

*Original Article*

# Tea Polyphenols Regulate Nicotinamide Adenine Dinucleotide Phosphate Oxidase Subunit Expression and Ameliorate Angiotensin II-Induced Hyperpermeability in Endothelial Cells

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Out-of-control reactive oxygen species (ROS) signaling is one of the key events in the pathogenesis of endothelial dysfunction and essential hypertension. We observed that tea polyphenols decreased the production of ROS *via* regulation of the protein expression of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase in bovine carotid artery endothelial cells (BCAECs). Both green tea polyphenols (GTP) and black tea polyphenols (BTP) down-regulated the expression of NADPH oxidase subunits p22phox and p67phox while up-regulating catalase expression ( $p < 0.05$ , respectively). Pre-treatment with GTP or BTP for 24 h significantly decreased the superoxide anion level ( $p < 0.05$ ) and permeable fluorescence intensities in Ang II-stimulated BCAECs. A decrease in cell permeability was also observed by pre-treatment with diphenylene iodonium chloride (DPI) or vitamin E ( $p < 0.05$ , respectively). The result demonstrates that tea polyphenols alleviate angiotensin (Ang) II-induced hyperpermeability mainly by decreasing ROS production. Our results suggest that tea polyphenols regulate ROS-related protein expression and may be beneficial in preventing endothelial cell dysfunction and development of cardiovascular diseases, including hypertension. (*Hypertens Res* 2003; 26: 823–828)

**Key Words:** tea polyphenols, nicotinamide adenine dinucleotide phosphate oxidase, endothelial cell permeability, angiotensin II

## Introduction

The vascular endothelium plays an important role in vascular function and endothelial dysfunction (ED), one of two characteristic alterations of the vascular wall in hypertension (1), and is also involved in the increased incidence of other cardiovascular events such as congestive heart failure (2), hypercholesterolemia (3, 4), diabetes mellitus (5, 6), and hyperhomocysteinemia (7). Although the exact mechanisms of ED

are extremely complicated and have not been clarified until recently, accumulating evidence has suggested that out-of-control reactive oxygen species (ROS) signaling and diminished bioavailability of NO are key events in the pathogenesis of endothelial dysfunction (8, 9).

ROS, especially superoxide anion and hydrogen peroxide, are important signaling molecules in cardiovascular cells (10) that influence both normal and abnormal cellular processes, including cellular growth, hypertrophy, remodeling, lipid oxidation, modulation of vascular tone, and inflam-

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mation (11). ROS accumulation has been reported in spontaneously hypertensive rats (SHR) and stroke-prone spontaneously hypertensive rats (SHRSP) (12), essential hypertension (13), and other types of hypertension (14, 15). In the vascular endothelial cells, gp91phox-containing nicotinamide adenine dinucleotide phosphate (NADPH) oxidase is the principal source of superoxide anion (16). A recent report has shown that regulation of NADPH oxidase expression by angiotensin (Ang) II is dose-dependent in human endothelial cells (17).

Antioxidant treatment has been shown to increase endothelium-dependent vasodilation and reduce blood pressure in Ang II-induced hypertension (18, 19). Previously, we reported that chronic ingestion of vitamin E and sesamin in SHRSP attenuated the elevations in blood pressure, oxidative stress, and thrombotic tendency (20). Emerging evidence also suggests an important role of antioxidants in modulating endothelial function (21). Tea polyphenols, the main compounds in tea, are considered to be antioxidants both from redox potential and *in vitro* data (22, 23). Some epidemiological studies have suggested an inverse relationship between tea consumption and cardiovascular disease risk (24–26), and a recent crossover trial by Duffy *et al.* showed that short- and long-term black tea consumption reverses endothelial vasomotor dysfunction in patients with coronary artery disease (27). However, the underlying mechanisms involved in ED improvement by tea polyphenols have not been demonstrated. Typical daily tea polyphenol intakes range from 2.6 mg/d to 68.2 mg/d, which is quite minimal (23), as is the free radical scavenging ability of tea polyphenols compared to the strong free radical scavenging ability of the human body itself (10). We hypothesized that the effect of tea polyphenols on ROS is mainly due to regulation of the enzymes related to ROS formation and degradation of vascular endothelial cells.

Our present study shows that tea polyphenols down-regulate the expression of the NADPH oxidase subunits p22phox and p67phox and lower the superoxide anion level inside the endothelial cells. To our knowledge, this is the first report on the regulation of flavonoids on NADPH oxidase expression. This mechanism may explain the beneficial effects of tea and other flavonoids.

## Methods

### Materials

Goat polyclonal antibody (anti-NADPH oxidase p22phox and p67phox) and rabbit anti- $\beta$ -actin antibody were from Santa Cruz Biotechnology, Inc. (Santa Cruz, USA). Rabbit anti-catalase polyclonal antibody was from Abcam, Ltd. (Cambridge, UK). Green tea polyphenols (GTP; catechins 70%, flavonols 10%, and polymeric flavonoids 20%) and black tea polyphenols (BTP; catechins 8%, flavonols 10%, thearubigins 70%, theaflavins 12%) were provided by

Unilever Health Institute (Vaardingen, The Netherland), Ang II was from Sigma (St. Louis, USA). FITC-dextran (40 kDa, anionic) was from Molecular Probes, Inc. (Eugene, USA). Other reagents used were of the highest grade commercially available.

### Cell Culture

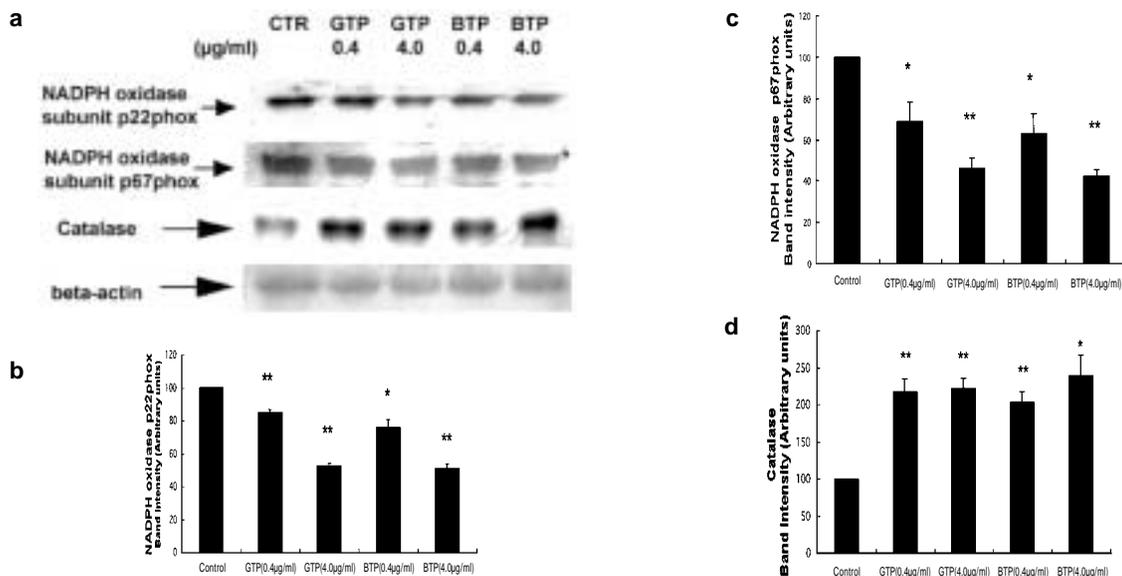
Bovine carotid artery endothelial cells (BCAECs) obtained from Health Science Research Resources Bank (Osaka, Japan) were maintained at 37°C in 5% CO<sub>2</sub> in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS). Confluent cultures were detached using trypsin/EDTA and plated on 60-mm-diameter dishes to determine cell proliferation, on 6-well plates for "scratch" wound assay (cell migration), and on 24-well filtration microplates for permeability determination.

### Electrophoresis and Immunoblotting

Whole cell extracts were prepared by lysing the cells in extraction buffer containing 50 mmol/l Tris/HCl, pH 8.0, 150 mmol/l NaCl, 1% Nonidet-P40, 1% sodium deoxycholate, 0.1% SDS, 0.1 mmol/l DTT, 0.05 mmol/l PMSF, 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 (Bio-Rad, Hercules, USA). Equal amounts of protein were mixed with SDS sample buffer and incubated for 3 min at 100°C before loading. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunological blotting were performed according to the method of Amersham Biosciences. Immunoreactive bands were detected by means of an ECL plus Western Blotting Detection System (Amersham Biosciences Little Chalford, UK) according to the manufacturer's instructions. The chemiluminescent signals were scanned from autoradiographic films (Nippon Polaroid K.K., Tokyo, Japan), and imported into Adobe Photoshop (Adobe, San Jose, USA). Quantitative analysis was performed by NIH Image 1.62 software.

### Quantitative Nitroblue Tetrazolium (NBT) Assay

An NBT assay (28) was carried out to measure the intracellular superoxide anion production in BCAECs grown to confluence in 100 mm dishes. The cells were washed with phosphate-buffered saline (PBS) (-), incubated in serum-free DMEM (phenol red-free) for 60 min at 37°C after being preincubated with GTP or BTP for 24 h, and challenged with Ang II (1  $\mu$ g/ml); NBT solution was then added to each well at a final concentration of 1 mg/ml. After incubation at 37°C for 30 min, the reaction was stopped by adding a mixture of 0.1 mol/l NaOH and 0.1% SDS in water containing 40 mg/l of diethylene triamine pentaacetic acid. The formazan pellets were suspended in 1 ml of pyridine at 100°C for 10 min. Re-



**Fig. 1.** Regulation of the expression of catalase and the NADPH oxidase subunits p22phox and p67phox by tea polyphenols. Bovine carotid artery endothelial cells (BCAECs) were incubated for 24 h with different concentrations of GTP or BTP, cells were collected and lysed, and 20  $\mu\text{g}$  of protein was dissolved by 12% SDS-PAGE for p22phox and  $\beta$ -actin, and 10% SDS-PAGE for p67phox and catalase. Protein levels were determined by Western blotting, and a representative study is shown (a). The bar graph represents three to four combined experiments. The results of the densitometric analysis (mean  $\pm$  SEM) of immunoblots of NADPH oxidase subunit p22phox (b), p67phox (c), and catalase (d) are expressed as band intensities. \*  $p < 0.05$  vs. the control; \*\*  $p < 0.01$  vs. the control. CTR, control; GTP, green tea polyphenols; BTP, black tea polyphenols.

ducing NBT was quantified by measuring the absorbance at 510 nm.

### Cell Permeability Assay

A previously described method was used for the cell permeability assay (29). Briefly, BCAECs were seeded in the upper chambers of 0.4  $\mu\text{m}$  polycarbonate Transwell filters of a 24-well filtration microplate (Whatman Inc., Clifton, USA). After reaching confluence, the cells were pre-incubated with or without tea polyphenols (0.4  $\mu\text{g}/\text{ml}$ ) for 24 h or pretreated with diphenylene iodonium chloride (DPI; an NADPH oxidase inhibitor) or vitamin E (an antioxidant) for 30 min. Medium was then replaced with fresh phenol red-free DMEM in the presence of Ang II (1  $\mu\text{g}/\text{ml}$ ) and FITC-dextran (2.5  $\mu\text{mol}/\text{l}$ ) in the upper chambers. Two hours after the start of the experiment, the filtration microplate was removed and fluorescence in the medium of the 24-well feeder tray was evaluated using a TECAN microplate reader (Wako, Osaka, Japan) at 494 nm excitation and 521 nm emission.

### Statistical Analysis

Quantitative values are expressed as the mean  $\pm$  SEM and were analyzed by ANOVA for repeated measures where appropriate. Between-group comparisons were made with the Bonferroni test with the level of statistical significance set at  $p < 0.05$ .

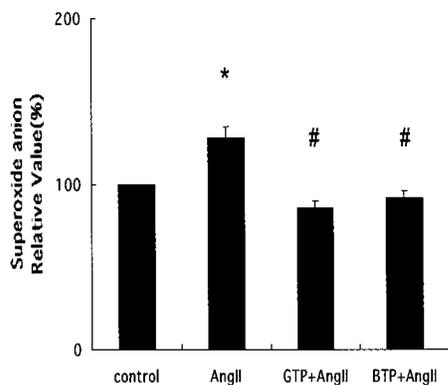
## Results

### ROS-Related Enzyme Protein Expression (Fig. 1)

NADPH oxidase has been identified as a major endothelial source of superoxide anions. BCAECs were incubated for 24 h with different concentrations of GTP or BTP. It was shown that both GTP and BTP down-regulated the expression of the NADPH oxidase subunits p22phox and p67phox (Fig. 1). For p22phox, decreases in expression from 100% (control) to 85% (GTP) and 76% (BTP) occurred at a dose level of 0.4  $\mu\text{g}/\text{ml}$  medium, and decreases to 53% (GTP) and 51% (BTP) occurred at a dose level of 4.0  $\mu\text{g}/\text{ml}$  medium. For p67phox, decreases from 100% (control) to 69% (GTP) and 63% (BTP) occurred at a dose level of 0.4  $\mu\text{g}/\text{ml}$  medium, and decreases to 46% (GTP) and 42% (BTP) occurred at a dose level of 4.0  $\mu\text{g}/\text{ml}$  medium. At the same time, catalase was up-regulated by either GTP or BTP to approximately 200% of the baseline concentration, but no dose-dependent difference was observed between the two tea polyphenols. In addition, no difference was seen in the expression of another enzyme, cytosolic Cu/Zn SOD (SOD-1) (data not shown).

### Effect on Ang II-Induced Superoxide Anion Production

Figure 2 shows that cells pretreated with tea polyphenols significantly decreased the production of superoxide anion stim-



**Fig. 2.** Effects of tea polyphenol pretreatments on Ang II-stimulated superoxide anion level. Cells were pretreated with tea polyphenols at the dose level of 0.4  $\mu\text{g/ml}$  medium for 24 h and then incubated with 1  $\mu\text{g/ml}$  Ang II and 1 mg/ml NBT solution for 30 min. The reductions in NBT were quantified by measuring the absorbance at 510 nm. Relative value of reducing NBT in each sample is shown with control group as 100%. The reductions in NBT in each sample are shown relative to the control value (100%). Data are expressed as the mean  $\pm$  SEM (n = 4). \* p < 0.05 vs. the control; # p < 0.05 vs. the Ang II group.

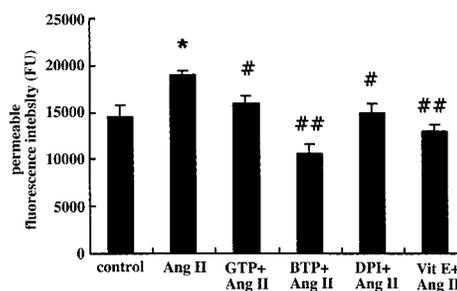
ulated by 1  $\mu\text{g/ml}$  Ang II. The reductions in NBT in each sample are shown relative to the control value (100%). Stimulation with 1  $\mu\text{g/ml}$  Ang II significantly increased the production of superoxide anion from 100% in the controls to  $128 \pm 7.1\%$  ( $p < 0.05$ ), and the level decreased to  $85.5 \pm 4.1\%$  ( $p < 0.05$  vs. Ang II stimulation group) with GTP pretreatment or to  $91.5 \pm 4.6\%$  ( $p < 0.05$  vs. Ang II stimulation group) with BTP pretreatment.

### Endothelial Cell Permeability

As shown in Fig. 3, Ang II induced a significant increase in permeable fluorescence intensity from  $14,577 \pm 1,273$  fluorescent unit (FU) in the control group to  $18,928 \pm 439$  FU by Ang II stimulation ( $p < 0.05$ ). The permeable fluorescence was significantly reduced to  $15,918 \pm 821$  FU in the cells pretreated with GTP ( $p < 0.05$  vs. Ang II group) or to  $10,599 \pm 1,040$  FU by BTP pretreatment ( $p < 0.01$  vs. Ang II group). A similar effect was also observed by pretreatment for 30 min with the NADPH oxidase inhibitor, diphenylene iodonium chloride (DPI, 10  $\mu\text{mol/l}$ ), or with the antioxidant, vitamin E (Vit. E, 100  $\mu\text{g/ml}$ ) ( $14,970 \pm 979$  FU for DPI, and  $p < 0.05$  vs. the Ang II group;  $13,003 \pm 658$  FU for Vit. E, and  $p < 0.01$  vs. the Ang II group).

### Discussion

Our present results (Fig. 1) demonstrated that either GTP or BTP down-regulated the expression of the NADPH oxidase subunits p22phox (a membrane-bound component) and



**Fig. 3.** Effects of tea polyphenol pretreatments on Ang II-induced hyper-permeability. After reaching confluence, the cells were pretreated with GTP or BTP (0.4  $\mu\text{g/ml}$ ) for 24 h, or pretreated with the NADPH oxidase inhibitor, diphenylene iodonium chloride (DPI, 10  $\mu\text{mol/l}$ ), or the antioxidant, vitamin E (Vit. E, 100  $\mu\text{g/ml}$ ), for 30 min, and then incubated with Ang II (1  $\mu\text{g/ml}$  in fresh serum-free DMEM) and FITC-dextran (2.5  $\mu\text{mol/l}$ ) in the upper chambers for 2 h. The permeable fluorescence was determined at 494 nm excitation and 521 nm emission. \* p < 0.05 vs. the control; # p < 0.05 vs. the Ang II group; ## p < 0.01 vs. the Ang II group. Data are expressed as the mean  $\pm$  SEM (n = 4).

p67phox (a cytosolic protein). The binding of these two essential subunits of NADPH oxidase with other parts of subunits of NADPH oxidase facilitates electron transfer from NADPH to molecular oxygen and leads to the production of superoxide anions. It has been proposed that polyphenols may achieve their beneficial effects in endothelial cells via their antioxidant properties (30). It has generally been thought that tea polyphenols, like other flavonoids, act as effective scavengers of ROS by direct reaction, by chelation of transition metal ions, or by acting as chain-breaking substances (31). However, our superoxide anion assay data (Fig. 2) showed that the decrease in ROS level still existed 24 h after pretreatment with tea polyphenols. This observation cannot be explained only by the direct ROS scavenging role of tea polyphenols. Furthermore, according to kinetic constants, the reaction between superoxide and antioxidant vitamins such as vitamin E and ascorbic acid is approximately 10,000 times slower than the reaction rate between superoxide and NO (32, 33). A recent study on vitamin C and E in SHRSP demonstrated a decreased activation of vascular NADPH oxidase, but no enzyme protein results or possible mechanisms were provided (34). Our present results indicate that the NADPH oxidase subunit proteins are down-regulated, which may explain the decreased production of superoxide by tea polyphenols. NADPH oxidase subunit expression was also reported to be inhibited by 17 $\beta$ -estradiol (35). The mechanism of regulation of NADPH oxidase subunits has not been fully clarified, and thus the redox-sensitive signal and consequent modifications of the NADPH oxidase subunit gene promoter activation should be considered. Previous evidence has shown that oxidants increase the nuclear levels of trans-acting factors in endothelial cells and that these in-

creases require oxidant-sensitive changes in both tyrosine and serine/threonine phosphorylations (36). Recent studies showing that antioxidants modify the activation of Ang II-induced MAP kinases in vascular smooth muscle cells (37) and that GTP(-)-epigallocatechin 3-gallate inhibits protein kinase C activation in human neuroblastoma cells may provide some insight in this area (38). Clearly, however, additional investigations will be needed to clarify the mechanism that tea polyphenols regulate promoter activation of NADPH oxidase subunits and relative signaling transduction.

Increased endothelial permeability is one of two aspects of endothelial dysfunction that may be important in determining the severity of vascular disorders (39). Another study has shown that persistent endothelial damage may synergistically increase the risk of early atherosclerosis under the presence of hypertension (40). Like other edemagenic mediators (e.g., thrombin, histamine, and TNF- $\alpha$ ), ROS cause intercellular gap formation, cell shape change, and actin filament reorganization (41). These morphological features are implicated in impaired cell-cell adhesion and, consequently, impaired intercellular junctions, as a primary determinant of increased paracellular permeability.

Specific studies directed at assessing the specific pathway of ROS synthesis activated by Ang II indicate that the NADPH oxidase system is the primary target of action (42, 43). It has also been reported that culturing in the presence of hydrogen peroxide (0.1 mmol/l) for 90 min increased endothelial permeability to 298% of the baseline value (44). Our protein expression results (Fig. 1) showed that both GTP and BTP down-regulated the p22phox and p67phox levels and up-regulated the catalase level, and that both GTP and BTP can decrease the paracellular permeability induced by Ang II. The Ang II-induced permeability was also abrogated by diphenylene iodonium chloride and vitamin E pretreatments, suggesting both that NADPH oxidase decreased ROS production and that ROS scavenging mechanisms are important. These results indicate that both decreases in NADPH oxidase and increases in catalase may be involved in the regulation of the endothelial ROS level by tea polyphenols.

It has long been proposed that flavonoids have antioxidant effects and are beneficial to endothelial dysfunction. As more than 5,000 flavonoids exist, the mechanisms of their antioxidant effects are likely different and may involve different pathways of interaction *in vivo* and *in vitro*. The present study shows that tea polyphenols alter the intracellular ROS mainly by regulating the expression of NADPH oxidase proteins. As suggested by Hamilton, *et al*, the NADPH oxidase pathway may be a novel target for drug intervention in cardiovascular treatment (45), and tea polyphenols may be valuable for preventing cardiovascular diseases such as hypertension.

In summary, our present study shows that tea polyphenols regulate the expression of the NADPH oxidase subunits p22phox and p67phox, as well as the expression of catalase in cultured endothelial cells. This may explain the beneficial

effects of tea on some cardiovascular diseases.

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