

# $\beta$ -Sitosterol oxidation products attenuate vasorelaxation by increasing reactive oxygen species and cyclooxygenase-2

Chao Yang<sup>1</sup>, Zhen Yu Chen<sup>1</sup>, Siu Ling Wong<sup>2</sup>, Jian Liu<sup>2</sup>, Yin Tong Liang<sup>1</sup>, Chi Wai Lau<sup>2</sup>, Hung Kay Lee<sup>3</sup>, Yu Huang<sup>2</sup>, and Suk Ying Tsang<sup>1\*</sup>

<sup>1</sup>School of Life Sciences, The Chinese University of Hong Kong, Hong Kong SAR, China; <sup>2</sup>Institute of Vascular Medicine, Li Ka Shing Institute of Health Sciences, School of Biomedical Sciences, Hong Kong SAR, China; and <sup>3</sup>Department of Chemistry, The Chinese University of Hong Kong, Hong Kong SAR, China

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**Aims**  $\beta$ -Sitosterol has become a popular cholesterol-lowering functional food product worldwide.  $\beta$ -Sitosterol can be oxidized to  $\beta$ -sitosterol oxidation products (SOPs) during food processing. Little is known about the impact of SOPs and  $\beta$ -sitosterol on the functionality of arteries. This study investigated the effects of SOPs and  $\beta$ -sitosterol on vasorelaxation and the possible cellular mechanisms involved.

**Methods and results** By isometric tension measurement, SOPs but not  $\beta$ -sitosterol blunted relaxation induced by acetylcholine or  $\text{Ca}^{2+}$  ionophore A23187 in endothelium-intact aortae. SOPs-impaired vasorelaxation was completely reversed by cyclooxygenase (COX)-2 inhibitor DuP-697, whereas the reversal by COX-1 inhibitor SC-560 was only partial. Western blotting and immunohistochemistry showed that SOPs increased the protein expression of COX-2 but not COX-1 in the endothelium. Using dihydroethidium staining and electron paramagnetic resonance spin trapping techniques, SOPs were found to elevate the level of reactive oxygen species in rat aortic endothelial cells, and the effects were reversed by antioxidants tempol, tiron, or diphenylene iodonium. Consistently, these antioxidants reversed SOPs-induced impairment of endothelium-dependent relaxation. Up-regulation of COX-2 expression by SOPs was also reversed by tempol. Moreover, SOPs attenuated nitric oxide donor sodium nitroprusside-induced relaxation in endothelium-intact, but not endothelium-denuded rings, confirming that SOPs act on the endothelium. Interestingly, a thromboxane-prostanoid (TP) receptor blocker S18886 reversed SOPs-impaired vasorelaxation, suggesting the involvement of TP receptor in mediating the downstream effect. SOPs decreased cGMP production, and the effect could be reversed by inhibiting COX-2 or TP receptor.

**Conclusion** This study provides novel experimental evidence showing the harmful effects of SOPs on endothelial function.

**Keywords** Beta-sitosterol • Beta-sitosterol oxidation products • Endothelium • Reactive oxygen species • Cyclooxygenase-2

## 1. Introduction

Phytosterols, the membrane constituents of plants, are present at high concentrations in vegetable oils, nuts, seeds, and grains. Phytosterols are structurally similar and functionally analogous to cholesterol.<sup>1,2</sup> Phytosterols can lower blood cholesterol by reducing the absorption of cholesterol,<sup>1,3</sup> which is one of major risk factors for the development of cardiovascular diseases.<sup>4–7</sup> Previous studies suggested that a daily intake of 1.5–3 g phytosterols could reduce 9–15% of the

serum low-density lipoprotein cholesterol level.<sup>8–11</sup> Owing to this health benefit, phytosterols have been used in the production of various functional foods.<sup>1,12–14</sup> Among different phytosterols, the most abundant one in nature is  $\beta$ -sitosterol.<sup>15–17</sup>  $\beta$ -Sitosterol shares the four-ring structure common to cholesterol and differs from cholesterol by having an extra ethyl group attached at C-24.<sup>2</sup>

Owing to the structural similarity, phytosterol is likely to be as susceptible as cholesterol to oxidation. Previous studies showed that phytosterol oxides are formed when plant oils are subjected to

\* Corresponding author. Tel: +852-39431020; fax: +852-26037732, Email: fayetsang@cuhk.edu.hk

heat treatment or under long-term storage.<sup>2,18</sup> Phytosterols can undergo oxidation in food-processing conditions, generating 7-hydroxysterols, 7-ketosterols, 5,6-epoxysterols, triols, and 25-hydroxysterols as the major oxidation products.<sup>2,19–21</sup> The amount of phytosterol oxides in vegetable oils increases after frying for 2 days<sup>18</sup> and French fries fried in vegetable oil was reported to contain higher concentrations of phytosterol oxides than those before frying.<sup>22</sup> There were also reports showing that  $\beta$ -sitosterol oxidation products (SOPs) can be formed in plants by biological procedures.<sup>22,23</sup> Indeed, commercially available phytosterol-enriched margarine contains  $\sim 0.1\%$  of phytosterol oxides.<sup>22</sup> With a daily intake of 2–4 g phytosterols, it is estimated that 2–4 mg phytosterol oxides could be ingested.<sup>24,25</sup> In fact, this quantity of phytosterol oxides ingested is similar to the quantities of cholesterol oxidation products (COPs) (3–4 mg) presented in the daily diets of people as documented in a study in the Netherlands and New Zealand.<sup>26</sup> Phytosterol oxides were found to be well-absorbed and accumulate in the body.<sup>22</sup> Since *in vitro* studies showed that the microsomal hydroxylase in the rat liver subcellular fractions can convert  $\beta$ -sitosterol into  $\beta$ -sitosterol epoxide,<sup>27</sup> similar process may occur *in vivo*. Indeed, phytosterol oxides are present in the plasma from healthy human subjects,<sup>28,29</sup> patients with phytosterolaemia<sup>28</sup> or patients with Waldenström macroglobulinaemia.<sup>30</sup>

Given that  $\beta$ -sitosterol is the most abundant phytosterol and that  $\beta$ -sitosterol exhibits a similar oxidation pattern as that of cholesterol in terms of oxidation products,<sup>2,31</sup> both SOPs and COPs are important constituents of our daily diets. Several studies investigated the effect of COPs on the vascular function. 7-Ketocholesterol induces apoptosis in rabbit vascular smooth muscle cells<sup>32</sup> and 7 $\beta$ -hydroxycholesterol and 7-ketocholesterol cause cell death and apoptosis vs. necrosis in cells of the human vascular wall.<sup>33</sup> Our recent investigation also reports that COPs attenuate endothelium-dependent relaxation of rat aortae.<sup>34</sup>

However, little is known about the effects of SOPs and  $\beta$ -sitosterol on the functionality of blood vessels. The main objective of this study was therefore to examine the effects of SOPs and  $\beta$ -sitosterol on vasorelaxation and the possible cellular mechanisms involved.

## 2. Methods

An expanded Materials and Methods section can be found in Supplementary material online.

### 2.1 Preparation and identification of SOPs

SOPs were prepared by heat treatment at 180°C. SOPs were identified by gas chromatography (GC)-mass spectrometry (MS). Individual SOPs were identified according to their relative retention times and specific characteristics of mass spectra ions as previously published.<sup>35–37</sup> The details of the preparation and identification of SOPs can be found in the Supplementary material online, Materials and Methods section.

### 2.2 Animals

#### 2.2.1 Aortic ring preparation

This study was approved by the Animal Ethics Committee, Chinese University of Hong Kong and conformed with the *Guide for the Care and Use of Laboratory Animals* published by the United States National Institutes of Health (NIH Publication, 8th Edition, 2011). Male Sprague–Dawley rats (260–280 g) were euthanized by CO<sub>2</sub> inhalation. Blood vessels were prepared as described<sup>38</sup> and the details were presented in the Supplementary material online, Materials and Methods section.

### 2.2.2 Isometric force measurement

Isometric tensions were recorded with force transducer connected to a Maclab analogue-to-digital converter system. Thirty min after mounting in organ baths, all rings were first contracted by 60 mM K<sup>+</sup> to test their contractile capacity. After washing twice, the rings were then contracted by 0.3  $\mu$ M phenylephrine followed by the addition of 3  $\mu$ M acetylcholine (ACh) to test the integrity of the endothelium (over 80% relaxation) or functional removal of the endothelium (no relaxation). All rings were subsequently rinsed in pre-warmed, oxygenated Krebs solution several times until baseline tone was restored and allowed to equilibrate for 30 min. Each experiment was carried out on rings prepared from different rats. Drugs or solvent, when used, was incubated with rings for either 30 min or 2 h depending on experiments prior to the addition of phenylephrine.

### 2.3 Western blotting

Isolated aortae were incubated with SOPs (30  $\mu$ g/mL) or solvent for 60 min. Some rings were pre-treated with tempol (30  $\mu$ M) before exposure to SOPs. In some experiments, primary endothelial cells were used. After treatment, rings or cells were frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  for later processing. Expressions of cyclooxygenase (COX)-1, COX-2, and housekeeping protein glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were determined by Western blot analysis (for details, see Supplementary material online, Materials and Methods section).

### 2.4 Immunohistochemical staining

Localization of COX-2 in the rat aorta and the effect of SOPs treatment on COX-2 expression were determined by immunohistochemistry. Details of the procedure involved in immunohistochemical staining were presented in the Supplementary material online, Materials and Methods section.

### 2.5 Primary culture of rat aortic endothelial cells

Endothelial cells were cultured from rat thoracic aortae as described.<sup>38</sup> Details of the procedure were presented in the Supplementary material online, Materials and Methods section. The cells were used within the first two passages. For transfection experiment, the cells were transfected with NOX4 short interfering RNA (siRNA) pool (SMARTpools, Thermo Scientific, Lafayette, CO, USA) or non-targeting siRNA as a control by electroporation using Nucleofector II machine (Amaxa/Lonza, Walkersville, MD, USA) according to the manufacturer's instruction.

### 2.6 Measurement of reactive oxygen species by dihydroethidium and electron paramagnetic resonance spin trapping

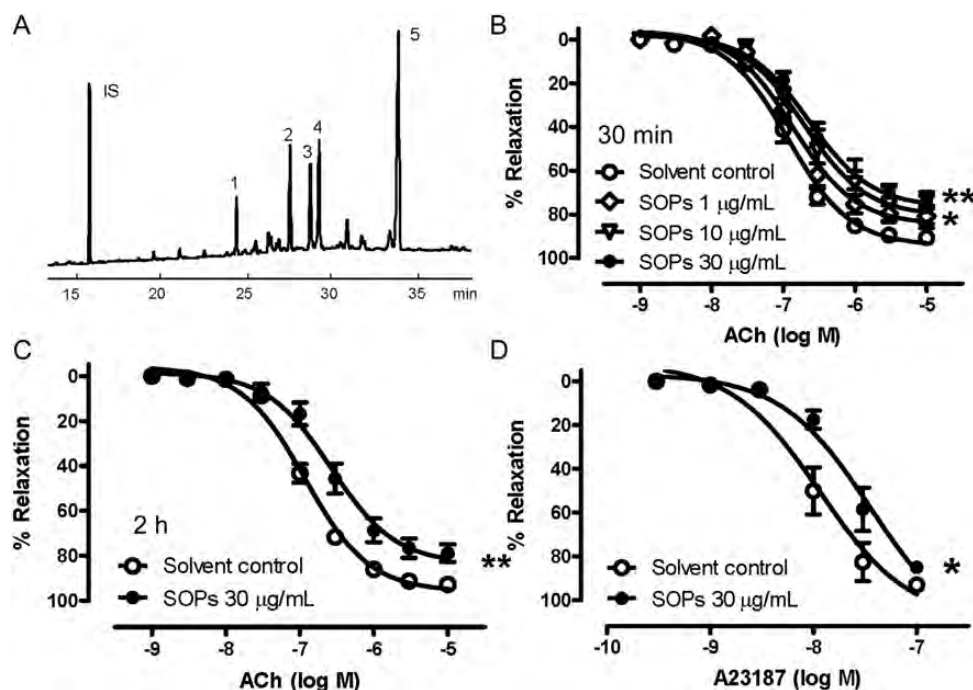
The intracellular level of reactive oxygen species (ROS) in primary rat aortic endothelial cells were determined by dihydroethidium (DHE) (Molecular Probes, Invitrogen) and electron paramagnetic resonance (EPR) spin trapping as described in the Supplementary material online, Materials and Methods section.

### 2.7 Measurement of nitric oxide

The intracellular level of nitric oxide in primary culture of rat aortic endothelial cells was determined by DAF-FM diacetate (Molecular Probes, Invitrogen) using confocal microscopy. Details were described in the Supplementary material online, Materials and Methods section.

### 2.8 Measurement of cyclic GMP

The levels of cyclic GMP (cGMP) in aortic tissues were measured by the direct cGMP ELISA kit (Enzo Life Sciences, Farmingdale, NY, USA) according to the manufacturer's instruction. In a myograph filled with oxygenated Krebs solution at 37°C, aortic rings were incubated with vehicle



**Figure 1** (A) GC spectrum of the main SOPs. IS, internal standard (5 $\alpha$ -cholestane), 1: 7 $\alpha$ -hydroxysitosterol, 2: 7 $\beta$ -hydroxysitosterol, 3: 5,6 $\beta$ -epoxysitosterol, 4: 5,6 $\alpha$ -epoxysitosterol, 5: 7-ketositosterol. (B) ACh-induced relaxation when the rat aortae were incubated with different concentrations of SOPs (1–30  $\mu$ g/mL) for 30 min. SOPs attenuated ACh-induced relaxation in a concentration-dependent manner. (C) ACh-induced relaxation in the absence or presence of SOPs (30  $\mu$ g/mL) for 2 h. Increasing the incubation time of SOPs from 30 min to 2 h did not further attenuate ACh-induced relaxation. (D) A23187-induced relaxation in the absence or presence of SOPs (30  $\mu$ g/mL) for 30 min. SOPs attenuated A23187-induced relaxation. Values are mean  $\pm$  SEM of five to nine experiments. \* $P$  < 0.05, \*\* $P$  < 0.01 vs. solvent control group.

or SOPs for 1 h and were then challenged by 1  $\mu$ M sodium nitroprusside (SNP) for 3 min before being removed from solutions, frozen and stored at  $-80^{\circ}\text{C}$  until assay. The result was expressed as cGMP production in pmol per mg protein.

## 2.9 Drugs

Chemicals and drugs used in this investigation can be found in the Supplementary material online, Materials and Methods section.

## 2.10 Data analysis

Data are mean  $\pm$  SEM of  $n$  rings from different rats. pEC<sub>50</sub> is the negative logarithm of the drug concentration needed to cause 50% of the maximal response (relaxation or contraction) ( $E_{\text{max}}$ ) as determined by nonlinear regression curve fitting (Graphpad Prism Software, version 5.0). Student's  $t$ -test (two-tailed) was used when two groups of means were compared. One-way ANOVA followed by the Bonferroni *post hoc* test were used when more than two groups were compared.  $P$  < 0.05 was considered statistically significant.

## 3. Results

### 3.1 GC-MS identification and the components of SOPs

Heat treatment of  $\beta$ -sitosterol produced SOPs. SOPs were identified by both GC (Figure 1A) and GC-MS (see Supplementary material online, Figure S1). The amount of 7-ketositosterol (peak 5) is the highest in SOPs, accounting for 41.8% of the total components.

7 $\alpha$ -hydroxysitosterol (peak 1), 7 $\beta$ -hydroxysitosterol (peak 2), 5,6 $\beta$ -epoxysitosterol (peak 3), and 5,6 $\alpha$ -epoxysitosterol (peak 4) accounted for 6.6, 13.6, 11.8, and 14.1%, respectively.

### 3.2 SOPs but not $\beta$ -sitosterol impaired ACh- or A23187-induced relaxations

Treatment with SOPs for 30 min attenuated ACh-induced endothelium-dependent relaxation in a concentration-dependent manner (Figure 1B, Table 1). Prolonged incubation (120 min) with SOPs did not cause further impairment in ACh-induced relaxation (Figure 1C, Table 1). Likewise, SOPs also reduced A23187-induced relaxations in aortae with endothelium (Figure 1D, Table 1). In contrast, the concentration-dependent relaxations to ACh were not affected by  $\beta$ -sitosterol (see Supplementary material online, Figure S2).

### 3.3 Inhibition of COX-2-thromboxane-prostanoid (TP) receptor pathway reversed SOPs-induced impairment in relaxation

Treatment with indometacin (1  $\mu$ M), a non-selective COX inhibitor, rescued the SOPs-impaired relaxation (Figure 2A, Table 1). Further examination showed that DuP-697 (a selective COX-2 inhibitor, 0.3  $\mu$ M) completely restored the SOPs-induced impairment in relaxation (Figures 2B, Table 1). In contrast, SC-560 (a selective COX-1 inhibitor, 0.3  $\mu$ M) only slightly alleviated the impaired relaxation

**Table 1** pEC<sub>50</sub> and E<sub>max</sub> (%) of various treatment on vasorelaxation

Treatment	pEC <sub>50</sub>	E <sub>max</sub>	n
ACh-induced relaxation after 30 min treatment with SOPs			
Solvent control	7.01 ± 0.06	90.76 ± 1.93	7
SOPs 1 µg/mL	6.86 ± 0.07	80.91 ± 3.46*	6
SOPs 10 µg/mL	6.70 ± 0.15	79.11 ± 7.29	5
SOPs 30 µg/mL	6.65 ± 0.08**	78.64 ± 3.15**	7
ACh-induced relaxation after 2 h treatment with SOPs			
Solvent control	6.93 ± 0.04	92.73 ± 1.73	9
SOPs 30 µg/mL	6.58 ± 0.08**	78.85 ± 3.85**	7
A23187-induced relaxation after 30 min treatment with SOPs			
Solvent control	7.94 ± 0.13	93.46 ± 1.09	6
SOPs 30 µg/mL	7.45 ± 0.13*	87.51 ± 1.99*	8
ACh-induced relaxation after 30 min treatment with β-sitosterol			
Solvent control (10 µL)	6.96 ± 0.05	91.77 ± 1.83	10
β-Sitosterol 1 µg/mL	6.70 ± 0.14	95.07 ± 7.18	4
β-Sitosterol 10 µg/mL	6.90 ± 0.10	88.49 ± 1.56	4
Solvent control (30 µL)	6.94 ± 0.06	94.75 ± 1.20	7
β-Sitosterol 30 µg/mL	6.83 ± 0.12	91.84 ± 4.60	5
ACh-induced relaxation after drug treatment for 30 min			
SOPs 30 µg/mL	6.65 ± 0.08	78.64 ± 3.15	7
SOPs 30 µg/ mL + indomethacin 1 µM	7.01 ± 0.08###	90.40 ± 3.92#	7
SOPs 30 µg/mL + SC-560 0.3 µM	7.00 ± 0.11#	78.55 ± 8.20	4
SOPs 30 µg/mL + DuP-697 0.3 µM	7.03 ± 0.09###	92.12 ± 2.79###	7
SOPs 30 µg/mL + S18886 0.1 µM	7.08 ± 0.12#	93.05 ± 4.63#	4
SOPs 30 µg/mL + tempol 30 µM	6.91 ± 0.07#	86.14 ± 1.93###	5
Solvent control	6.95 ± 0.09	102.95 ± 1.51	3
SOPs 30 µg/mL	6.66 ± 0.14	74.13 ± 4.00**	4
SOPs 30 µg/mL + tiron 100 µM	6.75 ± 0.08	87.25 ± 1.96#	3
SOPs 30 µg/mL + DPI 1 nM	7.10 ± 0.07#	91.66 ± 2.74#	4
SNP-induced relaxation after drug treatment for 30 min in endothelium-intact rings			
Solvent control	7.88 ± 0.08	101.86 ± 2.90	6
SOPs 30 µg/mL	8.24 ± 0.14*	74.76 ± 5.78**	4
SOPs 30 µg/mL + S18886 0.1 µM	8.40 ± 0.05	98.74 ± 3.34#	4
SOPs 30 µg/mL + DuP-697 0.3 µM	8.19 ± 0.06	98.46 ± 1.90#	5
SNP-induced relaxation after drug treatment for 30 min in endothelium-denuded rings			
Solvent control	8.11 ± 0.09	100.09 ± 2.51	6
SOPs 30 µg/mL	8.08 ± 0.09	100.08 ± 2.74	5

Significant difference between control and treatment groups is indicated by \**P* < 0.05, \*\**P* < 0.01, \*\*\**P* < 0.001 vs. solvent control; #*P* < 0.05, ###*P* < 0.01 vs. SOPs 30 µg/mL. Data are mean ± SEM of *n* experiments.

### 3.4 SOPs elevated endothelial COX-2 expression

Western blot analysis showed that SOPs increased the COX-2 expression in aortae with the endothelium ( $1.77 \pm 0.30$  vs.  $1.00 \pm 0.23$  in the control group, *P* < 0.05) (Figure 3A). On the contrary, SOPs did not alter COX-1 expression ( $0.96 \pm 0.06$  vs.  $1.00 \pm 0.06$  in the control group) (Figure 3B). The COX-2 expression level was not elevated in aortae of which the endothelium was removed after incubation ( $0.74 \pm 0.17$  vs.  $0.88 \pm 0.23$  for the endothelium-denuded group without SOPs vs. the endothelium-denuded group with SOPs, *P* > 0.05) (Figure 3C). Consistently, SOPs increased COX-2 expression in primary rat aortic endothelial cells ( $1.43 \pm 0.06$  vs.  $1.00 \pm 0.07$  in the control group, *P* < 0.05) (Figure 3D). Immunohistochemical staining also revealed that the expression of endothelial COX-2 was augmented in aortae treated with SOPs, whereas that of endothelial COX-1 was not altered upon SOPs treatment (see Supplementary material online, Figure S3).

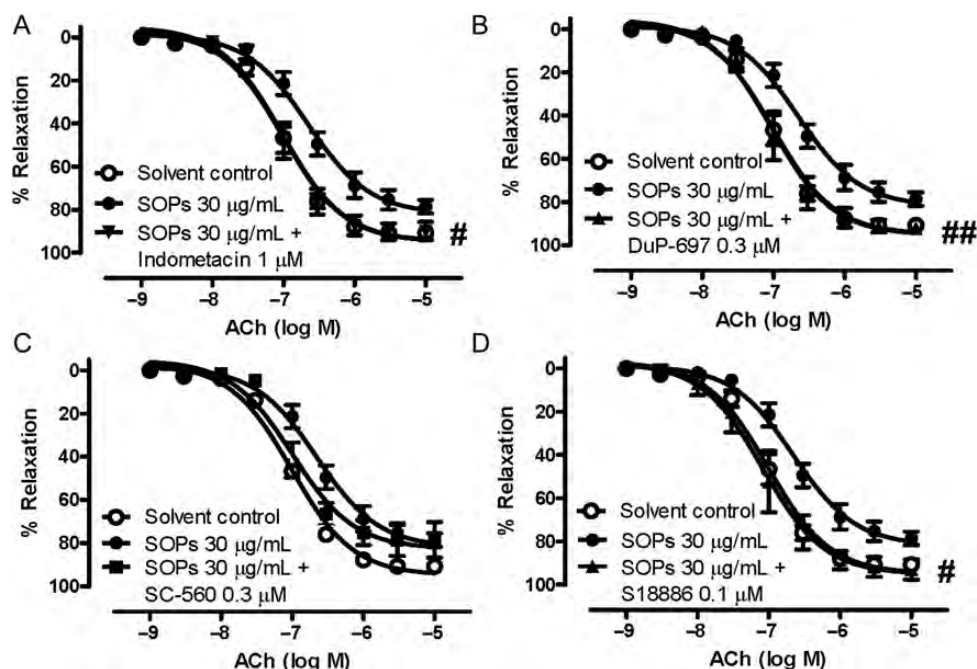
### 3.5 SOPs increased NADPH oxidase-derived ROS

SOPs (30 µg/mL) induced an early-on elevation in intracellular oxidative stress as reflected by a significant increase in DHE fluorescence in endothelial cells treated with SOPs for 10 min (see Supplementary material online, Figure S4). This SOPs-stimulated ROS level peaked at 15 min (see Supplementary material online, Figure S4), which was inhibited by the both the superoxide dismutase mimetic tempol (Figure 4A) and the free radical scavenger tiron (Figure 4B). Similarly, SOPs-stimulated increase in ROS was inhibited by NADPH oxidase inhibitor diphenylene iodonium (DPI) (Figure 4B). Consistent with the change in the ROS level as detected in the endothelial cells, tempol, tiron, and DPI reversed the attenuated ACh-induced relaxation in SOPs-treated aortic rings with the endothelium (Figures 4C–E and Table 1).

To examine the source of intracellular ROS stimulated by SOPs treatment, EPR spin trapping by 1-hydroxy-2,2,6,6-tetramethyl-4-oxo-piperidine hydrochloride (TEMPONE H) was used to detect superoxide anion generation (Figure 5A and B). SOPs were found to stimulate the production of superoxide anion in endothelial cells, and this SOPs-induced superoxide anions generation was inhibited by NADPH oxidase inhibitor DPI (Figures 5A and B). The results suggested that NADPH oxidase was the source of ROS. On the other hand, SOPs *per se* without any cell incubation did not generate superoxide anions (see Supplementary material online, Figure S5).

Both DHE measurement and EPR spin trapping experiment showed that SOPs-stimulated ROS could be inhibited by NADPH oxidase inhibitor DPI. On the other hand, many previous studies indicated that NOX4 is the most abundant NADPH oxidase isoform in endothelial cells of different species, including humans and rats.<sup>39–42</sup> To investigate whether NOX4 is involved in the generation of ROS by SOPs, RNA interference technology was used to knockdown the expression of NOX4 in rat aortic endothelial cells. siRNA against NOX4 reduced the expression of NOX4 expression in endothelial cells (see Supplementary material online, Figure S6). SOPs-induced ROS production was reduced after knockdown of NOX4 (Figure 5C), suggesting that NOX4 is the source of ROS production induced by SOPs.

(Figure 2C, Table 1). S18886 (0.1 µM), a thromboxane-prostanoid receptor (TP) receptor antagonist, also normalized the SOPs-induced inhibition of ACh-induced relaxation (Figure 2D and Table 1).



**Figure 2** Effect of (A) indometacin (1  $\mu\text{M}$ ), (B) DuP-697 (0.3  $\mu\text{M}$ ), (C) SC-560 (0.3  $\mu\text{M}$ ), and (D) S18886 (0.1  $\mu\text{M}$ ) on SOPs-induced impairment on ACh-induced relaxations. Indometacin, DuP-697, and S18886 completely reversed, whereas SC-560 partially reversed the effects of SOPs, suggesting that SOPs acted through a COX-2- and TP-receptor-mediated pathway. Values are mean  $\pm$  SEM of 4–7 experiments. # $P < 0.05$ , ## $P < 0.01$  vs. SOPs group.

### 3.6 SOPs increased endothelial COX-2 expression via an oxidative stress-sensitive pathway

To further investigate the possible association between SOPs-induced up-regulation of COX-2 and stimulated ROS production, Western blotting was performed to determine whether tempol could reverse the up-regulation of COX-2 induced by SOPs. Indeed, tempol normalized the SOPs-induced up-regulation of COX-2 expression ( $0.82 \pm 0.02$  vs.  $1.52 \pm 0.18$  for the SOPs and the tempol-cotreated group vs. the SOPs-treated group,  $P < 0.05$ ) (Figure 6), indicating that the increase in endothelial COX-2 expression by SOPs is oxidative stress sensitive.

### 3.7 SOPs did not affect the nitric oxide production in the endothelium

ACh (10  $\mu\text{M}$ ) triggered nitric oxide production in primary culture of rat endothelial cells (see Supplementary material online, Figure S7). SOPs exerted no effect on the production of nitric oxide (see Supplementary material online, Figure S7).

### 3.8 SOPs impaired SNP-induced relaxations in aortae with intact endothelium; this impairment could be reversed by COX-2 or TP receptor inhibitors

Treatment with SOPs attenuated SNP-induced relaxation in endothelium-intact rings (Figure 7A and Table 1) but not in endothelium-denuded rings (Figure 7B and Table 1). COX-2 inhibitor

DuP-697 (0.3  $\mu\text{M}$ ) or TP receptor inhibitor S18886 (0.1  $\mu\text{M}$ ) restored the impaired vasorelaxation induced by SOPs (Figures 7C and D, Table 1). Likewise, treatment with U46619 (1 nM), a widely adopted TP receptor agonist, attenuated SNP-induced relaxation (see Supplementary material online, Figure S8A). As expected, this attenuated relaxation by U46619 was not affected by DuP-697 (see Supplementary material online, Figure S8B) but was reversed by S18886 (see Supplementary material online, Figure S8C).

### 3.9 SOPs decreased the production of cGMP, which was reversed by pre-incubation with COX-2 or TP receptor inhibitors

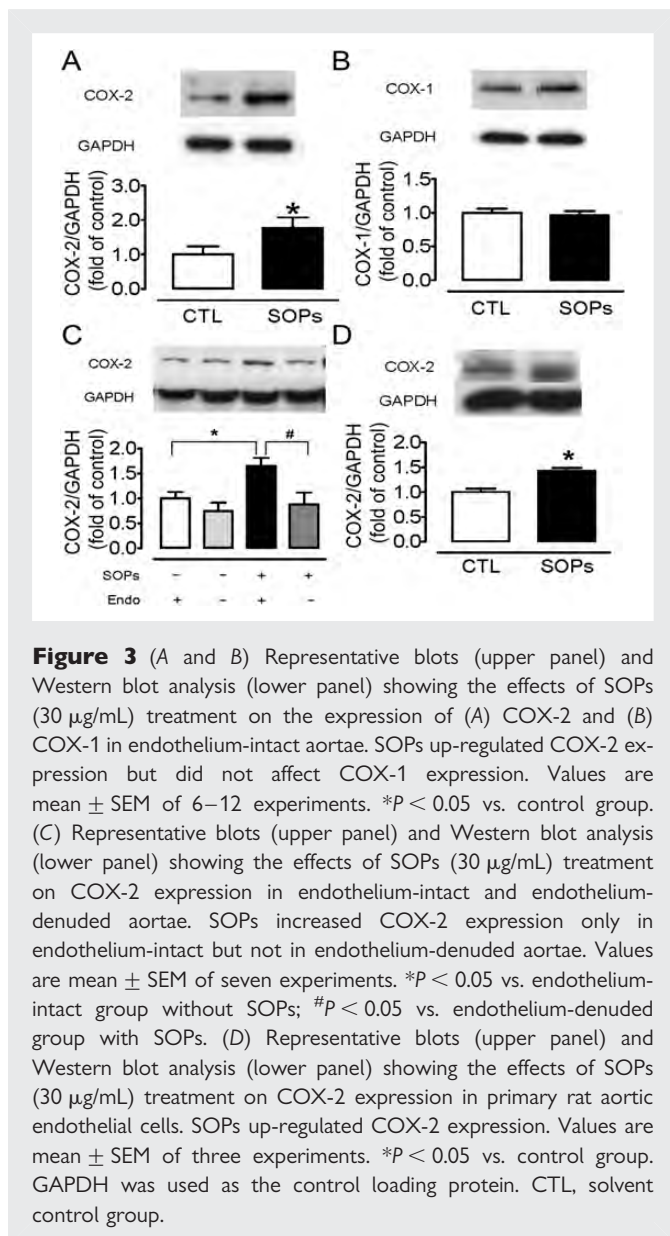
SOPs (30  $\mu\text{g}/\text{mL}$ ) markedly reduced the stimulatory effect of 1  $\mu\text{M}$  SNP on the production of cGMP when compared with the solvent control in rat aortae (Figure 7E). Treatment with S18886 (0.1  $\mu\text{M}$ ) or DuP-697 (0.3  $\mu\text{M}$ ) reversed the effect of SOPs on cGMP production (Figure 7E).

### 3.10 SOPs did not affect cGMP analogue-induced relaxations

Treatment with SOPs for 30 min did not affect 8-pCPT-cGMP-induced relaxation in endothelium-intact aortic rings (see Supplementary material online, Figure S9).

## 4. Discussion

The present study provides evidence for the impairment effect of SOPs on the functionality of rat aorta. Main findings of the present



**Figure 3** (A and B) Representative blots (upper panel) and Western blot analysis (lower panel) showing the effects of SOPs (30  $\mu\text{g}/\text{mL}$ ) treatment on the expression of (A) COX-2 and (B) COX-1 in endothelium-intact aortae. SOPs up-regulated COX-2 expression but did not affect COX-1 expression. Values are mean  $\pm$  SEM of 6–12 experiments. \* $P < 0.05$  vs. control group. (C) Representative blots (upper panel) and Western blot analysis (lower panel) showing the effects of SOPs (30  $\mu\text{g}/\text{mL}$ ) treatment on COX-2 expression in endothelium-intact and endothelium-denuded aortae. SOPs increased COX-2 expression only in endothelium-intact but not in endothelium-denuded aortae. Values are mean  $\pm$  SEM of seven experiments. \* $P < 0.05$  vs. endothelium-intact group without SOPs; # $P < 0.05$  vs. endothelium-denuded group with SOPs. (D) Representative blots (upper panel) and Western blot analysis (lower panel) showing the effects of SOPs (30  $\mu\text{g}/\text{mL}$ ) treatment on COX-2 expression in primary rat aortic endothelial cells. SOPs up-regulated COX-2 expression. Values are mean  $\pm$  SEM of three experiments. \* $P < 0.05$  vs. control group. GAPDH was used as the control loading protein. CTL, solvent control group.

investigation include: (i) SOPs but not  $\beta$ -sitosterol attenuated endothelium-dependent relaxation; (ii) SOPs-mediated attenuation of endothelium-dependent relaxation were reversed by scavenging ROS/inhibiting ROS production, by inhibiting COX-1 or COX-2, or by inhibiting TP receptor; (iii) SOPs increased ROS production in the endothelial cells via a NOX4-sensitive pathway; (iv) SOPs increased the expression of COX-2 in the endothelium which was reversed by scavenging ROS production; (v) SOPs attenuated nitric oxide donor-induced vasorelaxation and cGMP production, which could be reversed by inhibiting COX-2 or TP receptor.

For the first time, the present study demonstrated the effect of SOPs and  $\beta$ -sitosterol on the functionality of arteries. SOPs, but not  $\beta$ -sitosterol, were found to impair endothelium-dependent relaxation of rat aorta. Indeed, previous reports suggested that the oxidized forms of phytosterols or cholesterol were more cytotoxic than the non-oxidized form to various cell lines.<sup>43,44</sup> SOPs increased superoxide anion production<sup>12</sup> and decreased cellular glutathione.<sup>45</sup> This reduction in glutathione was accompanied by an increase in apoptosis which was not observed when cells were subjected to

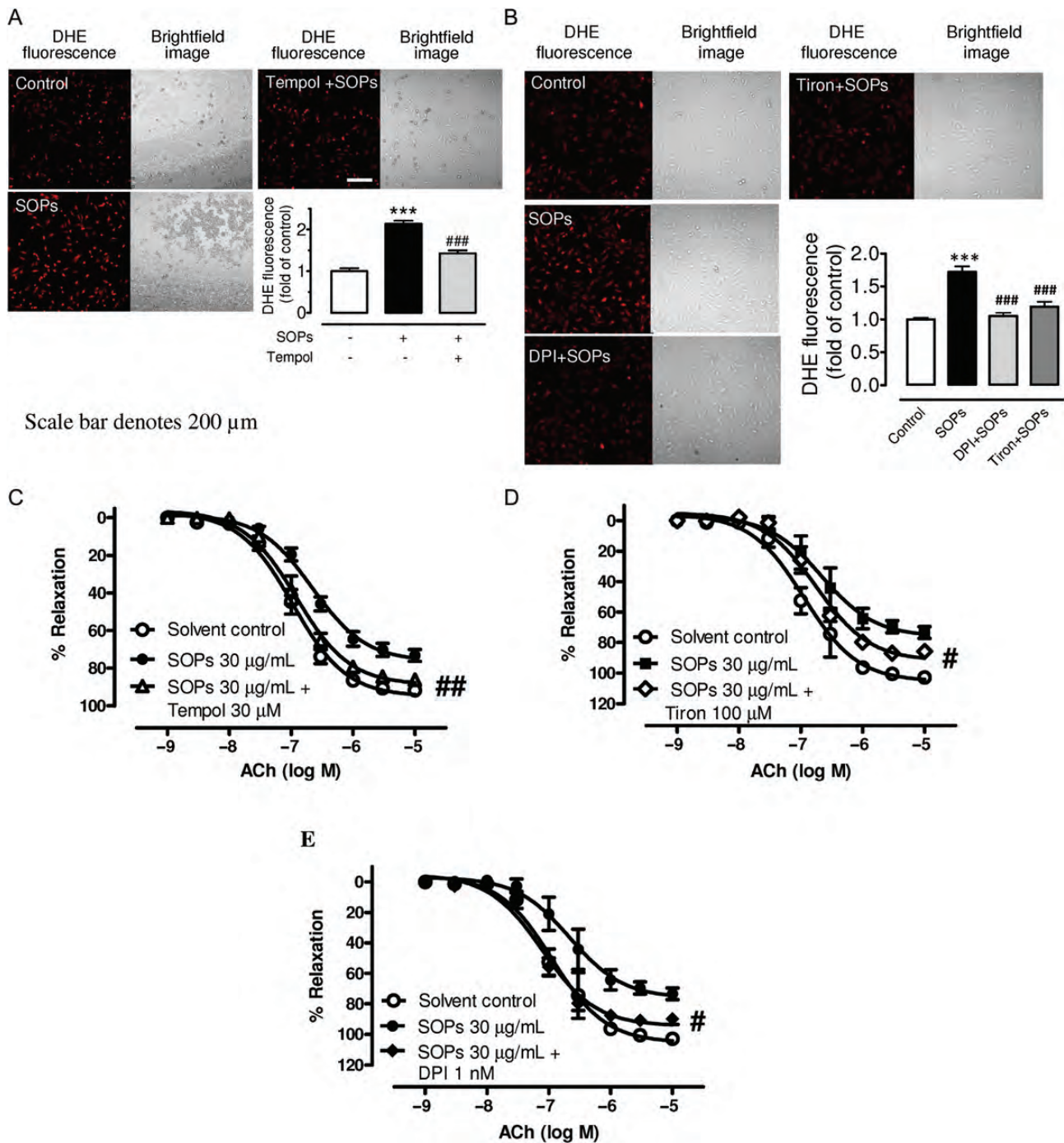
$\beta$ -sitosterol treatment.<sup>45</sup> These studies suggested that the oxidized forms of  $\beta$ -sitosterol, but not  $\beta$ -sitosterol *per se*, can produce oxidative stress and lead to detrimental cellular effects. Consistently, the present study showed that SOPs increased the intracellular ROS level in endothelial cells as shown by both DHE fluorescence measurement and EPR spectroscopy spin trapping. DHE fluorescence showed that SOPs induced a significant elevation in intracellular oxidative stress, which could be inhibited by ROS scavengers and NADPH oxidase inhibitor DPI. Moreover, the application of EPR spectroscopy spin trapping to determine the ROS production in rat aortic endothelial cells confirmed the endothelial origin of ROS production induced by SOPs. In addition, SOPs attenuated endothelium-dependent relaxation and the attenuated relaxation could be reversed by ROS scavengers (tempol, tiron) and DPI. Since SOPs *per se* do not release radicals, SOPs most likely induce oxidative stress by regulating the radical generating and scavenging enzymes. Taken together, SOPs mediate their effect through exerting oxidative stress originating from endothelium. More specifically, both the increase of ROS and the attenuation of vasorelaxation could be reversed by NADPH oxidase inhibitor DPI, suggesting the involvement of NADPH oxidase.

Although DPI was extensively applied as a NADPH oxidase inhibitor in many studies, DPI was also found to bind to other flavin-containing enzymes and may not be a specific inhibitor of NADPH oxidase.<sup>46</sup> On the other hand, NOX4 is the most abundant NADPH oxidase isoform in endothelial cells of different species, including humans and rats.<sup>39–42</sup> Ago *et al.*<sup>39</sup> examined the expression of different NADPH oxidase isoforms in rat aortic endothelial cells; it was found that NOX4 was strongly expressed in rat aortic endothelial cells, whereas NOX2/gp91<sup>phox</sup> and NOX1 were only expressed in a dramatically lower level. In addition, NOX3 and NOX5 were not detected. Therefore, in our present study, to further confirm the involvement of NADPH oxidase, intracellular ROS production was determined after silencing the expression of NOX4. SOPs-induced ROS production significantly decreased after knocking down of NOX4, suggesting that SOPs stimulated ROS by a NOX4-sensitive pathway.

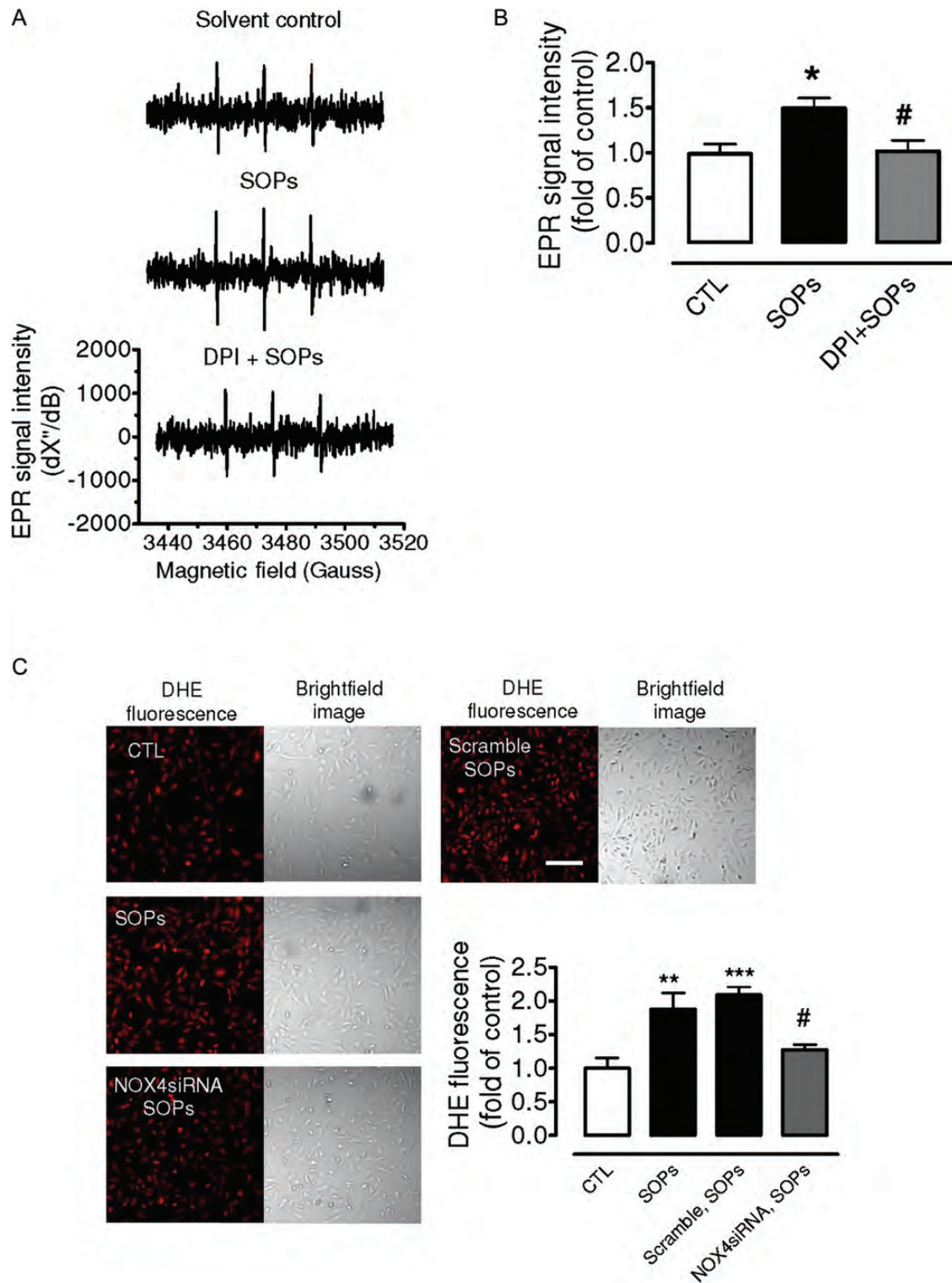
Similar to the well-studied NOX2, NOX4 is a p22<sup>phox</sup>-dependent enzyme. However, unlike NOX2, NOX4 does not require cytosolic subunits for its activity.<sup>47–50</sup> Interestingly, in heterologously NOX4-expressing cells, GTPase Rac is not required for the activity of NOX4,<sup>49</sup> while at least in some endogenously NOX4-expressing cells, Rac is required for the activity.<sup>51,52</sup> Therefore, more investigation is needed to clearly dissect the detailed activation mechanism of NOX4. In our study, SOPs-induced ROS production was abolished when NOX4 protein in the endothelial cells was down-regulated, suggesting that SOPs-induced ROS originates from NOX4. However, how SOPs activate NOX4 remains to be elucidated and is out of the scope of the present investigation.

On the other hand, although there are previous studies showing that ROS generated in the endothelium may react with nitric oxide and decrease the bioavailability of nitric oxide, our results showed that SOPs do not affect the nitric oxide production, suggesting that SOPs attenuate endothelium-dependent vasorelaxation through another pathway.

Previous studies showed that in hypertensive patients, vasodilation was impaired in the presence of COX-derived vasoconstrictor(s), such that infusion of indometacin improved the ACh-induced relaxation.<sup>53</sup> In our present investigation, whether COX is involved in the impact of SOPs on endothelial function is investigated by the

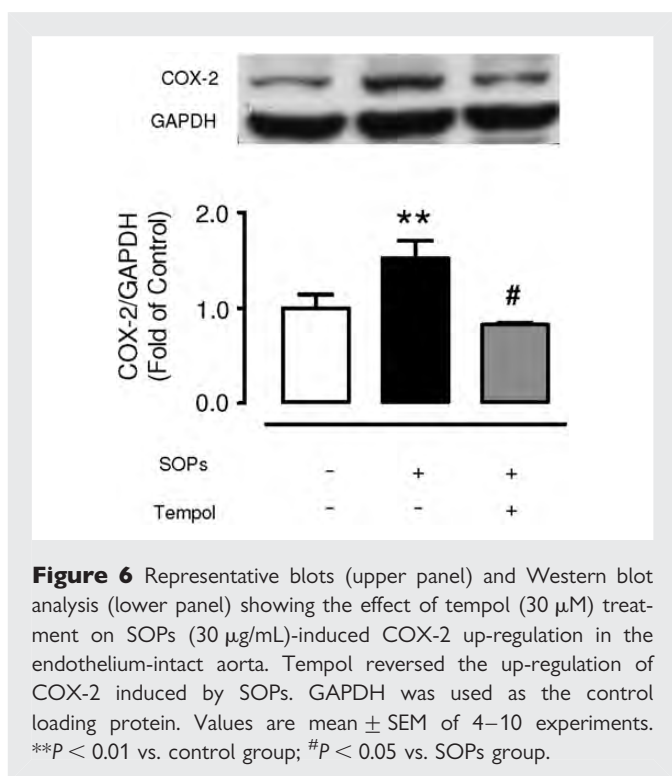


**Figure 4** (A and B) DHE fluorescence was elevated with 15 min incubation of SOPs (30 µg/mL) and was reversed by the ROS scavenger (A) tempol 30 µM, (B) DPI 1 nM, and tiron 100 µM in rat aortic endothelial cells. The results suggested that SOPs elevated the intracellular oxidative stress level. (C–E) Effect of (C) tempol, (D) tiron, and (E) DPI on SOPs-induced impairment on ACh-induced relaxations. Tempol completely reversed, whereas tiron partially reversed the effects of SOPs, suggesting that oxidative stress was involved in the SOPs-induced impairment of endothelium-dependent relaxation. In addition, DPI completely reversed the effects of SOPs, suggesting that the oxidative stress exerted by SOPs may originate from NADPH oxidase. Values are mean ± SEM of 3–12 experiments. #*p* < 0.05, ###*p* < 0.01 vs. SOPs group.



**Figure 5** (A) Representative EPR spectrum of radicals detected by spin trap in rat aortic endothelial cells treated by solvent, SOPs and DPI + SOPs; (B) Summarized data showing EPR signal intensity in rat aortic endothelial cells treated by solvent, SOPs and DPI + SOPs. SOPs increased the production of superoxide anions; this increase was reversed by DPI treatment, suggesting the SOPs increased NADPH oxidase-derived superoxide anions. Values are means  $\pm$  SEM of four to five experiments. \* $P < 0.05$  vs. solvent control group; # $P < 0.05$  vs. SOPs group. (C) Intracellular ROS was elevated after SOPs (30  $\mu$ g/mL) treatment; this increase in ROS was reversed by knocking down of NOX4 expression by NOX4 siRNA. On the other hand, scramble siRNA did not reverse SOPs-induced increase in ROS. Values are mean  $\pm$  SEM of five experiments. \*\* $P < 0.01$ , \*\*\* $P < 0.001$  vs. solvent control; # $P < 0.05$  vs. SOPs group.





application of different COX inhibitors. Indometacin, a non-selective COX inhibitor, was found to reverse the attenuated endothelium-dependent relaxations by SOPs. Notably, COX-2 inhibitor completely abolished the impact of SOPs, whereas the effect of COX-1 inhibitor was partial, indicating that SOPs may possibly attenuate relaxation by elevating prostanoids mainly derived from COX-2. The involvement of COX-2 was furthermore confirmed by Western blotting and immunostaining, both showing that SOPs treatment increased endothelial COX-2 expression. In contrast, there is no alteration of COX-1 expression upon SOPs treatment. Indeed, previous studies have shown that increased COX-2 expression in endothelial cells results in altered prostanoid profile, which furthermore lead to endothelial dysfunction and angiogenesis.<sup>54–58</sup> S18886, a TP receptor antagonist, completely normalized the effect of SOPs, indicating that the SOPs-induced inhibition on endothelium-dependent relaxation is mediated by activating TP receptor. SOPs attenuated vasorelaxation induced by the nitric oxide donor SNP in endothelium-intact but not endothelium-denuded rings, and this attenuated vasorelaxation could be reversed by COX-2 inhibitor DuP-697 or TP receptor inhibitor S18886, confirming the critical role of up-regulated endothelial COX-2 expression, and the prostanoids produced from the endothelial COX-2, which act on the TP receptors in the underlying vascular smooth muscle and impair the vasorelaxation. Consistently, the SNP-induced cGMP production was significantly inhibited in endothelium-intact aorta upon SOPs treatment and the inhibition of cGMP was reversed by DuP-697 or S18886. The results suggested that the prostanoids increased by SOPs act on the TP receptors which would in turn decrease cGMP production. Since the relaxation induced by cGMP analogue was not altered by SOPs, the decrease in production of cGMP is attributed to a decrease in guanylate cyclase activity. Taken the results at functional and molecular levels altogether, it is concluded that SOPs stimulate the expression of

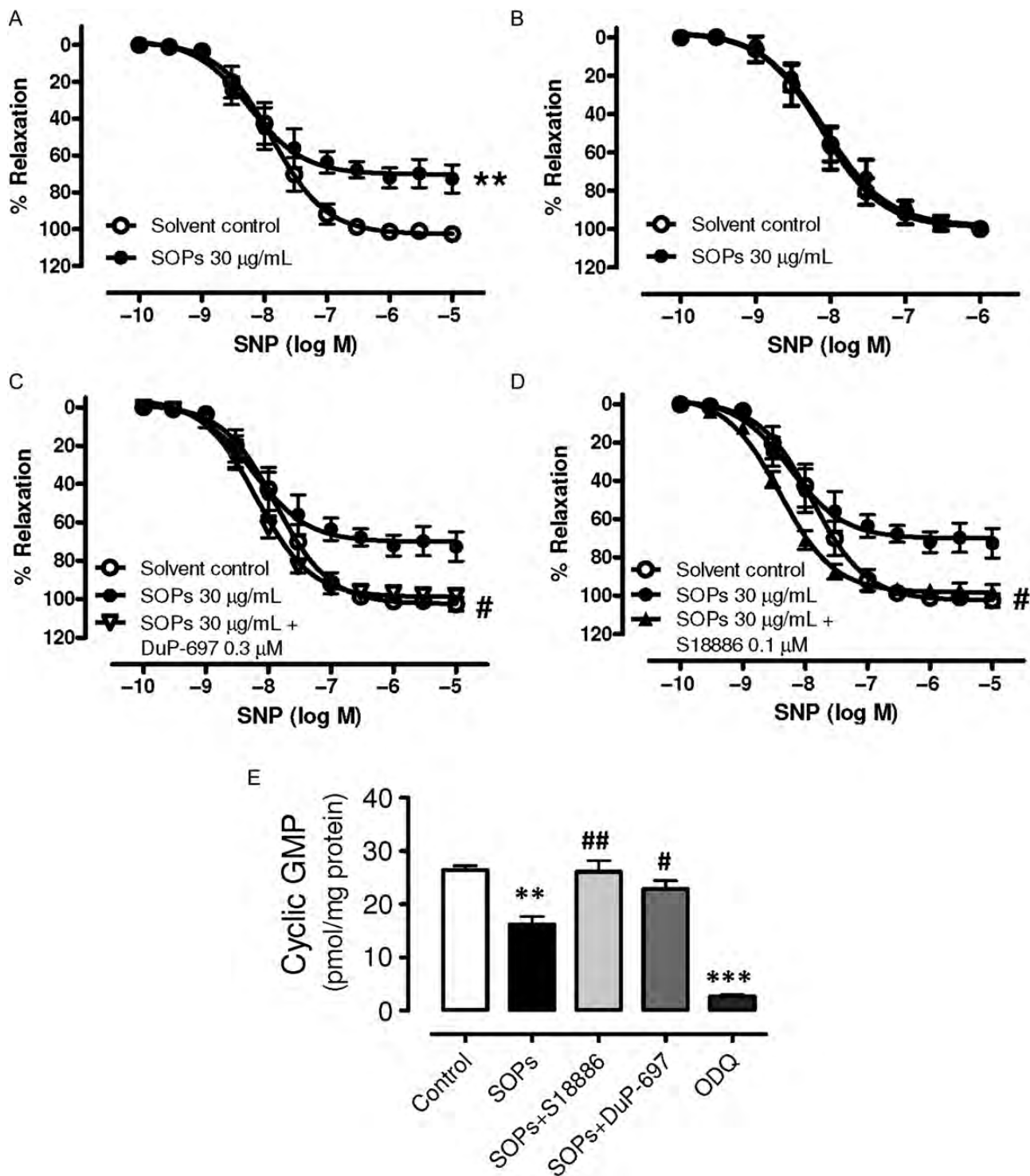
endothelial COX-2, resulting in the production of prostanoids which can act on the TP receptors located on the vascular smooth muscle cells. This activation of TP receptors would in turn attenuate vasorelaxation by inhibiting the activity of guanylate cyclase and decreasing the cGMP production. This finding is in agreement with previous studies which showed that the stimulation of COX2 expression accounts for the impairment of endothelium-dependent relaxation of the rat aorta.<sup>57,59</sup> Consistently, activating TP receptor by U46619 (TP receptor agonist) was also found to impair SNP-induced relaxation in the present study.

The present findings showed that the increased endothelial COX-2 expression by SOPs was reversed by scavenging ROS, suggesting that SOPs increase ROS production which in turn up-regulates endothelial COX-2 expression. These results are in line with previous studies showing that COX-2 expression is sensitive to oxidative stress in endothelial cells.<sup>60</sup> Although we cannot completely rule out the possibility that ROS can act directly to attenuate endothelium-dependent relaxation, our results showed that (i) SOPs increased intracellular ROS production; (ii) SOPs increased endothelial COX-2 expression which could be reversed by ROS scavenger; (iii) inhibition of ROS production or inhibition of COX-2 reversed the effect of SOPs on attenuating vasorelaxation to a very similar degree. It is therefore very likely that the involvement of ROS and COX-2 is in line and that ROS act to enhance endothelial COX-2 expression to mediate the downstream effect of attenuating vasodilation.

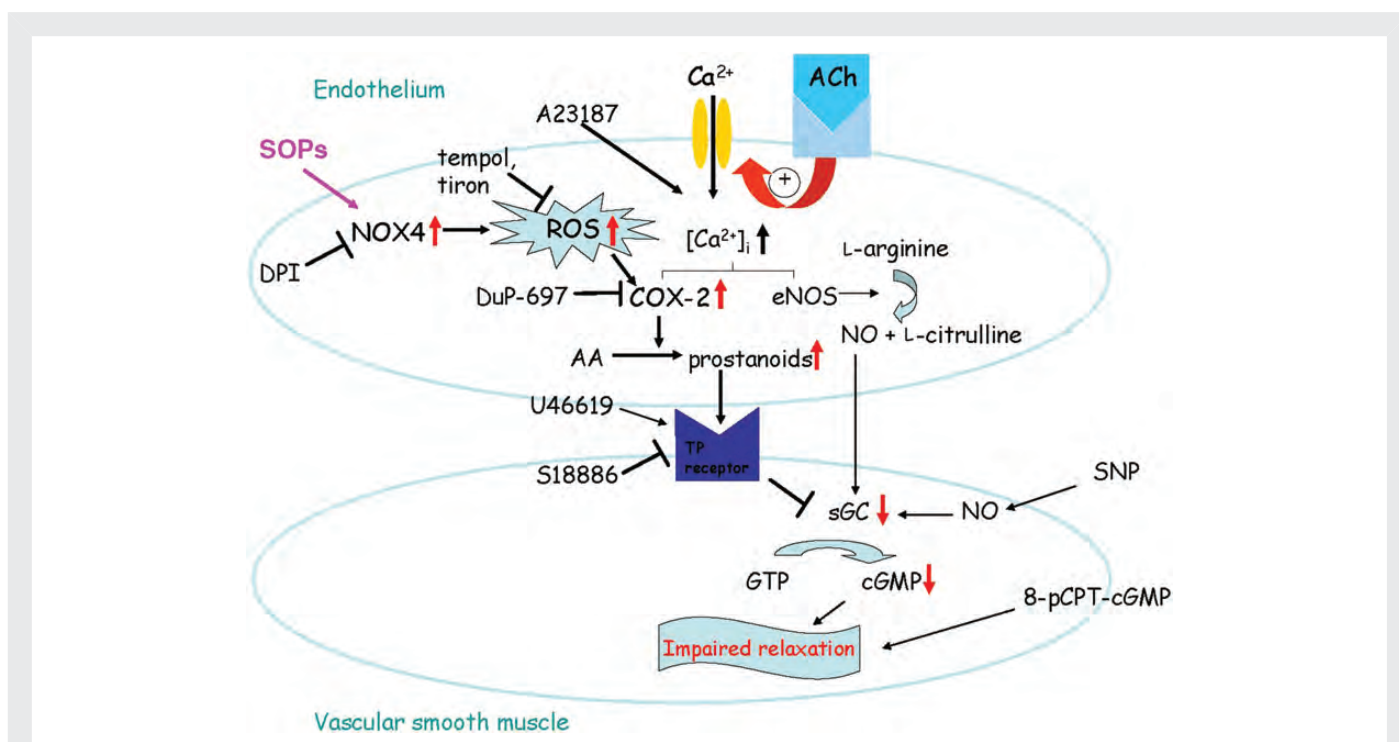
There were limited studies concerning the concentration of SOPs *in vivo*; previous studies showed that blood concentrations of normal and phytosterolaemia individuals were ~4.8–60 ng/mL<sup>22,29</sup> and 0.5–2.3 μg/mL,<sup>28</sup> respectively. Since there are several different types of SOPs in the blood, therefore, the actual concentration present should be a summation of individual SOPs. The concentrations of SOPs used in the present *in vitro* study are higher than those experienced *in vivo*.<sup>29</sup> Since the duration of exposure in the present study are much shorter than those experienced in animal models,<sup>61</sup> a higher concentration may be required to exert the effect that may happen *in vivo*, as in case of other *in vitro* studies.<sup>34,62</sup>

Endothelial dysfunction was found to be associated with the pathogenesis of a number of cardiovascular-related conditions and diseases, including atherosclerosis, hypertension, and diabetes.<sup>63–68</sup> In addition, enhanced ROS production was known to be detrimental to cardiovascular health.<sup>68,69</sup> COX-2 expression was augmented during ageing<sup>70</sup> and in hypertensive state,<sup>71,72</sup> suggesting that up-regulation of COX-2 expression is associated with a decreased cardiovascular performance. The present study showed that SOPs attenuate endothelium-dependent vasorelaxation via increasing the ROS production and COX-2 expression, suggesting that SOPs may lead to the pathogenesis of a number of cardiovascular diseases.

Since plant oils are important constituents of our diet, and that SOPs are generated when plant oils are subjected to frying and storage,<sup>2,18,22</sup> SOPs represent an important portion of our diet.<sup>24,25</sup> Interestingly, a recent study<sup>61</sup> also showed that hamster feeding with SOPs had impaired endothelium-dependent relaxations of the aorta and higher relative liver weight than hamsters feeding with β-sitosterol, suggesting that SOPs are possibly toxic. In addition, results showed that β-sitosterol reduced plasma total cholesterol, low-density lipoprotein cholesterol, and triacylglycerols while SOPs lost the capacity of lowering plasma lipids. Moreover, β-sitosterol but not SOPs were anti-atherosclerotic. Taken together with the information generated in the present investigation, SOPs may be as



**Figure 7** SNP-induced relaxation when (A) endothelium-intact or (B) endothelium-denuded rat aortae were incubated with SOPs (30 µg/mL) for 30 min. SOPs attenuated SNP-induced relaxation only in the presence of endothelium. Values are mean  $\pm$  SEM of four to six experiments.  $**P < 0.01$  vs. control group. (C and D) The effect of (C) DuP-697 (0.3 µM) and (D) S18886 (0.1 µM) on SOPs-induced impairment on SNP-induced relaxations. DuP-697 or S18886 completely reversed the effects of SOPs, suggesting that SOPs acted through a COX-2- and TP receptor-mediated pathway. Values are mean  $\pm$  SEM of four to six experiments.  $\#P < 0.05$  vs. SOPs group. (E) The inhibition effect of SOPs (30 µg/mL) on the production of cyclic GMP in endothelium-intact aortae. SOPs decreased cGMP production, and this effect was reversed by S18886 (0.1 µM) or DuP-697 (0.3 µM). Values are mean  $\pm$  SEM of four to six experiments.  $**P < 0.01$ ,  $***P < 0.001$  vs. solvent control group;  $\#P < 0.05$ ,  $###P < 0.01$  vs. SOPs group.



**Figure 8** Schematic diagram showing how SOPS attenuate vasorelaxation in the rat aorta. The present study shows that SOPS increase the production of ROS in the endothelium via a NOX4-sensitive pathway. The ROS would in turn increase the expression of COX-2, which is predicted to lead to an enhanced production of prostanoids. The prostanoids from the endothelium diffuse towards adjacent vascular smooth muscle cells, where they activate the TP receptors and attenuate the endothelium-dependent vasorelaxation by decreasing the activity of soluble guanylate cyclase and thereby decreasing the cGMP production.

equally harmful as COPs<sup>32–34</sup> to our cardiovascular health. Further investigations are needed to assess the suitability of SOPS consumption in our diet in long run.

## 5. Conclusion

In summary, SOPS attenuated endothelium-dependent relaxation by increasing the production of ROS via a NOX4-sensitive pathway in the endothelial cells. This increase in ROS up-regulated endothelial COX-2 expression, leading to the downstream activation of TP receptors. This in turn decreased the cGMP production by guanylate cyclase, and thereby leading to the attenuation of vasorelaxation (Figure 8). This study generated novel information on the action of SOPS in the vasculature, and shall provide important insights concerning the consumption of  $\beta$ -sitosterol and SOPS in our diet.

## Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

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