediated induction of select *CREB*-regulated antioxidant genes (*HO1* and *MnSOD*) by reducing transcriptional activity of *CREB via p66shc* in cultured renal proximal tubule cells.

Materials and Methods

Cell line and treatment. The rat renal proximal tubule cell line NRK52E was purchased from American Type Culture Collection (Manassas, VA, USA) and maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum and 1% antibiotic/antimycotic (Thermo Fisher Scientific, Waltham, MA, USA), in an atmosphere with 5% CO_2 at 37˚C. Cells were pre-

Correspondence to: Istvan Arany, Research Wing Room R116B, 2500 N. State St, Jackson, MS 39216, U.S.A. Tel: +1 6018159464, Fax: +1 6018155902, e-mail: iarany@umc.edu

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treated or not with 200 μM nicotine (Sigma-Aldrich, St. Louis, MO, USA) for 24 h (19) prior to treatment with 10 μM simvastatin (Sigma-Aldrich) (4). This line of work was approved by the Institutional Biohazard Committee (01/22/2010).

Reporter luciferase studies. Cells were transfected with either of the following reporter luciferase plasmids: *HO1* promoter (20); *CRE, ELK1* (Agilent Technologies, Santa Clara, CA) or *MnSOD* promoter (21). Cells were also co-transfected with a renilla luciferase plasmid (Promega, Madison, WI, USA) using Lipofectamine 3000 as suggested by the manufacturer (Thermo Fisher Scientific) and described elsewhere (19). After treatment, as described above, firefly and renilla luciferase activities were determined by a Dual Luciferase assay kit (Promega) and calculated as firefly/renilla ratios and expressed as a percentage that of the control.

Plasmid transfection. Some cells were also transfected with a short-hairpin *p66shc* (sh*p66shc*) (19) to knockdown *p66shc* expression or with a plasmid containing constitutively activated *CREB* (VP16*CREB*) (22) in order to overexpress *CREB*.

Statistical analysis. Continuous variables are expressed as means and standard deviations (S.D.). One-way ANOVA with Holm-Sidak *post-hoc* test was used to evaluate differences between groups. Differences between means were considered significant when $p<0.05$. All analyses were performed using the GraphPad InStat3 (La Jolla, CA, USA) software package.

Results

Chronic nicotine exposure attenuated simvastatin-dependent induction of the HO1 and MnSOD promoters. NRK52E cells were co-transfected with an HO1 promoter containing luciferase plasmid plus a renilla luciferase and treated with 200 μM nicotine for 24 h followed by treatment with 10 μM simvastatin. Luciferase activities were determined 24 h later. Figure 1A shows that pre-treatment with nicotine significantly reduced promoter activity of the *HO1* gene induced by simvastatin. In fact, nicotine alone slightly but significantly suppressed promoter activity of *HO1* compared with the control. Similar observations were made for the *MnSOD* gene promoter (Figure 1B): Simvastatin-mediated activation was significantly reduced by nicotine pretreatment.

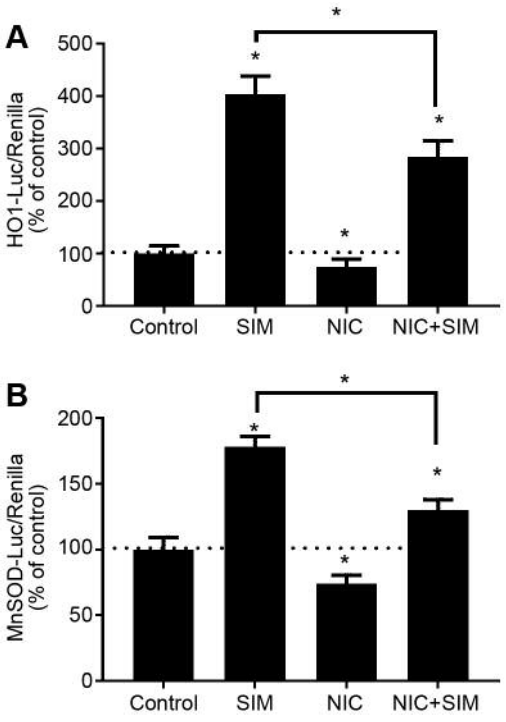

Figure 1. *Chronic nicotine (NIC) exposure suppresses simvastatin (SIM)-mediated induction of the heme oxygenase-1 (HO1) (A) and manganese superoxide dismutase (MnSOD) (B) gene promoters in cultured renal proximal tubule cells. NRK52E cells were co-transfected with an HO1 or MnSOD promoter luciferase and renilla luciferase plasmids. Twenty-four hours after transfection, one group was pre-treated with 200 μM NIC for 24 h followed by 10 μM SIM. Another group was treated with 10 μM SIM only. After 24 h, renilla activities were determined as described in the Materials and Methods. Results were calculated as firefly (promoter)/renilla ratios and expressed as percentages of the control group, n=3. Dotted line represents the control value. *Significantly different at p<0.05 compared to the control or as indicated.*

Chronic nicotine exposure suppressed simvastatin-mediated CREB activation via p66shc. Since both the HO1 (5) and *MnSOD* (7) promoters are induced, at least partly, by the *CREB* transcription factor, NRK52E cells were co-transfected with a *CRE* and a renilla luciferase plasmid and treated with 200 μM nicotine for 24 h followed by treatment with 10 μM simvastatin. Luciferase activities were determined 24 h later. Figure 2A shows that nicotine pretreatment significantly attenuated simvastatin-dependent induction of the *CRE* reporter. In previous work, we showed that nicotine exerts its adverse effects on renal proximal tubule cells by induction of *p66shc* (13). Hence, we determined whether *p66shc* mediates the above described adverse effect. Accordingly, NRK52E cells were co-transfected with a *CRE* and a renilla luciferase plasmid together with shp66shc plasmid in order to knockdown *p66shc* expression (19). These cells were treated with nicotine and simvastatin as described above and activity of the *CRE* reporter was determined and compared to the results obtained from cells with wild type (w.t.) *p66shc*. Figure 2A show that knockdown of *p66shc* significantly reversed negative effects of nicotine on simvastatin-dependent inducibility of the *CRE* reporter.

p66shc suppressed simvastatin-mediated CREB activation via ERK inhibition. Our next question was how p66shc suppresses simvastatin-mediated *CREB* activation.

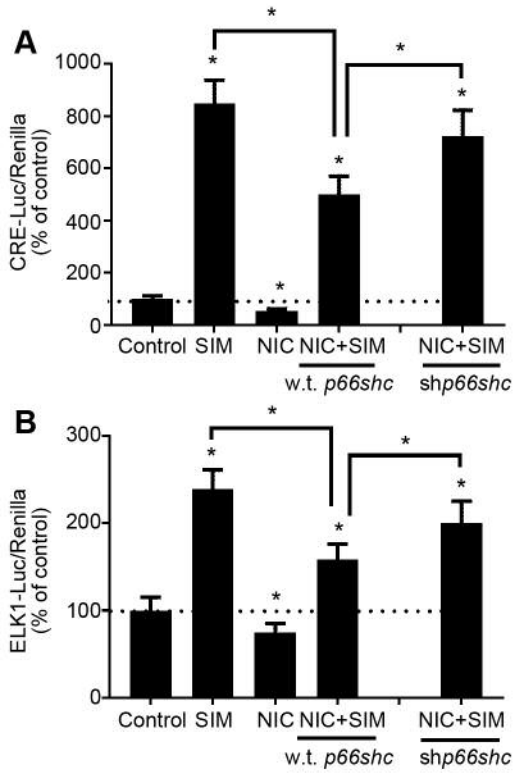

Figure 2. *Nicotine (NIC) interferes with the cAMP response element binding (CREB) protein (A) and extracellular signal regulated kinase (ERK) (B) pathways through p66shc. NRK52E cells were co-transfected with CRE or ELK1 and renilla luciferase plasmids and treated with either 10 μM simvastatin (SIM) or pretreated with 200 μM NIC prior to treatment with 10 μM SIM. Luciferase activities were determined 24 h later. Some cells were also co-transfected with a short-hairpin p66shc plasmid (shp66shc) to knockdown endogenous p66shc expression. n=3. Dotted line represents the control value. *Significantly different at p<0.05 compared to the control or as indicated. w.t.: Wild-type.*

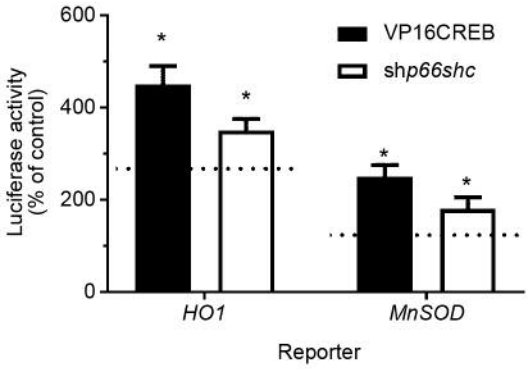

Figure 3. *Overexpression of cAMP-response element binding (CREB) protein or knockdown of p66shc restored simvastatin (SIM)-dependent induction of heme oxygenase-1 (HO1) and manganese superoxide dismutase (MnSOD) in the presence of nicotine (NIC). NRK52E cells were transfected with either an HO1 or MnSOD promoter plus renilla luciferase plasmids. In addition, some cells were also transfected with a VP16CREB (to overexpress activated CREB) or shp66shc (to knockdown p66shc) plasmid. Cells were treated with NIC+SIM and luciferase activities were measured/calculated. n=3. *Significantly different at p<0.05 compared to the NIC+SIM values. For comparison, dotted lines depict the relevant NIC+SIM data. Dotted lines represent respective levels of activity in the absence of VP16CREB or shp66shc.*

Previously, we determined that p66shc interferes with activation of *ERK* (17), which is a *CREB* kinase (23). To test this possibility, NRK52E cells were co-transfected with *ELK1* (a surrogate marker of *ERK*) and *CRE* plasmid together with renilla luciferase and treated as above. Figure 2B shows that while simvastatin activated *ELK1* (and hence *ERK*), nicotine pretreatment reduced this effect. Importantly, knockdown of p66shc ameliorated this suppression.

Overexpression of CREB or silencing of p66shc restored simvastatin-mediated induction of HO1 and MnSOD in the presence of nicotine. NRK52E cells were co-transfected with either a constitutively active *CREB* (VP16*CREB*) or shp66shc plasmid together with an *HO1* or *MnSOD* promoter reporter and renilla plasmids and treated with nicotine and simvastatin as above. Figure 3A demonstrates that promoter activity of both *HO1* and *MnSOD* was significantly enhanced by overexpression of constitutively active *CREB* or by knockdown of *p66shc* in the presence of nicotine and simvastatin.

Discussion

Chronic nicotine exposure, through smoking, E-cigarettes or nicotine replacement therapies, creates an environment in the kidney that interferes with several signaling pathways including antioxidant defenses (14, 15, 24, 25). The present study confirms this observation: chronic nicotine exposure suppressed the promoter of the *HO1* and *MnSOD* genes in cultured renal proximal tubule cells (Figure 1). Clinical studies have revealed that smoking adversely influences the effects of statins in ischemic heart disease (26). These studies, however, did not involve the kidney. It has been known for a while that besides their effects on dyslipidemia, statins also exert pleotropic effects, which are independent from their cholesterol-lowering effects (27). These pleiotropic properties include, among others, antioxidant effects (27), which may validate statin therapies for use in various kidney diseases (28, 29). We demonstrated that simvastatin induces the promoter of the antioxidant *HO1* gene *via* the *ERK–CREB* axis in cultured renal proximal tubule cells (4). Simvastatin-activated *HO1* led to remarkable protective effects against oleic acid-mediated lipotoxicity (4)

and pro-fibrotic events (30) by mitigating oxidative stress in renal proximal tubule cells. In our settings, pretreatment of renal proximal tubule cells with nicotine significantly attenuated simvastatin-mediated induction of *HO1* (Figure 1A) and *MnSOD* (Figure 1B). These results imply that pleiotropic (in this case antioxidant) effects of simvastatin might similarly be abolished in smokers. Our results are somewhat at odds with those that show protective effects of simvastatin against smoking-induced renal oxidative stress in rats (31) and mice (32). The main difference is that while we pre-treated cells with nicotine for 24 h prior to applying simvastatin, in those animal studies, both simvastatin and nicotine were applied together. In fact, when we applied nicotine and simvastatin together, we did not observe diminished *HO1*/*MnSOD* induction by simvastatin (data not shown). We believe that our experimental setting better emulates the impact of established smoking on simvastatin treatment.

Since both the *HO1* (5) and *MnSOD* (7) promoters are regulated, at least partly, *via CREB*, we also determined the activity of a *CRE*-luciferase reporter. Figure 2A shows that *CRE*-luc activity was highly stimulated by simvastatin, the extent of which diminished on pretreatment with nicotine. Interestingly, nicotine itself also suppresses *CRE* activity. These results suggest that nicotine suppresses *HO1* and *MnSOD via* suppression of CREB.

Our previous studies showed that chronic nicotine treatment increases the expression of the pro-oxidant *p66shc* gene in renal proximal tubule cells and in the kidney (13). We also showed that p66shc suppresses *HO1* (33) and *MnSOD* (15). Here we demonstrated that knockdown of p66shc alleviates the adverse effects of nicotine pretreatment on simvastatin-mediated *CRE* induction (Figure 2A). Conversely, knockdown of *p66shc* or overexpression of an activated *CREB* rescued simvastatin-mediated induction of *HO1* and *MnSOD* from adverse effects of chronic nicotine pretreatment (Figure 3A). These results demonstrate that nicotine suppresses *HO1* and *MnSOD via* suppression of *CREB* (Figure 4). It is unclear, however, how p66shc interferes with CREB activation. *p66shc* targets *ERK* (17), which is an activating kinase of *CREB* (4, 33). Indeed, nicotine attenuated simvastatin-dependent induction of *ERK* (measured by *ELK1* activation), which was relieved by p66shc knockdown (Figure 2B). Earlier we reported that inhibition of *ERK* inhibits simvastatin-dependent CREB activation (4). Hence, the nicotine-induced *p66shc* interferes with the *ERK*/*CREB* pathway, which leads to inhibition of *HO1* and *MnSOD*.

Our findings imply that antioxidant effects of simvastatin in the kidney might be attenuated in smokers and nicotine (E-cigarette) users. Hence, smoking cessation or other intervention that suppresses p66shc or augments *ERK*/*CREB* activation may increase therapeutic (antioxidant) efficiency of simvastatin in smokers.

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References

1 Gomez SI, Mihos CG, Pineda AM and Santana O: The pleiotropic effects of the hydroxy-methyl-glutaryl-CoA reductase inhibitors in renal disease. Int J Nephrol Renovasc Dis *7*: 123-130, 2014.

2 Sukhija R, Bursac Z, Kakar P, Fink L, Fort C, Satwani S, Aronow WS, Bansal D and Mehta JL: Effect of statins on the development of renal dysfunction. Am J Cardiol *101(7)*: 975-979, 2008.

3 Zhou MS, Schuman IH, Jaimes EA and Raij L: Renoprotection by statins is linked to a decrease in renal oxidative stress, TGF-beta, and fibronectin with concomitant increase in nitric oxide bioavailability. Am J Physiol Renal Physiol *295(1)*: F53-59, 2008.

4 Barnett M, Hall S, Dixit M and Arany I: Simvastatin attenuates oleic acid-induced oxidative stress through CREB-dependent induction of heme oxygenase-1 (HO-1) in renal proximal tubule cells. Pediatr Res *79(2)*: 234-250, 2015.

5 Astort F, Repetto EM, Rocha-Viegas L, Mercau ME, Puch SS, Finkielstein CV, Pecci A and Cymeryng CB: Role of CREB on heme oxygenase-1 induction in adrenal cells: Involvement of the PI3K pathway. J Mol Endocrinol *57(2)*: 113-124, 2016.

6 Chung YW, Kim HK, Kim IY, Yim MB and Chock PB: Dual function of protein kinase C (PKC) in 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced manganese superoxide dismutase (MnSOD) expression: Activation of CREB and FOXO3A by PKC-alpha phosphorylation and by PKC-mediated inactivation of AKT, respectively. J Biol Chem *286(34)*: 29681-29690, 2011.

7 Galeotti T, Pani G, Capone C, Bedogni B, Borrello S, Mancuso C and Eboli ML: Protective role of MnSOD and redox regulation of neuronal cell survival. Biomed Pharmacother *59(4)*: 197-203, 2005.

8 Orth SR, Viedt C and Ritz E: Adverse effects of smoking in the renal patient. Tohoku J Exp Med *194(1)*: 1-15, 2001.

9 Orth SR and Hallan SI: Smoking: A risk factor for progression of chronic kidney disease and for cardiovascular morbidity and mortality in renal patients – Absence of evidence or evidence of absence? Clin J Am Soc Nephrol *3(1)*: 226-236, 2008.

10 Nagasawa Y, Yamamoto R, Rakugi H and Isaka Y: Cigarette smoking and chronic kidney diseases. Hypertens Res *35(3)*: 261-265, 2012.

11 Arany I, Grifoni S, Clark JS, Csongradi E, Maric C and Juncos LA: Chronic nicotine exposure exacerbates acute renal ischemic injury. Am J Physiol Renal Physiol *301(1)*: F125-133, 2011.

12 Jain G and Jaimes EA: Nicotine signaling and progression of chronic kidney disease in smokers. Biochem Pharmacol *86(8)*: 1216-1223, 2013.

13 Arany I, Clark J, Reed DK and Juncos LA: Chronic nicotine exposure augments renal oxidative stress and injury through transcriptional activation of p66shc. Nephrol Dial Transplant *28(6)*: 1417-1425, 2013.

14 Arany I, Hall S, Reed DK, Reed CT and Dixit M: Nicotine enhances high-fat diet-induced oxidative stress in the kidney. Nicotine Tob Res *18(7)*: 1628-1634, 2016.
15 Arany I, Hall S, Reed DK and Dixit M: The pro-oxidant gene p66shc increases nicotine exposure-induced lipotoxic oxidative stress in renal proximal tubule cells. Mol Med Rep *14(3)*: 2771-2777, 2016.
16 Wei Z, Belal C, Tu W, Chigurupati S, Ameli NJ, Lu Y and Chan SL: Chronic nicotine administration impairs activation of cyclic AMP-response element binding protein and survival of newborn cells in the dentate gyrus. Stem Cells Dev *21(3)*: 411-422, 2012.
17 Arany I, Faisal A, Nagamine Y and Safirstein RL: p 66shc inhibits pro-survival epidermal growth factor receptor/ERK signaling during severe oxidative stress in mouse renal proximal tubule cells. J Biol Chem *283(10)*: 6110-6117, 2008.
18 Migliaccio E, Mele S, Salcini Anna E, Pelicci G, Lai K-Man V, Superti-Furga G, Pawson T, Paolo Di Fiore P, Lanfrancone L and Pelicci PG: Opposite effects of the p52shc/p46shc and p66shc splicing isoforms on the EGF receptor-MAP kinase-FOS signalling pathway. EMBO J *16(4)*: 706-716, 1997.
19 Arany I, Carter A, Hall S, Fulop T and Dixit M: Coenzyme Q10 protects renal proximal tubule cells against nicotine-induced apoptosis through induction of p66shc-dependent antioxidant responses. Apoptosis *22(2)*: 220-228, 2016.
20 Alam J and Cook JL: Transcriptional regulation of the heme oxygenase-1 gene *via* the stress response element pathway. Curr Pharm Des *9(30)*: 2499-2511, 2003.
21 Kuiperij HB, van der Horst A, Raaijmakers J, Weijzen S, Medema RH, Bos JL, Burgering BM and Zwartkruis FJ: Activation of FOXO transcription factors contributes to the antiproliferative effect of camp. Oncogene 24(12): 2087-2095, 2005.
22 Bonni A, Brunet A, West AE, Datta SR, Takasu MA and Greenberg ME: Cell survival promoted by the RAS-MAPK signaling pathway by transcription-dependent and-independent mechanisms. Science 286(5443): 1358-1362, 1999.
23 Johannessen M, Delghandi MP and Moens U: What turns CREB on? Cell Signal 16(11): 1211-1227, 2004.
24 Goven D, Boutten A, Lecon-Malas V, Boczkowski J and Bonay M: Prolonged cigarette smoke exposure decreases heme oxygenase-1 and alters NRF2 and BACH1 expression in human macrophages: Roles of the MAP kinases ERK(1/2) and JNK. FEBS Lett 583(21): 3508-3518, 2009.
25 Mercado N, Thimmulappa R, Thomas CM, Fenwick PS, Chana KK, Donnelly LE, Biswal S, Ito K and Barnes PJ: Decreased histone deacetylase 2 impairs NRF2 activation by oxidative stress. Biochem Biophys Res Commun 406(2): 292-298, 2011.
26 Milionis HJ, Rizos E and Mikhailidis DP: Smoking diminishes the beneficial effect of statins: Observations from the landmark trials. Angiology *52(9)*: 575-587, 2001.
27 Bedi O, Dhawan V, Sharma PL and Kumar P: Pleiotropic effects of statins: New therapeutic targets in drug design. Naunyn Schmiedebergs Arch Pharmacol *389(7)*: 695-712, 2016.
28 Honore PM, Jacobs R, Hendrickx I, De Waele E, Van Gorp V, De Regt J and Spapen HD: Statins and the kidney: Friend or foe? Blood Purif *43(1-3)*: 91-96, 2017.
29 Cachofeiro V, Goicochea M, de Vinuesa SG, Oubina P, Lahera V and Luno J: Oxidative stress and inflammation, a link between chronic kidney disease and cardiovascular disease. Kidney Int Suppl *111*: S4-9, 2008.
30 Clark JS, Carter AJ, Dixit M and Arany I: Simvastatin inhibits epithelial-to-mesenchymal transition through induction of HO-1 in cultured renal proximal tubule cells. In Vivo *30(4)*: 407-411, 2016.
31 Nasr MA, El-Gowilly SM and El-Mas MM: Comparable renovascular protective effects of moxonidine and simvastatin in rats exposed to cigarette smoke. Vascul Pharmacol *53(1-2)*: 53-60, 2010.
32 Ferreira TS, Lanzetti M, Barroso MV, Rueff-Barroso CR, Benjamim CF, de Brito-Gitirana L, Porto LC and Valenca SS: Oxidative stress and inflammation are differentially affected by atorvastatin, pravastatin, rosuvastatin, and simvastatin on lungs from mice exposed to cigarette smoke. Inflammation *37(5)*: 1355-1365, 2014.
33 Reed DK, Carter A, Dixit M and Arany I: P66shc-mediated toxicity of high-dose alpha-tocopherol in renal proximal tubule cells. J Physiol Biochem *73(2)*: 267-273, 2017.
34 Kisielow M, Kleiner S, Nagasawa M, Faisal A and Nagamine Y: Isoform-specific knockdown and expression of adaptor protein SHCA using small interfering rna. Biochem J *363(Pt 1)*: 1-5, 2002.

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