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Lack of modulatory effect of simvastatin on indoxyl sulfate-induced activation of cultured endothelial cells

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ABSTRACT

Aims: Endothelial dysfunction is a common manifestation of chronic kidney disease (CKD). The protein-bound uremic toxins have emerged as important factors associated with cardiovascular disease and the outcome of CKD. The effect of indoxyl sulfate (IS) on endothelial cells remains unclear.
Main methods: Human umbilical endothelial cells (HUVEC) were incubated using IS at two concentrations: 100 μM and 1000 μM over two periods of time: 16 and 48 h. HUVEC were also pre-treated with simvastatin to examine its effect. RT-PCR was used to assess changes in the gene expression of intracellular cell adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), Monocyte chemotactic protein-1 (MCP-1), E-selectin, and angiotensin receptor type 1 (AT1R). Protein abundance of the investigated molecules was assessed by immunoblotting.
Key findings: Treatment with 100 μM IS for 16 h induced a 2-fold increase in the expression of ICAM-1, VCAM-1, and MCP-1. At a concentration of 1000 μM, there was a 2–3-fold increase. An extended treatment period at low concentrations was associated with a 2–3 fold increase and the increase of ICAM-1 and VCAM-1 was more prominent under high concentration. Results of immunoblotting confirmed an increase in the abundance of ICAM-1, VCAM-1 and MCP-1. No significant change was noted in E-selectin and AT1R according to concentration or treatment duration. Pre-treatment with simvastatin did not alter IS-induced changes.
Significance: IS increased the expression of adhesion molecules of endothelial cells exhibiting a concentration and duration dependent pattern. Simvastatin did not demonstrate any effect on IS-associated endothelial activation.

Introduction

Chronic kidney disease (CKD) is associated with an increase in cardiovascular disease (Fort, 2005). Irrespective of underlying renal disease, patients with renal impairment manifest uremic symptoms as renal function progressively deteriorates. It has been postulated that renal failure syndrome is mainly due to a reduction in renal function, and the compounds retained in renal failure, so called uremic toxins produce a variety of deleterious effects involving many organ systems (Vanholder et al., 2008a). Protein-bound uremic toxins have been linked to long term outcomes both in pre- and chronic dialysis patients, indicating their pivotal role in CKD (Bammens et al., 2006; Barreto et al., 2009).

Endothelial dysfunction is a common feature of cardiovascular disease. With the high prevalence of cardiovascular disease in CKD, it is believed that renal impairment perpetuates endothelial injury, eventually leading to pathologic vasculature (Rabelink et al., 2010). The pathogenesis of endothelial dysfunction associated with renal disease is a multiple process and most of the factors also indicate a risk of cardiovascular disease. Nevertheless, information regarding whether uremic toxins can directly cause endothelial injury is limited (Tumur et al., 2010). Statins are competitive inhibitors of 3-hydroxy-3methylglutary coenzyme A (HMG-CoA) reductase. Clinical evidence strongly supports their administration to lower hyperlipidemia, to improve cardiovascular outcome. Furthermore, with their pleiotrophic properties, statins are able to modulate inflammatory reactions and have been considered vasculoprotective agents (Wang et al., 2008). In renal patients, accumulated evidence has supported the effectiveness of statins in reducing proteinuria, thereby providing renoprotection (Douglas et al., 2006).

The current study presents an investigation of the effect of protein-bound uremic toxin: indoxyl sulfate (IS) on endothelial cells. Leukocyte adhesion molecules expressed in endothelial cells were examined to

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evaluate the effect on endothelial activation. We further examined whether HMG-CoA reductase inhibitor, simvastatin influences IS-induced changes in endothelial cells.

Materials and methods

Materials

Human umbilical vein endothelial cells (HUVEC) were purchased from Bioresource Collection and Research Center (Hsinchu, Taiwan). The cells were seeded on 0.1% gelatin-coated culture flask (Sigma-Aldrich, Louis, MO, U.S.A.) and grown in M199 medium (GIBCO, California, U.S.A). In addition to 10% fetal bovine serum (FBS,GIBCO, California, U.S.A), 25 U/mL heparin and 30 μg/mL endothelial cell growth supplement (ECGS, Millipore Corporation, Billerica, MA, U.S.A.) was also added to the culture medium. The cultures were maintained at 37 °C in a fully humidified atmosphere of 5% CO_2 in air, and the culture medium was changed every 2 days. Cells were then detached using a trypsin-EDTA solution and subcultured to the next passage. All experiments were performed between cell passage 4 and 7.

Preparation of reagents

IS and simvastatin were purchased from Sigma (St. Louis, MO., USA). To examine the effects of IS, HUVEC were incubated at two concentrations: 100 μM and 1000 μM respectively for 2 periods of time: 16 h and 48 h. HUVEC was then collected to study gene expression and analyze proteins. To investigate the effect of simvastatin on IS-induced changes, HUVEC were pre-incubated with 0.5 μM and 2.5 μM respectively for 24 h and then treated with IS.

Gene expression study

Total RNA was extracted from the HUVEC using a Total RNA Mini kit following the manufacturer's instructions, and spectrophotometry at a wavelength of 260 nm was then used to detect total RNA concentrations. RNA was stored at −80 °C until use. A total of 1 μg RNA of each sample was reverse-transcribed using a First Strand cDNA Synthesis Kit. Real-time PCR was performed using the Light Cycler instrument with LightCycler ® TaqMan® Master, Universal ProbeLibrary Probe and primers of the target gene. The results of this study were normalized with housekeeping gene β-actin. The levels of mRNA expression were presented as the ratio of each mRNA to β-actin mRNA. The primer set of studied genes were as follows: ICAM-1(forward: AACCTCAGCCTCGCTATGG; reverse: ACTTTTGAGGGGGACACAGA), VCAM-1(forward: AAGGCAGGCTGTAAAAGAATTG; reverse: GTAGACCCTCGCTGGAACAG), MCP-1(forward: TTCTGTGCCTGCTGCTCAT; reverse: GGGGCATTGATTGCATCT), E-selectin (forward: AAAGGGTAGAATTCTGACAACTGG; reverse: TCCCTCTTGTTTTCCATTTCC), angiotensin receptor type 1 (ATR1, forward: ATTTTGTGAAAGAAGGAGCAAGA, reverse: TGCTCATTTGGTAGTGAAGTGC).

Protein abundance analysis (Western blotting)

All HUVEC (both the treated and control) were added to a protein lysis buffer solution containing 20-mM Tris–HCl (pH 7.4), 0.1% sodium dodecyl sulfate (SDS), 5-mM EDTA, 1% Triton X-100, and a protease inhibitor cocktail tablet (Roche, Penzberg, Germany). After determining the concentration, protein samples were then run on 6% SDS-polyacrylamide gel electrophoresis (PAGE) for ICAM-1, MCP-1, E-selectin and 10% for VCAM-1, ATR1, respectively, to transfer to the PVDF membranes. β-actin was used as the internal control in this study. For ICAM-1, the membrane was incubated with anti-ICAM-1 mouse monoclonal antibody (1:1000, ABCAM, UK) for 12 h after blocking with 5% skim milk in 0.1% Tris-buffered saline with Tween20 (TBS-T). For VCAM-1, the membrane was incubated with anti-VCAM-1 mouse monoclonal antibody (1:80, Santa Cruz, CA, USA), and with anti-MCP-1 mouse monoclonal antibody (1:150, Santa Cruz, CA, USA), anti-E-selectin rat monoclonal antibody (1:300, Santa Cruz, CA, USA) and anti-ATR1 mouse monoclonal antibody (1:200, Santa Cruz, CA, USA) for determination of MCP-1 E-selectin and ATR1. Finally, the membrane was incubated with goat anti-mouse IgG (1:5000 for ICAM-1 and ATR1; 1:3000 for VAM-1 and MCP-1, Jackson, USA) in conjunction with peroxidase-conjugated AffiniPure (1:5000). For E-selecin, goat anti-rat IgG (1:5000, Jackson, USA) was used as secondary antibody. The abundance of these molecules was then quantified by densitometric analysis. Changes in protein abundance were presented as percentages (%) of control animal values.

Statistic analysis

Data was presented as means ± SEMs. Statistical analysis of the data was performed using SPSS-PC software. Unpaired Student's *t* tests were used to compare differences between two groups. A *p* value of less than 0.05 was considered statistically significant for all tests.

Results

Effects on gene expression

Treatment with IS on HUVEC with different concentration and period (Figs. 1 and 2)

The results of RT-PCR are shown in Fig. 1. Cultured HUVEC treated with IS (100 μM) for 16 h induced a significant increase in the expression of ICAM-1 (177 ± 9%), VCAM-1 (171 ± 7%), MCP-1 (181 ± 10%) with no significant change in E-selectin (107 ± 2%) and AT1R (95 ± 4%). Treatment with higher dose (1000 μM) also elicited a marked increase in ICAM-1 (262 ± 9%), VCAM-1 (224 ± 8%), MCP-1 (261 ± 8%), but no change in E-selectin (129 ± 3%) and AT1R (124 ± 7%) was noted. An extension of the treatment period to 48 h with IS 100 μM was associated with a significant increase in ICAM-1 (286 ± 7%), VCAM-1 (243 ± 8%), MCP-1 (193 ± 9%) and expression of E-selectin and AT1R was not influenced (114 ± 3% and 111 ± 7%). A significant increase in ICAM-1 (597 ± 9%), VCAM-1 (502 ± 7%), MCP-1 (270 ± 10%) were observed with 1000 μM IS with no significant alteration in E-selectin (128 ± 6%) and AT1R (93 ± 4%).

Pre-incubation with simvastatin (Figs. 1 and 2)

Pre-incubation with simvastatin at a dosage of 0.5 μM followed by 16 h treatment with IS either at concentration of 100 μM or 1000 μM increased ICAM-1, VCAM-1 and MCP-1 significantly (ICAM-1: 183 ± 10%, 296 ± 5%; VCAM-1: 167 ± 8%, 209 ± 6%; MCP-1:199 ± 7%, 244 ± 6%, compared with the control, all $p<0.05$). The expression of E-selectin and AT1R was not altered (E-selectin: 117 ± 4%, 109 ± 2%; AT1R: 122 ± 10%, 89 ± 4%, all $p>0.05$). There was no significant difference between samples with and without simvastatin pre-incubation. With a higher concentration of simvastatin, 2.5 μM, followed by IS of 100 μM or 1000 μM, there was still significant increase in the expression of ICAM-1, VCAM-1 and MCP-1 (ICAM: 212 ± 10%, 281 ± 9%; VCAM: 183 ± 10%, 240 ± 5%; MCP-1: 163 ± 6%, 288 ± 4%; all $p<0.05$). We compared the expression of all molecules, and no significant difference was observed between the IS alone and the group having received pre-incubation with simvastatin. The expression of E-selectin and AT1R was not influenced (E-selectin: 98 ± 6%, 130 ± 4%; AT1R: 108 ± 6%, 94 ± 8%, all $p>0.05$).

After extending the treatment period to 48 h, pre-treatment with simvastatin 0.5 μM followed by 100 μM or 1000 μM of IS also induced a marked increase in gene expression (ICAM: 244 ± 8%, 622 ± 9%; VCAM: 271 ± 6%, 586 ± 10%; MCP-1: 183 ± 7%, 332 ± 9%; all $p<0.05$). No significant change was observed in E-selectin and AT1R (E-selectin: 101 ± 4%, 125 ± 6%; ATR1: 90 ± 3%, 129 ± 9%). Pre-