主論文の要旨

Indoxyl Sulfate Counteracts Endothelial Effects of Erythropoietin Through Suppression of Akt Phosphorylation

（インドキシル硫酸は、Aktリン酸化の抑制を介して
内皮細胞に対するエリスロポエチンの効果を阻止する）

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Introduction

Erythropoietin (EPO) is used to treat anemia in patients with chronic kidney disease (CKD). However, a wide variation in individual response to EPO is often observed, causing EPO resistance. EPO exhibits not only hematopoietic but also extra-hematopoietic functions such as endothelial effects. The endothelium was the first non-hematopoietic tissue to be identified as a physiological target of EPO, because EPO receptor (EPOR) is expressed on human vascular endothelial cells. The effects of EPO on endothelial cells are stimulation of proliferation, suppression of apoptosis, and increased production of nitric oxide due to activation of endothelial nitric oxide synthase (eNOS). Therefore, EPO might prevent progression of cardiovascular disease (CVD), because endothelial injury is the first step of the development of CVD. Furthermore, EPO induces production of several erythroid-stimulating factors such as thrombospondin-1 (TSP-1) in endothelial cells. Indoxyl sulfate, a uremic toxin, is involved in endothelial dysfunction, and consequently the pathogenesis of CKD-associated CVD.

The present study aimed to determine whether indoxyl sulfate affects EPO-induced extra-hematopoietic effects such as survival/proliferation, anti-apoptosis function, eNOS activation, and TSP-1 expression in endothelial cells, and how indoxyl sulfate affects the pathway of EPO signaling.

Methods

Human umbilical vein endothelial cells (HUVECs) were incubated with or without indoxyl sulfate, and stimulated with or without EPO. Cell survival/proliferation was measured by using a 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) reduction assay kit. The apoptosis was detected by evaluation of caspase 3/7 activity and terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay. Phospho-extracelluar signal-regulated kinases 1/2 (ERK 1/2), phospho-Akt, phospho-eNOS, TSP-1, and phospho-EPOR were measured by western blotting.

Results

Indoxyl sulfate inhibits EPO-induced survival/proliferation and anti-apoptosis function in HUVECs (Fig. 1). EPO induced phosphorylation of ERK1/2 and Akt with a peak at 10 min. Indoxyl sulfate did not affect EPO-induced ERK1/2 phosphorylation. However, indoxyl sulfate suppressed EPO-induced Akt phosphorylation (Fig. 2). Prior incubation with an Akt kinase inhibitor significantly suppressed EPO-induced cell proliferation and anti-apoptosis function in HUVECs (Fig. 3). EPO induced eNOS phosphorylation which increased at 10 min and sustained thereafter. Preincubation with indoxyl sulfate inhibited EPO-induced eNOS phosphorylation. In addition, either pretreatment with an Akt kinase inhibitor or transfection with Akt siRNA inhibited EPO-induced eNOS phosphorylation (Fig. 4). EPO increased the expression of TSP-1 in a time and dose-dependent manner. Indoxyl sulfate significantly inhibited EPO-induced expression of TSP-1.

Further, either treatment with an Akt kinase inhibitor or transfection with an Akt siRNA decreased the expression of TSP-1 (Fig. 5). EPO increased the tyrosine phosphorylation level of a protein between 61 kDa and 89 kDa. This level was reduced by indoxyl sulfate stimulation regardless of the presence or absence of EPO. Phosphorylation of EPOR by EPO increased from 5 min and reached a peak at 10 min. Pretreatment with indoxyl sulfate prevented EPO-induced phosphorylation of EPOR (Fig. 6). Knockdown of EPOR abolished EPO-induced cell proliferation, anti-apoptosis, Akt phosphorylation, eNOS phosphorylation, and TSP-1 expression (Fig. 7).

Discussion

In the present study, we demonstrated indoxyl sulfate counteracted EPO-induced survival/proliferation, anti-apoptosis function, and eNOS phosphorylation through suppression of Akt phosphorylation. Thus, indoxyl sulfate accumulated in serum of patients with CKD counteracts the beneficial effects of EPO on endothelial cells.

A main cause of anemia in advanced CKD is deficient production of EPO. Indoxyl sulfate suppresses EPO expression through reduced nuclear accumulation of hypoxia-inducible transcription factors and hypoxia-responsive element-luciferase activity following hypoxia in HepG2 cells. We focused on the expression of TSP-1, because TSP-1 stimulates erythroblast proliferation and counteracts the inhibitory action of insulin-like growth factor-binding protein 3（IGFBP-3). Indoxyl sulfate inhibited EPO-induced TSP-1 expression in HUVECs through suppression of Akt phosphorylation. In addition to high serum level of IGFBP-3 observed in patients with CKD, indoxyl sulfate might suppress erythroblast proliferation by deficient production of TSP-1.

Another cause of anemia is EPO resistance. However, little is known whether indoxyl sulfate is directly involved in EPO resistance in patients with CKD, although hemodialysis patients with increased serum level of indoxyl sulfate required a higher EPO dosage. The present study revealed that indoxyl sulfate inhibits EPO-induced phosphorylation of EPOR in HUVECs. Thus, we propose that indoxyl sulfate is a risk factor for EPO resistance in patients with CKD. However, the present study did not identify how indoxyl sulfate suppresses EPO-induced phosphorylation of EPOR.

Conclusion

Indoxyl sulfate negatively regulates EPOR-Akt pathway in endothelial cells, and might contribute to EPO resistance and endothelial dysfunction in patients with CKD.