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

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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.

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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

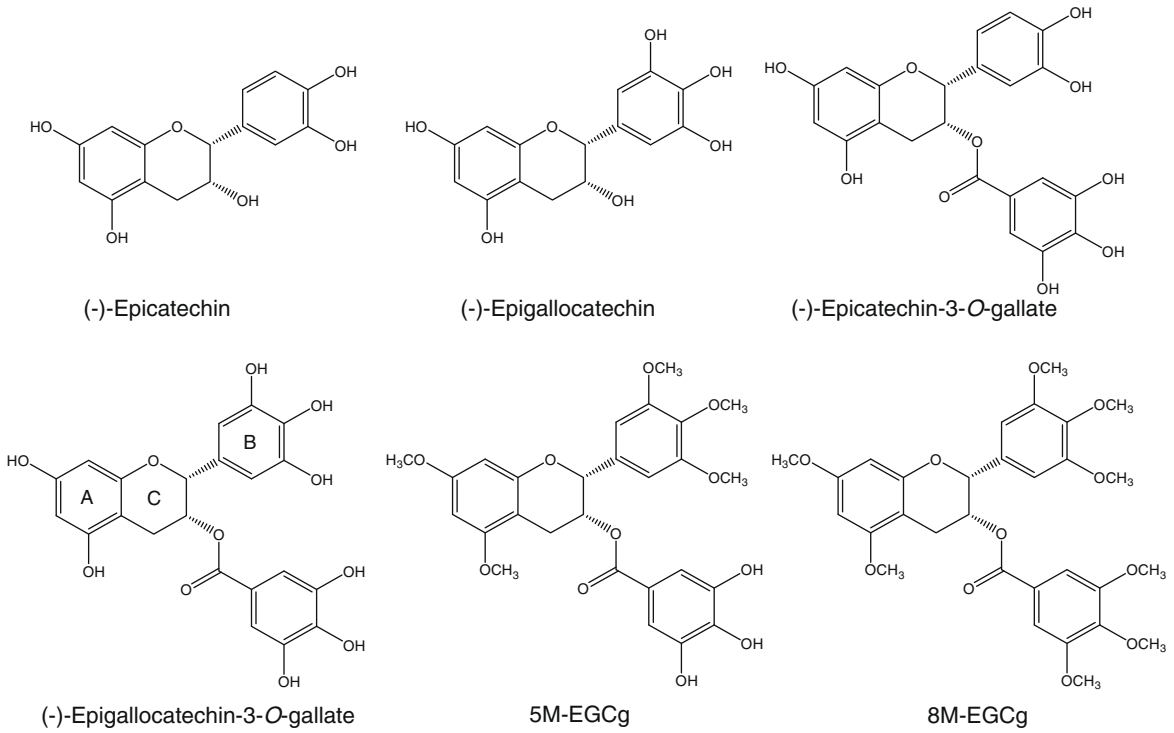

Fig. 1. Chemical structures of the epicatechins and hemisynthetic EGCg derivatives.