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Biphasic effect of sulforaphane on angiogenesis in hypoxia via modulation of both Nrf2 and mitochondrial dynamics

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Abstract: Sulforaphane (SFN) is an isothiocyanate (ITC) derived from a glucosinolate, glucoraphanin found in cruciferous vegetables. There are few studies that focus on the role of SFN in angiogenesis under hypoxic conditions. The effect of SFN on angiogenesis and the underlying mechanisms including the roles of Nrf2 and mitochondrial dynamics were investigated using cultured human umbilical vein endothelial cells (HUVECs) in hypoxia. SFN at low doses (1.25-5μM) increased hypoxia-induced HUVEC migration and tube formation, and alleviated hypoxia-induced retarded proliferation, but high doses (≥10μM) exhibited an opposite effect. Under hypoxia, the expression of Nrf2 and heme oxygenase-1 was up-regulated by SFN treatment. Nrf2 knockdown abrogated SFN (2.5μM)-induced tube formation and further potentiated the inhibitory effect of SFN (10μM) on angiogenesis. Meanwhile, the mitochondrial function, morphology and expression of dynamic-related proteins suggested that low dose SFN protected against hypoxia-induced mitochondrial injury and alleviated hypoxia-induced fission Nrf2-dependently without affecting the expression of key effector proteins (Drp1, Fis1, Mfn1/2 and Opa1), while high concentrations (≥10μM SFN) aggravated hypoxia-induced mitochondrial injury, fission and Drp1 expression, and inhibited Mfn1/2 expression. These findings suggest that SFN biphasically affected the angiogenic capacity of hypoxia challenged HUVECs potentially via mechanisms involving an integrated modulation of Nrf2 and mitochondrial dynamics.

Keywords: Sulforaphane · Isothiocyanates · Angiogenesis · Hypoxia · Nrf2 · Mitochondrial dynamics

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Introduction

The term "angiogenesis" is commonly used to reference the process of vessel growth but in its strictest sense it denotes vessels sprouting from pre-existing ones, and is a complex multistage process mainly involving extracellular matrix (ECM) degradation, proliferation, survival, migration, and morphological changes of endothelial cells (ECs) and their anastomosis to assemble into a vascular structure, as well as pericyte attachment. In physiological conditions, angiogenesis influences growth, development, tissue repair and menstrual cycle, but its disturbance can underlie many serious diseases, called angiogenesis-dependent diseases. Examples include: insufficient vessel growth or maintenance leading to stroke, myocardial infarction and neurodegeneration, abnormal vessel growth or remodeling contributing to pulmonary hypertension, inflammatory diseases, atherosclerosis and to most cancers. Historically, this has led to concepts of pro- and anti-angiogenic therapy, aiming to restore adequate vessel densities.[1,2]

Ischemic diseases, including myocardial infarction, stroke and peripheral vascular disease, have a high prevalence around the world and give rise to a high disability rate and mortality. Angiogenesis is both an essential adaptive response to physiological stress and an endogenous repair mechanism after ischemic injury. However, aging, diabetes, hypercholesterolemia and hypertension, the risk factors for ischemic diseases, can also constitute a deleterious macroenvironment that participate in the impairment or abrogation of post-ischemic revascularization and tissue regeneration. In turn, impaired angiogenesis can further lead to exacerbation of these diseases.[3,4] Identifying and using nutraceuticals and herbal medicines have become a potential therapeutic option to maintain an adequate vascularization and correct endothelial cell function or to blunt aberrant angiogenesis.[5]

Sulforaphane (4-methylsulfinybutyl isothiocyanate, SFN) is a bioactive isothiocyanate (ITC) derived from cruciferous vegetables such as broccoli, cauliflower, cabbage and kale. It has been intensively investigated *in vitro*, in a range of animal models, and clinical trials in diseases such as cancer, metabolic diseases and neurodegenerative diseases. The beneficial effects and the lack of serious side effects observed to date, together with high bioavailability make SFN a promising candidate in the adjuvant therapy of these diseases. Underlying the remarkable efficacy are its pleiotropic activities, for example, inducing antioxidant/detoxifying enzymes, anti-inflammation, mitostatic and pro-apoptotic

effects, epigenetic regulation of genes, improving metabolism.[6-9] There is also evidence indicating that SFN and its metabolites and analogues possess anti-angiogenic properties,[7,10] and the potential mechanisms, though not fully elucidated, may involve inducing ECs apoptosis,[11,12] inhibiting matrix metalloproteinase-2/9 (MMP-2/9),[13,14] hypoxia-inducible factor-1α (HIF-1α),[13,15,16] vascular endothelial growth factor (VEGF),[13,15,17,18] VEGF receptor-2 (VEGFR-2),[13,18] disrupting endothelial mitotic progression and microtubule polymerization,[19] anti-inflammation,[20] activating FOXO,[21] inhibiting STAT3[22,23] and inducing ROS.[24]

As a hormetic molecule, SFN displays characteristic biphasic dose response with a stimulatory or beneficial effect at low doses and an inhibitory or toxic effect at higher doses.[25,26] The hormetic property of SFN underlies many of its diverse biological activities and offers a reasonable explanation for its paradoxical effects. In regard to this, nuclear factor E2-related factor 2 (Nrf2) is an important factor.[27] Nrf2 is the master regulator of hundreds of transcripts, controlling cellular homeostasis through different and interconnecting effects. It can be induced by a variety of substances and stimuli, and in particular, SFN is recognized as the most classical and potent activator.[10,27] Evidence suggests that intracellular conjugation with GSH promotes SFN cellular uptake and concentration, and subsequently, moderate and transient cellular GSH pool depletion can adaptively induce the expressions of a battery of antioxidant/detoxifying enzymes via activation of Nrf2, providing powerful and prolonged protection against various stresses. Nevertheless, once above a concentration threshold, SFN may display cytotoxicity deriving from complex, concurring, and multiple mechanisms.[25] Interestingly, Nrf2 has also been confirmed to mediate pro-angiogenesis which has been documented to not only exert protective effects on several ischemic diseases but also contribute to the tumor growth, metastasis and chemo-resistant.[28-31]

Mitochondria have emerged as signaling hubs that modulate a wide range of endothelial functions including angiogenesis by coordinating reactive oxygen species, metabolism, apoptosis and calcium signaling.[32] Moreover, mitochondrial dynamics is also identified as a crucial determinant of angiogenesis,[33] which is regulated by opposing fusion and fission events via a complex protein machinery with mitofusin 1/2 (Mfn1/2) and optic atrophy (Opa1) as key pro-fusion proteins, and dynamin-related protein 1 (Drp1) and its receptors/adaptors like fission protein 1(Fis1) as pivotal pro-fission proteins. Mitochondrial dysfunction and dynamics disorder that can result from a wide variety

of factors such as aging, obesity, diseases, deprivation of mitochondrial fuel substrates or oversupply of nutrients including glucose and fatty acids, hypoxia-reoxygenation/ischemia-reperfusion, hemodynamic forces, environmental and chemical stressors, contribute to the pathogenesis of complex diseases including cardiovascular disease, cancer, neurodegenerative diseases and metabolic syndrome.[34-36] Therefore, improving mitochondrial function and preserving mitochondrial fusion have been considered as a potent defense against compromised angiogenesis and ischemic diseases,[37-39] whereas inducing mitochondrial dysfunction and fission can be taken as a potential strategy for anti-angiogenesic and anti-proliferative cancer management.[40-42] Just like the paradoxical effect on redox, modulation of mitochondrial functions by SFN is also a contradictory dual role.[7,43] As for mitochondrial dynamics, the investigations regarding the effect of SFN and its analogues on it and its related proteins are limited and discrepant.[44-49]

Hypoxia represents a typical characteristic of ischemia, and it is also a well-known background under which angiogenesis, no matter whether physiologically or pathologically derived, can adaptively take place. However, to date, there have been few studies investigating the effect of SFN and its analogues on angiogenesis under hypoxic conditions. Moreover, previous related research usually applies either *in vitro* culture of cancer cells or xenograft tumor models under high levels of SFN treatments,[7,9] because anti-angiogenesis and pro-apoptosis are the main focus of these studies. Additionally, the alteration of mitochondrial dynamics in ECs following SFN treatments and its potential role in angiogenesis have not been addressed. In the present study, we used *in vitro* primary HUVECs as a model to investigate the role of SFN in angiogenesis at a wide range of concentrations under a hypoxic condition, and to explore the potential mechanisms of Nrf2 and the mitochondrial dynamics involved.

Materials and methods

Reagents and antibodies

R, S-sulforaphane was purchased from LKT Laboratories (Minnesota, USA), with the purity ≥99%. WST-1 Cell Proliferation Reagent was purchased from Roche (Shanghai, China). Matrigel was purchased from BD Pharmingen (State of New Jersey, USA). Crystal violet was purchased from Beyotime Technology (Shanghai, China), JC-1 was purchased from Enzo Life Sciences, Inc (New

York, USA). Anti-Nrf2, anti-HO-1, anti-Mfn2, anti-Opa1, anti-Fis1, anti-Drp1, anti-β-actin and anti-mouse IgG were purchased from Santa Cruz Biotechnology (Dallas, USA), anti-Mfn1, goat anti-rabbit IgG H&L (HRP) were purchased from Abcam (Cambridge, UK).

Cell culture and hypoxic exposure

HUVECs were obtained from ScienCell Research Labs and cultured in Endothelial Cell Medium (ECM) containing 5% fetal bovine serum (FBS), 1% endothelial cell growth supplement (ECGS) and 1% penicillin/streptomycin (P/S) (ScienCell, San Diego, CA) at 37℃ in a humidified atmosphere containing 5% CO_2. Cells between passages 2 and 8 were used for all experiments. For hypoxia exposure, cells were subjected to hypoxia in a sealed incubator container (Unitech, Guangzhou, China) that was flushed with a gas mixture of 1% O_2, 5% CO_2, and balance N_2 by using Anoxomat MarkIII (Advanced instruments Inc., California, Pomona, USA). The container was then placed in a humidified 37°C incubator.

Cell proliferation assay

HUVECs proliferation was determined by a WST-1 cell proliferation assay. Cells were seeded in 96-well culture plates at a density of 1×10^4 per well and cultured in complete medium overnight, and then exposed to increasing concentrations of SFN (1.25, 2.5, 5, 10, 20,40, 80 or 100μM) for 24h under hypoxic conditions. The control groups were treated with a DMSO concentration equal to the highest percentage (1‰) under a hypoxic or normoxic condition. After treatment, 10μL WST-1 solution was added to each well, and the plates were incubated for an additional 4 h at 37℃, then shaken thoroughly for 1min on a shaker. Optical density was measured using a microplate reader (Bio-Tek instrument Inc, Vermont, USA) at a test wavelength of 440nm and a reference wavelength of 620nm.The IC_{50} was determined using CalcuSyn software version 2.0 (Biosoft, Cambridge, UK).

***In vitro* migration assay**

Cell migration assays were performed using 24-well modified Boyden chambers (Corning Life Science, Corning, NY, USA) containing a polycarbonate filter with a pore size of 8-μm, according to the manufacturer's protocol. HUVECs were added to the upper chamber of the insert in 200 μL of serum-free medium. Medium (600 μL) with 5% FBS was added to the lower chamber. To determine

the effect of SFN on cell migration, different concentrations of SFN were added to the lower chamber, then the transwell chambers were incubated for 24h under hypoxic conditions at 37℃ (DMSO treatments in hypoxia and normoxia were used as controls). After the incubation period, the non-migrated cells on the upper chambers were removed with cotton swabs. The cells that had migrated through the pores of the membrane to the bottom chambers were fixed with 4% paraformaldehyde for 15 min and then stained with 0.1% crystal violet for 15 min. Cell migration was quantified by counting the number of cells from five random fields for each membrane using an inverted fluorescence microscope (Nikon, Tokyo, Japan) at 100× magnification.

Capillary-like structure formation assay

Matrigel (70 μL/well) was added to a 96-well plate and incubated at 37°C for 1 h. HUVECs (3×10^4 cells) were seeded onto each well of the Matrigel-coated 96-well plate and then incubated in complete culture medium with various concentrations of SFN in hypoxia with DMSO (<1‰) treatments in hypoxia and normoxia used as controls. After incubation for 8 h, the formation of the endothelial cell tubular structures was visualized under an inverted microscope (Carl Zeiss AG, Aalen, Germany) and photographed at 100× and 200× magnification. Five randomly chosen fields for each well were photographed and analyzed with an image analysis system (ImageInside Ver 3.32) for the quantitation of the number of meshes and master junctions as well as the total length of branches.

Electron microscopy

HUVECs were fixed in 2.5% glutaraldehyde at 4 °C for 2h, then rinsed with 0.1 M phosphate buffer 6 times for 15 minutes each. After fixation in 1% osmic acid for 1.5h, the cells were subjected to gradient alcohol dehydration and resin penetration, then embedded in Epon812 pure resin. Ultrathin sections (50 nm) were generated by Ultrathin Microtome (Leica, Weztlar, Germany), then transferred to copper grids, and analysed by transmission electron microscopy (Japan Electron Optics Laboratory Co., Ltd., JEM-1400 PLUS). Images were taken at 30,000×magnification.

Measurement of mitochondrial membrane potential

Mitochondrial membrane potential (mtΔΨ) was detected by using a fluorescence probe JC-1 according to the manufacturer's instructions. In brief, HUVECs following different treatments for 24h were

incubated with 5 μg/ mL JC-1 at RT for 15 min, and then washed twice with PBS. The fluorescence of JC-1 was immediately detected at wavelengths of 530 nm (green) and 590 nm (red) by a microplate reader (Bio-Tek instrument Inc, Vermont, USA). The value of mtΔΨ was calculated as the ratio of red fluorescence intensity to green fluorescence intensity.

Mitochondrial morphology

To assess mitochondrial fusion and fission, mitochondria were stained and photographed. In brief, 2×10^5 cells were plated on 35 mm glass bottom microwell dishes (Mat-Tek, Ashland, MA) and cultured overnight, then treated with DMSO (<1‰) in normoxia or hypoxia or SFN (2.5, 10μM) in hypoxia for 24h. After treatment, the cells were washed twice with cold PBS and incubated with medium containing 100nM MitoTracker™ Green FM (Thermo Fisher Scientific, Carlsbad, USA) at 37 ℃ for 30 min in the dark, then washed with PBS. Mitochondria in live cells were visualized using laser confocal scanning microscopy (TCS SP5) with a HC×PL APO CS 40×1.3 OIL objective (Leica, Weztlar, Germany). The images were acquired with Leica confocal software and analyzed with an image analysis system (Image Inside Ver 3.32) to determine the mitochondrial lengths and circularity.

Cell transfection with Nrf2 siRNA

For RNA interfering knockdown, subconfluent HUVECs were transfected with 10 nM Nrf2 siRNA duplex or control siRNA (Santa Cruz Biotechnology, Dallas, USA) using lipo3000 reagent (Thermo Fisher Scientific, Massachusetts, USA) for 8 h. The medium was then replaced and incubation continued for 24h under normoxia, followed by SFN (2.5, 10μM) or DMSO (<1‰) intervention under hypoxia for 24h. Transfection efficiency was confirmed by Western blot.

Western blot analysis

After treatment for 24h, the total protein was extracted from the whole cells following standard procedures, and concentrations were quantified by a BCA protein assay kit (Beyotime Technology, Shanghai, China). Equal amounts of protein were separated by sodium dodecyl sulfate-polyacrylamide gels electrophoresis (SDS-PAGE), and then transferred onto a polyvinylidene fluoride (PVDF) membrane (Millipore, Bedford, MA). The membranes were blocked with 5% skimmed milk in TBST (0.1‰ Tween 20) at RT for 1.5 h, and then incubated with a specific primary antibody overnight at 4℃

at a dilution of 1:500 for Nrf2, 1:600 for HO-1, 1:1000 for Mfn1, Mfn2, Fis1, Drp1, Opa1 and β-actin. The membranes were washed three times for 15 min with TBST, followed by incubation with secondary antibody diluted at 1:10000 for goat anti-rabbit IgG and 1:4000 for anti-mouse IgG for 1 h at RT. After further washing, protein bands were visualized with ECL reagent (Thermo Fisher, Rockford, USA) and densitometry was measured by Image J.

Statistical analysis

The data are presented as means ± SD. Differences between groups were assessed by performing one-way ANOVA using SPSS 19.0 software (SPSS Inc, Chicago, IL, USA). $P<0.05$ was considered statistically significant.

Results

Biphasic effect of SFN on angiogenesis in HUVECs under hypoxia

ECs proliferation, migration and tube formation are the key steps of angiogenesis.[1,2] Therefore, the effect of a range of concentrations of SFN treatment (1.25-100 μM for 24 h) on proliferation of HUVECs was measured by a WST-1 assay under hypoxia. As presented in **Figure 1a**, hypoxia treatment alone suppressed HUVECs proliferative activities by 20.7% compared to normoxia control. Under hypoxic conditions, SFN treatment exerted a biphasic effect on HUVECs proliferative capacity. Low doses (1.25-5μM) of SFN had no cytotoxicity, but instead promoted cell proliferation to 127.0-146.8% of hypoxia control with the maximal induction occurring at 2.5μM. In comparison to hypoxia control, there was no significant difference in cell viability at 24h post 10 μM SFN exposure, however, once beyond this concentration threshold, a dose-dependent inhibitory effect was observed. These inhibition rates dramatically rose from 36.8 to 94.1% with increasing concentrations with the IC_{50} of about 27.6μM. Thus, to avoid severe cytotoxicity, a concentration range of 1.25-20 μM was used for the subsequent evaluation assays and the mechanistic studies.

The effect of SFN on HUVECs migration under hypoxia was then explored using transwell assays. The results showed that there was a small increase (about 22.2%) in the number of HUVECs migrating to the lower side of the filter through the transwell membrane (8μm pore size) after hypoxia exposure,

240 which was further potentiated by 2.5μM SFN co-exposure (an additional 35.8% increase or 129.3% of
241 hypoxia control) despite no significant alteration at 1.25 and 5μM SFN co-exposures. However, 10,
242 20μM SFN inhibited cell migration under hypoxia in a dose dependent manner (68.6% and 53.4% of
243 hypoxic control, respectively) (**Fig. 1b, c**).

244 The matrigel assay was performed to determine capillary-like structure formation capacity in the
245 model, which was quantified as the number of tube-like structures (meshes) and nodes and also as the
246 total branching length of tube networks. Similarly, as depicted in **Figure 1d-g**, the biphasic regulatory
247 effect was also found to be exerted by SFN on capillary-like structure formation in HUVECs under
248 hypoxia. A significant increase in the ability of HUVECs to form tube-like structures was detected
249 after 24h hypoxia treatment (140.2,130.7, and 121.5% increase in the number of meshes, nodes and the
250 total branching length, respectively), which was further substantially enhanced by lower doses (<5μM)
251 of SFN but markedly disrupted by higher doses (≥10μM) with no completely formed networks in 20
252 μM SFN treatment group. Compared to hypoxia control, the number of meshes and nodes was
253 increased by 46.0 to 92.4% and 21.3 to 56.7%, respectively, at 1.25-2.5 μM SFN, whereas decreased
254 by 25.6 to 98.7% and 17.8 to 89.9%, under 10-20 μM SFN treatments; the total length of tube networks
255 was increased by 15.7% at 2.5 μM SFN, whereas decreased by 11.6 to 83.4% at 10-20 μM SFN.

256 Collectively, these data suggest that SFN administration can display a dose-dependent biphasic effect
257 on hypoxia-induced angiogenesis *in vitro* HUVECs, reinforcing it at lower concentrations (<5 μM)
258 whereas inhibiting it at higher concentrations (≥10 μM).

259

260 **Nrf2/HO-1-mediated pro-angiogenesis contributes to the angio-modulatory property of SFN in**
261 **HUVECs under hypoxia**

262 Nrf2 and its down-stream target gene HO-1, the pivotal mediators of multiple biological activities of
263 SFN,[10,27] are also implicated in pro-angiogenesis.[28-31] Therefore the degree to which they interfere in
264 the regulation of angiogenesis by SFN under hypoxia was investigated. HUVECs were exposed to
265 either normoxia or hypoxia treatment or SFN treatment under hypoxia conditions for 24h, and then the
266 protein levels of Nrf2 and HO-1 in the whole cells were detected by immunoblotting analysis. As

expected, SFN (2.5-20μM) significantly up-regulated the expression of Nrf2 and HO-1 in a dose-dependent manner in hypoxia (**Fig. 2a, b**). Hypoxia treatment alone also led to a slight increase in Nrf2 expression as compared to normoxia, but the difference was not statistically significant.

In addition, the angiogenic capacity of HUVECs following SFN treatment under hypoxia was evaluated following knockdown of Nrf2. HUVECs were transfected with Nrf2 siRNA or scrambled siRNA for 8h, the medium was then replaced and incubation continued for 24h under normoxia, followed by 2.5 or 10 μM SFN treatment in hypoxia for a further 24h with DMSO (<1‰) as a solvent control. Compared with the scrambled control, transfection with Nrf2 siRNA significantly abated the protein expressions of Nrf2 and HO-1, indicating that knockdown of Nrf2 by siRNA transfection was effective in HUVECs (**Fig. 2c, d**). Next, as shown in **Fig. 2e-h**, knockdown of Nrf2 not only reduced the constitutive angiogenesis in hypoxia, but also inhibited angiogenesis following 2.5μM SFN treatment, and even exacerbated 10μM SFN-induced anti-angiogenesis. These results reveal that Nrf2/HO-1 mediated pro-angiogenesis contributes to the angio-modulatory property of SFN under hypoxia.

SFN treatment bilaterally affects hypoxia-induced mitochondrial injury and fission in HUVECs

Mitochondria are essential cellular organelles and are key players in a plethora of physiological and biochemical processes.[36] Accordingly, it is of great significance to analyze the change in their structures, forms and functions following hypoxia exposure and SFN co-exposure. Firstly, mitochondrial ultrastructures were examined by transmission electron microscopy (TEM). TEM images confirmed that hypoxia caused mitochondrial damage, including cristae disappearance, mitochondrial swelling, mitochondrial vacuolation, matrix dissolution, and mitochondrial pyknosis, which were relieved by 2.5μM SFN treatment but aggravated by a SFN treatment concentration of 10μM (**Fig. 3a**). For quantification, at least 300 mitochondria per condition were analyzed. Mitochondrial injuries were categorized into entirely damaged and partially damaged phenotypes, and the former was scored in the absence of cristae and the latter scored if some cristae were present, compared to intact mitochondria with normal cristae. The structural data verified the mitoprotection by the lower dose of SFN and the worsening effect by the higher dose in response to hypoxia-triggered

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mitochondrial injury (**Fig. 3b**).

Mitochondrial membrane potential (mtΔΨm) is a universal indicator of mitochondrial health and the loss of it indicates mitochondrial dysfunction or injury. The change of mtΔΨm during hypoxia and the influence of SFN administration were assessed using a voltage-sensitive JC-1 fluorescence probe and spectrofluorometry, and mtΔΨm values were calculated as the ratio of red to green fluorescence (aggregate/monomer). Consistent with the mitochondrial ultrastructure by TEM, quantitative analysis data (**Fig. 3c)** showed that there was significant loss (about 43.0%) of mtΔΨm in HUVECs subjected to hypoxia for 24h as compared to those in normoxia. SFN treatment displayed a dose-dependent opposite role in hypoxia-evoked mtΔΨm reduction. SFN treatments from 1.25 and 2.5μM tended to block the decrease in mtΔΨm induced by hypoxia with a statistically significant difference (x1.7 fold) from hypoxia control at 2.5μM. Conversely, 10 and 20μM SFN treatments further dose-dependently enhanced hypoxia-caused depolarization of mitochondria (36.3-46.3% of hypoxia control).

Mitochondria, as highly dynamic and sensitive organelles, constantly regulate the balance between fusion and fission in response to various stimuli.[35] Moreover, mitochondrial dynamics is not only a crucial determinant of angiogenesis,[33] but also emerges as a potential target of SFN and its analogues.[44-49] To examine the change of balance of mitochondrial dynamics in HUVECs following hypoxia treatment and the influence of SFN treatment, MitoTracker Green dye was used to visualize mitochondrial morphology by confocal microscopy and computer-assisted morphometric analysis was performed to measure mitochondrial lengths and circularity with higher values indicating more rounded mitochondria and a perfect circle equal to 1.0. As depicted in **Figure 3d-f**, mitochondrial morphology in the hypoxia-treated group differed from that in the normoxia group, and this was manifested as multiple, round, discriminative puncta and fewer networks which was quantified as a shorter average length and a higher circularity value. However, the abnormal mitochondrial morphology in HUVECs exposed to hypoxia was improved when co-exposed to 2.5μM SFN since the latter preserved a more elongated phenotype. Conversely, compared to hypoxia control, there was mild reduction in mitochondrial lengths and augmentation in circularity when exposed to 10μM SFN.

Taken together, these data suggest that hypoxia exposure results in mitochondrial injury and imbalances mitochondrial dynamics toward increased fission, and these disturbances are

bilaterally affected by SFN co-exposure with a protective effect at low doses but a deteriorative effect at high doses.

Inducing Drp1 and suppressing Mfn1/2 expression by SFN at high doses correlate with its promotion of hypoxia-induced fission in HUVECs

Mitochondrial dynamics is manipulated by the balance between fission, and fusion related proteins. To understand mechanistically the changes of mitochondrial morphology in different intervention conditions, the levels of five key mitochondrial dynamic regulatory proteins were determined, including pro-fissioned Drp1 and its adaptor Fis1 as well as pro-fused Mfn1/2 and Opa1. Representative blots and the average relative protein expressions are presented in **Figure 4**. Hypoxia significantly increased Drp1 levels, but had no effect on other proteins. Low concentrations (1.25-5μM) of SFN treatments did not affect any dynamics related protein expressions under hypoxia, while higher concentrations (10 and 20μM) treatments induced a further and remarkable dose-dependent increase in Drp1 expression in parallel with significant decreases in Mfn1/2. No significant changes were identified in Fis1 and Opa1 levels after high doses SFN treatments. These results, in combination with the above morphological changes of mitochondria, infer that high doses of SFN aggravate hypoxia-induced fission through a mechanism involving induction of Drp1 protein and inhibition of Mfn1/2 proteins. Low doses of SFN induced defense against hypoxia-caused fission may be dependent on regulating the detected dynamics related proteins activities via post-translational modification and/or implicate other undetected mitochondrial dynamics related proteins.

Effect of SFN on mitochondrial morphology after Nrf2 knockdown in HUVECs under hypoxia

Given the core role of Nrf2 in appropriate concentrations of SFN induced antioxidant defense[10] and the significance of redox modulation of fusion and/or fission proteins activities,[50,51] it can be speculated that the mechanism of alleviating hypoxia-induced fission by SFN at low doses is probably due to the modulation of mitochondrial dynamics related protein activities via Nrf2-dependent redox modification. This possibility was preliminarily explored by evaluating the mitochondrial

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morphology change elicited by SFN in hypoxia again after Nrf2 knockdown. HUVECs were transfected with Nrf2 siRNA or scrambled siRNA for 8h, the medium was then replaced and incubation continued for 24h under normoxia, followed by 2.5 or 10 μM SFN treatment in hypoxia for an additional 24h with DMSO (<1‰) as a solvent control. Subsequently, mitochondrial morphology was detected by MitoTracker Green staining. The results showed that, compared to the equivalent siCont group, the circularity values in both 2.5 and 10μM SFN-treated as well as DMSO-treated siNrf2 cells were all significantly elevated while the average mitochondrial lengths were all significantly diminished (**Fig. 5**). This is indicative of the importance of Nrf2 in both basal and low levels of SFN-induced defense against mitochondrial fission under hypoxia, as well as the contribution of Nrf2 to counteract to mitochondrial dynamic imbalance when concurrently suffering from high levels of SFN and hypoxia exposures.

Discussion

Like several other extensively studied dietary phytochemicals,[52] SFN has been documented to be a hormetic agent.[25,26] Low doses of these compounds may prepare cells to resist more severe stress by activating signaling pathways that result in increased expression of genes encoding cytoprotective proteins, but in contrast, high doses are cytotoxic. Some studies have also discovered that high doses of SFN and its analogues can have anti-angiogenic activities, however, few researchers have paid attention to what happens with treatments at low doses. Only two studies have been carried out on the direct effect of ITCs on angiogenesis under hypoxic conditions, showing an inhibitory effect on colon and glioma cancer cell migration.[15,16] In the present study, by using *in vitro* primary HUVECs as a model, we demonstrate for the first time that SFN displays a biphasic effect on angiogenesis in hypoxia and that the potential mechanisms involve an integrated modulation of Nrf2 and mitochondrial dynamics.

Hypoxia/Ischemia may result in EC injuries particularly manifested as mitochondrial dysfunction,[53] however, they can also adaptively trigger angiogenesis.[3] Both physiological and pathological angiogenesis can occur in a hypoxia/ischemia context, so *in vitro* hypoxia was adopted here to mimic the *in vivo* context to explore the effect of SFN on angiogenesis. The results showed that hypoxia

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promoted the migration and tube-like structure formation by HUVECs despite inhibiting their proliferation to a certain extent (**Fig. 1**). A similar finding was reported by Befani and Liakos in human microvascular endothelial cells (HMECs).[54] Hypoxia-induced migration and/or tube formation have also been observed in HUVECs,[55] human endothelial cell line EA.hy 926,[56] human pulmonary artery endothelial cells (PAECs)[57] and rat aortic endothelial spheroids[58] models, but conversely the opposite effects have also been reported.[59,60] Most but not all studies have shown that hypoxic exposure can inhibit the proliferative activity of ECs,[58,61,62] and some data suggest that normal ECs such as HUVECs are more sensitive than ECs from tumors.[63] Hence it will be of great significance to detect and mimic the specific hypoxic intensities in different physiopathology conditions and tissue types in the future since the influence of hypoxia on angiogenesis can be very different depending on the degree, duration and frequency of hypoxia as well as the cell types.

Under the hypoxic background, we demonstrated that SFN also has a hormetic effect on the angiogenic capacity of HUVECs, characterized by potentiating hypoxia-induced angiogenesis at low doses (1.25 and 2.5μM), but inhibiting it at high doses (≥10μM) (**Fig. 1**). Similarly, this bell-shaped effect of SFN and its analogues on angiogenesis in normoxia has also been found in our previous studies utilizing tumor cells and 3-D HUVECs and pericyte co-culture models.[17,22,24,64] The stimulating action of SFN on angiogenesis has been evidenced in ethanol-injected chick yolk sac membrane (YSM) and chorioallantoic membrane (CAM) models at 2.5-10μM co-treatment,[65] and in 2.5μM-pretreated endothelial colony forming cells (ECFCs).[66] In contrast, anti-angiogenic activities of SFN and some other ITCs have also been reported in HUVECs and rat aortic ring models at a single dose (**>** 10μM) of intervention in normoxia.[20,21] Additionally, dose-dependent inhibition of HUVECs proliferation and capillary-like tube formation in normoxia by SFN (≥10μM) has been demonstrated by Asakage *et al* and Nishikawa *et al*, but no effects were detected at intervention concentrations below 10μM.[11,12] Notably, compared to HUVECs, human microvascular ECs appeared much more sensitive to SFN-induced anti-angiogenesis since 0.3μM and 0.1μM SFN have been found capable of suppressing angiogenesis in these models.[13,14] Additionally, the anti-angiogenic thresholds of phenethyl isothiocyanate (PEITC) and benzyl Isothiocyanate (BITC) were found to be lower than that of SFN.[18,23] Given these limited reports and discrepant results, further studies are required to determine whether the biphasic effect of SFN on angiogenesis in hypoxic HUVECs can likewise exist in other hypoxic ECs

and tumor cells, whether other ITCs may also possess the similar angio-hormetic property, and what are the consequences *in vivo* under different physiological and pathological conditions.

Nrf2 is a major regulator of cellular response to diverse stresses, and mediates multiple biological effects of SFN.[10,27] The pro-angiogenic roles of Nrf2 and its target gene HO-1 as well as their protections on ischemic diseases and their implications in some pathogenic consequences like cancer, have all been demonstrated.[28-31] It has been reported that Nrf2 dysfunction may be a potential mechanism underlying impaired angiogenesis and microvascular rarefaction in aging.[4] Therefore, whether and how Nrf2 can interfere in the SFN-modulated angiogenesis in hypoxia was further explored. Firstly, as expected, SFN increased the levels of Nrf2 and HO-1 protein dose-dependently (**Fig. 2a, b**) and secondly, knockdown of Nrf2 and the consequent down-regulation of HO-1 protein expression not only reduced the constitutive angiogenesis in hypoxia, but also substantially inhibited the 2.5μM SFN-induced angiogenesis, and even exacerbated the anti-agiogenesis by10μM SFN in hypoxia (**Fig. 2c-h**). Nrf2-mediated angiogenesis has been reported to play an important role in the angiogenic capacity of ECFCs, particularly under conditions of increased oxidative stress, which constitutes the likely mechanism whereby SFN (2.5 μM) promoted basal angiogenesis and preserved ROS-inhibited angiogenesis in normoxia.[66] Additionally, it has been reported that silencing of Nrf2 abolished 2.5μM allyl isothiocyanate (AITC)-stimulated HepG2 cell migration[24] and 2.5μM SFN-induced vascular-like structures formation in normoxia[67] as well as hypoxia-induced angiogenesis.[68] The findings here offer further support that the positive role of Nrf2/HO-1 also mediates the angio-modulatory property of SFN in HUVECs under hypoxia.

Corresponding to the angio-hormetic effect, a mito-hormetic effect of SFN on HUVECs in hypoxia was also revealed in this study. Mitochondrial ultrastructure, membrane potential and morphology analysis showed that low doses of SFN improved hypoxia-induced mitochondrial injury, dysfunction and dynamic imbalance, while high doses worsened the abnormalities **(Fig. 3**). Modifications of mitochondrial signaling, functionality, and integrity by SFN have been widely reported, however, seemingly contradictory behaviors have been discovered. SFN can exert cyto- and mito-protective effect against ROS, toxins, ischemia, hypoxia and other attacks, but conversely it can cause deleterious changes in mitochondria that would eventually carry the cell to death.[43] Evidence also exists to indicate that the toxicity of SFN appears to be cancer cell specific,[25] nevertheless, our findings in HUVECs

suggested that this is not always the case. Apart from cell types, the paradoxical phenomena observed in our study suggested that exposure doses and context are crucial determining factors. Notably, most studies dealing with SFN toxicity report effects occurring at concentrations above 5-10μM.[25] Some investigations have shown that induction of mitochondrial dysfunction and fission can destroy angiogenesis[40-42] while improvement of mitochondrial function and promotion of fusion have the opposite effect.[33,37-39] Consequently, we speculate that the mito-hormetic effect of SFN might also contribute to its angio-hormetic effect in HUVECs under hypoxia.

Mitochondrial fusion and fission are coordinated by several dynamics related proteins, principally pro-fused Mfn1/2 and Opa1 and pro-fission protein Drp1 and its adaptor Fis1. The data on the effect of SFN and its analogues on the expression of these proteins is limited and inconsistent.[44-49] In addition, it was reported that Nrf2 can promote mitochondrial hyper-fusion through degradation of Drp1 levels[69] and some of its activators may ameliorate unbalanced mitochondrial dynamics caused by different pathogenic factors[70]. Therefore, the expressions of these key dynamics related proteins were further examined. In agreement to the above mitochondria morphology, hypoxia led to an up-regulated Drp1 expression despite no significant alteration in Mfn1/2, Opa1 and Fis1 observed. Similarly, hypoxia/ischemia induced-fission and Drp1 expression have also been reported to mediate stem cell apoptosis and tissue /cell damage.[71,72] Unexpectedly, SFN treatment did not improve hypoxia-induced abnormality in Drp1 expression, but conversely, 10 and 20μM SFN treatment potentiated the hypoxia-induced Drp1 expression and concurrently inhibited Mfn1/2 expression. Notably, in hypoxia, low doses (1.25-5μM) of SFN had no significant influence on any detected dynamics related protein levels, although the reversed hypoxia-induced fission was observed at 2.5μM SFN (**Fig. 3d-f and 4**). Hence, we infer that the phasic regulatory activity of SFN on mitochondrial dynamics in hypoxia might be ascribed to both transcriptional/translational regulation and posttranslational modification mechanism (PTM), that is improving hypoxia-induced fission via modulation of certain dynamics related proteins activities by PTM at low doses, whereas deteriorating that via up-regulation of Drp1 and down-regulation of Mfn1/2 levels as well as the non-excluded PTM.

Redox, phosphorylation, acetylation, and ubiquitylation are the main potential PTM in controlling certain fusion and fission proteins activities,[34-36,50] and most of their related signal pathways or effectors are also the targets of SFN and /or Nrf2. Given redox perturbance elicited by hypoxia, links between

ROS/RNS modification of certain dynamics related proteins and fission,[50,51] and the core role of Nrf2 in antioxidant defense, it can be speculated that the mechanism of alleviating hypoxia-induced fission by SFN at low doses is likely by Nrf2-dependent redox modulation of certain dynamics related proteins activities. This possibility was preliminarily validated here by findings that Nrf2 knockdown inhibited low dose SFN-induced fusion in hypoxia. The pro-fusion role of Nrf2 was not confined to low doses of SFN treatment and also existed when exposed to higher doses of SFN and hypoxia *per se* (**Fig. 5**), a finding compatible with the above positive mediated role of Nrf2 in angiogenesis. Further precise studies are needed to determine the redox homeostasis in cells and mitochondria, as well as to elucidate which dynamics related proteins and which amino acid residues are the key redox modification targets. The possibility that Nrf2-dependent non-redox PTM also requires consideration and confirmation.

Modulation of mitochondrial dynamics by SFN and its analogues has become a novel and intriguing research topic. Both their cytoprotective response by inhibiting fission and pro-apoptotic effects via inhibiting fusion have been reported.[40,44] Even circular mitochondria formed by granular mitochondria were detected in SFN treated cancer cells.[45] Moreover, the mechanisms whereby SFN and its analogues affect mitochondrial dynamics are not yet well understood. Mitigating the recruitment and /or retention of the soluble Drp1 to mitochondria without changing overall Drp1 abundances was reported to mediate inhibiting fission in human retinal pigment epithelial (RPE-1) cells by 50μM SFN treatment for 4h,[46] yet reversing iron-induced Drp1 protein reduction in hippocampus has also been found associated with neuroprotection by SFN.[47] In contrast, BITC (2.5 and 5μM for 6h) induced fission accompanied by both decreases in pro-fusion and pro-fission proteins, and this effect was cancer cell specific.[48] Nevertheless, although treating breast cancer cells with 20μM erucin for 6h can also induce fission and apoptosis, the mechanism was due to inducing mitochondrial translocation of Drp1.[49] The results here, together with other previous findings, suggest that the discrepancy in the effect of SFN and its analogues on mitochondrial dynamics may be due to the differences in study models and context, intervention duration and timing (pre, co- and post-treatment), exposure doses, as well as ITCs types, all of which deserve further exploration.

Conclusion

The present study innovatively demonstrates that SFN exhibits a dose-dependent biphasic effect on angiogenesis in hypoxia challenged HUVECs via mechanisms involving an integrated modulation of Nrf2 and mitochondrial dynamics. Nrf2 activation by SFN at moderate doses (1-5μM, achievable in human plasma *via* dietary intake of cruciferous vegetables [9]) contributes to the protection against hypoxia-evoked mitochondrial injury and fission thus boosting the angiogenic capacity of HUVECs. However, under high pharmacological doses of SFN exposure, the induction of mitochondrial fission resulting from up-regulating Drp1 and down-regulating Mfn1/2, coupled with aggravated mitochondrial injury, overwhelms Nrf2-mediated defenses, hence generating an angio-inhibitory effect. Our findings enrich the knowledge of the hormetic character of SFN from the angle of angiogenesis in hypoxia. The discovery of crosstalk between mitochondrial dynamics and Nrf2 in mediating SFN action in angiogenesis, and the complex regulatory mechanism of SFN on mitochondrial dynamic infer that, mitochondrial dynamics deserves further focusing when exploring and interpreting SFN bio-efficacy. Given the limitation of cell-based *in vitro* experiments and the different consequence of angiogenesis in therapeutic/reparative angiogenesis and aberrant angiogenesis related diseases, further *in vivo* studies with exposure to a wide range of SFN concentrations under specific angiogenesis-related disease backgrounds are warranted.

Author contributions

Dan Li and Yongping Bao contributed to the study design and manuscript writing. Yaqian Wang, Fangfang Chen and Yuan Zhang performed the experiments. Yaqian Wang and Xiangyu Zheng conducted data analysis. Shiyan Liu drew the graphic for proposed mechanism. Meijuan Tang, Ziling Wang and Pan Wang revised the manuscript.

Conflicts of interest

The authors declare no conflict of interest.

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Figure legends

Fig. 1. SFN treatment bilaterally affects hypoxia-induced angiogenesis in HUVECs. HUVECs were treated with indicated concentrations of SFN under hypoxia with DMSO (≦1‰) treatment under normoxia or hypoxia as different controls. **a** HUVECs proliferation was measured by a WST-1 assay at 24h. Data are presented as mean ± SD of six independent experiments carried out in sextuplicate. **p*<0.05, ***p* <0.01 vs. normoxia control; $^{\#}p$<0.05, $^{\#\#}p$ <0.01 vs. hypoxia control. **b,c** HUVECs migration was determined by transwell assays at 24h. Representative pictures depicting HUVECs migration (scale bar = 100 μm) and quantitative analysis of the migrated cells numbers are presented. Data represents mean ± SD of three independent experiments carried out in triplicate. **p*<0.05, ***p* <0.01 vs. normoxia control; $^{\#}p$<0.05, $^{\#\#}p$ <0.01 vs. hypoxia control. **d-g** Capillary-like structure formation by HUVECs was detected at 8h. Representative images of capillary-like structures (scale bar = 200 μm) (**d**) and quantification of the number of meshes (**e**) and nodes (**f**) as well as the total lengths of branches (**g**) are shown. All values are expressed as mean ± SD of three independent experiments carried out in triplicate. **p*<0.05, ***p* <0.01 vs. normoxia control; $^{\#}p$<0.05, $^{\#\#}p$ <0.01 vs. hypoxia control.

Fig. 2. Nrf2/HO-1-mediated pro-angiogenesis contributes to the angio-modulatory property of SFN in HUVECs under hypoxia. **a, b** Representative immunoblots and relative densitometric analysis of Nrf2 and HO-1 in the whole HUVECs exposed to 1.25-20μM SFN for 24h under hypoxia with DMSO (<1‰) intervention under normoxia and hypoxia as different controls. The results are presented as mean ± SD of three independent experiments. **p*<0.05, ***p* <0.01 vs. normoxia control; $^{\#}p$<0.05, $^{\#\#}p$ <0.01 vs. hypoxia control. HUVECs were transfected with 10nM Nrf2 siRNA or con siRNA for 8h. The medium was then replaced and incubation continued for 24h under normoxia, followed by SFN (2.5, 10μM) or DMSO (<1‰) intervention under hypoxia. **c, d** The protein levels of Nrf2 and HO-1 were detected by Western blots analysis after 24h intervention. The results are presented as mean ± SD of three independent experiments. **p*<0.05, ***p* <0.01 vs. DMSO-treated

siCON; $^{\#}p<0.05$, $^{\#\#}p<0.01$ between equivalent SFN or DMSO-treated siNrf2 and siCON. **e-h** Tube formation was determined by *in vitro* Matrigel angiogenesis assay after 8h treatment. Representative images of capillary-like structures (scale bar = 200 μm) (**e**) and quantification of the number of meshes (**f**) and nodes (**g**) as well as the total lengths of branches (**h**) are shown. All values are expressed as mean ± SD of three independent experiments carried out in triplicate. $^{*}p<0.05$, $^{**}p<0.01$ vs. DMSO-treated siCON; $^{\#\#}p<0.01$ between equivalent SFN or DMSO-treated siNrf2 and siCON.

Fig. 3. SFN treatment bilaterally affects hypoxia-induced mitochondrial injury and fission in HUVECs. HUVECs were exposed to the indicated concentration of SFN under hypoxia for 24h with DMSO (<1‰) intervention under normoxia and hypoxia as different controls. **a, b** Representative ultrastructure of mitochondria by transmission electron microscopy (magnification×30,000; scale bar = 500 nm) and percentage of mitochondrial injuries in each condition. For quantification, at least 300 mitochondria per condition were analyzed. Mitochondrial injuries were categorized into entirely damaged and partially damaged phenotypes, and the former was scored in the absence of cristae and the latter scored if some cristae were present, compared to intact mitochondria with normal cristae. **c** Quantification of the mitochondrial membrane potential (mtΔΨ) was done using fluorescence probe JC-1 and a microplate reader with mtΔΨ values calculated as the ratio of red/green fluorescence intensity. Data are presented as mean ± SD of three independent experiments carried out in quintuplicate. $^{*}p<0.05$, $^{**}p<0.01$ vs. normoxia control; $^{\#}p<0.05$, $^{\#\#}p<0.01$ vs. hypoxia control. **d** Representative morphology of mitochondria by confocal microscopy after staining with mitochondrial fluorescent dye MitoTracker Green (panels on the left: scale bar = 25 μm; middle: scale bar =15μm). **e, f** Quantification of mitochondrial circularity and lengths in the fluorescence photograph by computer-assisted morphometric analysis. Data are presented as mean ± SD of three independent experiments carried out in triplicate and at least 60 cells per group were analyzed. $^{*}p<0.05$, $^{**}p<0.01$ vs. normoxia control; $^{\#}p<0.05$, $^{\#\#}p<0.01$ vs. hypoxia control.

Fig. 4. Effect of SFN on hypoxia-induced alteration in dynamics related protein levels in HUVECs. HUVECs were exposed to normoxia or hypoxia treatment alone or hypoxia in

combination with SFN (1.25-20μM) treatment, respectively, for 24h. Representative Western blots images and bands densitometric quantification showing the levels of the pro-fission proteins Drp1 (**a**) and Fis1 (**b**), the pro-fusion proteins Mfn1/2 (**c, d**) and Opa1(**e**), with β-actin as loading control. Data are presented as mean ± SD of three independent experiments. **p*<0.05, ***p* <0.01 vs. normoxia control; $^{\#}p$<0.05, $^{\#\#}p$ <0.01 vs. hypoxia control.

Fig. 5. Effect of SFN on mitochondrial morphology after Nrf2 knockdown in HUVECs under hypoxia. HUVECs were transfected with 10nM nrf2 siRNA or con siRNA for 8h. The medium was then replaced and incubation continued for 24h under normoxia, followed by SFN (2.5, 10μM) or DMSO (<1‰) treatment for 24h under hypoxia. Mitochondria morphology was detected by MitoTracker Green staining. **a** Representative fluorescence images captured by confocal microscopy (panels on the left: scale bar = 25 μm; middle: scale bar =15μm). **b, c** Statistical data of the quantified mitochondrial circularity and lengths were shown as histograms. Data are means ± SD of triplicate determinations and at least 30 cells per group were analyzed. **p*<0.05, ***p* <0.01 vs. DMSO-treated siCON; $^{\#}p$<0.05, $^{\#\#}p$ <0.01 between equivalent SFN or DMSO-treated siNrf2 and siCON.

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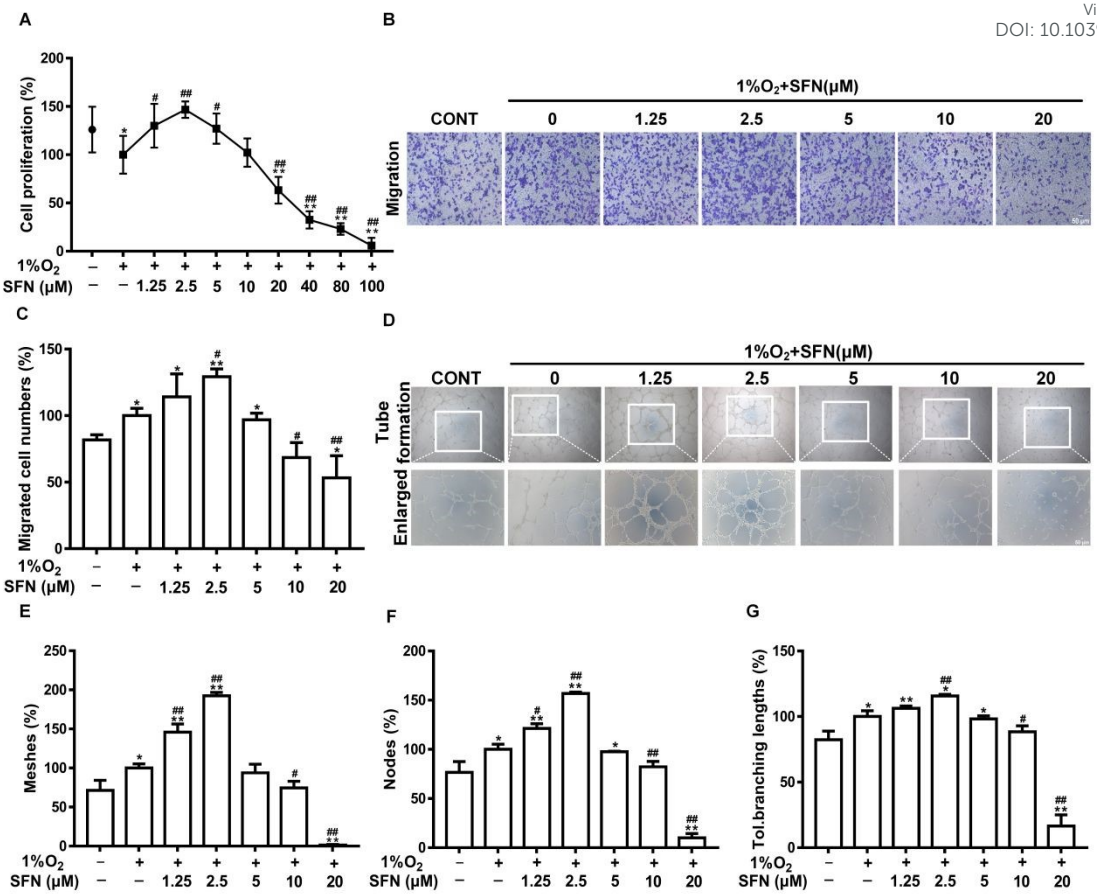

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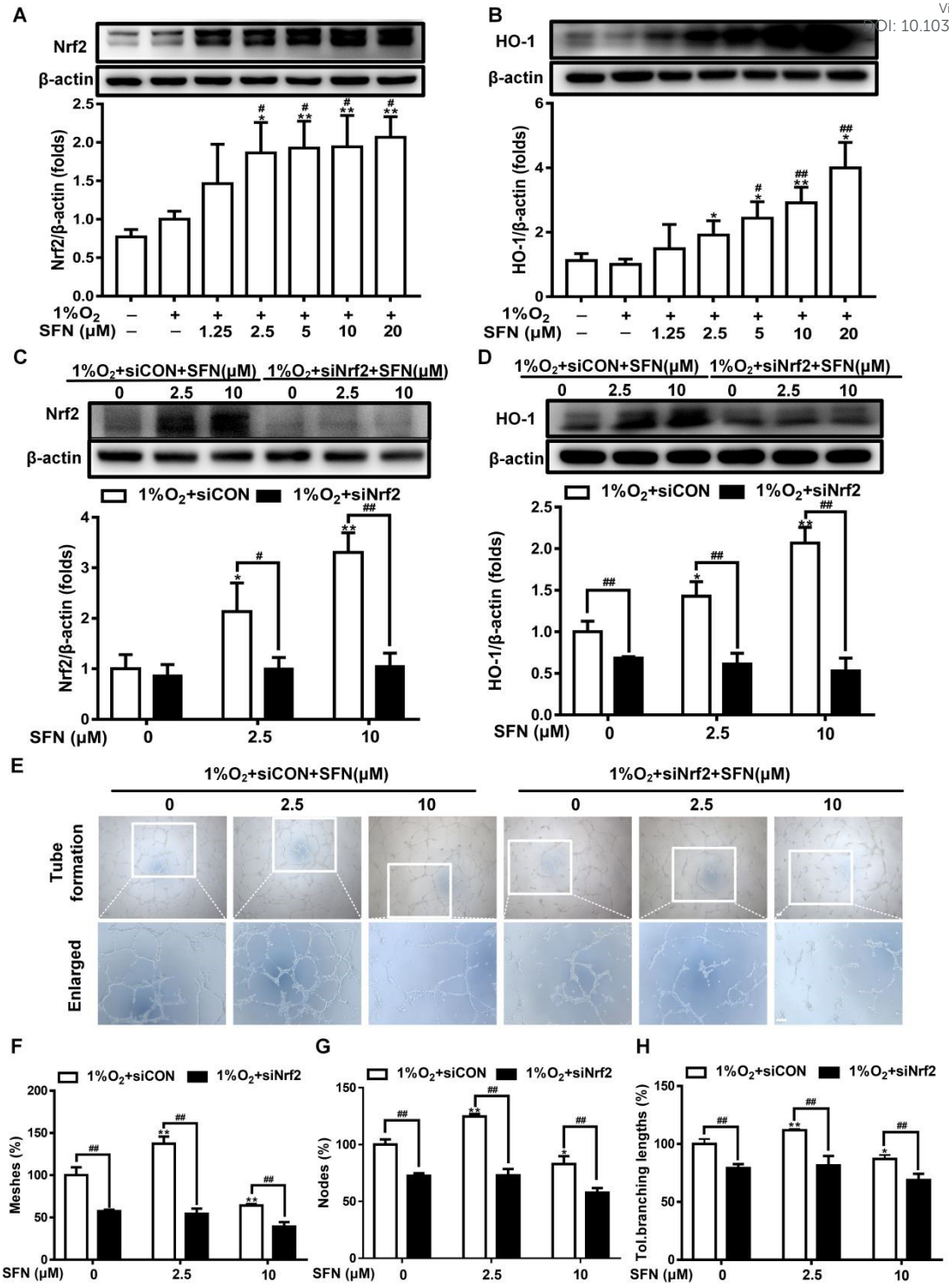

804

805 **Fig. 2. Nrf2/HO-1-mediated pro-angiogenesis contributes to the angio-modulatory property of**
806 **SFN in HUVECs under hypoxia.**

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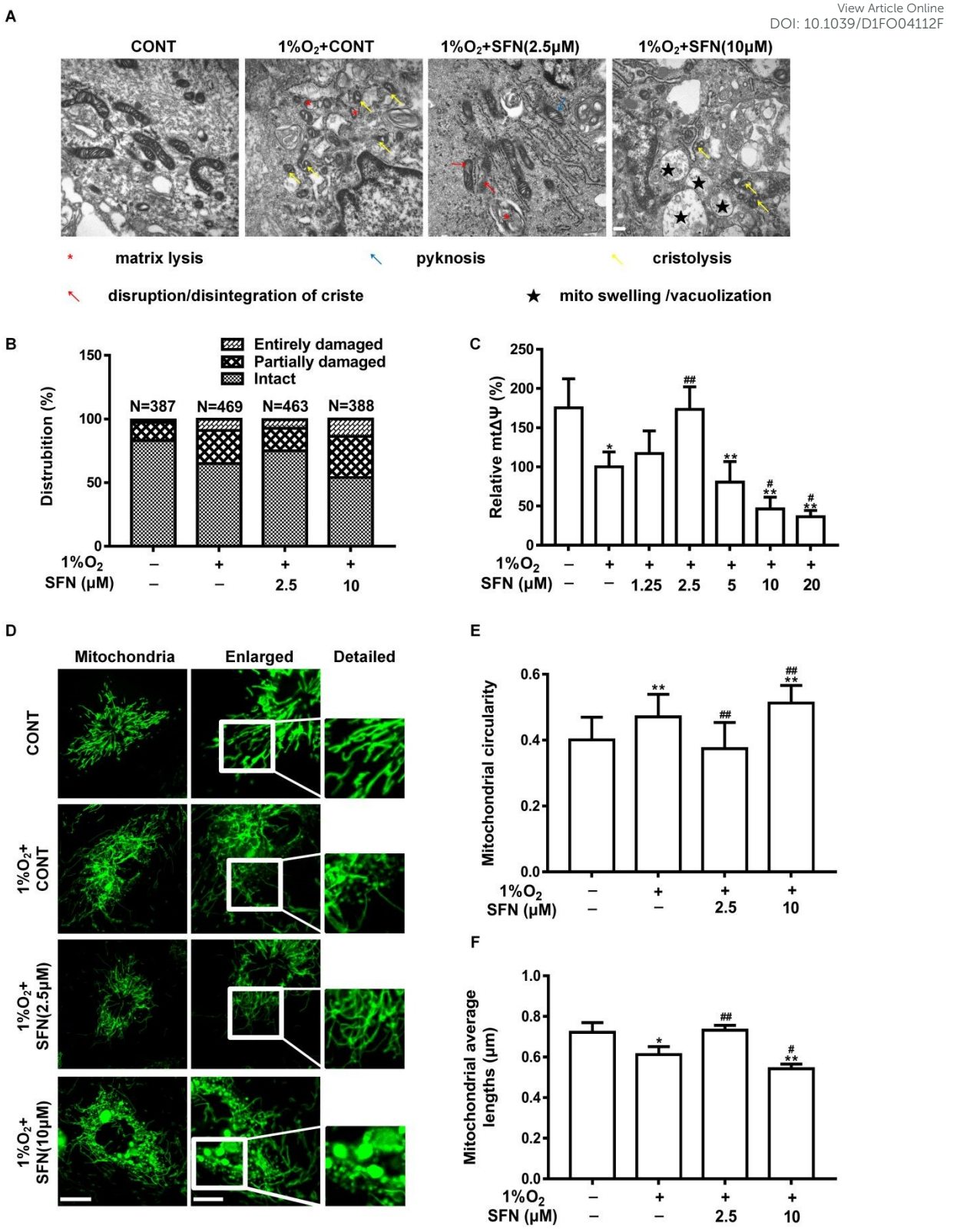

807

808 **Fig. 3. SFN treatment bilaterally affects hypoxia-induced mitochondrial injury and fission in**
809 **HUVECs.**

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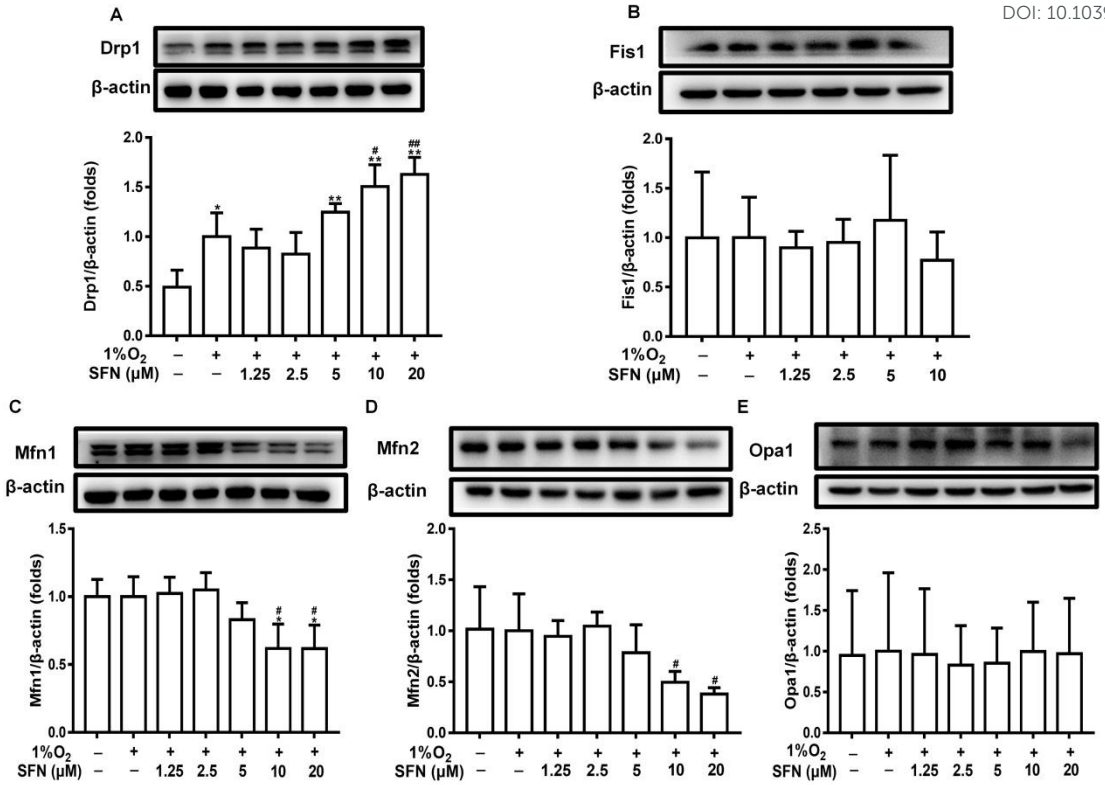

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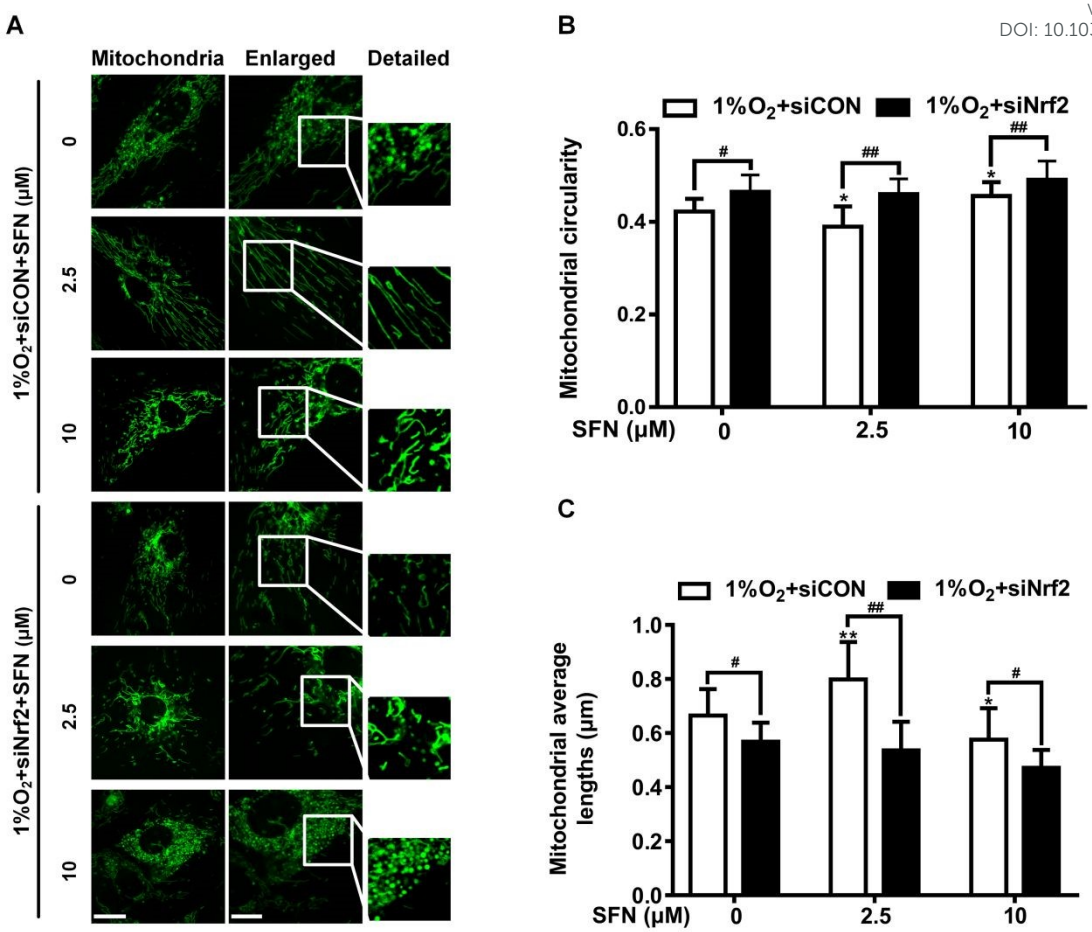

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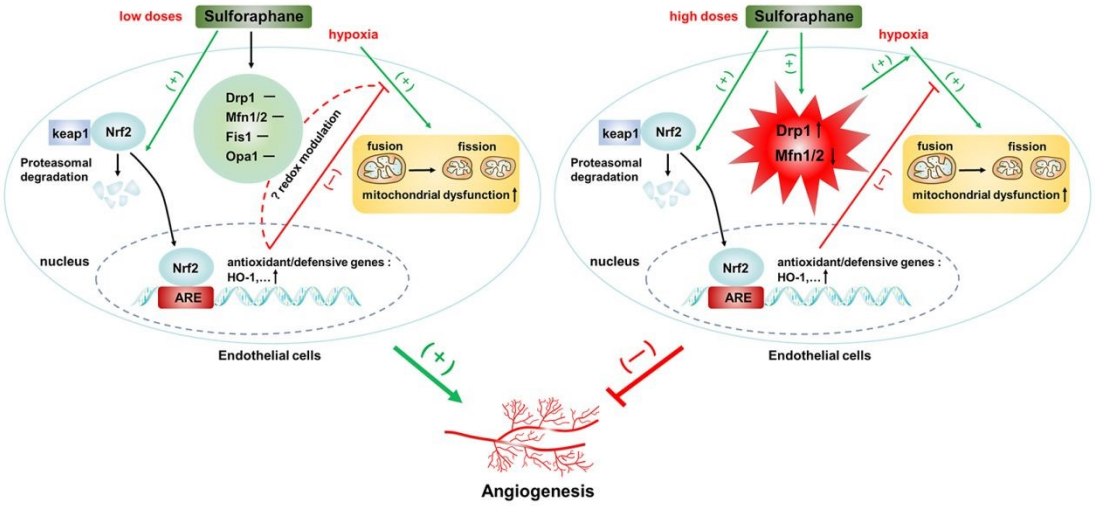

819

820 **Fig. 6. Proposed mechanism of biphasic effect of SFN on angiogenesis in hypoxia.**

821 The angio-hormetic effect of SFN in hypoxia may be mainly ascribed to its mito-hormetic effect via an
822 integrated modulation of Nrf2 and mitochondrial dynamics. Nrf2 activation by moderate doses of SFN
823 contributes to the protection against hypoxia-evoked mitochondrial injury and fission thus boosting
824 the angiogenic capacity of vascular endothelial cells. However, under high dose of SFN exposure, the
825 induction of mitochondrial fission resulting from up-regulating Drp1 and down-regulating Mfn1/2,
826 coupled with aggravated mitochondrial injury, overwhelms Nrf2-mediated defense, hence generating
827 an angio-inhibitory effect.
828