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Research Paper

Dimethylfumarate attenuates restenosis after acute vascular injury by cell-specific and Nrf2-dependent mechanisms

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ABSTRACT

Excessive proliferation of vascular smooth muscle cells (VSMCs) and incomplete re-endothelialization is a major clinical problem limiting the long-term efficacy of percutaneous coronary angioplasty. We tested if dimethylfumarate (DMF), an anti-psoriasis drug, could inhibit abnormal vascular remodeling via NF−E2-related factor 2 (Nrf2)-NAD(P)H quinone oxidoreductase 1 (NQO1) activity. DMF significantly attenuated neointimal hyperplasia induced by balloon injury in rat carotid arteries via suppression of the G1 to S phase transition resulting from induction of p21 protein in VSMCs. Initially, DMF increased p21 protein stability through an enhancement in Nrf2 activity without an increase in p21 mRNA. Later on, DMF stimulated p21 mRNA expression through a process dependent on p53 activity. However, heme oxygenase-1 (HO-1) or NQO1 activity, well-known target genes induced by Nrf2, were dispensable for the DMF induction of p21 protein and the effect on the VSMC proliferation. Likewise, DMF protected endothelial cells from TNF-α-induced apoptosis and the dysfunction characterized by decreased eNOS expression. With knock-down of Nrf2 or NQO1, DMF failed to prevent TNF-α-induced cell apoptosis and decreased eNOS expression. Also, CD31 expression, an endothelial specific marker, was restored *in vivo* by DMF. In conclusion, DMF prevented abnormal proliferation in VSMCs by G1 cell cycle arrest via p21 upregulation driven by Nrf2 and p53 activity, and had a beneficial effect on TNF-α-induced apoptosis and dysfunction in endothelial cells through Nrf2–NQO1 activity suggesting that DMF might be a therapeutic drug for patients with vascular disease.

Introduction

Neointimal hyperplasia and incomplete re-endothelialization are major clinical mechanisms known to limit the long-term efficacy of percutaneous coronary angioplasty (PTCA) [1]. Although the introduction of drug-eluting stents has significantly reduced the re-stenosis rate compared to bare-metal stents [2,3], the incidence of late stent thrombosis in recipients of drug-eluting stents is largely dependent on the degree of re-endothelialization and intimal proliferation.

Dimethylfumarate (DMF) is the main active component of oral formulations of fumaric acid esters that have been successfully used for treating psoriasis in chronic inflammatory skin disease [4]. Previous studies have demonstrated that DMF inhibits multiple sclerosis by reducing inflammation [5,6] and increases cardioprotection via activation of the NF−E2-related factor 2 (Nrf2) antioxidant pathway [7]. In addition, DMF attenuates renal fibrosis via Nrf2-mediated inhibition of transforming growth factor-β (TGF-β)/Smad signaling and suppresses adipogenic differentiation in 3T3-L1 preadipocytes through inhibition of STAT3 activity [8,9]. It was reported that DMF inhibited PDGF-induced proliferation in airway smooth muscle cells through p38-heme oxygenase-1 (HO-1) [10]. However, the potential effects of DMF on vascular injury after balloon angioplasty *in vivo* have not been studied.

Abbreviations: DMF, dimethylfumarate; VSMCs, vascular smooth muscle cells; HAECs, human aortic endothelial cells; LST, late stent thrombosis; Nrf2, nuclear-factor-E2-related factor-2; NQO1, NAD(P)H quinone oxidoreductase 1; HO-1, heme oxygenase-1; PTCA, percutaneous coronary angioplasty; PFT-α, Pifithrin-α.

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Neointimal hyperplasia is caused by growth factors and cytokines released by platelets and leukocytes at sites of injury after balloon angioplasty [11]. In the endothelial cell, DMF decreases tissue factor and expression of adhesion molecules such as vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1) through the obstruction of tumor necrosis factor-α (TNF-α)-induced nuclear entry of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB)/p65 [12]. Furthermore, the blockade of TNF-α accelerates functional endothelial recovery after balloon angioplasty [13,14]. Therefore, we investigated the effects of DMF on TNF-α mediated suppression of eNOS and on TNF-α mediated apoptosis in endothelial cells.

Previous reports have demonstrated that Nrf2 protects against tissue fibrosis, diabetic nephropathy and non-alcoholic fatty liver, presumably through enhancement of cellular antioxidant capacity such as by increased NAD(P)H quinone oxidoreductase 1 (NQO1) and heme oxygenase-1 (HO-1) [15–17]. DMF and its primary metabolite monomethylfumarate can activate Nrf2, a well-known Cap-N-Collar transcription factor that is essential for antioxidant responsive element (ARE)-mediated transcription such as for NQO1 and HO-1 [8,9]. Also, several studies have shown that Nrf2 overexpression prevents neointimal hyperplasia by inhibiting the proliferation of VSMCs after vascular injury through HO-1 dependent antioxidant and anti-inflammatory effects [18,19]. The beneficial effect of DMF induced Nrf2–NQO1 activity has not been reported with regard to endothelial dysfunction occurring during abnormal vascular remodeling. Basically, we have investigated the mechanism by which DMF could prevent abnormal VSMC proliferation by modulation of the cell cycle via p21 protein upregulation through Nrf2 and p53 activity, and protect against TNF-α-induced apoptosis and dysfunction in endothelial cells through Nrf2–NQO1 activity, respectively.

Materials and methods

Animals

The procedures used in this study were approved by the Animal Care and Use Committee of Kyungpook National University School of Medicine and conducted according to the Guide for the Care and Use of Laboratory Animals published by the United States National Institutes of Health (NIH Publication, 8th Edition, 2011). Ten-week-old male Sprague-Dawley (SD) rats (Hyochang, Daegu, Korea) weighing 280–320 g were used for *in vivo* experiments. All animals were provided *ad libitum* access to food (standard chow diet, Research diets, New Brunswick, NJ, USA) and water before the study.

Materials

Dimethylfumarate was purchased from Sigma-Aldrich (St. Louis, MO, USA). The antibodies against Nrf2, Cyclin D and Cyclin E were purchased from Santa Cruz Biotechnologies (Santa Cruz, CA, USA). The antibodies for p21 and p27 were from BD Bioscience (San Jose, CA, USA); antibodies for Keap1, p53, p-p53, Rb, and p-Rb from Cell signaling (Beverley, MA, USA) were used for Western blotting. Pifithrin-α (PFT-α) was purchased from Alexis Biochemicals (San Diego, CA, USA) and TNF-α was purchased from R&D Systems (Minneapolis, MN, USA). All other chemicals were of the highest purity commercially available.

Balloon injury in rat carotid artery

The rat carotid artery balloon-injury method was described previously [20,21]. Rats were pretreated with vehicle or DMF (each $n=5$ per group) using gavage, 1/day for 3 days. DMF at 5 or 15 mg/kg was dissolved in DMSO/corn oil (3:7 ratio) which was used as vehicle. After the balloon injury (BI), rats were treated with vehicle, DMF 5 mg/kg/day or 15 mg/kg/day for 2 weeks continuously. At the conclusion of the study, the tissue samples were harvested after pentobarbital sodium injection (50 mg/kg i.p.). The left common carotid arteries were harvested and fixed with 4% paraformaldehyde. Sections were used for hematoxylin & eosin (H&E) staining (ScyTek Laboratories, Logan, UT, USA) and further immunohistochemical staining. The representative sections in the middle of the artery from control, BI, BI+DMF (5 mg), and BI+DMF(15 mg), $n=2$, 3, 4, and 4, respectively, were used for the statistical analysis. IA/MA ratio was determined as intimal area divided by medial area. IA/MA ratios between the groups were analyzed by one way ANOVA and nonparametric followed by Bonferroni's test. $P<0.05$ was considered statistically significant.

Histological analysis

Immunohistochemical staining was performed using antibodies for Ki67 (1:100 dilution, NCL-Ki67p, Novocastra Laboratories Ltd, UK) and CD31 (1:100 dilution, MAB1393, Millipore Corporation, USA) using the method described in a previous study [20,21]. An Ultravision LP Large Volume Detection System and DAB plus substrate system were used for signal detection according to the manufacturer's instruction (Lab Vision, USA).

Cell culture

Primary VSMCs were isolated from thoracic aorta of 4-week-old male SD rats [20,21]. The cells were cultured in DMEM (Low glucose) supplemented with 10% (v/v) FBS and antibiotics (100 units/mL penicillin and 100 μg/mL streptomycin) at 37 °C in humidified 5% CO_2 incubator. Cells between the third and sixth passage were used in all experiments. Primary human aortic endothelial cells (HAECs) were purchased from Lonza (Walkersville, MD, USA). The cells were cultured in 2% FBS endothelial growth medium (EGM)-2 Bullet Kit (Lonza) and used for the experiments at passages 5–10. DMF was dissolved in DMSO which was used as vehicle.

Cell proliferation assay

Rat VSMCs were plated in 6-well culture plates at 1×10^4 cells/well and were grown until 30% confluence. The VSMCs were rendered quiescent by incubation for 24 h with serum-free media followed by the stimulation with 15% FBS including vehicle or indicated dose of DMF for the next 48 h. The cells were detached using 0.25% Trypsin–EDTA and were counted using hematocytometer after trypan blue staining.

Cell viability assay

HAECs were seeded into 96-well culture plates at 5×10^3 cells/well with 0.1% FBS EGM-2 media and incubated for 24 h. The media for the HAECs was changed to 0.1% FBS EGM-2 media including TNF-α (50 ng/mL), vehicle or indicated dose of DMF for next 24 h. Cell viability was measured using Cell Counting Kit-8 (Dojindo, Rockville, MD, USA).

DNA synthesis assay

5-Bromo-2′-deoxy-uridine (BrdU) labeling and detection kit III was purchased from Roche (Mannheim, Germany). VSMCs were seeded into 96-well culture plates at 1×10^3 cells/well. At 30% confluence, the cells were starved with serum-free media for 48 h.

The cells were stimulated with 15% FBS and pretreated with vehicle or indicated dose of DMF for 24 h. Cells were incubated with BrdU 10 μmol/L for an additional 6 h. The incorporation of BrdU was measured according to the manufacturer's instruction.

Western blot analysis

Cells were washed with PBS and harvested by scraping in the lysis buffer (20 mmol/L Tris–HCl (pH 7.4), 1% NP-40, 5 mmol/L EDTA, 2 mmol/L Na_3VO_4, 100 mmol/L NaF, 10 mmol/L $Na_4P_2O_7$, 100 μmol/L PMSF, 7 μg/mL aprotinin, and 7 μg/mL leupeptin). Proteins were separated on 10–12% SDS-polyacrylamide gel, transferred to PVDF membranes (Millipore, USA) and subjected to immunoblot analysis using appropriate antibodies.

Cell cycle analysis

The VSMCs were seeded at a density of 3×10^5 cells into 100 mm culture dishes. At 30% confluence, the cells were starved in serum-free media for 48 h and stimulated with 15% FBS for 20 h. Then, the cells were trypsinized, centrifuged at 1000 rpm for 5 min, washed once with cold PBS including 1% BSA, fixed in 70% cold ethanol, and stained with 1 mL of staining solution containing 50 mg/mL propidium iodide, 1 mg/mL RNase A and 1.5% Triton X-100 for at least 1 h in the dark at 4 °C. DNA content of the cells was analyzed by flow cytometry (FACS Calibur, BD Bioscience, San Jose, CA, USA).

Promoter assay and plasmids

The reporter plasmid p21-Luc and was kindly provided by Dr. Xiao-Fan Wang (Duke University Medical Center, Durham, NC, USA) and Dr. Taeg Kyu Kwon (Keimyung University, Daegu, Rep, Korea). The mammalian expression plasmid pcDNA3-mouse Nrf2 was a kind gift from Dr. Carl-Henrik Heldn (Ludwig Institute for Cancer Research, Uppsala, Sweden) and Dr. Mi-Kyoung Kwak (Yeungnam University, Daegu, Rep, Korea). For promoter assay, AD-293 cells at 3×10^4 cells/well were seeded on 24-well plates, incubated for 24 h, and transfected with 300 ng of reporter plasmids and mammalian expression plasmids encoding Nrf2 (100 or 300 ng/well) using the TransIT-LT1 transfection reagent (Mirus Bio Incorporation, Madison, WI, USA). After 24 h, cells were harvested in lysis buffer (0.2 mol/L Tris–HCl, pH 8.0, 0.1% Triton X-100). Cytomegalovirus (CMV)-β-galactosidase plasmids were cotransfected as an internal control. Luciferase activity was normalized to that of β-galactosidase.

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA from the cells was isolated using Trizol reagent (Invitrogen, USA) and reverse-transcribed with the First Strand cDNA synthesis kit (Fermentas, Lithuania) according to manufacturer's instructions. RT-PCR was performed using the primer sequences as follows: (1) rat Nrf2, sense primer: 5′-CCA TTT ACG GAG ACC CAC CGC-3′, antisense primer: 5′-GCC CAA GTC TTG CTC CAG CTC-3′; (2) rat Keap1: sense primer: 5′-TGC TCA ACC GCT TGC TGT ATG-3′, antisense primer: 5′-CCA AGT GCT TCA GCA GGT ACA-3′; (3) human eNOS: sense primer: 5′-CTG TGG AAA GAC AAG GCA GC-3′, antisense primer: 5′-CTG TTC TTC CTG GTG ATG CC-3′; (4) human Nrf2: sense primer: 5′-AGA CAA ACA TTC AAG CCG CT-3′; human Nrf2: antisense primer: 5′-CCA TCT CTT GTT TGC TGC AG-3′; (5) human NQO1 sense primer: 5′-TTA CTA TGG GAT GGG GTC CA-3′, antisense primer: 5′-TCT CCC ATT TTT CAG GCA AC-3′.

siRNA transfection

HAECs were seeded into 60 mm culture dishes at a density of 2×10^5 cells/well and were transfected simultaneously with human Nrf2-siRNA, human NQO1-siRNA and control siRNA duplex (Bioneer Corporation, Daejeon, Korea) using Lipofectamine™ RNAiMAX according to the manufacturer's instruction. At 24 h after transfection, the cells were incubated with 0.1% FBS media including DMF ± TNF-α for 24 h.

Preparation of recombinant adenovirus

A full length mouse Nrf2 was inserted into KpnI and XhoI sites of the pAdTrack-CMV shuttle vector. The recombinant adenoviral plasmid was generated as described previously [20,21] and recombinant adenoviruses were amplified using HEK-293 cells and subsequently purified by CsCl banding.

Statistical analysis

Numerical data are represented as means ± SD or means ± SE. Statistical analyses were performed using an unpaired Student's *t*-test or one way ANOVA as appropriate and a value of $P < 0.05$ was considered statistically significant (GraphPad Software, Inc., La Jolla, CA, USA).

Results

DMF prevents neointimal hyperplasia in rat carotid artery after balloon injury

We examined the beneficial effects of DMF on abnormal vascular remodeling using a rat carotid artery balloon injury model. Neoinitimal hyperplasia was observed in the vessel at 14 days after injury (Fig. 1A). DMF significantly reduced neointimal formation in the injured artery in a dose-dependent manner (Fig. 1B). The expression of Ki67, a marker of cell proliferation, was strongly increased in neointimal formation but not in the medial area of balloon-injured arteries at 14 days, an effect which was attenuated by DMF treatment (Fig. 1C). These results indicate that DMF reduces neointimal hyperplasia induced by balloon injury in rat carotid arteries through inhibition of abnormal VSMC proliferation.

DMF inhibits VSMC proliferation through G0/G1 phase cell cycle arrest

Based on viable cell counting and DNA synthesis analysis for cell proliferation rate, DMF treatment decreased cell numbers and DNA synthesis in VSMCs significantly (Fig. 2A and B). Cell cycle analysis using flow cytometry showed that the dramatic induction of S phase by serum compared to quiescent cells was reduced by DMF treatment; the cells at G0/G1 phase were increased in DMF-treated cells compared to serum only treated cells, indicating that DMF arrests the cells at G0/G1 phase (Fig. 2C and D). In addition, DMF significantly decreased serum-induced expression of cell cycle promoting proteins such as p-Rb, cyclin D, and cyclin E which are involved in G1 to S phase transition in VSMCs (Fig. 2E–G). Taken together, these data suggest that DMF suppresses VSMC proliferation through the induction of G0/G1 phase cell cycle arrest.

DMF upregulates p21 protein through both p53-independent (early) and p53-dependent (late) pathways

Because p21 and p27 repress cyclins/cyclin dependent kinase (CDK) complexes, which play a critical role in G1 to S cell cycle progression by increasing p-Rb, we examined the effect of DMF on the expression of these cell-cycle inhibitory proteins. As shown in Fig. 3A–C, DMF significantly increased p21 and p-p53 protein level in a time-dependent manner implying the involvements of p21

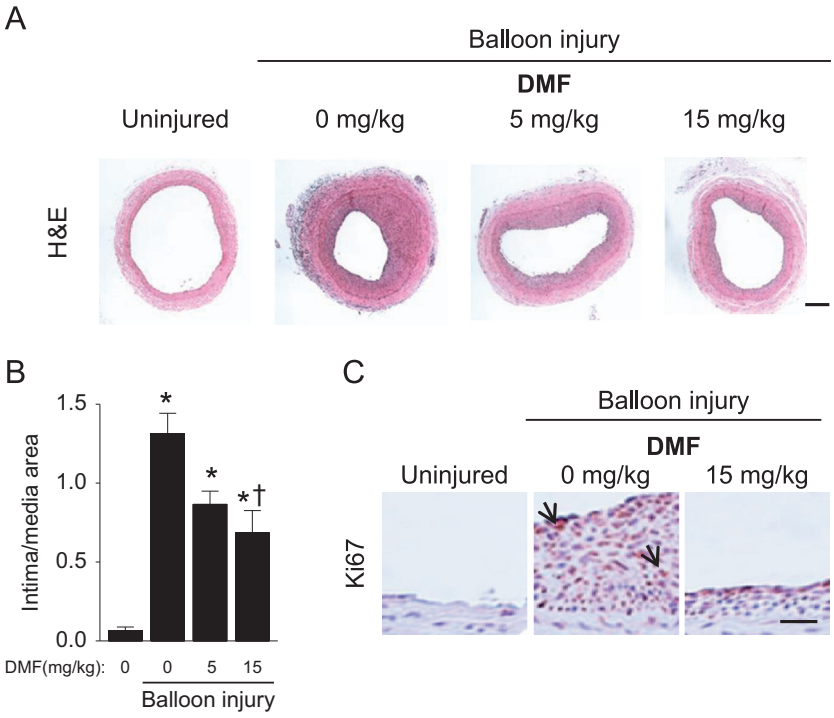

Fig. 1. Dimethylfumarate inhibits balloon injury induced neointimal hyperplasia in rat carotid arteries. (A) Representative histological cross-sections using H&E staining from uninjured, and neointimal formation after balloon injury in carotid arteries from rats orally given either vehicle or indicated concentrations of DMF (100 ×), scale bar (100 μm). (B) Morphometric analysis based on computerized image system for the carotid arteries from 4 different rat groups ($n=2$, 3, 4, and 4). Bars represent the mean ± SE. *$P<0.05$ vs. uninjured group, †$P<0.05$ vs. balloon injured group treated with vehicle. The statistical analyses were performed by Bonterroni's test. (C) Immunohistochemical staining for Ki67 antigen, a proliferation marker. Arrows indicate Ki67-positive cells (shown in brown) during neointimal formation after balloon injury (200 ×), scale bar (100 μm).

and p53 in the growth inhibitory effect of DMF in VSMCs. Also, DMF treatment markedly stimulated p21 promoter luciferase activity in a dose dependent manner (Fig. 3D). Next, we determined if the p21 promoter activity induced by DMF was regulated by PFT-α, an inhibitor of p53 transcriptional activity. The addition of PFT-α diminished DMF-enhanced p21 promoter activity in a dose dependent manner (Fig. 3E). Likewise, pharmacological inhibition of p53 attenuated the growth inhibition of VSMCs by DMF significantly (Fig. 3F) suggesting that anti-proliferative effect of DMF was partially mediated by p53 activity. Intriguingly, we observed that DMF caused a very rapid increase in p21 protein expression, which occurred earlier than p53 phosphorylation (Fig. 3A–C). Concomitantly, p53 pharmacological inhibition had little effect on early induction of p21 protein at 1 h after DMF treatment, whereas it strongly inhibited p21 expression at 6 h (Fig. 3G–J). These findings suggest that DMF inhibits VSMC proliferation by upregulating p21 via p53-independent and -dependent pathways at different time points.

Nrf2 plays an important role in the DMF-induced p21 upregulation, independent of p53 activity

To identify the mediator of p53-independent induction of p21 proteins, we investigated the involvement of Nrf2, which has been shown to be activated by DMF [7,8]. DMF significantly augmented Nrf2 protein expression in VSMCs from 30 min to 12 h in a time-dependent manner (Fig. 4A), whereas it had no effect on Nrf2 mRNA levels (data not shown). Based on immunofluorescence microscopic analysis, Nrf2 protein expression was significantly increased in the nucleus and the cytosol at 1 h after DMF treatment (Fig. 4B). Nrf2 overexpression by adenovirus delivery increased p21 protein, but not phosphorylated p53 protein expression (Fig. 4C). To explore the mechanism for Nrf2-induced p21 upregulation, we overexpressed Nrf2 and assessed p21 promoter activity. Nrf2 overexpression did not activate p21 promoter activity, but it did stimulate promoter activity of NQO1, a known target of Nrf2 (Fig. 4D). Likewise, HO-1 and NQO1, genes known as direct targets of Nrf2, took at least 2 hours for induction, which might not interfere with the p21 upregulation occurring within 1 h of DMF treatment (Fig. 4E) [22,23]. Also, p21 protein expression was unchanged by HO-1 inhibition with tin protoporphyrin (SnPP), an inhibitor of HO-1 activity, or by NQO1 inhibition with ES936, an inhibitor of NQO1 activity (Fig. 4F and G). Additional quantitative analyses for Fig. 4A–G are shown in Supplemental Fig. 1. Increased Nrf2 activity suppressed serum-induced VSMC proliferation significantly (Fig. 4H). To verify the requirement of Nrf2 for DMF-induced p21 expression, we assessed the effect of adenovirus overexpressing Keap1 (Ad-Keap1), a negative regulator of Nrf2. As shown in Fig. 4 I and J, Keap1 overexpression markedly down-regulated DMF-induced p21 protein levels at 1 h after DMF treatment, suggesting that Nrf2 activity plays an important role in regulation of p21 protein by DMF, independent of NQO1 activity.

DMF accelerates vascular re-endothelialization by ameliorating endothelial cell dysfunction induced by TNF-α

To explore the prospective effects of DMF on vascular re-endothelialization, we examined the effect of DMF on CD31 expression, a specific maker of endothelial cells, using immunohistochemical analysis in rat carotid artery after balloon injury. In Fig. 5A, DMF reversed the decrease in CD31 expression in carotid artery after balloon injury. Although we assessed the effect of DMF on HAEC proliferation, we did not observe any inhibitory effect of DMF on the proliferation of HAEC or any change in expression of cell cycle proteins (Fig. 5B and C). Previous studies demonstrated that TNF-α

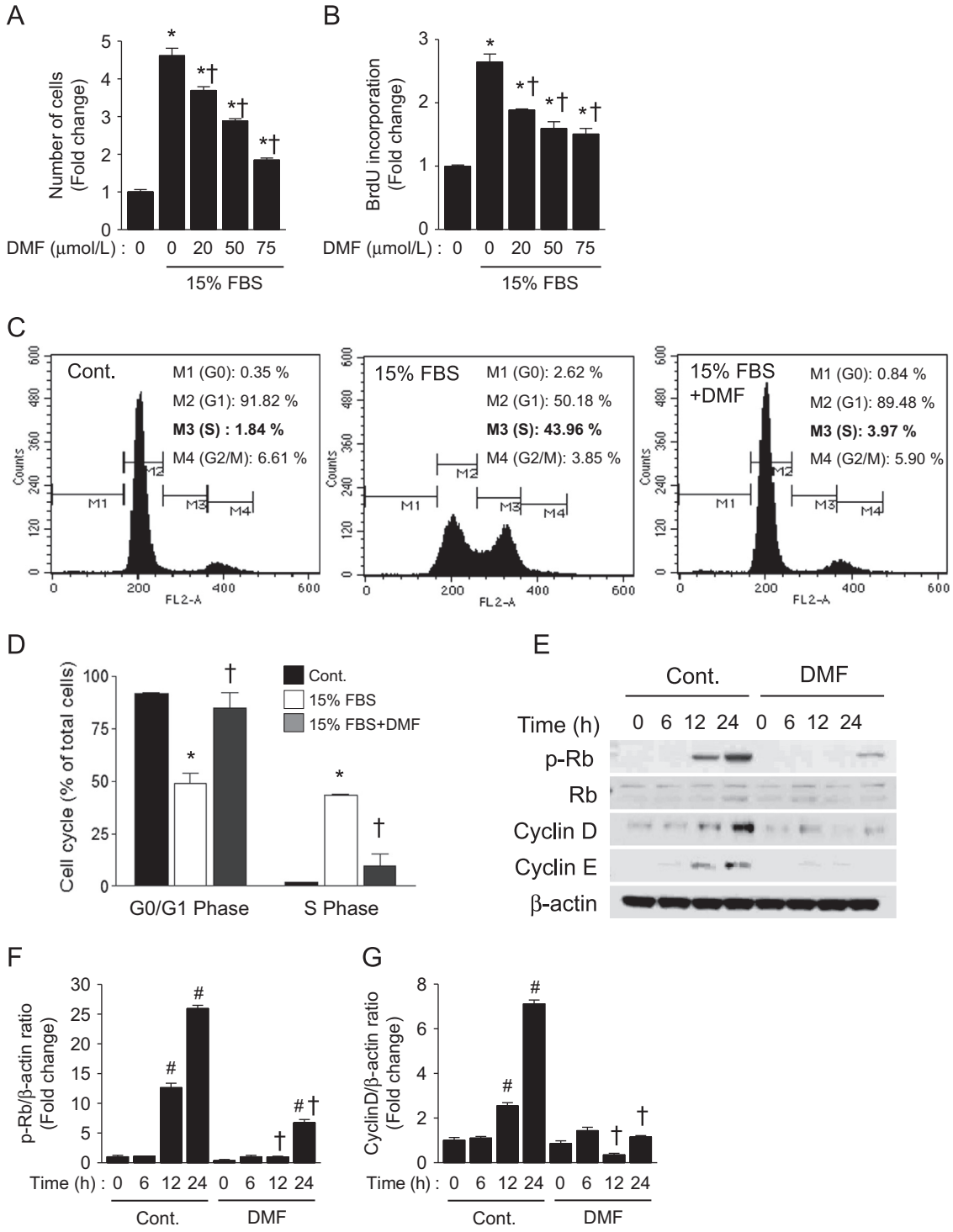

Fig. 2. DMF attenuates vascular smooth muscle cells (VSMCs) proliferation through the induction of G1 cell cycle arrest. (A) Cell proliferation rate, (B) DNA synthesis analysis, and (C, D) cell cycle analysis in rat primary VSMCs. The quiescent cells were stimulated with 15% FBS in the presence of indicated concentrations or 75 μmol/L of DMF for (A) 48 h or (B–D) 20 h (E) representative western blot and (F–G) quantitative analysis for phospho-Rb (p-Rb), cyclin D, cyclin E and β-actin as a control. The quiescent cells were stimulated with 15% FBS in the absence or presence of 75 μmol/L DMF for indicated times and whole cell extracts were subjected to Western blotting. All experiments were repeated 3 independent times. Bars represent the mean ± SD. $^{*}P < 0.05$ vs. control, $^{\dagger}P < 0.05$ vs. 15% FBS only, $^{\#}P < 0.05$ vs. zero time point.

blockade accelerated functional endothelial recovery after balloon angioplasty [13,14]. So, we confirmed that TNF-α-induces endothelial cell dysfunction by assaying for eNOS expression, which was significantly improved by DMF (Fig. 5D). Additionally, we showed that Nrf2–NQO1 expression mediated the beneficial effect of DMF on endothelial cells (Fig. 5E–H). Overall, DMF regulates eNOS expression mediated by Nrf2–NQO1 activity, which can improve vascular function and re-endothelialization after vascular injury *in vivo*.

DMF protects against endothelial cell apoptosis induced by TNF-α mediated by Nrf2–NQO1 axis

To explain the stimulatory effect of DMF on re-endothelialization, we also investigated whether DMF could inhibit apoptosis of endothelial cells induced by TNF-α [24]. As expected, DMF protected endothelial cells from TNF-α induced apoptosis in a dose-dependent manner (Fig. 6A). Consistent with Fig. 6A, after

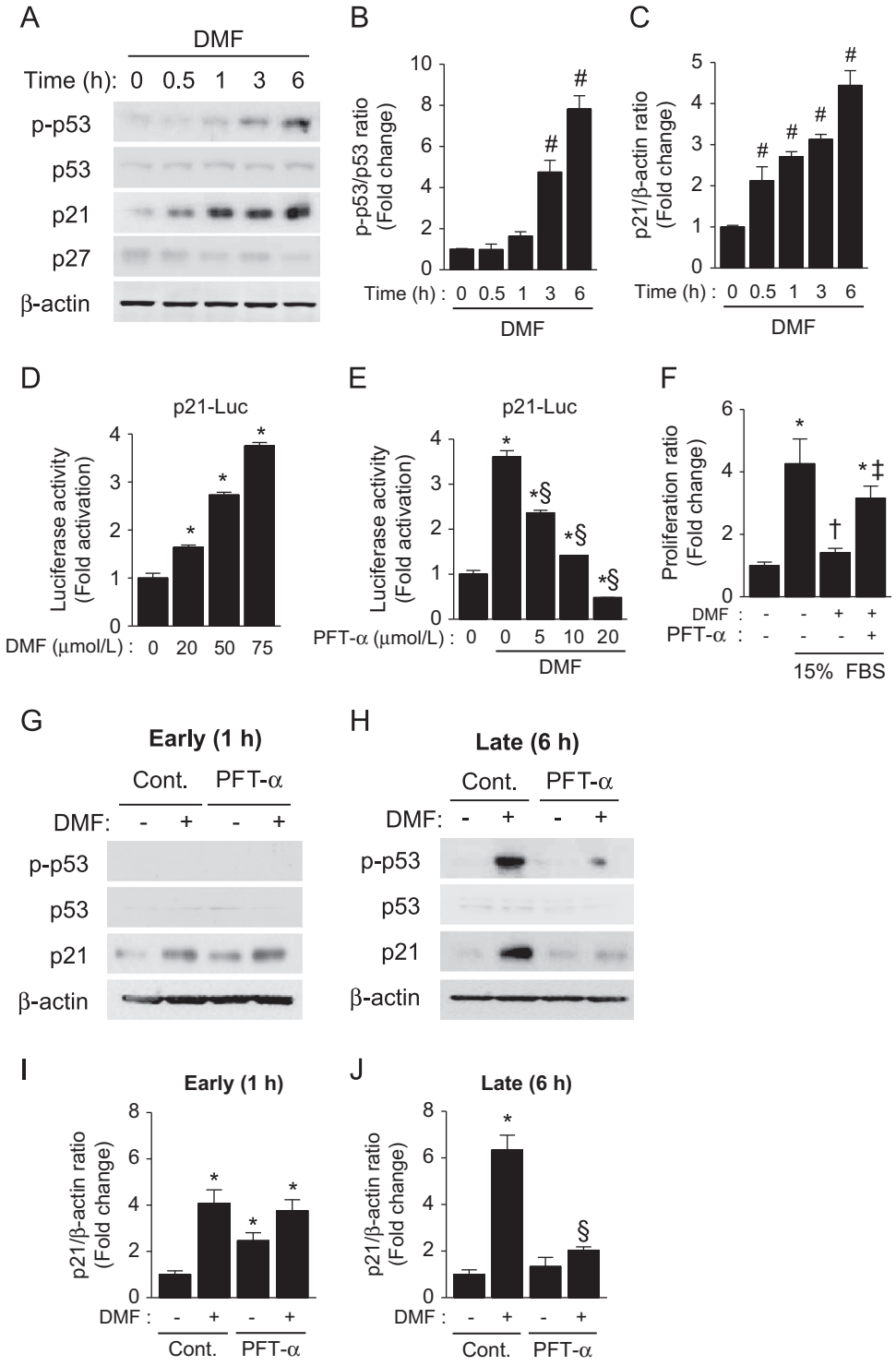

Fig. 3. DMF suppresses vascular smooth muscle cells (VSMCs) proliferation by p21 overexpression via both p53-independent (early) and dependent (late) pathways. (A) Western blot and (B–C) quantitative analysis for phospho-p53 (p-p53), p53, p21, p27 and β-actin in a time dependent manner in VSMCs at 75 μmol/L DMF. (D) p21 Promoter activity in AD293 cells at different concentrations of DMF (0–75 μmol/L). (E) Effect of Pifithrin-α (PFT-α), a known inhibitor of p53 activity, on increased p21 promoter activity by DMF treatment. AD-293 cells were transfected with p21-Luc plasmid. (F) PFT-α effect on proliferation rate in VSMCs. (G–J) PFT-α (20 μmol/L) effects on p-p53, p53, p21, and β-actin at Early (E: 1 h) and late (F: 6 h) time points assessed by Western blot. All experiments were repeated 3 independent times. Bars represent the mean ± SD. $^{\#}P<0.05$ vs. time point zero $^{*}P<0.05$ vs. control, $^{\S}P<0.05$ vs. DMF only, $^{\dagger}P<0.05$ vs. 15% FBS only, $^{\ddagger}P<0.05$ vs. 15% FBS + DMF.

Nrf2 or NQO1 knockdown, DMF failed to prevent TNF-α induced cell apoptosis (Fig. 6B and C). Also, morphological data by microscopy showed that DMF increased low cell density affected by TNF-α, which was attenuated with siRNA for Nrf2 or NQO1 in HAECs (Fig. 6D). In general, enhanced Nrf2 or NQO1 activity by DMF can decrease the apoptosis of endothelial cells induced by TNF-α.

Discussion

In the present study, we demonstrated that DMF inhibited the proliferation of VSMCs via induction of G1 phase arrest leading to the suppression of neointimal formation caused by balloon injury *in vivo*. This protective effect of DMF against neointimal hyperplasia was

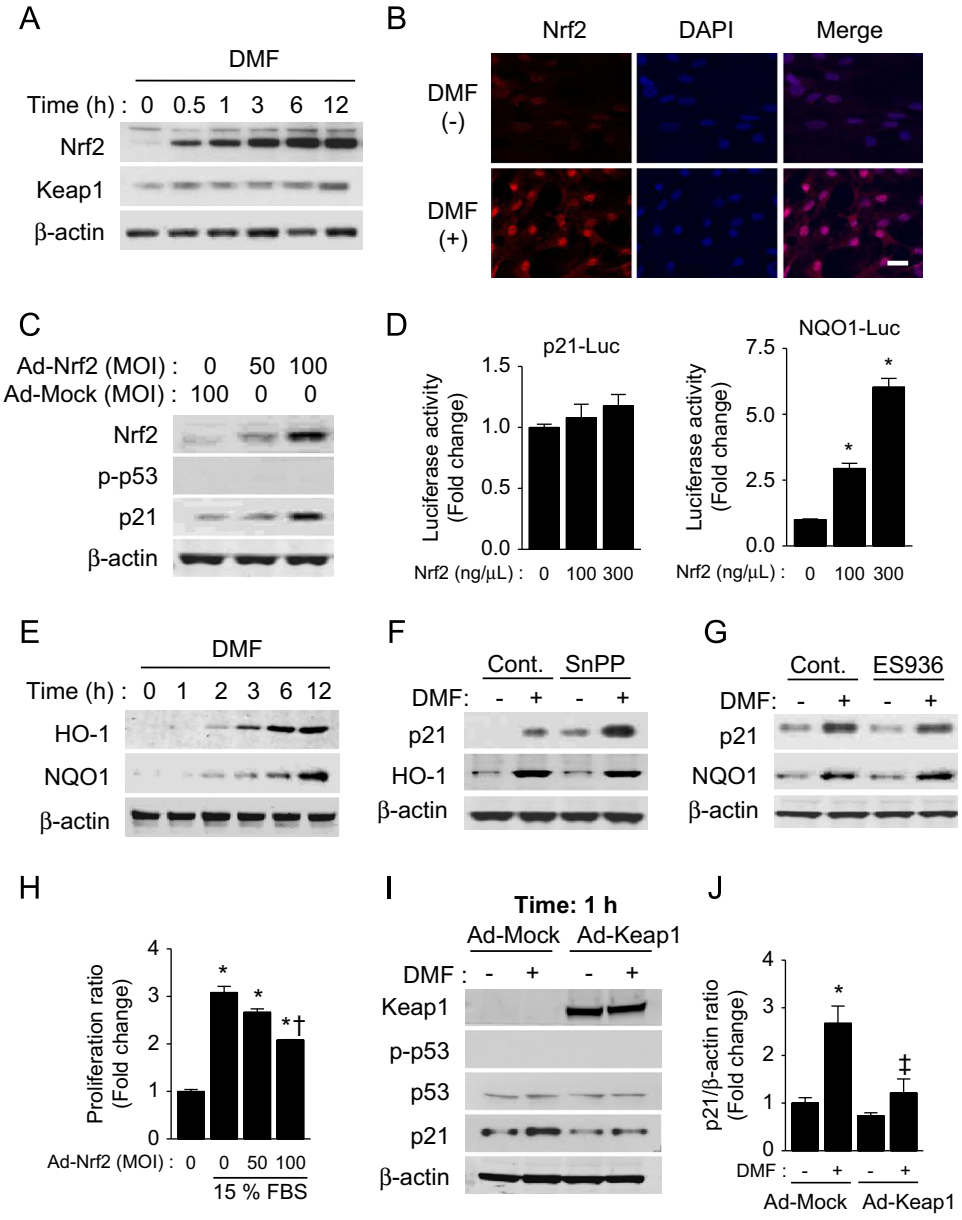

Fig. 4. Nrf2 plays a critical role in DMF-induced upregulation of p21 protein, independent of p53 activation in vascular smooth muscle cells (VSMCs). (A) DMF effects on time dependent expression of Nrf2, Keap1 in VSMCs. (B) Immunofluorescent staining with Nrf2 antibody after 75 μmol/L DMF treatment (1 h) compared to control (400 ×), scale bar (25 μm). (C) Western blot for Nrf2 and p-p53 after adenoviral delivery of Nrf2 gene (Ad-Nrf2). (D) Effect of Nrf2 overexpression on the promoter activities of p21 and NQO1 in a dose dependent manner. AD-293 cells were transfected with the plasmids for 24 h (E) DMF effect on HO-1 and NQO1 expression in a time dependent manner in VSMCs. (F) Effect by HO-1 pharmacological inhibitor, SnPP on p21 expression. (G) Effect by NQO1 pharmacological inhibitor, ES936 on p21 expression. (H) Nrf2 overexpression effect on proliferation rate in a dose dependent manner. (I–J) Effect of Keap-1 overexpression on p-p53, p53, p21 and β-actin at 75 μmol/L DMF for 1 h. All experiments were repeated 3 independent times. Bars represent the mean ± SD. $^{*}P < 0.05$ vs. control, $^{\dagger}P < 0.05$ vs. 15% FBS only, $^{\ddagger}P < 0.05$ vs. Ad-Mock+DMF.

dependent on the cooperative induction of p21 by activation of Nrf2 and p53 activity. Likewise, we observed that DMF treatment accelerated re-endothelialization after balloon injury *in vivo* and DMF protected the endothelial cell from TNF-α-induced apoptosis via Nrf2–NQO1 activity. Excessive proliferation of VSMCs is a primary source of vascular restenosis after PTCA [1]; therefore, new drug development and better understanding of the molecular mechanisms are very important for treating patients. Originally, DMF was used as an anti-psoriatic ointment and orally for the treatment of multiple sclerosis, inflammatory skin disease and several cancers [25,26]. In this study, we found that DMF could inhibit VSMCs proliferation through p21 induction which led to G1 cell cycle arrest. We verified that p21 protein expression was upregulated by DMF, mediated by both p53-independent (early) and p53-dependent (late) pathways. Initially, DMF increases p21 protein stability via stimulated Nrf2 activity. Overall, p21 expression was regulated by DMF by actions on both protein stability (p53-independent pathway) and transcription (p53-dependent pathway).

Even though inhibition of abnormal VSMCs proliferation has been a primary target to prevent neointimal hyperplasia after PTCA, delayed re-endothelialization contributes to the acceleration of neointimal hyperplasia. It is well accepted that apoptosis, impaired re-endothelialization, and vascular endothelial cell dysfunction play important roles in the development of atherosclerosis and restenosis [27]. In the case of DMF, previous studies have shown that DMF inhibited TNF-α-induced expression of inflammation markers such as VCAM-1 and ICAM-1 via inhibition of NF-κB signaling [28,29] and attenuated TNF-α-induced tissue factor expression in endothelial cells

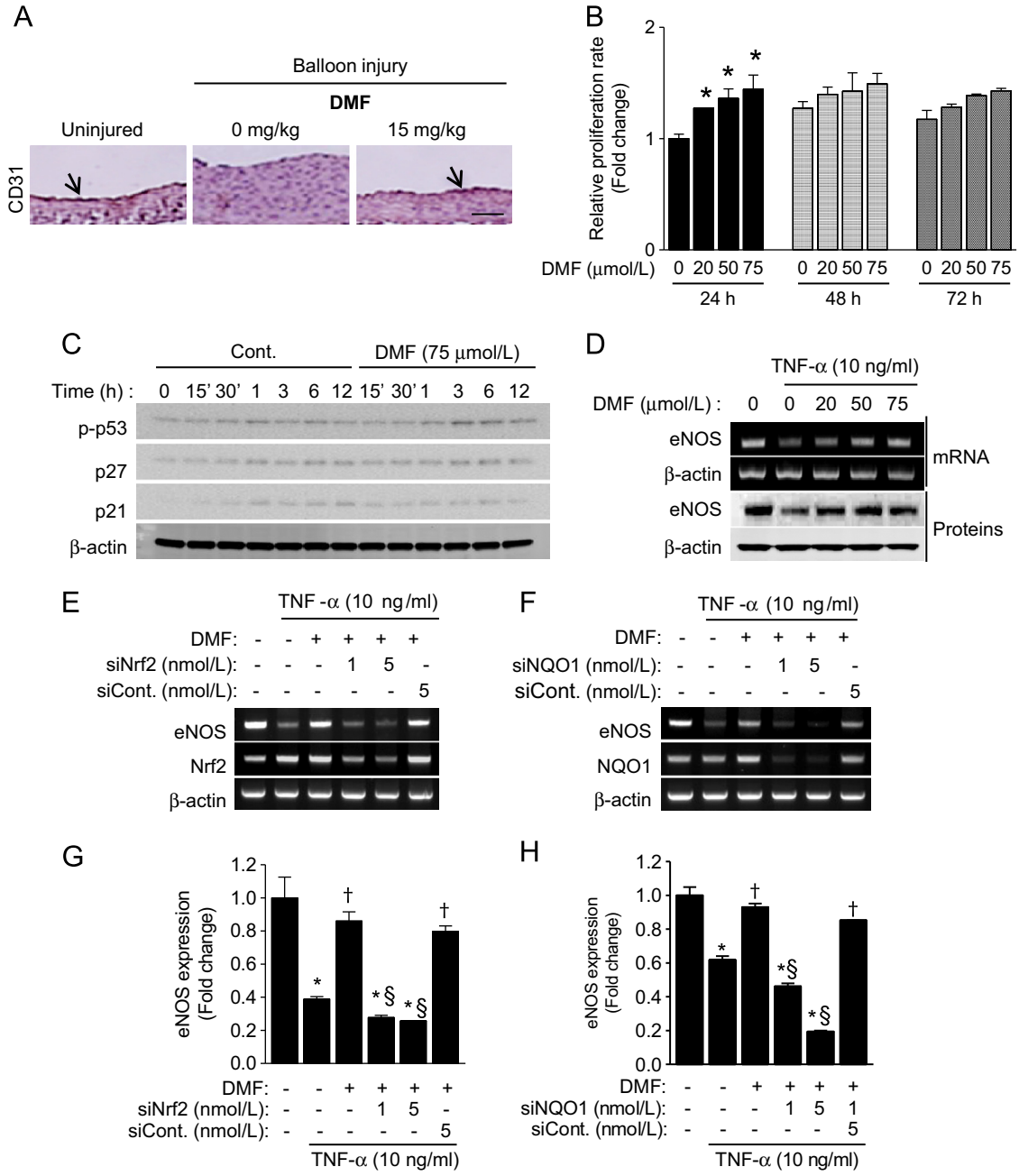

Fig. 5. DMF increases vascular re-endothelialization by reducing endothelial cell dysfunction induced by TNF-α. (A) Immunohistochemical staining for CD31, endothelial marker at 14 days after balloon injury (100 ×), scale bar (50 μm). (B) Relative proliferation rate of HAECs and (C) representative western blot in different concentrations of DMF treatment. (D) Representative RT-PCR and western blot for TNF-α-stimulated mRNA and protein expression of eNOS at different concentrations of DMF in treated HAECs. Representative RT-PCR (E–F) and quantitative analysis (G–H) for eNOS, Nrf2 and β-actin as a control after knockdown of Nrf2 or NQO1 using siRNA. The quiescent cells were stimulated with TNF-α (10 ng/mL) in the presence of indicated concentrations of DMF for 24 h. All experiments were repeated 3 independent times. Bars represent the mean ± SD. $^{*}P < 0.05$ vs. control, $^{\dagger}P < 0.05$ vs. TNF-α, $^{\S}P < 0.01$ vs. TNF-α supplemented with DMF.

[12], indicating that DMF might inhibit VSMC migration and thrombosis after balloon angioplasty. Also, DMF protected against TNF-α-induced endothelial cell apoptosis and dysfunction such as reduced eNOS expression via Nrf2–NQO1 activity in our study. Previous studies have shown that TNF-α induces endothelial cell apoptosis, while it increases the proliferation of VSMCs [24,30]. Taken together, DMF might be used as a therapeutic drug for vascular diseases including atherosclerosis and PTCA-associated restenosis.

HO-1 and NQO1 are well-known target genes stimulated by Nrf2. The by-products of HO-1 (hemin and carbon monoxide) have been reported to inhibit proliferation and induce apoptosis of VSMCs [31]. Previously, we showed that β-lapachone inhibited proliferation of VSMCs by activating NQO1 [22]. Thus, we examined the involvement of HO-1 or NQO1 on DMF-induced p21 expression and inhibition of VSMC proliferation. HO-1 and NQO1 proteins were not detected clearly until 2 h after DMF treatment, in contrast to p21 induction which was detectable at an early time. Similarly, the inhibition of HO-1 by SnPP and NQO1 activities by ES936 did not have any considerable inhibitory effect on DMF-induced p21 expression, implying that HO-1 and NQO1 activities were not required for p21 induction by DMF. DMF failed to reverse TNF-α-induced apoptosis and inhibition of eNOS expression in endothelial cells after knockdown of NQO1. Thus, NQO1 may be a crucial factor in the survival and function of vascular endothelial cells. Collectively, our data suggest that DMF may protect against restenosis

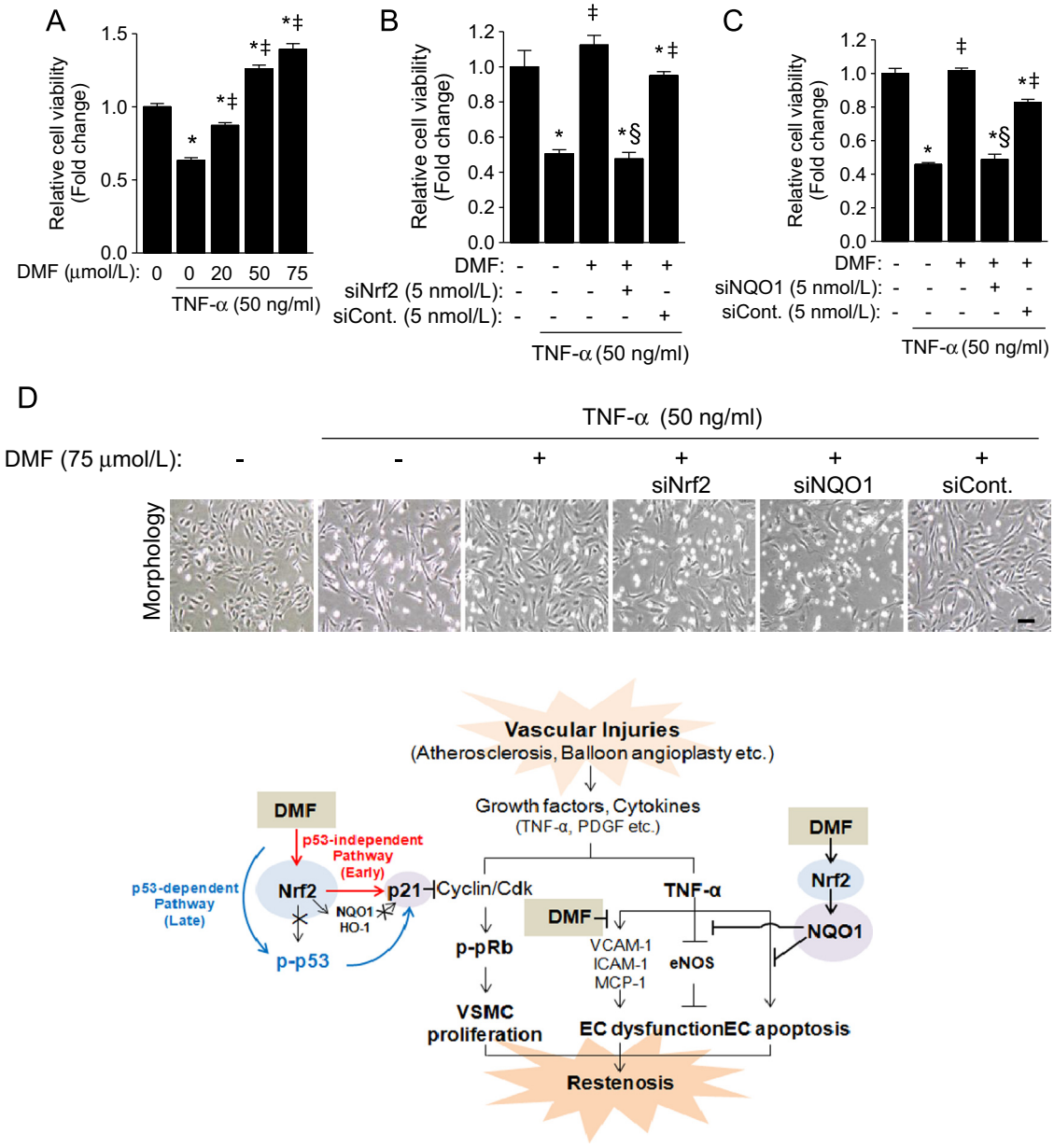

Fig. 6. DMF protects TNF-α induced cell apoptosis mediated by Nrf2–NQO1 activity in human aortic cells (HAECs). (A) Quantitation of cell viability by TNF-α associated apoptosis in a dose dependent manner (0–75 μmol/L) in HAECs. (B, C) Quantitative cell viability assay using siRNA knock-down of Nrf2 or NQO1 in the presence of DMF after TNF-α- induced apoptosis. (D) Morphological images by microscope showing cell density after TNF − α ± DMF in the presence of siNrf2 or siNQO1 (40x), scale bar (100 μm). All experiments were repeated 3 independent times. Bars represent the mean ± SD. $^{*}P < 0.05$ vs. control, $^{‡}P < 0.05$ vs. TNF-α, $^{§}P < 0.01$ vs. TNF-α supplemented with DMF.

after balloon angioplasty or stenting by inhibiting neointimal hyperplasia and by accelerating re-endothelialization by blocking the apoptosis and dysfunction of endothelial cells induced by TNF-α.

Conclusion

Vascular remodeling after balloon injury can be ameliorated by DMF via 2 distinct mechanisms in VSMCs and endothelial cells, respectively. Firstly, DMF stimulates Nrf2 activity leading to p21 upregulation, which blocks the G1 to S phase transition in cell cycle immediately, independent of p53 activity. Later on, p21 expression can be regulated by enhanced p53 transcriptional activity in VSMCs. The other mechanism accounting for the beneficial effect by DMF on vascular injury may be explained by a protective role on endothelial apoptosis and eNOS expression followed by accelerated re-endothelization via Nrf2–NQO1 activity. Therefore, these results suggest that DMF could be a useful therapeutic for patients with vascular restenosis.

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Supporting material

Supplementary material associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.redox.2014.06.003.

References

[1] M.B. Preisack, K.R. Karsch, The paradigm of restenosis following percutaneous transluminal coronary angioplasty, European Heart Journal 14 (Suppl. i) (1993) S187–S192.

[2] M.C. Morice, P.W. Serruys, J.E. Sousa, J. Fajadet, E. Ban Hayashi, M. Perin, A. Colombo, G. Schuler, P. Barragan, G. Guagliumi, F. Molnar, R. Falotico, A randomized comparison of a sirolimus-eluting stent with a standard stent for coronary revascularization, New England Journal of Medicine 346 (2002) 1773–1780, http://dx.doi.org/10.1016/j.redox.2014.06.003 12050336.

[3] J.W. Moses, M.B. Leon, J.J. Popma, P.J. Fitzgerald, D.R. Holmes, C. O'Shaughnessy, R.P. Caputo, D.J. Kereiakes, D.O. Williams, P.S. Teirstein, J.L. Jaeger, R.E. Kuntz, Sirolimus-eluting stents versus standard stents in patients with stenosis in a native coronary artery, New England Journal of Medicine 349 (2003) 1315–1323, http://dx.doi.org/10.1016/j.redox.2014.06.003 14523139.

[4] H.J. Bovenschen, A.M. Langewouters, P.C. van de Kerkhof, Dimethylfumarate for psoriasis: pronounced effects on lesional T-cell subsets, epidermal proliferation and differentiation, but not on natural killer T cells in immunohistochemical study, American Journal of Clinical Dermatology 11 (2010) 343–350, http://dx.doi.org/10.1016/j.redox.2014.06.003 20553063.

[5] R.J. Fox, D.H. Miller, J.T. Phillips, M. Hutchinson, E. Havrdova, M. Kita, M. Yang, K. Raghupathi, M. Novas, M.T. Sweetser, V. Viglietta, K.T. Dawson, Placebo-controlled phase 3 study of oral BG-12 or glatiramer in multiple sclerosis, New England Journal of Medicine 367 (2012) 1087–1097, http://dx.doi.org/10.1016/j.redox.2014.06.003 22992072.

[6] L. Kappos, R. Gold, D.H. Miller, D.G. Macmanus, E. Havrdova, V. Limmroth, C. H. Polman, K. Schmierer, T.A. Yousry, M. Yang, M. Eraksoy, E. Meluzinova, I. Rektor, K.T. Dawson, A.W. Sandrock, G.N. O'Neill, Efficacy and safety of oral fumarate in patients with relapsing-remitting multiple sclerosis: a multicentre, randomised, double-blind, placebo-controlled phase IIb study, Lancet 372 (2008) 1463–1472, http://dx.doi.org/10.1016/j.redox.2014.06.003 18970976.

[7] H. Ashrafian, G. Czibik, M. Bellahcene, D. Aksentijević, A.C. Smith, S.J. Mitchell, M.S. Dodd, J. Kirwan, J.J. Byrne, C. Ludwig, H. Isackson, A. Yavari, N.B. Støttrup, H. Contractor, T.J. Cahill, N. Sahgal, D.R. Ball, R.I. Birkler, I. Hargreaves, D. A. Tennant, J. Land, C.A. Lygate, M. Johannsen, R.K. Kharbanda, S. Neubauer, C. Redwood, R. de Cabo, I. Ahmet, M. Talan, U.L. Günther, A.J. Robinson, M. R. Viant, P.J. Pollard, D.J. Tyler, H. Watkins, Fumarate is cardioprotective via activation of the Nrf2 antioxidant pathway, Cell Metabolism 15 (2012) 361–371, http://dx.doi.org/10.1016/j.redox.2014.06.003 22405071.

[8] C.J. Oh, J.Y. Kim, Y.K. Choi, H.J. Kim, J.Y. Jeong, K.H. Bae, K.G. Park, I.K. Lee, Dimethylfumarate attenuates renal fibrosis via NF-E2-related factor 2-mediated inhibition of transforming growth factor-β/Smad signaling, PloS One 7 (2012) e45870, http://dx.doi.org/10.1016/j.redox.2014.06.003 23056222.

[9] H.J. Kang, H.A. Seo, Y. Go, C.J. Oh, N.H. Jeoung, K.G. Park, I.K. Lee, Dimethylfumarate suppresses adipogenic differentiation in 3T3-L1 preadipocytes through inhibition of STAT3 activity, PloS One 8 (2013) e61411, http://dx.doi.org/10.1016/j.redox.2014.06.003 23637829.

[10] P. Seidel, S. Goulet, K. Hostettler, M. Tamm, M. Roth, DMF inhibits PDGF-BB induced airway smooth muscle cell proliferation through induction of heme-oxygenase-1, Respiratory Research 11 (2010) 145, http://dx.doi.org/10.1016/j.redox.2014.06.003 20961405.

[11] T.F. Lüscher, J. Steffel, F.R. Eberli, M. Joner, G. Nakazawa, F.C. Tanner, R. Virmani, Drug-eluting stent and coronary thrombosis: biological mechanisms and clinical implications, Circulation 115 (2007) 1051–1058, http://dx.doi.org/10.1016/j.redox.2014.06.003 17325255.

[12] R. Loewe, W. Holnthoner, M. Gröger, M. Pillinger, F. Gruber, D. Mechtcheriakova, E. Hofer, K. Wolff, P. Petzelbauer, Dimethylfumarate inhibits TNF-induced nuclear entry of NF-kappa B/p65 in human endothelial cells, Journal of Immunology 168 (2002) 4781–4787, http://dx.doi.org/10.1016/j.redox.2014.06.003.

[13] H. Tanaka, G. Sukhova, D. Schwartz, P. Libby, Proliferating arterial smooth muscle cells after balloon injury express TNF-alpha but not interleukin-1 or basic fibroblast growth factor, Arteriosclerosis, Thrombosis, and Vascular Biology 16 (1996) 12–18, http://dx.doi.org/10.1016/j.redox.2014.06.003 8548411.

[14] K. Krasinski, I. Spyridopoulos, M. Kearney, D.W. Losordo, *in vivo* blockade of tumor necrosis factor-alpha accelerates functional endothelial recovery after balloon angioplasty, Circulation 104 (2001) 1754–1756, http://dx.doi.org/10.1016/j.redox.2014.06.003 11591609.

[15] H.Y. Cho, S.P. Reddy, M. Yamamoto, S.R. Kleeberger, The transcription factor NRF2 protects against pulmonary fibrosis, FASEB Journal: Official Publication of the Federation of American Societies for Experimental Biology 18 (2004) 1258–1260 15208274.

[16] H. Zheng, S.A. Whitman, W. Wu, G.T. Wondrak, P.K. Wong, D. Fang, D.D. Zhang, Therapeutic potential of Nrf2 activators in streptozotocin-induced diabetic nephropathy, Diabetes 60 (2011) 3055–3066, http://dx.doi.org/10.1016/j.redox.2014.06.003 22025779.

[17] W. Xu, L. Shao, C. Zhou, H. Wang, J. Guo, Upregulation of Nrf2 expression in non-alcoholic fatty liver and steatohepatitis, Hepato-Gastroenterology 58 (2011) 2077–2080 22024078.

[18] J.Y. Kim, H.J. Cho, J.J. Sir, B.K. Kim, J. Hur, S.W. Youn, H.M. Yang, S.I. Jun, K. W. Park, S.J. Hwang, Y.W. Kwon, H.Y. Lee, H.J. Kang, B.H. Oh, Y.B. Park, H.S. Kim, Sulfasalazine induces haem oxygenase-1 via ROS-dependent Nrf2 signalling, leading to control of neointimal hyperplasia, Cardiovascular Research 82 (2009) 550–560, http://dx.doi.org/10.1016/j.redox.2014.06.003 19234301.

[19] J.D. Ahn, R. Morishita, Y. Kaneda, S.J. Lee, K.Y. Kwon, S.Y. Choi, K.U. Lee, J.Y. Park, I.J. Moon, J.G. Park, M. Yoshizumi, Y. Ouchi, I.K. Lee, Inhibitory effects of novel AP-1 decoy oligodeoxynucleotides on vascular smooth muscle cell proliferation in vitro and neointimal formation *in vivo*, Circulation Research 90 (2002) 1325–1332, http://dx.doi.org/10.1016/j.redox.2014.06.003 12089071.

[20] H.J. Kim, E.K. Yoo, J.Y. Kim, Y.K. Choi, H.J. Lee, J.K. Kim, N.H. Jeoung, K.U. Lee, I.S. Park, B.H. Min, K.G. Park, C.H. Lee, B.J. Aronow, M. Sata, I.K. Lee, Protective role of clusterin/apolipoprotein J against neointimal hyperplasia via antiproliferative effect on vascular smooth muscle cells and cytoprotective effect on endothelial cells, Arteriosclerosis, Thrombosis, and Vascular Biology 29 (2009) 1558–1564, http://dx.doi.org/10.1016/j.redox.2014.06.003 19696405.

[21] H.J. Kim, J.Y. Kim, S.J. Lee, H.J. Kim, C.J. Oh, Y.K. Choi, H.J. Lee, J.Y. Do, S.Y. Kim, T.K. Kwon, H.S. Choi, M.O. Lee, I.S. Park, K.G. Park, K.U. Lee, Alpha-lipoic acid prevents neointimal hyperplasia via induction of p38 mitogen-activated protein kinase/Nur77-mediated apoptosis of vascular smooth muscle cells and accelerates postinjury reendothelialization, Arteriosclerosis, Thrombosis, and Vascular Biology 30 (2010) 2164–2172, http://dx.doi.org/10.1016/j.redox.2014.06.003 20829507.

[22] S.Y. Kim, N.H. Jeoung, C.J. Oh, Y.K. Choi, H.J. Lee, H.J. Kim, J.Y. Kim, J.H. Hwang, S. Tadi, Y.H. Yim, K.U. Lee, K.G. Park, S. Huh, K.N. Min, K.H. Jeong, M.G. Park, T. H. Kwak, G.R. Kweon, K. Inukai, M. Shong, I.K. Lee, Activation of NAD(P)H: quinone oxidoreductase 1 prevents arterial restenosis by suppressing vascular smooth muscle cell proliferation, Circulation Research 104 (2009) 842–850, http://dx.doi.org/10.1016/j.redox.2014.06.003 19229058.

[23] A.I. Rodriguez, A. Gangopadhyay, E.E. Kelley, P.J. Pagano, B.S. Zuckerbraun, P.M. Bauer, HO-1 and CO decrease platelet-derived growth factor-induced vascular smooth muscle cell migration via inhibition of Nox1, Arteriosclerosis, Thrombosis, and Vascular Biology 30 (2010) 98–104, http://dx.doi.org/10.1016/j.redox.2014.06.003 19875720.

[24] S. Rastogi, W. Rizwani, B. Joshi, S. Kunigal, S.P. Chellappan, TNF-alpha response of vascular endothelial and vascular smooth muscle cells involve differential utilization of ASK1 kinase and p73, Cell Death and Differentiation 19 (2012) 274–283, http://dx.doi.org/10.1016/j.redox.2014.06.003 21738216.

[25] U. Mrowietz, P. Altmeyer, T. Bieber, M. Rocken, R.E. Schopf, W. Sterry, Treatment of psoriasis with fumaric acid esters (Fumaderm), Journal der Deutschen Dermatologischen Gesellschaft (Journal of the German Society of Dermatology) 5 (2007) 716–717.

[26] R. Loewe, T. Valero, S. Kremling, B. Pratscher, R. Kunstfeld, H. Pehamberger, P. Petzelbauer, Dimethylfumarate impairs melanoma growth and metastasis, Cancer Research 66 (2006) 11888–11896, http://dx.doi.org/10.1016/j.redox.2014.06.003 17178886.

[27] A.K. Mitra, D.K. Agrawal, In stent restenosis: bane of the stent era, Journal of Clinical Pathology 59 (2006) 232–239, http://dx.doi.org/10.1016/j.redox.2014.06.003 16505271.

[28] M. Vandermeeren, S. Janssens, M. Borgers, J. Geysen, Dimethylfumarate is an inhibitor of cytokine-induced E-selectin, VCAM-1, and ICAM-1 expression in human endothelial cells, Biochemical and Biophysical Research Communications 234 (1997) 19–23, http://dx.doi.org/10.1016/j.redox.2014.06.003 9168952.

[29] S.A. Rubant, R.J. Ludwig, S. Diehl, K. Hardt, R. Kaufmann, J.M. Pfeilschifter, W.H. Boehncke, Dimethylfumarate reduces leukocyte rolling *in vivo* through modulation of adhesion molecule expression, Journal of Investigative Dermatology 128 (2008) 326–331 17671516.

[30] D.A. Goukassian, R. Kishore, K. Krasinski, C. Dolan, C. Luedemann, Y.S. Yoon, M. Kearney, A. Hanley, H. Ma, T. Asahara, J.M. Isner, D.W. Losordo, Engineering the response to vascular injury: divergent effects of deregulated E2F1 expression on vascular smo oth muscle cells and endothelial cells result in endothelial recovery and inhibition of neointimal growth, Circulation Research 93 (2003) 162–169, http://dx.doi.org/10.1016/j.redox.2014.06.003 12829616.

[31] J.A. Moraes, P. Barcellos-de-Souza, G. Rodrigues, V. Nascimento-Silva, S.V. Silva, J. Assreuy, M.A. Arruda, C. Barja-Fidalgo, Heme modulates smooth muscle cell proliferation and migration via NADPH oxidase: a counter-regulatory role for heme oxygenase system, Atherosclerosis 224 (2012) 394–400, http://dx.doi.org/10.1016/j.redox.2014.06.003 22954673.