Erythropoietin and Hypertension

Erythropoietin Increases Expression and Function of Transient Receptor Potential Canonical 5 Channels

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Abstract—Hypertension is a common complication in hemodialysis patients during erythropoietin (EPO) treatment. The underlying mechanisms of EPO-induced hypertension still remain to be determined. Increased transient receptor potential canonical (TRPC) channels have been associated with hypertension. Now, TRPC gene expression was investigated using quantitative real-time RT-PCR and immunoblotting in cultured human endothelial cells and in monocytes from hemodialysis patients. EPO dose-dependently increased TRPC5 mRNA in endothelial cells. EPO increased TRPC5 mRNA stability, that is, EPO prolonged the half-life period for TRPC5 mRNA from 16 hours (control) to 24 hours (P<0.05). The poly(A) tail length was measured by rapid amplification of cDNA ends-poly(A) test. Increased TRPC5 mRNA stability was attributed to longer 3' poly(A) tail lengths after EPO administration. EPO also significantly increased TRPC5 channel protein abundance by 70% (P<0.05). Whole-cell patch clamp showed that angiotensin II–induced, TRPC5-mediated currents were dramatically increased in endothelial cells treated with EPO. Fluorescent dye techniques confirmed that increased calcium influx after EPO treatment was abolished after TRPC5 knockdown (P<0.05). EPO also significantly increased intracellular reactive oxygen species production. Knockdown of TRPC5 alleviated EPO-induced reactive oxygen species generation in endothelial cells (P<0.05). In vivo, EPO-treated hemodialysis patients showed significantly increased amounts of TRPC5 mRNA in monocytes compared with EPO-free hemodialysis patients (6.0±2.4 [n=12] versus 1.0±0.5 [n=9]; P<0.01). Patients undergoing EPO treatment also showed significantly elevated systolic blood pressure (160±7 versus 139±6 mm Hg; P<0.05). Our findings suggest that upregulated functional TRPC5 gene may be one cause of EPO-induced hypertension in patients with chronic kidney disease. (Hypertension. 2011;58:317-324.)

Key Words: transient receptor potential channel ■ erythropoietin ■ hypertension

Elevated arterial blood pressure is a common adverse effect of erythropoietin (EPO) administration in patients and animal models with chronic kidney disease.1-4 Several observations indicate that EPO-induced hypertension is not only mediated by changes in erythrocyte mass but also by direct adverse effects of EPO. The underlying mechanisms of EPO-induced rise in blood pressure still remain to be determined.4-5 Considerable evidence has been accumulated for impaired cellular calcium homeostasis in EPO-associated hypertension. EPO significantly increases cytosolic free calcium concentration and expands sarcoplasmic calcium stores in platelets from essential hypertensive or EPO-associated hypertension patients and cultured vascular smooth muscle cells from rats.6-8

Transient receptor potential canonical (TRPC) channels are calcium-permeable nonselective cation channels that had been identified in many cell types, including endothelial cells and peripheral blood cells.9 We previously showed significantly increased expression of functional TRPC5 channel protein in patients with essential hypertension and in spontaneously hypertensive rats.10-12 In the present study, we hypothesized that EPO increases the expression and function of the TRPC5 gene, finally contributing to the pathogenesis of hypertension. To test this hypothesis, we examined the effects of EPO on TRPC5 channel expression and function in cultured human endothelial cells and peripheral blood cells.

Experimental Procedures

Cell Culture

Human umbilical vein endothelial cell–derived EA.hy926 cells (American Type Culture Collection) were maintained at 37°C and 5% CO2 in DMEM containing 10% FCS, 100 U/mL of penicillin, and 100 µg/mL of streptomycin (BIOCHROM AG, Berlin, Germany). After detachment with 0.25% trypsin, cells were seeded in plates as appropriate, that is, 6-well plates for RNA and protein isolation, 12-well plates for patch clamp experiments, and 96-well...
plates for intracellular calcium measurement. All of the experiments were performed using cells between passages 5 and 8. Cells were serum starved and incubated with different doses of EPO for 24 to 48 hours, as indicated.

Isolation of RNA and cDNA Synthesis
Total RNA was isolated from cells using the RNeasy minikit (Qiagen, Hilden, Germany). RNA was used to synthesize first-strand cDNA using the Transcriptor First Strand cDNA Synthesis kit (Roche Diagnostics, Mannheim, Germany). PCR was performed using a reverse transcription mixture consisting of 1 μg of total RNA template, anchored-oligo(dT)18 primer, and transcriptase reverse transcriptase and incubated according to the following procedure, denaturation at 65°C for 10 minutes, followed by 50°C for 60 minutes and heating at 85°C for 5 minutes.

Quantitative Real-Time RT-PCR
Quantitative real-time RT-PCR was performed as described by our group. The primers for coding regions of TRPC type 3 (TRPC3), type 5 (TRPC5), type 6 (TRPC6), or GAPDH were as follows: TRPC3 (NM_001130698.1) forward GCGCCGGAGGAGGAGGAAG-GAAG and reverse AGGTCCGGCCCGTGGGAGAA; TRPC5 (NM_012471.2) forward CCACCAGCTATCAGATAAGG and reverse AGGTCCGGCCCGTGGGAGAA; TRPC6 (NM_001130698.1) forward GCGCCGGAGGAGGAG-GAAG and reverse AGGTCCGGCCCGTGGGAGAA; TRPC5 (NM_001130698.1) forward GCGCCGGAGGAGGAGGAAG and reverse AGGTCCGGCCCGTGGGAGAA; and GAPDH (NM_002046.3) forward TGTTCGACAGTCAGCCGCATCTTC and reverse CTTC-CCCATCTTGCTGCATGGAGC; and GAPDH (NM_002046.3) forward TGTTCGACAGTCAGCCGCATCTTC and reverse GTTGACACCAGGCGCGCCAATACG.

Quantitative real-time RT-PCR was performed using FastStart DNA Master SYBR Green I (Roche Diagnostics). PCR amplification conditions were 30 to 40 cycles of denaturation at 95°C for 10 seconds, annealing at 60°C for 10 seconds, and extension at 72°C for 15 seconds. After quantification, melting curves were generated with 1% serum for 10 hours, actinomycin D was added, and the decrease of TRPC5 mRNA abundance was determined by quantitative real-time RT-PCR.

mRNA Decay Analysis
The effect of EPO on TRPC5 mRNA stability was examined by inhibiting mRNA transcription using actinomycin D (1 μmol/L). After the cells had been incubated with EPO or vehicle in DMEM with 1% serum for 10 hours, actinomycin D was added, and the reduction of TRPC5 mRNA abundance was determined by quantitative real-time RT-PCR.

Rapid Amplification of cDNA Ends-Poly(A) Test
To measure the 3′ poly(A) tail lengths of TRPC5 mRNA, rapid amplification of cDNA ends-poly(A) test was performed using a commercially available ALL-TAIL kit following the manufacturer’s instructions (Bioo Scientific, Austin, TX). First, T4-RNA-ligase2 was used to join an adenylated adaptor oligonucleotide 5′rAppTT-TAACCCCGGAATTCAG(3d) dC′3′ to the 3′ end of total RNA. Next, reverse transcription was performed using a primer specific to the adenylated adaptor to initiate the production of cDNA. The primers used for amplification of the poly(A) tail and a short stretch of the 3′ untranslated region of TRPC5 transcripts were forward AGAACCTGCGCTACATCTCC and reverse CGTGAATTCCGC-GTTAAA. PCR products were separated on 1.8% agarose gels and were visualized by ethidium bromide.

Knockdown of TRPC5 by Small Interfering RNA
Endothelial cells were transfected with small interfering RNA (siRNA) specific for TRPC5 for 48 hours using a silencer siRNA transfection kit (Ambion, Cambridgeshire, United Kingdom). Briefly, endothelial cells in DMEM containing 10% FBS were incubated with siPORT amine (Ambion) and chemically synthesized siRNA (10 nmol/L; Ambion) specific for TRPC5. The target sequence for TRPC5 was 5′-GGAGGCGGAGAAGCUACU/AUt-3′ (sense) and 5′-AUAGUAGAUCU CAGCCUCCg-3′ (antisense). In control experiments, negative control siRNA (Ambion) that has no significant homology to any known human gene sequence did not affect TRPC5 expression.

Immunoblotting of TRPC5
Immunoblotting was performed as described by our group. Cells were washed with ice-cold PBS and were collected in 500 μL of ice-cold lysis buffer containing Tris-HCl (25 mmol/L; pH 8.0), NaCl (150 mmol/L), EDTA (1 mmol/L), 3-[3-cholamidopropyl]-dimethylammonio-1-propane sulfonate (20 mmol/L), phenylmethylsulfonyl fluoride (1 mmol/L), and complete protease inhibitor mixture (Roche Diagnostics). Proteins were separated by 10% SDS-PAGE at 100 V for 30 minutes followed by 150 V for 60 minutes and transferred to Hybond-ECL nitrocellulose membranes (NEN Life Science, Boston, MA). Membranes were blocked with Odyssey blocking buffer (Licor Biosciences, Bad Homburg, Germany) overnight at 4°C. Membranes were incubated with primary antibodies, that is, rabbit antirat TRPC5 antibody (1:500, Alomone Labs, Jerusalem, Israel) together with goat antirat GAPDH antibodies (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4°C. The membranes were washed 3 times with PBS containing 0.1% Tween 20 and were subsequently incubated with secondary IRDye800CW-infrared fluorescent dye-labeled sheep antirabbit antibodies (1:1000, Biomer, Hamburg, Germany) and Alexa Fluor680-allophycocyanin-fluorescence-labeled goat antibovine antibodies (1:1000; Invitrogen, Eugene, OR) for 60 minutes at room temperature with gentle agitation. After washing, imaging was performed using the Odyssey infrared imaging system (Licor Biosciences) at 810 nm emission with an excitation wavelength of 780 nm and at 700 nm emission with an excitation wavelength of 680 nm. The predicted molecular weights are 97 kDa for TRPC5 and 37 kDa for GAPDH, respectively.

Quantitative In-Cell Western Assay of TRPC5
Quantitative in-cell Western assays of proteins were performed using the Odyssey infrared imaging system (Licor biosciences), as described by our group. Cells were grown on 96-well plates and fixed with 3.7% formaldehyde. Measurements were performed in quadruplicate and averaged.

Intracellular Calcium Measurements Using Fluorescence Spectrophotometry
Endothelial cells were seeded onto 96-well plates and incubated with the calcium indicator fluo-4AM (5 μmol/L) for 60 minutes at room temperature. Cells were then washed with physiological saline solution containing 134 mmol/L of NaCl, 6 mmol/L of KCl, 2 mmol/L of CaCl2, 1 mmol/L of MgCl2, 10 mmol/L of glucose, and 10 mmol/L of HEPES (pH 7.4 with NaOH) to remove extraneous dye. Fluorescence was measured using a fluorescent plate reader (Victor3, Helsinki, Finland) at 535 nm emission with excitation wavelength of 485 nm. TRPC5 was activated by angiotensin II (Ang II). The amplitudes were expressed as fractional fluorescence increase (F/F0).

Patch Clamp Experiments
Membrane currents were recorded using the whole-cell configuration of the patch-clamp technique as described by our group. Experiments were performed at room temperature in the tight-seal whole-cell configuration of the patch-clamp technique with heat-polished,
patch pipettes with resistances of 4 to 6 mol/L. Series resistances were in the range of 10 to 35 mol/L, and seal resistances were in the range of 1 to 2 GΩ. High-resolution membrane currents were recorded with an EPC-9 patch-clamp amplifier (HEKA) controlled by PULSE software on a Power Macintosh computer (G3). High-resolution currents were low-pass filtered at 2.9 kHz. The reference electrode was an Ag/AgCl pellet connected to the bath solution through a 150-mmol/L NaCl/agar bridge. Whole cell currents were elicited by voltage-ramps from −120 to +120 mV (300-ms duration) applied every 15 seconds from a holding potential of −40 mV. Pipettes for whole cell recordings were filled with a solution composed of 130 mmol/L of CsCl, 2 mmol/L of MgCl₂, and 10 mmol/L of HEPES (pH 7.2 with CsOH). The standard bath solution contained 140 mmol/L of NaCl, 2.8 mmol/L of KCl, 2 mmol/L of CaCl₂, 1 mmol/L of MgCl₂, 11 mmol/L of glucose, 10 mmol/L of sucrose, and 10 mmol/L of HEPES (pH 7.4 with NaOH). Ang II (200 nmol/L) was added to the cell via an application pipette positioned in close proximity to the cell.

Assessment of Intracellular Reactive Oxygen Species

Intracellular reactive oxygen species (ROS) generation was assessed in endothelial cells using 5-(6)-chloromethyl-2',7'-dichlorodihydroflourescein diacetate (CM-H₂DCFDA), a cell-permeable indicator for ROS. In brief, endothelial cells were seeded equally in a 96-well plate and treated with 10 μmol/L of CM-H₂DCFDA (Invitrogen, Eugene, OR) for 40 minutes at 37°C in the dark. For cells washing and loading, the following physiological saline solution was used: 134 mmol/L of NaCl, 6 mmol/L of KCl, 2 mmol/L of CaCl₂, 1 mmol/L of MgCl₂, 10 mmol/L of glucose, and 10 mmol/L of HEPES (pH 7.4 with NaOH). Mean fluorescence intensity was measured using a fluorescent plate reader (Victor3) at 535 nm emission with excitation wavelength of 485 nm. Untreated cells were used to determine background fluorescence, which was subtracted from the treated samples.

Patients

A total of 21 hemodialysis patients (17 men and 4 women; mean age: 63±2 years; dialysis vintage: 44±12 months) were investigated. Patient history was raised using a standardized questionnaire and was confirmed by personal histories, smoking habits, cause of kidney disease, months of hemodialysis treatment, preceding cardiovascular disease (ie, history of myocardial infarction, need for coronary angioplasty or coronary bypass surgery, ischemic stroke, or peripheral vascular disease with the need for amputation or angioplasty), presence of diabetes mellitus, and current medication, including angiotensin-converting enzyme inhibitors, β-blockers, lipid-lowering agents, or EPO. Blood pressure was measured predialysis after a rest period of 10 minutes of recumbency. The study was approved by the local ethics committee. All of the patients gave written, informed consent. The cause of end-stage renal disease was diabetic nephropathy in 6 cases (28%), nephrosclerosis in 5 cases (24%), chronic glomerulonephritis in 2 cases (10%), polycystic kidney disease in 1 case (5%), and other/unknown in 7 cases (33%). Angiotensin-converting enzyme inhibitors were prescribed in 2 cases (10%), β-blockers in 13 cases (62%), calcium channel blockers in 3 cases (14%), and EPO therapy in 12 cases (57%). Mean duration of hemodialysis at inclusion was 44±12 months. All of the patients were routinely dialyzed for 4 to 5 hours 3 times weekly using biocompatible membranes with no dialyzer reuse. The dialysates used were bicarbonate based. All of the patients were ambulatory and free of acute intercurrent illness. Mean leukocyte count was 9.3±0.6×10⁹/L, mean hemoglobin level 9.9±0.3 mg/dL, mean platelet count 253±27×10⁹/L, serum creatinine 6.5±0.6 mg/dL, blood urea 85±10 mg/dL, serum albumin 3.3±0.2 g/L, serum calcium 2.24±0.05 mmol/L, and serum phosphate 1.71±0.16 mmol/L. Mean dose of dialysis (kV) was 1.2±0.1.

Preparation of Monocytes

Human monocytes were prepared using antibody coated superparamagnetic polystyrene beads coated with a primary monoclonal antibody specific for the CD14 membrane antigen expressed on human monocytes (DynaBiotec, Hamburg, Germany). Briefly, mononuclear cells were obtained from heparinized blood using HISTOPAQUE-1077 and purified by superparamagnetic polystyrene beads. Cells were then resuspended in Hank’s balanced salt solution containing the following: NaCl, 136 mmol/L; KCl, 5.40 mmol/L; KH₂PO₄, 0.44 mmol/L; NaHCO₃, 0.34 mmol/L; d-glucose, 5.6 mmol/L; CaCl₂, 1 mmol/L; and HEPEs, 10 mmol/L (pH 7.4).

Materials

Ang II, phosphoinositide 3-kinase (PI3K) inhibitor LY294002, Rho-associated protein kinase inhibitor Y27632, and mitogen-activated protein kinase inhibitor PD98059 were purchased from Sigma-Aldrich (Deisenhofen, Germany). EPO (epoetin-β) was purchased from Roche (Mannheim, Germany).

Statistical Analysis

All of the data were expressed as the mean±SEM. Comparisons between groups were analyzed using the Mann-Whitney test or ANOVA and Bonferroni multiple comparison test as appropriate. A 2-tailed P value <0.05 was considered statistically significant.

Results

Dose-Dependent Effect of EPO on TRPC mRNA Levels

Using quantitative real-time RT-PCR, we detected TRPC3, TRPC5, and TRPC6 mRNA in cultured endothelial cells. PCR products appeared at expected molecular weight on the agarose gel. Melting analysis confirmed the presence of a single peak for all 3 of the TRPC channel members (Figure 1A and 1B). Incubation of endothelial cells with EPO for 24 hours resulted in a dose-dependent upregulation of TRPC5 mRNA (Figure 1C). EPO at 100 U/mL significantly increased TRPC5 mRNA by 79%.

Effect of EPO on TRPC5 mRNA Stability

The increased TRPC5 mRNA could be because of increased mRNA stability. To address this question, we examined EPO mRNA stability by inhibiting new mRNA transcription with actinomycin D. After the cells had been incubated with EPO, actinomycin D (1 μmol/L) was added, and the time-dependent decay of TRPC5 mRNA added, and the time-dependent decay of TRPC5 mRNA was measured by real-time RT-PCR. The increased TRPC5 mRNA stability in endothelial cells, that is, EPO prolonged the half-life period for TRPC5 mRNA from 16 hours under control conditions to 24 hours after EPO treatment (each n=3; P<0.05; Figure 1D).

Identification of TRPC5 mRNA With Long Poly(A) Tails With Rapid Amplification of cDNA Ends-Poly(A) Test

The 3’ poly(A) tail plays an important role in determining mRNA stability. Longer 3’ poly(A) tail length causes increased mRNA stability because of reduced degradation. Thus, we examined the 3’ poly(A) tail lengths of the TRPC5 mRNA in endothelial cells treated with or without EPO. As shown in Figure 1E, EPO increased 3’ poly(A) tail lengths of TRPC5 mRNA, as indicated by separating PCR products on
agabase. In endothelial cells exposed to EPO for 48 hours, there was a significant increase in the ratio of long (≈200-bp bands) to short (≈150-bp bands) poly(A) of TRPC5 fragments (2.8±0.4 versus 1.0±0.1; each n=4; P<0.05).

Effect of EPO on TRPC5 Protein Expression
To confirm that the increased TRPC5 mRNA abundance facilitated gene translation, we investigated TRPC5 protein abundance using both immunoblotting and quantitative in cell Western techniques. In accordance with transcripts data, TRPC5 protein expression was also significantly higher in endothelial cells after incubation with EPO (100 U/mL) for 48 hours compared with control (1.0±0.1 versus 1.7±0.1; each n=4; P<0.05; Figure 2).

Effect of EPO on TRPC5-Mediated Currents and Calcium Influx
Patch clamp studies indicated that Ang II (200 nmol/L) could induce TRPC-like channel-mediated currents in endothelial cells (Figure 3A). Cells pretreated with EPO for 48 hours exhibited a pronounced increase in such currents, whereas cell capacitance was unchanged. Furthermore, Ang II–induced calcium influx was significantly increased in EPO-treated endothelial cells compared with control conditions (EPO 1.1±0.1 versus control 0.5±0.1; each n=5; P<0.05).

After TRPC5 knockdown, the increased calcium influx after EPO treatment was abolished (siRNA against TRPC5 plus EPO, 0.6±0.1; each n=5; P<0.05 compared with EPO alone; Figure 3B and 3C).

Inhibition of PI3K Attenuates the EPO-Enhanced Ang II–Induced Calcium Influx
As shown in Figure 3D, the enhanced Ang II–induced calcium influx after EPO was blocked in the presence of the PI3K inhibitor LY294002. On the other hand, Rho-associated protein kinase inhibitor Y27632 and mitogen-activated protein kinase kinase inhibitor PD98059 did not affect the Ang II–induced calcium influx after EPO.

Effect of EPO on Intracellular ROS Generation in Endothelial Cells
As shown in Figure 3E, incubation of EPO in endothelial cells for 48 hours significantly increased ROS generation by 70% compared with the control (EPO: 1.7±0.1; control: 1.0±0.1; each n=4; P<0.05). After TRPC5 knockdown, the EPO-induced intracellular ROS production was not significantly different compared with control conditions (each n=4; P>0.05). There was no significant difference between the EPO-free control and EPO-free control treated with negative control siRNA, which has no significant homology to any known human gene sequence.
Hemoglobin: 9.7 ± 0.5 g/dL versus 10.3 ± 0.5 g/dL; platelets: 147 ± 10^9/L versus 125 ± 10^9/L; leukocytes: 9.7 ± 1.1 mg/dL versus 10.3 ± 0.5 g/dL; blood urea: 76 ± 6.6 mg/dL versus 6.4 ± 3.9 mg/dL; serum creatinine: 1.81 ± 0.30 mmol/L; each P > 0.05 between the groups).  

**Discussion**  
In the present study we showed that EPO increases TRPC5 gene expression and function. Treatment with EPO resulted in a dose-dependent increase in TRPC5 mRNA by enhancing TRPC5 mRNA stability via increased 3′ polyadenylation. Consequently, upregulated TRPC5 protein abundance was accompanied by increased currents, calcium influx, and enhanced oxidative stress in endothelial cells.

EPO is a well-known growth factor causing maturation of erythroblasts. EPO receptors have been identified in several cell types, including endothelial cells. In the literature there are several examples showing that EPO can increase gene expression in several tissues. d’Uscio et al. showed that EPO increased the expression of copper- and zinc-containing superoxide dismutase in vascular tissue. Grossi et al. showed that EPO upregulates the expression of the EPO receptor in a TF-1 cell line. As reported by Acquaviva et al., EPO administration enhanced frataxin protein in primary fibroblasts.

Results from RT-PCR using subtype-specific primers indicate that TRPC3, TRPC5, and TRPC6 were expressed in endothelial cells. Our results showed that EPO selectively increases TRPC5 but not TRPC3 or TRPC6 mRNA in a dose-dependent manner. The specific EPO-TRPC5 interaction underscores TRPC subtype-specific characteristics of TRPC5 compared with TRPC3 and TRPC6. Based on their different structure, TRPC5 belongs to the TRPC4/5 subfamily, whereas TRPC3 and TRPC6 belong to TRPC3/6/7 subfamily. Furthermore, these TRPC channels show different electrophysiological characteristics. TRPC5 channels can be activated by lanthanum (La^{3+}) and do not respond to diacylglycerol. By contrast, TRPC3 and TRPC6 channels are inhibited by micromolar concentrations of La^{3+} and can be activated by diacylglycerol. We showed that EPO increases TRPC5 mRNA by enhancing its stability. A long 3′ poly(A) tail is important for mRNA stabilization. A long 3′ poly(A) tail facilitates binding of multiple poly(A) binding protein molecules, which protects against ribonucleolytic attack and allows mRNA-ribosome interactions. Using the rapid amplification of cDNA ends-poly(A) test we observed that EPO increased TRPC5 mRNA with long 3′ poly(A) tails. These findings shed light on a novel mechanism by which EPO affects gene transcription. We also showed that increased TRPC5 mRNA caused increased TRPC5 protein abundance. Taken together, we provide direct evidence that EPO upregulates TRPC5 gene expression at both transcriptional and translational levels, likely because of increased polyadenylation in the 3′ poly(A) tail of TRPC5 mRNA.

We further investigated the functional consequences of increased expression of the TRPC5 gene. Our in vitro studies indicated that the administration of EPO increased TRPC5-mediated currents and calcium influx in endothelial cells. Altered TRPC channel function may contribute to hypertension. TRPC5 channels are nonselective cationic channels that
can be activated by G protein–coupled receptors.\textsuperscript{23} Several reports showed an increased agonist-induced calcium influx in hypertension.\textsuperscript{10–12} In line with previous reports, we show that Ang II induced a strong increase of calcium influx through TRPC-like channels in endothelial cells. Furthermore, Ang II–induced calcium influx was significantly attenuated after specific TRPC5 knockdown using gene silence techniques. The TRPC5-mediated calcium influx underlies a key signaling mechanism that stimulates the calcium-dependent release of several endothelium-derived vasoconstrictive agents, including endothelin, urotensin, or epoxyeicosatrienoic acids, which contributes to the development of hypertension.\textsuperscript{24–26} A recent study also showed that TRPC5 is activated by pressure-induced membrane stretch associated with hypertension.\textsuperscript{27}

EPO has been shown to initiate a cascade of downstream signaling pathways, for example, PI3K, Rho-associated protein kinase, and mitogen-activated protein kinase, to affect endothelial cell function.\textsuperscript{28–31} Our data showed that the enhanced Ang II–induced calcium influx in EPO-treated cells could be blocked by the PI3K inhibitor LY294002 but not by Rho-associated protein kinase inhibitor Y27632 or mitogen-activated protein kinase inhibitor PD98059. These data are consistent with previous reports showing growth factors induced incorporation of functional TRPC5 channels in a PI3K-dependent manner.\textsuperscript{32}

Figure 3. Erythropoietin (EPO) affects transient receptor potential canonical (TRPC) 5–associated calcium influx and reactive oxygen species (ROS) generation in cultured endothelial cells. A, TRPC-like currents in endothelial cells pretreated with vehicle (control) or EPO. To exclude potassium channel currents, pipettes for whole cell recordings were filled with solution containing cesium. B, Immunoblots confirming knockdown of TRPC5 channel proteins using gene silence techniques. Control indicates negative control small interfering RNA (siRNA) that has no significant homology to any known human gene sequence. C, Representative recordings and summary data (bar graph) of Ang II–induced calcium influx. siRNA\textsuperscript{−}EPO indicates siRNA against TRPC5 plus EPO; control indicates negative control siRNA that has no significant homology to any known human gene sequence. *P < 0.05. D, Inhibition of the phosphoinositide 3-kinase (PI3K) using LY294002 attenuated calcium influx in EPO-treated cells. n = 5 in each group. *P < 0.05. E, Measurement of intracellular ROS generation in cultured endothelial cells. Cells were subject to EPO treatment for 48 hours. Cells were then loaded with the intracellular fluoroprobe CM-H\textsubscript{2}DCFDA (10 \textmu mol/L), and fluorescence intensity was measured. Intracellular ROS formation was expressed as the mean fluorescence intensity and normalized to control. Experiments were performed in quadruplicate. *P < 0.05.
Using the fluorescent dye techniques, we observed that endothelial cells incubated with exogenous EPO showed enhanced oxidative stress, as indicated by increased intracellular ROS production. These findings are supported by several studies, showing that EPO generates significant oxidative stress and oxidant damage both in vivo and in vitro.\(^{33-35}\) We have further shown that knockdown of TRPC5 is accompanied by reduced intracellular ROS production, which was induced by EPO in endothelial cells. Recently, it was reported that agonist-stimulated cytosolic calcium increase is followed by an increase in ROS formation in endothelial cells.\(^{36}\) Because TRPC5 channels are calcium permeable, elevated agonist-stimulated calcium influx through upregulated TRPC5 channels in endothelial cells during EPO treatment may contribute to the ROS formation, which underlies pathogenesis of EPO-induced hypertension.

The present results may put light on the mechanisms underlying development of hypertension during EPO treatment in hemodialysis patients. Large clinical studies uncovered that elevated blood pressure is a frequent adverse effect of EPO treatment.\(^{1-3}\) In line with these findings we gave evidence that hemodialysis patients treated with EPO had higher systolic blood pressure. Furthermore, these patients had significantly increased expression of TRPC5 compared with EPO-free hemodialysis patients. We, therefore, speculate that upregulated TRPC5 might have functional relevance in the pathogenesis of EPO-associated hypertension in patients with chronic kidney disease. The TRPC5-mediated calcium influx underlies a key signaling event that stimulates not only the calcium-dependent release of several endothelin-derived vasconstrictive agents, for example, endothelin, urotensin, or epoxyeicosatrienoic acids, but also calcium-dependent generation of ROS. These mediators can contribute to the development of hypertension.

Future studies should examine the effects of EPO, for example, on the intact endothelium in humans. Presently there is no drug available for humans that specifically blocks TRPC5 channels. When novel drugs blocking TRPC5 activity are available, they should be tested in a model of EPO-induced hypertension for further validation of the observed mechanisms.

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**Disclosures**

None.

**References**


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