Original Research Article

Indoxyl sulfate stimulates oxidized LDL uptake through up-regulation of CD36 expression in THP-1 macrophages

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\textbf{Introduction}

Cardiovascular disease is the major cause of death in patients with chronic kidney disease (CKD) (Gansevoort et al., 2013). In particular, accelerated atherosclerosis leading to severe coronary artery disease has been observed in patients with CKD (Baber et al., 2012; Liu et al., 2012). Although the pathophysiology of accelerated atherosclerosis in patients with CKD is not completely understood, accumulation of uremic toxins is partly involved in the condition (Balla et al., 2013).
Indoxyl sulfate (IS) is a uremic toxin resulting from the bacterial metabolism of tryptophan in the colon (Evenepoel et al., 2009). In the setting of CKD, IS is accumulated in body fluids owing to the kidney function impairment, exerting renal and vascular toxicity (Niwa, 2010; Laibeuf et al., 2011). Due to its high binding affinity for albumin, IS cannot be efficiently removed by current dialysis techniques (Laibeuf et al., 2011). Serum IS level has been shown to be associated with risk factors of atherosclerosis in hemodialysis patients (Taki et al., 2007) and correlate with severity of coronary atherosclerosis in patients with stable angina (Hsu et al., 2013). As a result, AST-120, an oral absorbent to reduce serum level of IS, has been purchased from Yiyuan Biotech (Guangzhou, GD, China). Cell Counting Kit-8 (CCK-8) was purchased from Dojindo Laboratories (Kamimashiki, KU, Japan). Antibodies to phosphorylated and total ERK1/2 were obtained from Cell Signaling Technology (Beverly, MA, USA).

Flow cytometry

Both Ox-LDL uptake assay (Yang et al., 2011) and CD36 expression assay (Luan and Griffiths, 2006) were determined by flow cytometry as described previously. For Ox-LDL uptake assay, Dil-Ox-LDL was used as the ligand. Cells were treated for indicated time with various doses of IS in the presence or absence of 2 h pre-treatment with 250 μM SSO, 30 μM PD98059 or 2 μM U0126. Then cells were exposed to 20 mg/L Dil-Ox-LDL in the last 4 h of incubation. Cells were then harvested with trypsin, washed twice with cold PBS, and suspended in PBS. For CD36 expression assay, cells were treated for 24 h with 250 μM IS in the presence or absence of 2 h pre-treatment with 30 μM PD98059 or 2 μM U0126. Cells were then harvested with trypsin, washed twice with cold PBS. Subsequently, cells were incubated for 15 min in the dark with appropriate dilutions of FITC conjugated anti-human CD36.

The mean fluorescence intensities of Dil-ox-LDL and CD36 were analyzed by FACSCalibur flow cytometer (BD, Becton New Jersey, USA) using Flowjo software. At least 10,000 individual live cells were determined for each sample. Data were represented as a percentage of the control cells.

Western blotting

THP-1 macrophages were pre-treated with or without 30 μM PD98059 or 2 μM U0126 for 2 h prior to 250 μM IS stimulation for 15 min. And then cells were harvested and lysed with modified RIPA buffer in the presence of protease and phosphatase inhibitors (Sigma-Aldrich, St. Louis, MO, USA). After centrifuged for 15 min, the cleared cell lysates were collected. The protein content of the supernatants was measured by the Bradford assay (Bio-rad, Hercules, CA, USA). Equal amounts of protein were subjected to SDS-polyacrylamide gel electrophoresis followed by immunoblotting with specific antibodies. Band intensity was analyzed using program Imagej software. Data were represented as a percentage of the control cells.

Materials and methods

Cell culture

THP-1 cells were obtained from American Type Culture Collection (Manassas, VA, USA). THP-1 monocytes were differentiated into macrophages by incubation with 160 nM phorbol 12-myristate 13-acetate (PMA) for 72 h as described previously (Daigneault et al., 2010). THP-1 macrophages were cultured in RPMI 1640 medium containing 10% fetal calf serum, penicillin (100 units/ml) and streptomycin (100 μg/ml) at 37 °C in a humidified 5% CO2 incubator.

Reagents and antibodies

IS, PMA, and FITC conjugated anti-human CD36 were purchased from Sigma-Aldrich (St. Louis, MO, USA). Sulfo-succinimidyl oleate (SSO), a CD36 inhibitor, was purchased from Toronto Research Chemicals (Toronto, ON, Canada). PD98059 and U0126, were purchased form Cell Signaling Technology (Beverly, MA, USA) and Calbiochem (La Jolla, CA), respectively. 1,1’-Dioctadecyl-3,3,3’,3’-tetramethylindocarbocyanine-labeled ox-LDL (DiL-ox-LDL) was purchased from Yiyuan Biotech (Guangzhou, GD, China). Antibodies to phosphorylated and total ERK1/2 were obtained from Cell Signaling Technology (Beverly, MA, USA).

Western blotting

THP-1 macrophages were pre-treated with or without 30 μM PD98059 or 2 μM U0126 for 2 h prior to 250 μM IS stimulation for 15 min. And then cells were harvested and lysed with modified RIPA buffer in the presence of protease and phosphatase inhibitors (Sigma-Aldrich, St. Louis, MO, USA). After centrifuged for 15 min, the cleared cell lysates were collected. The protein content of the supernatants was measured by the Bradford assay (Bio-rad, Hercules, CA, USA). Equal amounts of protein were subjected to SDS-polyacrylamide gel electrophoresis followed by immunoblotting with specific antibodies. Band intensity was analyzed using program Imagej software. Data were represented as a percentage of the control cells.
Statistical analysis

Statistical analysis was performed using the GraphPad Prism 6 (GraphPad Software, La Jolla, CA, USA). Multiple comparisons were made using one-way ANOVA followed by a post hoc Newman–Keuls test at the significance level $\alpha = 0.05$.

Results

**IS stimulates Ox-LDL uptake in THP-1 macrophages in both dose- and time-dependent manners**

The concentration range of IS tested with THP-1 macrophages for 24 h incubation was from 1 to 500 μM. At concentrations of 100–500 μM, IS significantly stimulated Ox-LDL uptake in THP-1 macrophages in a dose-dependent manner. The stimulations were 128.05%, 140.56% and 161.26% of untreated control at the concentration of 100, 250 and 500 μM, respectively (Fig. 1A). In addition, 250 μM IS time-dependently stimulated Ox-LDL uptake in THP-1 macrophages from 12 to 48 h. The stimulations were 127.35%, 148.34%, 170.82% and 188.05% of untreated control at the treatment time of 12, 24, 36 and 48 h, respectively (Fig. 1B).

Further, the CD36 inhibitor, SSO (250 μM), and the ERK1/2 inhibitors, PD98059 (30 μM) and U0126 (2 μM), significantly inhibited Ox-LDL uptake increased by IS (250 μM) after 24 h of incubation, indicating that IS may stimulate Ox-LDL uptake in THP-1 macrophages at least partly via CD36 and ERK1/2 MAPK pathway (Fig. 1C).

**IS increases expression of CD36 in THP-1 macrophages**

Flow cytometric analysis revealed that 250 μM IS significantly increased protein expression of CD36, indicating that IS might stimulate Ox-LDL uptake in THP-1 macrophages via increasing CD36 expression (Fig. 1D). Further, inhibition of ERK1/2 MAPK pathway with 30 μM PD98059 and 2 μM U0126 significantly decreased IS-increased CD36 expression, indicating IS may increase CD36 expression partly via ERK1/2 MAPK pathway (Fig. 1D).

![Fig. 1 - Effects of IS on Ox-LDL uptake and CD36 expression in THP-1 derived macrophages. Ox-LDL uptake and CD36 expression were analyzed by flow cytometry as described in 'Materials and methods' section.](image-url)

(A) IS dose-dependently stimulated Ox-LDL uptake in THP-1 macrophages after 24 h of incubation at concentrations indicated. (B) IS (250 μM) time-dependently stimulated Ox-LDL uptake in THP-1 macrophages after indicated time of incubation. (C) The stimulating effect of IS (250 μM) on Ox-LDL uptake in THP-1 macrophages after 24 h of incubation was inhibited by SSO (250 μM), PD98059 (30 μM) and U0126 (2 μM). (D) IS (250 μM) stimulated CD36 expression in THP-1 macrophages after 24 h of incubation, and the increase was inhibited by SSO (250 μM), PD98059 (30 μM) and U0126 (2 μM). Data presented as means ± SD from 3 experiments with triplicates. *Statistically significant as compared with controls, #statistically significant versus IS.
IS activates ERK1/2 MAPK pathway in THP-1 macrophages

Western blotting analysis demonstrated that 250 μM IS activated ERK1/2 MAPK pathway by significantly increasing its phosphorylation level in THP-1 macrophages after 15 min of incubation (Fig. 2A and B). Furthermore, the activation of ERK1/2 MAPK by IS was significantly inhibited by 30 μM PD98059 and 2 μM U0126 (Fig. 2A and B). Western blotting analysis also showed that IS treatment did not affect the total protein level of ERK1/2 MAPK under the same experimental condition (Fig. 2A and C), indicating that IS did not affect protein phosphorylation by changing the protein level. Thus, IS may affect THP-1 macrophages through activation of ERK1/2 MAPK pathway.

IS does not affect cell viability of THP-1 macrophages

CCK-8 assay revealed that IS co-culture at various doses of up to 500 μM for 24 h did not affect cell viability of THP-1 macrophages (Fig. 3A). Incubation with IS (250 μM) for 12, 24, 36, 48 h did not affect cell viability of THP-1 macrophages (Fig. 3B). In addition, the presence of 250 μM SSO, 30 μM PD98059 or 2 μM U0126 did not affect cell viability of THP-1 macrophages (Fig. 3C).

Discussion

The present study provides evidence, for the first time, that IS, a protein-bound uremic toxin, may directly mediate potentially pro-atherogenic effect in macrophages. In particular, we have observed direct stimulation of Ox-LDL uptake in THP-1 macrophages by this toxin in cell culture. These findings have considerable implications for atherosclerosis in patients with CKD, where levels of this toxin are elevated.

IS has been shown to accelerate the progression of CKD (Meijers and Evenepoel, 2011), but data are limited about its actions on cardiovascular system. A recent clinical study has shown that serum IS levels are correlate with the severity of coronary atherosclerosis in patients with stable angina, suggesting that IS may be involved in the pathogenesis of atherosclerosis (Hsu et al., 2013). Given that uptake of Ox-LDL in macrophages play a crucial role in the development of atherosclerosis (Moore and Tabas, 2011), we sought to determine whether IS may stimulate the uptake of Ox-LDL in THP-1 macrophages. The present study found that IS increased Ox-LDL uptake in THP-1 macrophage in both dose- and time-dependent manners. The increase was observed at concentrations of IS from 100 to 500 μM, which were consistent with circulating levels of IS in patients with CKD (Miyamoto et al., 2011). Thus, it is possible that IS-stimulated Ox-LDL uptake in macrophages is one of the causes of atherosclerosis in patients with CKD.

Macrophage scavenger receptor family, mainly the type A scavenger receptor (SR-A) and the type B scavenger receptor CD36, provide a high-affinity uptake mechanism for the accumulation of lipid derived from Ox-LDL (Kunathoor et al., 2002; Kzhyshkowska et al., 2012). Previous data have showed that Ox-LDL was primarily mediated by CD36 in macrophages (Nicholson and Hajjar, 2004; Kzhyshkowska et al., 2012). A study comparing aortic lesions in apolipoprotein E-null mice with knockout of SR-A, CD36 and both of these receptors demonstrated that the absence of CD36 protects against atherosclerosis with no additional protection provided...
by absence of scavenger receptor A I/II, suggesting a limiting role of CD36 in lesion formation (Kuchibhotla et al., 2008). In present study, we found that the CD36 inhibitor SSO had an inhibitory effect on IS-stimulated Ox-LDL uptake in THP-1 macrophages, indicating that IS-stimulated Ox-LDL uptake in THP-1 macrophages is CD36-dependent. We also confirmed that IS stimulated a significant increase on CD36 expression in THP-1 macrophages, indicating that IS may stimulate Ox-LDL uptake in THP-1 macrophages at least partly via increasing CD36 expression. Thus, these findings suggest that CD36 may play an important role in the IS-stimulated Ox-LDL uptake in THP-1 macrophages.

ERK1/2 MAPK pathway has been shown to be involved in Ox-LDL uptake in macrophages and the development of atherosclerosis (Li et al., 2010). Previous studies have also shown that ERK1/2 MAPK pathway was involved in activation of peroxisome proliferator-activated receptor gamma (PPARγ) (Yang et al., 2010) and nuclear factor (erythroid-derived 2)-like 2 (Nrf2) (Zipper and Mulcahy, 2000), both of which mediated the CD36 expression at the transcriptional level in macrophages (Ishii et al., 2004; Nicholson and Hajjar, 2004). Therefore we evaluated the involvement of ERK1/2 MAPK in the effect of IS on THP-1 macrophages, and we found that pretreatment of THP-1 macrophages with ERK1/2 MAPK inhibitors, PD98059 and U0126, could both cause inhibition of both CD36 expression and Ox-LDL uptake in THP-1 macrophages. We also found that IS induced ERK1/2 MAPK phosphorylation, which was inhibited by PD98059 and U0126. These findings indicate that IS appears to stimulate CD36 expression and Ox-LDL uptake in macrophages at least partly via activation of ERK1/2 MAPK pathway. Of note, all of the above observations occurred in the absence of any significant reduction in cell viability, as assessed by the CCK-8 assay.

IS is the product of diet protein being converted by intestinal bacteria to indole and finally IS in the body (Evenepoel et al., 2009). Thus, inhibition of its biosynthesis with protein restriction, prebiotics, probiotics and oral absorbent, may be a potential therapeutic approach to the treatment of atherosclerosis in patients with CKD. As mentioned, oral absorbent has been reported to protect against atherosclerosis accelerated by kidney disease (Yamamoto et al., 2011). In addition, recent data have shown the effects of very low protein diet (Marzocco et al., 2013), prebiotics and probiotics (Rossi et al., 2012) on reducing IS levels in CKD. As food and its composition are very important environmental influences on gut microbiome activities and human health (Berger, 2013; Tuohy et al., 2014), we propose that studies with protein restriction, dietary prebiotics and probiotics in atherosclerosis with co-morbid CKD may be of considerable therapeutic interest.
In conclusion, the present study in vitro showed that IS, a uraemic toxin, increased THP-1 macrophage Ox-LDL uptake at least partly through up-regulation of CD36 expression, partly via ERK1/2 MAPK pathway. This might be one of the mechanisms underlying the progression of atherosclerosis in patients with CKD. Studies investigating the potential effects of IS on foam cell formation and the mechanism involved, such as expression of other scavenger receptors, activation of signal pathways, cholesterol metabolism and efflux, will enable us to further understanding the potential role of IS in atherogenesis.

Conflict of interest statement

None.

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References


