Circulation Journal
Official Journal of the Japanese Circulation Society
http://www.j-circ.or.jp

ORIGINAL ARTICLE
Vascular Biology and Vascular Medicine

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

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Background: Erythropoietin (EPO) is used to treat anemia in patients with chronic kidney disease (CKD). A wide variation in individual response to EPO, however, is often observed, causing EPO resistance. EPO exhibits not only hematopoietic but also extra-hematopoietic functions such as endothelial effects. Indoxyl sulfate, a uremic toxin, is involved in endothelial dysfunction, and consequently, the pathogenesis of CKD-associated cardiovascular disease. The aim of the present study was to determine the effect of indoxyl sulfate on the extra-hematopoietic functions of EPO in human umbilical vein endothelial cells (HUVECs).

Methods and Results: HUVECs were incubated with or without indoxyl sulfate or an Akt inhibitor, and then stimulated with or without EPO. Indoxyl sulfate suppressed EPO-induced survival/proliferation, anti-apoptosis function, phosphorylation of endothelial nitric oxide synthase, and the expression of thrombospondin-1, an erythroid-stimulating factor, in HUVECs. Although EPO induced phosphorylation of both Akt and extracellular signal-regulated kinases (ERK) in HUVECs, indoxyl sulfate suppressed phosphorylation of Akt but not ERK. An Akt kinase inhibitor or Akt small interfering RNA suppressed all the EPO-induced cellular effects in HUVECs. As a site of action of indoxyl sulfate on EPO signaling, indoxyl sulfate attenuated EPO-induced tyrosine phosphorylation of EPO receptor (EPOR) in HUVECs.

Conclusions: Indoxyl sulfate negatively regulates the EPOR-Akt pathway in endothelial cells, and might contribute to EPO resistance and endothelial dysfunction in patients with CKD. (*Circ J* 2013; **77:** 1326–1336)

Key Words: Cardiovascular disease; Chronic kidney disease; Endothelial cells; Erythropoietin resistance; Indoxyl sulfate

Erythropoietin (EPO) is an erythropoiesis-stimulating cytokine, and is produced mainly by the adult kidney in response to hypoxia.[1] The relative deficiency of EPO is a major cause of anemia in patients with chronic kidney disease (CKD).[2,3] The use of recombinant EPO has improved anemia in patients with CKD, reduced mortality and improved quality of life.[4] EPO stimulates production of red blood cells by promoting proliferation and differentiation of erythroid progenitors and preventing apoptosis of late erythroid progenitors.[5–7] Furthermore, EPO exhibits extra-hematopoietic effects beyond its hematopoietic function. 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 from coronary, pulmonary, and cerebral arteries, umbilical veins and dermal vessels.[8] The effects of EPO on endothelial cells are stimulation of proliferation, suppression of apoptosis of endothelial cells, and increased production of nitric oxide due to activation of endothelial nitric oxide synthase (eNOS).[9,10] Therefore, EPO might prevent progression of cardiovascular disease (CVD),[11] because endothelial injury is the first step in the development of CVD. Furthermore, EPO induces production of several erythroid-stimulating factors such as thrombospondin-1 (TSP-1) in endothelial cells. TSP-1 prevents inflammation, stimulates erythroblast proliferation, and counteracts insulin-like growth factor-binding protein 3 (IGFBP-3)-mediated suppression of erythroblast proliferation.[12,13]

Part of the dietary protein-derived tryptophan is metabolized into indole by tryptophanase in intestinal bacteria, which is absorbed into the blood from the intestine, and is metabolized to indoxyl sulfate in the liver. Indoxyl sulfate is normally excreted into urine, whereas an inadequate renal clear-

Received July 10, 2012; revised manuscript received November 5, 2012; accepted December 12, 2012; released online January 19, 2013 Time for primary review: 19 days

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ISSN-1346-9843 doi:10.1253/circj.CJ-12-0884

ance of indoxyl sulfate during CKD leads to elevated serum levels.[14–17] In addition, indoxyl sulfate cannot be efficiently removed by hemodialysis due to its high binding ratio with albumin, because the indoxyl sulfate-albumin complex is larger than the pores in the dialysis membrane. Overload of indoxyl sulfate taken up by proximal tubular cells through organic anion transporters 1 and 3 generates reactive oxygen species (ROS), and consequently causes activation of nuclear factor-κB (NF-κB).[18–20] Activation of NF-κB by indoxyl sulfate induces cellular senescence and expression of transforming growth factor-β1 (TGF-β1), α-smooth muscle actin (α-SMA), and monocyte chemotactic protein-1 (MCP-1).[20–22] Indoxyl sulfate leads to epithelial-mesenchymal transition.[23] These changes facilitate interstitial fibrosis and glomerular sclerosis, accelerating the progression of CKD.[14–17,23,24] Besides its renal impact, serum indoxyl sulfate level is associated with CVD.[24–26] In endothelial cells, indoxyl sulfate suppresses the nitric oxide level and cell proliferation.[27] In addition, indoxyl sulfate induces the expression of MCP-1 and intercellular adhesion molecule-1 (ICAM-1) through ROS-induced activation of NF-κB,[28] and also leads to cellular senescence through upregulation of p53 expression.[29] Indoxyl sulfate inhibits the viability of endothelial progenitor cells.[30]

In the present study, we focused on the cross-talk between indoxyl sulfate and EPO signaling in endothelial cells, because hemodialysis patients with increased serum indoxyl sulfate level required a higher dosage of EPO.[31] Therefore, the aim of this study was 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

Reagents

Antibodies and the other reagents were obtained from the following suppliers: anti-α-tubulin, from Calbiochem (La Jolla, CA, USA); anti-phospho-Akt (Ser473), anti-Akt, anti-phospho-extracellular signal-regulated kinases 1/2 (anti-phospho-ERK1/2), anti-ERK1/2, anti-phospho-eNOS (Ser1177) as well as anti-rabbit IgG horseradish peroxidase (HRP)-linked antibody and anti-mouse IgG, HRP-linked antibodies, from Cell Signaling Technology (Beverly, MA, USA); anti-eNOS, from BD Biosciences (Mississauga, ON, Canada); anti-phosphotyrosine (4G10), from Upstate Biotechnology (Lake Placid, NY, USA); anti-TSP-1, from Thermo Scientific (Waltham, MA, USA); anti-phospho-EPOR (Tyr456), anti-EPOR, from Santa Cruz Biotechnology (Santa Cruz, CA, USA); indoxyl sulfate, from Sigma Chemical (St. Louis, MO, USA); EGM-2-MV BulletKit, from Lonza (Walkersville, MD, USA); trypsin-EDTA, from Gibco (Invitrogen, Grand Island, NY, USA); penicillin and streptomycin, from Nacalai Tesque (Kyoto, Japan); human EPO, from Cell Signaling Technology (Beverly, MA, USA); CellTiter 96® Aqueous One Solution Cell Proliferation Assay and Apo-ONE Homogenous Caspase-3/7 Assay, from Promega (Madison, WI, USA); Akt kinase inhibitor, from WAKO (Osaka, Japan); terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) kit, from Takara (Tokyo, Japan); and Lipofectamine RNAiMAX Reagent, from Invitrogen (Tokyo, Japan).

Cell Culture

Human umbilical vein endothelial cells (HUVECs) purchased from Cascade Biologics (Portland, OR, USA) were maintained in EGM-2 medium containing 5% fetal bovine serum (FBS) under standard cell culture conditions (humidified atmosphere, 5% CO_2, 37°C). The response of HUVECs to indoxyl sulfate (250 μmol/L) and EPO (10U/ml) was analyzed between passages 4 and 6 after a 24-h incubation in EGM-2 medium containing 0.5% FBS.

Measurement of Cell Survival/Proliferation

The survival/proliferation of HUVECs was measured using a 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) reduction assay kit (CellTiter 96® Aqueous One Solution Cell Proliferation Assay, Promega). Serum-starved HUVECs (2.5×10^3 cells/well) in a 96-well plate were incubated with or without indoxyl sulfate (250 μmol/L) for 24 h before EPO (10 U/ml) stimulation for 48 h. In another experiment, serum-starved HUVECs (2.5×10^3 cells/well) in a 96-well plate were incubated with or without an Akt kinase inhibitor (1 μmol/L) for 30 min before EPO (10 U/ml) stimulation for 48 h. For gene knockdown experiments, HUVECs in a 96-well plate were transfected with small interfering RNA (siRNA) for 24 h before EPO (10 U/ml) stimulation for 48 h. Thereafter, cells were subjected to the MTS assay according to the manufacturer's instruction.

Apo-ONE Homogenous Caspase-3/7 Assay

The apoptosis was detected by evaluation of caspase 3/7 activity. Serum-starved HUVECs (5×10^3 cells/well) in a 96-well plate were incubated with or without indoxyl sulfate (250 μmol/L) for 24 h before EPO (10 U/ml) stimulation for 48 h. In another experiment, serum-starved HUVECs (5×10^3 cells/well) in a 96-well plate were incubated with or without an Akt kinase inhibitor (1 μmol/L) for 30 min before EPO (10 U/ml) stimulation for 48 h. Thereafter, cells were subjected to this assay according to the manufacturer's instruction.

TUNEL Assay

Serum-starved HUVECs were incubated with or without indoxyl sulfate (250 μmol/L) for 24 h before EPO (10 U/ml) treatment for 48 h in 3.5-cm dishes. Cellular apoptosis was detected by TUNEL assay, using an apoptosis detection TUNEL kit according to the manufacturer's protocol. TUNEL-positive cells were counted in 6 randomly representative fields under light microscopy. Representative pictures were captured under microscopy (×100).

Transfection of siRNA

A small interfering RNA specific to Akt (Akt siRNA) was purchased from Nippon Gene Material (Tokyo, Japan); siRNA specific to EPOR (EPOR siRNA) was purchased from Qiagen (Tokyo, Japan). Lipofectamine was used to transfect the siRNA into the HUVECs according to the manufacturer's protocol. HUVECs were treated with or without Akt siRNA (10 nmol/L) or EPOR siRNA (10 nmol/L) for 24 h. Protein expression of Akt and EPOR was determined by immunoblotting.

Immunoblotting

After starvation, HUVECs were incubated with or without indoxyl sulfate (250 μmol/L) before EPO (10 U/ml) stimulation. In another experiment, HUVECs were incubated with or without an Akt kinase inhibitor (1 μmol/L) before EPO (10 U/ml) stimulation. For gene knockdown experiments, HUVECs were transfected with siRNA for 24 h before EPO (10 U/ml) stimulation. Cells were lysed in lysis buffer (1% NP-40, 150 mmol/L NaCl, 50 mmol/L Tris-HCl, complete in-

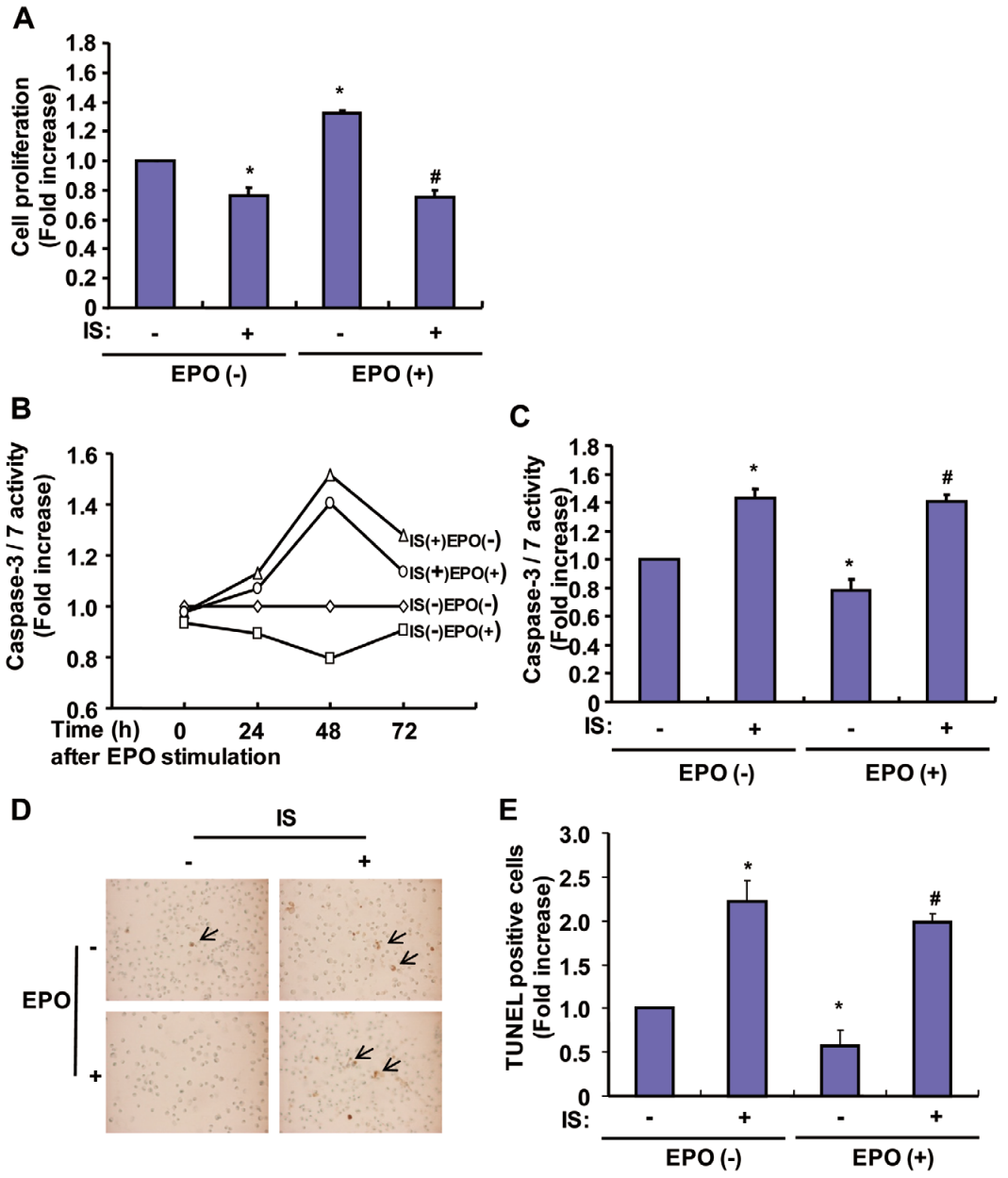

Figure 1. Indoxyl sulfate (IS) inhibits erythropoietin (EPO)-induced cell survival/proliferation and anti-apoptosis function in human umbilical vein endothelial cells (HUVECs). (**A**) Serum-starved HUVECs were incubated with or without IS (250 μmol/L) for 24 h, and then stimulated with or without EPO (10 U/ml) for 48 h. Thereafter, survival/proliferation of HUVECs was measured by MTS cell proliferation assay. (**B**) Serum-starved HUVECs were incubated with or without IS (250 μmol/L) for 24 h, and then stimulated with or without EPO (10 U/ml) for 0, 24, 48, 72 h. Apoptosis was measured by Apo-ONE Homogenous Caspase-3/7 Assay. (**C**) Experimental conditions as described in (**A**) except measurement of caspase 3/7 activity. (**D**,**E**) Experimental conditions as described in (**A**) except for detection of apoptosis by TUNEL assay. TUNEL-positive cells were counted in 6 randomly selected fields under light microscopy (×100). Data given as mean±SE from (**A**,**C**) 4 independent experiments or (**B**,**E**) 3 independent experiments. *P<0.05 vs. untreated cells; #P<0.05 vs. EPO-treated cells.

hibitor, 10% glycerol, phosphatase inhibitor) and clarified by centrifugation, and the supernatant was collected. After cell lysates were fractionated by SDS-PAGE on polyacrylamide gels, proteins were transferred to PVDF membranes (Immobilon-P, Millipore, Bedford, MA, USA). Phospho-ERK1/2, phospho-Akt, and phospho-eNOS, TSP-1, and phospho-EPOR were detected using specific antibodies and normalized to ERK1/2, Akt, eNOS, α-tubulin, and EPOR, respectively. The protein bands were visualized using the enhanced Chemi-Lumi One system (Nacalai Tesque, Kyoto, Japan).

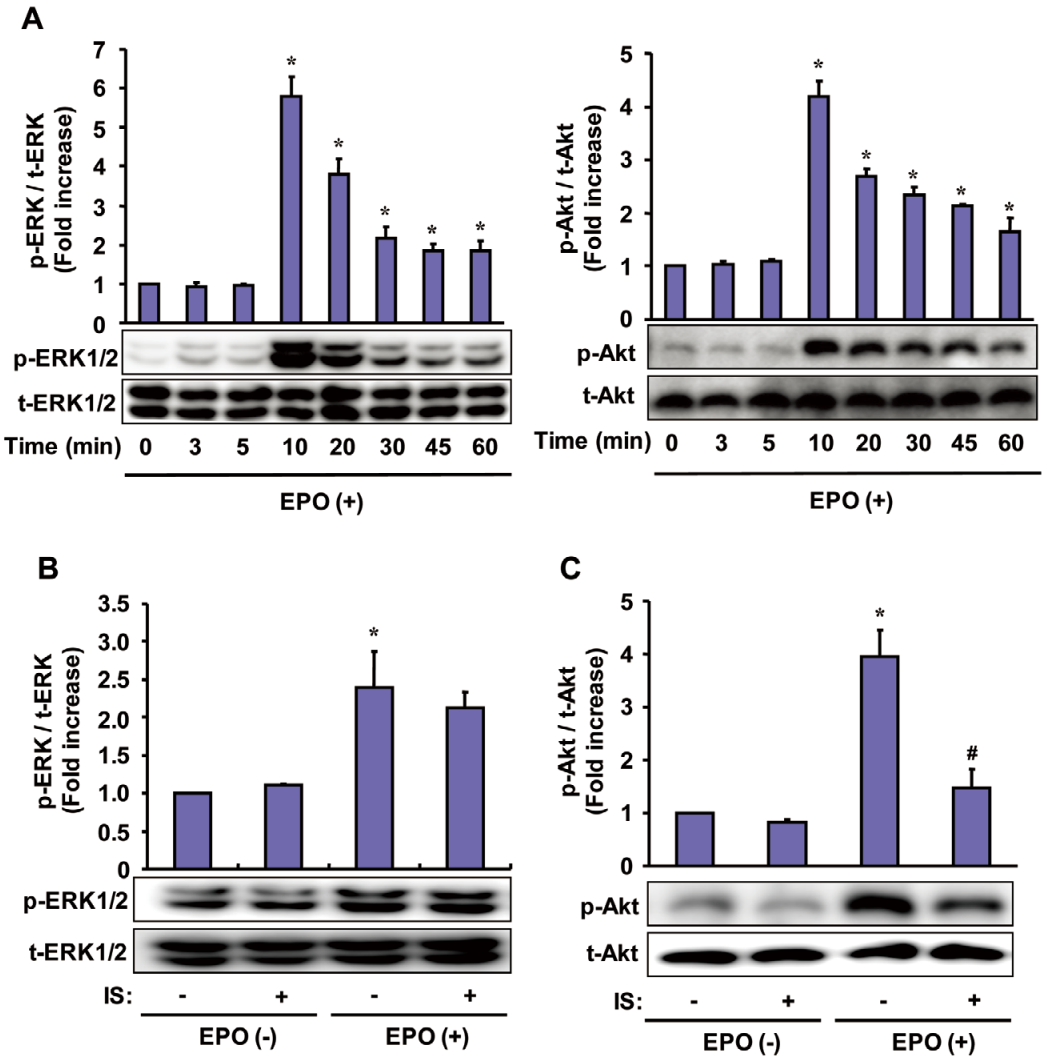

Figure 2. Indoxyl sulfate (IS) suppresses erythropoietin (EPO)-induced phosphorylation of Akt but not extracellular signal-regulated kinases (ERK) in human umbilical vein endothelial cells (HUVECs). (**A**) Serum-starved HUVECs were treated with EPO (10 U/ml) for 0, 3, 5, 10, 20, 30, 45, and 60 min. Cell lysates were immunoblotted using anti-phospho-ERK1/2, anti-ERK1/2, anti-phospho-Akt, and anti-Akt antibodies. (**B**) Serum-starved HUVECs were incubated with IS (250 μmol/L) for 24 h, and then stimulated with or without EPO (10 U/ml) for 10 min. Cell lysates were immunoblotted using anti-phospho-ERK1/2 and anti-ERK1/2 antibodies. (**C**) Cell lysates were immunoblotted using anti-phospho-Akt and anti-Akt antibodies. (**A**–**C**) Intensity of the phospho-ERK bands and phospho-Akt bands was quantified, and results normalized to amount of ERK and Akt, respectively, are expressed as ratios (fold increase) of the control value. Data given as mean±SE of (**A**) 3 independent experiments, and (**B**,**C**) 4 independent experiments. *P<0.05 vs. 0 min (**A**), and untreated cells (**B**,**C**); #P<0.05 vs. EPO-treated cells (**B**,**C**).

Statistical Analysis

Results are expressed as mean±SE. Data between groups were compared using analysis of variance and Fisher's protected least significance difference test. Results were considered statistically significant at P<0.05.

Results

Indoxyl Sulfate Inhibits EPO-Induced Survival/Proliferation and Anti-Apoptosis Function in HUVECs

The serum concentration of indoxyl sulfate in uremic patients is 50–90-fold higher than in healthy subjects.[16,32] The present study used indoxyl sulfate at a concentration of 250 μmol/L, which is comparable to the mean serum concentration in hemodialysis patients.[16,32] Because EPO induces survival/proliferation of HUVECs,[10] we first determined the effect of indoxyl sulfate on EPO-induced cell survival/proliferation. HUVECs were incubated with or without indoxyl sulfate for 24 h, and then the cells were stimulated with or without EPO for 48 h. Survival of HUVECs was significantly suppressed in indoxyl sulfate-treated cells even in the presence of EPO (Figure 1A). Further, cell survival in indoxyl sulfate+EPO was not significantly different from that in indoxyl sulfate alone. Thus, prior incubation with indoxyl sulfate significantly inhibited EPO-induced cell proliferation. Based on these results, we hypothesized that indoxyl sulfate induces endothelial cell death by

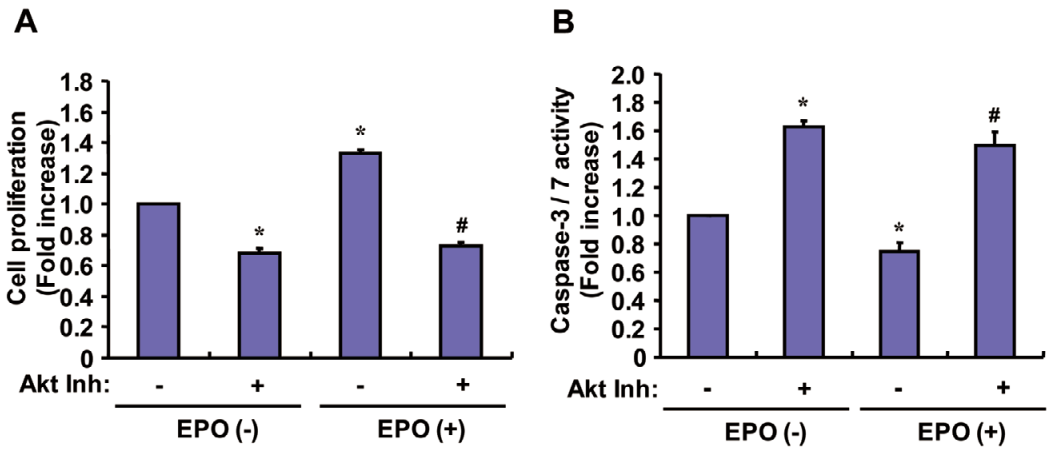

Figure 3. Akt kinase inhibitor suppresses erythropoietin (EPO)-induced survival/proliferation and anti-apoptosis function in human umbilical vein endothelial cells (HUVECs). (**A**) Serum-starved HUVECs were treated with an Akt kinase inhibitor (1 μmol/L) for 30 min before incubation with or without EPO (10 U/ml) for 48 h. Thereafter, survival/proliferation of HUVECs was measured by MTS assay. (**B**) Experimental conditions as described in (**A**) except for measurement of caspase 3/7 activity. Data given as mean±SE of 4 independent experiments. *P<0.05 vs. untreated cells; #P<0.05 vs. EPO-treated cells.

stimulating apoptosis. Apoptosis was evaluated by measurement of caspase 3/7 activity. HUVECs were incubated with or without indoxyl sulfate for 24 h, and then stimulated with or without EPO for 0, 24, 48 and 72 h. The most prominent difference in caspase 3/7 activity among groups was observed at 48 h after EPO stimulation (**Figure 1B**). At 48 h after EPO stimulation the activity of caspase 3/7 was significantly increased in indoxyl sulfate-treated cells even in the presence of EPO (**Figure 1C**). Furthermore, activity of caspase 3/7 in indoxyl sulfate+EPO was not significantly different from that in indoxyl sulfate alone, although EPO suppressed its activity. These findings were confirmed by TUNEL staining. Indoxyl sulfate significantly increased the number of TUNEL-positive cells in HUVECs. EPO significantly suppressed the number of TUNEL-positive cells, while indoxyl sulfate+EPO increased it (**Figures 1D,E**). Thus, indoxyl sulfate inhibits survival/proliferation of HUVECs through induction of apoptosis even when HUVECs are stimulated by EPO.

Indoxyl Sulfate Inhibits EPO-Induced Cell Survival/Proliferation and Anti-Apoptosis Function Through Suppression of Akt Phosphorylation

EPO-induced survival/proliferation is caused by activation of ERK1/2 and Akt.[10,33–35] Therefore, we examined the effect of indoxyl sulfate on phosphorylation of ERK and Akt. Before analysis of these effects, we confirmed that EPO induced phosphorylation of ERK and Akt with a peak at 10 min (**Figure 2A**). Based on these results, HUVECs were incubated with or without indoxyl sulfate for 24 h, and then the cells were stimulated with or without EPO for 10 min. We first focused on whether indoxyl sulfate suppresses EPO-induced ERK phosphorylation, because it suppressed vascular endothelial growth factor-induced ERK phosphorylation.[36] Indoxyl sulfate did not affect EPO-induced ERK1/2 phosphorylation (**Figure 2B**). Indoxyl sulfate, however, suppressed EPO-induced Akt phosphorylation (**Figure 2C**). These results suggest that inhibitory effects of indoxyl sulfate on EPO-induced survival/proliferation of HUVECs may be due to suppression of Akt phosphorylation.

To determine the role of Akt activation in EPO-induced cell survival/proliferation and anti-apoptosis function, HUVECs were incubated with or without an Akt kinase inhibitor for 30 min, and then the cells were stimulated with or without EPO for 48 h. Survival of HUVECs was significantly suppressed in the Akt kinase inhibitor-treated cells even in the presence of EPO (**Figure 3A**). Thus, prior incubation with an Akt kinase inhibitor significantly suppressed EPO-induced cell proliferation. Furthermore, activity of caspase 3/7 was significantly increased in the Akt kinase inhibitor-treated cells even in the presence of EPO (**Figure 3B**). In addition, activity of caspase 3/7 in Akt kinase inhibitor+EPO was not significantly different from that in the Akt kinase inhibitor alone, although EPO suppressed its activity (**Figure 3B**). Taken together, indoxyl sulfate inhibits EPO-induced cell survival/proliferation and anti-apoptosis function in HUVECs through suppression of Akt phosphorylation.

Indoxyl Sulfate Inhibits EPO-Induced eNOS Activation Through Suppression of Akt Phosphorylation

Activated Akt phosphorylates (activates) eNOS in response to various stimuli.[37–40] EPO induced eNOS phosphorylation on Ser1177, which increased at 10 min and was sustained thereafter (**Figure 4A**). We then examined the effect of indoxyl sulfate on EPO-induced eNOS phosphorylation. HUVECs were incubated with or without indoxyl sulfate for 24 h, and then the cells were stimulated with or without EPO for 10 min. Preincubation with indoxyl sulfate for 24 h inhibited EPO-induced eNOS phosphorylation (**Figure 4B**). In addition, either pretreatment with an Akt kinase inhibitor or transfection with Akt siRNA inhibited EPO-induced eNOS phosphorylation (**Figures 4C,D**). Thus, indoxyl sulfate inhibits EPO-induced eNOS activation in HUVECs through suppression of Akt phosphorylation.

Indoxyl Sulfate Inhibits EPO-Induced TSP-1 Expression Through Suppression of Akt Phosphorylation

TSP-1 expression is decreased in endothelial cells of Akt1-

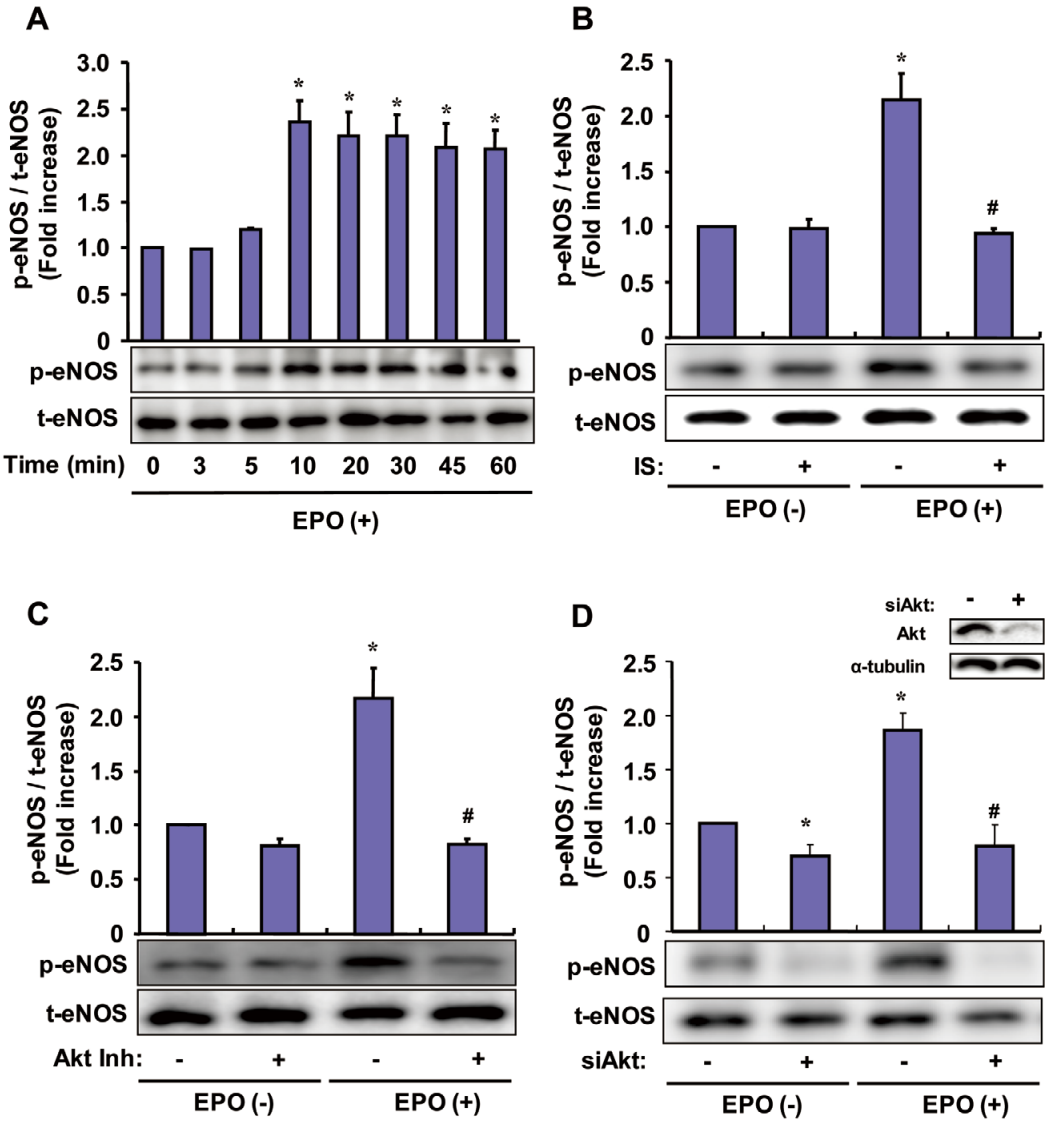

Figure 4. Indoxyl sulfate (IS) inhibits endothelial nitric oxide synthase (eNOS) phosphorylation through suppression of Akt phosphorylation in human umbilical vein endothelial cells (HUVECs). (**A**) Experimental conditions as described in **Figure 2A**. (**B**) Experimental conditions as described in **Figure 2B**. (**C**) Experimental as described in **Figure 3A** except for treatment with or without EPO for 10 min. (**D**) HUVECs were treated with or without Akt small interfering RNA (siRNA; 10 nmol/L), and then stimulated with or without EPO (10 U/ml) for 10 min. Cell lysates from EPO-untreated cells were immunoblotted using anti-Akt and anti-α-tubulin antibodies. (**A**–**D**) Cell lysates were immunoblotted using anti-phospho-eNOS and anti-eNOS antibodies. Intensity of phospho-eNOS bands was quantified, and results normalized to amount of eNOS are expressed as ratios (fold increase) of the control value. Data given as mean±SE of (**A**,**D**) 3 independent experiments; and (**B**,**C**) 4 independent experiments. *P<0.05 vs. 0 min (**A**) or untreated cells (**B**–**D**); #P<0.05 vs. EPO-treated cells (**B**–**D**).

knockout mice.[40] We confirmed that EPO increased the expression of TSP-1 in a time- and dose-dependent manner (**Figures 5A**,**B**). To determine the effects of indoxyl sulfate on EPO-induced expression of TSP-1, HUVECs were incubated with or without indoxyl sulfate for 24 h, and then the cells were stimulated with or without EPO for 24 h. Indoxyl sulfate significantly inhibited EPO-induced expression of TSP-1 (**Figure 5C**) Further, either treatment with an Akt kinase inhibitor or transfection with Akt siRNA decreased the expression of TSP-1 (**Figures 5D**,**E**). Thus, indoxyl sulfate inhibits EPO-induced expression of TSP-1 through suppression of Akt phosphorylation.

Indoxyl Sulfate Inhibits EPO-Induced Phosphorylation of EPOR

To determine the effects of indoxyl sulfate on EPO signaling, we analyzed the phospho-tyrosine state of proteins in cell lysates, because activated EPOR induces tyrosine phosphorylation of EPOR itself and of downstream molecules. HUVECs were incubated with or without indoxyl sulfate for 24 h, and then the cells were stimulated with or without EPO for 10 min. Thereafter, cell lysates were immunoblotted with an anti-

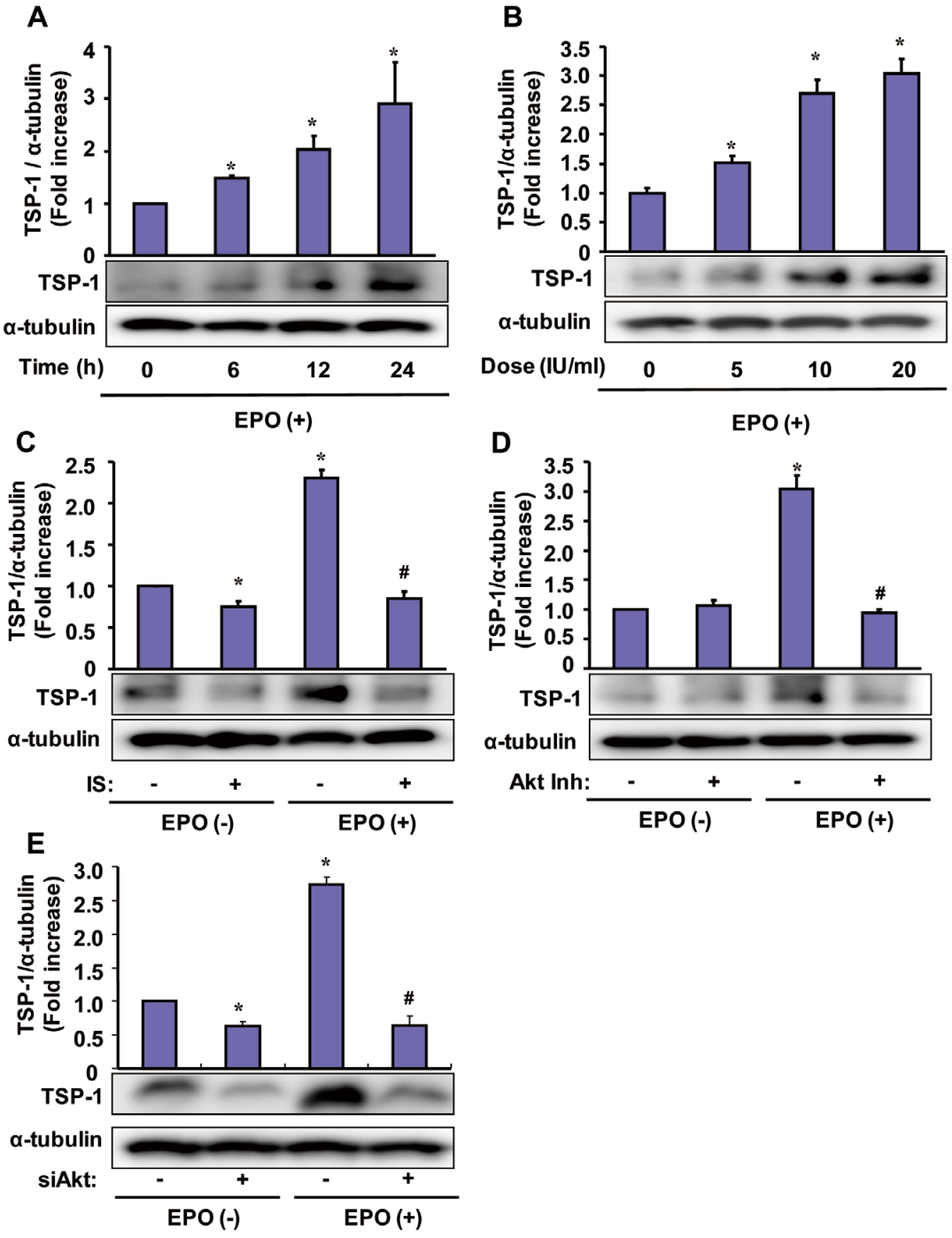

Figure 5. Indoxyl sulfate (IS) inhibits erythropoietin (EPO)-induced thrombospondin-1 (TSP-1) expression in human umbilical vein endothelial cells (HUVECs). (**A**) Serum-starved HUVECs were incubated with EPO (10 U/ml) for 0, 6, 12, and 24 h. (**B**) Serum-starved HUVECs were incubated with EPO at 0, 5, 10 and 20 U/ml for 24 h. (**C**) Experimental conditions as described in **Figure 2B** except for treatment with or without EPO for 24 h. (**D**) Experimental conditions as described in **Figure 3A** except treatment with or without EPO for 24 h. (**E**) Experimental conditions as described in **Figure 4D** except treatment with or without EPO for 24 h. (**A**–**E**) Cell lysates were immunoblotted using anti-TSP-1 and anti-α-tubulin antibodies. Intensity of TSP-1 bands was quantified, and results normalized to amount of α-tubulin are expressed as ratios (fold increase) of the control value. Data given as mean±SE of (**A**–**D**) 4 independent experiments or (**E**) 3 independent experiments. *P<0.05 vs. 0 h (**A**,**B**) or untreated cells (**C**–**E**); #P<0.05 vs. EPO-treated cells (**C**–**E**).

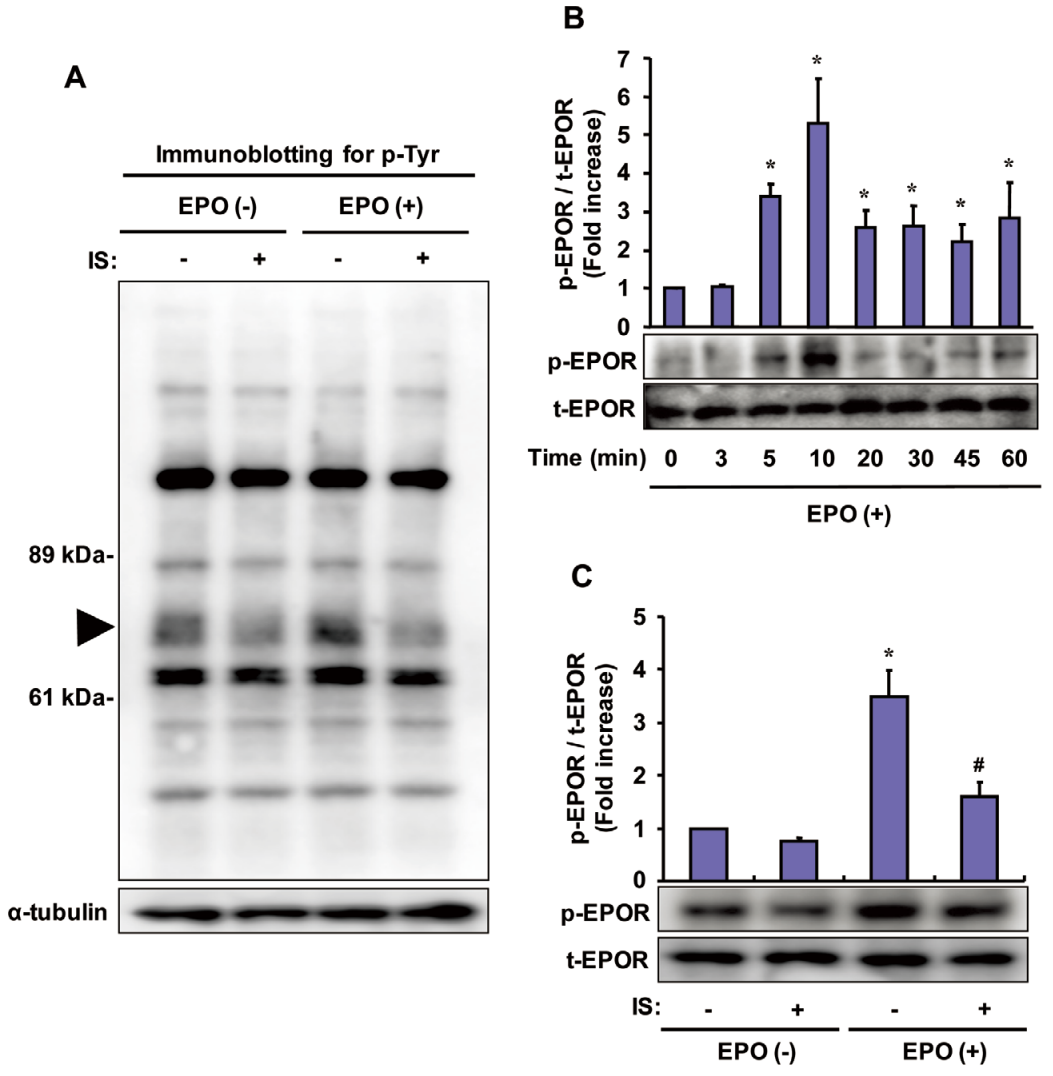

Figure 6. Indoxyl sulfate (IS) inhibits erythropoietin (EPO)-induced phosphorylation of EPO receptor (EPOR) in human umbilical vein endothelial cells (HUVECs). (**A**) Experimental conditions as described in **Figure 2B**. Cell lysates were immunoblotted using anti-phospho-tyrosine (4G10) and anti-*α*-tubulin antibodies. (**B**) Experimental conditions as described in **Figure 2A**. (**C**) Experimental conditions as described in **Figure 2B**. (**B**,**C**) Cell lysates were immunoblotted using anti-phospho-EPOR and anti-EPOR antibodies. Intensity of phospho-EPOR bands was quantified, and results normalized to amount of EPOR are expressed as ratios (fold increase) of the control value. Data given as mean±SE of (**A**,**C**) 4 independent experiments or (**B**) 3 independent experiments. *P<0.05 vs. untreated cells (**A**,**C**) or 0 min (**B**); #P<0.05 vs. EPO-treated cells (**C**).

phospho-tyrosine antibody. EPO increased the phosphorylation level of a protein between 61 kDa and 89 kDa (**Figure 6A**). This level was reduced by indoxyl sulfate stimulation regardless of the presence or absence of EPO (**Figure 6A**). Based on the molecular weight (64–78 kDa), we hypothesized that this protein is EPOR. Phosphorylated EPOR protein is observed even in the absence of EPO, and this might be due to the effect of basal medium (0.5% FBS).[42] We confirmed that phosphorylation of EPOR by EPO increased from 5 min and reached a peak at 10 min (**Figure 6B**). Pretreatment with indoxyl sulfate prevented EPO-induced phosphorylation of EPOR (**Figure 6C**). These findings suggest that indoxyl sulfate inhibits EPO-induced tyrosine phosphorylation of EPOR with subsequent suppression of Akt phosphorylation.

We also determined the role of EPOR in EPO-induced beneficial effects by using specific EPOR siRNA (**Figure 7A**). Knockdown of EPOR abolished EPO-induced cell proliferation (**Figure 7B**), anti-apoptosis (**Figure 7C**), Akt phosphorylation (**Figure 7D**), eNOS phosphorylation (**Figure 7E**), and TSP-1 expression (**Figure 7F**). Taken together, indoxyl sulfate counteracts endothelial effects of EPO through suppression of EPOR phosphorylation.

Discussion

The novel findings of the present study are: (1) indoxyl sulfate inhibits EPO-induced cell survival/proliferation, anti-apoptosis function, eNOS phosphorylation, and TSP-1 expression

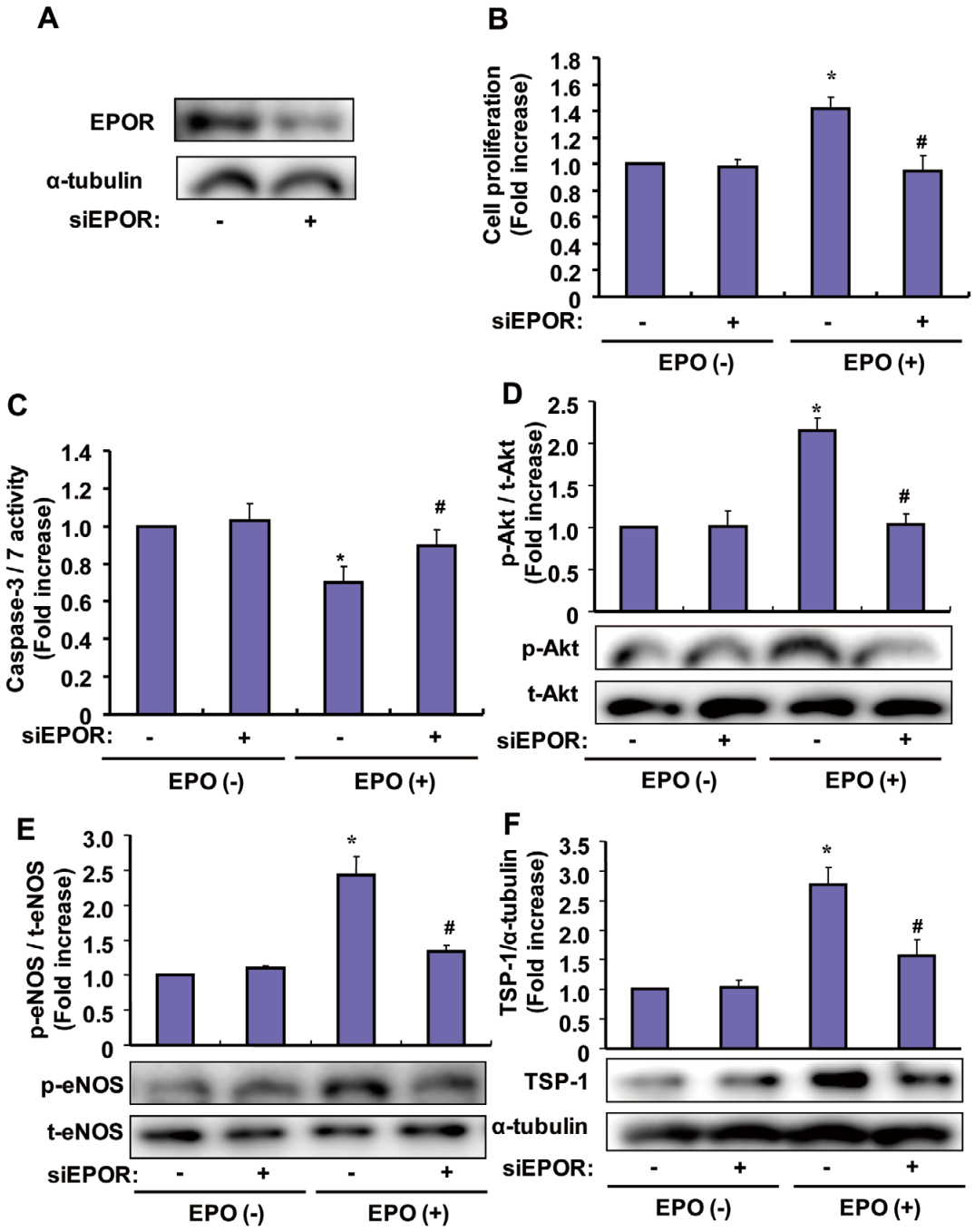

Figure 7. (**A**) Human umbilical vein endothelial cells (HUVECs) were treated with or without erythropoietin receptor (EPOR) small interfering RNA (siRNA; 10 nmol/L) for 24 h. Cell lysates were immunoblotted using anti-EPOR and anti-α-tubulin antibodies. (**B**) HUVECs were treated with or without EPOR siRNA (10 nmol/L) for 24 h, and then stimulated with or without erythropoietin (EPO; 10 U/ml) for 48 h. Thereafter, survival/proliferation of HUVECs was measured by MTS cell proliferation assay. (**C**) Experimental conditions as described in (**B**) except for measurement of caspase 3/7 activity. (**D**–**E**) Experimental conditions as described in (**B**) except for treatment with or without EPO (10 U/ml) for 10 min. (**F**) Experimental conditions as described in (**B**) except for treatment with or without EPO (10 U/ml) for 24 h. (**D**–**F**) Cell lysates were immunoblotted using anti-phospho-Akt, anti-Akt, anti-phospho-eNOS, anti-eNOS, anti-TSP-1 and anti-α-tubulin antibodies. Intensity of phospho-Akt bands, phospho-eNOS bands and TSP-1 bands were quantified, and results normalized to the amounts of Akt, eNOS and α-tubulin, respectively, are expressed as ratios (fold increase) of control value. Data given as mean±SE from 3 independent experiments. *P<0.05 vs. untreated cells; #P<0.05 vs. EPO-treated cells. eNOS, endothelial nitric oxide synthase; TSP-1, thrombospondin-1.

through suppression of Akt phosphorylation in HUVECs; and (2) indoxyl sulfate suppresses EPO-induced tyrosine phosphorylation of EPOR in HUVECs. Thus, indoxyl sulfate negatively regulates the EPOR-Akt pathway in endothelial cells, and might be involved in EPO resistance and endothelial dysfunction in patients with CKD.

Patients with CKD often suffer from anemia, and require erythropoiesis-stimulating agents for the treatment of anemia. Recombinant human EPO ameliorates progression of arteriosclerosis in addition to improvement of anemia in patients with CKD.[11] Large clinical trials, however, demonstrated that targeting higher hemoglobin levels with EPO is not cardioprotective.[43,44] In the present study, we examined whether indoxyl sulfate affects the beneficial effects of EPO on HUVECs, because endothelial injury induces initiation and development of arteriosclerosis. Indoxyl sulfate prevents EPO-induced survival/proliferation, anti-apoptosis function, and eNOS phosphorylation through suppression of Akt phosphorylation. Thus, indoxyl sulfate accumulated in the serum of CKD patients counteracts the beneficial effects of EPO on endothelial cells.

A main cause of anemia in advanced CKD is deficient production of EPO.[2,3] 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.[45] We hypothesized, however, that another mechanism also exists in anemia associated with CKD, because hemodialysis patients with increased serum indoxyl sulfate require a higher dosage of EPO.[31] We focused on the expression of TSP-1, because TSP-1 stimulates erythroblast proliferation and counteracts the inhibitory action of IGFBP-3.[12,13] Indoxyl sulfate inhibited EPO-induced TSP-1 expression in HUVECs through suppression of Akt phosphorylation (**Figure 5**). In addition to high IGFBP-3 serum level observed in patients with CKD,[46] indoxyl sulfate might suppress erythroblast proliferation by deficient production of TSP-1.

Another cause of anemia is EPO resistance.[47] Little is known, however, whether indoxyl sulfate is directly involved in EPO resistance in patients with CKD, although hemodialysis patients with increased indoxyl sulfate serum level required a higher EPO dosage.[31] The present study showed that indoxyl sulfate inhibits EPO-induced phosphorylation of EPOR in HUVECs (**Figure 6**). Thus, we propose that indoxyl sulfate is a risk factor for EPO resistance in patients with CKD. The present study, however, did not identify how indoxyl sulfate suppresses EPO-induced phosphorylation of EPOR. We speculate that indoxyl sulfate upregulates activation or expression of protein tyrosine phosphatase (PTP), which dephosphorylates EPO-induced phosphorylation of EPOR, because there was no change in expression of EPOR between untreated and indoxyl sulfate-treated cells (**Figure 6**), and some PTPs cause insulin resistance by dephosphorylation of insulin receptor, a kind of receptor-type tyrosine kinase, as well as EPOR.[48] We suggest that indoxyl sulfate induces site-selective dephosphorylation of EPOR, because it suppressed EPO-induced phosphorylation of Akt but not ERK (**Figure 2**). In addition, phosphatidylinositol 3-kinase (an upstream molecule of Akt) is site-selectively associated with phosphorylated EPOR, and does not affect EPO-induced ERK phosphorylation in human erythroid progenitors and UT7 cells.[33] Therefore, the upregulated PTP activity or expression might site-selectively dephosphorylate the phosphorylated EPOR. The issue of site selectivity in the dephosphorylation of EPOR by PTP, however, remains poorly understood, although site-selective dephosphorylation of platelet-derived growth factor β-receptor, a kind of receptor-type tyrosine kinase, as well as EPOR, by PTPs has been investigated in detail.[49–52] Further study is required to clarify what kind of PTP activity or expression is upregulated by indoxyl sulfate, and site-selectively dephosphorylates the phosphorylated EPOR in HUVECs.

Acknowledgments

Grants: This work was supported by a grant for a pathophysiological research conference in CKD from the Kidney Foundation, Japan and a research grant from the Japanese Association of Dialysis Physicians. H. Shimizu, S. Saito and T. Niwa belong to the endowed department (Kureha Co).

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