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 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 (DME) containing 10% fetal bovine serum and 1% antibiotic/antimycotic (Thermo Fisher Scientific, Waltham, MA, USA), in an atmosphere with 5% CO2 at 37°C. Cells were pre-

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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 (shp66shc) (19) to knockdown p66shc expression or with a plasmid containing constitutively activated CREB (VP16CREB) (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.

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 promoter activity of CREB compared with the control. Similar observations were made for the MnSOD gene promoter (Figure 2B). Simvastatin-mediated activation was significantly reduced by nicotine pretreatment.

determined whether p66shc mediates the above described adverse effect. Accordingly, NRK52E cells were cotransfected 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.
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 (VP16CREB) 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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