Green Tea Attenuates Angiotensin II-Induced Cardiac Hypertrophy in Rats by Modulating Reactive Oxygen Species Production and the Src/Epidermal Growth Factor Receptor/Akt Signaling Pathway1,2

Italia Papparella, Giulio Ceolotto, Domenico Montemurro, Michele Antonello, Spiridione Garbisa, GianPaolo Rossi, and Andrea Semplicini*

Department of Clinical and Experimental Medicine and Experimental Biomedical Sciences, University of Padova, 35128 Padova, Italy

Abstract

We previously documented a clear-cut antihypertensive effect of green tea extract (GTE), which was associated with correction of endothelial dysfunction and prevention of left ventricular hypertrophy in an angiotensin II (Ang II)-dependent model of hypertension, but the molecular mechanisms remain to be defined. As several effects of Ang II involve production of reactive oxygen species (ROS) and activation of 2nd messengers, such as mitogen-activated protein kinase (MAPK) and Akt, we investigated the effect of GTE on these signal transduction pathways in Ang II-treated rats. Rats were treated for 2 wk with Ang II infusion (700 μg·kg⁻¹·d⁻¹; n = 6, via osmotic minipumps), Ang II plus GTE (6 g/L) dissolved in the drinking water; n = 6), or vehicle (n = 6) to serve as controls. Blood pressure was monitored by telemetry throughout the study. The activation and expression of NAD(P)H oxidase subunits, protein kinase C isoforms, Src, epidermal growth factor receptor (EGFR), Akt, and MAPK were determined in the heart in vitro through immunoprecipitation and western blot analysis with specific antibodies. NAD(P)H oxidase enzymatic activity was measured by cytochrome c reduction assay. GTE blunted Ang II-induced blood pressure increase and cardiac hypertrophy. In Ang II-treated rats, GTE decreased the expression of the NAD(P)H oxidase subunit gp91phox and the translocation of Rac-1, as well as NAD(P)H oxidase enzymatic activity. Furthermore, it specifically reduced Ang II-induced Src, EGFR, and Akt phosphorylation. These results show that GTE blunts Ang II-induced cardiac hypertrophy specifically by regulating ROS production and the Src/EGFR/Akt signaling pathway activated by Ang II. J. Nutr. 138: 1596–1601, 2008.

Introduction

Epidemiological evidence suggests that the green tea extract (GTE)3 prevents cardiovascular diseases, particularly atherosclerosis and coronary heart disease (1,2). These favorable effects have been attributed to the antioxidant, antiproliferative, anti-inflammatory properties of the catechins, which are the major components of GTE (3). GTE also blunts the development of cardiac hypertrophy, but the molecular mechanisms are not clear (4,5).

One of the main signal transduction mechanisms leading to the development of cardiac hypertrophy is the production of reactive oxygen species (ROS), derived from NAD(P)H oxidase, a multi-enzymatic complex formed by 4 oxidase subunits, gp91phox, p22phox, p47phox, and p67phox (phox stands for phagocyte oxidase), which are required for its activity (6). Additional components of the enzyme include p40phox subunit, which is not essential for oxidase activity, and the small G proteins Rac. gp91phox and p22phox, together, form an integral membrane complex termed cytochrome b₅₅₈, located in the plasma membrane. Another protein complex, composed of p47phox, p67phox, and p40phox, is cytosolic and does not interact with the cytochrome in resting cells. The function of p40phox subunit, which is not essential for oxidase activity, is controversial (7). Two events are required for oxidase activation: exchange of GDP for GTP and the phosphorylation of the p47phox subunit by protein kinase C (PKC), which triggers conformational change of the cytosolic complex and its association with Rac. This activated cytoplasmic complex associates with the cytochrome in

1 Supported by research grants from the Italian Society of Hypertension and the Italian Ministry of University and Research.
2 Author disclosures: I. Papparella, G. Ceolotto, D. Montemurro, M. Antonello, S. Garbisa, GP. Rossi, and A. Semplicini, no conflicts of interest.
3 Abbreviations used: Ang II, angiotensin II; DBP, diastolic blood pressure; EGFR, epidermal growth factor receptor; ERK1/2, extracellular signal-regulated kinase 1/2; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GTE, green tea extract; JNK, c-Jun N-terminal kinase; LV, left ventricle; MAPK, mitogen-activated protein kinase; PI3K, phosphatidylinositol 3-kinase; PKC, protein kinase C; ROS, reactive oxygen species; SBP, systolic blood pressure; SOD, superoxide dismutase.

* To whom correspondence should be addressed. E-mail: andrea.semplicini@unipd.it.
the membrane to form a functional enzyme (8). Cardiac NAD(P)H oxidase activity is increased by angiotensin II (Ang II), endothelin-1, α-adrenergic agonists, cyclic stretch, during development of pressure-overload hypertrophy and in several animal models of hypertension (9–11). Furthermore, gp91phox or Rac-1 null mice have reduced NAD(P)H oxidase activity, lower myocardial oxidative stress, and blunted hypertrophic and cell growth responses to Ang II (12).

Phosphatidylinositol 3-kinase (PI3K) and extracellular signal-regulated kinase 1/2 (ERK1/2) pathways are other mediators that are independently activated but are both required for Ang II-induced cell growth (13). Akt, also known as protein kinase B, is a major downstream target of PI3K, activated in response to various stimuli, growth factors, and hormones. It is activated by phosphorylation, and, once activated, it phosphorylates many cytosolic and nuclear substrates that are involved in numerous cellular responses, including promotion of cell survival, control of cell cycle progression, and regulation of cell growth (14). ERK1/2 belong to the mitogen-activated protein kinase (MAPK) family and lie downstream of the cascade of Ras/Raf/MEK/ERK, nuclear translocation of ERK1/2 is a critical step to promote cell growth (15). The stimulation of both pathways by Ang II raises the possibility of a cross-talk between the PI3K/Akt and ERK1/2 pathways, mediated by ROS production, which plays a major role in regulating cell proliferation under oxidative stress.

ROS-generating enzymes are inhibited by the scavenging properties of GTE, which increases total plasma antioxidant capacity. In vitro, in rat cardiomyocytes, GTE reduced Ang II-induced ROS production by decreasing the expression of NAD(P)H oxidase subunits (5) and in vivo, in a pressure overload induced hypertrophy rat model with a high level of ROS, GTE attenuated the generation of ROS (4).

These findings suggest that GTE has direct and indirect antioxidant effects in the cardiovascular system, but the molecular mechanisms are not clear. Therefore, the aim of our study was to investigate the effects of GTE on ROS-mediated intracellular signal transduction of Ang II in the rat heart.

Materials and Methods

**Study protocol**

The investigation followed the Guide for the Care And Use of Laboratory animals, published by the U.S. NIH (NIH publication no. 85–23, revised 1996). The protocol of the experiment has already been reported in detail elsewhere (16). Briefly, 18 male, normotensive, Sprague-Dawley rats 3 mo old were used. Six rats were treated for 15 d with Ang II (700 μg·kg⁻¹·d⁻¹) through minialzet pumps (implanted in the midsapular region), 6 rats were treated for 15 d with Ang II (700 μg·kg⁻¹·d⁻¹) plus GTE (SOFAR, Trezzano Rosa, 6 g/L) dissolved in the drinking water, and 6 rats were used as controls and were infused with physiological NaCl solution (0.9%) for 15 d. GTE was administered every day and the treatment started 48 h before Ang II infusion and lasted for 2 wk. All the rats consumed a commercial pelleted maintenance diet (19% protein, 25% fat, 5% fiber). The study started 48 h before Ang II infusion and lasted for 2 wk. All the rats consumed a commercial pelleted maintenance diet (19% protein, 25% fat, 5% fiber).

**In vivo studies**

One week before treatment, the rats were anesthetized with isoflurane to insert into the abdominal aorta a catheter with a small transducer (Telemetry system, Dataquest IV, DATA Sciences International) to measure blood pressure. The telemetry system is based on a transducer inserted in the animal and a metallic panel that functions as an electric monitor located at the base of the cage and directly connected to the animal. Electric impulses are transformed into digital signals, data collected by a computer, and analyzed by a specific software. During the study, systolic (SBP) and diastolic (DBP) blood pressure were monitored every 5 min over 24 h and the mean values calculated.

**In vitro studies**

At the end of treatment, the rats were killed by rapid decapitation after administering light anesthesia (CO2 gas) and the heart was quickly removed for ex vivo evaluations. The rats had a body weight (cardiac mass index) and left ventricle:body weight (LV mass index) were determined.

**Preparation of cytosolic and membrane fractions**

Each heart was homogenized separately in 1 mL of lysis buffer (10 mmol/L Tris-HCl pH 7.4, 1 mmol/L EGTA, 25 mmol/L β-glycerophosphate, 2 mmol/L Na3VO4, 10 μmol/L phenylmethylsulphonyl fluoride, 1 μmol/L leupeptin, and 1 μmol/L aprotinin) and the cytosolic and membrane fractions separated by centrifugation according to the previously reported protocol (11).

**NAD(P)H oxidase subunits expression and activation**

Expression of gp91phox, p22phox, p47phox, p67phox, and Rac-1. Each membrane and cytosolic fraction (30 μg of lysate) were immunoblotted by western blot with rabbit polyclonal antibodies for the detection of gp91phox, p22phox, p47phox, p67phox, and Rac-1. The proteins were separated by electrophoresis following the protocol previously reported (11). The membranes were exposed to the primary antibody against gp91phox, p22phox, p47phox, p67phox, and Rac-1 (1:3000 dilution, Santa Cruz Biotechnology) overnight at 4°C. The membranes were washed (4 times for 5 min) with the same buffer and then incubated with horse-radish peroxidase-conjugate secondary antibody (1:10,000, Amersham Biosciences). Detection was made with Enhanced Chemiluminescence Detection system from Pierce (CELBIO). Blots were analyzed by the Quantity One program of VersaDOC 3000 (Bio-Rad). The results were adjusted to control(s) on the same blot, set at 100%, and expressed as the densitometric ratio between the specific protein and the housekeeping protein glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Translocation of Rac-1, p47phox, p67phox, and Rac-1, p47phox, p67phox translocation from the cytosol to the membrane was assessed by separate measurement in the cytosolic and in the membrane fractions through western blot. The proteins were loaded in a polyacrylamide gel according to the above reported protocol. The results were adjusted to control(s), set at 100%, and expressed as membrancycytosol ratio.

**NAD(P)H oxidase enzyme activity**

The enzymatic activity of NAD(P)H oxidase (NADPH-dependent O2⁻ production) in tissue homogenates was determined by using superoxide dismutase (SOD)-inhibitable cytochrome c reduction assay, as previously described (20). Cytocrome c (500 μmol/L) and NADH (100 μmol/L) were added to the tissue homogenate in the presence or absence of SOD (200 U, Sigma) and incubated at room temperature for 30 min. Cytocrome C reduction was measured photometrically at 550 nm. NAD(P)H-dependent O2⁻ production was calculated from the difference between absorbance with or without SOD.

**Translocation of α, βII, δ, ε, and ζ PKC subunits**

PKC subunit translocation from the cytosol to the membrane was calculated by measuring each subunit both in the cytosolic and in the membrane fractions by western blot. The membranes were exposed to the primary antibodies against PKCα, PKCβ II, PKCβ, PKCγ, and PKCζ (1:3000 dilution, Santa Cruz Biotechnology) and, later, to the secondary horseradish conjugated anti-mouse or anti-rabbit antibody (1:7000).
dilution, Amersham Biosciences) before Enhanced Chemiluminescence system revelation. The results were adjusted to control(s) on the same blot, set as 100%, and expressed as membrane/cytosolic ratio.

**Epidermal growth factor receptor and Src activity**

Homogenates of rat heart were immunoprecipitated with antibodies to epidermal growth factor receptor (EGFR) and Src (Santa Cruz Biotechnology). Immunocomplexes were recovered by the addition of protein A/G PLUS-Agarose (Santa Cruz Biotechnology). Samples were centrifuged and the beads washed with lysis buffer, solubilized in Laemmli buffer, and subjected to immunoblotting. Membranes were probed with anti-phosphotyrosine antibody PY20 (1:1000, Santa Cruz Biotechnology) and immunoreactive proteins were detected by chemiluminescence.

**MAPK and Akt activation**

MAPK and Akt activation were determined by measuring the level of phosphorylation in the cytosolic fraction by western blot. The membranes were exposed to the primary antibodies against ERK1/2, p38, c-Jun N-terminal kinase (JNK), Akt (1:3000 dilution, Santa Cruz Biotechnology) and against phospho-ERK1/2, phospho-p38, phospho-JNK, and phospho-Akt (1:3000 dilution, Cell Signaling, CELBIO). The results were normalized to the control(s), set as 100%, and expressed as the ratio between the phosphorylated:nonphosphorylated target protein.

**Statistical analysis**

Results are presented as means ± SEM. Differences between the control and the experimental groups (Ang II and Ang II + GTE) were performed by ANOVA followed by Bonferroni’s post hoc test, using the SPSS version 13.0. The statistical comparison between basal and final blood pressure was conducted by Student’s paired test. A P-value < 0.05 was accepted as significant.

**Results**

**Blood pressure and cardiac mass index.** Ang II (700 μg·kg⁻¹·d⁻¹) significantly increased SBP and DBP compared with basal values and to control rats and the increase was blunted by GTE administration (Table 1). The LV mass index was greater in Ang II-treated rats than in control rats (P < 0.01) and the increase was attenuated by GTE to a value that remained higher than in controls (P < 0.01) (Table 1).

**NAD(P)H oxidase subunits expression.** Several studies have documented that Ang II induces oxidative stress through NAD(P)H oxidase-dependent ROS production (6). When NAD(P)H oxidase is stimulated, the cytosolic subunits translocate from the cytosol to the membrane and promote ROS production. Ang II increased gp91phox protein expression 1.5-fold compared with controls and GTE treatment prevented this effect (Fig. 1A,C). Total p47phox, p22phox, p67phox, and Rac-1 expression were unaffected (data not shown). Furthermore, Ang II infusion markedly stimulated the translocation of Rac-1 to the membrane compared with controls (P < 0.05) and green tea reduced this effect by 80% (P < 0.01) to a value that was 50% less than that of control rats (P < 0.05) (Fig. 1B,D). The treatments did not affect p47phox and p67phox activation (data not shown).

**NAD(P)H oxidase activity.** NADPH oxidase activity was 2-fold greater in Ang II-treated rats (4.4 ± 0.3 μm/mg protein) compared with controls (1.5 ± 0.2 μm/mg protein; P < 0.01) and the increase was abolished by GTE (2.1 ± 0.3 μm/mg protein).

**PKC and EGFR/Src activation.** The main upstream mediators that mediate the activation of NAD(P)H oxidase induced by Ang II involve the PLD/PKC/p47phox phosphorylation and Src/EGFR/P3K/Rac-1 pathway (21). Therefore, to characterize which molecular mechanism is responsible for Ang II-dependent NAD(P)H oxidase activation and whether it may be modulated by GTE, we determined the activation of PKC and EGFR/Src kinase.

We measured the translocation from the cytosol to the membrane of several isoforms of PKC, such as α and β, which are Ca²⁺ and DAG dependent; δ and ε, which are DAG dependent; and ζ, which is Ca²⁺ and DAG independent. Ang II-infusion for 2 wk increased PKCα translocation from the cytosol to the membrane by 1.5-fold compared with untreated rats (Fig. 2A,C). However, this change was not affected by GTE. The treatments did not affect other PKC isoforms (data not shown).

Ang II increased EGFR activation and Src kinase phosphorylation by 1- and 2-fold, respectively, compared with controls (P < 0.01) and this effect was prevented by GTE (Fig. 2B,D). These results show that Ang II-induced EGFR/Src pathway activation is specifically inhibited by GTE.

**Akt and MAPK activation.** The redox-sensitive downstream targets of NADPH oxidase-derived ROS induced by Ang II are several and mainly entail the MAPK family: ERK1/2, p38, JNK1/2, and the serine-threonine kinase Akt (21).

Ang II increased the activation of Akt (Fig. 3A,C) and ERK1/2 (Fig. 3B,D) by 2-fold compared with controls (P < 0.01), whereas the activation of p38 and JNK was unaffected (data not shown). Green tea consumption for 2 wk eliminated the Ang II-induced Akt phosphorylation but did not affect Ang II-induced ERK phosphorylation.

**Discussion**

We recently found that GTE exerts prominent cardiovascular protective effects in Ang II-dependent hypertension by scavenging of superoxide anion and restoration of endothelial function (16). However, the relevance of the antioxidant effect of GTE in the prevention of development of cardiac hypertrophy was unclear. The present results highlight the molecular mechanisms that might be involved. We found that 2-wk exposure to GTE antagonizes in vivo the cardiac effects of Ang II through specific inhibition of the Src/EGFR/Rac-1 kinase pathway, which activates NAD(P)H oxidase, and of Akt kinase (also called PKB, protein kinase B), which is critical for the hypertrophic adap-
Ang II is a potent hypertrophic agent that activates numerous growth-related signal transduction pathways that are crucial for regulating cardiomyocyte size. Recent evidence indicates that many detrimental actions of Ang II occur via activation of NAD(P)H oxidase, generating ROS (24,25). NAD(P)H oxidase is a multimeric enzyme that consists of a membrane-bound cytochrome b558 (composed of 1 gp91phox and 1 p22phox subunit), which forms the catalytic core of the enzyme, and 4 cytosolic regulatory subunits (p47phox, p67phox, p40phox, and Rac-1), which translocate to the cytochrome b558 to activate the enzyme (8). Activated NAD(P)H oxidase is a major source of Ang II-induced ROS production in the cardiovascular system. We found that 2-wk treatment with Ang II increased NAD(P)H oxidase activation through overexpression of gp91phox and translocation of Rac-1 from the cytosol to the membrane, whereas the expression of the other NAD(P)H subunits was unaffected. These results are in accordance with previous studies showing that gp91phox and Rac-1 are the major source of ROS in the cardiovascular system. In fact, Ang II specifically increases mRNA gp91phox expression in cardiomyocytes and induces protein synthesis de novo (26), vascular gp91phox is markedly increased in Ang II-dependent animal models of hypertension, and inactivation of gp91phox blunts hypertension and cardiac hypertrophy (12).

Rac-1 is a small GTP-binding protein, which mediates the heterodimerization of gp91phox with p67phox. Its translocation from the cytosol to the membrane is critical for the assembly and function of the multicomponent NAD(P)H oxidase (27). The activation of Rac-1 in response to Ang II plays a critical role in the development of cardiac hypertrophy by modulating the expression of fetal genes (24). Deletion or inhibition of Rac-1 leads to diminished Rac-1-gp91phox complex formation in the membrane, diminished activation of NAD(P)H oxidase, reduction of ROS production, and attenuation of cardiac hypertrophy (28).

GTE specifically blunted the Ang II-induced increase of gp91phox and Rac-1 translocation, thus showing that GTE attenuates oxidative stress by specific inhibition of NAD(P)H oxidase. Antioxidant action of GTE has been attributed both to direct scavenging and/or chelation of redox-active metal ions, to the inhibition of ROS generation and of redox-sensitive transcription factors, as well as to the induction of antioxidant enzymes (3). We previously showed that the increase of plasma oxidative stress was blunted by GTE administration in Ang II-treated rats. As the
impairment of endothelium-dependent relaxation was also corrected in a fashion that closely mimicked the effect of the superoxide anion scavenger tempol, we suggested that GTE mainly acted as a free radical scavenger (16). The present findings highlight an additional antioxidant action of GTE that involves the direct inhibition of NAD(P)H oxidase. This contention accords with findings from other groups that demonstrated that in endothelial cells, green tea metabolites protect vascular endothelial cells against ROS induced by Ang II through inhibition of NAD(P)H oxidase activity (29). The chemical structure of these metabolites of green tea is similar to that of synthetic NAD(P)H inhibitors and therefore they might affect the assembly of the multiprotein complex, both of the membrane-linked components and of the cytosolic proteins (30), and this can mechanistically account for this beneficial effect of GTE.

The 2nd aim of this study was to elucidate which molecular signaling pathways, linking overactivation of NAD(P)H oxidase and ROS production with cardiac hypertrophy, are affected by GTE. In fact, Ang II binding to AT1 receptor leads to ROS generation via NAD(P)H oxidase activation through a PKC-dependent (7) or a Src-dependent mechanism (31). In turn, Ang II-induced ROS generation triggers cellular dysfunction, matrix remodeling, and myocardial growth by activating several other signaling pathways (32), such as p38MAP kinase activation or EGFR transactivation (7). Transactivation of EGFR is a major mechanism by which Ang II influences growth-related signaling pathways via the activation of PI3K/Akt and ERK pathways.

According to our results, Ang II activates both the Src/EGFR/ Akt pathway and ERK pathway, but GTE prevents the activation of the former and not of the latter. Src kinases belong to a family of nonreceptor tyrosine kinases that mediate responses of extracellular stimuli by phosphorylation of downstream substrates and are involved in cell differentiation and proliferation. Ang II-induced Src kinase activation promotes the transactivation of EGF receptor (EGFR), which initiates both the activation of ERK1/2 and of PI3K and Akt kinase, that mediate the hypertrophic response (33,34). The serine-threonine kinase Akt is a target of ROS and a key downstream mediator of a number of agonists that induce cellular hypertrophy (23). Overexpression of a constitutively active mutant of Akt increases cell surface area and the expression of atrial natriuretic peptide, a marker of cardiac hypertrophy (35). In vivo, pressure overload leads to rapid activation of Akt in the myocardium and these changes are correlated with cardiac hypertrophy (36). Furthermore, transgenic mice overexpressing Akt from a cardiomyocyte-specific promoter exhibit cardiac hypertrophy and enhanced myocardial contractility (37). In our study, GTE reduced cardiac hypertrophy and strongly inhibited Ang II-induced Akt activation, thus confirming its dual activation by ROS and Src/EGFR, both inhibited by GTE. This suggests that GTE affects a feed-forward mechanism, triggered by Ang II and leading to excessive ROS production; ROS produced by NAD(P)H oxidase activate EGFR and Src phosphorylation, which induce again NAD(P)H oxidase activation and Akt phosphorylation. Long-term GTE administration affects this feed-forward mechanism by inhibiting the Ang II-induced activation of NAD(P)H oxidase and EGFR/Src/Akt. ERK1/2, which are required for promoting Ang II-induced cell growth, were not significantly affected by GTE, indicating that they are not a target of GTE and play a permissive rather than a dominant role in the effect of GTE on Ang II-induced cardiac hypertrophy.

The dose of GTE used in our experiments was rather high, up to 2 orders of magnitude higher than that which might be expected to be consumed by avid green tea drinkers. However, in rodents, the main catechins of GTE (epigallocatechin-3-gallate and epicatechin) are largely conjugated as glucuronates and sulfate, and inactive (which might not occur at the same extent in humans) and relatively larger doses are, therefore, required in rat studies (17,18). The need to investigate within an experimentally reasonable time period the effects that human beings can obtain in their life span with a long-term consumption was also taken into account when choosing the dosage.

In conclusion, our study demonstrates in vivo that the beneficial effects of long-term GTE administration on Ang II-induced hypertrophy are specifically mediated by inhibition of ROS production and EGFR/Src/Akt signaling and suggests the existence of a feed-forward mechanism between EGFR/Src/Akt and NAD(P)H-derived ROS, which is blunted by GTE.

### Literature Cited


