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RESEARCH ARTICLE

Zinc deficiency promotes endothelin secretion and endothelial cell migration through nuclear hypoxia-inducible factor-1 translocation

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Morand J, Briançon-Marjollet A, Lemarie E, Gonthier B, Arnaud J, Korichneva I, Godin-Ribuot D. Zinc deficiency promotes endothelin secretion and endothelial cell migration through nuclear hypoxia-inducible factor-1 translocation. *Am J Physiol Cell Physiol* 317: C000–C000, 2019. First published May 22, 2019; doi:10.1152/ajpcell.00460.2018.—Zinc is involved in the expression and function of various transcription factors, including the hypoxia-inducible factor-1 (HIF-1). HIF-1 and its target gene endothelin-1 (ET-1) are activated by intermittent hypoxia (IH), one of the main consequences of obstructive sleep apnea (OSA), and both play a key role in the cardiovascular consequences of IH. Because OSA and IH are associated with zinc deficiency, we investigated the effect of zinc deficiency caused by chelation on the HIF-1/ET-1 pathway and its functional consequences in endothelial cells. Primary human microvascular endothelial cells (HMVEC) were incubated with submicromolar doses of the zinc-specific membrane-permeable chelator *N*,*N*,*N*′,*N*′-tetrakis(2-pyridylmethyl)-ethylene diamine (TPEN, 0.5 μM) or ET-1 (0.01 μM) with or without bosentan, a dual ET-1-receptor antagonist. HIF-1α expression was silenced by transfection with specific siRNA. Nuclear HIF-1 content was assessed by immunofluorescence microscopy and Western blot. Migratory capacity of HMVEC was evaluated with a wound-healing scratch assay. Zinc chelation by TPEN exposure induced the translocation of the cytosolic HIF-1α subunit of HIF-1 to the nucleus as well as an HIF-1-mediated ET-1 secretion by HMVEC. Incubation with either TPEN or ET-1 increased endothelial wound-healing capacity. Both HIF-1α silencing or bosentan abolished this effect. Altogether, these results suggest that zinc deficiency upregulates ET-1 signaling through HIF-1 activation and stimulates endothelial cell migration, suggesting an important role of zinc in the vascular consequences of IH and OSA mediated by HIF-1-ET- signaling.

endothelial cell migration; endothelin; HIF-1α; TPEN; zinc

INTRODUCTION

Zinc is an essential trace element that is vital for cell function as shown by the severe consequences of zinc deficiency (5). More specifically, zinc deficiency has been linked to various diseases associated with endothelial cell migration and defective angiogenesis, such as atherosclerosis, pulmonary hypertension (7), or cancer (22). The mechanisms by which zinc may affect endothelial cell function are multifaceted, since zinc has structural, catalytic, or signaling regulatory properties (19). Zinc binding or release from zinc-containing proteins can modulate their activity, as shown for protein kinase C (13), matrix metalloproteinases (21), or multiple transcription factors (19). In addition, zinc can also regulate the activity of transcription factors that do not contain structural zinc. Hence, zinc depletion has been shown to increase the activity of NF-κB and activator protein-1, leading to proinflammatory effects such as enhanced endothelial cell permeability and adhesion to monocytes (2) but also to increased expression of proangiogenic molecules such as VEGF, IL-8, and matrix metalloproteinase-9, by cancer cells (27).

The hypoxia-sensitive HIF-1 transcription factor is well known to play a key role in normal and defective angiogenesis. It was first identified in the 90s by Semenza, Kaelin, and Ratcliffe (11) as an heterodimer consisting of an oxygen-sensitive HIF-1α subunit and a constitutive HIF-1β subunit. In well-oxygenated cells, proline hydroxylation of the HIF-1α subunit by oxygen-dependent prolyl-hydroxylase domain enzymes (PHDs) leads to its proteasomal degradation. In hypoxic conditions, reduced PHD activity promotes HIF-1α stabilization, translocation to the nucleus, and dimerization with the HIF-1β subunit, triggering the transcription of several angiogenic genes such as placental growth factor, TGF-β1, and VEGF (11).

The link between zinc homeostasis and HIF-1 regulation is suggested by the fact that the HIF-1 pathway plays a major role in the diseases associated with zinc deficiency, such as atherosclerosis, pulmonary hypertension, and cancer (18). Despite a lack of direct experimental evidence, zinc supplementation has been shown to promote HIF-1α degradation and decreased VEGF expression in tumor cells (20). At the same time, zinc deficiency is associated with inhibition of HIF-1α ubiquitination and induction of VEGF expression in cancer cells (8).

Zinc deficiency is observed in obstructive sleep apnea (OSA) syndrome (6), a well-known risk factor for cardiovascular disease (17) and cancer (16). Interestingly, OSA patients display increased monocyte and tissue expression of HIF-1 (10, 12) and of HIF-1 gene products involved in endothelial dysfunction, cell migration, and angiogenesis, such as VEGF (3, 15) and endothelin-1 (ET-1) (9). We have previously shown that HIF-1 activation and ET-1 expression are major contributors to the vascular remodeling and enhanced atherosclerosis

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induced by chronic exposure to intermittent hypoxia (IH), a hallmark of OSA (1). Moreover, we observed that intermittent hypoxia exposure induces a significant decrease in plasma zinc levels in rodents (10.31 ± 0.53 μM after 35 days of IH versus 13.01 ± 1.19 μM after 35 days of normoxia, $P < 0.05$, unpublished observations).

In the present study, we established a link between zinc deficiency and the HIF-1/endothelin-1 pathway and demonstrated that it had physiological consequences in terms of cell migration. Using human microvascular endothelial cells, we provide here the first evidence that zinc deficiency caused by specific zinc chelation promotes HIF-1 translocation to the nucleus, leading to HIF-1-dependent ET-1 secretion and endothelial cell migration.

METHODS

Endothelial cells. Primary human adult dermal microvascular endothelial cells (HMVEC-D; Lonza) were cultured in EGM2-MV according to the supplier's recommendations. The cells were used between passages 4 and 8.

Immunofluorescence. Cells cultured on fibronectin-coated glass slides (1.5 μg/cm^2; Sigma) in 24-well dishes were exposed to vehicle (0.05‰ DMSO) or to the membrane-permeable zinc chelator *N*,*N*,*N*′,*N*′-tetrakis(2-pyridylmethyl)-ethylene diamine (TPEN, 0.5 μM in 0.05‰ DMSO; Fluka Analytical) for 2 and 5 h. They were then fixed with 4% paraformaldehyde and permeabilized in PBS-0.1% Triton. HIF-1α primary antibody (1:100; Abcam) and secondary antibody (Alexa Fluor 546, 1:500; Invitrogen) were incubated at 37°C for 60 and 45 min, respectively. 4′,6-Diamidino-2-phenylindole (DAPI, 0.5 μg/ml) was incubated during 10 min at room temperature. Slides were assembled with fluorescence mounting medium (Dako) and analyzed by fluorescence (AxioImager; Zeiss) or confocal (LSM710; Zeiss) microscopy. Quantification of the nuclear-to-cytosolic HIF-1 ratio was performed using ImageJ (NIH, Bethesda, MD) software.

Intracellular zinc imaging. Fluorescent staining of intracellular free zinc was performed using 15 μM *N*-(6-methoxy)-8-quinolyl-toluene sulfonamide (TSQ) as previously described (13). HMVECs were cultured in four-well Laboratory-Tek plates previously coated with fibronectin. At least 24 h after seeding, cells were loaded with 15 μM TSQ (Molecular Probes) and placed for 30 min in a CO_2 incubator at 37°C. Thereafter, extracellular TSQ was rinsed two times with serum-free cell medium. Cell fluorescence was analyzed under fluorescence microscopy (AxioVert, Zeiss, HBO 100 W; Metamorph Software) in controlled CO_2 and temperature conditions. Fluorescence excitation and emission wavelengths were 362 and 400–450 nm, respectively. In each well, fluorescence was measured at preselected positions. The first image (baseline) was recorded in serum-free cell medium before incubation with 30 μM TPEN. Images of each preselected position were recorded every 10 min during the 1-h exposure with TPEN. Whole cell fluorescence quantification was performed using ImageJ image processing software. All measurements were normalized to the baseline value of the corresponding position.

HIF-1α gene silencing. HMVEC were transfected with 20 nM scrambled siRNA coupled to fluorescein (Santa Cruz) or with 20 nM of a pool of three target-specific siRNAs directed against HIF-1α (Santa Cruz). In accordance, transfected cells are referred to as control and HIF-1α-neg, respectively. Transfection was performed at ~70% confluence using the TransIT-TKO reagent (Mirus) according to the supplier's recommendations. Transfection efficiency was assessed by fluorescein detection (see Fig. 2*A*) and by Western blot analysis of HIF-1α protein expression (see Fig. 2*B*).

Western blot analysis. After transfection (24–48 h), cultured cells were collected, centrifuged (1,000 revolutions/min, 5 min, 4°C), and stored at −80°C. Protein extraction was performed in RIPA buffer (50 mM Tris·HCl, pH 7.2, 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, 1 mM PMSF) with a protease inhibitor cocktail (Roche Diagnostics). Protein concentration of total protein extracts was determined using the Pierce BCA protein assay kit (Thermo Scientific). Quantification of HIF-1α nuclear content was performed on nuclear extracts (Nuclear Extract Kit; Active Motif) from HMVEC exposed to DMSO, TPEN, or $CoCl_2$ for 16 h.

Ten micrograms of total or cytosolic protein extracts were separated on 9% SDS-PAGE and transferred to nitrocellulose membranes (Amersham Biosciences). After blocking (0.1% TBS-Tween with 5% dry milk), membranes were incubated overnight at 4°C with rabbit anti-HIF-1α (no. 39665; Active Motif), mouse anti-β-actin (no. A3853; Sigma), and mouse anti-TATA-binding protein (no. ab818; Abcam) primary antibodies (1:1,000 in 0.1% TBS-Tween with 5% dry milk). Horseradish peroxidase-coupled anti-rabbit (Santa Cruz) and anti-mouse (Sigma) secondary antibodies (1:2,500 in 0.1% TBS-Tween with 5% dry milk) were applied during 1 h under gentle agitation at room temperature before enhanced chemiluminescence revelation (Thermo Scientific).

Wound-healing experiments. HMVEC were seeded at 40 × 10^3 cells/well in 48-well dishes (Nunclon) and allowed to reach 100% confluence. Thereafter, a scratch was performed by crossing the wells from side to side with a sterile 10- to 100-μl cone (Starlab; TipOne). Detached cells were removed by changing the culture medium. Nontransfected cells were incubated overnight with either vehicle, 0.01 μM ET-1, or 0.5 μM TPEN with or without 1 μM bosentan (graciously provided by Actelion Pharmaceuticals). Control or HIF-1α-neg cells were seeded 24 h after transfection and submitted to the same conditions. Wound repair was recorded during 16 h with a dynamic microscope (AxioVert 200M; Zeiss) under controlled temperature and CO_2 conditions. Images of two positions per well were taken every 30 min under ×10 magnification (Metamorph). Size of filled areas was determined by subtracting the cell-free area at the end of the experiment from the initial cell-free area (Image J software). Filled area values were normalized relative to control (vehicle-treated wells) values.

Quantitative PCR. For quantitative PCR analysis, HMVEC were cultured for 16 h with DMSO (control) or TPEN (0.5 μM). Next, cells were scrapped on ice, and dry pellets were used for mRNA isolation. Total mRNA was extracted from cell pellets using Tri-Reagent RNA isolation reagent (Sigma Aldrich). Endothelin-1 (*Edn1*) and *HIF-1α* gene expression was assessed by quantitative reverse transcription-polymerase chain reaction, normalized to β-actin and RPL27 rRNA content as internal controls, and expressed as fold change compared with DMSO control cell pellet using the ΔΔCT method. Primer sequences were as follows: *Edn1*: 5′-TGGCTTTCCAAGGAGCTCCAG-3′; 3′-GACGCGCTCGGGAGTGTTGA-5′; HIF-1α: 5′-TGGCAGCAACGACACAGAAACT-3′; 3′-TTGGCGTTTCAGCGGTGGGT-5′; β-actin: 5′-GCTGTGCTACGTCGCCCTGG-3′; 3′-ACAGGACTCCATGCCCAGGAAGG-5′; and RPL27: 5′-GGTGGTTGCTGCCGAAATG-3′; 3′-CTGTAGGGGCGATCTGAGGT-5′. AQ: 5

Endothelin-1 assay. ET-1 was measured in the culture medium of cells exposed to TPEN for 16 h using an ELISA kit (Enzo Life Sciences) according to the supplier's recommendations.

Statistical analysis. Data (expressed as means ± SE) were analyzed using GraphPad Prism 6 Software (San Diego, CA). The various statistical tests used are described in the figures. Statistical significance was set at $P < 0.05$.

RESULTS

TPEN induces the nuclear translocation of HIF-1α in HMVEC. Cell exposure to TPEN led to HIF-1α translocation from cytosol to nucleus. Fluorescent images presented in Fig. 1*A* demonstrate a significant proportion of HIF-1α in the nuclear areas well defined by DAPI staining on the correspond- F1

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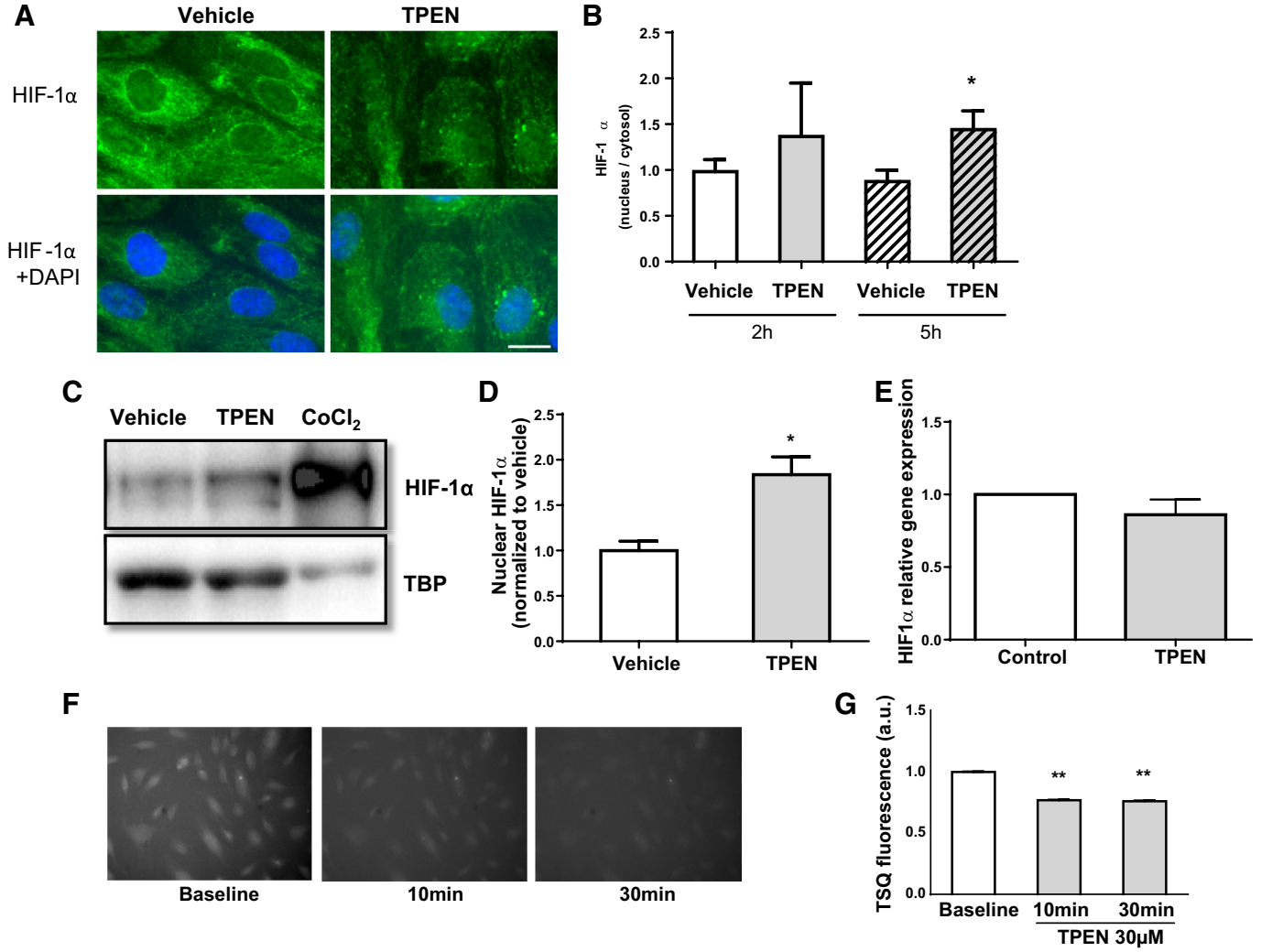

Fig. 1. *N,N,N′,N′*-tetrakis(2-pyridylmethyl)-ethylene diamine (TPEN) induces hypoxia-inducible factor-1α (HIF-1α) nuclear translocation in human microvascular endothelial cells (HMVEC). *A*: representative images showing HIF-1α alone (in green, *top*) or HIF-1α superposed with nuclear staining [DAPI in blue, *bottom*] in HMVEC treated for 5 h with vehicle (0.05‰ DMSO) or TPEN (0.5 μM). Bar = 10 μm. *B*: quantification of HIF-1α nucleus-to-cytosol ratios in HMVEC treated for 2 or 5 h with vehicle or TPEN. $N = 4$. $^*P < 0.05$ vs. vehicle-treated cells (Kruskal-Wallis rank-sum test). *C*: Western blot images of nuclear HIF-1α in HMVEC treated overnight with vehicle, TPEN, or $CoCl_2$ (as indicated above). TATA-binding protein (TBP) was used as a loading control. *D*: quantification of nuclear HIF-1α contents relative to TBP and normalized to vehicle-treated cells. $N = 4$. $^*P < 0.05$ vs. vehicle-treated cells (Mann-Whitney rank-sum test). *E*: HIF-1α gene expression assessed by quantitative RT-PCR in control cells and cells exposed to TPEN for 16 h. No significant difference was detected ($N = 3$). *F*: representative images showing fluorescent staining of zinc with *N*-(6-methoxy)-8-quinolyl-toluene sulfonamide (TSQ) probe at baseline or after 10 and 30 min of exposure to TPEN (30 μM), thus confirming zinc chelation by TPEN. *G*: quantification of TSQ fluorescence intensity. $N = 6$. $^{**}P < 0.02$ vs. baseline (one-way ANOVA followed by Tukey's post hoc tests for multiple comparisons).

COLOR

ing images. The quantitative analysis of distribution of HIF-1α between nuclear and cytosolic compartments is shown in the bar graphs of Fig. 1*B*. Indeed, the nuclear-to-cytosolic HIF-1α ratio observed by immunofluorescence was significantly increased after 5 h of incubation with TPEN ($P < 0.05$ versus vehicle-exposed cells). $CoCl_2$ was used as a positive control; it inhibits PHDs, resulting in abundant levels of HIF-1α translocated to the nuclei (data not shown). Immunofluorescent data were confirmed biochemically. Figure 1*C* displays typical Western blot with quantification given in Fig. 1*D*. It further shows that nuclear HIF-1α content was increased after an overnight exposure to TPEN ($P < 0.05$ versus vehicle-exposed cells). By contrast, quantitative PCR experiments showed that HIF-1α gene transcription is not affected by TPEN (Fig. 1*E*), suggesting that the increase in HIF-1α nuclear content is regulated at the protein level. It should be noted that the efficacy of TPEN for causing zinc deficiency was assessed in the fluorescent assay using the highly sensitive zinc-specific fluorescent probe TSQ. Figure 1*F* shows verification of TPEN efficacy on intracellular free zinc levels determined with TSQ, a zinc-specific fluorescent probe. Fluorescence microscopy images demonstrate intracellular zinc decrease in the cells exposed to TPEN. Figure 1*G* is the quantification of the TSQ imaging data. AQ: 6

Thus, zinc deficiency induced by TPEN provokes nuclear relocalization of HIF-1α.

TPEN promotes endothelin secretion by HMVEC in a HIF-1-dependent manner. The ET-1 pathway is one of the major signaling events activated by HIF-1α. Quantitative PCR analysis showed that TPEN induced a 24% increase of ET-1 gene

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transcription (Fig. 2*A*) that was reversed by zinc pyrithione supplementation (data not shown). Consistently, as assessed by ELISA of cellular media, ET-1 levels were increased by 28.5% in supernatant of cells exposed to TPEN for 16 h compared with those of vehicle-exposed cells ($P < 0.05$) (Fig. 2*B*). To confirm the role of HIF-1α in ET-1 secretion, we downregulated its expression using specific siRNA treatment. Transfection efficiency was assessed with Western blotting showing a 60% reduction of HIF-1 protein expression after transfection with anti-HIF-1α siRNA (Fig. 2*C*). Moreover, we used fluorescein-tagged siRNA and observed that almost 100% of the cells were indeed transfected (Fig. 2*D*, green dots). Finally, we further demonstrate that HIF-1α silencing prevented the effect of TPEN on ET-1 secretion (Fig. 2*B*). This suggests that TPEN-induced ET-1 secretion is mediated by HIF-1.

Both endothelin-1 and TPEN promote cell migration. The wound-healing assay was performed on the cells treated with either ET-1 or TPEN. Cell migration in the scratch area was recorded by videomicroscopy. The wound closure was significantly increased when cells were cultured with either ET-1 or TPEN ($P < 0.05$, Fig. 3*A*). The magnitude of the effects of ET-1 and TPEN on cell migration were similar (26.8 and 26.1% increase, respectively). To confirm ET-1 action through its membrane receptors, we used the dual ET_A- and ET_B-receptor-antagonist bosentan (1 μM). Although bosentan per se did not affect wound healing, it prevented both ET-1 and TPEN effects, suggesting that both agents involve the ET-1 receptor to activate cell migration (Fig. 3). Thus, both ET-1 and TPEN induce cell migration through the ET-1 receptor.

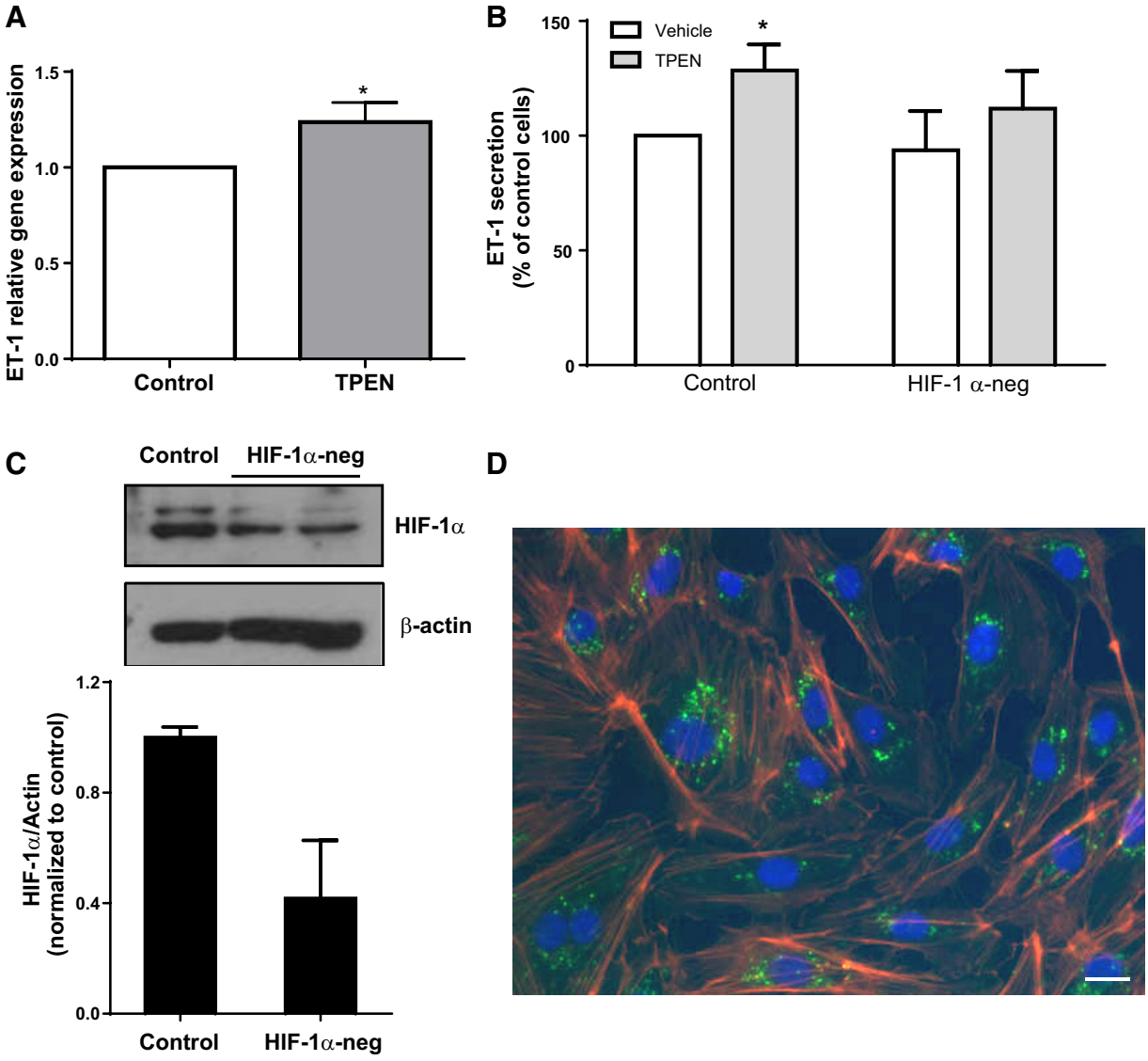

Fig. 2. *N*,*N*,*N*′,*N*′-tetrakis(2-pyridylmethyl)-ethylene diamine (TPEN) triggers hypoxia-inducible factor-1α (HIF-1α)-dependent endothelin (ET)-1 secretion by human microvascular endothelial cells (HMVEC). *A*: effect of TPEN (0.5 μM) on ET-1 gene expression assessed by quantitative PCR (Q-PCR). $N = 4$. *$P = 0.03$ versus vehicle-treated cells, Mann-Whitney rank-sum test. *B*: effect of TPEN (0.5 μM) on ET-1 secretion in culture medium of HMVEC transfected with scrambled (Control) or anti-HIF-1α (HIF-1α-neg) siRNA. $N = 6$. *$P < 0.05$ vs. vehicle-treated cells, Wilcoxon-signed rank test. *C*: representative Western blots and quantification of total cellular HIF-1α expression after transfection with scrambled or anti-HIF-1α siRNA, normalized to actin as a control. $N = 3$. *D*: representative image showing transfection efficacy observed by fluorescence microscopy in HMVEC transfected with scrambled siRNA coupled to fluorescein. Red, actin (phalloidin-TRITC); green, fluorescein-coupled-scrambled siRNA; blue, nuclei (DAPI). Bar = 10 μm.

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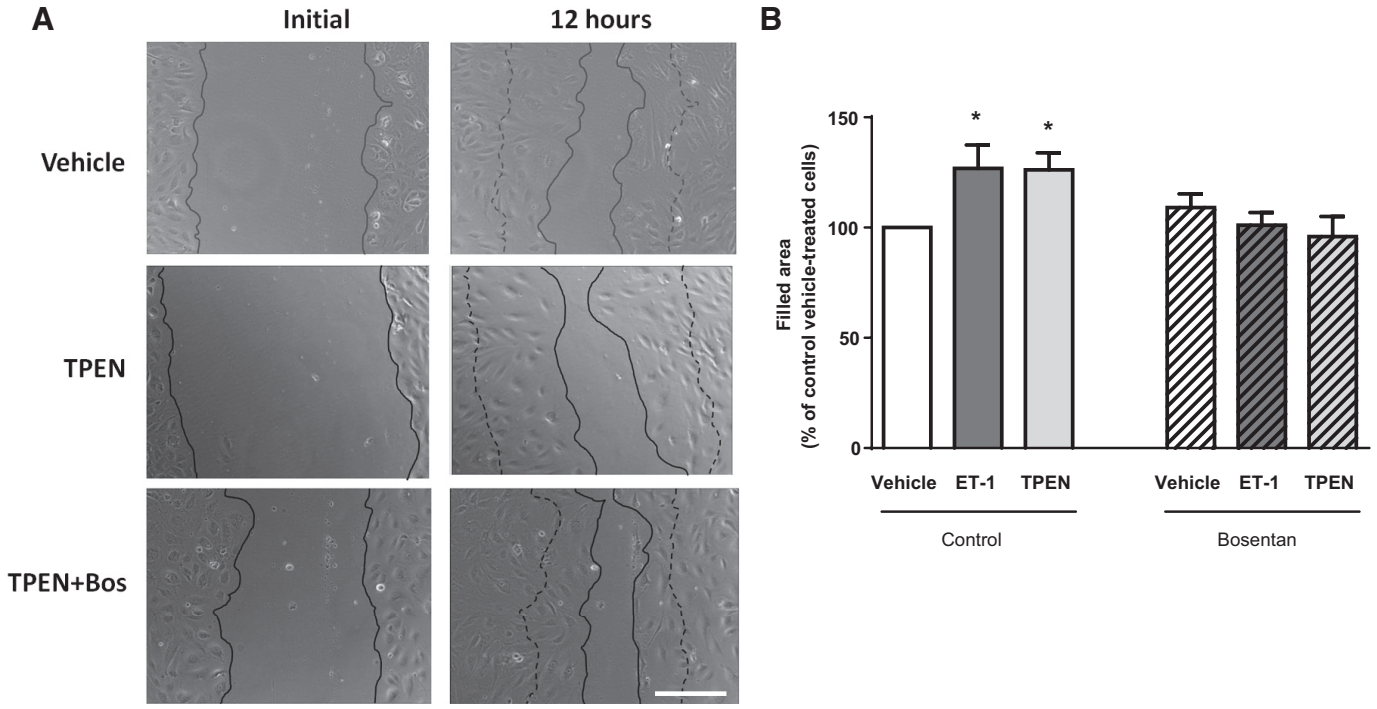

Fig. 3. Endothelial cell wound closure is induced by endothelin-1 (ET-1) and *N*,*N*,*N*′,*N*′-tetrakis(2-pyridylmethyl)-ethylene diamine (TPEN) and is abolished by bosentan. *A*: representative phase-contrast images (×10 magnification) showing wound at *time* (*t*) = *0* and wound closure after 12 h for cells treated with vehicle, TPEN (0.5 μM), or TPEN + bosentan (1 μM). Bar = 50 μm. *B*: quantification of wound healing for cells treated with vehicle, TPEN (0.5 μM), or ET-1 (10 nM) with or without bosentan (1 μM), normalized to control cells. $N > 5$. *$P < 0.05$ vs. vehicle-treated cells (Wilcoxon signed-rank test).

HIF-1α gene silencing abolishes endothelin- and TPEN-induced cell migration. To further trace the pathway of cell migration to HIF-1α, gene silencing by siRNA was applied before the wound-healing assay. Figure 4 presents images of the cells along with the quantification of the areas using ImageJ software. It demonstrates that siRNA transfection itself decreased wound-healing capacity by almost 40% compared with control cells ($P < 0.05$). In addition, it abolished both ET-1 and TPEN effects on cell migration ($P < 0.05$). Thus, we suggest that HIF-1α mediates TPEN effects on cell migration.

DISCUSSION

Zinc deficiency is a condition that relates to numerous pathologies and to aging. In the present study, we provide evidence that cellular zinc deficiency promotes the activation of the HIF-1/endothelin-1 pathway, resulting in increased migration of endothelial cells (Fig. 5).

Translocation of the HIF-1α subunit from the cytosol to the nucleus is necessary for the activation of HIF-1 and transcription of its target genes (11). We have shown that zinc chelation by TPEN does not modify HIF-1α gene transcription but rapidly induces nuclear translocation of HIF-1α protein in HMVEC, thus suggesting that zinc and/or zinc signaling are involved in the regulation of the HIF-1 expression and function in endothelial cells. This is in accordance with abundant literature suggesting that HIF-1α regulation is rather observed at the posttraductionnal level with mechanisms involving protein ubiquitination and proteasome degradation, rather than at the transcriptional level (24). Studies performed in cancer cells have proposed certain mechanisms behind such regulation. Specifically, Choi et al. showed that zinc chelation stabilizes HIF-1α by inhibiting its ubiquitination and by preventing the inhibitory hydroxylation of the Asp^{803} residue by the factor inhibiting HIF-1 (8). In accordance, Nardinocchi et al. observed that zinc supplementation decreases cytosolic HIF-1α levels by promoting its proteasomal degradation (20). In both studies, modulation of cytosolic HIF-1α levels was accompanied by changes in transcriptional activity of its target genes. Our study, however, is the first to confirm that stabilization of HIF-1α by zinc chelation effectively induces its translocation to the nucleus.

Dimerization of HIF-1α with HIF-1β in the nucleus, along with recruitment of transcriptional coactivators, leads to HIF-1 DNA binding and transcription of numerous genes, such as erythropoietin, VEGF, and ET-1 (1). In our study, TPEN induced HIF-1α translocation to the nucleus. HIF-1 transcriptional activity was too low to be detected by commercial kits under these normoxic conditions; however, when cells were under hypoxic conditions (3% O_2), TPEN addition provoked an almost threefold increase of HIF-1 transcriptional activity (data not shown). Moreover, TPEN-induced zinc chelation enhances ET-1 gene transcription, and TPEN-induced HIF-1α translocation increased ET-1 protein secretion, since it was abolished by HIF-1α silencing. It should be mentioned that HIF-1α silencing did not affect ET-1 secretion by nontreated HMVEC. This is in agreement with the observation showing that HIF-1 is not involved in the transcriptional control of ET-1 synthesis under control quiescent conditions (23).

To evaluate the functional consequences of the increase in ET-1 secretion induced by TPEN, we analyzed cell migration capacity using wound-healing scratch assay. Both TPEN and ET-1 accelerated wound closure to the same degree, suggesting that they use the same pathway to increase cell migration

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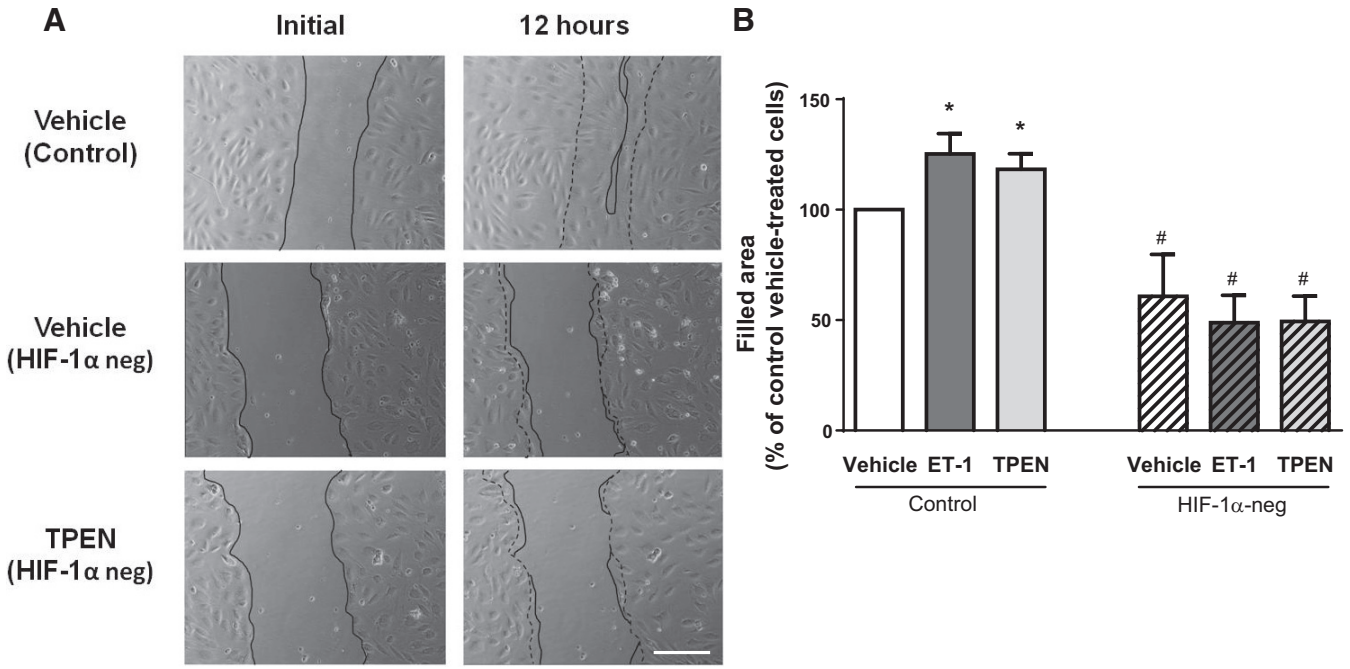

Fig. 4. Endothelial cell wound closure induced by *N,N,N′,N′*-tetrakis(2-pyridylmethyl)-ethylene diamine (TPEN) is hypoxia-inducible factor-1α (HIF-1α)-dependent *A*: representative phase-contrast images (×10 magnification) showing wound at *time* (*t*) = *0* and wound closure after 12 h for cells transfected with anti-HIF-1α siRNA and treated with TPEN (0.5 μM). Bar = 50 μm. *B*: quantification of wound healing for cells treated with vehicle, TPEN (0.5 μM), or ET-1 (10 nM) with or without anti-HIF-1α siRNA transfection, normalized to control cells. $N > 3$. *$P < 0.05$ vs. vehicle-treated cells (Wilcoxon signed-rank test). #$P < 0.05$ vs. corresponding control cells (two-way ANOVA).

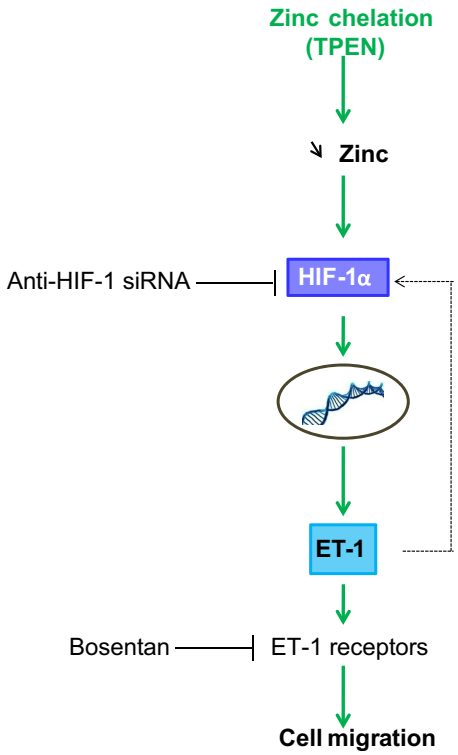

Fig. 5. Proposed pathway linking zinc deficiency and cell migration. *N,N,N′,N′*-tetrakis(2-pyridylmethyl)-ethylene diamine (TPEN) provokes zinc deficiency, which activates hypoxia-inducible factor-1 (HIF-1) nuclear localization. HIF-1 upregulates endothelin-1 (ET-1) secretion and ET-1, acting through its ET_A and/or ET_B receptors, activates cell migration. A retroactive feed-forward loop may link ET-1 and HIF-1 (dotted line).

capacity to promote angiogenic processes (14). The wound-healing capacity of cultured cells can potentially be a factor of cell migration and/or cell proliferation. However, because primary HMVEC have a very slow proliferation rate and the wound-healing effects of TPEN or ET-1 appeared very rapidly, we did not find a TPEN effect on proliferation in our system (data not shown), and we assume that wound-healing potentiation effects in our experiments were because of cell migration.

To add to the well-known effect of ET-1 on endothelial cell migration (26), our data are the first to show that zinc deficiency evokes the same consequences and suggest upstream regulation by zinc. Furthermore, the effects of both ET-1 and TPEN were blocked by dual endothelin receptor antagonist and by HIF-1 silencing, confirming that activation of the HIF-1/ET-1 pathway by TPEN is responsible for its effect on endothelial cell migration.

Interestingly, HIF-1α silencing also abolished ET-1-induced wound healing. This could be because of a cross talk between HIF-1 and ET-1 signaling. Indeed, ET-1 has been shown to reduce PHD2 activity and to enhance HIF-1α synthesis and stability in various cell types, including endothelial cells (4), thus promoting its secretion in a HIF-1-dependent feed-forward mechanism.

On the mechanistic level, zinc deficiency is often linked to oxidative stress (13). It appears to associate with prolonged IH, as suggested by our unpublished data. Oxidative stress induced by IH activates HIF-1 and its target gene, *ET-1*, which play a key role in the pathophysiological consequences of this IH (1). It may be suggested that certain pathways should be employed to protect the tissues from the impact of oxidative stress, and one of them is angiogenesis. The cell migration induced by zinc chelation and ET-1 could be of major importance in this angiogenesis.

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Finally, while our data clearly define the HIF-1-ET-1 pathway that activates HMVEC migration, it should be mentioned that the increased HIF-1 activity by zinc chelation may also promote cell migration through other HIF-1-dependent angiogenic factors, such as VEGF (11). Indeed, the angiogenic effect of VEGF is well recognized (25), and we recently showed that it promotes wound healing in HMVEC (3). Moreover, even though we verified that TPEN induced zinc deficiency in our cells, we cannot exclude that TPEN may have effects independent of zinc regulation. However, incubation with TPEN + zinc pyrithione reversed TPEN-induced ET-1 gene expression as well as HIF-1α nuclear relocalization and transcriptional activity (data not shown). Altogether, we suggest that TPEN effects on HIF-1 regulation are indeed the result of its zinc chelation properties.

In summary, the importance of this work is suggested by discovery of a new potentially compensatory pathway contributing to angiogenesis under the condition of oxidative stress, while the detailed molecular mechanism remains to be elucidated. This mechanism should be considered in aging and age- and stress-related pathologies, including intermittent hypoxia and OSA.

GRANTS

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DISCLOSURES

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AUTHOR CONTRIBUTIONS

J.M., A.B-M., I.K. and D. G-R. conceived and designed research; J.M., A.B.-M., E.L., B.G., and J.A. performed experiments; J.M., A.B.-M., E.L., B.G., and J.A. analyzed data; J.M. and A.B.-M. interpreted results of experiments; J.M. and A.B.-M. prepared figures; J.M., A.B.-M., and D.G.-R. drafted manuscript; J.M., A.B.-M., I.K., and D.G.-R. edited and revised manuscript;
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