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The EGCg-induced redox-sensitive activation of endothelial nitric oxide synthase and relaxation are critically dependent on hydroxyl moieties

Cyril Auger [a], Jong-Hun Kim [a], Philippe Chabert [b], Mehdi Chaabi [b], Eric Anselm [a], Xavier Lanciaux [a], Annelise Lobstein [b], Valérie B. Schini-Kerth [a,*]

[a] UMR CNRS 7213, Laboratoire de Biophotonique et Pharmacologie, Faculté de Pharmacie, Université de Strasbourg, Illkirch, France
[b] UMR CNRS 7200, Laboratoire d'Innovation Thérapeutique, Faculté de Pharmacie, Université de Strasbourg, Illkirch, France

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ABSTRACT

Several rich sources of polyphenols stimulate the endothelial formation of nitric oxide (NO), a potent vasoprotecting factor, via the redox-sensitive activation of the PI3-kinase/Akt pathway leading to the phosphorylation of endothelial NO synthase (eNOS). The present study examined the molecular mechanism underlying the stimulatory effect of epicatechins on eNOS. NO-mediated relaxation was assessed using porcine coronary artery rings in the presence of indomethacin, and charybdotoxin plus apamin, inhibitors of cyclooxygenases and EDHF-mediated responses, respectively. The phosphorylation level of Akt and eNOS was assessed in cultured coronary artery endothelial cells by Western blot, and ROS formation using dihydroethidine. (−)-Epigallocatechin-3-*O*-gallate (EGCg) caused endothelium-dependent relaxations in coronary artery rings and the phosphorylation of Akt and eNOS in endothelial cells. These responses were inhibited by membrane-permeant analogues of superoxide dismutase and catalase, whereas native superoxide dismutase, catalase and inhibitors of major enzymatic sources of reactive oxygen species including NADPH oxidase, xanthine oxidase, cytochrome P450 and the mitochondrial respiration chain were without effect. The EGCg derivative with all hydroxyl functions methylated induced neither relaxations nor the intracellular formation of ROS, whereas both responses were observed when the hydroxyl functions on the gallate moiety were present. In conclusion, EGCg causes endothelium-dependent NO-mediated relaxations of coronary artery rings through the Akt-dependent activation of eNOS in endothelial cells. This response is initiated by the intracellular formation of superoxide anions and hydrogen peroxide, and is critically dependent on the gallate moiety and on the presence of hydroxyl functions possibly through intracellular auto-oxidation.

Introduction

Several epidemiological studies have indicated an inverse correlation between consumption of polyphenol-rich sources of food including red wine and green tea, and mortality from cardiovascular diseases [1–4]. The protective effects of polyphenols on the cardiovascular system has been attributable to several mechanisms, including improvement of the lipid profile, anti-atheroscelotic, anti-hypertensive, and anti-inflammatory effects [5–8]. Polyphenols and polyphenol-rich sources of food have also been shown to protect the endothelial function by acting directly on endothelial cells [9–11]. Indeed, red wine polyphenols and green tea polyphenols induce endothelium-dependent relaxations of isolated arteries involving a NO-mediated component and also, in some arteries, an endothelium-derived hyperpolarizing factor (EDHF)-mediated component [11–13]. The polyphenol-induced endothelial formation of NO is mediated by Src kinase leading to the subsequent PI3-kinase/Akt-dependent phosphorylation of eNOS, and this response does not seem to involve estrogen receptors, insulin receptors, laminin receptors and VEGF receptors [11,14–16]. The fact that intracellular scavengers of ROS prevented the grape-derived polyphenol-induced activation of Src and the subsequent PI3-kinase/Akt-dependent activation of eNOS implies a redox-sensitive event [14,17]. Although ROS formation is an early key event in the signal transduction pathway leading to eNOS activation, the cellular and molecular sources of the endothelial formation of ROS in response to polyphenols remain unclear. Therefore, the aim of the present study was to determine whether the redox-sensitive activation of eNOS by polyphenols involves major enzymatic sources of ROS including NADPH oxidase, xanthine oxidase, cytochrome P450 and the mitochondrial respiration chain, and/or auto-oxidation of

Abbreviations: EGCg, (−)-epigallocatechin-3-*O*-gallate; 5M-EGCg, pentamethylated EGCg; 8M-EGCg, permethylated EGCg; eNOS, endothelial nitric oxide synthase; NO, nitric oxide.

* Corresponding author. Address: UMR CNRS 7213 – Laboratoire de Biophotonique et Pharmacologie, Université de Strasbourg – Faculté de Pharmacie, 74, route du Rhin, 67401 Illkirch, France. Fax: +33 3 68 85 43 13.
E-mail address: valerie.schini-kerth@unistra.fr (V.B. Schini-Kerth).

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hydroxyl functions of the polyphenol structure [18–20]. Indeed, hydroxyl functions of polyphenols can be oxidized to form semiquinone and then quinones with the concomitant generation of superoxide anions at each oxidation step [19]. Since grape-derived polyphenols are a complex mixture of several hundreds of polyphenols, the present investigations were performed with authentic tea catechins including (−)-epigallocatechin-3-*O*-gallate (EGCg), and EGCg derivatives, which have been chemically modified to replace the oxidable hydroxyl functions by non-oxidable methoxy functions (Fig. 1).

Materials and methods

Vascular reactivity studies were done in isolated porcine coronary arteries as described previously [14,21]. Briefly, coronary artery rings were suspended in organ baths and constricted with U46619 before a concentration–relaxation curve to EGCg or an EGCg derivative was constructed. All experiments were performed in the presence of indomethacin (10 μM), an inhibitor of cyclooxygenases, and the combination of charybdotoxin (100 nM) and apamin (100 nM), two inhibitors of the EDHF pathway, to assess only the NO-mediated relaxation. In some experiments, rings were incubated with a pharmacological agent for 30 min before addition of U46619.

Porcine coronary artery endothelial cells were isolated and cultured using methods previously described [22]. Briefly, endothelial cells were isolated from porcine coronary arteries by collagenase treatment (type I, Worthington, 1 mg/mL for 12 min at 37 °C), and cultured in culture dishes containing medium MCDB 131 (Invitrogen) with 15% foetal calf serum supplemented with penicillin (100 U/mL), streptomycin (100 U/mL), fungizone (250 μg/mL), and L-glutamine (2 mM) (all from Cambrex). All experiments were performed with confluent cultures of cells used at first passage.

Western blot analyses were performed as previously described by Anselm et al. [22] using selected primary antibody (eNOS, p-Akt Ser473 and p-eNOS Ser1177, Cell Signalling Technology; dilution of 1:1000).

The oxidative fluorescent dye dihydroethidine (DHE, Sigma–Aldrich) was used to evaluate the production of ROS in cultured endothelial cells as described previously [23]. Cultured coronary endothelial cells in Hanks' balanced salt solution were loaded with DHE (2.5 μM) for 20 min before treatment either with solvent (ethanol 0.5%), EGCg or an EGCg derivative (100 μM) for 15 min at 37 °C. In some experiments, cells were exposed to MnTMPyP (100 μM) for 30 min before treatment. Images were obtained with a Leica DM 4000 fluorescence microscope equipped with CY3 filter and analysed using ImageJ software (National Health Institute, USA).

To obtain permethylated EGCg (8M-EGCg), EGCg was permethylated with dimethylsulfate and potassium carbonate in anhydrous acetone under reflux (yield 90%) [24]. Pentamethylated EGCg (5M-EGCg) or (−)-(2*R*,3*R*)-5,7-dimethoxy-2-(3,4,5-trimethoxyphenyl)chroman-3-yl-(3,4,5-trihydroxy)benzoate was prepared in a four step procedure (overall yield 58%) starting from EGCg according to a modified method [25]. Briefly, permethylation of the EGCg followed by saponification of the ester moiety with sodium hydroxide in methanol gave (−)-(2*R*,3*R*)-5,7-dimethoxy-2-(3,4,5-trimethoxyphenyl)chroman-3-ol (yield 81%). Acylation with DCC/DMAP in anhydrous dichloromethane using 3,4,5-tribenzyloxybenzoic acid yielded an ester linkage (yield 72%). Deprotection of all benzyl groups was quantitative in ethyl acetate under a hydrogen atmosphere using palladium hydroxide as catalyst.

For 8M-EGCg and 5M-EGCg, ^{1}H and ^{13}C NMR spectra were obtained on a Bruker Avance 300 MHz instrument with $CDCl_3$ and MeOD as solvent respectively.

Reagents were obtained from Sigma–Aldrich (St. Quentin Fallavier, France) except 9,11-dideoxy-11α,9α-epoxymethano-prostaglandin $F_{2\alpha}$ (U46619) from Cayman Chemical (Ann Arbor, MI, USA), and the SOD mimetic Mn (III) tetrakis (1-methyl-4-pyridyl)porphyrin (MnTMPyP) from Alexis Biochemicals (Coger, France). Organic solvents were obtained from Carlo Erba Chemicals (Peypin, France). EGCg was kindly provided by DSM Nutritional Products (Basel, Switzerland).

Values are expressed as means ± SEM. Statistical evaluation was performed with a paired *t*-test and ANOVA for paired data followed

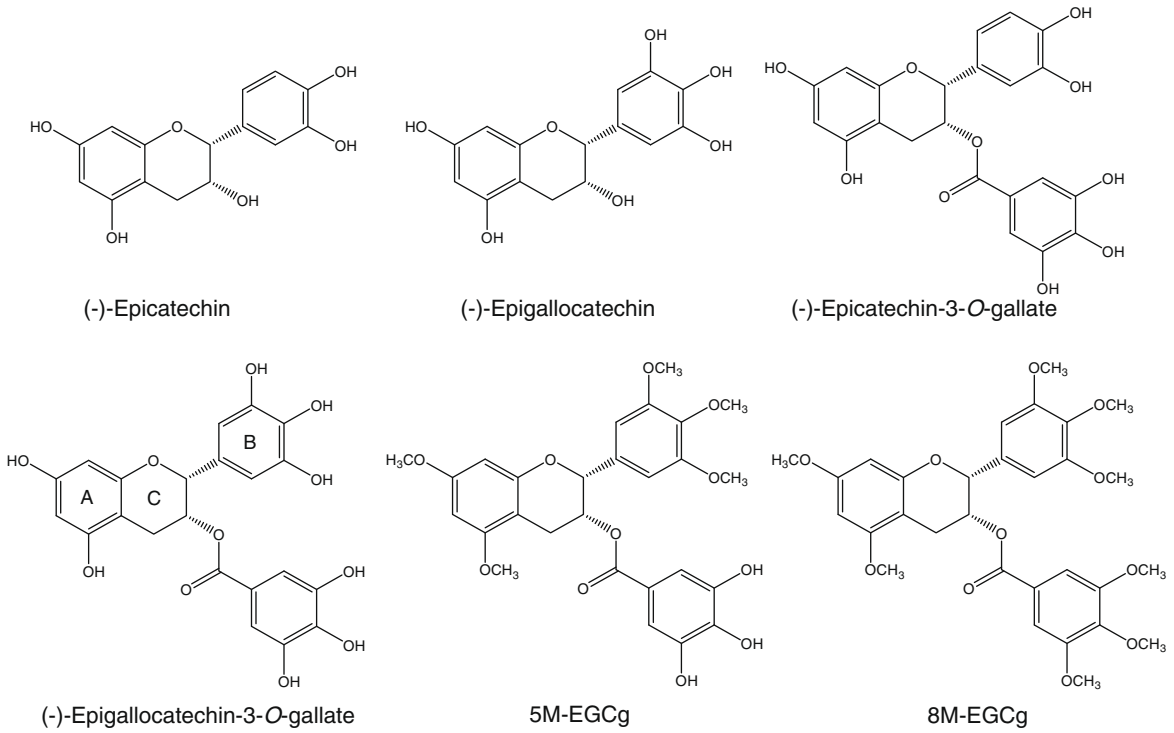

Fig. 1. Chemical structures of the epicatechins and hemisynthetic EGCg derivatives.

by Fischer's protected least significant difference test as appropriate. Values of $P < 0.05$ were considered statistically significant.

Results

In the presence of indomethacin, and charybdotoxin plus apamin to inhibit the formation of vasoactive prostanoids and EDHF-mediated responses, respectively, EGCg-induced concentration-dependent relaxations in porcine coronary artery rings with endothelium whereas only minor relaxations were observed in rings without endothelium (Fig. 2 A). Endothelium-dependent relaxations to EGCg were markedly reduced by N^{ω}-nitro-L-arginine, a competitive eNOS inhibitor, demonstrating the involvement of NO (Fig. 2A).

Since the NO-mediated relaxation to grape-derived polyphenols is a redox-sensitive event [11,14,17], experiments were performed to determine the role of ROS in the EGCg-induced NO-mediated relaxation. Relaxations to EGCg were markedly reduced by the membrane-permeant analogue of SOD, MnTMPyP, and also, to a smaller extent, by the membrane-permeant analogue of catalase, PEG-catalase (Fig. 2B). In contrast, native superoxide dismutase and native catalase, which are unable to cross membranes, had only minor effects (Fig. 2B). These results indicate that the EGCg-induced NO-mediated endothelium-dependent relaxation is a redox-sensitive event requiring the intracellular formation of superoxide anions and also, to some extent, hydrogen peroxide.

In endothelial cells, several enzymatic sources of ROS have been identified including NADPH oxidase, xanthine oxidase, cytochrome P450 and the mitochondrial respiration chain [18]. Therefore, the possibility that these potential endogenous sources of ROS contribute to the redox-sensitive activation of eNOS by EGCg has been examined. Inhibitors either of NADPH oxidase (apocynin), xanthine oxidase (allopurinol), cytochrome P450 (sulfaphenazole) or the mitochondrial respiratory chain (KCN, myxothiazol, and rotenone) affected only minimally relaxations to EGCg indicating that these endogenous sources of ROS are not involved (Fig. 2C).

Alternatively, the EGCg structure itself may contribute to the endogenous formation of ROS following auto-oxidation of hydroxyl functions present either on the B ring or the gallate moiety (Fig. 1) [19]. Such a mechanism can generate superoxide anions during oxidation of EGCg into a semi-quinone and also during its subsequent oxidation into a quinone [21]. To test such a hypothesis, a permethylated EGCg (8M-EGCg) with all eight hydroxyl functions replaced by methoxy groups has been synthesized to prevent the auto-oxidation process. In contrast to EGCg, 8M-EGCg was unable to induce relaxations in coronary artery rings (Fig. 2D). In addition, to determine the importance of hydroxyl functions on the flavan-3-ol structure and those on the gallate structure, a pentamethylated EGCg derivative (5M-EGCg) with all hydroxyl functions on the flavan-3-ol structure replaced by methoxy functions was synthesized and tested (Fig. 1). As indicated in panels D and E of Fig. 2, 5M-EGCg was still able to cause concentration-dependent relaxations in coronary artery rings with endothelium although to a smaller extent as EGCg whereas only small relaxations were observed in rings without endothelium. In addition, relaxations to 5M-EGCg in rings with endothelium were significantly reduced

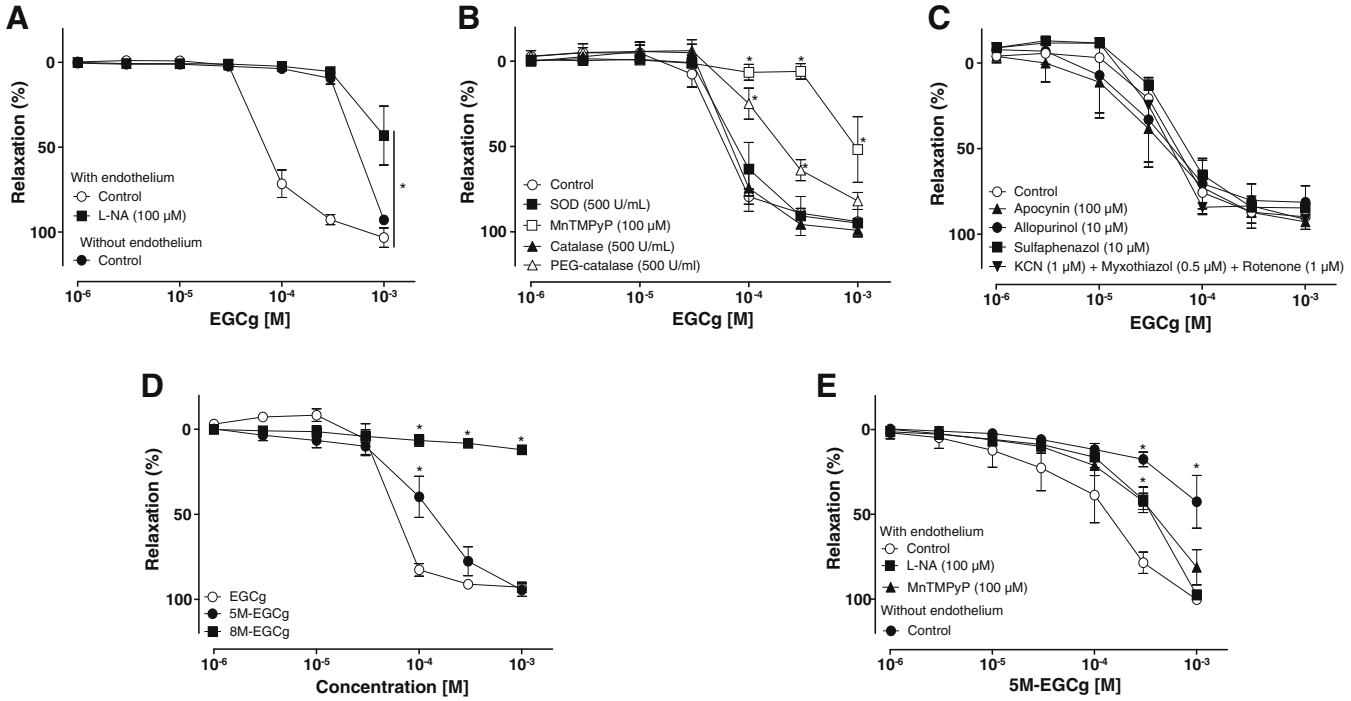

Fig. 2. Vascular reactivity studies in porcine coronary artery rings. (A) (−)-Epigallocatechin-3-*O*-gallate (EGCg) causes endothelium-dependent NO-mediated relaxations in coronary artery rings. Rings with endothelium were exposed the NO synthase inhibitor N^{ω}-nitro-L-arginine (L-NA; 100 μM) for 30 min before addition of increasing concentrations of EGCg. (B) EGCg causes redox-sensitive endothelium-dependent NO-mediated relaxations in coronary artery rings. Rings with endothelium were exposed to either native superoxide dismutase (SOD), native catalase, membrane-permeant SOD mimetic (MnTMPyP), or membrane-permeant catalase (PEG-catalase) for 30 min before addition of increasing concentrations of EGCg. (C) Role of several major enzymatic sources of ROS in the redox-sensitive endothelium-dependent NO-mediated relaxations to EGCg. Arterial rings with endothelium were exposed to either apocynin (NADPH oxidase inhibitor, 100 μM), allopurinol (xanthine oxidase inhibitor, 10 μM), sulfaphenazole (cytochrome P450 inhibitor, 10 μM) or inhibitors of the mitochondrial respiration chain (KCN 1 μM, myxothiazol 0.5 μM, and rotenone 1 μM) for 1 h before addition of increasing concentrations of EGCg. (D) Role of hydroxyl functions in the EGCg-induced redox-sensitive endothelium-dependent NO-mediated relaxations in coronary artery rings. Arterial rings with endothelium were exposed to increasing concentrations of either EGCg, pentamethylated EGCg (5M-EGCg) or permethylated EGCg (8M-EGCg). (E) Characterization of the 5M-EGCg-induced relaxations. Some rings with endothelium were exposed to either N^{ω}-nitro-L-arginine (L-NA; 100 μM) or MnTMPyP (100 μM) for 1 h before addition of increasing concentrations of 5M-EGCg. All experiments were performed in the presence of indomethacin (10 μM) and charybdotoxin (100 nM) + apamin (100 nM) to prevent the formation of prostanoids and endothelium-derived hyperpolarizing factor-mediated responses, respectively. Results are means ± SEM of 5 different experiments. $^{*}P < 0.05$ vs. control.

by L-NA and MnTMPyP, indicating the involvement of NO and superoxide anion, respectively (Fig. 2E).

The further characterization of the signalling pathway mediating the stimulatory effect of EGCg on eNOS was done in cultured coronary artery endothelial cells by determining the phosphorylation level of both Akt on serine 473 and eNOS on serine 1177. The importance of the gallate group was assessed by studying the effect of (−)-epicatechin, (−)-epigallocatechin, (−)-epicatechin-3-*O*-gallate and of EGCg. The data indicate that (−)-epicatechin-3-*O*-gallate and EGCg significantly induced phosphorylation of Akt and eNOS, whereas no such effects were observed with (−)-epicatechin and (−)-epigallocatechin, indicating a key role for the gallate moiety (Fig. 3A). The EGCg-induced activation of Akt and eNOS is significantly inhibited by membrane-permeant analogues either of SOD (MnTMPyP and PEG–SOD) or catalase (PEG-catalase) whereas corresponding native enzymes did not have such an effect (Fig. 3B and C). These results indicate that the intracellular formation of superoxide anions and hydrogen peroxide plays a crucial role in the stimulatory effect of EGCg on eNOS activation. Moreover, treatment of endothelial cells with specific inhibitors of endogenous enzymatic sources of ROS including apocynin, allopurinol, sulfaphenazole and rotenone did not affect the EGCg-induced phosphorylation of Akt and eNOS (data not shown) indicating that these enzymatic sources of ROS are not involved in the EGCg-induced redox-sensitive phosphorylation of Akt and eNOS.

The redox-sensitive fluorescent probe dihydroethidine was used to obtain direct evidence that EGCg causes ROS formation in endothelial cells. As indicated in Fig. 4, EGCg significantly increased the fluorescence signal in endothelial cells and this effect is prevented by MnTMPyP indicating the involvement of superoxide anions. The stimulatory effect of EGCg is mimicked by 5M-EGCg but not by 8M-EGCg (Fig. 4).

Discussion

The present findings indicate that the EGCg-induced endothelium-dependent NO-mediated relaxation of coronary artery rings is a redox-sensitive event involving intracellular superoxide anions and hydrogen peroxide. They further indicate that EGCg caused generation of superoxide anions in endothelial cells as an early signal to trigger the PI-3-kinase/Akt-dependent activation of eNOS through phosphorylation of Ser1177. The characterization of the pro-oxidant response of endothelial cells to EGCg indicated a major role of hydroxyl functions in particular those on the gallate moiety rather than major enzymatic sources of ROS including NADPH oxidase, xanthine oxidase, cytochrome P450 and the mitochondrial respiration chain. It is likely that the hydroxyl functions of EGCg are auto-oxidized in endothelial cells to form a semi-quinone and then a quinone with the concomitant generation of superoxide anions, which, in turn, mediate the stimulatory effect of EGCg to eNOS through the PI3-kinase/Akt pathway. However, the present findings do not rule out the possibility that hydroxyl functions contribute to either the interaction of EGCg with essential endothelial cell targets or its stability.

Several rich sources of polyphenols including green tea, red wine and chocolate have been shown to improve flow-mediated vasodilatation in both healthy humans and subjects with coronary artery disease by increasing the endothelial formation of NO [26–29]. Green tea- and grape-derived polyphenols also effectively induced endothelium-dependent relaxations of isolated arteries by increasing the endothelial formation of NO and, also in some arteries, that of EDHF [14,16,17]. In contrast to physiological agonists such as bradykinin, the ability of tea- and grape-derived polyphenols to induce the formation of NO is a sustained event, which occurs for several hours rather than several minutes [17,30,31]. Such a time course of the formation of NO in endothelial cells may help to promote beneficial effects on the vascular system. The characterization of the signalling pathway has indicated that both green tea polyphenols and grape-derived polyphenols caused the PI3-kinase-dependent activation of Akt by phosphorylation of Ser 473, which in turn activates eNOS by phosphorylation of Ser1177, an activator site, and the dephosphorylation of eNOS on Thr495, an inhibitor site [11,14,16,31]. Moreover, Src kinase has been identified as an upstream mediator of the PI3-kinase/Akt signalling

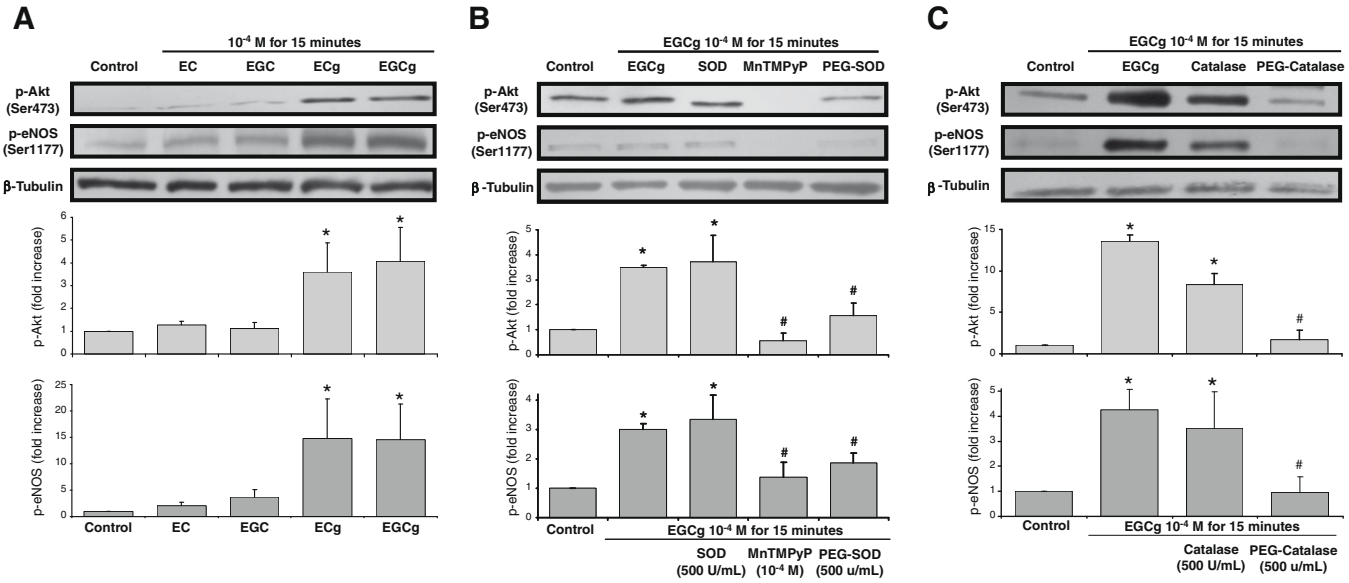

Fig. 3. Western blot analyses in cultured coronary artery endothelial cells. (A) Effect of several epicatechins on the phosphorylation of Akt at Ser473 and eNOS at Ser1177 in cultured endothelial cells. Cells were exposed to an epicatechin for 15 min at 37 °C. EC: (−)-epicatechin; EGC: (−)-epigallocatechin; ECg: (−)-epicatechin-3-*O*-gallate; EGCg: (−)-epigallocatechin-3-*O*-gallate. (B and C) Role of superoxide anions and hydrogen peroxide in the EGCg-induced phosphorylation of Akt and eNOS in endothelial cells. Cells were incubated either (B) with solvent, SOD (500 U/mL), MnTMPyP (100 μM), PEG–SOD (500 U/mL), or either (C) with catalase (500 U/mL) or PEG-catalase (500 U/mL) for 30 min before the addition of EGCg. The level of p-Akt and p-eNOS was determined by Western blot analysis. Representative immunoblots, and corresponding cumulative data. Results are shown as means ± SEM of 5 different experiments. $^{*}P < 0.05$ versus control. $^{\#}P < 0.05$ versus EGCg.

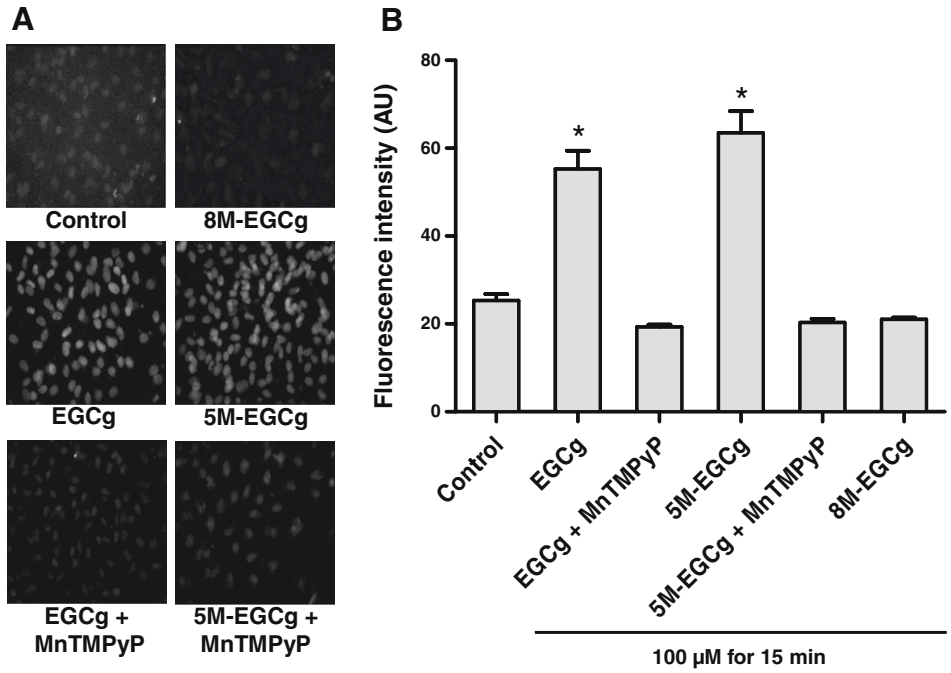

Fig. 4. Effect of EGCg and the 5- and 8-methylated EGCg derivatives on the formation of ROS in endothelial cells. Dihydroethidine (DHE, 2.5 µM)-loaded cells were exposed to either solvent or MnTMPyP (100 µM) for 30 min before the addition of an epicatechin for 15 min. (A) Representative original micrographs, and (B) corresponding cumulative data. Results are shown as means ± SEM of 3 different experiments. $^{*}P < 0.05$ versus control.

pathway of the green tea polyphenol and the grape-derived polyphenol-induced activation of eNOS [14,16,17]. Although polyphenols have antioxidant properties [32], the present findings indicate that ROS are required for the EGCg-induced activation of eNOS. Indeed, EGCg-induced endothelium-dependent NO-mediated relaxations and phosphorylation of eNOS are markedly reduced by membrane-permeant analogues of either SOD or catalase whereas native SOD and native catalase were inactive. Similar findings were observed previously with grape-derived polyphenols [11,14,17]. In contrast, NO-mediated relaxations to bradykinin are only minimally affected by the membrane-permeant analogue of SOD [11]. The antioxidant *N*-acetylcysteine also prevented EGCg-induced phosphorylation of eNOS [16]. Altogether the present findings in conjunction with those previous ones indicate that the intracellular formation of superoxide anions and hydrogen peroxide is a key event in both green tea polyphenol- and grape-derived polyphenol-induced endothelial formation of NO. Moreover, direct evidence that EGCg and grape-derived polyphenols stimulate the formation of superoxide anions and hydrogen peroxide specifically in endothelial cells has been obtained using redox-sensitive fluorescent probes in cultured endothelial cells and also in arterial sections [11,14,16,17, present findings]. In addition, ROS, predominantly superoxide anions, have been shown to act as upstream activators of Src kinase leading to the subsequent PI3-kinase/Akt-dependent phosphorylation of eNOS in response to EGCg and grape-derived polyphenols [14,16,17]. Despite their key role in the signal transduction pathway leading to eNOS activation, the source of the polyphenol-induced intracellular formation of ROS in endothelial cells remains unclear. Several enzymes are known to generate intracellular superoxide anions in a controlled way in endothelial cells. The most important ones are the membrane-located NADPH oxidase, xanthine oxidase, arachidonic acid–metabolizing enzymes such as cytochrome P450, and the mitochondrial electron transport chain [18]. The present findings showing that inhibitors of these major enzymatic sources of superoxide anions did affect neither the EGCg-induced NO-mediated relaxation nor the phosphorylation of eNOS imply the involvement of another source of ROS, which, however, remains to be determined. Alternatively, the polyphenolic structure itself can also generate superoxide anions through an auto-oxidation process.

During auto-oxidation of a phenolic structure, a group of two ortho hydroxyl functions provides through a two-step oxidation process first a semi-quinone and, then, a quinone [21]. Each step of oxidation generates superoxide anions [20]. Auto-oxidation of EGCg has been reported to occur *in vitro*, both in cultured cells and in physiological solution, either yielding an EGCg quinone or generating various dimeric structures like theasinensins [20,21,33]. Thus, the formation of ROS through an auto-oxidation process is critically dependent on conjugated hydroxyl functions such as those on the B ring and the gallate moiety of EGCg, both featuring three conjugated hydroxyl functions.

The present findings indicate that the gallate moiety on C3 is a key feature for the stimulatory effect of catechin on eNOS since (−)-epicatechin-3-*O*-gallate and EGCg significantly induced the phosphorylation of Akt and eNOS whereas (−)-epicatechin and (−)-epigallocatechin were inactive. To further assess the role of hydroxyl functions in the EGCg-induced activation of eNOS, two methylated EGCg derivatives were prepared by hemisynthesis: a permethylated EGCg (8M-EGCg) with all hydroxyl functions methylated, and a pentamethylated EGCg (5M-EGCg) with the 5 hydroxyl functions on the flavan-3-ol structure methylated whereas the 3 hydroxyl functions on the gallate moiety remain free. In contrast to EGCg, 8M-EGCg did not induce NO-mediated relaxations in coronary artery rings. These findings suggest a key role of hydroxyl functions in the EGCg-induced NO-dependent relaxation. A previous study has also shown that methylation of a grape seed extract abolished its ability to cause endothelium-dependent relaxations in rabbit aortic rings [34]. In contrast to 8M-EGCg, the 5M-EGCg induced, although to a slightly lesser extent as EGCg, significant NO-mediated endothelium-dependent relaxations in coronary artery rings that require superoxide anions. These findings indicate that hydroxyl functions on the flavan-3-ol structure are not essential whereas those on the gallate moiety are critical for the NO-mediated vasorelaxation. They are also in agreement with the present

findings that in contrast to 8M-EGCg, 5M-EGCg caused the generation of superoxide anions in endothelial cells.

In conclusion, the present findings indicate that EGCg-induced endothelium-dependent and NO-mediated relaxation in coronary artery rings, which is dependent on the intracellular formation of both superoxide anions and hydrogen peroxide. They further demonstrate that the pro-oxidant response of endothelial cells to EGCg is critically dependent of the presence of hydroxyl functions and in particular on those of the gallate moiety in C3 rather than on major endogenous enzymatic sources of ROS. Further studies are required to determine whether EGCg generates ROS directly from its structure by an auto-oxidation process occurring specifically in endothelial cells, and to identify the key hydroxyl functions in EGCg.

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