

## ROS-related Enzyme Expressions in Endothelial Cells Regulated by Tea Polyphenols<sup>1</sup>

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**Objective** Elevation of reactive oxygen species (ROS), especially the level of superoxide is a key event in many forms of cardiovascular diseases. To study the mechanism of tea polyphenols against cardiovascular diseases, we observed the expressions of ROS-related enzymes in endothelial cells. **Methods** Tea polyphenols were co-incubated with bovine carotid artery endothelial cells (BCAECs) *in vitro* and intracellular NADPH oxidase subunits p22phox and p67phox, SOD-1, and catalase protein were detected using Western blot method. **Results** Tea polyphenols of 0.4 µg/mL and 4.0 µg/mL (from either green tea or black tea) down-regulated NADPH oxidase p22phox and p67phox expressions in a dose-negative manner ( $P<0.05$ ), and up-regulated the expressions of catalase ( $P<0.05$ ). **Conclusions** Tea polyphenols regulate the enzymes involved in ROS production and elimination in endothelial cells, and may be beneficial to the prevention of endothelial cell dysfunction and the development of cardiovascular diseases.

**Key words:** Tea polyphenols; Endothelial cells; NADPH oxidase; Catalase; Western blot

### INTRODUCTION

Reactive oxygen species (ROS), especially superoxide anion and hydrogen peroxide, are important signaling molecules in cardiovascular cells that influence both normal and abnormal cell processes, including cellular growth, hypertrophy, remodeling, lipid oxidation, modulation of vascular tone, endothelial permeability and adhesion for leukocytes<sup>[1]</sup>. Elevation of ROS, especially the level of superoxide, is a key event in many forms of cardiovascular disease, such as essential hypertension and atherosclerosis. In vascular endothelial cells, angiotensin II-regulated NADPH oxidase was the prominent source of superoxide anions<sup>[2]</sup>, and NADPH oxidase expression regulated by Ang II was in a dose-dependent manner in human endothelial cells<sup>[3]</sup>. The superoxide anions derived from NADPH oxidase are converted by cytosolic superoxide dismutase (SOD-1) into hydrogen peroxide, which may be converted into water by enzyme catalases. ROS accumulation has been reported in spontaneous hypertensive rats (SHR) and stroke-prone spontaneous

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hypertensive rats (SHRSP)<sup>[4]</sup>, essential hypertension<sup>[5]</sup>, and other types of hypertension<sup>[6,7]</sup>.

Emerging evidence also suggests an important role of antioxidants in modulating endothelial function, and antioxidant treatment has been shown to increase endothelium-dependent vasodilation and reduce blood pressure in Ang II-induced hypertension<sup>[8]</sup>. Recently, a chronic ingestion study of vitamin E and sesamin in SHRSP has shown vitamin E and sesamin can increase blood pressure, oxidative stress and thrombosis<sup>[9]</sup>. Tea polyphenols, the main components of tea, were considered to be antioxidants both from redox potential and *in vitro* data<sup>[10]</sup>. Several epidemiological studies have suggested an inverse relationship between tea consumption and cardiovascular disease risk<sup>[11,12]</sup>, and a recent crossover trial by Duffy *et al.* showed that short- and long-term consumption of black tea reversed endothelial vasomotor dysfunction in patients with coronary artery disease<sup>[13]</sup>. However, the underlying mechanisms of cardiovascular beneficial effects of tea polyphenols have not been demonstrated. As the amount of daily tea polyphenol intake (2.6 mg/d-68.2 mg/d) was quite minimal<sup>[12]</sup>, the free radical scavenging ability of tea polyphenols should be limited compared with the strong free radical scavenging ability of the human body<sup>[14]</sup>. We here hypothesize that the effect of tea polyphenols on ROS is mainly due to the regulation of the enzymes related to ROS formation or degradation in vascular endothelial cells. To justify this hypothesis, protein expressions of NADPH oxidase subunits p22phox and p67phox, cytosolic Cu/Zn SOD (SOD-1), and catalases in endothelial cells *in vitro* were evaluated.

## MATERIALS AND METHODS

### Materials

Goat polyclonal antibody (anti-NADPH oxidase p22phox and p67phox), rabbit anti-SOD-1 antibody, and rabbit anti-beta-actin antibody were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Rabbit anti-catalase polyclonal antibody was from Abcam Ltd. (Cambridge, UK). Anti-goat and anti-rabbit horseradish peroxidase-conjugated antibodies were obtained from Amersham, Japan. Green tea polyphenols (GTP) and black tea polyphenols (BTP) were kindly provided by Unilever Health Institute with the components listed in Table 1. Dulbecco's modified Eagle medium (DMEM) was from Life Technologies. Other reagents used were of the highest grade commercially available.

TABLE 1

	Components of Tea Polyphenols (%)	
	Green Tea Polyphenols	Black Tea Polyphenols
Catechin	70	8
Flavonols	10	10
Thearubigins	—	70
Theaflavins	—	12
Polymeric Flavonoids	20	—

### Cell Culture

Bovine carotid artery endothelial cells (BCAECs) were purchased from Health Science Research Resources Bank (Osaka, Japan). The cells were grown at 37°C in 5% CO<sub>2</sub> in DMEM supplemented with 10% FBS and passaged twice a week by harvesting with trypsin EDTA and seeding into 100 mm dishes. For experiments, the cells between passages 6 and 15 were used at confluence.

### *Electrophoresis and Immunoblotting*

Total cell extracts were prepared by lysing the cells in extraction buffer containing 50 mmol/L Tris/HCL, pH8.0, 150 mmol/L NaCl, 1% nonidet-P40, 1% sodium deoxycholate, 0.1% SDS, 0.1 mmol/L DTT, 0.05 mmol/LPMSF, 0.002 mg/mL aprotinin, 0.002 mg/mL leupeptin, and 1 mmol/L NaVO<sub>3</sub> after stimulation. The protein concentration was quantified with BIO-RAD Dc protein assay reagent (Hercules, CA). Equal amounts of protein were mixed with SDS sample buffer and incubated for 3 min at 100°C before loading. Protein electrophoresis separation (SDS-PAGE) and immunological blotting were performed according to the method of Amersham Biosciences. Immunoreactive bands were detected by enhanced chemiluminescence (Amersham) following the manufacturer's instructions. The chemiluminescent signals were scanned from autoradiographic films (Nippon Polaroid K.K., Tokyo, Japan), and imported into Adobe Photoshop. Quantitative analysis was performed by NIH Image 1.62 software.

### *Statistical Analysis*

Quantitative values were expressed as  $\bar{x} \pm s$  and analyzed by ANOVA for repeated measures when appropriate. Between-group comparisons were made with the Bonferroni test with a significance level set at  $P < 0.05$ .

## RESULTS

### *Expression of NADPH Oxidase Subunit p22phox and p67phox*

Proteins in BCAECs were determined after 24 h incubation at different concentrations of GTP or BTP (Fig.1). Both GTP and BTP down-regulated the expression of NADPH oxidase subunits p22phox and p67phox. For p22phox, the expression from 100% (control) to 85% (GTP) and 76% (BTP) occurred at a dose level of 0.4  $\mu\text{g/mL}$  medium, and to 53% (GTP) and 51% (BTP) at a dose level of 4.0  $\mu\text{g/mL}$  medium. And for p67phox, the expression

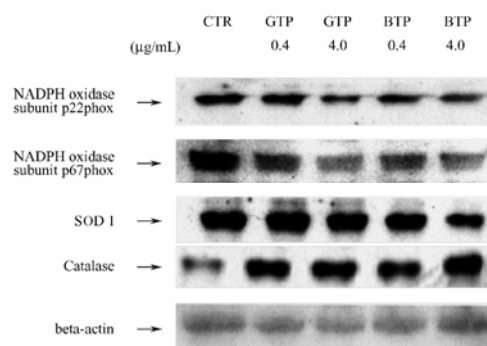


FIG. 1. Expressions of ROS-related enzymes regulated by tea polyphenols. BCAECs were incubated for 24 h at different concentrations of GTP or BTP. Cells were collected and lysed, and 20  $\mu\text{g}$  of protein was dissolved in 12% SDS-PAGE for p22phox, SOD-1 and beta-actin, and 10% SDS-PAGE for p67phox and catalase. Protein was determined by Western blotting method and a representative study was shown with beta-actin as reference. CTR: control, GTP: green tea polyphenols, BTP: black tea polyphenols.

from 100% (control) to 69% (GTP) and 63% (BTP) occurred at a dose level of 0.4  $\mu\text{g/mL}$  medium, and to 46% (GTP) and 42% (BTP) at a dose level of 4.0  $\mu\text{g/mL}$  medium. NADPH oxidase subunit expressions in three combined experiments are shown in Figs. 2 and 3 ( $P<0.05$ ).

The bar graph represents three combined experiments ( $n=3$ ) and the densitometric analysis ( $\bar{x}\pm s$ ) of immunoblots of NADPH oxidase subunit p22phox. \* $P<0.05$  versus control, \*\* $P<0.01$  versus control. GTP: green tea polyphenols, BTP: black tea polyphenols.

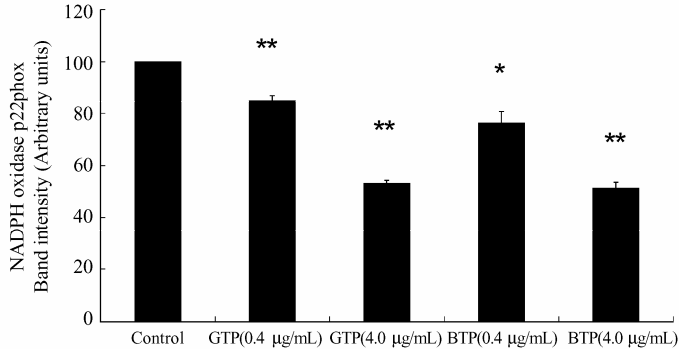


FIG. 2. Bar graph of NADPH oxidase subunit p22phox.

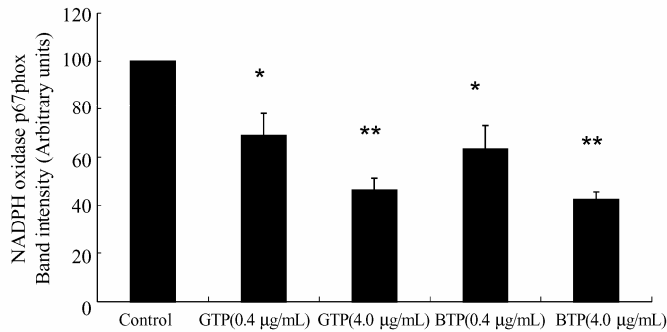


FIG. 3. Bar graph of NADPH oxidase subunit p67phox.

The bar graph represents three combined experiments ( $n=3$ ) and the densitometric analysis ( $\bar{x}\pm s$ ) of immunoblots of NADPH oxidase subunit p67phox. \* $P<0.05$  versus control, \*\* $P<0.01$  versus control. GTP: green tea polyphenols, BTP: black tea polyphenols.

### Expressions of SOD-1 and Catalase

The protein expression demonstrated that catalase was up-regulated by either GTP or BTP to about 200% ( $P<0.05$ ), but no difference of SOD-1 was observed after 24 h of pretreatment with both GTP and BTP (shown in Fig.1). The densitometric analysis ( $\bar{x}\pm s$ ) for four combined experiments of SOD-1 (Fig. 4,  $P>0.05$ ) and catalase expressions are shown in Fig. 5. The expressions of catalase increased from 100% (control) to 216.8% (GTP) and 204.5% (BTP) at a dose level of 0.4  $\mu\text{g/mL}$  medium, and to 223.1% (GTP) and 239.4% (BTP) at a dose level of 4.0  $\mu\text{g/mL}$  medium. There were significances between GTP and control ( $P<0.01$ ) and between BTP and control ( $P<0.05$ ).

The bar graph represents three combined experiments ( $n=4$ ) and the densitometric analysis ( $\bar{x}\pm s$ ) of immunoblots of SOD-1. GTP: green tea polyphenols, BTP: black tea polyphenols.

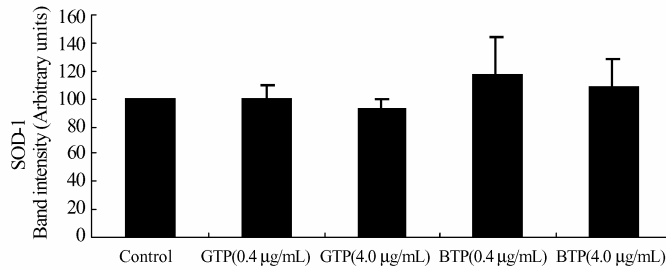


Fig. 4. Bar graph of SOD-1.

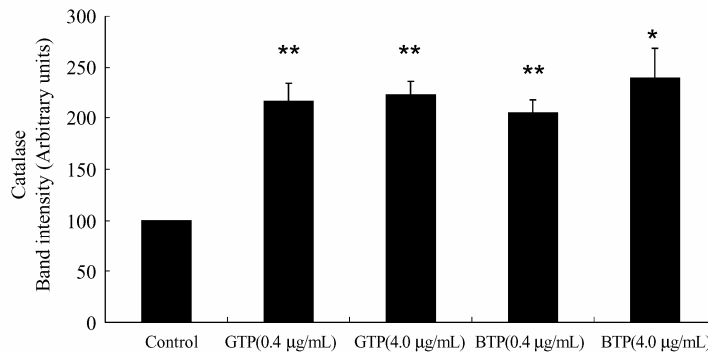


FIG. 5. Bar graph of Catalase.

The bar graph represents four combined experiments ( $n=4$ ) and the densitometric analysis ( $\bar{x}\pm s$ ) of immunoblots of catalase. \* $P<0.05$  versus control, \*\* $P<0.01$  versus control. GTP: green tea polyphenols, BTP: black tea polyphenols.

## DISCUSSION

In non-vascular tissues, xanthine oxidase, mitochondrial oxidases and arachidonic acid were the major sources of oxidative molecules, whereas membrane associated NADPH oxidase appeared to be the most important source of superoxide in vascular cells<sup>[15]</sup>. The enzyme comprises five subunits: three cytosolic subunits, namely, p22phox, p47phox, and a low molecular weight G protein (Rac1 or Rac 2), and two membrane associated subunits p22phox and gp91phox. The assembling of cytosolic subunits of NADPH oxidase to the membrane facilitates electron transfer from NADPH to molecular oxygen and leads to the production of superoxide anions. A recent study on relative mRNA expression of NADPH subunits in human umbilical vein endothelial cells (HUVECs) and leukocytes showed that subunits p67phox (2.5%) and gp91phox (1.1%) were expressed at a much lower level in endothelial cells than in leukocytes<sup>[16]</sup>. It is suggested that the expression level of these two subunits may be rate-limiting factors for superoxide production. Our present results (Fig. 1) demonstrated that either GTP or BTP down-regulated the expressions of the NADPH oxidase subunits p22phox and p67phox, suggesting that tea polyphenols possess the property

of limiting the superoxide production.

One proposed mechanism explaining the beneficial effect of polyphenols in endothelial cells was their antioxidant properties<sup>[17]</sup>. Tea polyphenols like other flavonoids were traditionally considered as effective scavengers of ROS by direct reaction, chelation of transition metal ions or action as chain-breaking substances<sup>[18]</sup>. However, the data of our superoxide anion assay showed that a decreasing effect on the ROS level still existed 24 h after pretreatment with tea polyphenols. The phenomenon observed could not be explained mainly by the direct ROS scavenging role of tea polyphenols. Besides, according to kinetic constants, the reaction between superoxide and antioxidant vitamins such as vitamin E and ascorbic acid was approximately 10 000 times slower than that of the reaction between superoxide and NO<sup>[19,20]</sup>. Furthermore, a recent study of vitamins C and E in stroke-prone SHR showed that a decreased activation of vascular NADPH oxidase was observed, though no enzyme protein or possible mechanisms were provided<sup>[21]</sup>. From our present study and others, it can be concluded that tea polyphenols can change the intracellular ROS mainly by regulating the NADPH oxidase protein expressions. And this may explain the beneficial role of tea polyphenols in preventing cardiovascular diseases such as hypertension.

In this study, the up-regulation of catalase expression was also observed though no significant difference was found in the expression of SOD-1. There existed three kinds of SOD, in which SOD-1 was located in cytosol of endothelial cells and the other two at mitochondrion and extracellular, respectively<sup>[22]</sup>. The different locations of these SODs may explain the specificity of their functions.

The mechanism of regulation of NADPH oxidase subunits has not been completely shown, the redox-sensitive signal and thereby a consequence of modifications in NADPH oxidase subunit gene promoter activation should be considered. Evidences showed that oxidants increased nuclear levels of trans-acting factors in endothelial cells and that these increases required oxidant-sensitive changes in both tyrosine and serine/threonine phosphorylations<sup>[23]</sup>. A recent study showed that antioxidants could modify the activation of Ang II-induced MAP kinases in vascular smooth muscle cells<sup>[24]</sup>, and another study showed that green tea polyphenol (-)-epigallocatechin 3-gallate could inhibit protein kinase C activation in human neuroblastoma cells, which may provide some insights in this field<sup>[25]</sup>. Further researches in this field may give more evidence.

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