Sulfasalazine induces haem oxygenase-1 via ROS-dependent Nrf2 signalling, leading to control of neointimal hyperplasia

Ju-Young Kim†, Hyun-Jai Cho†, Jung-Ju Sir, Baek-Kyung Kim, Jin Hur, Seock-Won Youn, Han-Mo Yang, Soo-In Jun, Kyung-Woo Park, Seok-Jae Hwang, Yoo-Wook Kwon, Hae-Young Lee, Hyun-Jae Kang, Byung-Hee Oh, Young-Bae Park, and Hyo-Soo Kim*

1National Research Laboratory on Cardiovascular Stem Cell, Seoul National University, College of Medicine, Seoul, Korea; 2Cardiovascular Center, Department of Internal Medicine, Seoul National University Hospital, 28 Yangdong-dong, Chongno-gu, Seoul 110-744, Korea; and 3Innovative Research Institute for Cell Therapy, Seoul National University Hospital, Seoul, Korea

Received 6 December 2008; revised 3 February 2009; accepted 4 February 2009; online publish-ahead-of-print 21 February 2009

Aims Inflammation, and the subsequent proliferative activity of vascular smooth muscle cells (VSMCs), is one of the major pathophysiological mechanisms associated with neointimal hyperplasia following vascular injury. Although sulfasalazine (SSZ) has been used as an anti-inflammatory and immune-modulatory agent in various inflammatory diseases, its primary targets and therapeutic effects on vascular disease have not yet been determined. We investigated whether SSZ could suppress VSMC growth and prevent neointimal hyperplasia.

Methods and results SSZ was found to have pro-apoptotic and anti-proliferative activity in cultured VSMCs. Unexpectedly, these effects were not mediated by nuclear factor kappa B (NF-κB) inhibition, which has been suggested to be the anti-inflammatory mechanism associated with the effects of SSZ. Instead, cell-cycle arrest of the VSMCs was observed, which was mediated by induction of haem oxygenase-1 (HO-1) followed by an increased expression of p21waf1/Cip1. The underlying mechanism for SSZ-induced HO-1 expression was by reactive oxygen species (ROS)-dependent nuclear translocation and activation of nuclear factor erythroid-2-related factor 2 (Nrf2). In a rat carotid artery balloon injury model, administration of SSZ significantly suppressed neointimal growth. In a series of reverse experiments, inhibition of HO-1 by shRNA, ROS by N-acetylcysteine (NAC) or Nrf2 by dominant-negative Nrf2 abrogated the beneficial effects of SSZ.

Conclusion Our data demonstrate that SSZ inhibits VSMC proliferation in vitro and in vivo through a novel signalling pathway and may be a promising therapeutic option for the treatment of proliferative vascular disease.

Key words Sulfasalazine; Vascular injury; Reactive oxygen species; haem oxygenase-1; Nuclear factor erythroid-2-related factor 2

1. Introduction

Proliferative vascular disease such as post-angioplasty neointimal hyperplasia or restenosis has been a persistent problem in clinical practice, even in the drug-eluting stent (DES) era.1,2 The inflammatory and proliferative responses of vascular smooth muscle cells (VSMCs) following injury is one of the major pathophysiological mechanisms underlying vascular disease.3 Sulfasalazine (SSZ) is an anti-inflammatory and immune-modulatory agent used for chronic inflammatory diseases such as rheumatoid arthritis and inflammatory bowel disease.4,5 However, the underlying mechanisms of action of SSZ have remained a matter of debate, and its possible therapeutic effects on proliferative vascular disease remain to be determined.

Oxidative stress and the production of intracellular reactive oxygen species (ROS), such as superoxide (O2•−), hydrogen peroxide (H2O2), and hydroxyl radical (OH•), have been implicated in the pathogenesis of cardiovascular disease.6,7 Several key findings support the concept that ROS can stimulate VSMC proliferation, contributing to the pathogenesis of neointimal hyperplasia after arterial injury. However, recent studies have challenged the dogma that oxidants are detrimental. When the ROS level exceeds the defence capacity, it may induce damage.8 By contrast, low levels of ROS may stimulate defence networks and play an essential role in adaptation, leading to prevention of further damage.9 In this context, intracellular antioxidants are important for the regulation of cell adaptation in
response to oxidative stress, which stimulates key cellular processes such as proliferation and apoptosis. A number of antioxidant and detoxification genes including haem oxygenase-1 (HO-1) have the antioxidant response element (ARE) in their promoter region and are under the control of nuclear factor erythroid-2-related factor 2 (Nrf2).10,11 Recently, in neural cells, Nrf2 and endogenous antioxidant enzymes were reported to be activated by low dose 4-hydroxy-trans-2-nonenal (4-HNE).9,12 Prior studies showed that 4-HNE, in the VSMCs, was induced by SSZ,13,14 which inhib-
ited glutathione-S-transferases (GSTs), a major determinant of the intracellular concentration of 4-HNE.15,16 Thus, there appears to be a high probability that the activity of SSZ plays a role in the regulation of the antioxidant system in VSMCs.

In this study, we investigated whether SSZ could suppress VSMC growth in vitro and in vivo rat carotid artery injury model. To investigate the underlying mechanisms, we studied whether SSZ could activate the regulator of antioxidant genes and induce endogenous antioxidant enzymes in the VSMCs, to provide protection against oxidative stress, which would parallel the inflammatory and proliferative phases of vascular injury.

2. Methods
Expanded full methods and associated references are described in the Supplementary material.

2.1 Cell culture and HO-1 shRNA design
VSMCs were isolated from the thoracic aorta of Sprague–Dawley rats using a previously described method.17 To silence rat HO-1 expression, a target site of rat HO-1 mRNA was used to design CMV-HO-1-shRNA (see Supplementary material online, Figure S1).18

2.2 Viability, proliferation, apoptosis, and cell-cycle analysis
Cell viability after 48 h of SSZ treatment was evaluated using the Trypan Blue exclusion assay. Cell proliferation after 24 h of SSZ treatment was measured by the incorporation of bromodeoxyuridine. Apoptosis and the cell-cycle status after 18 and 48 h of SSZ treatment were evaluated by flow cytometry.

2.3 Reverse transcriptase–polymerase chain reaction (RT–PCR) and immunoblot
Total RNA was extracted from the VSMCs and PCR was performed using specific primers. Western blotting was performed as described previously.19

2.4 Electrophoretic mobility shift assay (EMSA) and chromatin immunoprecipitation assay (ChIP assay)
Nuclear extracts from the cells exposed to SSZ or in combination with TNF-α (10 ng/mL), for 40 min, were prepared. The EMSA for nuclear factor kappa B (NF-κB) and Nrf2 was performed as previously described.20 To evaluate whether Nrf2 binds to the ARE of the rat HO-1 promoter, a ChIP assay was carried out following the ChIP assay kit protocol (Upstate, MA, USA).

2.5 Detection of in situ reactive oxygen species
To measure the generation of ROS, in vitro, we added the fluorescent indicator dihydroethidium (DHE) to the media. Within the cells, the superoxide (O$_2^-$) oxidized the indicator, which resulted in fluorescent products that were trapped by intercalation into the DNA.21

2.6 Carotid artery injury model, drug treatment, and adenoviral vector transfection
All animal experiments were performed after receiving approval from the Institutional Animal Care and Use Committee (IACUC) of Clinical Research Institute at Seoul National University Hospital and all protocols complied with the US National Institutes of Health ‘Guide for the Care and Use of Laboratory Animals’ (NIH publication No. 85–23, revised 1996). Sprague–Dawley rats received SSZ intraperitoneally. Right common carotid arteries were injured by an intraluminal 2F arterial catheter. For the viral and plasmid transfection, Adv-GFP or Adv-Nrf2DN and control shRNA plasmid or HO-1 shRNA plasmid was instilled for 30 min as described previously.22 Three and 14 days after injury, the tissues were harvested, and morphometric analysis and immunohistochemistry were performed.

2.7 Statistical analyses
All data were expressed as mean ± SE. Comparisons of the means were performed using the Student’s t-test for each paired experiments and ANOVA followed by Bonferroni corrections for multiple comparisons. A P < 0.05 was considered statistically significant.

3. Results

3.1 SSZ modulates apoptosis and proliferation of the VSMCs
In order to examine the effects of SSZ on the VSMCs in the context of vascular injury, first, we performed an immunohistochemical analysis of the injured arterial tissue and found that TNF-α was significantly increased in the medial VSMC layer (see Supplementary material online, Figure S2), consistent with the previous report.23 We examined the effects of SSZ in vitro in the presence of TNF-α. The cell viability test showed that after 48 h of exposure to SSZ treatment, SSZ decreased the number of viable VSMCs in a dose-dependent manner, and this was accentuated by the presence of TNF-α (Figure 1A). The apoptosis analysis showed that SSZ increased the population of apoptotic cells (sub-G1) at concentrations of 100 µM or higher, and this was also exaggerated by the presence of TNF-α (Figure 1B). In addition to the pro-apoptotic activity, SSZ significantly inhibited the induction of DNA synthesis in the VSMCs stimulated by platelet-derived growth factor (PDGF)-BB in a dose-dependent manner, whereas it did not at basal conditions without proliferative stimuli. A statistically significant anti-proliferative effect was observed at concentrations of 100 µM or higher (P < 0.05) (Figure 1C). To clarify the cellular mechanisms associated with the anti-proliferative effects on the VSMCs, cell-cycle profiles were examined. The flow cytometric analyses of the DNA content demonstrated that SSZ reduced the number of cells at the S-phase, which was increased by PDGF-BB (Figure 1D) in a dose-dependent manner. The reduction of the number of cells at the S-phase was accompanied by an increase in the fraction of G1 cells, suggesting that SSZ suppressed cell-cycle progression.

3.2 SSZ inhibits TNF-α-induced NF-κB activation only at extreme concentrations
The results of previous reports suggested that SSZ has a potent inhibitory effect on NF-κB activation.24,25 Therefore, we examined the inhibitory effects of SSZ on the NF-κB binding activity in nuclear preparation by EMSA to determine whether the anti-proliferative effects of SSZ observed above
Figure 1 The effects of SSZ on apoptosis and proliferation of VSMCs. Cells were exposed to different concentrations of SSZ in the presence or absence of TNF-α or PDGF-BB. (A) In the absence of TNF-α, SSZ reduced the viability of VSMCs at high concentrations (200 μM), whereas in the presence of TNF-α, SSZ even at relatively low concentrations (100 μM) reduced the viability (n = 3). *P < 0.05 compared with vehicle. (B) The apoptotic cell fraction significantly increased at concentrations of 100 μM or higher, which was accentuated by the presence of TNF-α. (C) SSZ decreased PDGF-induced VSMC proliferation in a dose-dependent manner (n = 3). *P < 0.05 compared with vehicle. (D) SSZ blocked PDGF-induced cell-cycle progression at the S-phase in a dose-dependent manner. *P < 0.05 compared with vehicle with PDGF.
were mediated by the inhibition of NF-kB activation. TNF-α increased NF-kB DNA binding activity in the nuclear extracts, which were significantly inhibited by SSZ (Figure 2). However, with regard to the effective concentration of SSZ, the inhibition of NF-kB binding to DNA was negligible when the TNF-α-stimulated cells were exposed to SSZ at concentrations up to 100 μM. The inhibition of NF-kB DNA binding activity was only evident at high concentrations, at least 200 μM or higher. The serum SSZ concentration, after a tolerable dose of 3–6 g per day, has been reported to be <100 μM.26 This suggests that the anti-proliferative effects of SSZ on the VSMCs, within the in vivo therapeutic window, might be mediated through a different mechanism that is unrelated to the NF-kB axis. Therefore, in further in vitro experiments, we selected 50–100 μM as the concentration of SSZ.

3.3 Inhibition of VSMC proliferation by SSZ is mediated through HO-1 and p21

First, we tested whether SSZ could activate genes associated with antioxidant enzymes such as HO-1, NAD(P)H:quinone oxidoreductase (NQO)-1 and GST-4. The VSMCs were treated with SSZ at 50 μM for 6 h. SSZ markedly induced HO-1 mRNA, whereas GST-4 and NQO-1 were mildly induced (Figure 3A and see Supplementary material online, Figure S3A). The real-time PCR results showed that the induction of HO-1 mRNA by SSZ was evident as early as 6 h (Figure 3B). HO-1, a stress–response protein, is known to confer protection against stress and has anti-proliferative effects.27 We examined the dose–response of HO-1 induction. SSZ induced HO-1 expression at a low concentration and in a concentration-dependent manner (Figure 3C and see Supplementary material online, Figure S3B). We also investigated the temporal response of HO-1 to the VSMCs treated with SSZ. The induction of HO-1 by SSZ was evident as early as 6 h, and augmentation lasted for at least 24 h (Figure 3D and see Supplementary material online, Figure S3C). This was also evident on the immunofluorescent staining of HO-1 in the VSMCs (Figure 3E).

We next investigated whether HO-1 mediates the anti-proliferative effects of SSZ on the VSMCs. We added the shRNA specific to HO-1 to confirm that the anti-proliferative effects were mediated through HO-1. The inhibitory effect of SSZ, on PDGF-BB-induced cell proliferation, was significantly reversed by HO-1 silencing by the HO-1 shRNA plasmid (Figure 3F). In addition, the flow cytometry demonstrated that cell-cycle arrest in the G1/G2 transition phase, by SSZ, was also abrogated by HO-1 silencing (Figure 3G). The anti-proliferative effect of HO-1 is known to be mainly due to the downstream modulation of cell-cycle check point proteins such as cyclin-dependent kinase inhibitors p21\(^{\text{waf1/Cip1}}\) and p27\(^{\text{kip1}}\). Therefore, we investigated the effects of SSZ on the protein expression of p21\(^{\text{waf1/Cip1}}\) and p27\(^{\text{kip1}}\). The expression of p21\(^{\text{waf1/Cip1}}\), but not p27\(^{\text{kip1}}\), was significantly increased in the SSZ-treated VSMCs (Figure 3H). These effects were also significantly attenuated.
by HO-1 silencing. Additionally, we performed an experiment using a HO-1 chemical inhibitor, zinc protoporphyrin IX (ZnPP), to demonstrate HO-1-mediated anti-proliferative and pro-apoptotic effects of SSZ (see Supplementary material online, Figure S6).

Taken together, SSZ-induced HO-1 expression resulted in p21 up-regulation and subsequent cell-cycle arrest.

3.4 HO-1 induction by SSZ is mediated by ROS-dependent ARE/Nrf2 signalling

Since HO-1 is known to respond to a variety of oxidative stresses, we investigated whether the anti-proliferative effects of SSZ were dependent on intracellular ROS generation. Intracellular ROS generation was determined by fluorescence microscopy and fluorometer using the fluorescent
SSZ significantly increased ROS generation in the VSMCs (Figure 4A and B). One hour after stimulation, the cells were lysed and fluorescence intensities determined. SSZ mildly but significantly increased ROS production, which was abrogated by NAC (n = 3, each). *P < 0.05. (C) SSZ significantly increased Nrf2 in the nuclear fraction compared with the cytosolic fraction, which was reversed by NAC treatment. Lamin-C and β-actin were used as internal controls. (D) Immunofluorescent staining showed nuclear translocation of Nrf2 (red) with SSZ treatment. DAPI (blue) for nuclei. (n = 3, each). *P < 0.05 compared with vehicle, †P < 0.05 compared with SSZ-treated group. (E) EMSA showed that SSZ significantly increased Nrf2 binding to ARE in the promoter region of the HO-1 gene (n = 3, each). *P < 0.05 compared with vehicle. (F) ChiP assay revealed that SSZ promoted Nrf2 binding to the promoter region of the HO-1 gene. (G) RT-PCR (upper panels) and immunoblotting (lower panels) confirmed that SSZ induced HO-1 expression, which was augmented by Nrf2 up-regulation (by adenoviral Nrf2 vector) and reversed by Nrf2 down-regulation (by adenoviral dominant-negative Nrf2 vector).

Figure 4 Induction of HO-1 by SSZ is mediated by ROS-dependent nuclear translocation of Nrf2. (A) The ROS synthesis in VSMCs was determined by the measurement of DHE sensitive fluorescence. Representative images showed that SSZ treatment increased ROS production. NAC inhibited SSZ-induced ROS production. (B) One hour after stimulation, the cells were lysed and fluorescence intensities determined. SSZ mildly but significantly increased ROS production, which was abrogated by NAC (n = 3, each). *P < 0.05. (C) SSZ significantly increased Nrf2 in the nuclear fraction compared with the cytosolic fraction, which was reversed by NAC treatment. Lamin-C and β-actin were used as internal controls. (D) Immunofluorescent staining showed nuclear translocation of Nrf2 (red) with SSZ treatment. DAPI (blue) for nuclei. (n = 3, each). *P < 0.05 compared with vehicle, †P < 0.05 compared with SSZ-treated group. (E) EMSA showed that SSZ significantly increased Nrf2 binding to ARE in the promoter region of the HO-1 gene (n = 3, each). *P < 0.05 compared with vehicle. (F) ChiP assay revealed that SSZ promoted Nrf2 binding to the promoter region of the HO-1 gene. (G) RT-PCR (upper panels) and immunoblotting (lower panels) confirmed that SSZ induced HO-1 expression, which was augmented by Nrf2 up-regulation (by adenoviral Nrf2 vector) and reversed by Nrf2 down-regulation (by adenoviral dominant-negative Nrf2 vector).

indicator DHE as a probe to detect ROS in the VSMCs. SSZ significantly increased ROS generation in the VSMCs (Figure 4A and B). SSZ-induced ROS production was strongly attenuated by pre-incubation of the cells with the antioxidant, N-acetylcysteine (NAC). Upon exposure to ROS, Nrf2 in the cytoplasm was reported to be dissociated, released, and then translocated to the nucleus.30 In the next experiment, to determine the effects of SSZ on Nrf2, we found that it increased the intracellular protein levels of Nrf2 (Figure 4C), especially in the nuclear rather than in the cytosolic fraction. Nuclear translocation was blocked by the antioxidant, NAC, suggesting that the effects were mediated by ROS. The increased Nrf2 expression, induced by SSZ, was confirmed by immunofluorescent staining, which showed that Nrf2 had a nuclear localization after 3–6 h of SSZ treatment (Figure 4D).

To further assess whether SSZ increases the binding activity of Nrf2 to the ARE, a nuclear extract isolated from SSZ-treated
VSMCs was probed with a radiolabelled ARE sequence. The EMSA revealed that SSZ increased Nrf2 binding activity to ARE (Figure 4E). To confirm the binding of Nrf2 to the ARE of HO-1, we performed ChIP assays (Figure 4F). SSZ treatment increased the PCR products for ARE of the HO-1 gene using nuclear extracts immunoprecipitated with antibodies to Nrf2. These findings suggest that the recruitment and binding of Nrf2, to the promoter region of HO-1 gene, were significantly increased after SSZ treatment.

We then performed experiments to demonstrate that Nrf2 is required for HO-1 induction by SSZ. We tested whether Nrf2 up- or down-regulation could augment or reverse HO-1 induction by SSZ. Transfection with Ad-Nrf2 significantly augmented SSZ-induced HO-1 expression at both the mRNA and protein levels (Figure 4G). By contrast, transfection with Ad-Nrf2DN reversed the SSZ-induced HO-1 expression.

Taken together, these findings suggest that the underlying mechanism of HO-1 induction by SSZ was found to be mediated by ROS production, which activated the nuclear translocation and binding of Nrf2 to ARE of the HO-1 promoter region, leading to increased HO-1 expression.

3.5 SSZ suppresses neointimal hyperplasia after arterial injury

On the basis of the in vitro data that SSZ suppressed VSMC proliferation, we investigated the anti-proliferative effects of SSZ in vivo using a rat carotid artery balloon injury model. Administration of SSZ markedly reduced the development of neointimal hyperplasia 2 weeks after balloon injury (Figure 5A). Quantitative morphometric analysis showed that SSZ significantly reduced neointimal hyperplasia and increased the lumen area (Figure 5B). To examine the effects of SSZ on VSMC proliferation in vivo, we performed immunohistochemistry for the proliferating cell nuclear antigen (PCNA), a marker for cell proliferation. On Day 3, 2.6 ± 0.4% of the nuclei in the vehicle-treated group were PCNA-positive, whereas only 1.8 ± 0.6% in the SSZ-treated group were PCNA-positive (P < 0.05) (Figure 5C and D). To evaluate apoptosis in vivo, we used TUNEL staining. SSZ significantly increased the fraction of apoptotic cells (39.1 ± 3.3%) compared with the vehicle-treated group (9.5 ± 6.16%, P < 0.05) (Figure 5E and F). To verify whether the administration of SSZ induced ROS production in the balloon-injured vessels similar to the above in vitro experiments, we estimated the superoxide levels by fluorescence indicator staining. The ROS were significantly increased in the injured carotid arteries of the SSZ-treated rats on Day 3 after the balloon-injury (Figure 5G and H).

In addition, we performed immunohistochemistry using anti-Nrf2 and anti-HO-1 antibodies to examine whether Nrf2 and HO-1 were activated after SSZ treatment. With SSZ treatment, the number of VSMCs, with nuclear staining of Nrf2, was significantly higher in the medial layer of the injured arteries (Figure 5I) and there was significant induction of HO-1 expression (Figure 5I).

3.6 Inhibition of Nrf2 or HO-1 abrogates SSZ's effect on neointimal hyperplasia

To verify the hypothesis that Nrf2-mediated HO-1 induction by SSZ has a major role in the suppression of neointimal formation in vivo, we then examined whether the down-regulation of Nrf2, using Ad-Nrf2DN, reversed the suppression of neointimal hyperplasia by SSZ. Successful adenoviral transfection, to the artery, was measured by the presence of GFP (data not shown). Representative sections and morphometric analysis 2 weeks after injury showed that SSZ treatment significantly reduced intima-to-media (I/M) ratio compared with the vehicle-treated group, which was significantly reversed by Nrf2 blocking (Figure 6A and B). The immunoblots from the balloon-injured tissues confirmed HO-1 expression induced by SSZ and its reversal by Nrf2 blocking (Figure 6C).

We performed an additional experiment using selective HO-1 shRNA plasmid to verify whether inhibition of neointimal hyperplasia by SSZ was again reversed through HO-1 inhibition in vivo. The transfection efficiency and knock-down efficiency were confirmed (see Supplementary material online, Figures S7 and S8). SSZ reduced the neointimal hyperplasia in the control shRNA transfected group. But such inhibitory effect of SSZ on neointimal hyperplasia was not observed in HO-1 shRNA transfected group, indicating that SSZ inhibits neointimal hyperplasia through HO-1 induction (Figure 6D).

Therefore, these findings suggest that the Nrf2 and its downstream molecule, HO-1, which be activated and increased by SSZ, inhibit neointimal hyperplasia.

4. Discussion

In the present study, we addressed the effects of SSZ on proliferative vascular disease. We demonstrated that SSZ has pro-apoptotic and anti-proliferative action on the VSMCs. Such effects were independent of TNFα-induced NF-κB modulation, since the concentration of SSZ at which these effects were observed was relatively low and did not significantly antagonize NF-κB activation. Therefore, we investigated another possible mechanism by which SSZ modulates the proliferation of VSMCs. We found that SSZ induced HO-1, leading to p21 up-regulation and cell-cycle arrest. The mechanism of HO-1 induction, by SSZ, was observed to be by ROS production, which activated nuclear translocation of Nrf2, a transcription factor that binds to ARE of the HO-1 promoter region. We also demonstrated that SSZ treatment significantly inhibited neointimal hyperplasia of balloon-injured carotid arteries. Such effects correlated with increased ROS generation or induction of Nrf2 and HO-1 in the injured vasculature of the SSZ-treated animals. Finally, we confirmed that Nrf2 or HO-1 blocking blunted SSZ's inhibitory action on neointimal hyperplasia. This is the first report on the novel effects of SSZ on VSMC biology. Treatment with SSZ resulted in ROS-dependent, Nrf2-mediated, HO-1 expression followed by p21 induction, and cell-cycle arrest. The schematic illustration of the proposed mechanism of action is presented in Figure 7.

4.1 The role of ROS as 'bona fide' second messengers in VSMC signalling

ROS are key components for the integration of VSMC signalling events. Our data showed that ROS are important mediators that regulate proliferation and cell-cycle progression in VSMCs. Recently, investigators have come to recognize the role of ROS as ‘bona fide’ second messengers in cell signalling as well as the modulation of gene expression. Several studies have shown that ROS can
positively and negatively regulate VSMCs. We supposed that this paradoxical effect of ROS on VSMC growth might be related to the synthetic level of ROS. Recent studies have shown that endogenous low levels of ROS modulate neointimal hyperplasia; micromolar concentrations (μM) of ROS induced cell-cycle arrest; and millimolar concentrations (mM) of ROS resulted in apoptosis. However, apoptosis of the VSMCs caused by excessive ROS stimulated the inflammatory response and this reversed the effects of modulating neointimal hyperplasia.31

We could not directly measure the absolute levels of the ROS, because different types of ROS can be induced by SSZ.

Figure 5 SSZ inhibits neointimal hyperplasia in a rat carotid artery balloon injury model. (A) Representative sections of the common carotid artery 14 days after injury. The arrows indicate neointimal hyperplasia. SSZ, SSZ-treated rat. (B) I/M ratio and lumen area of the arteries after balloon injury (n = 5 for vehicle, n = 9 for SSZ treated). * P < 0.05. (C) PCNA immunostaining 3 days after injury. Arrows indicate PCNA-positive, brown–black nuclei. (D) The number of PCNA-positive cells in the neointimal region of the SSZ-treated group was significantly lower than that of the vehicle (n = 6, each). * P < 0.05. (E) TUNEL staining 3 days after injury. Arrows indicate TUNEL-positive nuclei. (F) The number of TUNEL-positive cells in the neointimal region of the SSZ-treated group was significantly higher than that of the vehicle (n = 4, each). * P < 0.05. (G) In situ detection of superoxide with the O2•−-sensitive dye DHE (red fluorescence) at 3 days. (H) ROS levels were significantly increased in the SSZ-treated group. Mean fluorescence intensity (n = 4, each). * P < 0.05. (I) Representative sections of the immunohistochemistry for Nrf2 (left panel) and HO-1 (right panel) in the neointima. Arrows indicate Nrf2-positive cells.
treatment. However, we could measure the relative levels of ROS using hydrogen peroxide (H$_2$O$_2$) as a positive control. The ROS level induced by SSZ was similar to the levels of ROS induced by micromolar concentrations of H$_2$O$_2$ (see Supplementary material online, Figure S5). Oxidative stress at levels that are 'too low' to cause apoptosis can in certain cases induce growth arrest. H$_2$O$_2$ in the micromolar range has been shown to induce cell-cycle arrest in the G0/G1 phase, whereas higher concentrations in the millimolar range have been associated with apoptosis.$^{31,32}$

Because SSZ has been identified as a potent inhibitor of GSTs, and thus as an inducer of 4-HNE,$^{13,14}$ the underlying mechanism associated with SSZ activation of the

Figure 6  Blocking of Nrf2 or HO-1 reverses SSZ's inhibitory action on neointimal hyperplasia. (A) Representative sections showed significant reduction of the neointima in SSZ with control virus-treated rats (SSZ + Ad-GFP, middle panel) compared with the control rats (vehicle + Ad-GFP, upper panel). Transfection of the dominant-negative Ad-Nrf2 (SSZ + Ad-Nrf2DN, lower panel) significantly reversed SSZ’s inhibitory action on neointimal hyperplasia. The arrows indicate the neointima. (B) I/M ratio of the carotid artery 14 days after the balloon injury ($n$ = 3, each). *$P$, 0.05. (C) Immunoblot analysis showed that SSZ administration significantly increased HO-1 expression in the carotid artery, which was partially reversed by Nrf2 blocking. (D) Representative sections showed that the reduction of neointima with SSZ in the control rats was significantly reversed in the HO-1 shRNA plasmid DNA transfected rats. The arrows indicate neointima. The bar graph represents the I/M ratio of the carotid artery 14 days after the balloon injury ($n$ = 4, each). *$P$, 0.05.
endogenous antioxidant pathway, Nrf2 and HO-1, as noted in the present study, might be through 4-HNE. Recently, it has been shown that HNE induces Nrf2-dependent HO-1 expression and elicits adaptive responses to oxidative stress in several cell types. Treatment with 4-HNE was shown to arrest the cell cycle at the G0/G1 phase by p21 induction. The results of these previous experiments corroborate our results and suggest that 4-HNE may be an endogenous mediator of SSZ that activates antioxidant genes.

4.2 The novel mechanism of SSZ: ROS-dependent Nrf2-mediated HO-1 induction

SSZ was developed as an anti-rheumatic drug in the 1940s, but its actual therapeutic mechanism remains elusive. The therapeutic benefits of SSZ for inflammatory bowel disease and rheumatoid arthritis have been suggested to be due to its ability to inhibit NF-κB activation, resulting in the down-regulation of pro-inflammatory cytokine mRNA expression. In previous studies, SSZ has been shown to inhibit NF-κB activation via direct inhibition of IκB kinase in cultured colon cells. In addition, SSZ has been shown to specifically inhibit the activation of NF-κB, block the cell cycle, and induce apoptosis in several cancer cell lines.

However, the underlying mechanisms involved in its anti-inflammatory effects remain unclear and controversial. The serum SSZ concentration, after an average oral dose of 3–6 g per day, is reported to be 10–15 μg/mL (equivalent to 25–38 μM). The concentration range for effective inhibition of NF-κB in vitro has been reported to be 500–2000 μM; 20- to 100-fold higher than the plasma concentration range. In our study, SSZ at lower concentrations (50 μM) suppressed VSMC proliferation through Nrf2-mediated HO-1 induction. Therefore, the major mechanism of action of SSZ for regulation of inflammation and proliferation in vivo may not be NF-κB inhibition, but rather the induction of HO-1.

HO-1, an inducible enzyme by various stimuli, such as cytokines and oxidants, is the rate-limiting enzyme in the catabolism of haem into bilirubin, which exerts additional antioxidant effects. We showed that the level of HO-1 was significantly increased in the neointima and tunica adventitia after treatment with SSZ. Overexpression of HO-1 in the arterial walls has been shown to reduce neointimal hyperplasia subsequent to vascular injury in animals. Our findings combined with the previous reports suggest a novel anti-proliferative mechanism associated with HO-1 induction by SSZ. These results might have clinical benefit for patients with coronary artery disease. The efficacy of SSZ for the treatment of coronary artery disease as an additional candidate for DES merits further investigation to determine its therapeutic significance in patients with vascular proliferative disorders.

In conclusion, SSZ induces HO-1 via ROS-dependent Nrf2 signalling, leading to inhibition of VSMC proliferation and subsequent reduction of neointimal hyperplasia. Our findings suggest that SSZ modulates endogenous antioxidant enzymes in VSMC against oxidative stress following vascular injury and may be a potential therapeutic option for the treatment of patients with proliferative vascular disease.

**Supplementary material**

Supplementary material is available at Cardiovascular Research online.

**Acknowledgements**

We thank Timothy H. Murphys (Kinsmen Laboratory of Neurological Research and Departments of Psychiatry and Psychology, University of British Columbia, Vancouver, British Columbia V6T 1Z3, Canada) for generously providing Nrf2 and dominant-negative Nrf2 adenovirus.

Conflict of interest: none declared.

**Funding**

Grant from the National Research Laboratory for Cardiovascular Stem Cell and the Innovative Research Institute for Cell Therapy, Republic of Korea.

**References**


13. He NG, Awasthi S, Singhal SS, Trent MB, Boor PJ. The role of glutathione
14. Cao Z, Hardej D, Trombetta LD, Li Y. The role of chemically induced glu-
17. Yang HM, Kim HS, Park KW, You HJ, Jeon SI, Youn SW
19. Park KW, Kim DH, You HJ, Sir JJ, Jeon SI, Youn SW
20. Hautmann MB, Thompson MW, Swartz EA, Olson EN, Owens GK. Angioten-
vasa recta pericytes by PKC and reactive oxygen species. Am J Physiol
22. Park KW, Yang HW, Youn SW, Yang HJ, Chae IH, Oh BH et al. Constitutively
active glycogen synthase kinase-3beta gene transfer sustains apoptosis, inhibits proliferation of vascular smooth muscle cells, and reduces neoin-
tima formation after balloon injury in rats. Arterioscler Thromb Vasc Biol
2003;23:1364–1369.
TNF alpha induces expression of transcription factors c-fos, Egr-1, and
Ets-1 in vascular lesions through extracellular signal-regulated kinases
In vitro and in vivo activity of the nuclear factor-kappaB inhibitor
sulfasalazine in human glioblastomas. Clin Cancer Res 2004;10:
559–5603.
Sulfasalazine reduces bile acid induced apoptosis in human hepato-
27. Togane Y, Morita T, Suematsu M, Ishimura Y, Yamazaki Ji, Katayama S.
Protective roles of endogenous carbon monoxide in neointimal develop-
278:H623–H632.
oxygenase-1 attenuates glucose-mediated cell growth arrest and apopto-
sis in human microvesSEL endothelial cells. Circ Res 2003;93:
507–514.
29. Shen XL, Kunsch C. Induction of cytoprotective genes through Nrf2/
antioxidant response element pathway: a new therapeutic approach for
the treatment of inflammatory diseases. Curr Pharm Des 2004;10:
879–891.
30. He X, Lin GX, Cheng MG, Zhang JX, Ma Q, Protection against chromium (VI)-induced oxidative stress and apoptosis by Nrf2. Recruiting Nrf2 into
the nucleus and disrupting the nuclear Nrf2/Keap1 association. Toxicol
Mechanism of hydrogen peroxide-induced cell cycle arrest in vascular
primary human fibroblast cell line to H2O2: senescence-like growth arrest
nuclear factor erythroid 2-related factor 2 signaling. Am J
34. Barrera G, Pizzimenti S, Dianzani MU. 4-Hydroxynonenal and regulation
of cell cycle: effects on the pRb/E2F pathway. Cell Growth Differ
Sulfasalazine reduces bile acid induced apoptosis in human hepato-
37. Togane Y, Morita T, Suematsu M, Ishimura Y, Yamazaki Ji, Katayama S.
Protective roles of endogenous carbon monoxide in neointimal develop-
278:H623–H632.
oxygenase-1 attenuates glucose-mediated cell growth arrest and apopto-
sis in human microvesSEL endothelial cells. Circ Res 2003;93:
507–514.
39. Shen XL, Kunsch C. Induction of cytoprotective genes through Nrf2/
antioxidant response element pathway: a new therapeutic approach for
the treatment of inflammatory diseases. Curr Pharm Des 2004;10:
879–891.
40. He X, Lin GX, Cheng MG, Zhang JX, Ma Q, Protection against chromium (VI)-induced oxidative stress and apoptosis by Nrf2. Recruiting Nrf2 into
the nucleus and disrupting the nuclear Nrf2/Keap1 association. Toxicol
Mechanism of hydrogen peroxide-induced cell cycle arrest in vascular
42. Bladier C, Wolvetang EJ, Hutchinson P, de Haan JB, Kola I. Response of a
primary human fibroblast cell line to H2O2: senescence-like growth arrest
43. Ishii T, Itoh K, Ruiz E, Leake DS, Unoki H, Yamamoto M et al. Role of Nrf2 in
the regulation of CD36 and stress protein expression in murine macro-
phages: activation by oxidatively modified LDL and 4-hydroxynonenal.
44. Barrera G, Pizzimenti S, Dianzani MU. 4-Hydroxynonenal and regulation
45. Mairesse MD. The heme oxygenase system: a regulator of second messenger