Cyanidin-3-glucoside suppresses TNF-α-induced cell proliferation through the repression of Nox activator 1 in mouse vascular smooth muscle cells: involvement of the STAT3 signaling

Xiaoqin Luo · Shi Fang · Yunjun Xiao ·
Fenglin Song · Tangbin Zou · Min Wang ·
Min Xia · Wenhua Ling

Abstract Cyanidin-3-glucoside (C3G) is a member of the anthocyanin family which belongs to the flavonoid class and possesses antiatherogenic properties. Many studies have demonstrated the protective effects of C3G on vascular endothelial cells and monocytes, however, the precise effects on vascular smooth muscle cells (VSMCs) have been less thoroughly studied. Hence, we investigated the role of C3G in TNF-α-induced VSMCs proliferation and explored the possible mechanisms. TNF-α stimulated VSMCs proliferation, and pretreatment with C3G inhibited the proliferation in dose- and time-dependent manners. Then, we found that C3G attenuated TNF-α-induced ROS over generation by Dihydroethidium staining. The combination of 50 μM C3G and 100 μM apocynin significantly reduced ROS generation. Moreover, C3G pretreatment significantly suppressed the expression of Nox activator 1, a subunit of NADPH oxidase in mouse VSMCs. C3G also inhibited TNF-α-induced signal transducer and activator of transcription (STAT3) phosphorylation, and the inhibitory effect was more prominent in C3G and apocynin co-pre-treated cells than that pretreated with C3G or apocynin alone. Administration of the ROS scavenger catalase (2,000 U/ml) remarkably inhibited TNF-α-induced cell proliferation and STAT3 activation. These data suggest that C3G exerts its antiproliferative effect on TNF-α-induced VSMCs proliferation through inhibiting STAT3 activation by attenuating NoxA1-derived ROS over production.

Abbreviations

C3G	Cyanidin-3-glucoside
DHE	Dihydroethidium
JAK/STAT	Janus kinase/signal transducer and activator of transcription
NoxA1	Nox activator 1
HBSS	Hank's balanced salt solution
PVDF	Polyvinylidene fluoride
ROS	Reactive oxygen Species
STAT3	Signal transducer and activator of transcription 3
VSMCs	Vascular smooth muscle cells

S. Fang is the co-first author.

X. Luo · Y. Xiao · F. Song · T. Zou · M. Wang · M. Xia · W. Ling (✉)
Department of Nutrition, School of Public Health, Sun Yat-sen University, 74 Zhongshan 2nd Road, Guangzhou 510080, People's Republic of China
e-mail: lingwh@mail.sysu.edu.cn

S. Fang
Department of Nutrition, The First Affiliated Hospital of Sun Yat-sen University, 58 Zhongshan 2nd Road, Guangzhou 510080, People's Republic of China

Introduction

Epidemiological data suggest that consumption of fruits and vegetables, in which many phytochemicals present, has been associated with a lower incidence of cardiovascular disease [1–3]. Cyanidin-3-glucoside (C3G), which belongs to the flavonoid class of molecules and is a member of the anthocyanin family, is widely distributed in plant-based daily diets and fruits with antiatherogenic effects [4]. For example, wine polyphenols were shown to modulate blood

pressure, promote vasodilatation, inhibit smooth muscle cell migration and proliferation, and inhibit platelet aggregation [5]. In addition, black currant anthocyanins activated the endothelial nitric oxide synthase (eNOS) in vitro in human endothelial cells [6]. Many studies have also shown that C3G has an important role in the prevention of oxidative damage caused by active oxygen radicals in living systems as a dietary antioxidant [7, 8]. However, the precise molecular mechanisms are still unknown.

The proliferation of VSMCs is an important event during the development of atherosclerosis. The cytokines, such as tumor necrosis factor-α (TNF-α), play an important role in inducing VSMCs migration and proliferation [9]. It has now become clear that ROS play a crucial role in VSMCs proliferation both directly and indirectly by inducing auto/paracrine growth mechanisms [10]. In atherosclerotic lesions, TNF-α is secreted by VSMCs in the neointimal after balloon-injury as well as by macrophages and is considered as a common stimulus for ROS production [11]. NADPH oxidases (Nox) are the most important ROS producing enzymes for vascular pathologies such as hypertension and atherosclerosis [12]. Nox enzymes differ in both tissue distributions and mechanisms by which their activities are regulated [13]. Nox1 may be the most likely source of immediate NADPH oxidase activity in response to TNF-α in non phagocytic cell types. The Nox1-based NADPH oxidase contains regulatory cytosolic components p47phox, Nox activator 1 (NoxA1), and Rac1, in addition to Nox1 and p22phox, which form a functional NADPH oxidase. The activation of Nox1 relies on regulatory proteins p67phox, or its homology NoxA1 [14–16]. In mouse VSMCs, however, NoxA1 was detected instead of p67phox. NoxA1 is expressed in the cytoplasm of VSMCs, and treatment of cells with epidermal growth factor induces the translocation of NoxA1 to the cell membrane, which then serves as activating regulatory components in Nox1-mediated superoxide generation [15].

It has been previously shown that TNF-α induced the activation of redox-sensitive protein kinases and transcription factors in VSMCs [17]. The activation of Janus kinase/signal transducers and activators of transcription (JAK/STAT) pathway, one of the most important redox-sensitive signaling, is an essential intracellular mechanism of TNF-α and other stimulis that regulates gene expression and cellular activation, proliferation, and differentiation [18]. Inhibition of JAK/STAT pathway reduced atherogenesis [19]. However, the effects of C3G on the regulation of this pathway during oxidative stress-induced vascular damage are not clearly elucidated. Therefore, the present study was designed to investigate the effects of C3G on cell proliferation, oxidative stress, NoxA1 expression and the JAK/STAT3 signaling pathway in TNF-α-treated VSMCs.

Materials and methods

Materials

C3G was purchased from Polyphenol AS (Sandnes, Norway). Antibodies used were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA) unless otherwise noted. All other reagents and kits were obtained from Sigma-Aldrich (St. Louis, MO) and Invitrogen (Invitrogen, Carlsbad, Calif) unless otherwise noted.

Cell culture

VSMCs derived from C57BL/6J mouse aorta were obtained from the American Type Culture Collection (ATCC, Rockville, MD, Cat#CRL1476) and cultured routinely in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), penicillin (50 U/ml), and streptomycin (50 μg/ml) at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. The cells grew to 80% confluence before overnight serum starvation and were treated with vehicle or C3G diluted in 0.1% DMSO and stimulated with TNF-α.

Assessment of cell viability

Cell viability was determined by the trypan blue exclusion assay. Briefly, cells were treated with different concentration of C3G and incubated in a humidified atmosphere of 5% CO_2 in air at 37°C in DMEM for 18 h. After cell detachment using trypsin–EDTA, an equal volume of 0.4% Trypan Blue Stain (Gibco BRL, Burlington, Ontario, Canada) was added to the cell suspension and the proportion of viable cells was evaluated under the field microscope. About 100 cells were counted and cells stained dark blue were not considered viable.

Cell proliferation assay

The cell proliferation reagent Cell Counting Kit-8 (CCK-8) (Roche, Germany) was used for the quantitative determination of cellular proliferation as previously described [20]. In brief, the cells (5×10^3 cells/well) were cultivated for 24 h in quadruplicate into 96-well microliter plates and starved for another 18 h in a humidified 5% CO_2 incubator at 37°C, then treated with different concentration of C3G. After incubation for 24 h, the cells were incubated with TNF-α (100 ng/ml) for 2 additional hours. Then, the CCK-8 labeling mixture (10 μl per well) was added for another 2 h. The absorbance of the samples, against a background control (medium alone) as a blank, was measured at 450 nm using a microliter plate (ELISA) reader (Molecular Devices, Sunnyvale CA).

Measurement of reactive oxygen species

Superoxide levels in mouse aortic VSMCs were measured by staining with dihydroethidium (DHE; Invitrogen). The cells were quiesced for 24 h and treated with TNF-α (100 ng/ml) for 30 min after the preincubation for 24 h with 50 μM C3G or 100 μM apocynin, an inhibitor of the NADPH oxidase. Then, the cells were washed with Hank's balanced salt solution (HBSS), and incubated with 10 μM DHE at 37°C for 15 min. The DHE fluorescence was obtained at 590 nm in several visual fields using Leica DMIRB fluorescent microscope.

Real-time polymerase chain reaction

The first-strand cDNA synthesis was carried out from total RNA isolated from VSMCs with Super Script II reverse transcriptase (Invitrogen, Carlsbad, Calif) according to the manufacturer's instructions. Real-time RT PCR with SYBR Green PCR Master Mix (Applied Biosystems, USA) was performed using ABI Prism 7500 Sequence Detector (Applied Biosystems). Primers against mouse NoxA1 (forward primer, 5′-AGATACGGGACTGGCACCG-3′; reverse primer, 5′-CATCCTAGCCAGCGGCTCTC-3′) were designed using the sequence information of the NCBI database. The PCR conditions were as follows: initial denaturation: 95°C, 7 min; 40 cycles of denaturation (95°C, 30 s), annealing (60°C, 30 s), and elongation (72°C, 60 s). The fluorescent signals were collected during the extension phase, Ct values of the sample were calculated, and the NoxA1 mRNA levels were analyzed by $2^{-\Delta\Delta Ct}$ method.

SDS-PAGE and western blots

Cellular protein was extracted with 1$\times$ cell lysis buffer. The protein concentration was determined using the BCA™ assay kit from Thermo Fisher Scientific Inc. (Huntsville, AL) according to the user manual. Protein (50 μg) from each sample was separated by 10% SDS-PAGE and electrotransferred to Polyvinylidene Fluoride (PVDF) membranes. The membranes were blocked with 5% BSA for 1 h at room temperature and incubated overnight at 4°C using the following 1:1,000 primary antibodies: rabbit anti NoxA1 (Abcam, USA), rabbit anti β-actin, rabbit anti phosphor-STAT3, and mouse anti total STAT3, followed by 1:2,000 dilution of goat anti-rabbit or goat anti-mouse horseradish peroxidase-labeled antibody. The bands were visualized using the ECL system, and the band density was determined by Image J software (NIH, USA).

Statistical analysis

All of the experiments were repeated at least three times. Data are expressed as means $\pm$ SE. The significance of differences was determined by One-way ANOVA using SPSS13.0 software (SPSS, Chicago, IL). A value of $P < 0.05$ was considered to be statistically significant.

Results

C3G inhibits TNF-α-induced VSMCs proliferation

TNF-α (100 ng/ml) markedly stimulated the proliferation of VSMCs, as measured by CCK-8 assay. The proliferation was significantly inhibited by C3G in a dose-dependent manner while 50 μM C3G decreased the CCK-8 signal to the basal level (Fig. 1a). Accordingly, 50 μM was chosen as the proper dose of C3G for the following experiments. C3G alone or vehicle (0.1% DMSO) had no effect on basal levels of CCK-8 signals. Moreover, TNF-α-stimulated VSMCs proliferation was reduced by pretreatment with C3G (50 μM) in a time-dependent manner (Fig. 1b).

C3G attenuates TNF-α-induced ROS production

To further explore the mechanism of inhibitory effect of C3G on TNF-α-induced proliferation, we examined the ROS generation and investigated whether C3G can affect the production of O_2^- in VSMCs. Dihydroethidium (DHE), which reacts with O_2^- and produces ethidium, was used to measure ROS production. The results (Fig. 2) showed that treatment with TNF-α (100 ng/ml) for 30 min significantly increased ROS production ($P < 0.01$). TNF-α-induced ROS generation was largely inhibited by the pretreatment with C3G (50 μM, 24 h). Preincubation with apocynin (100 μM, 24 h), an inhibitor of the NADPH oxidase, also significantly reduced TNF-α-induced ROS generation ($P < 0.05$) and inhibited TNF-α-induced VSMCs proliferation. Interestingly, combination of C3G and apocynin suppressed ROS production more strikingly than C3G or apocynin alone (Fig. 2c).

C3G suppresses NoxA1 expression

It has been reported that ROS derived from vascular NADPH oxidases serve as second messengers to activate multiple intracellular proteins that play an important role in the physiology and pathophysiology of VSMCs [21]. Since C3G decreased TNF-α-induced ROS generation, we then focus on whether C3G can suppress the NoxA1 expression in VSMCs. As shown in Fig. 3, TNF-α (100 ng/ml) caused a marked increase of the NoxA1 expression at mRNA level (3.14 $\pm$ 0.24-fold increase; $P < 0.05$) and protein level (1.85 $\pm$ 0.21-fold increase) compared with the basal level. However, C3G failed to reduce the expression of NoxA1 to the basal level. Expectedly, both the mRNA and protein levels of NoxA1 expression decreased in the presence of
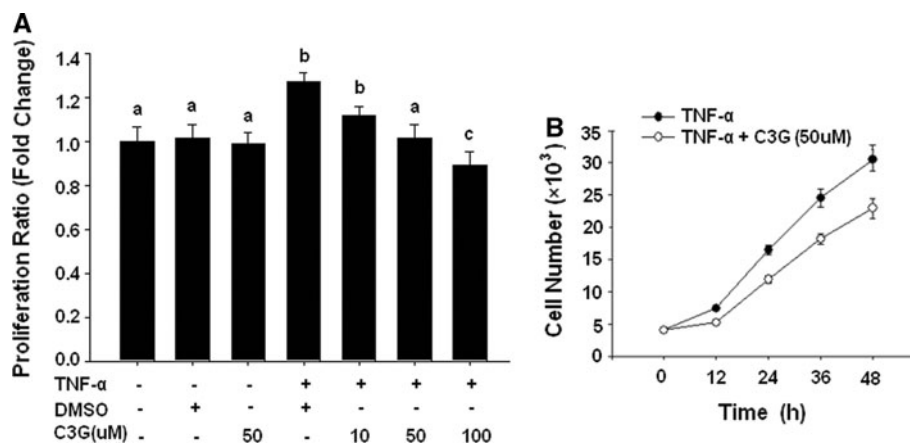
Fig. 1 C3G inhibits TNF-α-stimulated VSMCs proliferation. **a** VSMCs proliferation was measured by CCK-8 assay in response to C3G (10, 50, 100 μM) for 24 h followed by treatment with TNF-α (100 ng/ml) for 2 h. C3G (50 μM) alone or vehicle (0.1% DMSO) had no effect on basal levels of CCK-8 signals. **b** VSMCs were

incubated with 50 μM C3G at the indicated time periods; followed by treatment with TNF-α (100 ng/ml) for 2 h and CCK-8 signals were measured; Each point represents the means ± SE of three experiments. *a, b, c* Within each graph, means without a common letter differ, $P < 0.05$
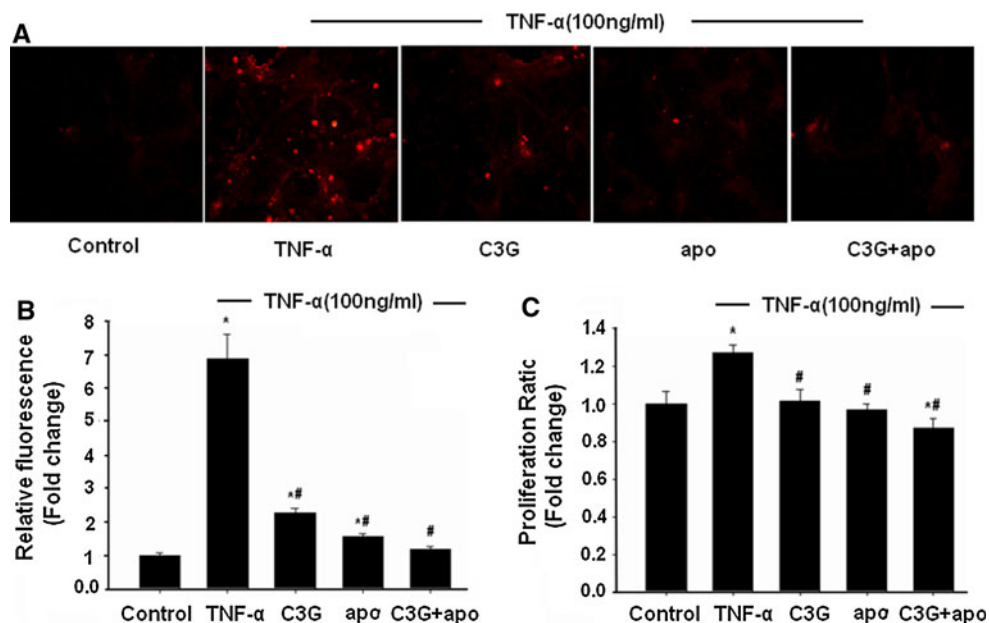

Fig. 2 C3G attenuates TNF-α-induced ROS production. **a** Cells were treated with C3G (50 μM), apocynin (100 μM), or the combination of C3G (50 μM) and apocynin (100 μM) for 24 h and then incubated with TNF-α (100 ng/ml) for 30 min. ROS production of cells was detected by DHE staining. Results are typical of representative staining. **b** Digital scans of DHE-stained cells were quantified using

NIH Image software. Results shown are means ± SE. **c** C3G (50 μM), apocynin (100 μM), or the combination of C3G and apocynin suppressed TNF-α-induced cell proliferation. Values are means ± SE of three independent experiments. *$P < 0.05$ compared with control; #$P < 0.05$ compared with TNF-α-stimulated VSMCs

apocynin and the combination of C3G and apocynin significantly reduced TNF-α-induced NoxA1 expression compared with the basal level.

ROS-stimulated activation of STAT3 is involved in TNF-α-induced cell proliferation

Oxidative stress has been suggested to be an important regulator in TNF-α-induced redox-sensitive protein kinases

activation, which is in turn controlled by antioxidant availability in the cellular milieu [22]. To investigate whether the JAK/STAT pathway, one of the most important redox-senstive signaling pathway, is involved in TNF-α-induced proliferation, VSMCs were pretreated with AG490 (20 μM), the JAK-2 protein tyrosine kinase-specific inhibitor [23], for 24 h and then co-treated with TNF-α for another 2 h. As shown in Fig. 4a, a significant decrease of cell proliferation was observed in VSMCs
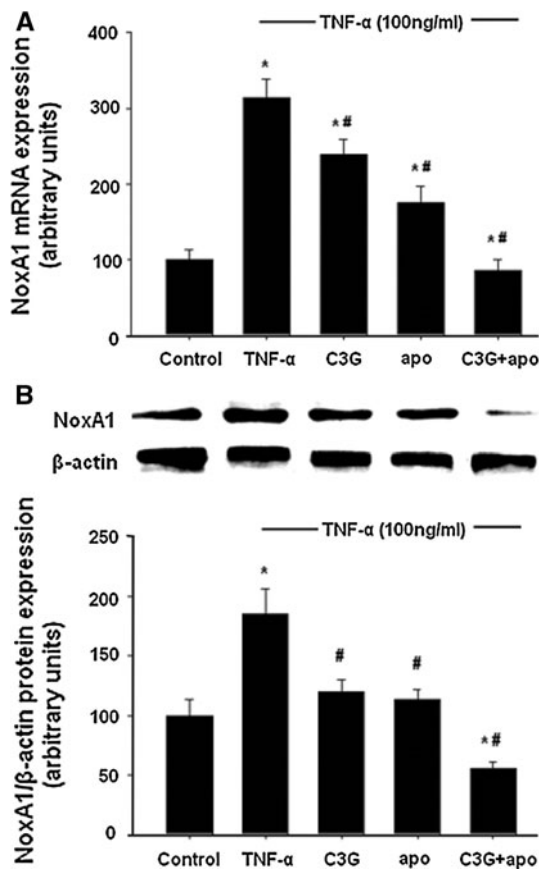
Fig. 3 Effect of C3G on TNF-α-induced NoxA1 expression in VSMCs. After being incubated with C3G (50 μM), apocynin (100 μM), or the combination of C3G and apocynin for 24 h and then with TNF-α (100 ng/ml) simulation for 4 h, the total RNA was extracted and the NoxA1 mRNA expression was measured by Real-time polymerase chain reaction. **b** Similarly, the cell lysates were collected and the NoxA1 expression was analyzed by Western blots. Values are means ± SE of three independent experiments. *$P < 0.05$ compared with control; #$P < 0.05$ compared with TNF-α-stimulated VSMCs

exposed to AG490. Furthermore, pretreatment with the ROS scavenger catalase (2,000 U/ml) remarkably inhibited the TNF-α-induced proliferation. We further examined the effect of ROS overproduction increased by TNF-α on the regulation of STAT3 signaling. As shown in Fig. 4b, administration of catalase significantly prevented the TNF-α-induced phosphorylation of STAT3.

C3G suppresses TNF-α-induced STAT3 activation

Next, we determined the effects of C3G on STAT3 activation. After being incubated with C3G for 24 h, cell lysates were prepared for western blots. As shown in Fig. 5, C3G significantly suppressed TNF-α-induced phosphorylation of STAT3 while the total level of STAT3 remained unchanged. To determine the effect of NoxA1 on STAT3 activation, cells were co-treated with C3G and

apocynin. The combination of C3G and apocynin significantly suppressed phosphorylation of STAT3 compared with C3G or apocynin treated alone.

Discussion

The proliferation of VSMCs is a critical event contributing to the pathogenesis of atherosclerosis. It has become clear that the overproduction of ROS is implicated in the processes associated to vascular plaque formation such as VSMCs proliferation [24]. Cytokines, such as TNF-α, stimulate ROS generation, including superoxide and hydrogen peroxide, which serve as signals facilitating the redox-sensitive protein kinases (JAK/STAT and others) activation and mediating VSMCs injury [25]. NoxA1 activates Nox1 expression, which is the most important of the super oxide-(O_2^-) producing enzyme. C3G is endowed with antioxidant properties and we speculated that C3G may act as a modulator of gene regulation and signal-transduction pathways associated with NoxA1-derived ROS production. The present study showed that C3G inhibits TNF-α-induced VSMCs proliferation through diminishing the upregulated NoxA1-derived ROS production, in which the STAT3 signaling is involved.

Previous study demonstrated that C3G protected against TNF-α-induced endothelial dysfunction as a potent antioxidant and free radical scavenger [26]. Our laboratory has also shown that C3G reduced the monocyte infiltration in mice [27]. Although previous study demonstrated that red wine polyphenols, of which anthocyanin presents the largest group of pigments, showed antiproliferative effects on VSMCs [28], the precise biochemical effects of C3G on VSMCs remain unknown. In the present study, C3G significantly inhibited TNF-α-induced VSMCs proliferation both in a dose-dependent and in a time-dependent manner (Fig. 1). This antiproliferative effect is because of the attenuated ROS generation by the addition of C3G, which is consistent with a recent study that delphinidin, another dietary anthocyanidin, directly scavenged ROS and prevented the PDGF (AB)-induced formation of ROS in VSMCs [29].

NADPH oxidases represent a family of enzymes that catalyze the regulated formation of ROS [21]. In addition to basal ROS production, NADPH oxidase activity in cardiovascular cells is acutely up-regulated by a large number of stimuli such as Ang II, VEGF, and TNF-α [30, 31]. NoxA1 is a central component of the smooth muscle NADPH oxidase in mice [32]. It has been demonstrated that treatment with 20 ng/ml TNF-α for 4 h significantly increased NoxA1 expression [33]. Recently, it has been reported that an antisense plasmid of NoxA1 attenuated the ROS formation and adenovirus-mediated over expression of NoxA1 in guide
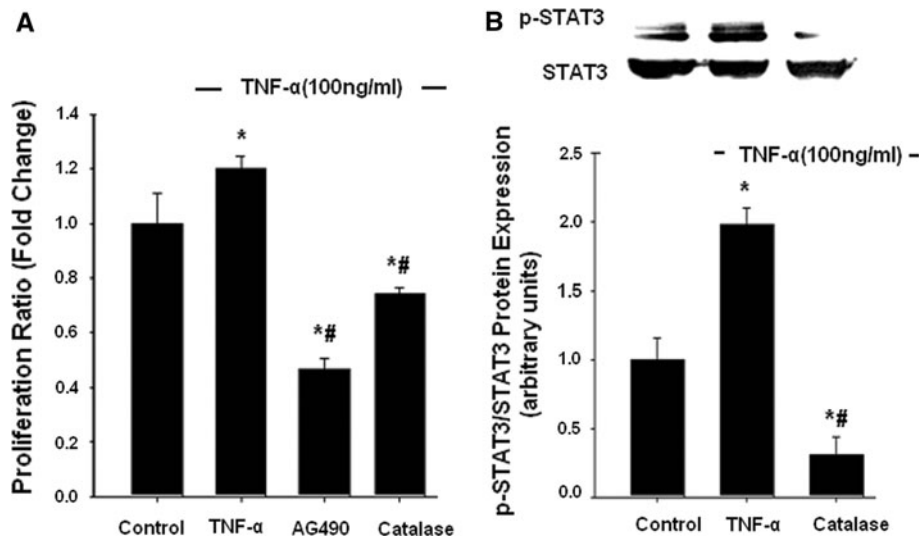
Fig. 4 ROS-stimulated activation of STAT3 is involved in TNF-α-induced proliferation. **a** Cells were treated with AG490 (20 μM), catalase (2,000 U/ml) for 24 h and then co-incubated with TNF-α (100 ng/ml) for another 2 h, and the CCK-8 signals were measured. **b** Cells were treated with catalase (2,000 U/ml) for 24 h and then co-incubated with TNF-α (100 ng/ml) for 30 min. The proteins isolated from cells were prepared for Western blots using anti-phospho-STAT3 and total STAT3 antibodies. The bottom panel is the densitometric analysis of the two proteins. Results are presented as the ratio of phospho-STAT3 to total STAT3 (means ± SE; $n = 3$). *$P < 0.05$ compared with control; #$P < 0.05$ compared with TNF-α-stimulated VSMCs

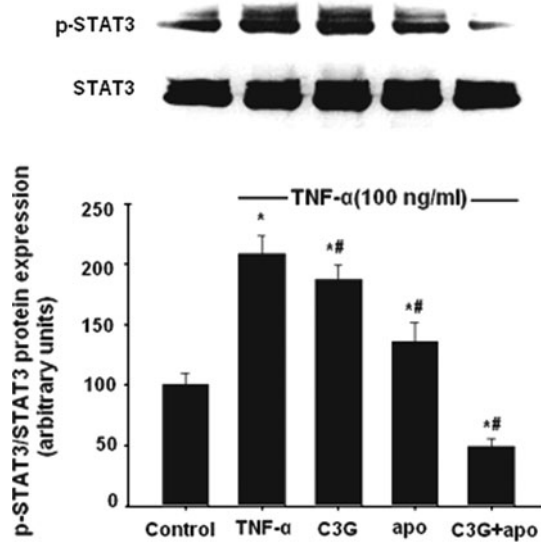
Fig. 5 C3G suppresses TNF-α-induced activation of STAT3. Cells were treated with C3G (50 μM), apocynin (100 μM), or the combination of C3G (50 μM) and apocynin (100 μM) for 24 h and then incubated with TNF-α (100 ng/ml) for 30 min. The cell lysates were prepared for Western blots using anti-phospho-STAT3 and total STAT3 antibodies. The bottom panel is the densitometric analysis of STAT3 phosphorylation and total STAT3 protein. Results are presented as the ratio of phospho-STAT3 to total STAT3 (means ± SE; $n = 3$). *$P < 0.05$ compared with control; #$P < 0.05$ compared with TNF-α-stimulated VSMCs

wire-injured mouse carotid arteries significantly increased superoxide production in medial VSMCs and enhanced neointimal hyperplasia [32]. Consistent with the previous study, the results in our present study suggested that C3G inhibits TNF-α-induced NoxA1 expression both at mRNA and protein levels (Fig. 3). Interestingly, the combination of C3G with a relatively specific inhibitor of NADPH, apocynin, reduced TNF-α-induced NoxA1 expression, ROS generation and cell proliferation more significantly than in cells treated with C3G or apocynin alone. It would be likely that the combined application of C3G and apocynin has a synergistic inhibitory effect on TNF-α-induced NoxA1 expression and subsequent ROS overproduction. These results demonstrate that the suppression of NoxA1-mediated ROS generation by C3G plays an important role in inhibiting TNF-α-induced VSMCs proliferation.

Activation of Janus tyrosine kinase/signal transducers and activators of transcription (JAK/STAT) signaling pathway is an essential pathogenic mechanism leading to VSMCs hyperplasia and activated STAT3 was detected in atherosclerotic lesions [34]. In this regard, we hypothesized that STAT3 signaling is a potential signal-transduction mediator that regulates VSMCs proliferation. As expected, the inhibitor of JAK/STAT cascade, AG490, significantly inhibited TNF-α-induced proliferation. Moreover, the results that eliminated ROS by catalase inhibited TNF-α-induced STAT3 activation and cell proliferation suggest that ROS are required for the activation of STAT3. C3G significantly abolished TNF-α-induced STAT3 activation, which indicated that C3G plays a negative regulatory role in the STAT3 signaling. A recent study suggested that suppression of NoxA1 expression decreases thrombin-induced activation of JAK/STAT signaling [33]. Our result

that C3G diminished the activation of STAT3 more strikingly when co-pretreated with apocynin (Fig. 5) also indicated that C3G exerts its antiproliferative effect on TNF-α-induced VSMCs through inhibiting STAT3 activation by attenuating NoxA1-derived ROS overproduction.

In conclusion, the present findings demonstrate that C3G inhibits TNF-α-induced VSMCs proliferation through down regulating of the NoxA1-ROS-STAT3 pathway. It is noteworthy that NoxA1 is a potential target for modulation of vascular ROS in atherosclerotic arteries. Hence, further understanding of the antioxidative property of C3G in NoxA1-containing NADPH oxidase and the detailed mechanisms should provide the basis for devising novel therapies in cardiovascular disorders.

References

1. Craig WJ (2010) Nutrition concerns and health effects of vegetarian diets. Nutr Clin Pract 25:613–620
2. Ruel G, Couillard C (2007) Evidences of the cardioprotective potential of fruits: the case of cranberries. Mol Nutr Food Res 51:692–701
3. Arai Y, Watanabe S, Kimira M, Shimoi K, Mochizuki R, Kinae N (2000) Dietary intakes of flavonols, flavones and isoflavones by Japanese women and the inverse correlation between quercetin intake and plasma LDL cholesterol concentration. J Nutr 130:2243–2250
4. Williams CA, Grayer RJ (2004) Anthocyanins and other flavonoids. Nat Prod Rep 21:539–573
5. Dell'Agli M, Busciala A, Bosisio E (2004) Vascular effects of wine polyphenols. Cardiovasc Res 63:593–602
6. Edirisinghe I, Banaszewski K, Cappozzo J, McCarthy D, Burton-Freeman BM (2011) Effect of black currant anthocyanins on the activation of endothelial nitric oxide synthase (eNOS) in vitro in human endothelial cells. J Agric Food Chem 59:8616–8624
7. Galvano F, La Fauci L, Vitaglione P, Fogliano V, Vanella L, Felgines C (2007) Bioavailability, antioxidant and biological properties of the natural free-radical scavengers cyanidin and related glycosides. Ann Ist Super Sanita 43:382–393
8. Stintzing FC, Stintzing AS, Carle R, Frei B, Wrolstad RE (2002) Color and antioxidant properties of cyanidin-based anthocyanin pigments. J Agric Food Chem 50:6172–6181
9. Kim H, Lee MJ, Kim JE, Park SD, Moon HI, Park WH (2010) Genistein suppresses tumor necrosis factor-alpha-induced proliferation via the apoptotic signaling pathway in human aortic smooth muscle cells. J Agric Food Chem 58:2015–2019
10. Taniyama Y, Griendling KK (2003) Reactive oxygen species in the vasculature molecular and cellular mechanisms. Hypertension 42:1075–1081
11. Jovinge S, Hultgardh-Nilsson A, Regnstrom J, Nilsson J (1997) Tumor necrosis factor-alpha activates smooth muscle cell migration in culture and is expressed in the balloon-injured rat aorta. Arterioscler Thromb Vasc Biol 17:490–497
12. Cai H, Griendling KK, Harrison DG (2003) The vascular NAD(P)H oxidases as therapeutic targets in cardiovascular diseases. Trends Pharmacol Sci 24:471–478
13. Krause KH (2004) Tissue distribution and putative physiological function of NOX family NADPH oxidases. Jpn J Infect Dis 57:S28–S29
14. Geiszt M, Lekstrom K, Witta J, Leto TL (2003) Proteins homologous to p47phox and p67phox support superoxide production by NAD(P)H oxidase 1 in colon epithelial cells. J Biol Chem 278:20006–20012
15. Banfi B, Clark RA, Steger K, Krause KH (2003) Two novel proteins activate superoxide generation by the NADPH oxidase NOX1. J Biol Chem 278:3510–3513
16. Ueyama T, Geiszt M, Leto TL (2006) Involvement of Rac1 in activation of multicomponent Nox1- and Nox3-based NADPH oxidases. Mol Cell Biol 26:2160–2174
17. Goetze S, Kintscher U, Kaneshiro K, Meehan WP, Collins A, Fleck E, Hsueh WA, Law RE (2001) TNFalpha induces expression of transcription factors c-fos, Egr-1, and Ets-1 in vascular lesions through extracellular signal-regulated kinases 1/2. Atherosclerosis 159:93–101
18. Yoshimura A, Naka T, Kubo M (2007) SOCS proteins, cytokine signalling and immune regulation. Nat Rev Immunol 7:454–465
19. Manea A, Tanase LI, Raicu M, Simionescu M (2010) Jak/STAT signaling pathway regulates nox1 and nox4-based NADPH oxidase in human aortic smooth muscle cells. Arterioscler Thromb Vasc Biol 30:105–112
20. Neumann S, Huse K, Semrau R, Diegeler A, Gebhardt R, Buniatian GH, Scholz GH (2002) Aldosterone and D-glucose stimulate the proliferation of human cardiac myofibroblasts in vitro. Hypertension 39:756–760
21. Lee HS, Son SM, Kim YK, Hong KW, Kim CD (2003) NAD(P)H oxidase participates in the signaling events in high glucose-induced proliferation of vascular smooth muscle cells. Life Sci 72:2719–2730
22. Na HK, Surh YJ (2006) Transcriptional regulation via cysteine thiol modification: a novel molecular strategy for chemoprevention and cytoprotection. Mol Carcinog 45:368–380
23. Abe J, Berk BC (1999) Fyn and JAK2 mediate Ras activation by reactive oxygen species. J Biol Chem 274:21003–21010
24. Cai H, Harrison DG (2000) Endothelial dysfunction in cardiovascular diseases: the role of oxidant stress. Circ Res 87:840–844
25. Au-Yeung KK, Woo CW, Sung FL, Yip JC, Siow YL, OK (2004) Hyperhomocysteinemia activates nuclear factor-kappaB in endothelial cells via oxidative stress. Circ Res 94:28–36
26. Speciale A, Canali R, Chirafisi J, Saija A, Virgili F, Cimino F (2010) Cyanidin-3-O-glucoside protection against TNF-alpha-induced endothelial dysfunction: involvement of nuclear factor-kappaB signaling. J Agric Food Chem 58:12048–12054
27. Wang D, Zou T, Yang Y, Yan X, Ling WH (2011) Cyanidin-3-O-beta-glucoside with the aid of its metabolite protocatechuic acid, reduces monocyte infiltration in apolipoprotein E-deficient mice. Biochem Pharmacol 82:713–719
28. Iijima K, Yoshizumi M, Hashimoto M, Kim S, Eto M, Ako J, Liang YQ, Sudoh N, Hosoda K, Nakahara K, Toba K, Ouchi Y (2000) Red wine polyphenols inhibit proliferation of vascular smooth muscle cells and downregulate expression of cyclin A gene. Circulation 101:805–811
29. Oak MH, Bedoui JE, Madeira SV, Chalupsky K, Schini-Kerth VB (2006) Delphinidin and cyanidin inhibit PDGF(AB)-induced VEGF release in vascular smooth muscle cells by preventing activation of p38 MAPK and JNK. Br J Pharmacol 149:283–290
30. Vendrov AE, Madamanchi NR, Niu XL, Molnar KC, Runge M, Szyndralewiez C, Page P, Runge MS (2010) NADPH oxidases regulate CD44 and hyaluronic acid expression in thrombin-treated vascular smooth muscle cells and in atherosclerosis. J Biol Chem 285:26545–26557
31. Touyz RM, Cruzado M, Tabet F, Yao G, Salomon S, Schiffrin EL (2003) Redox-dependent MAP kinase signaling by Ang II in

vascular smooth muscle cells: role of receptor tyrosine kinase transactivation. Can J Physiol Pharmacol 81:159–167

32. Ambasta RK, Schreiber JG, Janiszewski M, Busse R, Brandes RP (2006) Noxa1 is a central component of the smooth muscle NADPH oxidase in mice. Free Radic Biol Med 41:193–201

33. Niu XL, Madamanchi NR, Vendrov AE, Tchivilev I, Rojas M, Madamanchi C, Brandes RP, Krause KH, Humphries J, Smith A, Burnand KG, Runge MS (2010) Nox activator 1: a potential target for modulation of vascular reactive oxygen species in atherosclerotic arteries. Circulation 121:549–559

34. Seki Y, Kai H, Shibata R, Nagata T, Yasukawa H, Yoshimura A, Imaizumi T (2000) Role of the JAK/STAT pathway in rat carotid artery remodeling after vascular injury. Circ Res 87:12–18