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Synthesis of novel (−)-epicatechin derivatives as potential endothelial GPER agonists: Evaluation of biological effects

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ABSTRACT

To potentially identify proteins that interact (i.e. bind) and may contribute to mediate (−)-epicatechin (Epi) responses in endothelial cells we implemented the following strategy: 1) synthesis of novel Epi derivatives amenable to affinity column use, 2) *in silico* molecular docking studies of the novel derivatives on G protein-coupled estrogen receptor (GPER), 3) biological assessment of the derivatives on NO production, 4) implementation of an immobilized Epi derivative affinity column and, 5) affinity column based isolation of Epi interacting proteins from endothelial cell protein extracts. For these purposes, the Epi phenol and C3 hydroxyl groups were chemically modified with propargyl or mesyl groups. Docking studies of the novel Epi derivatives on GPER conformers at 14 ns and 70 ns demostrated favorable thermodynamic interactions reaching the binding site. Cultures of bovine coronary artery endothelial cells (BCAEC) treated with Epi derivatives stimulated NO production via Ser1179 phosphorylation of eNOS, effects that were attenuated by the use of the GPER blocker, G15. Epi derivative affinity columns yielded multiple proteins from BCAEC. Proteins were electrophoretically separated and inmmunoblotting analysis revealed GPER as an Epi derivative binding protein. Altogether, these results validate the proposed strategy to potentially isolate and identify novel Epi receptors that may account for its biological activity.

Flavonoids are an important class of widely distributed natural products that posses a diverse range of biological activities.[1,2] Accumulating evidence indicates that the consumption of flavanol-rich foods such as those found in cacao-based products, protects against cardiometabolic diseases.[3–5] (−)-Epicatechin (**Epi**) is the main flavanol present in cacao seeds and its oral intake mimicks the beneficial vascular effects observed after the consumption of cocoa products.[6,7] A proposed mechanism through which **Epi** mediates its vascular effects include the stimulation of nitric oxide (NO) production via endothelial NO synthase (eNOS) activation.[8] Evidence indicates that eNOS activation can occur secondary to the stimulation of cell surface receptors including those from the tyrosine kinase and G-protein-coupled receptor (GPCRs) families.[9,10] Due to the healthy effects triggered by Epi, there is an increasing interest in elucidating the mechanisms by which this flavanol mediates its cardiometabolic protective effects.[11,12]

We recently demonstrated that **Epi** stimulates NO production through the involvement of the G-protein coupled estrogen (GPER) and epidermal growth factor receptors (EGFR).[13] However, the use of selective blockers or receptor gene silencing approaches resulted in a partial blockade of Epi stimulated NO production. Thus, other cell membrane receptors are likely involved in mediating the effects of Epi and there is little knowledge about the identity of

Abbreviations: Epi, (−)-epicatechin; eNOS, endothelial nitric oxide synthase; NO, nitric oxide; GPER, G-protein coupled estrogen receptor; BCAEC, bovine coronary artery endothelial cells; GPCRs, G-protein coupled receptors; EPI-COLUMN, affinity column with Epi covalently bound; Epi-4-prop, 3,5,7,3′,4′-penta-*O*-propargyl-(−)-epicatechin; Epi-Ms, 3-*O*-mesyl-(−)-epicatechin; Epi-5-prop, 5,7,3′,4′-tetra-*O*-propargyl-(−)-epicatechin; Epi-prop, 3-*O*-propargyl-(−)-epicatechin; G15, GPER antagonist; BPY, 5,5-difluoro-1,3,7,9-tetramethyl-N-(prop-2-yn-1-yl)-5H-4λ4,5λ4-dipyrrolo[1,2-*c*:2′,1′-*f*][1,3,2]diazaborinin-10-amine; SAR, structure–activity relationship.

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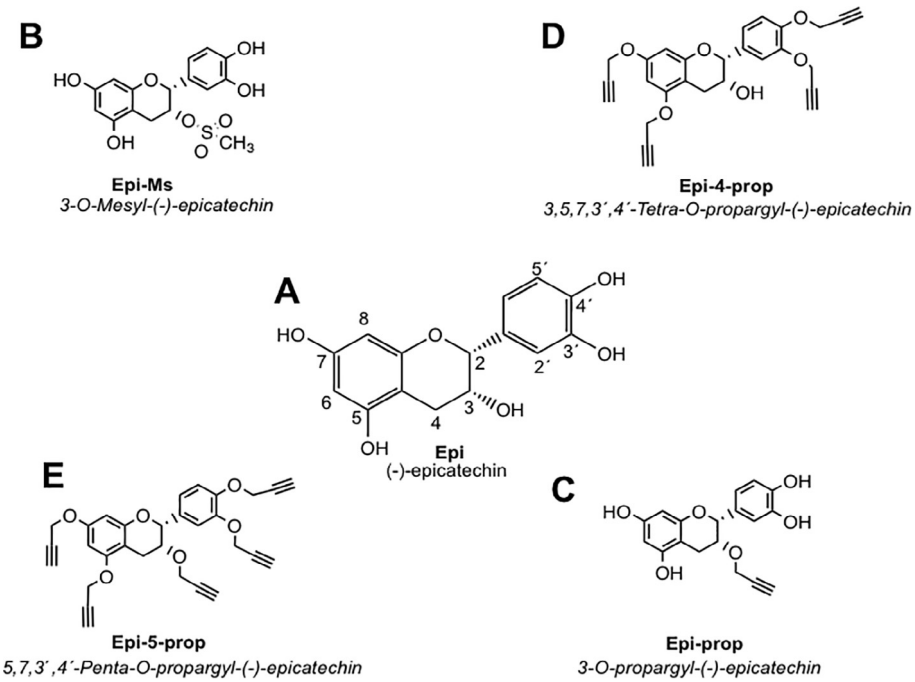

Fig. 1. Structures of (A) (−)-epicatechin (**Epi**) and its synthetized derivatives; **Epi-5-prop (E), Epi-4-prop (D), Epi-Ms. (B)** and **Epi-prop (C)**.

such structures. Interestingly, several studies have suggested that the biological properties of flavonoids are largely dependent on the availability of "free" phenol groups on their structure (Fig. 1).[14,15]

We thus, implemented a rational strategy comprising the following steps: 1) synthesis of novel Epi derivatives (which relied on the introduction of mesyl or propargyl groups) that may be optimal for the generation of affinity columns and purification of Epi binding proteins, 2) *in silico* molecular docking of the novel Epi derivatives on a previously validated GPER platform, 3) *in vitro* analysis of the novel Epi derivatives on NO production and, 4) implementation of an inmobilized Epi derivative affinity column to isolate binding Epi proteins from endothelial cell protein extracts. Flavonoid effects appear to be structure-dependent and a major determinant factor is the presence of hydroxyl (i.e. phenols and alcohol groups) moieties.[14,15] The esterification and alkylation of the hydroxyl groups are commonly used methods used to generate flavonoid derivatives. Using this strategy, others and we have synthesized novel flavonoid derivatives by targeting their phenol groups.[16–19]. In this study, we modified the structure of **Epi** by targeting its phenol (3′,4′, 5 and 7 position) and alcohol (C-3 position) groups (Fig. 1A). For the synthesis of the derivatives, native **Epi** was used as a starting material. The detailed synthetic procedures used to obtain each **Epi** derivative are presented in Supplementary data. As a first step, we introduced mesyl or propargyl group substituents in the **Epi** molecule at the C-3 alcohol group in order to keep the four phenolic groups available (Fig. 1B and C respectively). We also alkylated the four phenol groups of **Epi**, and kept free the 3-alcohol group (Fig. 1D). Finally, we alkylated the four phenolic and the alcohol groups of **Epi**, which led to a molecule with no free hydroxyl groups (Fig. 1E).

The resultant **Epi** derivatives were 3-*O*-mesyl-(−)-epicatechin (**Epi-Ms**), 5,7,3,4′-tetra-*O*-propargyl-(−)-epicatechin (**Epi-4-prop**), 3,5,7,3′,4′-penta-*O*-propargyl-(−)-epicatechin (**Epi-5-prop**), and 3-*O*-propargyl-(−)-epicatechin (**Epi-prop**).

On the other hand, to ascertain for their possible bioactivity and coupling to a known receptor, the novel **Epi** derivatives were evaluated *in silico*. For this purpose, molecular docking and dynamics studies were implemented as previously described (see Supplementary data).[20]

Docking results (Fig. 2) suggest that the interactions between **Epi** derivatives and GPER are energetically favorable and that the type of interactions generated are via hydrogen and π-π bonding. In general, the interactions of Epi derivatives on the GPER conformer at 14 ns are similar to those of **Epi**. In contrast, Epi derivatives docking results on GPER conformer at 70 ns evidenced different binding modes between Epi derivatives and **Epi**. In the 14 ns GPER conformer, **Epi** ($\Delta G = -7.9$ kcal/mol)[13] and **Epi-Ms**. ($\Delta G = -7.74$ kcal/mol) reach some common aminoacid residues L137, M141 by hydrophobic interaction whereas under with F208, F223, W272 there are π-π interactions and with S317 and A313 there are hydrogend bonds, **Epi-Ms**. also interacts with S112 residue via hydrogen bonds.

Epi-5-prop ($\Delta G = -9.2$ kcal/mol) reaches the aminoacid residues L108 and L137 by hydrophobic interactions and W272, F208 and Y142 by interactions and with S112 and Q138 under hydrogen bonds (Fig. 2 upper panel). **Epi-prop** shows a $\Delta G = -8.05$ kcal/mol and makes π-π interactions with F278 and hydrogen bonds with N310. **Epi-4-prop** ($\Delta G = -8.68$ kcal/mol) reaches the aminoacid residues Q138, E218, Q215, E275 by hydrogen bonds; Y142, F208 and F206 by π-π interactions and; R286 by a π-cation interaction.

In contrast, using GPER conformer at 70 ns, docking analyses demonstrate that Epi derivatives interact with aminoacid residues distinct than those observed with **Epi** derivatives at 14 ns (Fig. 1 bottom panel). **Epi-Ms**. establishes hydrogend bonds with N310, S62, Q54, E115 and C205 and π-π interactions with Y123. **Epi-5-prop** makes hydrogen bonds with P226, T220 and W150; π-π interactions with F146, W150 and; hydrophobic interactions with L221, V225, F146 and L176. **Epi-prop**, establishes hydrogen bonds with S317, D111 and D105; π-π interactions with F268 and W272 and; hydrophobic interactions with L108. **Epi-4-prop** recognized Q138 and C207 using hydrogen bonds. Additionally, this Epi derivative established π-π interactions with F208 and Y123 as well as hydrophobic interactions with L129, V196 and M133. Using both GPER conformers, docking modeling estimates that **Epi-4-**