NF-κB-dependent increase in tissue factor expression is responsible for hypoxic podocyte injury

（NFκB活性化に伴う組織因子の発現増強は低酸素状態におけるポドサイト障害に関与する）

申請者

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Abstract

Background. Fibrin deposition within glomeruli is commonly seen in kidney biopsy specimens, suggesting enhanced coagulant activity. Tissue factor (TF) is a coagulation factor which is also related to various biological effects, and TF is upregulated by hypoxia in cancer cells. Recently, hypoxic podocyte injury has been proposed, therefore, we investigated TF expression in hypoxia.

Methods. Conditionally immortalized human podocytes were differenciated and treated under hypoxic or normoxic conditions. mRNA expressions of TF and tissue factor pathway inhibitor (TFPI) were analyzed by quantitative RT-PCR. Protein levels of TF and TFPI were tested by enzyme-linked immunosorbent assay. We employed small interfering RNA (siRNA) to temporary knockdown early growth response protein 1 (Egr-1), hypoxia-inducible factor-1α (HIF-1α) and TF. The expression of CD2-associated protein (CD2AP) mRNA and phalloidin staining was examined to assess podocyte injury.

Results. Hypoxia increased mRNA expression of TF (6h: 2.3±0.05 fold, $p<0.001$, 24h: 5.6±2.4 fold, $p<0.05$) and suppressed TFPI (6h: 0.54±0.04 fold, $p<0.05$, 24h: 0.24±0.06 fold, $p<0.001$) compared with normoxia. Similarly, protein levels of TF were increased and TFPI were decreased. Egr-1 siRNA did not change TF mRNA expression. Pyrrolidine dithiocarbamate (PDTC), a nuclear factor kappa B (NF-κB) inhibitor significantly reduced hypoxia induced TF expression, and HIF-1α knockdown further increased TF. Hypoxia resulted in the decreased CD2AP and actin reorganization in podocytes, and these changes were attenuated by TF siRNA.

Conclusion. Hypoxia increased the expression of TF in human podocytes NF-κB dependently. TF may have a critical role in the hypoxic podocyte injury.

Keywords: hypoxia; podocytes; tissue factor

Introduction

Fibrin deposition within the glomeruli is commonly seen in kidney biopsy specimens, particularly in crescentic glomerulonephritis, chronic glomerulonephritis, diabetic nephropathy, and malignant hypertension. Thus, enhanced coagulant activity is suggested in glomerular injury.

Tissue factor (TF), also known as coagulation factor III or CD142, is a 46-kDa transmembrane glycoprotein. As well as being an initiator of extrinsic blood coagulation cascade, TF play an important role in numerous physiologic and pathologic conditions such as development, inflammation, proliferation, cell adhesion/migration and cancer growth [1,2,3]. Tissue factor pathway inhibitor (TFPI) is a natural antagonist of TF which inhibits the complex formation of factor Xa and TF/factor Ⅶa. Therefore, the effect of TF is regulated by a balance between TF and TFPI.

We have previously shown that TF and TFPI are expressed by intrinsic kidney cells, such as mesangial cells, proximal tubular epithelial cells and human podocytes [4,5,6,7]. In the kidney, TF is primarily expressed in the podocytes and parietal epithelial cells of glomeruli [8].

Hypoxia contributes to renal damage in acute kidney injury, as well as chronic kidney disease (CKD) [9]. However, most studies have focused on tubulointerstitial injury, while little attention has been paid to glomerular damage. Recently, Neusser et al showed that hypoxia regulated gene expressions were increased in glomeruli from human kidney biopsy specimens from patients with hypertensive nephrosclerosis, which is one of the most important causes of CKD. HIF is a key transcription factor which regulates adaptive responses to hypoxia. They demonstrated that the HIF target gene, chemokine receptor C-X-C motif receptor (CXCR4),

was prominently increased in podocytes, and HIF-1α showed nuclear localization, which was indicative of HIF activation [10]. Ding et al also showed that forced HIF stabilization by deletion of the Von Hippel-Lindau gene induced CXCR4 in the glomeruli and resulted in necrotizing crescentic glomerulonephritis [11]. In vitro studies as well have been reported hypoxic podocyte injury. It was demonstrated that podocytes appeared swollen with narrowed filtration slits [12,13], and the expressions of slit diaphragm proteins, such as podocin, CD2-associated protein (CD2AP) and nck were reduced under hypoxic conditions [14]. Furthermore, podocytes treated in hypoxia for 48 h developed actin disarrangement [14].

In cancer cells, such as glioma, lung cancer, and breast cancer hypoxia induces TF [15,16,17]. On the other hand, the expression of TF in podocytes under hypoxic conditions is not known.

In the present study, we hypothesized that TF is induced in the hypoxic conditions in podocytes and contributes to podocyte damage. We investigated the expression of TF and TFPI under hypoxic conditions, and further tested its regulatory mechanisms.

Materials and methods

Cell culture and hypoxic conditions

Immortalized human podocytes were maintained in RPMI 1640 containing 10% fetal bovine serum (FBS), 1% insulin-transferrin-selenium-A supplement (Invitrogen, Carlsbad, CA), penicillin (100 U/mL) and streptomycin (100 μg/mL). Cells were grown at 33℃ in 95% air, 5% CO_2 and then were converted to differentiated cells by incubating at 37℃ for 10 days. Cells were kept in 1% FBS for 24 h and then treated

under normoxic or hypoxic conditions. The hypoxic condition (an atmosphere of 1% oxygen) was achieved using an anaerobic chamber equipped with an aerating agent (Anaeropack, Mitsubishi Gas Chemical, Tokyo, Japan). For analysis of the regulatory mechanism, podocytes were incubated with 100 μM of pyrrolidine dithiocarbamate (PDTC), a nuclear factor kappa B (NF-κB) inhibitor. Cells were used between passages 15-18.

Small interfering RNA

Cultured podocytes were transfected using small interfering RNA (siRNA) as described by the manufacturer. A siRNA targeted to an irrelevant mRNA served as the non-specific control. Cells were transfected with siRNA (100 nM) without antibiotics for 24 h using Dharmafect transfection reagent (GE Healthcare), according to the manufacturer's protocol. Then the cells were further incubated for 24h under hypoxic or normoxic cinditions. Depletion of target gene was determined by real-time polymerase chain reaction (RT-PCR).

Assay of tissue factor and tissue factor pathway inhibitor

The protein levels of TF in the cell lysate and TFPI in the culture supernatant were quantified by enzyme-linked immunosorbent assay (ELISA) using Total TF ELISA Kit and IMUBIND Total TFPI ELISA Kit (IMUBIND, Sekisui Diagnostics), respectively. These are sandwiched ELISA employing murine anti-human TF monoclonal antibody and rabbit anti-human TFPI polyclonal antibody, respectively as the capture antibodies. Podocytes were cultured on 6-well plates (Becton Dickinson, Oxford, CA). Podocytes were washed twice with Hank's balanced salt solution (HBSS; Gibco Laboratories) and incubated with 1%

FBS for 24 h before treatment, then the cells were incubated under normoxic or in hypoxic condition for 6 h or 24 h. TF was measured in the cell lysates. The cells from each well were disrupted by repeated freeze-thaw cycles with a buffer of Tris-buffered-saline (50 mM Tris, 100 mM NaCl), at pH 7.4 and containing 0.1% Triton X-100. TFPI was measured in the culture supernatants. After removal of the supernatant, cells were dissolved in NaOH and protein content was measured by the method of Lowry et al [18]. The levels of TFPI were normalized by the protein content in each well.

Analysis of mRNA expressions by quantitative RT-PCR

Total RNA was extracted from the cells using QIA shredder and RNeasy Protect Mini Kit (QIAGEN, Valencia, CA). RNA was transcribed into the firststrand cDNA with the Omniscript RT kit (QIAGEN) according to the manufacturer's description. Quantitative RT-PCR was performed using an ABI PRISM 7700 Sequence Detector (Applied Biosystems, Foster City, CA) with TaqMan Universal PCR Master Mix (Applied Biosystems). The specific primers and probes (Applied Biosystems) were acquired to detect human TF (Assay ID: Hs01076029_m1), human TFPI (Assay ID: Hs00196731_m1), CD2AP (CD2-associated protein) (Assay ID: Hs00961451_m1), synaptopodin (Assay ID: Hs00702468_s1), human glyceraldehyde-3-phosphatasedehydrogenase (GAPDH) (Assay ID: Hs99999905_m1), Egr-1 (Assay ID: Hs00152928_m1), and hypoxia-inducible factor-1α (HIF-1α) (Assay ID:Hs00468869_m1). Results were normalized by the expression levels of GAPDH.

Western blot analysis

For nuclear and cytoplasm extraction, NE-PER nuclear and cytoplasmic extraction reagents (Thermo

Scientific, Rockford, IL) were used according to the instruction. Sample protein content was determined by the BCA protein assay (Thermo Scientific). Twenty micrograms of total protein were loaded per lane for sodium dodecyl sulfate–polyacrylamide gel electrophoresis (10% wt/vol) analysis and transferred to polyvinyliden difluoride membranes. Membranes were incubated with rabbit anti phospho- NF-κB p65 and total NF-κB p65 antibody (Cell signaling, Danvers, MA) and visualized by using a horseradish peroxidase (HRP) secondary antibody (Santa Cruz Biotechnology). The protein bands were detected by Amersham ECL Prime Western Blotting Detection Reagents (GE Healthcare, Buckinghamshire, UK). Densitometric analysis was performed with image J software. Blots were also analyzed for tubulin for cytoplasmic fraction and lamin A/C for nucleus fraction as a loading control.

Immunofluorescence study

Podocytes were grown and differentiated on glass bottom culture dishes (MatTec, Ashland, MA). Cells were pretreated in 1% reduced serum for 24 h and incubated under hypoxic or normoxic coditions. The cells were fixed with ice-cold methanol for 30 min, permeabilized with 0.1% Triton X-100 in phosphate-buffered saline (PBST) for 20 min. The cells were incubated with anti-human TF mouse monoclonal antibody (Abnova) or anti-human HIF-1α mouse monoclonal antibody (abcam) diluted 1:200 in blocking buffer for 1 h and then incubated with an Alexa-Fluor 488 goat anti mouse antibody for 2 h. For the staining of p65, cells were incubated overnight with anti phospho- NF-κB rabbit monoclonal antibody (Cell Signaling), diluted 1:100 in blocking buffer and then incubated with an Alexa-Fluor 488 goat anti rabbit antibody for 2 h. For F-actin staining, cells were incubated after fixation with an Alexa Fluor 488-conjugated phalloidin antibody

(Molecular Probes, Carlsbad, CA) for 1 h. Samples were covered with antifading mounting medium with or without 4',6-diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA). Preparations were image using a fluorescence microscope (model BZ-X700; Keyence).

Effect of cobalt chloride on tissue factor and tissue factor pathway inhibitor production

To verify the effect of hypoxia, podocytes were treated with cobalt chloride ($CoCl_2$) (75 μM and 150 μM), which is a strong inducer of HIF activation and mimicry of a hypoxic condition. Podocytes were incubated with $CoCl_2$ for 6 h and 24 h; the mRNA expressions of TF and TFPI were then examined by quantitative RT-PCR.

Statistical analysis

All data were expressed as mean ± standard deviation (SD). The results were compared using Student's independent samples t-test or one-way analysis of variance (ANOVA) followed Turkey's honestly significant difference test and the two-way repeated-measures ANOVA. $P < 0.05$ was considered to be significant.

Results

Expression of tissue factor and tissue factor pathway inhibitor mRNA under hypoxic conditions

The time course of TF mRNA expression in podocytes is shown in Figure 1A. The relative TF mRNA expression under hypoxic conditions was significantly higher than normoxic conditions both at 6 h and 24 h (6 h: 2.29 ± 0.05 fold, $p<0.001$; 24 h: 5.6 ± 2.4 fold, $p<0.05$). The time course of TFPI mRNA expression in podocytes is shown in Figure 1B. The relative TFPI mRNA expression under hypoxic conditions was

significantly lower than normoxic conditions both at 6 h and 24 h (6 h: 0.54 ± 0.04 fold, $p<0.05$; 24 h: 0.24 ± 0.06 fold, $p<0.001$).

Expression of tissue factor and tissue factor pathway inhibitor mRNA after treatment with cobalt chloride

As shown in Figure 2A, the expression of TF significantly increased after treatment with $CoCl_2$ which mimics hypoxia. $CoCl_2$ suppressed the expression of TFPI mRNA (Figure 2B)

Expression of tissue factor and tissue factor pathway inhibitor under hypoxic conditions by enzyme-linked immunosorbent assay

Hypoxia increased the expression of TF in the cell lysate (6 h: 1152.8 ± 125.7 pg/mL protein in the controls and 1561.7 ± 12.6 pg/mL protein in the hypoxia-stimulated cells, $p=0.08$; 24 h: 1032.5 ± 67.18 pg/mL protein in the controls and 2845.1 ± 618.1 pg/mL protein in the hypoxia-stimulated cells, $p<0.05$; 36 h: 1556 ± 158.2 pg/mL protein in the controls and 3447.9 ± 137.9 pg/mL protein in the hypoxia-stimulated cells, $p<0.05$) (Figure 3A). Hypoxia suppressed TFPI in the supernatant (6 h: 1.18 ± 0.5 ng/mL protein in the controls and 0.25 ± 0.1 ng/mL protein in the hypoxia-stimulated cells , $p<0.05$; 24 h: 1.88 ± 0.72 ng/mL protein in the controls and 0.3 ± 0.13 ng/mL protein in the hypoxia-stimulated cells, $p<0.05$, 36 h: 1.18 ± 0.11 ng/mL protein in the controls and 0.64 ± 0.07 ng/mL protein in the hypoxia-stimulated cells, $p<0.05$) (Figure 3B).

Immunofluorescence study

In the immunofluorescent study, the enhancement of TF staining was observed in the cytoplasm of podocyte exposed to hypoxia for 6 h (Figure 4B) compared with normoxia (Figure 4A). HIF-1 was also strongly stained in the cell nuclei of podocytes exposed to hypoxia for 6 h (Figure 4D) compared with normoxia (Figure 4C).

Effect of hypoxia-inducible factor-1α small interfering RNA

The mRNA levels of HIF-1α were reduced by transfection with siRNA, suggesting that the knockdown of target gene was achieved by RNA interference (Figure 5A). The results showed that HIF-1α siRNA further increased the expression of TF mRNA under hypoxic conditions (Figure 5B). On the other hand, decreased expression of TFPI mRNA by hypoxic condition was significantly recovered by HIF-1 siRNA (Figure 5C).

Effect of early growth response protein 1 small interfering RNA

To determine whether siRNA targeted against Egr-1 altered the expression of TF, podocytes were transfected with Egr1 siRNA for 24 h. The mRNA expression of Egr-1 was successfully reduced by transfection with siRNA (Figure 6A). However, Egr-1 siRNA did not have an influence on the expressions of TF and TFPI (Figures 6B and 6C).

Tissue factor induction by hypoxia was attenuated by nuclear factor kappa B inhibition

To evaluate the activation of NF-κB in the podocytes, cells were incubated under normoxia or hypoxia for 1 h. The expression of phosphorylated NF-κB p65 was enhanced in the nucleus (Figure 7B) compared with normoxia (Figure 7A), documenting the activation of NF-κB. The expression of phosphorylated p65 was increased in the nucleus. The amount of phosphorylated p65 in the cytoplasm as well was significantly increased, on the other hand, total amount of p65 in the cytoplasm was not altered (Figure 7C and D). Furthermore, the NF-κB inhibitor PDTC significantly reduced the expression of TF in hypoxia (Figure 7E), showing that TF upregulation in podocytes was dependent on NF-κB activation. On the other hand, the expression of TFPI mRNA tend to recover but there was no significant difference (Figure 7F).

Temporary knockdown of tissue factor attenuates hypoxia induced podocyte injury

To access the role of TF in podocyte damage, the expression of slit diaphragm molecules CD2AP and synaptopodin in podocytes were evaluated by quantitative RT-PCR. The expression of CD2AP mRNA was significantly decreased by 24h incubation in hypoxia, and it was significantly recovered by TF siRNA (Figure8A). On the other hand, the expression of synaptopodin mRNA was significantly decreased by 24h incubation in hypoxia, but there was not a significant change by TF siRNA (data not shown).

In addition, podocytes undergo actin reorganization in response to hypoxia. By phalloidin staining, untreated differentiated podocytes demonstrated the characteristic central stress fibers of actin (Figure 8B) which are disrupted by hypoxia, resulting in actin reorganization (Figure 8C). A temporary knockdown of TF by siRNA prevented actin reorganization (Figure 8G). Cells transfected with control siRNA showed actin reorganization in response to hypoxia (Figure 8E). Under normoxia, the cells transfected with control siRNA (Figure 8D) and TF siRNA (Figure 8F) showed the characteristic central stress fibers. (Data are representative one of three independent experiments).

Discussion

In the present study, we showed that hypoxia increased the expression of TF and decreased the expression of TFPI in differentiated human podocytes in culture.

According to previous knowledge in other cell types [19], we tested the roles of Egr-1 and NF-κB as candidate transcription factors for the regulation of TF. Also, we tested the role of HIF, which is a key

transcription factor in hypoxia. As a result, temporary knockdown of Egr-1 by siRNA did not affect the expression levels of TF. NF-κB is an important rapid-acting transcription factor found in all cell types and is involved in cellular responses to stimuli, such as stress, hypoxia, cytokines, and infections.

In podocytes, hypoxia increased the expression of phosphorylated NF-κB p65 in the nucleus compared with normoxia, which signified NF-κB activation. PDTC, an inhibitor of NF-κB, partially suppressed TF expression induced by hypoxia.

As expected, HIF-1α expression in the cell nuclei was increased by hypoxic conditions in podocytes, showing HIF-1α activation. Interestingly, temporary knockdown of HIF-1α by siRNA further increased the hypoxia induced TF expression, thus, HIF-1α may act to prevent excessive expression of TF in the podocytes.

We confirmed that hypoxia for 24 h decreased the expression of slit diaphragm protein CD2AP and synaptopodin in podocytes, and the expression of CD2AP was significantly recovered by temporary knockdown of TF by siRNA, and TF siRNA did not change the expressions of synaptopodin. These results were compatible with the previous report that the alteration of CD2AP was greater than that of synaptopodin [14]. In addition, hypoxia for 24 h induced the reorganization of actin cytoskeleton in podocytes, which may correspond to changes in foot process effacement. Temporary knockdown of TF also attenuated hypoxia induced actin reorganization at least in part. Thus, we showed here for the first time, that TF plays a significant role in hypoxic podocyte injury. Some previous works in other cell types suggest a relationship between TF and actin cytoskeleton. The interaction of the cytoplasmic domain of TF with the cytoskeleton [20,21], as well as that of TF with α3β1-integrin which is involved in the adhesion of the sole of podocyte foot processes to

laminin-5 in the glomerular basement membrane (GBM), have been demonstrated [22]. In addition, studies on cultured cancer cells revealed that interaction between the cytoplasmic domain of TF and actin-binding protein (ABP-280) induced cytoskeletal rearrangement. Therefore, it is reasonable to infer that TF may play a critical role in the maintenance of the actin cytoskeleton in podocytes.

On the contrary, mice lacking the cytoplasmic domain of TF caused albuminuria, podocyte effacement and reduced podocyte number [1]. These results suggest that either excess or reduced TF function may lead to podocyte injury.

Proteinuria is an independent risk factor for end-stage renal disease regardless of the underlying pathology [23]. It has been reported that the risks of mortality, myocardial infarction, and progression to kidney failure for a given level of eGFR were independently increased in patients with higher levels of proteinuria [24].

Thus, podocyte injury which is a critical cause of proteinuria, is an important clinical problem. Podocyte injury is difficult to recognize in regular light microscopy analysis, therefore, hypoxia-induced podocyte injury may have been underestimated, compared with tubulointerstitial injury. Our results suggested that podocyte injury under hypoxic conditions deserves more attention in further studies.

Fibrin depositions within the glomeruli are commonly seen in kidney biopsy specimens. It is reasonable that the activation of intraglomerular coagulation is related to the rupture of capillary loop. However, the results of the present study suggests that upregulation of TF in the podocyte may not need the disruption of capillary loop.

There are some limitations in this study. This is an *in vitro* study, and it is not known how hypoxia acts on

podocytes *in vivo*. Besides, it is not clear how important is the local expression of TF in podocytes compared with systemic TF levels in the blood. Phalloidin staining is a generally accepted methods to access podocyte injury, however, it is a visual determination which lacks in objectivity and quantativity. Our results warrant the importance of further studies on TF in the podocytes.

In summary, we showed that hypoxia increased the expression of TF in differentiated human podocytes in culture. It was dependent on NF-κB, whereas HIF-1α negatively regulated these changes. In addition, hypoxia induced podocyte injury which was attenuated by temporary knockdown of TF, showing that TF may play a significant role in podocytes.

Conflict of interest: The authors have declared that no conflict of interest exists.

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Legend to figures

Figure1

Human podocytes were incubated in normoxic or hypoxic conditions for 6h and 24h. Hypoxia significantly increased mRNA expression levels of TF (A) and TFPI (B). (n=3: **P*<0.05 v.s. normoxia, ***P* < 0.001 v.s. normoxia).

Figure2

Human podocytes were incubated with 75 μM and 150 μM of Cobalt Chloride ($CoCl_2$) for 6 h and 24 h. $CoCl_2$ significantly increased mRNA expression levels of TF (A) and significantly decreased mRNA expression levels of TFPI (B) (n=3: *P<0.05 v.s. control).

Figure3

Human podocytes were incubated in normoxic or hypoxic conditions for 6 h, 24h and 36 h. Hypoxia (square circles) significantly increased protein levels of TF in the cell lysate compared with normoxia (open circles) (n=4: *P<0.05 vs normoxia) (A). Hypoxia (square circles) significantly decreased protein levels of TFPI in the cell supernatant compared with normoxia (open circles) (n=4: *P<0.05 v.s. normoxia) (B).

Figure4

Immunofluorescent study. Human podocytes were incubated in normoxic or hypoxic conditions for 6 h. In hypoxia, TF staining was enhanced in the cytoplasm (B) in comparison with normoxia (A). Hypoxia enhanced hypoxia inducible factor-1α (HIF-1α) expression in the cell nuclei (D) (nuclei stained with 4’,6-diamidino-2-phenylindole and shown in blue in the right column) in comparison with normoxia (C). (Data are representative one of three independent experiments).

Figure5

The expression of HIF-1α mRNA was effectively suppressed by siRNA in human podocytes (A). Temporary knockdown of HIF-1α expression by siRNA further increased the expression of TF (B). Whereas, mRNA expression of TFPI returned to baseline (C). (n=3: *P < 0.05 v.s. control siRNA, **P < 0.001 v.s. control siRNA).

Figure6

The expression of Egr1 mRNA was effectively suppressed by siRNA in human podocytes (A). Temporary knockdown of Egr1 expression by siRNA did not alter the expression of TF (B), and TFPI (C). (n=3: *P < 0.05 v.s. control siRNA).

Figure7

Human podocytes were incubated in normoxic or hypoxic conditions for 1h. In normoxia, the expression of phosphorylated p65 is weak as noted by the light red fluorescence in the left column (A) (nuclei stained with 4’,6-diamidino-2-phenylindole and shown in blue in the right column). In hypoxia, the expression of phosphorylated p65 was enhanced in the nucleus (B). (Data are representative one of three independent experiments). Western blotting analysis demonstrated that phosphorylated p65 was increased in the nucleus in hypoxic condition. The amount of phosphorylated p65 in the cytoplasm as well was significantly increased, on the other hand, total amount of total p65 in the cytoplasm was not altered (C) and (D). (n =3: *P < 0.05). Then,

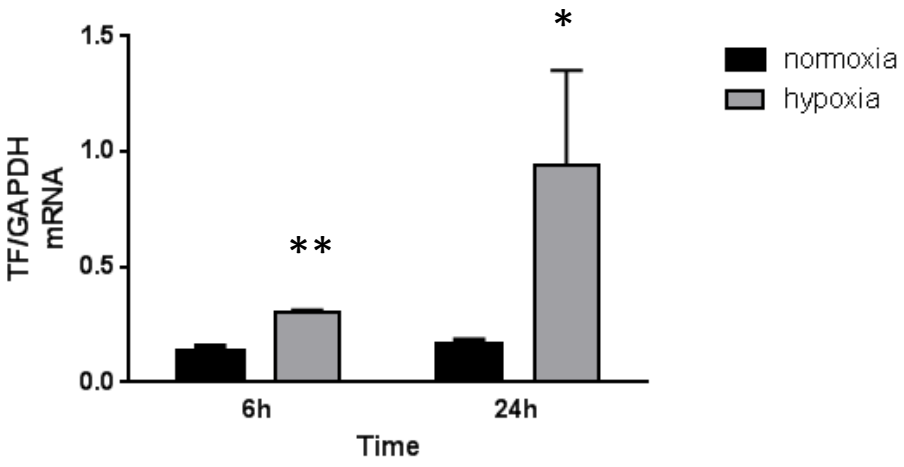
human podocytes were incubated in normoxic or hypoxic conditions for 24 h with or without NF-κB inhibitor PDTC (100 μM). PDTC significantly reduced the expression of TF both at basal level and in response to hypoxia (E). TFPI mRNA was not significantly changed by PDTC (F). (n=3: *P < 0.05 v.s. control).

Figure8

The expression of CD2AP mRNA was significantly decreased by 24h incubation in hypoxia, and it was significantly recovered by TF siRNA (A) (n=3: *P < 0.05 v.s. control siRNA). Podocytes undergo actin reorganization in response to hypoxia. By phalloidin staining, untreated differentiated podocytes demonstrate characteristic central stress fibers of actin (B) which are disrupted by hypoxia, resulting in actin reorganization (C). Temporary knockdown of TF by siRNA prevented actin reorganization (G). Cells transfected with control siRNA show actin reorganization in response to hypoxia (E). In normoxia, the cells transfected with control siRNA (D) and TF siRNA (F) showed characteristic central stress fibers. (Data are representative one of three independent experiments).

Figure1

A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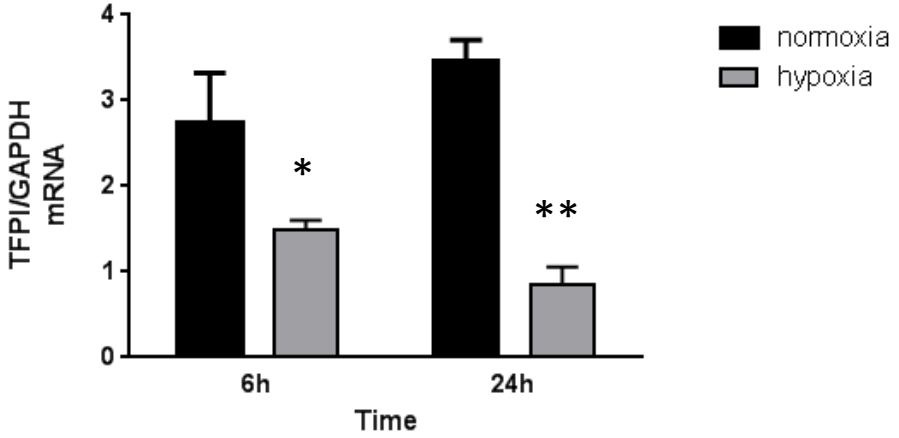
B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TFPI/GAPDH mRNA
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Figure2

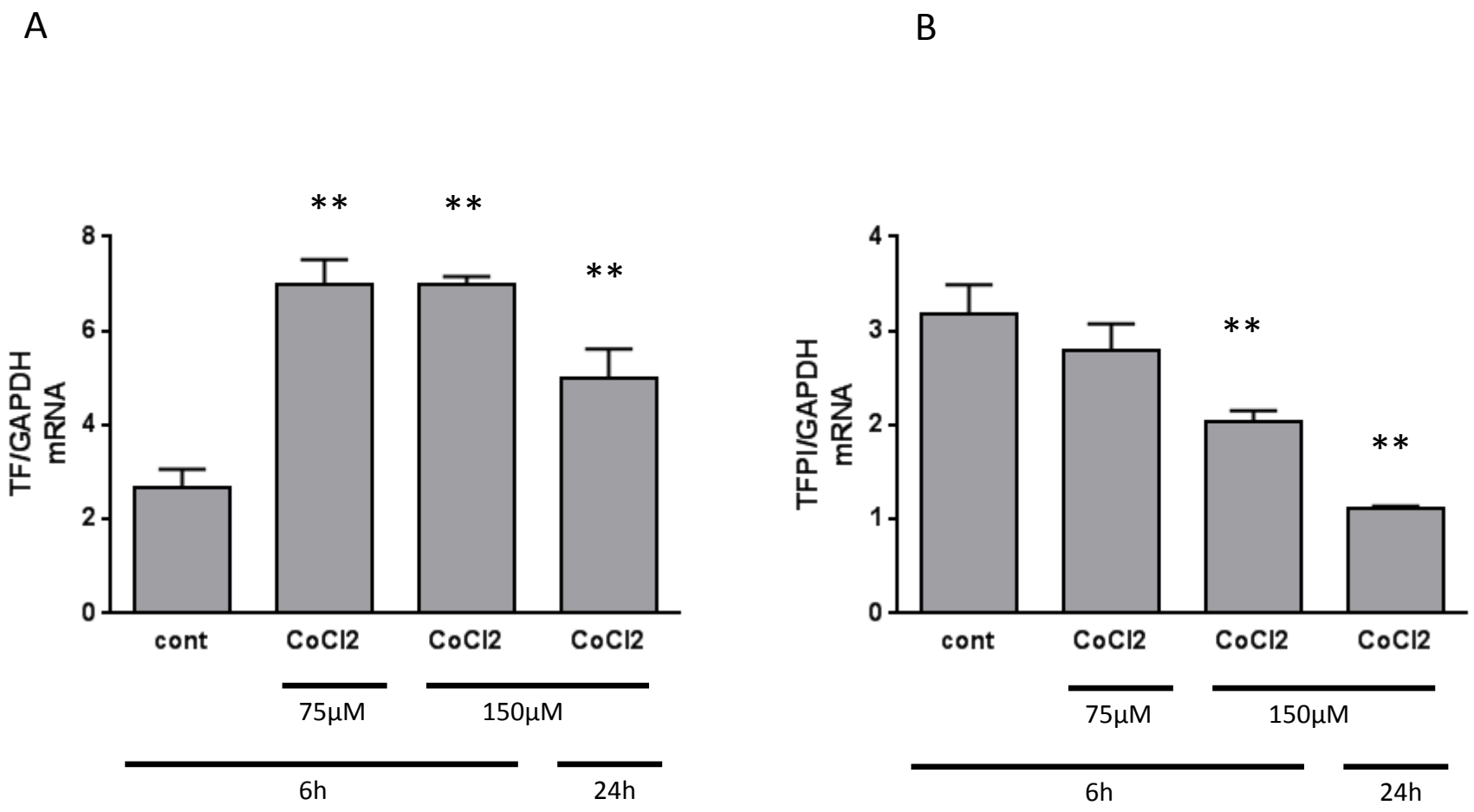


Figure3A

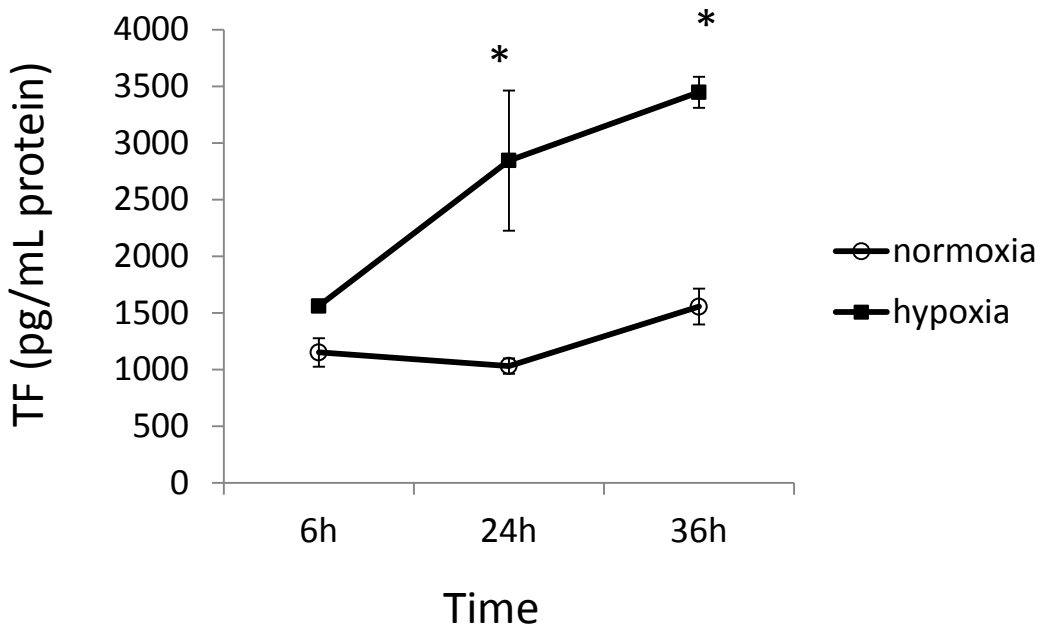

4000
3500
3000
2500
2000
1500
1000
500
0
TF (pg/mL protein)
*
*
6h
24h
36h
Time
normoxia
hypoxia

Figure3B

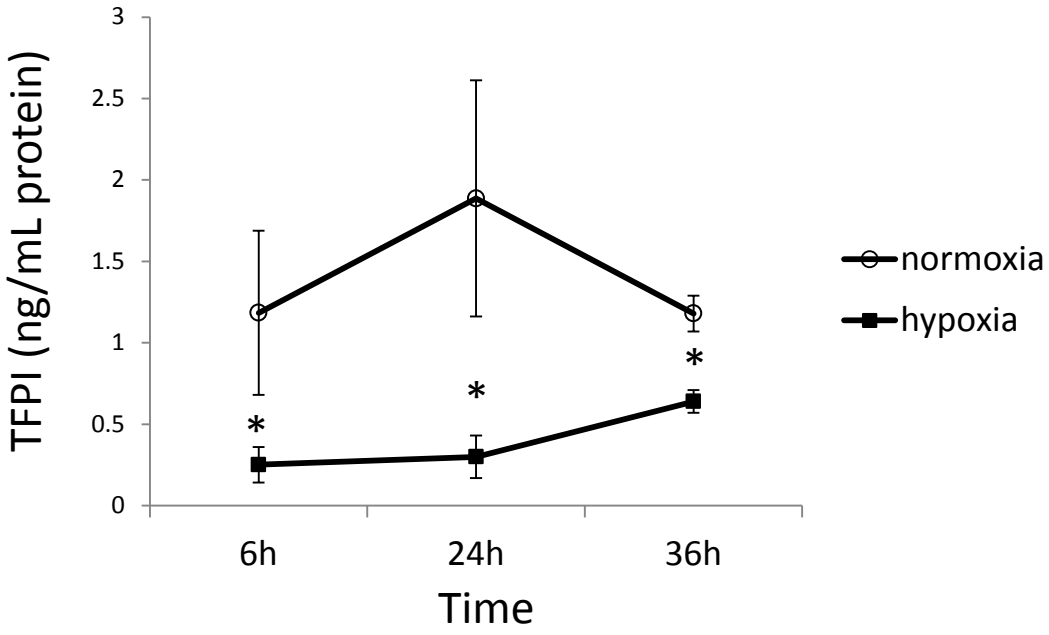
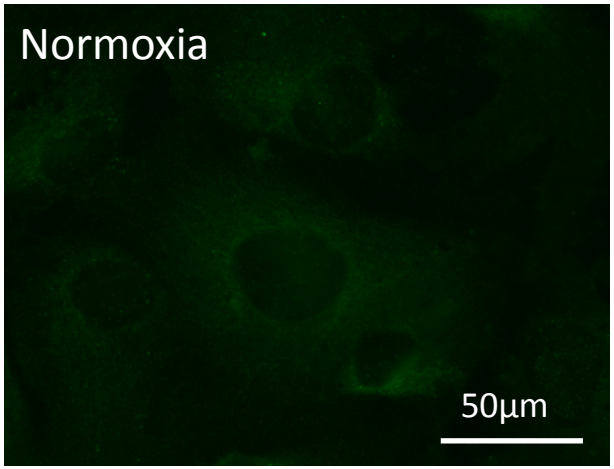
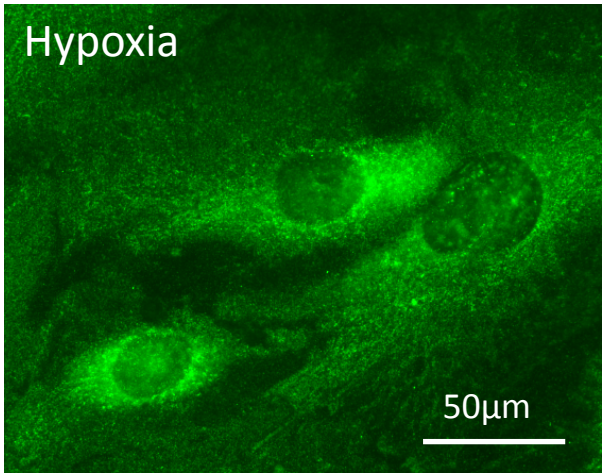

TFPI (ng/mL protein)
3
2.5
2
1.5
1
0.5
0
6h
24h
36h
Time
normoxia
hypoxia
*
*
*

Figure4

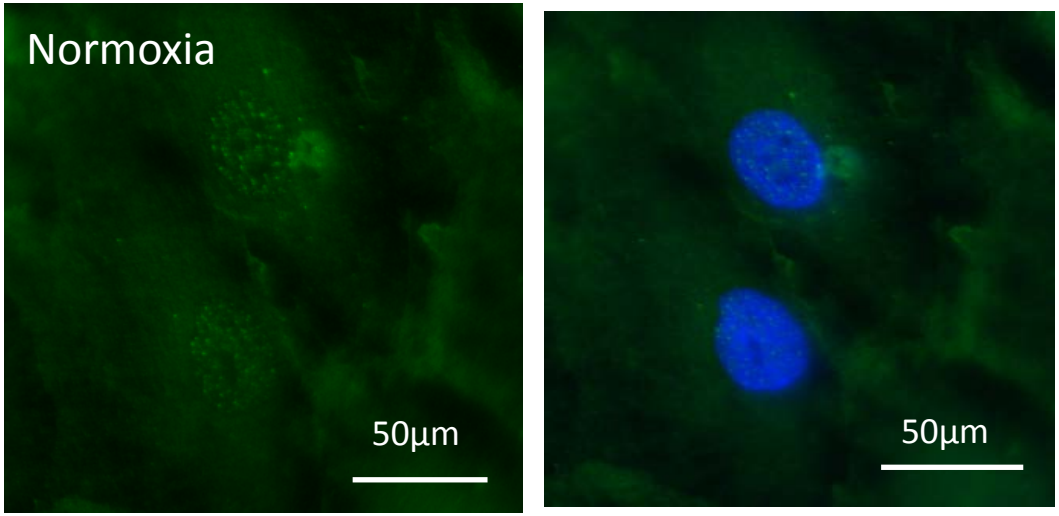
A



B



C



D

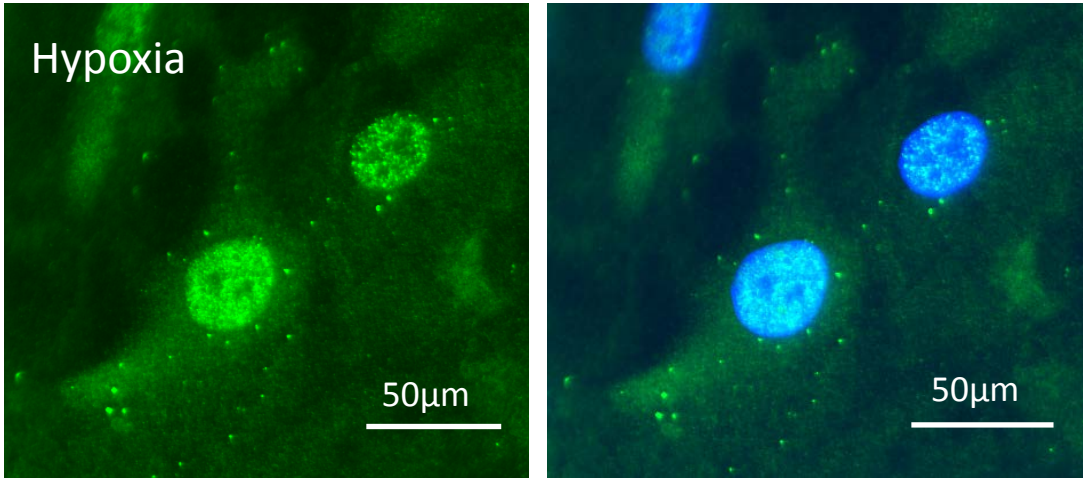
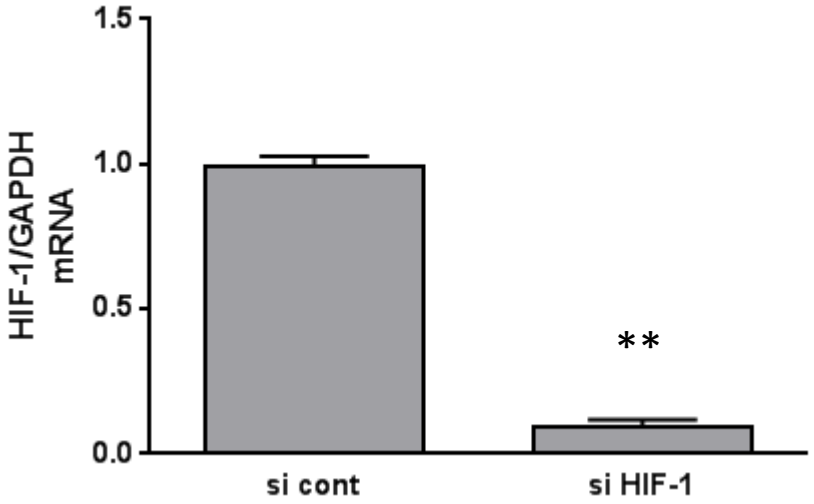
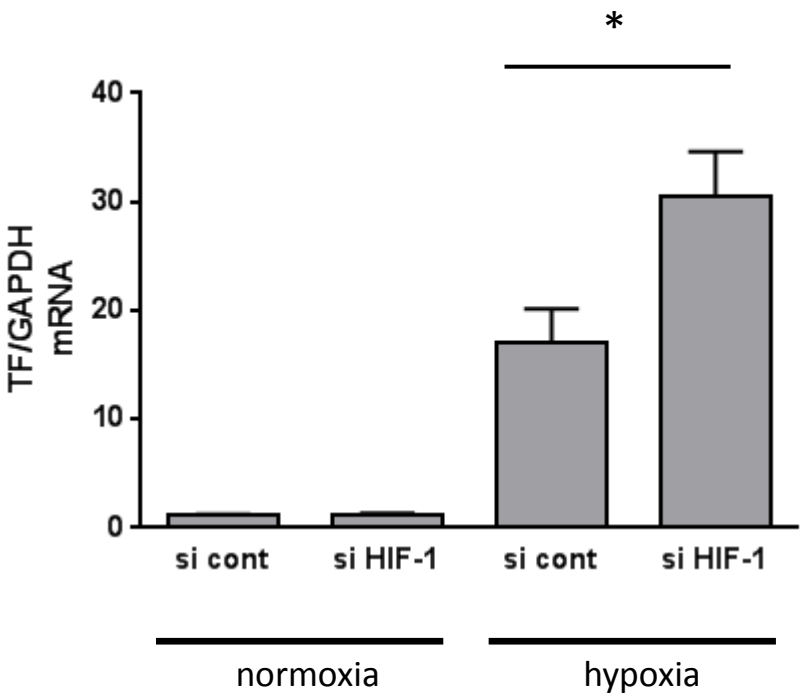


Figure5

A



B


*
40
30
20
10
0
TF/GAPDH mRNA
si cont
si HIF-1
si cont
si HIF-1
normoxia
hypoxia

C

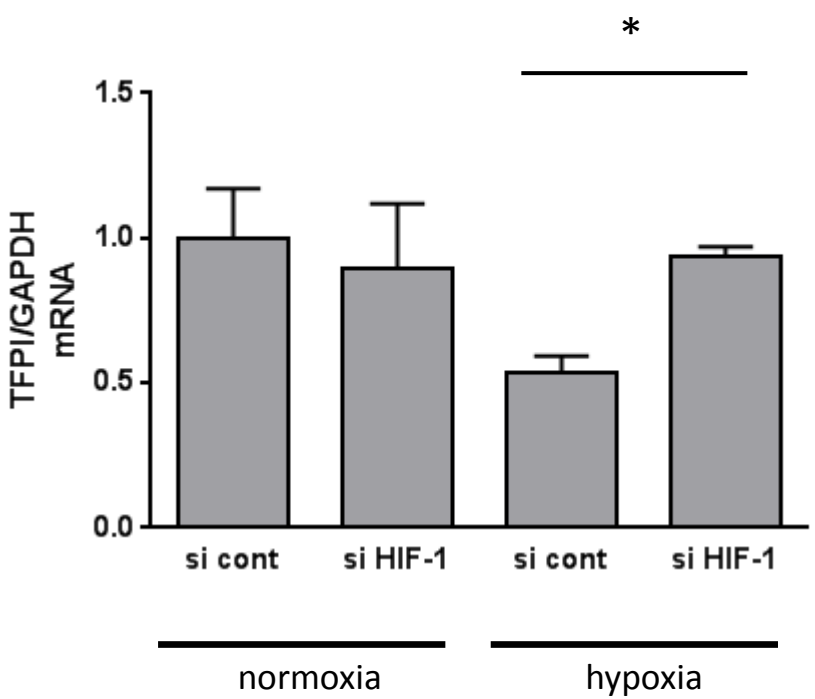
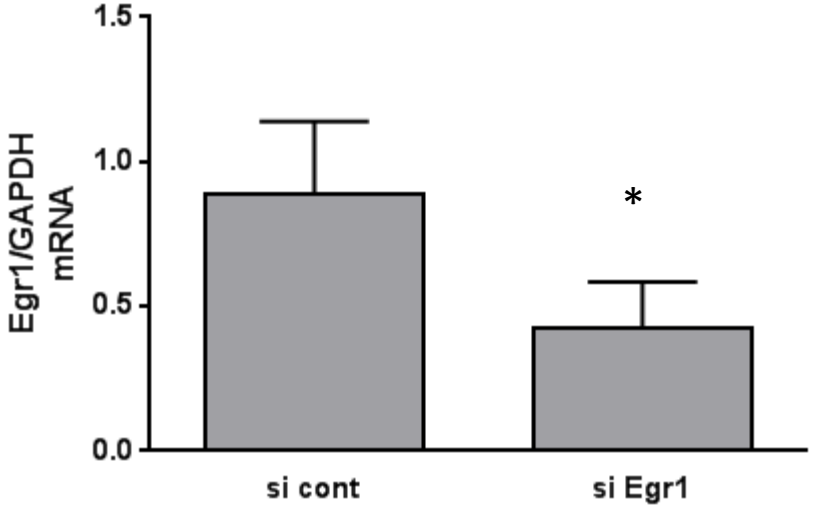
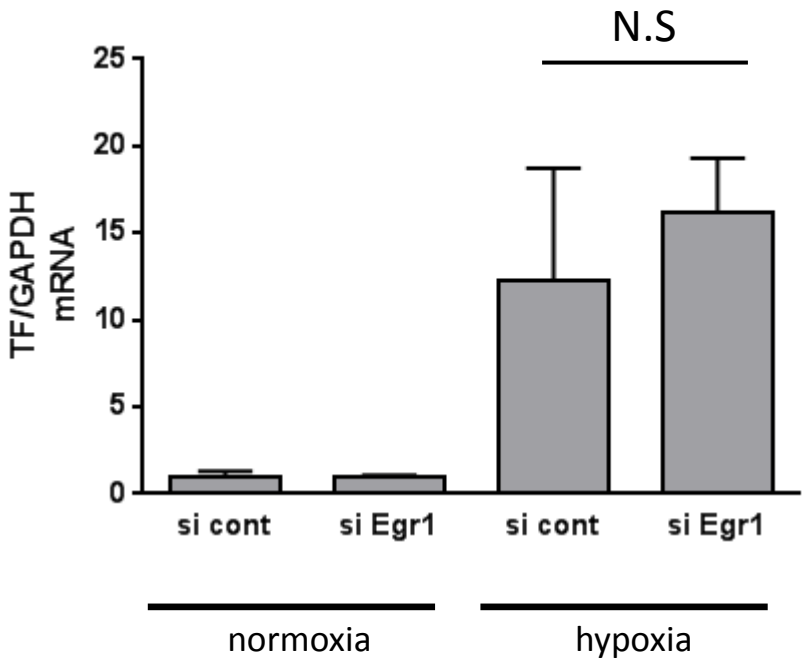

*
1.5
1.0
0.5
0.0
TFPI/GAPDH mRNA
si cont
si HIF-1
si cont
si HIF-1
normoxia
hypoxia

Figure6

A



B


N.S
25
20
15
10
5
0
TF/GAPDH mRNA
si cont
si Egr1
si cont
si Egr1
normoxia
hypoxia

C

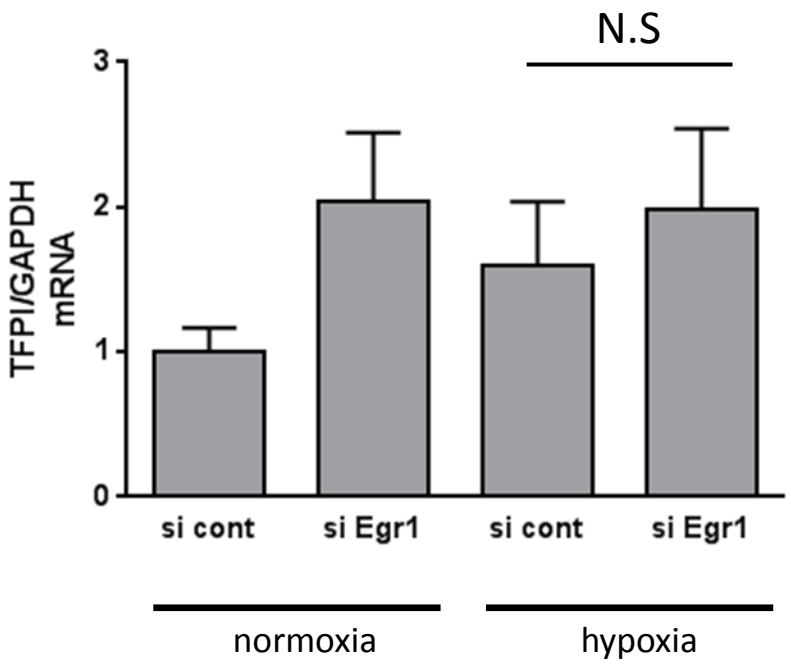
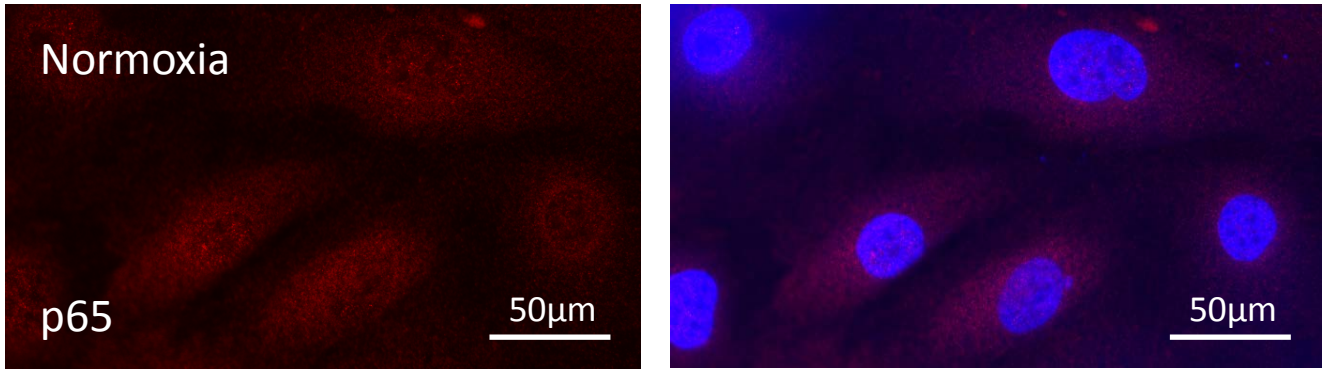
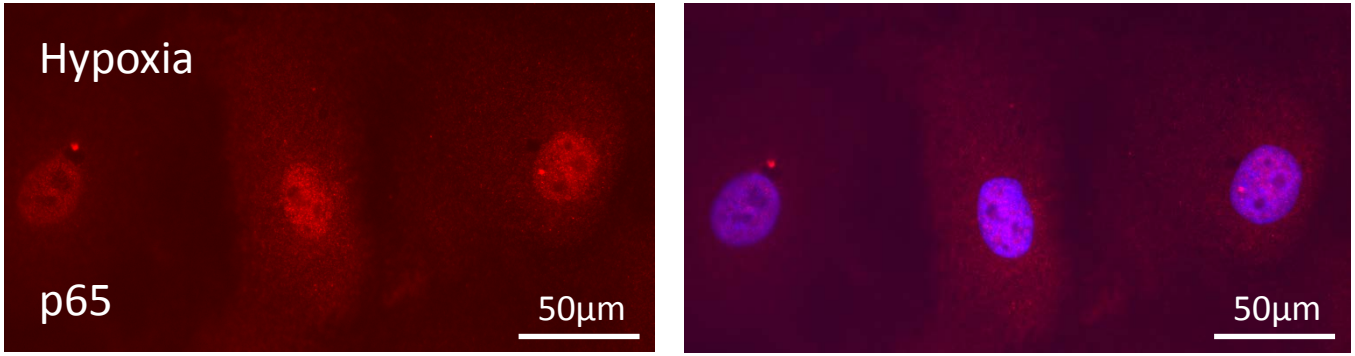

N.S
3
2
1
0
TFPI/GAPDH mRNA
si cont
si Egr1
si cont
si Egr1
normoxia
hypoxia

Figure7

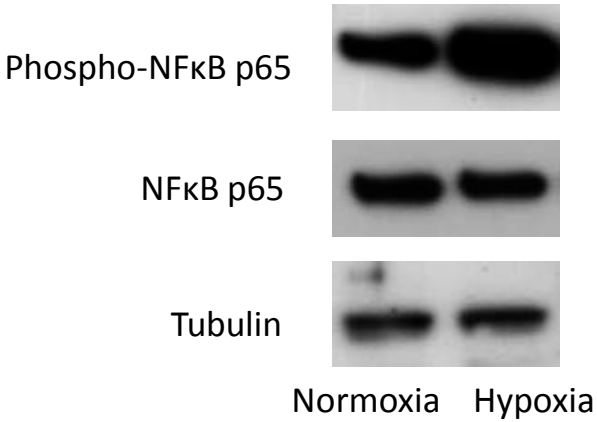
A



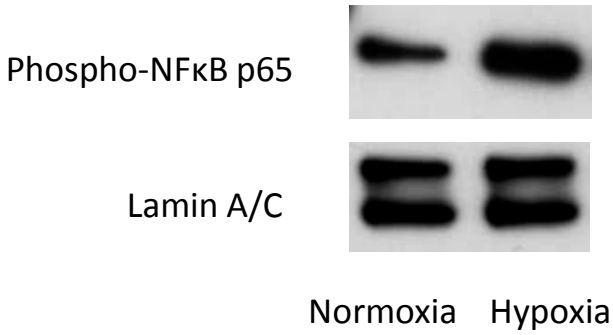
B



C

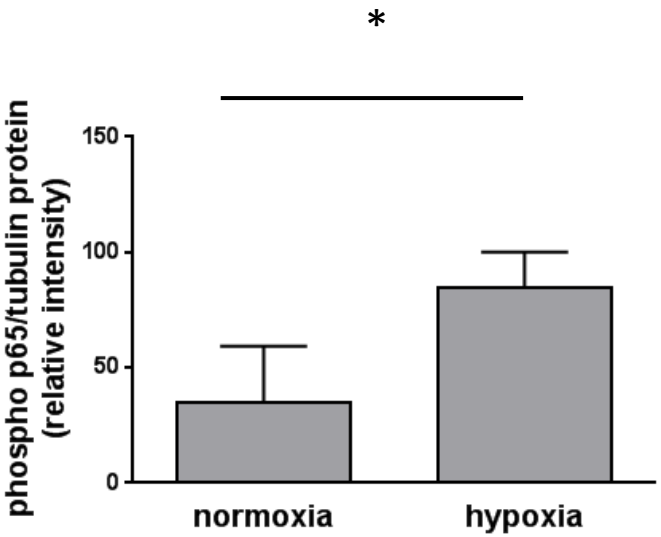


Cytoplasmic Fraction

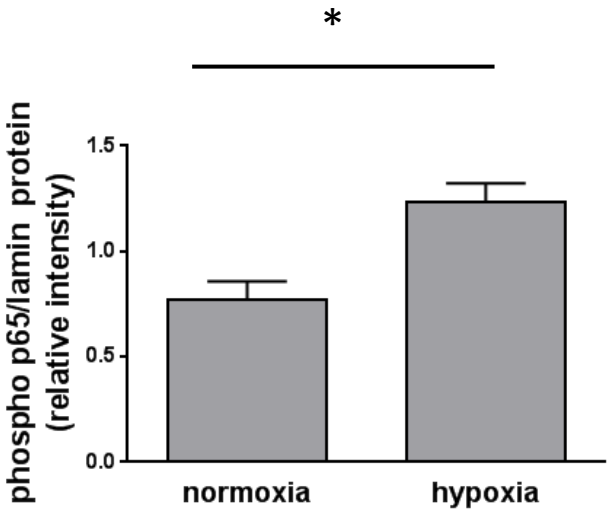


Nuclear Fraction

D

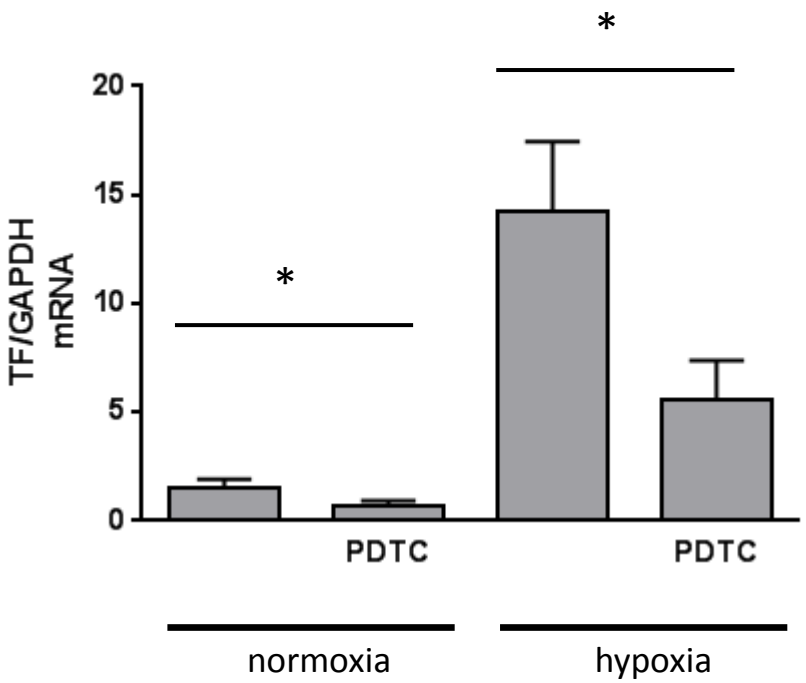


Cytoplasmic Fraction



Nuclear Fraction

E



F

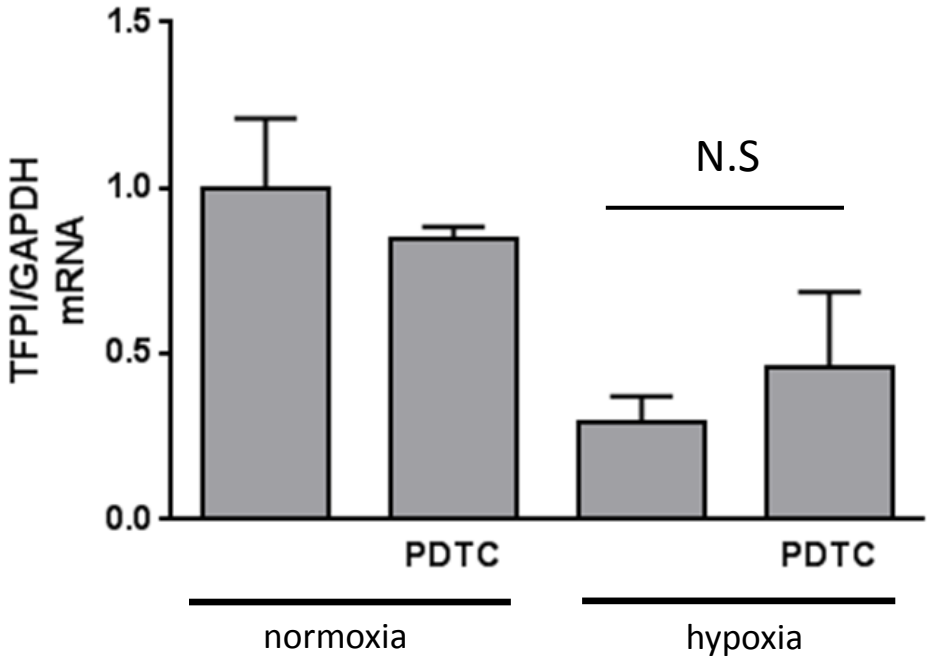


Figure8

A

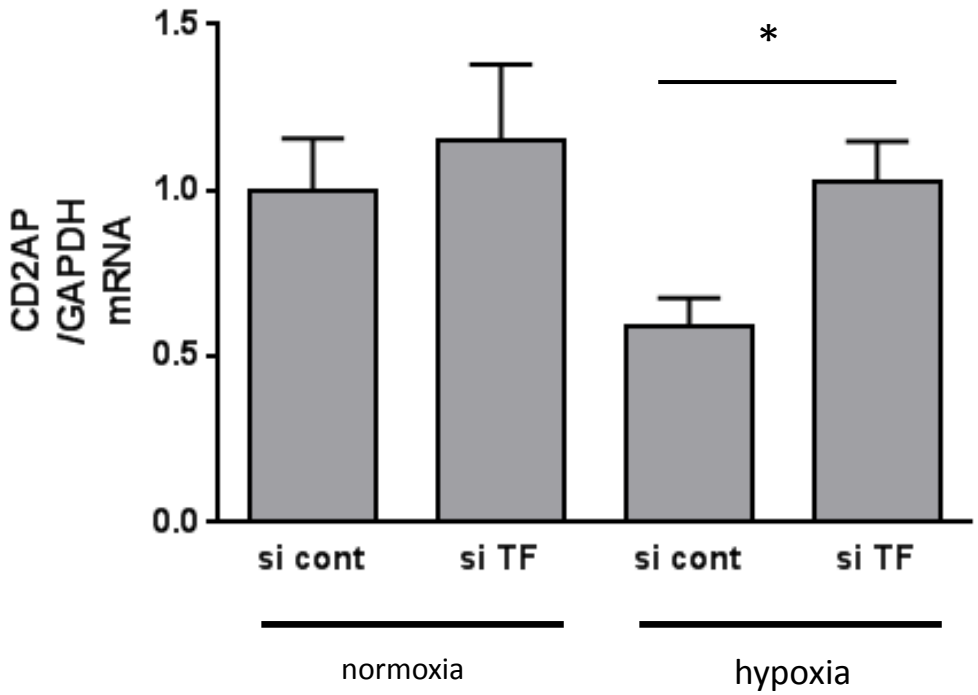
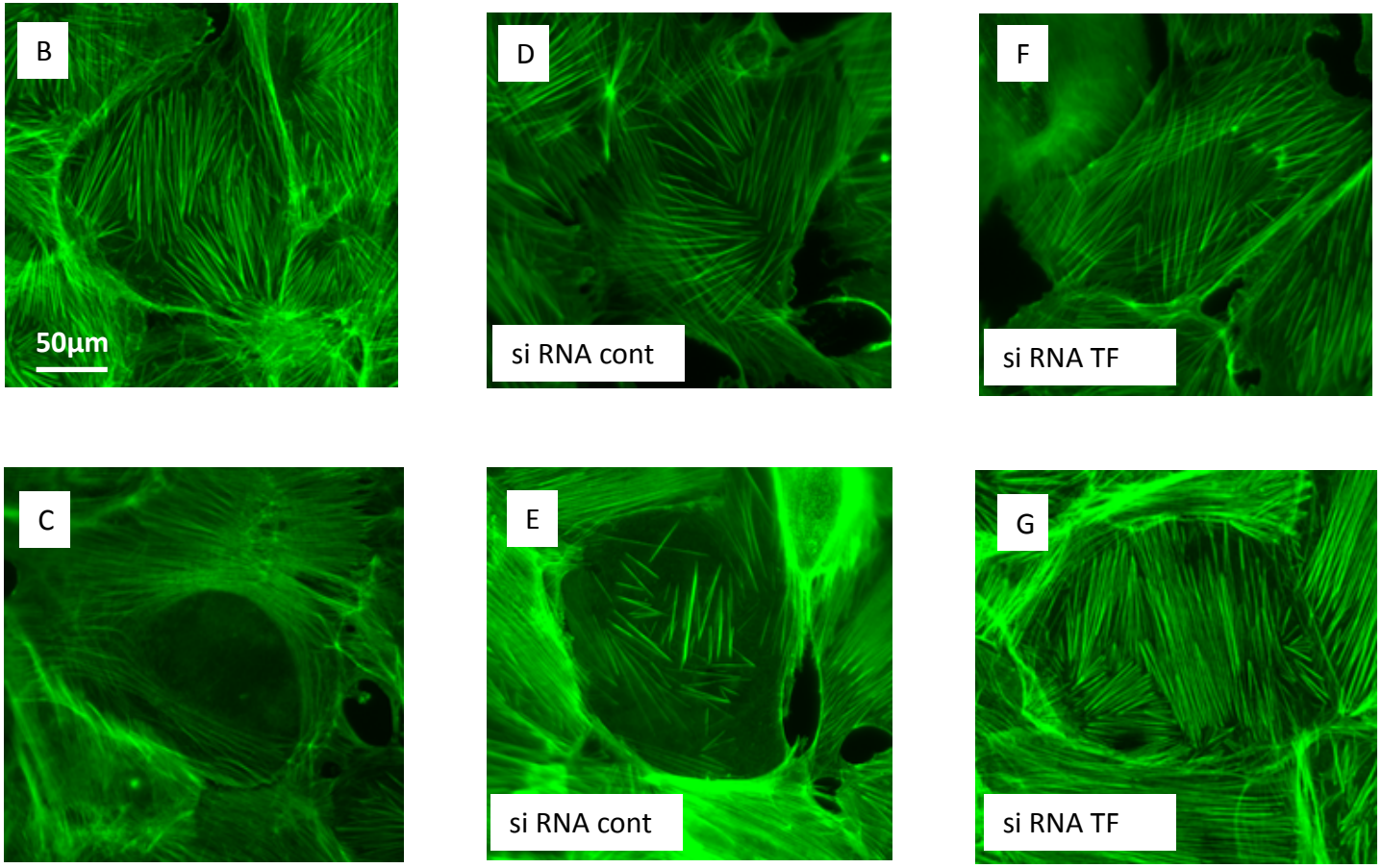

1.5
1.0
0.5
0.0
CD2AP /GAPDH mRNA
*
si cont
si TF
si cont
si TF
normoxia
hypoxia

Figure8


Normoxia
Hypoxia
B
50μm
D
si RNA cont
F
si RNA TF
C
E
si RNA cont
G
si RNA TF