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Epigallocatechin-3-gallate Regulates NADPH Oxidase Expression in Human Umbilical Vein Endothelial Cells

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Vascular NADPH oxidase plays a pivotal role in producing superoxide in endothelial cells and thus acts in the initiation and development of inflammatory cardiovascular diseases such as atherosclerosis. Epigallocatechin-3-gallate (EGCG), the major catechin derived from green tea, has multiple beneficial effects for treating cardiovascular disease but the effect of EGCG on the expression of vascular NADPH oxidase remains unknown. In this study, we investigated the mechanism(s) by which EGCG might inhibit the expression of subunits of NADPH oxidase, namely $p47^{phox}$, $p67^{phox}$ and $p22^{phox}$, induced by angiotensin II (Ang II) in human umbilical vein endothelial cells. Ang II increased the expression levels of $p47^{phox}$, $p67^{phox}$, and $p22^{phox}$, but EGCG counteracted this effect on $p47^{phox}$. Moreover, EGCG did not affect the production of reactive oxygen species induced by Ang II. These data suggest a novel mechanism whereby EGCG might provide direct vascular benefits for treating inflammatory cardiovascular diseases.

Key Words: EGCG, NADPH oxidase, Angiotensin II, ROS, HUVEC

INTRODUCTION

Atherosclerosis is a chronic systemic disease of the vasculature with an inflammatory component. Elevated vascular superoxide anion formation has been implicated in the initiation and progression of hypertension and atherosclerosis [1]. The production of reactive oxygen species (ROS) such as superoxide is increased by cytokines, peptide hormones and shear stress after vascular injury. The major source of ROS in vascular endothelial cells is NADPH oxidase (Nox). Nox4 produces mainly H_2O_2, while Nox1 generates mostly $O_2^{\cdot -}$ that is later converted to H_2O_2. Nox4 is responsible for basal H_2O_2 production, while $O_2^{\cdot -}$ production in nonstimulated and Ang II-stimulated cells depends on Nox1 [2]. ECs express all the components of Nox including $gp91^{phox}$, $p22^{phox}$, $p47^{phox}$, $p67^{phox}$, $p40^{phox}$ and the small G protein Rac1 [3].

Numerous studies have demonstrated that activation of the angiotensin II (Ang II) type 1 (AT1) receptor plays an important role in the pathogenesis of cardiovascular diseases. Ang II, an important stimulant for vascular NADPH oxidase, generates superoxide and other ROS, which stimulates IkappaB degradation and NF-kB activation. This activation was shown to enhance the expression of vascular cell adhesion molecule 1 involved in the early stages of atherosclerosis [4,5]. Ang II-induced ROS can cause peroxynitrite generation- and lipid oxidation. They also lead to the activation of redox-sensitive genes controlling chemotaxis- and the production of adhesion molecules, proinflammatory cytokines and matrix metalloproteinases, all of which are involved in the initiation and progression of atherosclerosis [6]. Moreover, overproduction of ROS leads to endothelial nitrous oxide synthase uncoupling, mitochondrial dysfunction and impaired antioxidant defenses resulting from the depletion of intracellular NADPH [7]. Recently, Ang II type 2 receptor upregulation reduced atherogenesis, possibly by modulating oxidative stress and the pro-inflammatory cascade, mediated via Akt-1 [8].

Flavonoids are polyphenolic compounds widely distributed in plants and their consumption might be associated with decreased risks for some chronic degenerative diseases in humans [9]. Epidemiological research in Japan has shown that green tea consumption can be protective against coronary atherosclerosis [10]. Recent lines of evidence have shown that epigallocatechin-3-gallate (EGCG), a flavanol that is enriched in green tea, reduces vascular adhesion molecule-1 expression [11,12] and monocyte chemotactic protein-1 production in vascular endothelium [13]. However, the effect of EGCG on the expression of NADPH oxidase, the major enzyme for ROS production in vascular endothelium, remains unknown. Specific inhibitors of NADPH oxidase might be helpful for the treatment of atherosclerosis.

In this study, we hypothesized that EGCG reduces the expression of NADPH oxidase and ROS production by Ang II in human umbilical vein endothelial cells (HUVECs).

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ABBREVIATIONS: EGCG, epigallocatechin-3-gallate; NADPH oxidase, nicotinamide adenine dinucleotide phosphate-oxidase; HUVEC, human umbilical vein endothelial cell; Ang II, angiotensin II; MAPK, mitogen-activated protein kinase; ROS, reactive oxygen species.

METHODS

Materials

All antibodies for western blotting were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The culture medium was obtained from Invitrogen (Carlsbad, CA, USA). Ang II, EGCG and other reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA), unless otherwise specified.

Cell culture

HUVECs were obtained from Clonetics (Walkersville, MD, USA). They were grown in medium 199 containing 0.1 mg/ml heparin, 25 μg/ml endothelial cell growth factor (Biomedical Technologies: Stoughton, MA, USA), 2 mM L-glutamine, 100 U/ml penicillin G, 100 μg/ml streptomycin and 20% fetal bovine serum (FBS). The medium was renewed every two days until confluence, when cells were subcultured at a 1 : 3 ratio and then cultured in an atmosphere of 95% air and 5% CO_2 at 37°C.

Western blot analysis

HUVEC cultures were starved for 12 h and treated with the desired drugs for desired times. Cells were lysed in ice-cold buffer (20 mM Tris-HCl pH 7.4, 1% Triton X-100, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 2.5 mM sodium pyrophosphate, 1 mM β-glycerol phosphate, 1 mM Na_3VO_4, 1 mM PMSF and 1 μg/ml leupeptin). The lysates were sonicated and centrifuged (12,000 rpm, 20 min). Protein concentration was measured by the Bradford method. Equal amounts of protein (10 μg) were run on 12% SDS-PAGE and blotted onto polyvinylidene difluoride membranes. These were incubated with goat polyclonal antibodies (1 : 1,000) for subunits of NADPH oxidase. Secondary antigoat antibodies and enhanced chemiluminescence (ECL) Plus reagent kits (Amersham: Little Chalfont, Buckinghamshire, UK) were used for detection and subsequently exposed to ECL hyperfilms.

Detection of ROS

The cells were starved in phenol red-free M199 medium containing 1% FBS for 12 h and stimulated with Ang II and 2',7'-dichlorofluorescein diacetate for 2 h. Fluorescence signals were quantified (Molecular Devices: Sunnyvale, CA, USA).

Statistical analysis

Results are shown as the means±SEM from at least three independent experiments. Statistical significance between the means was assessed by one-way ANOVA followed by Turkey's multiple comparison test; $p<0.05$ was taken as statistically significant.

RESULTS

Ang II increased the levels of p47^{phox}, p67^{phox} and p22^{phox} in HUVECs

To determine whether the expression of NADPH oxidase, the major enzyme for ROS production in vascular endothelium, would be affected by Ang II, HUVECs were treated with Ang II. Ang II (100 nM) increased the expression of p47^{phox}, a non heme-containing, regulatory subunit of NADPH oxidase, in a time-dependent manner; the levels peaked at 12 h and were sustained until 24 h. However, p67^{phox}, a non heme-containing, regulatory subunit of NADPH oxidase, levels peaked at 6 h and returned to basal levels at 12 h after Ang II treatment; p22^{phox}, a heme-containing, catalytic subunit of NADPH oxidase, levels peaked at 6 h and decreased with time but were above basal levels at 24 h after Ang II treatment (Fig. 1). Thus, Ang II increases the protein level of endothelial NADPH oxidase subunits. However, the changes observed in p67^{phox}, p22^{phox} protein level are contrast to p47^{phox}.

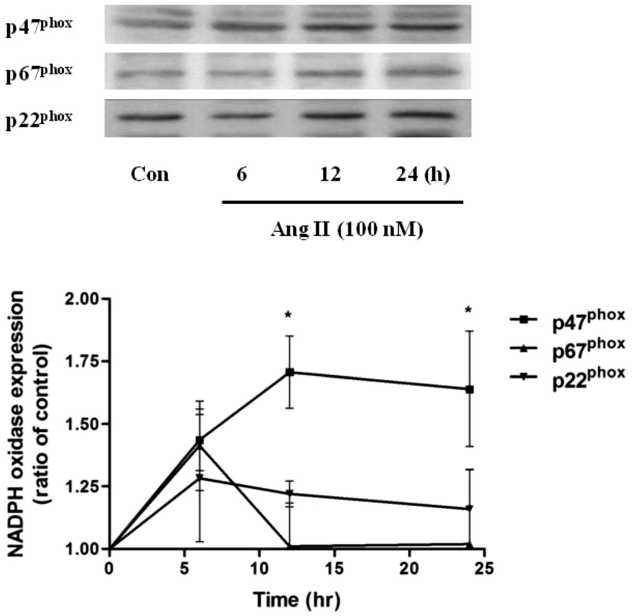

Fig. 1. Effect of Ang II treatment (100 nM, 0～24 h) on the expression levels of NADPH oxidase subunits p47^{phox}, p67^{phox} and p22^{phox} in HUVECs. Summary data are shown as the mean±SEM. *$p<0.05$ by Turkey's multiple comparison test.

Effect of EGCG on the expression of p47^{phox} induced by Ang II in HUVECs

To determine whether Ang II-stimulated p47^{phox}, a non heme-containing, regulatory subunit of NADPH oxidase, expression would be affected by EGCG, HUVECs were pretreated for 0.5 h with 0～50 μM EGCG prior to treatment with Ang II (100 nM) for 24 h. Increasing concentrations of EGCG inhibited Ang II-induced p47^{phox} expression (Fig. 2). Thus, EGCG, a major catechin obtained from green tea leaf, decreases the protein level of p47^{phox} in a concentration-dependent manner.

Effect of EGCG on the production of ROS induced by Ang II in HUVECs

To determine whether Ang II-induced ROS production would be affected by EGCG, HUVECs were pretreated with EGCG prior to treatment with Ang II. Ang II (100 nM) in-

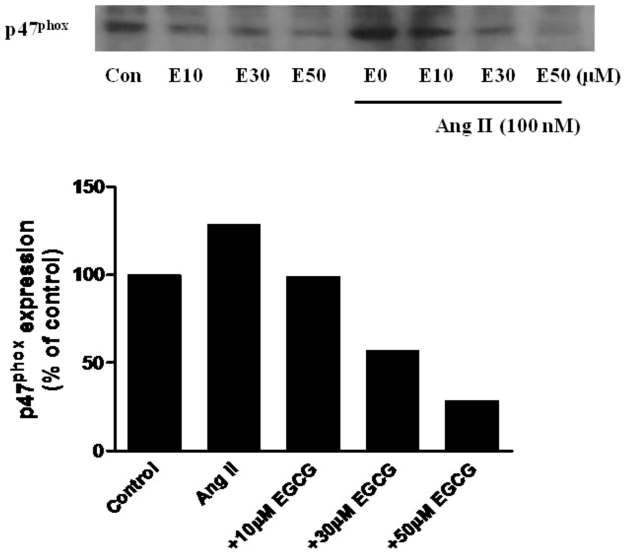

Fig. 2. Effect of EGCG on $p47^{phox}$ expression in HUVECs treated with Ang II (100 nM) for 24 h, 30 min after treatment with EGCG (10～50 μM).

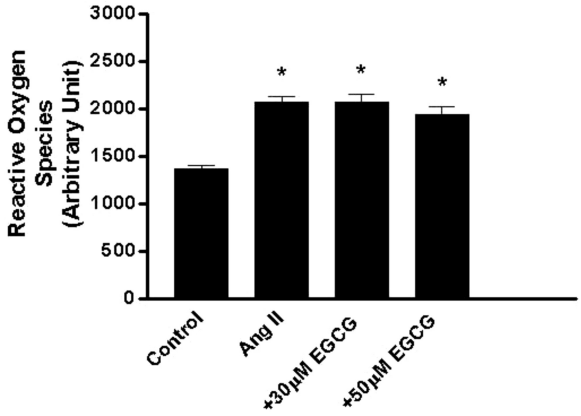

Fig. 3. Effect of EGCG (30～50 μM) on ROS induced by Ang II (100 nM) for 24 h in HUVECs. EGCG was pretreated 30 min before Ang II stimulation. Bars represent the mean±SEM. *$p<0.05$ by Turkey's multiple comparison test.

creased ROS production (151.5±4.2%, n=12, $p<0.05$) compared with control (no treatment) and this was not prevented by 30～50 μM EGCG (151.0±6.6%, n=12, $p<0.05$; 141.3±6.3%, n=12, $p<0.05$, respectively; Fig. 3). These data suggest that EGCG decreases the protein level of $p47^{phox}$, a non heme-containing, regulatory subunit of NADPH oxidase, without affecting ROS.

DISCUSSION

Numerous lines of evidence suggest that increased oxidative stress causes cardiovascular diseases. ROS such as superoxide and H_2O_2 produce vascular inflammation, resulting in atherosclerosis. Vascular NADPH oxidases are predominant sources of superoxide in the vasculature [14]. NADPH oxidase is composed of two essential membrane-bound components, $gp91^{phox}$/Nox2 and $p22^{phox}$, which compose flavocytochrome b_{558}- and four cytosolic components, $p47^{phox}$, $p67^{phox}$, $p40^{phox}$, and the small G protein rac1/2 [15]. Among the four cytosolic components of NADPH oxidase, $p47^{phox}$ is important for vascular ROS production [16]. Moreover, Nox4, a homologue of $gp91^{phox}$/Nox2, might function as the major catalytic component of an endothelial NADPH oxidase [17]. The NADPH oxidases, Nox4 and Nox2, are major sources of ROS in endothelial cells and are implicated both in vasodilator dysfunction [18].

A peptide hormone, Ang II, a major stimulus for vascular NADPH oxidase, also plays an important role in atherosclerosis. AT 1 receptor enhanced the activation of nuclear factor NF-kappaB, which stimulated the production of pro-inflammatory cytokines. Activation of AT 1 receptor via inducible cyclooxygenase promoted biosynthesis of matrix metalloproteinases [19]. Ang II increased superoxide production in endothelial cells from wild-type mice, but not in those from $p47(^{phox-/-})$ knockout mice [20]. Candesartan, an AT 1 receptor antagonist, reduced NADPH activity, attenuated the diabetes-induced over-expression of $p22^{phox}$ and led to a substantial improvement of endothelium-dependent vasodilatation in Sprague Dawley rats [21]. Irbesartan, an AT 1 receptor antagonist, attenuated atherosclerosis, and this effect was partly related to the inhibition of oxidative stress and inflammatory signal transduction pathways in high cholesterol-diet apolipoprotein E knock-out mice [22]. Ang II upregulated the expression levels of Nox4 and $p22^{phox}$ and enhanced Nox4 translocation to the cell membrane in human endothelial cells [23].

Catechin, a flavanol derived from green tea, possesses antioxidant, antiangiogenesis and antiproliferation activities to the prevention and treatment of cardiovascular diseases [24]. EGCG, the major component of green tea catechins, is known to have many effects that will translate to vascular benefits including suppression of vascular hypertrophy [25]. EGCG decreased the risk of cardiovascular disease by reducing inflammatory markers in rats fed an atherogenic diet [26]. EGCG inhibited endothelin-1-stimulated generation of C-reactive protein in vascular smooth muscle cells, which relieved the inflammatory response and oxidative stress by blocking ROS signals and producing an anti-atherosclerotic effect [27]. EGCG inhibited cytosolic subunits of NADPH oxidase from translocating into membrane suggesting inhibition of NADPH oxidase activity in cutaneous mastocytoma cells [28]. Flavonoids prevented oxidative stress in activated monocytes. These inhibitory effects may involve downregulation of PKC-dependent NADPH oxidase pathway, phosphorylation of p38 mitogen-activated protein kinase (MAPK) and extracellular signal-regulated protein kinase in human monocytic THP-1 cells [29].

In this study, we investigated whether EGCG could reduce Ang II-mediated NADPH oxidase expression in HUVECs. Ang II increased the levels of subunits of NADPH oxidase, namely $p47^{phox}$, $p67^{phox}$ and $p22^{phox}$. However, EGCG prevented the $p47^{phox}$, non heme-containing, regulatory subunit of NADPH oxidase, expression induced by Ang II in a concentration-dependent manner. This novel finding for the effects of green tea/EGCG is complemented by findings that the expressional suppression of NADPH oxidase might be an important component of the vascular protective effect of resveratrol, an important antioxidant found in grapes and wine [30]. Moreover, dietary flavonoid, quercetin pre-

vented Ang II-induced endothelial dysfunction by inhibiting the overexpression of $p47^{phox}$ and the subsequent increased $O_2^{\cdot -}$ production, resulting in increased nitric oxide bioavailability [31]. Quercetin and catechin regulated S100B-activated oxidant stress-sensitive pathways through blocking $p47^{phox}$ protein expression in human monocytes [32]. Together, these results show that flavonoids have a common inhibitory effect on NADPH oxidase expression in a number of different cell types in a variety of conditions.

To understand the potential mechanism(s) for EGCG attenuation of $p47^{phox}$ expression by Ang II, we confirmed that ROS attenuation in HUVECs induced by EGCG led to an inhibition of $p47^{phox}$ expression. Our data demonstrated that EGCG did not affect ROS induced by Ang II. However, EGCG was effective in reducing $CoCl_2$-derived ROS [33]. Ang II activated p38 MAPK and increased ROS in the vasculature [34]. Therefore, an inhibition of NADPH oxidase expression by EGCG may be insufficient to affect ROS production in HUVECs. And an interference on ROS by EGCG may not be necessary for an inhibition of endothelial NADPH oxidase expression.

In conclusion, the antiatherosclerotic activity of EGCG is likely not associated with decreased ROS production although there may be some benefit through inhibiting the expression of NADPH oxidase induced by Ang II.

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