Vascular Injury Involves the Overoxidation of Peroxiredoxin Type II and is Recovered by the Peroxiredoxin-Activity Mimetic that Induces Reendothelialization

Running title: Kang et al.; Repair of vascular injury by 2-Cys Prx mimetic

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Abstract:

Background—Typical 2-Cys peroxiredoxin (Prx) is inactivated by overoxidation of the peroxidatic cysteine residue under oxidative stress. However, the significance in the context of vascular disease is unknown.

Methods and Results—The immunohistochemical analyses revealed that 2-Cys Prxs, particularly Prx type II, are heavily overoxidized in balloon-injured rodent carotid vessels and in human atherosclerotic lesions. Consistent with this observation, the selective depletion of Prx II exacerbated neointimal hyperplasia in injured carotid vessels. We also found that the epipolythiodioxopiperazine (ETP) class of fungal metabolites exhibited an enzyme-like activity mimicking 2-Cys Prx peroxidase and manifestly eliminated the intracellular H$_2$O$_2$ in the vascular cells. Functionally, the ETPs reciprocally regulated the PDGFRβ- and VEGFR2-mediated signaling in these vascular cells by replacing Prx II. As a consequence, the ETPs inhibited proliferative and migratory activities of smooth muscle cells, while they promoted those of endothelial cells in vitro. Moreover, administration of the ETPs to the injured carotid vessels resulted in a successful recovery by inhibiting neointimal hyperplasia without causing cytotoxicity and simultaneously inducing reendothelialization.

Conclusions—This study reveals for the first time the involvement of the 2-Cys Prx overoxidation and hereafter the therapeutic use of their activity mimetic in vascular injuries like stenting.

Key words: peroxiredoxin-1, reactive oxygen species, signal transduction, restenosis, endothelialization, Tyrosine kinase receptor
Introduction

The arterial wall is mainly composed of vascular smooth muscle cells (VSMC) and endothelial cells (VEC). The endothelial damage by inflammation, high blood pressure, or lipid deposition activates the VSMC to de-differentiate and re-enter cell cycles, thereby contributing to the thrombosis and neointimal hyperplasia 1,2. In particular, the clinical use of a balloon catheter to remove the plaques and dilate the occluded vessel area is always accompanied by massive denudation of endothelium in the lesion. Therefore, the repair of the injured vasculature requires the inhibition of SMC hyperplasia and simultaneous induction of endothelialization 3,4. Given that the platelet-derived growth factor receptor-β (PDGFRβ) and vascular endothelial growth factor receptor-2 (VEGFR2) are major receptor tyrosine kinases (RTK) orchestrating proliferation, chemotactic migration, and survival of VSMCs and VECs, respectively 5,6, the coordinated control of these similar RTKs is essential for restoration of the injured arterial vessels.

Mammalian 2-Cys peroxiredoxins (Prx, gene locus Prdx) comprise the family of peroxidase enzymes that catalyze reduction reaction converting the H₂O₂ to water. This is achieved with two electrons derived from NADPH via an electron-conveying system comprised of thioredoxin (Trx) and thioredoxin reductase (TR) 7. Recently, we reported that Prx II among various isoforms is the specific enzyme that negatively regulates PDGFRβ-mediated signaling in VSMCs and suppresses neointimal hyperplasia 8. Our subsequent studies using vascular endothelial cells led to an unexpected result that reveals how Prx II protects VEGFR2 from oxidative inactivation in VECs 9. In the absence of Prx II, VEGFR2 kinase activation was impaired by oxidation. These studies together suggest that Prx II may be the mediator of reciprocal regulation in the two types of vascular cells, VSMCs and VECs. On the other hand,
the etiology of vascular diseases like atherosclerosis and restenosis implicates the involvement of reactive oxygen species (ROS) in the disease progression\textsuperscript{10, 11}. The reason is because the endothelial damage leads to the recruitment of platelets and macrophages to the injured lesion. Given that these cells are robust ROS producers, the endothelial damage may render the 2-Cys Prx enzymes in neighboring vascular cells to be overoxidized. Therefore, it is possible that if Prx II is inactivated by overoxidation, it will aggravate vascular damage response by perturbing the major RTK signalings in both vascular cell types.

In this study, we showed for the first time that the 2-Cys Prxs were overoxidized in balloon-injured carotid artery of rodent models and in human atherosclerotic lesions. In particular, inactivation of Prx II exacerbated neointimal thickening in the balloon-injured carotid arteries. We then discovered an enzyme-like activity of the fungal secondary metabolites called epipolythiodioxopiperazine (ETP) class mimicking 2-Cys Prx peroxidase. Subsequently, we applied these ETP compounds to treat the vascular injury. Surprisingly, the ETPs prevented the neointimal SMC hyperplasia and simultaneously induced re-endothelialization in the injured lesions by reciprocally regulating the PDGFR\textbeta and VEGFR2-mediated signaling in VSMCs and VECs, respectively. Therefore, the study reveals a therapeutic efficacy of the natural fungal metabolites to treat the injured arterial vessels, where the 2-Cys Prxs, particularly Prx II, are overoxidized.

Methods

Animals

Animal experiments were performed in compliance with the guidelines of the Institutional Animal Care and Use Committee (IACUC) of Ewha Womans University. The ten-week-old male
Sprague-Dawley rats were used for a balloon-induced injury model. Prx II−/− knockout C57BL/6 mice were used as a genetic model. The mice were maintained and genotyped as previously described. The animal experiments were repeated twice or three times, and the detailed group sizes in each experiment are described in the figure legends.

**Balloon-induced injury of rat carotid artery**

A balloon injury was created using an infiltrated 2F Fogarty balloon embolectomy catheter in the left common carotid artery as follows: The rats were anaesthetized by inhalation of isoflurane gas (N₂O:O₂/70%:30%); the left external carotid artery was exposed; and its branches were electro-coagulated. A catheter was pushed 1 cm through the transverse arteriotomy of the external carotid artery, and the endothelial denudation was achieved by three passes along the common carotid artery. After removal of the catheter, the punched area was sealed and the clapped common carotid artery was opened to resume the blood flow. Unless otherwise stated, the rats were then recovered in the cages for 10 days. For histological analyses, rats were anesthetized and the common carotid arteries were excised after transcardia perfusion-fixation with heparinized saline containing 3.7% formaldehyde. The vessels were paraffin embedded and sectioned by rotary microtome (Leica RM2255). The two serial tissue sections (4 μm in thickness) were obtained from the middle area of common carotid arteries and stained with haematoxylin and eosin (HE). The luminal, internal elastic laminal, and external elastic laminal areas were measured using NIH Image v1.62. The intimal and medial areas were determined by subtraction of the luminal area from the internal elastic area and by subtraction of the internal elastic area from the external elastic area. The values from two serial sections per rat were averaged for analysis.

**Catheter-mediated intramural delivery of siRNAs or compounds into carotid artery**
The rat Prx-specific siRNAs (200 nM) were premixed with siPORT\textsuperscript{TM} NeoFX\textsuperscript{TM} reagent (Ambion). After operation of the balloon-induced injury, the transfection mixture (200 l) was administered into the injured area of the carotid lumen through the catheter. The vessel was incubated for 15 mins to allow the efficient transfection and then sealed. A fluorescent dye-conjugated control siRNA, siGLO-Red (Dharmacon), was used for optimizing the intramural transfection efficiency. Similarly, the ETP solutions (200 nM in dimethyl sulfoxide) were administered into the injured area of the carotid lumen through the catheter and then incubated for 30 mins to allow the efficient drug diffusion.

**Immunohistochemistry and immunofluorescence staining**

Immunohistochemistry for the overoxidized 2-Cys Prxs was performed on paraffin sections using anti-Prx-SO\textsubscript{2}/SO\textsubscript{3} antibody (1:1000 dilution). The antibody was generated as described previously.\textsuperscript{13} For immunohistochemistry, the antisera were subjected to the affinity purification through the antigenic peptide (DFTFVC(SO\textsubscript{2}/SO\textsubscript{3})PTEI)-conjugated agarose column. Briefly, sections were de-waxed in xylene and rehydrated in ethanol. Subsequently, the antigen retrieval was performed by boiling the sections in a citric buffer (pH 6.0). Sections were then incubated with the primary antibody for 48 hrs at 4\textdegree C. After washing three times with PBS, sections were incubated with a peroxidase-conjugated secondary antibody and stained with a 3’,3’-diaminobenzidine (DAB) substrate solution. The nuclei were further stained with haematoxylin. For negative staining, the Prx-SO\textsubscript{2}/SO\textsubscript{3} antibody was blocked with the corresponding antigenic peptides. The DAB-stained images were obtained and quantified using HistoFAXS Tissue Analysis System (TissueGnostics, USA).

Paraffin sections of human coronary arteries with atherosclerotic lesions were obtained from patients who underwent heart transplantations at Emory University Hospital, as approved...
by the Emory human subjects review committee. Paraffin sections of human carotid arteries with atherosclerotic lesions were purchased from Origin Technologies (Rockville, USA).

For indirect immunofluorescence staining, the paraffin sections were blocked with 5% normal rabbit serum (Vector Laboratories) in PBST (0.3% Triton X-100 in PBS) for 1 hr at room temperature. The sections were then incubated overnight at 4°C with antibodies for rat smooth muscle α-actin (1:300 dilution) and rat CD31 (1:200 dilution). Nuclei were labeled with DAPI. After several PBST washes, the samples were incubated for 2 hrs at room temperature with Alexa Fluor 568-conjugated donkey anti-mouse and Alexa Fluor 488-conjugated donkey anti-rabbit IgG antibodies. The fluorescence images were obtained on three random fields per tissue section at a screen magnification of 100x using a LSM 510 Meta confocal microscope equipped with argon and helium-neon lasers (Carl Zeiss).

Statistical analyses
Data were analyzed using the Student's *t*-test for comparisons between two groups or one-way ANOVA with Tukey’s ‘honestly significant difference’ *post hoc* test for multiple comparisons (SPSS 12.0K for Windows, SPSS, Chicago, IL, USA) to determine the statistical significance (*P* value). A *P* < 0.05 was considered to be significant.

Results

**Inactivation of Prx II is involved in the neointimal SMC hyperplasia**

We initially hypothesized the possibility of the 2-Cys Prx inactivation by overoxidation in injured arterial vessels. To access this possibility, we employed the balloon-induced injury of rat carotid artery, which is a clinically-relevant *in vivo* model for vascular remodeling induced by endothelial damage (**Figure 1A**). Then, the *in vivo* overoxidation of the 2-Cys Prxs in the
balloon-injured carotid arteries was evaluated using an anti-Prx-SO$_{2/3}$ antibody that specifically recognizes the 2-Cys Prx isoforms containing active-site cysteine sulfinic/sulfonic acids (Cys-SO$_{2/3}$) $^{13}$. This antibody has been used for immunoblotting but never been characterized for immunohistochemistry of animal tissues. However, we found that the antibody generated positive signals in the rat carotid vessels soaked in H$_2$O$_2$ solution and the immune-positive signals were completely blocked by preincubation with the corresponding antigenic peptide (DFTFVC(SO$_{2/3}$)PTEI) used for the antibody generation (Figure 1B, left panels). This control experiment indicates that the anti-Prx-SO$_{2/3}$ antibody specifically recognized the overoxidized 2-Cys Prxs in the tissue paraffin sections. Subsequently, the immunohistochemical analyses of the balloon-injured carotids using the same antibody indicated that the overoxidation of 2-Cys Prxs was profound in the neointimal SMC layers and, to some extent, in the medial layers at 5 and 7 days post-injury (Figure 1B, right panels). Since the antibody does not distinguish the overoxidized forms of different 2-Cys Prx isoforms in the tissue staining, we then dissected the overoxidation of each 2-Cys Prx isoform by immunoblotting. As a result, the immunoblot analyses showed that the overoxidation of Prx I thru III among the isoforms occurred rapidly within a day after injury and persisted along with neointimal thickening (Figure 1C). When the two cytosolic 2-Cys Prxs, Prx I and Prx II similar in size, were further separated by immunoprecipitation, it turned out that the Prx II overoxidation was transiently and noticeably increased during neointimal thickening. Based on this observation, we wanted to address whether Prx II plays a more prominent role than the other isoforms in the neointimal hyperplasia. The expression of each Prx isoform was knocked down regionally by administrating the siRNA-transfection complexes into the injured luminal area of the carotid vessel as described in the Methods. Indeed, the neointimal hyperplasia induced by the balloon injury was exacerbated in
the carotid vessels where only Prx II, not Prx I and Prx III, had been knocked down (Figure 1D).

We further assessed the relevance to human vascular disease by examining the 2-Cys Prx overoxidation in human atherosclerotic lesions. As a result, the anti-Prx-SO$_{2}$/3 antibody strongly detected the overoxidized 2-Cys Prxs in the endothelial and neointimal layers of human coronary arterial sections with different degrees of atherosclerosis (Figure 2 and Supplemental Figure 1A). The medial layers were discretely stained near the areas where the internal elastic laminas were disrupted. Furthermore, the 2-Cys Prxs were overoxidized in the human carotid arterial sections with atherosclerotic lesions (Supplemental Figure 1B). Collectively, the data indicate that the overoxidation of 2-Cys Prxs, particularly Prx II, may be a causative factor for neointimal hyperplasia in injured vessel walls as well as it can be a hallmark of vascular injury, including atherosclerosis.

The ETP class of fungal metabolites exhibits thioredoxin-dependent peroxidase activity

Next, we sought a drug-like small molecule that enables to substitute the overoxidized Prx II in the clinically-relevant in vivo systems. The ETP class of fungal secondary metabolites is represented by a common core structure known as the dithio-diketopiperazine ring with an internal disulfide bridge$^{15}$. Therefore, we have tested whether the disulfide bridge in the piperazine ring may serve as an active site for peroxidatic reaction$^{16}$. To strengthen this idea, the three representative ETPs (GT, chaetocin, and chetomin) were chosen by availability (Figure 3A) and subjected to the activity assay coupled with either of the two major cellular electron-conveying systems, i.e. Trx/TR or glutathione (GSH)/glutathione reductase (GR). Surprisingly, they all exhibited the apparent H$_2$O$_2$-reducing activity in the presence of only the Trx/TR system (Figure 3B - D). Interestingly, the activities of chaetocin and chetomin (8.18 ± 0.24 and 8.62 ± 0.51 nmole/min, respectively) were approximately two times higher than that of GT (3.72 ± 0.53
nmole/min), which correspond to the number of the disulfide-containing piperazine rings present in the compounds. Chaetocin and chetomin also exhibited marginal H$_2$O$_2$-reducing activities (1.51 ± 0.39 and 2.53 ± 0.79 nmole/min, respectively) in the presence of GSH/GR system, as compared to those in the presence of the Trx/TR system. Furthermore, the H$_2$O$_2$-reducing activities of ETPs required all three components of the Trx/TR/NADPH system and were proportional to the incremental concentration (Figure 3E). A derivative of gliotoxin with methylated thiols in the diketopiperazine ring (bis(methylthio)-GT) had no peroxidase activity, indicating that the oxidation-reduction cycle of dithiols in the ring is essential for the peroxidase activity. Thus, the results imply that the ETPs are the first natural compounds mimicking a typical 2-Cys Prx activity coupled with Trx/TR/NADPH system.

**ETPs inhibit PDGFRβ signaling in SMCs but promote VEGFR2 signaling in VECs**

We previously reported that Prx II is the specific peroxidase enzyme that reciprocally regulates the PDGFRβ-mediated signaling in VSMCs and VEGFR2-mediated signaling in VECs $^8,^9$. Accordingly, we accessed the cellular activity of the ETP compounds in human aortic vascular cells with no Prx II. Since most ETPs are known to be cytotoxic to animal cells $^{15}$, we determined the optimal non-toxic concentrations of ETPs for the *in vitro* studies. The cytotoxicity test turned out that the human aortic SMCs (HASMCs) and ECs (HAECs) were viable in the GT treatment at the concentrations below 200 nM and 50 nM, respectively (Supplemental Figure 2A, B). Chaetocin and chetomin were less toxic than GT. The toxicity with higher concentrations of GT appeared to be due to the NF-$\kappa$B inhibition as previously known $^{17}$ (Supplemental Figure 2C, D). It was also known that ETPs can be intracellularly enriched because only the oxidized form of the ETP compound with disulfide penetrates the cell membrane and therefore its reduced form is trapped in the cytoplasm $^{18}$. To assess whether this is
the case in vascular cells, we measured the cellular GT concentration by the HPLC purification from the methanol extracts of HASMCs and HAECs treated with a low non-toxic concentration as described in the *Methods* (Supplemental Figure 3). The cellular GT amounts estimated from a standard curve were 31.9 ± 4.8 and 59.8 ± 12.4 pmoles /10^5 cells in SMCs and ECs, respectively, indicating that almost all GT compounds treated were promptly taken up by the cells. Given the average volume of a single cell (~5 picoliters) 19, the actual GT concentration per cell reached approximately 100 μM, which is similar to the average cellular concentration (about 10 ~ 100 μM) of 2-Cys Prxs estimated in cell lines 20. Such estimation suggests that the nanomolar GT can sufficiently exert the effective peroxidase activity in cells.

First, we examined the peroxidase activity of ETPs in vascular cells by monitoring the intracellular H₂O₂ level in the ETP-treated cells using an oxidant-sensitive fluorescent dye (2’,7’-dihydro-chlorofluorescein diacetate, H₂DCF-DA). H₂O₂ was intracellularly generated by PDGF stimulation in HASMCs and its level was further increased by the Prx II knockdown. However, the GT treatment completely reduced the level of intracellular H₂O₂ in a dose-dependent manner (Figure 4A). In HAECs, the Prx II knockdown *per se* elevated the basal level of intracellular H₂O₂, which was again eliminated by GT in a dose-dependent manner (Figure 4B). In addition, none of GT and chaetocin did inhibit the NADPH oxidase (NOX) activities in HASMCs and HAECs stimulated with cognate growth factors (Supplemental Figure 4), which excluded the possibility that the ETPs could block the source of H₂O₂ in the cells 21. Collectively, the data indicate that the GT representing ETPs acts as a peroxidase in vascular cells.

Secondly, we examined the regulatory effect of GT on PDGFRβ- and VEGFR2-mediated signaling pathways. The PDGF-induced tyrosine phosphorylation in HASMCs that had been enhanced by the Prx II knockdown was completely returned to the level in the stimulated control
cells by the GT pretreatment (Figure 4C). Conversely, the VEGF-induced tyrosine phosphorylation in HAECs was impaired by the Prx II knockdown, which was restored by the GT pretreatment (Figure 4D). Specifically, GT reduced the activation-dependent phosphorylation of PDGFRβ and PLC 1 in HASMCs by the levels in the stimulated control cells, whereas it restored the activation-dependent phosphorylation of VEGFR2 and ERK in HAECs at a comparable level to the stimulated control cells. In latter case, the VEGFR2 oxidation induced by PrxII knockdown in endothelial caveolae 9 was completely inhibited by GT treatment (Supplemental Figure 5A). It is also noteworthy that other antioxidant compounds, such as N-acetylcysteine and butylated hydroxyanisole, did not restore the VEGFR2 and downstream ERK activation in the Prx II-depleted HAECs even though they were treated at 103 ~ 104-fold higher concentrations than that of GT or chaetocin (Supplemental Figure 5B). These control experiments strongly support the selective cellular function of GT via a 2-Cys Prx-like activity, not a non-specific oxidant-scavenging activity. More importantly, such reciprocal regulatory effect of GT on PDGF and VEGF signaling was constantly reproduced using mouse aortic SMCs and ECs isolated from the aortas of WT and Prx II−/− mice, respectively (Figure 4E, F) and abolished by co-treatment of the TR inhibitors (Supplemental Figure 6), indicating that GT substituted for the Trx/TR-dependent peroxidase Prx II in vascular cells.

Thirdly, we evaluated the efficacy of GT in the vascular cell function. The Prx II knockdown enhanced the proliferation and chemotactic migration of HASMCs in response to PDGF stimulation, which were attenuated by the GT pretreatment in a dose-dependent manner (Figure 5A). In contrast, the same treatment improved the VEGF-dependent proliferation and chemotactic migration of HAECs, which had been impaired by the Prx II knockdown (Figure 5B). Another ETP compound, chaetocin, was also tested for its effectiveness on vascular cell
function. Likewise, the chaetocin treatment markedly reduced the PDGF-dependent proliferation and chemotactic migration in Prx II-depleted HASMCs (Figure 5C); whereas, the same treatment significantly increased the VEGF-dependent proliferation and chemotactic migration in the Prx II-depleted HAECs (Figure 5D). It is noteworthy that the nanomolar concentrations of ETPs were sufficient for regulation of receptor tyrosine kinase (RTK) signaling and cell function in VSMCs and VECs. Collectively, the data indicate that the ETPs almost perfectly restored PDGF- and VEGF-dependent vascular function perturbed by the absence of Prx II, a key RTK signal regulator in vascular cells.

**ETPs inhibit neointimal SMC hyperplasia in the injured carotid vessels**

We then evaluated the *in vivo* efficacy of ETPs for preventing SMC hyperplasia in the balloon-injured rat carotid arteries, where the overoxidized forms of 2-Cys Prxs, primarily Prx II, were accumulated in the injured arterial vessels. To do so, we administered the ETP solution into the injured luminal area of the carotid vessel immediately after a balloon injury as described in the Methods. The incubation of GT, not the inactive bis(methylthio)-GT, profoundly suppressed the neointimal hyperplasia in a dose-dependent manner (Figure 6A, B) without a noticeable cell death up to micromolar concentrations. The inhibitors of coupling enzyme TR, e.g. 2,4-dinitrochlorobenzene (DNCB) and auranozin, abolished the *in vivo* activity of GT (Figure 6B), indicating that the GT activity was dependent on the Trx/TR system *in vivo*. More importantly, the GT treatment suppressed the neointimal hyperplasia in the injured carotid vessels of Prx II-/- mice (Figure 6C) and reduced the PDGFRβ autophosphorylation (pY857) in the injured carotid vessels (Supplemental Figure 7A), which confirm the *in vivo* effect of GT as the *bona fide* substitute for Prx II. Furthermore, the incubation of chaetocin and chetomin comparably suppressed the neointimal hyperplasia in the injured vessels (Figure 6D, E), indicating a general
biological efficacy of the ETP compounds.

In addition, the GT treatment did not prevent the 2-Cys Prx overoxidation in the injured lesion (Supplemental Figure 8). This result gave us an important message that the ETP compounds acted as an auxiliary peroxidase after the 2-Cys Prx overoxidation occurred. Considering that the specific activities of the ETP compounds were in the range of 0.16 ~ 0.32 nmole/min/μM compound, which are much lower than those of 2-Cys Prxs (~22 nmole/min/μM enzyme), it is very true that the 2-Cys Prxs with high affinity to H2O2 are the primary antioxidant enzymes that combat against the prolonged ROS burst from vascular injury.

**ETPs induce re-endothelialization in the injured carotid vessels**

We further attempted to determine whether ETPs can induce reendothelialization in the injured lesions, which was also inhibited by the PrxII knockdown (Supplemental Figure 9). The co-immunofluorescence staining of the endothelial cell marker CD31 (PECAM1) with α-smooth muscle actin (SMA) revealed a uniform EC monolayer above the medial SMC layer in the normal carotid vessel (Figure 7A, upper panels). Only the GT-treated injured vessel, not the control injured vessel, showed the recovery of the EC monolayer (Figure 7A, lower panels). In addition, the VEGFR2 autophosphorylation (pY1059) was increased along with EC monolayer in the GT-treated injured vessels (Supplemental Figure 7B), indicating the restoration of VEGF-dependent endothelial signaling by GT. The chaetocin and chetomin also induced the formation of EC monolayer in the injured lesions (Figure 7B, C). We then examined the functionality of the recovered endothelium by using Evans blue extravasation. The balloon injury induced an extensive extravasation of Evans blue dye compared to the healthy collateral carotid vessels, indicating complete loss of permeability control by denudation (Figure 7D). In contrast, the GT treatment markedly blocked the Evans blue extravasation in injured vessels, which was
countered by a VEGFR2 kinase-specific inhibitor (SU5416), suggesting that the GT effect was accomplished by preventing the oxidative inactivation of VEGFR2 kinase induced by the Prx II inactivation. The structural integrity of the EC layer on the luminal surface of the injured carotid vessels was visualized by a scanning electron microscope. The ECs in the GT-treated carotid vessels were adhered to each other in a monolayer, whereas those in the control carotid vessels were shrunk and patched (Figure 7E). The data together indicate that the endothelium recovered by the GT treatment is functional barrier capable of controlling vascular permeability.

Discussion

The microbial secondary metabolites belonging to the epipolythiodioxopiperazine (ETP) class (i.e. gliotoxin, chaetocin, chetomin, etc) are produced only by fungi, including some pathogenic species, and exhibit intrinsic cytotoxicity to the mammalian cells. Gliotoxin (GT), the first-characterized ETP, is a virulence factor for invasive aspergillosis in immunocompromised human patients. Chetomin is also produced from Chaetomium globosum, a systemic pathogen of the immunocompromised human patients. Sporidesmin causes the facial eczema and liver disease in herbivores such as sheep. The scabrosin ester ETP impaired the mitochondrial function in the mammalian cells. The cytotoxicity of these compounds was proposed based on the ability to induce the ROS-dependent damages in proteins and DNAs and/or the formation of inactive adducts with cellular protein thiols. Thus, it is a paradox that some ETPs are considered as potential therapeutic agents based on their cytotoxicity. For example, GT has been shown to exert the immune suppressive and anti-cancer activities. Chaetocin and chetomin were also shown to possess anti-cancer activity. Moreover, the molecular targets for these natural products are actively sought. GT was shown to inhibit several enzymes such as NF-κB,
farnesyltransferase, and phagocytic NOX2 \(^{17,21,25}\); chaetocin inhibited TR or histone methyltransferase \(^{30,31}\). Chetomin has been shown to disrupt the interaction between HIF-1 and p300 \(^{29}\). Nonetheless, it is difficult to deduce a logical correlation between the chemical structures and cellular activities of the ETP compounds. More specifically, the cellular function of the dithioketopiperazine ring structure, despite being the core structure of the ETPs, has never been uncovered. Our study showed for the first time that the dithiol group in the ETPs exhibits an H\(_2\)O\(_2\)-reducing peroxidase activity \textit{in vitro} and \textit{in vivo}, which exclusively relies on the Trx/TR system. Given that 2-Cys Prx is the only mammalian peroxidase coupled with a Trx/TR redox system, the ETPs are the first natural compounds mimicking the 2-Cys Prx. In particular, the ETP’s cellular efficacy is unique in that they played an effective regulatory role in VSMCs and VECs by substituting the Prx II at nanomolar concentrations without substantial cytotoxicity (Figure 7F).

The ETP compounds are known to be highly cytotoxic to the mammalian cells \textit{in vitro}. Therefore, a number of ETPs produced from pathogenic fungi (e.g. \textit{Aspergillus fumigatus}, \textit{Chaetomium globosum}, \textit{Pithomyces chartarum}) are presumed to be involved in the mammalian diseases. Particularly, the ETP’s cytotoxicity becomes obvious when the pathogenic fungi are enriched in immunocompromized human patients. However, our study indicated that the ETP’s cytotoxicity is highly a concentration-dependent phenomenon, such that at non-toxic level the dithiol group in the ETPs plays an H\(_2\)O\(_2\)-reducing enzymatic role in mammalian cells. In mammalian system, they can specifically substitute the pool of 2-Cys Prxs inactivated by overoxidation under stressed conditions. The cellular enrichment of ETPs due to the molecular characteristics overcomes their intrinsic peroxidase activity lower than those of the 2-Cys Prx enzyme. Therefore, our finding is the first case for the toxicity-independent beneficial use of
fungal toxins. It becomes of more interest if we notice that non-pathogenic members of these fungal genera, including *Penicillium* and *Aspergillus* species useful for food fermentation, also produce the ETP metabolites: that is, some type of ETPs might already have given us a health benefit as an ingredient of the fermented foods.

Another important finding in this study is that the 2-Cys Prxs were overoxidized in the pathological condition of aortic vessels, i.e. balloon-induced endothelial damage in rodent model clinically relevant to the percutaneous transluminal angioplasty and human atherosclerotic lesions. This is the first observation of the 2-Cys Prx overoxidation in *in vivo* tissues under pathological conditions. Specifically, the inactivation of Prx II among 2-Cys Prxs turned out to be the key factor for neointimal SMC hyperplasia. In fact, Prx II was proposed to be the reciprocal regulator of the RTK signaling in vascular SMCs and ECs. Regarding coronary or peripheral artery diseases, the unfavorable vascular remodeling is related to sub-acute thrombosis after percutaneous transluminal angioplasty. To prevent this poor prognosis, there is a medical need for agents with differential effects on VSMCs and VECs, by which they circumvent the thrombotic problem. The ETPs successfully reduced the neointimal hyperplasia and simultaneously induced the reendothelialization in the injured vessels. This evidence supports the therapeutic usefulness of ETPs as the coating materials for drug-eluting stents.

In conclusion, the unique *in vivo* effectiveness of the ETPs represents the first example of the mechanism-based druggable compounds useful for normalization of the injured arterial vessels in the circulatory system. Considering the ubiquitous expression of 2-Cys Prxs in human organs, the ETPs could be broadly applicable for repairing tissue damage involving the overoxidation of 2-Cys Prxs.
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Conflict of Interest Disclosures: None.

References:


**Figure Legends:**

**Figure 1.** Overoxidation of 2-Cys Prxs in the balloon-injured rat carotids and human atherosclerotic lesions. (A) Neointimal thickening during recovery time in balloon-injured carotid artery. Representative HE-stained images are shown. Data in the graph are means ± SEM.
of intima versus media ratio measured from HE-stained carotid samples (n=8 rats per group). (B) Overoxidation of 2-Cys Prxs in carotid vessels induced by balloon injury. Immunohistochemistry was performed using anti-overoxidized 2-Cys Prx (Prx-SO2/3) antibody. Normal carotid vessels soaked in H2O2 solution for 10 mins were stained for positive control (Note: The blocking antigenic peptide eliminated the immune-positive signal). The DAB-stained images are representatives of four independent carotid samples. Data in the graph are means ± SD of mean intensities of the Prx-SO2/3-positive signal per cell (n=8 - 9 rats per group, *P<0.01). (C) Immunoblot analyses of the overoxidation of intracellular 2-Cys Prxs using the injured carotid arterial extracts. Extracts from H2O2-treated normal carotid vessels are loaded as a positive control. The precipitate (ppt) and corresponding supernatant (sup) obtained from the Prx I immunoprecipitation (IP) were analyzed by immunoblotting. A representative blot from three experiments with similar results is shown. (D) Exacerbation of neointimal SMC hyperplasia by the Prx II absence. Representative HE-stained images are shown. Data in the graph are means ± SEM of intima versus media ratio measured from HE-stained carotid artery sections (n=8 - 10 rats per group, *P<0.01, N.S. not significant). Immunoblot shows the Prx level in the carotid vessels transfected with control or rat Prx-specific siRNAs. Note that the rats were recovered for 7 days post injury to better compare the change of neointimal hyperplasia by the Prx knockdown.

**Figure 2.** Overoxidation of 2-Cys Prxs in human coronary arterial sections with Type II (A) and Type IV (B) atherosclerotic lesions. Immunohistochemistry was performed using anti-Prx-SO2/3 antibody. For negative control, a serial section was stained after blocking with the antigenic peptide (+Peptide). Each image was labeled with patient identification numbers (11-07-06 and 10-26-06).
Figure 3. H2O2-reducing activity of ETPs coupled with thioredoxin and thioredoxin reductase system. (A) Chemical structures of GT, chaetocin, and chetomin. (B – D) H2O2-reducing activities of ETPs. The *in vitro* peroxidase reaction was carried out with 25 μM of each ETP compound in the presence of components of coupling redox systems as indicated. A representative reaction curve of three experiments is shown. (E) Concentration-dependent peroxidase activities of ETPs. The peroxidase activity is presented as mean ± SD of initial rates from three independent assays. A methylated derivative of GT, bis(methylthio)-GT, was used as negative control.

Figure 4. Reciprocal regulation of PDGF- and VEGF-dependent signaling by GT. HASMCs and HAECs were transfected with control or Prx II siRNA for 24 hrs and then pretreated with vehicle alone (DMSO) or GT (50 nM for SMCs; 25 nM for ECs) for 2 hrs. (A and B). Intracellular H2O2 level in HASMCs (A) and HAECs (B). DCF fluorescence images are shown. Bars in the graph are means ± SD of the relative DCF fluorescence values averaged from 50 - 80 cells (n=3, *P<0.01, **P<0.001). (C and D) Immunoblot analyses of PDGF-induced tyrosine phosphorylation in HASMCs (C) and VEGF-induced tyrosine phosphorylation in HAECs (D). Total tyrosine phosphorylation (pTyr) was detected by anti-phosphotyrosine antibody (4G10). A representative blot from three experiments with similar results is shown. (E and F) Effect of GT on PDGF and VEGF signaling in Prx II+/− vascular cells. Mouse aortic SMCs (E) and ECs (F) isolated from the wild type (WT) and Prx II+/− mouse aortas were treated with PDGF-BB and VEGF-A, respectively, for 10 mins in the absence and presence of GT. Activation of PDGFRβ, PLCγ1, VEGFR2, and ERK were analyzed using phoso-specific antibodies as indicated. For loading control, the blots were stripped and re-probed with corresponding proteins. A representative blot from three experiments with similar results is shown.
Figure 5. Reciprocal regulation of proliferation and chemotactic migration of SMCs and ECs by ETPs. (A) Effect of GT on proliferation and migration of HASMCs in response to PDGF. (B) Effect of GT on proliferation and migration of HAECs in response to VEGF. (C) Effect of chaetocin on proliferation and migration of HASMCs in response to PDGF. (D) Effect of chaetocin on proliferation and migration of HAECs in response to VEGF. HASMCs and HAECs were transfected with control or Prx II siRNA for 24 hrs and then pretreated with vehicle alone (DMSO) or various concentrations of GT for 2 hrs as indicated. Bars in graphs are means ± SEM of fold increases versus unstimulated control sample (n=3, *P<0.01, **P<0.005, #P<0.001).

Figure 6. Suppression of neointimal hyperplasia by ETPs in the injured carotid arteries. (A and B) Inhibition of neointimal hyperplasia by GT in balloon-injured carotids. The injured carotid arteries were incubated with control vehicle or indicated concentrations of GT for 30 mins. Representative HE-stained images are shown. Data (filled circle) in the graph are means ± SEM of intima-to-media ratio measured from HE-stained carotid samples (n=9 rats per group, *P<0.01 versus untreated sample). The cell death (open circle) was monitored by TUNEL staining and presented as the percent of TUNEL-positive cells versus DAPI-positive cells. Control experiments (B) with bis(methylthio)-GT and TR inhibitors were also performed (n=7 rats per group, N.S. not significant). DNCB and auranofin were used at 5 μM and 0.5 μM, respectively. Arrow indicates the thickened neointimal layer. (C) Inhibition of neointimal hyperplasia by GT in the injured carotids of Prx II−/− mice. The left carotid arteries in the Prx II−/− mice were injured using a flexible wire as previously described. After injury, the control vehicle or GT (0.2 μM) was externally infused through the surface of the adventitia with a strip of Whatman No.1 filter paper for 30 mins. The mice were recovered for 10 days post injury.
Representative HE-stained images are shown. Data in the graph are means ± SEM of intima versus the media ratio measured from HE-stained carotid artery sections (n=8 mice per group, *P<0.01). (D and E) Inhibition of neointimal hyperplasia by chaetocin and chetomin in balloon-injured carotids. The injured carotid arteries were incubated with chaetocin or chetomin (0.2 μM each). Representative HE-stained images are shown. Arrows indicate the thickened neointimal layer. Bars in the graph are means ± SEM of intima versus media ratio measured from HE-stained carotid samples (n=7 rats per group, *P<0.01).

**Figure 7.** Induction of re-endothelialization by ETPs in the injured carotid arteries. (A – C) Co-immunofluorescence staining of smooth muscle α-actin (SMA) and CD31 in the injured carotid arteries treated with control or ETPs as indicated. Typical endothelial monolayers are indicated in normal and ETP-treated carotid vessels (*Arrowheads*). Images shown are representatives of three independent samples. DAPI indicates nuclei. (D) Evans blue extravasation in balloon-injured carotid vessels treated with control and GT. Uninjured collateral carotid vessels are shown for comparison. The VEGFR2 inhibitor (SU5416) was incubated together with GT. A representative image is shown. Data in the graph are means ± SEM of the light absorbance values at 620 nm (n=7 rats per group, *P<0.01). (E) Scanning electron microscopic examination of luminal surfaces of balloon-injured carotid arterial vessels treated with control and GT. A representative image from three independent experiments is shown (*Boxed* indicates zoom-in area). (F) A schematic representation for the mode of ETP action in normal and injured arterial vasculature.
Figure 1
Figure 3
Figure 5
Figure 6
Figure 7
Figure 7, cont’d
Vascular Injury Involves the Overoxidation of Peroxiredoxin Type II and is Recovered by the Peroxiredoxin-Activity Mimetic that Induces Reendothelialization

Dong Hoon Kang, Doo Jae Lee, Jiran Kim, Joo Young Lee, Hyun-Woo Kim, Kihwan Kwon, W. Robert Taylor, Hanjoong Jo and Sang Won Kang

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Vascular injury involves the overoxidation of peroxiredoxin type II and is recovered by the
peroxiredoxin-activity mimetic that induces reendothelialization

Dong Hoon Kang, PhD¹*, Doo Jae Lee, PhD¹*, Jiran Kim, MS¹, Joo Young Lee, PhD¹, Hyun-
Woo Kim, PhD³, Kihwan Kwon, MD, PhD⁴, W. Robert Taylor, MD⁵, Hanjoong Jo, PhD⁶, and
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* These authors contributed equally to this work.
Supplemental Methods

Reagents

Gliotoxin, chaetocin, chetomin, and 2,4-dinitrochlorobenzene (DNCB) were purchased from Sigma-Aldrich. Auranofin was purchased from Alexis Biochemicals. Bis(methylthio)gliotoxin and anti-PDGFR-β (M-20) and KDR/Flk-1 (VEGFR2) antibodies were purchased from Santa Cruz Biotechnology. Anti-PLCγ1 (pY783), PLCγ1, and VEGFR2 (pY1175 and pY1059) antibodies were purchased from Cell Signaling Technology. Anti-phosphotyrosine (4G10) and PDGF-BB were from Upstate Biotechnology. VEGF-A (human VEGF165) was purchased from R&D systems. Anti-rat smooth muscle actin (CP47) was purchased from Calbiochem. Mouse anti-rat CD31 antibody was purchased from BD Bioscience. Alexa Fluor 488-conjugated donkey anti-mouse and Alexa Fluor 568-conjugated donkey anti-rabbit secondary antibodies were purchased from Invitrogen. Biotinylated goat anti-rabbit IgG, avidin-HRP, and DAB substrate were purchased from Vector Laboratories. Rabbit polyclonal antibodies against Prx I, Prx II, Prx-SO2/3, and PDGFRβ (pY857) were produced as previously described.1,2

The SMART pools of four siRNA duplexes for rat Prx I (Cat No. 095300), rat Prx II (Cat No. 089973), and rat Prx III (Cat No. 095706) from Dharmacon were used for the rat carotid balloon injury experiment. A firefly luciferase-specific siRNA from Dharmacon was used for control siRNA.
**Cell culture**

Human aortic ECs (HAEC) and human aortic SMCs (HASMC) were purchased from Clonetics-Bio Whittaker (Venders, Belgium) and grown on 0.1% gelatin-coated plate at 37°C in a humidified incubator containing 5% CO₂ in Endothelial Basal Medium (EBM™-2) and Smooth Muscle Cell Basal Medium (SmBM™) with 10% fetal bovine serum (FBS) and full supplements (Clonetics-BioWhittaker; Cat no. cc-4176 for HAEC and Cat no. cc-4149 for HASMC), respectively. They were used for the study in passages 5 to 7.

**Peroxidase activity assay**

A standard peroxidase assay was carried out in a 200 μl reaction mixture containing 250 μM NADPH, 3 μM yeast Trx, 1.5 μM yeast TR, 25 μM ETP, and 1.2 mM H₂O₂ in 50 mM Hepes-NaOH buffer (pH 7.0) containing 1 mM EDTA. For a glutathione-dependent peroxidase reaction, GSH (1 mM) and yeast GR (1 Unit) were added instead of yeast Trx and TR. The mixture (minus H₂O₂) was pre-incubated for 3 mins to allow the reduction of the oxidized ETP, and then the reaction was initiated by adding H₂O₂. The NADPH oxidation was monitored for 12 mins at 30°C by following absorbance reduction at 340 nm in an Agilent UV8453 spectrophotometer (Hewlett Packard, USA). The initial rate of reaction was calculated using the
linear portion of the curve and expressed as the amount of NADPH oxidized per minute.

**In vitro cell function assays**

For proliferation assay, the HAECs were seeded at a density of 4000 cells/well in a final volume of 100 μl onto 96-well plates containing siRNA-transfection reagent mixtures. After a 24-hr transfection, the cells were serum-starved for 18 hrs, and then placed in an EBM-2 basal medium supplemented with VEGF-A165 (25 ng/ml, R&D systems) for an additional 24 hrs. The extent of cell proliferation was measured using a WST-1 cell proliferation assay kit (Roche Diagnostics, USA). The cell number was expressed as absorbance at 450 nm, which was averaged from triplicate wells after subtracting the turbidity at 600 nm.

The migration assay was performed in 24-well Transwell culture chambers (Costar; 8-μm pore size). The bottom of the filter was coated with gelatin B (1 mg/ml) and air-dried for 1 hr. HAECs (6 × 10³) were added to the upper chambers, which contained transfection complexes. After 24 hrs, the transfected cells were serum-starved overnight. Solutions of VEGF-A (25 ng/ml) were prepared in basal media with 0.5% BSA and added to the bottom chambers. The upper chamber wells were filled with basal media containing 0.5% BSA. Transwell chambers were incubated at 37°C/5% CO₂ for 8 hrs. After incubation, the non-migrated cells were removed from the top of the filters, and the cells that migrated onto the bottom of filters were
fixed and stained with 0.6% hematoxylin and 0.5% eosin. The stained cells were photographed and counted. The number of migrating cells was averaged from triplicate wells.

The *in vitro* cell assays using HASMCs were essentially the same as above, except that PDGF-BB (25 ng/ml) was used as a stimulant.

**Immunoblot analysis**

Cells were rinsed once with ice-cold phosphate-buffered saline (PBS) and then lysed in an extraction buffer containing 20 mM Hepes (pH 7.0), 1% Triton X-100, 150 mM NaCl, 10% glycerol, 1 mM EDTA, 2 mM EGTA, 1 mM DTT, 5 mM Na$_3$VO$_4$, 5 mM NaF, 1 mM AEBSF, aprotinin (5 μg/ml), and leupeptin (5 μg/ml). After centrifugation at 12,000×g, the clarified cell extract was used for immunoblotting. When necessary, the membranes were stripped by shaking them for 60 mins at 37°C in 67 mM Tris (pH 6.7), 2% SDS, 100 mM 2-mercaptoethanol and reprobed with the appropriate pan antibody.

**TUNEL assay**

The paraffin sections were incubated in PBS containing 0.1% Triton X-100 for 10 mins. Then TUNEL reactions were performed for 60 mins at 37°C using *In Situ* Cell Death Detection Kit, Fluorescein (Roche Diagnostics Corp.), according to the manufacturer’s instructions. Cell nuclei
were counterstained with DAPI.

**Intracellular H$_2$O$_2$ measurement**

After stimulation, cells were washed with a phenol red-free basal medium and incubated with 5 μM 5,6-chloromethyl-2’,7’-dichlorodihydrofluorescein diacetate (CM-H$_2$DCFDA) (Molecular Probe) at 37°C for 5 mins. The fluorescence images were taken by a fluorescence microscope (Axiovert 200 Basic standard, Zeiss, Germany). The relative DCF fluorescence was calculated by averaging the fluorescence levels from 50 - 80 cells after subtracting the background fluorescence.

**NADPH oxidase activity**

Whole cell superoxide production was measured using an enhanced luminescence system (Diogenes, National Diagnostics). For the assay, serum-starved cells were pre-incubated with a 100 μl of Diogene reagent at 37°C for 5 mins. After stimulated by the indicated growth factor, chemiluminescence was detected every second for 10 mins with TD-20/20 Luminometer (Turner Biosystems).

**GT purification and quantification**
The HASMCs and HAECs (1 × 10⁵ cells each) were plated on 6-well plates and then treated for 2 hrs with 50 nM GT in 1-ml culture media. The cells were extracted with 500 μl methanol followed by centrifugation at 10,000×g. The extract was dried, dissolved in the 200 μl of 0.1% trifluoroacetic acid solution, and then separated by a reverse-phase high performance liquid chromatography (Agilent Series 1100 HPLC system with diode array). The samples (20 μl each) were injected onto a C₁₈ column (4.6×250 mm ID, 5μm spherical packing; Vydac) and eluted with an ascending gradient of acetonitril (5 ~ 50% for 20 mins) in 0.1 % trifluoroacetic acid at a flow rate of 1 ml/min. The GT-containing fractions were detected at 254 nm and its concentration in the extracts was determined on a standard curve (y=10.495x+ 177.86, R²=0.972) of peak area versus GT concentration that was constructed using standard GT solution (31.25, 62.5, 125, 250, 500 nM).

**Vascular permeability test**

Mice were injected intravenously with 100 μl of 5% Evans blue for 30 mins and then perfused with PBS for 5 mins. The common carotid arteries were removed from both uninjured collateral and injured ipsilateral vessels. They were dissected, opened longitudinally, and examined on a phase contrast microscope with a magnification of 20×. For quantification, extravasated Evans blue was extracted from the vessel fragments with formamide at 55°C overnight and centrifuged
for 10 mins at 12,000 rpm. The supernatant was collected and its absorbance was measured at 620 nm and normalized by subtracting the background values at 740 nm. For VEGFR2 inhibition, SU5416 (20 mg/kg) was injected intraperitoneally three times before and after balloon injury. Control injection was made of 200 μl of PBS containing vehicle (5% DMSO).

**Scanning Electron Microscopy**

The carotid vessels were taken from animals, opened longitudinally, and fixed with 2.5% glutaraldehyde for 24 hrs. The tissues were rinsed with PBS, incubated with 1% osmium tetroxide, and then dehydrated through a series of ethanol dilutions. The tissue was dried to a critical point and mounted on scanning electron microscopy stubs with colloidal silver paste. After being sputter-coated with gold/palladium, the specimens were examined with a scanning electron microscope (Hitachi, Japan).
Supplemental Figure I

A

Coronary Artery

03-31-08 + peptide  03-31-08  01-31-08  11-12-08  06-12-08  12-03-07

B

Carotid Artery

FC8 + peptide  FC8  10B5  FD3  7051  213C
Supplemental Figure II

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Supplemental Figure III

Reduced GT Standard

EC MeOH extract

SMC MeOH extract

Time (min)
Supplemental Figure V

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Ponceau S

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- Caveolin-1
- VEGFR2
- Prx II

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Red

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siRNA:
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- Prx II

Compounds:
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- NAC
- BHA
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- 250 μM
- 500 μM

VEGF:
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</table>
Supplemental Figure VIII

After injury

<table>
<thead>
<tr>
<th></th>
<th>0 day</th>
<th>5 day</th>
<th>7 day</th>
</tr>
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<td><img src="control_5.png" alt="Image" /></td>
<td><img src="control_7.png" alt="Image" /></td>
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<tr>
<td>GT</td>
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<td><img src="gt_5.png" alt="Image" /></td>
<td><img src="gt_7.png" alt="Image" /></td>
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50 µm

- Control
- GT

Prx-SO2/3 mean intensity

0 25 50 75 100

0 5 7 (Days)

N.S.
Supplemental Figure I. Hyperoxidation of 2-Cys Prxs in human coronary and carotid arterial sections with atherosclerotic lesions.

Immunohistochemistry was performed using anti-Prx-SO$_{2/3}$ antibody. A serial section was immuno-stained after blocking with the antigenic peptide as a negative control (+peptide). Each image was labeled with patient identification numbers. The letter “L” indicates the vascular lumen.

Supplemental Figure II. Cytotoxicity test of GT and chaetocin in vitro and in vivo.

(A and B) HASMCs (A) and HAECs (B) were serum-starved in basal medium containing 0.5% FBS and then pretreated for 2 hrs with the indicated concentrations of GT or chaetocin. The cells were collected and stained by trypan blue for checking viability. The live cells are presented as the percent of unstained cells versus total cells. Note: Presence of serum markedly reduced the GT cytotoxicity, which hence appeared above the micromolar range for HASMCs and HAECs (not shown).

(C and D) Effect of GT on NF-κB transcriptional activities. HASMCs (C) or HAECs (D) were infected with adenovirus encoding NF-κB-luciferase (Luc) sequence (The University of Iowa Gene Transfer Vector Core) for 24 hrs. The cells were pretreated with a control vehicle or GT
for 2 hrs in the presence of serum and then stimulated with the indicated ligands (15 ng/ml TNF-α) for 6 hrs. The relative luciferase activity was normalized to the β-galactosidase activity and is presented as mean ± S.D. of fold increase from three independent experiments (*P<0.01, **P<0.005, N.S. not significant).

Supplemental Figure III. Quantification of cellular GT concentration from HASMCs and HAECs.

As described in the Methods, the methanol extracts from the GT-treated SMCs and ECs were separated by a reverse-phase HPLC. The standard GT compound (asterick) was reduced with 5 mM dithiothreitol, injected into the C\textsubscript{18} column, and then eluted at 36.2% acetonitrile in 0.1% trifluoroacetic acid solution. The experiments were repeated twice with same results.

Supplemental Figure IV. Effect of GT and chaetocin on NADPH oxidase activity in HASMCs and HAECs.

HASMCs (A) and HAECs (B) were serum-starved in a basal medium containing 0.5% FBS and then pretreated for 30 mins with various concentrations of GT or chaetocin. Cells were treated with PDGF-BB or VEGF-A for additional 10 mins. Recombinant superoxide dismutase (SOD, 300 U/ml) or diphenyliodonium (DPI, 10 μM) was added into the culture media 10 mins before
growth factor stimulation. Data are means ± SD of the percent of superoxide amount in stimulated cells versus unstimulated cells ($n=3$, *$P<0.05$, **$P<0.001$, N.S. not significant).

Supplemental Figure V. Effect of antioxidant compounds on VEGF signaling in HAECs.

(A) HAECs were transfected with Prx II siRNA and then pretreated with or without GT. The caveolae fractions were isolated from HAECs by a combination of bicarbonate-buffered extraction and sucrose-gradient centrifugation (left panel), as previously described 3. Fraction numbers and sucrose concentrations are indicated. Ponceau S-stained membrane is shown for protein loading. Representative fractions for caveolae (#4) and non-caveolae (#11) were used for analyzing the VEGFR2 oxidation on non-reducing denaturing gels (right panel). The fast- and slow-migrating bands for VEGFR2 represent the oxidized (Oxi) and reduced (Red) forms, respectively. NR, non-reducing gel; R, reducing gel.

(B) HAECs were transfected with Prx II siRNA and then pretreated with increasing concentrations of the indicated compounds (NAC, N-acetylcysteine; BHA, butylated hydroxyanisole) for 2 hrs. The cells were treated with VEGF for 10 mins and then analyzed by immunoblotting. A representative blot from three experiments is shown.

Supplemental Figure VI. TR inhibitors abrogate the GT effect on PDGF and VEGF
signaling.

HASMCs and HAECs were transfected with the Prx II siRNA for 24 hrs and then serum-starved for 18 hrs. The serum-starved cells were pretreated with a control vehicle (DMSO) or 2,4-dinitrochlorobenzen (DNCB) or auranofin for 1 hr, followed by the GT treatment. The SMCs and ECs were stimulated with PDGF-BB and VEGF-A, respectively, for 10 mins. Activation of PDGFRβ and VEGFR2 were analyzed using phospho-specific antibodies as indicated. The Prx I blot is shown for equal loading. A representative blot from three experiments is shown.

Supplemental Figure VII. Immunofluorescence staining of PDGFRβ and VEGFR2 phosphorylation in the injured carotid vessels.

The injured rat carotid vessels treated with or without GT were subjected to the immunofluorescence staining for PDGFRβ and VEGFR2 phosphorylation using anti-PDGFRβ-pY857 and anti-VEGFR2-pY1059 antibodies, respectively. Smooth muscle actin (SMA) and CD31 were co-stained in serial tissue sections as control for neointimal layer (Arrows) and endothelial monolayer (Arrowheads), respectively. Images shown are representatives of three independent samples. The phospho-stained images were merged with DAPI (Nuclei). The graphs show the average intensity of p-PDGFRβ+ or p-VEGFR2+ pixels per 20×20 μm² field (n=3, P<0.01).
Supplemental Figure VIII. GT does not block the 2-Cys Prx hyperoxidation in balloon-injured carotid arteries.

Immunohistochemistry was performed using anti-hyperoxidized 2-Cys Prx (Prx-SO$_{2/3}$) antibody as described in Methods. The DAB-stained images are representatives of five independent carotid samples. Data in the graph are means ± SD of mean intensities of the PrxII-SO$_{2/3}$-positive signal per cell ($n=5$ per group). No a significant difference between the control and GT-treated samples were observed.

Supplemental Figure IX. Inhibition of re-endothelialization by the Prx II knockdown.

The balloon-injured carotid vessels were transfected with control and Prx II siRNA and recovered for 7 days, as in Fig. 1D. The 0 day represents no recovery. Vascular permeability was measured by Evans blue extravasation as described in Methods. Uninjured collateral carotid vessels are shown for comparison. A representative image is shown. Data in the graph are means ± SEM of the light absorbance values at 620 nm ($n=6$ per group, *$P<0.05$).
Supplemental References

