VE-cadherin cleavage in sleep apnoea: new insights into intermittent hypoxia-related endothelial permeability

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Shareable abstract (@ERSpublications)
This study demonstrates for the first time that VE-cadherin is cleaved in sleep apnoea patients, in volunteers exposed to 14 nights of intermittent hypoxia and in endothelial cells exposed to *in vitro* intermittent hypoxia, leading to increased endothelial permeability https://bit.ly/3sAy5sc

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Abstract

Background Obstructive sleep apnoea (OSA) causes intermittent hypoxia that in turn induces endothelial dysfunction and atherosclerosis progression. We hypothesised that VE-cadherin cleavage, detected by its released extracellular fragment solubilised in the blood (sVE), may be an early indicator of emergent abnormal endothelial permeability. Our aim was to assess VE-cadherin cleavage in OSA patients and in *in vivo* and *in vitro* intermittent hypoxia models to decipher the cellular mechanisms and consequences.

Methods Sera from seven healthy volunteers exposed to 14 nights of intermittent hypoxia, 43 OSA patients and 31 healthy control subjects were analysed for their sVE content. Human aortic endothelial cells (HAECs) were exposed to 6 h of intermittent hypoxia *in vitro*, with or without an antioxidant or inhibitors of hypoxia-inducible factor (HIF)-1, tyrosine kinases or vascular endothelial growth factor (VEGF) pathways. VE-cadherin cleavage and phosphorylation were evaluated, and endothelial permeability was assessed by measuring transendothelial electrical resistance (TEER) and fluorescein isothiocyanate (FITC)–dextran flux.

Results sVE was significantly elevated in sera from healthy volunteers submitted to intermittent hypoxia and OSA patients before treatment, but conversely decreased in OSA patients after 6 months of continuous positive airway pressure treatment. OSA was the main factor accounting for sVE variations in a multivariate analysis. In *in vitro* experiments, cleavage and expression of VE-cadherin increased upon HAEC exposure to intermittent hypoxia. TEER decreased and FITC–dextran flux increased. These effects were reversed by all of the pharmacological inhibitors tested.

Conclusions We suggest that in OSA, intermittent hypoxia increases endothelial permeability in OSA by inducing VE-cadherin cleavage through reactive oxygen species production, and activation of HIF-1, VEGF and tyrosine kinase pathways.

Introduction

Obstructive sleep apnoea (OSA) is one of the most frequent chronic diseases, affecting up to nearly 1 billion individuals worldwide [1], and is characterised by the repetitive occurrence of apnoeas and hypopnoeas during sleep [2]. With the key feature of repeated episodes of hypoxia–reoxygenation, the main intermediary mechanism for the deleterious consequences of OSA is intermittent hypoxia.

OSA is associated with elevated cardiovascular morbidity and mortality due to an increased risk of hypertension, atherosclerosis, coronary heart disease and cerebrovascular disease [2]. Intermittent hypoxia is recognised as the key intermediary mechanism leading to cardiovascular morbidity and mortality. Indeed, intermittent hypoxia induces various processes such as oxidative stress, activation of hypoxia-inducible factor (HIF)-1, low-grade chronic inflammation and sympathetic activation, which in

turn are responsible for endothelial dysfunction, hypertension and atherosclerosis [2–4]. Importantly, the link between OSA/intermittent hypoxia and the structural and functional impairment of the vascular endothelium has been addressed both in patients and in animal models, in which elevated oxidative stress, higher production of inflammatory cytokines and pro-atherogenic adhesion molecules have been reported [3–7]. However, although the regulation of endothelial permeability is of major importance in the initiation and development of early atherogenesis, this aspect has been sparsely studied in OSA so far. Cellular and molecular mechanisms underlying the regulation of endothelial permeability in OSA have been investigated in a limited number of studies both in OSA patients and experimental models such as rodents [8] or cell culture [9, 10] exposed to intermittent hypoxia.

Endothelial barrier permeability is controlled by adherens junctions in which VE-cadherin, through its capability for homophilic adhesion, plays a pivotal role [11]. Targeting VE-cadherin *in vitro* using specific antibodies directed against epitopes in its extracellular domain impedes the formation of adherens junctions and increases endothelial permeability [12]. Two mechanisms explaining the reduction in VE-cadherin's homophilic adhesion have been described and are known to increase endothelial permeability: the first is the internalisation of the whole molecule [13] and the second, more recently described, is the cleavage of its extracellular domain by proteases such as matrix metalloproteinases. This cleavage releases a 90-kDa soluble fragment (sVE) detectable in blood [14, 15] and elevated serum levels of sVE have been documented in several human disorders characterised by endothelial dysfunction, such as atherosclerosis, systemic vasculitis and cancer metastasis [16, 17].

The phosphorylation of the cytoplasmic domain of VE-cadherin at a specific tyrosine site (Y685) by the tyrosine kinase Src is an early event of major importance in the mechanism of VE-cadherin cleavage and regulation of endothelial permeability [15, 18–20]. These phosphorylation processes are induced by cytokines such as vascular endothelial growth factor (VEGF) [18] and tumour necrosis factor (TNF)-α [15].

To the best of our knowledge, VE-cadherin regulation in OSA has never been studied. Several signalling pathways involved in VE-cadherin cleavage are activated in individuals with OSA or in intermittent hypoxia models, in which elevated VEGF [6, 21, 22] and TNF-α [23] levels are found. Thus, we hypothesised that intermittent hypoxia could activate signalling pathways leading to VE-cadherin cleavage and thereby increase endothelial permeability. These intermittent hypoxia-induced pathways could include generation of oxidative stress, activation of HIF-1 by reactive oxygen species (ROS) [2, 24], expression of VEGF as a target gene of HIF-1 [25, 26], and activation of Src kinases by VEGF signalling [18] and/or directly by ROS [27].

Our objectives were 1) to study sVE levels in OSA patient's sera before and after treatment with continuous positive airway pressure (CPAP) as well as in healthy volunteers exposed to 14 nights of intermittent hypoxia, and 2) to dissect *in vitro* the molecular mechanisms involved in VE-cadherin cleavage and increased permeability in an endothelial cellular model of intermittent hypoxia.

A visual overview of the study can be found in the supplementary material.

Methods

Study populations

Healthy volunteers exposed to 14 nights of intermittent hypoxia

We used sera from a double-blind randomised crossover study (ClinicalTrials.gov: NCT02058823) in which 12 healthy volunteers were exposed to intermittent hypoxia (inspiratory oxygen fraction (F_{IO_2}) cycling between 0.21 and 0.13; 120 s hypoxia–reoxygenation cycles), 8–9 h per night, for 14 nights, or to intermittent air (F_{IO_2} 0.21) with similar airflow as a control [28]. Among them, seven volunteers completed the two arms of the crossover trial. Blood samples were collected from these seven fasted volunteers in the morning, before and after 2 weeks of overnight exposure to intermittent air or to intermittent hypoxia, and stored at −80°C. More details can be found in the supplementary material.

OSA patients before and after CPAP treatment

Biological samples from a case-controlled study that included 43 OSA patients without any known cardiovascular comorbidity and 31 control subjects without OSA or cardiovascular disease (ClinicalTrials.gov: NCT00764218) were analysed [29]. Serum samples were taken fasted in the morning after overnight polysomnography performed before and after 6 months of CPAP treatment, or at 6 months follow-up without intervention in control subjects. Sera were stored at −80°C until use. VEGF was measured in sera by ELISA (Quantikine; R&D Systems, Minneapolis, MN, USA) [6].

Ethics

For both studies, ethical approval was obtained from our Institutional Review Board (Comité de Protection des Personnes Sud-Est V, Grenoble, France) and all subjects or patients gave written informed consent. Protocols conformed to the principles of the Declaration of Helsinki.

Cell culture

Cells

Cryopreserved primary human aortic endothelial cells (HAECs) (Thermo Fisher Scientific, Waltham, MA, USA) were used before passage 8. Cells were seeded at a density of 1.6×10^4 cells·cm^{-2} in M200 medium supplemented with Large Vessel Endothelial Supplement (LVES; Thermo Fisher Scientific) and penicillin/streptomycin according to the supplier's recommendations, using a conventional incubator for cell culture (37°C/5% CO_2). Cell culture reagents were purchased from Gibco Thermo Fisher Scientific (Illkirch, France).

Intermittent hypoxia exposure

Cells were exposed to fast intermittent hypoxia cycles using a recently developed device that mimics the hypoxia–reoxygenation cycles of OSA patients, as previously described [24]. An intermittent hypoxia cycle includes a 5-min normoxia phase (16% O_2) followed by a 5-min hypoxia phase (2% O_2). More details can be found in the supplementary material.

Chemicals and inhibitors

Details can be found in the supplementary material.

Measurement of transendothelial electrical resistance

Confluent HAECs seeded in transwell inserts (supplementary material) were placed in M200 without LVES, treated with dimethyl sulfoxide (DMSO) or inhibitors and exposed to intermittent hypoxia or normoxia for 6 h. Endothelial permeability was assessed by measuring transendothelial electrical resistance (TEER) at 0 and 6 h of exposure using an EVOM resistance meter (World Precision Instruments, Sarasota, FL, USA). Three values of TEER were recorded per insert and a blank insert (devoid of cells) was used to subtract the blank TEER value. Data are expressed as percentage of the TEER value of inserts containing confluent normoxic cells.

Measurement of fluorescein isothiocyanate–dextran flux

Confluent HAECs seeded in transwell inserts (supplementary material) were placed in M200 without LVES and exposed to intermittent hypoxia or normoxia. After 4 h of exposure, 40-kDa fluorescein isothiocyanate (FITC)–dextran (Sigma-Aldrich, Darmstadt, Germany) was added at 200 µg·mL^{-1} to the upper chamber of the transwell. The cells were then placed in the intermittent hypoxia device for another 2 h. The quantification of FITC–dextran flux through the endothelial monolayer was monitored by measuring the fluorescence in the lower chamber underneath the culture. Data are expressed as percentage of fluorescence of normoxic inserts.

Immunofluorescence

Cells (previously fixed with 4% paraformaldehyde in PBS for 10 min) were permeabilised and stained with mouse monoclonal anti-VE-cadherin primary antibody directed against the extracellular domain (BV9 clone; Merck Millipore, Darmstadt, Germany) followed by Alexa Fluor 488 anti-mouse secondary antibody (Thermo Fisher Scientific). Actin was stained with phalloidin and cell nuclei were stained with 4′,6-diamidino-2-phenylindole. Cells were examined by fluorescence microscopy (Axio Imager; Zeiss, Oberkochen, Germany) and fluorescence in the membrane area was quantified using ImageJ software (Fiji; imagej.net/Fiji) by subtracting cytoplasmic and nuclear staining from whole-cell staining.

VE-cadherin phosphorylation detection

Cells were placed in M200 without LVES for 1 h, then treated with the inhibitors and exposed to intermittent hypoxia or normoxia for 6 h. After exposure, cells were scrapped on ice using PBS and centrifuged at 250×*g*, at 4°C for 5 min. The presence of phosphorylated VE-cadherin in cell extracts was assessed by Western blotting with a rabbit anti-phospho-Y685-VE-cadherin antibody (1 µg·mL^{-1}). More details are available in the supplementary material.

sVE detection

Participant's sera and concentrated HAEC supernatants were analysed for sVE content. Sera were diluted 1:50 in PBS supplemented with 0.5% Triton. HAEC supernatants were collected after intermittent hypoxia exposure and concentrated using Amicon Ultra-4 centrifugal filter columns with 30-kDa molecular weight

cut-off (Sigma-Aldrich, Darmstadt, Germany). The presence of sVE was assessed by Western blotting with a mouse monoclonal anti-VE-cadherin primary antibody (BV9 clone; 1 μg·mL^{-1}). Investigators were blinded to the source of sera. More details about the Western blot protocol are available in the supplementary material.

Statistical analysis

Unpaired comparisons between two groups were made using the t-test or Mann–Whitney test and paired comparisons were performed using the Wilcoxon signed-rank test. Comparisons between several groups were performed by two-way ANOVA followed by the *post hoc* Tukey test. The choice of parametric or nonparametric tests depended on the normal distribution of values and on the equality of variances. Repeated measures ANOVA was performed when applicable (*i.e.* for subjects at baseline and month 6). A univariable linear model was used to identify factors associated with sVE. Since sVE was not normally distributed, a logarithmic transformation was performed. Variables with p<0.2 in the univariable analysis were considered in the multivariable linear regression, in which we adjusted for confounding parameters (age, sex, heart rate, arterial pressure, waist/hip ratio, cholesterol, glycaemia and creatinine). Finally, to assess the evolution of serum sVE under CPAP, a multivariate mixed linear regression with a random effect on patient was performed, adjusting on age, sex, CPAP adherence and duration between sVE measurements.

In cell culture experiments, to take into account the fact that several wells were usually used in a single independent experiment, the experiment was included as a random factor in a linear mixed model, where fixed effects were intermittent hypoxia and pharmacological treatment exposure. We verified that there was no significant impact of experiment on the effect of intermittent hypoxia or pharmacological treatment exposure.

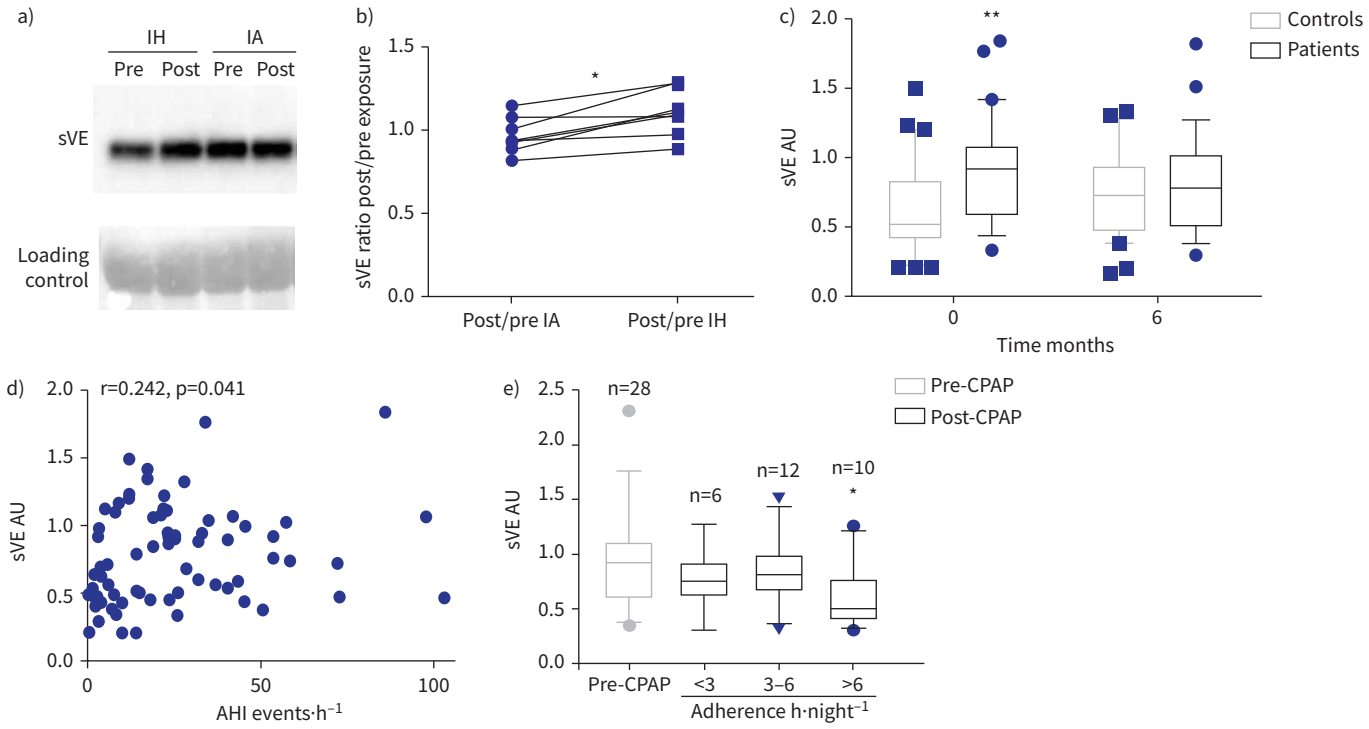

FIGURE 1 Circulating soluble VE (sVE) was increased in sera from healthy volunteers (controls) subjected to intermittent hypoxia (IH) and obstructive sleep apnoea (OSA) patients. IA: intermittent air; AHI: apnoea–hypopnoea index; CPAP: continuous positive airway pressure. a) Western blot showing a representative example of sVE (90 kDa) in the serum of a healthy volunteer before and after 14 nights of exposure to IH or IA. b) Ratio of sVE post/pre IH or IA exposure calculated after quantification of Western blots for seven individuals. *: p=0.015 *versus* IA; Wilcoxon test. c) sVE was significantly higher in patients than in control subjects at baseline (month 0) (**: p=0.0058; repeated measures two-way ANOVA) but not after 6 months of CPAP treatment (p=0.78). 30 subjects for each column, because only subjects for which both month 0 and month 6 samples were available were included in this paired analysis. d) Spearman correlation between AHI and sVE at baseline. 72 subjects. e) Patients with high adherence to CPAP therapy (>6 h·night^{-1}) significantly decreased their serum level of sVE after 6 months (*: p=0.034 *versus* sVE level before CPAP treatment) unlike less adherent patients (<6 h·night^{-1}; p>0.99 *versus* sVE level before CPAP treatment); Kruskal–Wallis test. Tukey plots indicate median and interquartile range.

TABLE 1 Univariate analysis for factors contributing to soluble VE variations at baseline

Variables	Items, classes	Estimator±SE	p-value
Group	OSA patients	0.413±0.12	0.001#,*
Age years	47–<53.5	0.335±0.18	0.18#
	53.5–<61.75	0.126±0.18	
	⩾61.75	−0.014±0.18	
Sex	Male	0.481±0.15	0.002#,*
BMI kg·m^{-2}	23.2–<24.9	−0.215±0.19	0.46
	24.9–<27.3	0.038±0.18	
	⩾27.3	0.065±0.02	
Neck circumference cm		0.056±0.18	0.01#,*
Waist/hip ratio		1.98±0.88	0.03#,*
Tobacco status	Current or ex-smoker	0.068±0.13	0.60
Alcohol consumption	No alcohol	−0.174±0.15	0.25
AHI events·h^{-1}	6.5–<21	0.19±0.17	0.04#,*
	21–<35.2	0.46±0.17	
	⩾35.2	0.39±0.17	
Epworth score	5–<9	−0.114±0.19	0.63
	9–<13	−0.175±0.19	
	⩾13	0.034±0.19	
Mean nocturnal S_{aO_2} %	93–<94	0.16±0.15	0.46
	94–<95	0.31±0.21	
	⩾95	0.07±0.18	
Minimum nocturnal S_{aO_2} %	94–<95	−0.021±0.009	0.03#,*
	⩾95		
Time spent S_{aO_2} <90% % TST	0–<2	0.08±0.16	0.62
	⩾2		
24-h SBP mmHg		0.021±0.005	0.0002#,*
24-h DBP mmHg		0.026±0.006	0.0002#,*
Heart rate beats·min^{-1}	64–<69	0.371±0.19	0.09#
	69–<76	0.217±0.18	
	⩾76	0.449±0.18	
VEGF pg·mL^{-1}		0.001±0.0002	0.04#,*
Ultrasensitive CRP mg·L^{-1}	0.5–<1.05	0.035±0.20	0.94
	1.05–<1.9	0.086±0.22	
	⩾1.9	−0.03±0.21	
Total cholesterol g·L^{-1}	1.8–<2	−0.180±0.18	0.13#
	2–<2.3	−0.082±0.19	
	⩾2.3	0.221±0.18	
Triglycerides g·L^{-1}	0.7–<1	0.074±0.18	0.81
	1–<1.4	0.162±0.19	
	⩾1.4	0.151±0.18	
Glycaemia mmol·L^{-1}		0.295±0.13	0.03#,*
Creatinine µmol·L^{-1}		0.0008±0.003	0.04#,*
Insulin µIU·mL^{-1}	3–<3.85	0.074±0.18	0.82
	3.85–<5.5	0.162±0.19	
	⩾5.5	0.151±0.18	

OSA: obstructive sleep apnoea; BMI: body mass index; AHI: apnoea–hypopnoea index; S_{aO_2}: arterial oxygen saturation; TST: total sleep time; SBP: systolic blood pressure; DBP: diastolic blood pressure; VEGF: vascular endothelial growth factor; CRP: C-reactive protein. Variables were categorised in three classes corresponding to terciles or in two categories to compare severe OSA *versus* other patients. #: variables with p<0.2 in the univariable analysis were considered in the multivariable linear regression. *: p<0.05.

Outcomes were considered significant when p<0.05. Values are reported as mean±SEM or median (interquartile range (IQR)), depending on their normal distribution.

Results

Intermittent hypoxia stimulates VE-cadherin cleavage in healthy individuals

We assessed sVE levels in the sera of seven healthy volunteers randomly assigned to 14 nights of intermittent hypoxia or intermittent air. The subjects' characteristics were: mean age 23.8±3.5 years and body mass index (BMI) 21.3±2.4 kg·m^{-2}, and five out of the seven (~70%) were male. sVE levels

TABLE 2 Baseline parameters independently associated with soluble VE as determined by a multivariate analysis

Variables	Items, classes	Estimator±SE	p-value
Group	OSA patients	0.346±0.12	0.005*
Sex	Male	0.387±0.15	0.01*
Age years	47–<53.5	0.277±0.16	0.23
	53.5–<61.75	0.075±0.16	
	⩾61.75	−0.022±0.16	
VEGF pg·mL^{-1}		0.0004±0.0002	0.06

OSA: obstructive sleep apnoea; VEGF: vascular endothelial growth factor. *: p<0.05.

remained stable after exposure to 14 nights of intermittent air (median post/pre intermittent air ratio 0.94) while they increased by 19% after 14 nights of intermittent hypoxia exposure (median post/pre intermittent hypoxia ratio 1.12; p=0.015 *versus* intermittent air) (figure 1a and b).

sVE levels are higher in OSA patients than in healthy control subjects

Baseline characteristics of the study population are shown in supplementary table S1. BMI, triglycerides and diastolic blood pressure were slightly higher in individuals with OSA, without clinical relevance. At baseline, the median sVE was significantly higher (+70%) in patients than in control subjects (0.90 (0.57–1.06) *versus* 0.52 (0.43–0.92) AU; p=0.0058) (figure 1c and supplementary table S1). Univariate analysis showed that among parameters significantly associated with sVE values were the OSA severity indices apnoea–hypopnoea index (AHI) and minimal oxygen saturation, sex, systolic and diastolic blood pressure, and the concentration of VEGF in sera (table 1). AHI positively correlated with sVE (r=0.242, p=0.041) (figure 1d). In multivariate analysis, OSA status was independently associated with a 34.6% increase in sVE (p=0.005). Male sex was associated with a 38.7% increase in sVE (p=0.01) (table 2).

sVE decreases after CPAP treatment

After 6 months of CPAP treatment, there was no difference in sVE levels between control subjects and patients (0.77 (0.51–0.92) AU for patients *versus* 0.72 (0.47–0.93) AU for controls; p=0.78) (figure 1c). The level of sVE in control subjects tended to increase between month 0 and month 6, but this increase remained nonsignificant (p=0.59). In a *post hoc* analysis, a decrease in sVE level only occurred in patients with the highest nightly CPAP usage (p=0.034 when CPAP was used for >6 h·night^{-1} *versus* nonsignificant for CPAP used <3 or 3–6 h·night^{-1}) (figure 1e).

Intermittent hypoxia increases VE-cadherin expression and cleavage in vitro *leading to endothelial permeability*

Western blot analysis showed a significant increase in sVE in supernatants of HAECs after 6 h of exposure to intermittent hypoxia compared with normoxia (+39%; p<0.05) (figure 2a). This result was accompanied by an apparent decrease in the presence of the extracellular domain of VE-cadherin at the cell membrane as shown by immunofluorescence (figure 2b). There was a significant 37.5% reduction in the TEER of HAEC monolayers exposed to 6 h of intermittent hypoxia compared with normoxia (figure 2c), reflecting an increase in endothelial permeability. This was confirmed by an increase in transendothelial FITC–dextran flux after intermittent hypoxia (+19.5%) (figure 2d).

We also demonstrated that intermittent hypoxia induced a 1.3-fold increase in the total amount of VE-cadherin (figure 3a and b) accompanied by a 2.5-fold increase in the amount of phosphorylated VE-cadherin tyrosine (Y685) compared with β-actin (figure 3c). The phosphorylated VE-cadherin/total VE-cadherin ratio tended to be elevated (median (IQR) ratio 1.3 (1.0–2.4)) although it did not reach significance (p=0.12) (figure 3d).

Signalling pathways involved in intermittent hypoxia-induced VE-cadherin cleavage

We investigated the role of oxidative stress, HIF-1 activation, VEGF signalling and tyrosine kinase activity. First, the role of oxidative stress was addressed by using Tempol, a potent ROS scavenger acting as a superoxide dismutase mimetic. Tempol significantly increased TEER values and decreased sVE levels in HAEC supernatants under intermittent hypoxia compared with DMSO, suppressing the intermittent hypoxia effects and returning TEER and sVE levels to baseline (figure 4a and b). Tempol had no effect on TEER and sVE levels in normoxia-exposed cells (figure 4a and b).

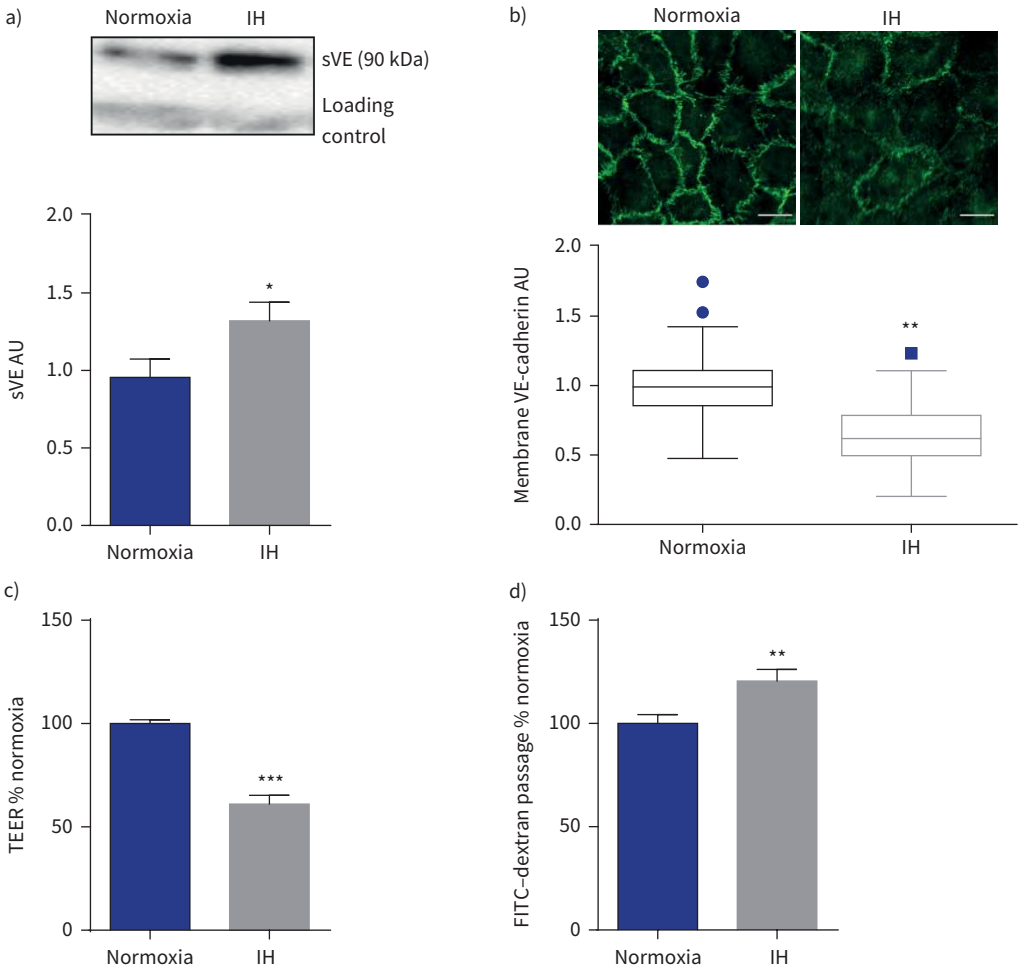

FIGURE 2 Intermittent hypoxia (IH) alters the endothelial barrier. sVE: soluble VE; TEER: transendothelial electrical resistance; FITC: fluorescein isothiocyanate. a) Representative example of Western blotting showing a more intense sVE band in the supernatant of human aortic endothelial cells exposed to IH than to normoxia (an immunoglobulin band was used as a loading control for normalisation) and quantification of sVE measured in cell supernatants after 6 h of IH. *: p<0.05 *versus* normoxia; t-test, n=15. b) Immunofluorescence and quantification of anti-VE-cadherin staining performed with an antibody directed against the VE-cadherin extracellular domain showed decreased expression at the membrane after IH. **: p=0.01 *versus* normoxia; Mann–Whitney rank sum test, four independent experiments and 10–14 cells per experiment. Scale bar: 20 µm. c) Increase in endothelial permeability measured by TEER after 6 h of IH. ***: p<0.001 *versus* normoxia; Welch test, 16 transwells from four independent experiments. d) Increase in endothelial permeability measured by FITC–dextran transendothelial flux (passage). **: p<0.01 *versus* normoxia; Welch test, 33 transwells from 12 independent experiments. Data are presented as mean±SEM except for the Tukey plot in b) where data are presented as median and interquartile range due to nonnormality of data distribution.

Similarly, inhibition of HIF-1 activity by 2-methoxyestradiol or acriflavine totally prevented any effect of intermittent hypoxia on endothelial permeability and VE-cadherin cleavage (figure 4c and d).

Moreover, inhibition of the tyrosine kinase activity of the VEGF receptor by pazopanib or by using an anti-human VEGF blocking antibody both abolished the effects of intermittent hypoxia on endothelial permeability and VE-cadherin cleavage (figure 5a and b).

Finally, we investigated the role of the Src family tyrosine kinases in the effects induced by intermittent hypoxia. Genistein, a broad-spectrum inhibitor of tyrosine kinases, as well as PP2 and AZD0530 (saracatinib), two specific inhibitors of Src kinases, blocked the intermittent hypoxia-induced decrease in TEER and VE-cadherin cleavage (figure 5c and d).

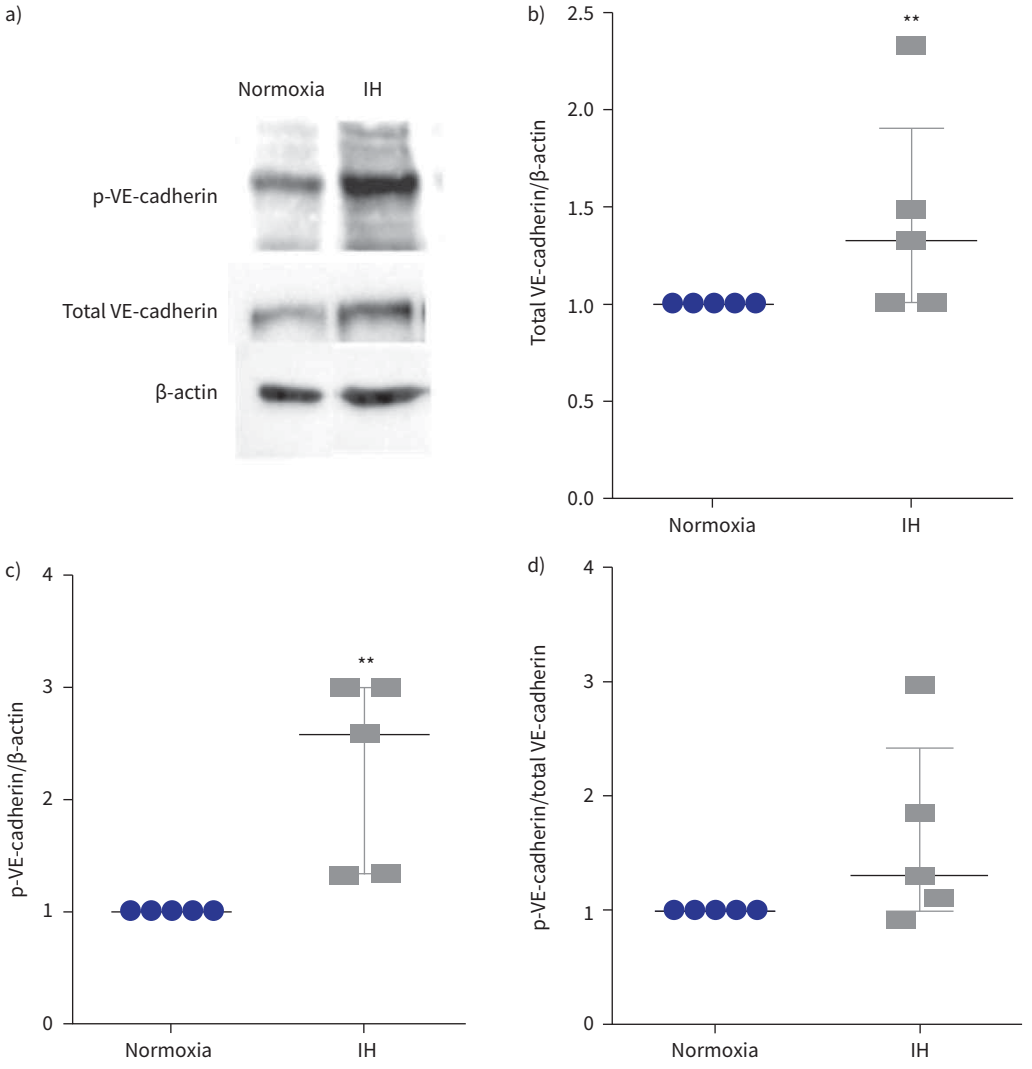

FIGURE 3 Expression and phosphorylation of tyrosine Y685 of VE-cadherin after intermittent hypoxia (IH). p: phosphorylated. a) Representative example of Western blotting showing a more intense p-VE-cadherin band in cell extracts of human aortic endothelial cells exposed to IH compared with normoxia. β-actin was used as a loading control for normalisation. b) Quantification of levels of total VE-cadherin normalised to β-actin measured in cell lysates. **: p<0.01 *versus* normoxia; Mann–Whitney test, n=5. c) Quantification of levels of p-VE-cadherin normalised to β-actin measured in cell lysates. **: p<0.01 *versus* normoxia; Mann–Whitney test, n=5. d) p-VE-cadherin/total VE-cadherin ratio. p=0.12; Mann–Whitney test, n=5. Data are presented as median and interquartile range.

Discussion

The present study investigates for the first time the cleavage of VE-cadherin in sleep apnoea patients and the specific involvement of the different intermittent hypoxia-activated pathways underlying the increase in endothelial permeability. We demonstrated an elevated level of sVE in sera of both healthy volunteers without any confounders upon exposure to intermittent hypoxia and OSA patients without any known cardiovascular comorbidity. This elevation in serum sVE correlated with AHI and was reversed by CPAP treatment only in the OSA subgroup with high adherence. In cultured cells exposed to intermittent hypoxia, we demonstrated that signalling pathways classically involved in VE-cadherin cleavage (VEGF and Src family tyrosine kinase activation) as well as pathways known to be activated by intermittent hypoxia (ROS and HIF-1 signalling) are implicated in the intermittent hypoxia-induced endothelial barrier dysfunction.

The description of VE-cadherin cleavage in OSA patients due to intermittent hypoxia is original and new. In multivariate analysis, the range of increase in sVE levels in OSA is nearly 40%, which is certainly of

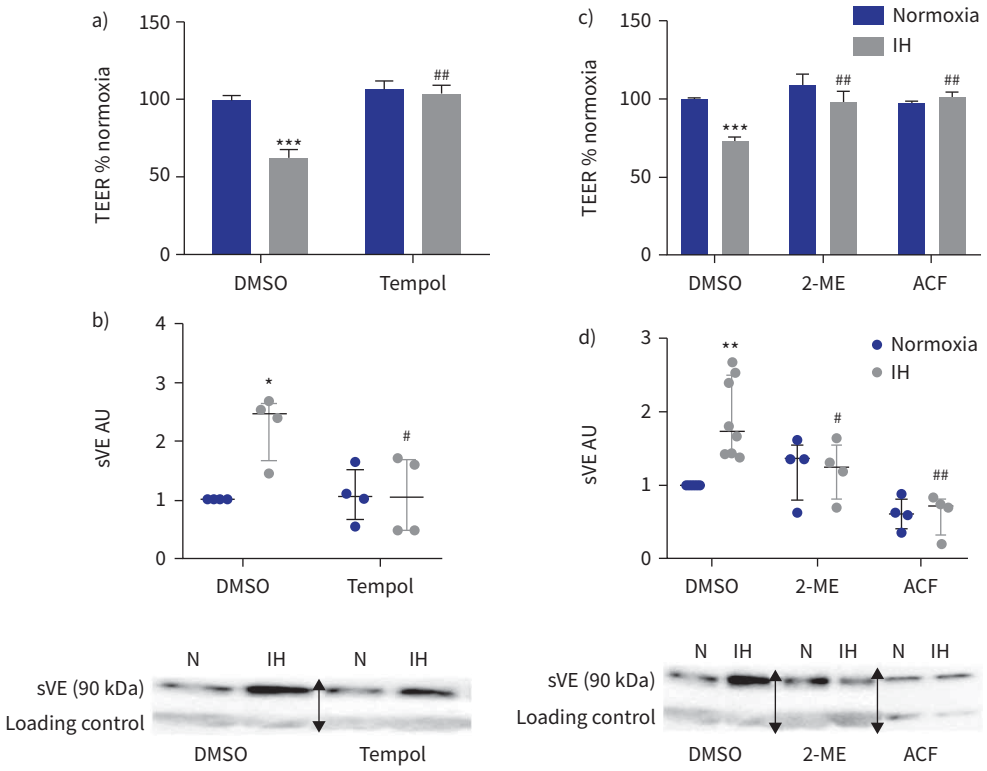

FIGURE 4 Impact of a reactive oxygen species scavenger (Tempol) and hypoxia-inducible factor (HIF)-1 inhibition on the endothelial barrier under intermittent hypoxia (IH) conditions. TEER: transendothelial electrical resistance; DMSO: dimethyl sulfoxide; sVE: soluble VE; 2-ME: 2-methoxyestradiol; ACF: acriflavine; HAEC: human aortic endothelial cell. a) Tempol (100 µM) abolished the effect of IH on endothelial permeability assessed by TEER. ***: p<0.001 *versus* normoxia+DMSO; ##: p<0.01 *versus* IH+DMSO; two-way ANOVA, 16 transwells from four independent experiments. Data are presented as mean±SEM. b) Levels of sVE measured in supernatants of HAECs treated with Tempol. *: p<0.05 *versus* normoxia+DMSO; #: p<0.05 *versus* IH+DMSO; two-way ANOVA, n=4. Data are presented as median and interquartile range. Below are shown representative examples of Western blotting showing a less intense sVE band in the supernatant of HAECs treated with IH +Tempol compared with IH+DMSO (an immunoglobulin band is used as a loading control for normalisation). N: normoxia. c) Inhibiting HIF-1 by 2-ME (1 µM) or ACF (1 µM) prevented the effect of IH on endothelial permeability assessed by TEER. ***: p<0.001 *versus* normoxia+DMSO; ##: p<0.01 *versus* IH+DMSO; two-way ANOVA, 16 independent transwells from four independent experiments. Data are presented as mean±SEM. d) Quantification of sVE levels measured in supernatants of HAECs treated with HIF-1 inhibitors. **: p<0.01 *versus* normoxia+DMSO; ##: p<0.01 and #: p<0.05 *versus* IH+DMSO; two-way ANOVA, n=4. Data are presented as median and interquartile range. Below are shown representative examples of Western blotting showing a less intense sVE band in the supernatant of HAECs treated with IH+HIF-1 inhibitors compared with IH+DMSO (an immunoglobulin band is used as a loading control for normalisation). N: normoxia. On Western blots, arrows indicate where images were grouped from different parts of the same gel or from different gels.

clinical relevance. The presence of sVE in serum is recognised as a marker of endothelial dysfunction in several cardiovascular and inflammatory diseases (for review, see [16]), including atherosclerosis [30]. VE-cadherin cleavage leads to the destabilisation of adherens junctions and increases endothelial permeability. This endothelial disorganisation and increased permeability is one of the early steps in the atherogenic process. In our clinical study, patients were free of known cardiovascular comorbidities often occurring with sleep apnoea and our results suggest that in this population sVE might represent an early biomarker revealing initiation of the endothelial dysfunction process. The impact of CPAP treatment on reducing incident late cardiovascular events is still debated and the majority of well-designed randomised controlled trials have been negative (for review, see [31]). Patients included in these trials exhibited prevalent cardiovascular disease and any benefit of CPAP intervention was limited by already existing irreversible lesions. Our results suggest that 6 months of CPAP treatment significantly decreases VE-cadherin cleavage in OSA patients, exerting a protective effect on endothelial function in patients at an

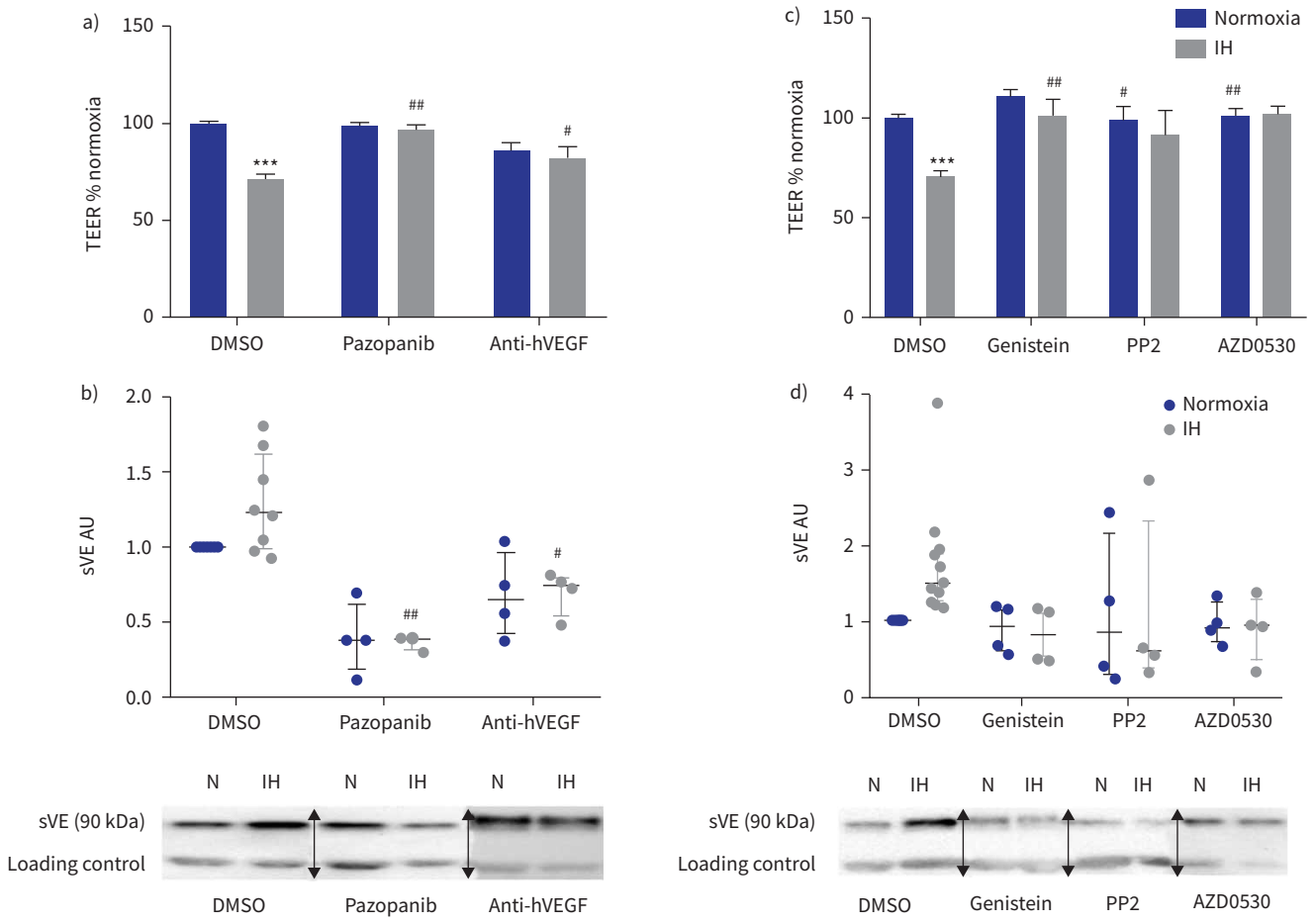

FIGURE 5 Impact of inhibition of vascular endothelial growth factor (VEGF) signalling and tyrosine kinase inhibition on the endothelial barrier under intermittent hypoxia (IH) conditions. TEER: transendothelial electrical resistance; DMSO: dimethyl sulfoxide; h: human; sVE: soluble VE; HAEC: human aortic endothelial cell. a) Inhibiting VEGF receptor tyrosine kinases by pazopanib (5 μg·mL^{-1}) and blocking hVEGF by anti-hVEGF blocking antibody (0.5 μg·mL^{-1}) prevented the effect of IH on endothelial permeability as assessed by TEER. ***: p<0.001 *versus* normoxia+DMSO; $^{\#\#}$: p<0.01 and $^{\#}$: p<0.05 *versus* IH+DMSO; two-way ANOVA, 19 transwells from four independent experiments. Data are presented as mean±SEM. b) Quantification of levels of sVE measured in supernatants of HAECs treated with VEGF signalling inhibitors. $^{\#\#}$: p<0.01 and $^{\#}$: p<0.05 *versus* IH +DMSO; two-way ANOVA, n=4. Data are presented as median and interquartile range. Below are shown representative examples of Western blotting showing a less intense sVE band in the supernatant of HAECs treated with IH+VEGF signalling inhibitors compared with IH+DMSO (an immunoglobulin band is used as a loading control for normalisation). N: normoxia. c) Inhibiting tyrosine kinases by genistein (30 μM) and Src kinases by PP2 (10 μM) and AZD0530 (saracatinib, 1 μM) prevented the effect of IH on endothelial permeability assessed by TEER. ***: p<0.001 *versus* normoxia+DMSO; $^{\#\#}$: p<0.01 and $^{\#}$: p<0.05 *versus* IH+DMSO; two-way ANOVA, 16 transwells from at least four independent experiments. Data are presented as mean±SEM. d) Quantification of sVE levels measured in HAEC supernatants showing that treatment by tyrosine kinase inhibitors abolished the effect of IH on VE-cadherin cleavage. n=4. Data are presented as median and interquartile range. Below are shown representative examples of Western blotting showing a less intense sVE band in supernatants of HAECs treated with tyrosine kinase inhibitors+IH compared with DMSO+IH (an immunoglobulin band is used as a loading control for normalisation). N: normoxia. On Western blots, arrows indicate where images were grouped from different parts of the same gel or from different gels.

early stage of development of cardiovascular dysfunction. This is of major importance as the field is currently desperately seeking biomarkers to predict cardiovascular response to CPAP so as to personalise prescription [32].

OSA is characterised by intermittent hypoxia associated with sleep fragmentation and increased respiratory efforts during sleep. Among these components, our data suggest that intermittent hypoxia could be the main contributing factor involved in VE-cadherin cleavage. A model of exposure to intermittent hypoxia in healthy volunteers without any confounders robustly demonstrated an increase in sVE levels in sera. Despite a small number of participants, we were able to demonstrate a significant increase of sVE in a

paired analysis, indicating that this effect is homogeneous among patients and consistent with our hypotheses and the other *in vitro* and *in vivo* results. Accordingly, intermittent hypoxia applied to endothelial cells in culture also led to VE-cadherin cleavage. Although a previous publication suggested that membrane expression of VE-cadherin decreased after intermittent hypoxia in cultured human endothelial cells [9], the mechanism involved (*i.e.* internalisation or cleavage) was never investigated. We demonstrate for the first time that VE-cadherin is cleaved *in vivo* in OSA patients, as well as after intermittent hypoxia in cultured human endothelial cells, releasing a soluble fragment in cell supernatant as well as in patients' blood. Regarding the cascade of molecular mechanisms, in OSA patients multivariate analysis found that VEGF tended to be independently associated with sVE levels ($p=0.06$). These findings are in line with the known mechanisms of VE-cadherin cleavage after VEGF binding to its receptor [18] and support the hypothesis that VEGF, which is known to be elevated in OSA patients [6, 21, 22], may be a key cytokine signalling VE-cadherin cleavage. This was confirmed by the demonstration that both an anti-VEGF blocking antibody and an inhibitor of the tyrosine kinase activity of the VEGF receptor (pazopanib) prevented intermittent hypoxia-induced sVE release and endothelial permeability *in vitro*. Similarly, tyrosine kinase inhibitors, either nonspecific (genistein) or targeting the Src family tyrosine kinases (PP2 and saracatinib), were able to prevent intermittent hypoxia-initiated consequences. Finally, we showed that intermittent hypoxia induces an increase in VE-cadherin phosphorylation at the Y685 tyrosine, confirming that intermittent hypoxia activates the major signalling pathways leading to sVE release [15, 19].

Since VEGF is a target of the HIF-1 transcription factor, which is known to be activated in individuals with OSA as well as in murine models of intermittent hypoxia (for review, see [33]), we investigated the involvement of HIF-1 in our *in vitro* model. None of the currently available HIF-1α inhibitors are truly specific. We thus used two different inhibitors, 2-methoxyestradiol (an HIF-1 inhibitor devoid of any oestrogenic activity) [34, 35] and acriflavine [36], to strengthen our approach and demonstrated that HIF-1 is involved in intermittent hypoxia-induced VE-cadherin cleavage.

Finally, in OSA and intermittent hypoxia, HIF-1 is stabilised and activated by repeated hypoxia–reoxygenation cycles and associated ROS production [33, 37]. We thus used a superoxide dismutase mimetic, Tempol, to demonstrate that ROS are indeed necessary for intermittent hypoxia-induced sVE release and endothelial permeability. Our results are consistent with results from Makarenko *et al.* [9] who showed that intermittent hypoxia-induced permeability and decrease of VE-cadherin membrane expression were dependent on ROS. Interestingly, beyond the known activation of HIF-1 by ROS, ROS have also been suggested as directly activating tyrosine kinases [28]. HIF-1-independent pathways may thus be involved in intermittent hypoxia effects on endothelial permeability. Similarly, we cannot exclude that other cytokines and pathways may lead to VE-cadherin cleavage in our model, such as TNF-α [15]. This needs to be explored in future studies.

To conclude, we demonstrated that intermittent hypoxia induces VE-cadherin cleavage at least partly mediated by ROS, HIF-1, VEGF and tyrosine kinase pathways. The sVE-cadherin fragment was found at elevated levels in healthy volunteers exposed to intermittent hypoxia as well as in OSA patients, and might provide an innovative biomarker of increased endothelial permeability and early stages of related disorders.

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