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EFFECTS OF (−)-EPICATECHIN AND DERIVATIVES ON NITRIC OXIDE MEDIATED INDUCTION OF MITOCHONDRIAL PROTEINS

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Abstract

Impaired mitochondrial function represents an early manifestation of endothelial dysfunction and likely contributes to the development of cardiovascular diseases (CVD). The stimulation of mitochondrial function and/or biogenesis is seen as a means to improve the bioenergetic and metabolic status of cells and thus, reduce CVD. In this study we examined the capacity of the flavanol (–)-epicatechin and two novel derivatives to enhance mitochondrial function and protein levels in cultured bovine coronary artery endothelial cells. As nitric oxide production by endothelial cells is suspected in mediating mitochondria effects (including biogenesis), we also examined the dependence of responses on this molecule using an inhibitor of nitric oxide synthase. Results indicate that the flavanol (–)-epicatechin and derivatives are capable of stimulating mitochondria function as assessed by citrate synthase activity as well as induction of structural (porin, mitofilin) and oxidative phosporylation protein levels (complex I and II). Effects were blocked by the use of the chemical inhibitor of the synthase thus, evidencing a role for nitric oxide in mediating these effects. The results observed indicate that the three agents are effective in enhancing mitochondria function and protein content. The effects noted for (–)-epicatechin may serve to explain the healthy effects on cardiometabolic risk ascribed to the consumption of cocoa products.

Cardiovascular diseases (CVD) are a leading cause of mortality and morbidity worldwide. The health of the endothelium is central to the underlying pathophysiology of many CVD [1]. Endothelial health is partly dependent on the physiological production of nitric oxide (NO) mainly by the endothelial nitric oxide synthase (eNOS) [2]. Disruption in the bioavailability of NO facilitates a vasoconstricting and procoagulating state characteristic of many CVD. Impaired mitochondrial function represents an early manifestation of endothelial dysfunction and likely contributes to the development of CVD [3, 4]. Physiological increases in NO have been linked with mitochondrial biogenesis and thus, mitochondrial function [5–8]. Therefore, strategies that increase physiological NO production may enhance mitochondrial biogenesis in endothelial and other cell types and are promising for the preservation of cardiovascular health.

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In vitro and *in vivo* studies suggest that the flavan-3-ol (−)-epicatechin (**EPI**) can exert CVD protection [9–11], potentially via improving mitochondrial structure/function [12–14]. These effects have been partly ascribed to the capacity of **EPI** to activate eNOS and thereby increase NO production [15, 16]. As previously reported by us, a possible means by which EPI (and possibly derivatives) triggers these responses can involve a putative receptor entity, as there is indirect evidence for its presence [11,15]. Under physiological conditions, native **EPI** is prone to oxidation secondary to the reactivity of the phenolic hydroxyl groups (OH) [17]. The pharmacological effects of native **EPI** are also limited by its susceptibility to phase II metabolism (sulfation and glucuronidation of the OH groups), which can lead to a short half-life [18]. We propose that the development of more stable derivatives of **EPI** is a useful strategy to retain biological activity and enhance potency.

Two strategies may be useful in protecting **EPI** from oxidation. First, **EPI** *in vitro* and *in vivo* is mono-methylated by the endothelium in the -OH of the catechol ring (ring B, see figure 1), leading to a mixture of 3′- and 4′-O-methyl epicatechin [19, 20], which stabilizes the molecule against oxidation. Although, 3′-O-methyl epicatechin (3′-O-MeEPI) and **EPI** have different H-donating potentials, they appear to posses the same biological capacities. For example, both molecules inhibit cell death induced by H_2O_2, implying a mechanism of action independent of their antioxidant properties as Spencer et al proposed [21]. Second, the acetylation of flavanols such as (−)-epigallocatechin gallate confers protection to all OH groups and improves the chemical stability of the compound under physiological conditions [22], which translates into enhanced biological actions [23].

The goal of the current study was to rationally develop compounds derived from **EPI** with improved effects on NO production and thence on mitochondrial enzyme activity and protein content. We hypothesized that **EPI** acetylation or alkylation will confer stability to the molecule and thereby increase its efficacy on endothelial NO production and NO-mediated effects on mitochondrial endpoints. To test our hypothesis, we modified **EPI** via alkylation or acetylation of OH groups. For our first approach, we used an ethyl group as the alkylating agent, which mimics (but with an additional $-CH_2$) the biotransformation of **EPI** into 3′-O-MeEPI, which should yield similar or enhanced biological activity as **EPI,** likely with improved stability. For our second approach, we acetylated all OH groups of **EPI**. The three compounds used were tested for biological activity using a cell culture model of bovine coronary artery endothelial cells (BCAEC).

For the acetylation modification, all OH groups of **EPI** were acetylated, leading to 3, 3′, 4′, 5, 7-O-penta-acetyl (−)-epicatechin (**PEPI**). The chemical structures of **PEPI** and **Ethyl EPI** are illustrated in figure 1. For the alkylation modification, **EPI** was ethylated at the position 3′ (ring B), leading to 3′-O-ethyl (−)-epicatechin (**Ethyl EPI**) (for synthesis procedures and spectra characterization see supplemental methods). In all experiments, **EPI** was used as a positive control. Administration of **EPI** to cultured endothelial cells stimulates NO synthesis secondary to the activation of eNOS; the effect peaks at 10 minutes and 1 μM EPI [15]. As a prelude to determine the effects of NO on mitochondria-related endpoints, we generated concentration-response curves of NO production for **EPI**, **PEPI**, and **Ethyl EPI** (figure 2). The rank order of potency (EC_{50}) derived from the curves was **PEPI** (0.006 nmol/L) > **Ethyl EPI** (1 nmol/L) **>** **EPI** (2 nmol/L) with respect to their capacities to increase NO production in BCAEC.

The regulatory role of NO on mitochondrial structure/function has been previously studied [24, 25]. The sustained exposure of cells to NO can trigger mitochondrial biogenesis [5]. However, mitochondrial biogenesis also appears to be only partly dependent on NO signaling. [5, 8, 26–29]. We used the EC_{50} concentrations to evaluate effects of these compounds on mitochondrial endpoints over a 48 h treatment. We first examined citrate

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synthase activity (CS). Citrate synthase is the initial enzyme of the tricarboxylic acid (TCA) cycle [30]. CS has been routinely used to assess the oxidative and respiratory capacity as well as mitochondrial volume and density in cells and tissues [31–33]. All compounds significantly increased CS by ~ 30 % (figure 3A). This increase in CS suggests an augmented endothelial mitochondrial activity and/or density elicited by the epicatechin compounds. EC_{50} concentrations of **PEPI**, **Ethyl EPI** and **EPI** achieved similar increases in activity.

We then examined for changes in constitutive members of mitochondrial structure, specifically, porin, the most abundant outer mitochondrial membrane protein that often is used as a marker for cellular mitochondrial mass [34]. **PEPI** and **Ethyl EPI** but not **EPI** significantly increased porin levels over control (figure 3B). We also examined changes in mitofilin, a protein from the inner mitochondrial membrane that promotes/induces cristae and cristae junction formation [35]. **EPI** and **PEPI** significantly increase mitofilin protein levels (figure 3C), suggesting augmented inner mitochondrial mass. To assess changes in the oxidative phosphorylation system, we assessed levels of mitochondrial complex I (CI), the major entry point for electrons from NADH produced by the TCA cycle [36]. **EPI**, and **PEPI**, stimulated CI by ~20 % and 60 % over control, respectively, while **Ethyl EPI** produced no significant effect (figure 3D). Complex II (CII) was also evaluated as a direct link between the TCA cycle and the respiratory chain [37], **EPI** and **PEPI** increased CII protein levels by ~25 % increase over control (figure 3E). Thus, **PEPI** treatment increased all the mitochondrial endpoints measured; **EPI** and **Ethyl EPI** appear to exert a semi-selective effect on specific mitochondrial proteins. Perhaps, a higher concentration is needed for **EPI** and **Ethyl EPI** to increase all the mitochondrial endpoints, as we only used the EC_{50} for NO production.

In order to determine the role of **EPI** and derivatives stimulated NO in mediating compound effects on mitochondrial endpoints, we used the nitric oxide synthase (NOS) inhibitor L-N^G-Nitroarginine methyl ester (L-NAME). Treatment of BCAEC for 48 h with L-NAME by itself induced no significant change but blocked the effects of **EPI**, **PEPI**, and **Ethyl EPI** on CS and all assessed mitochondrial protein levels (porin, mitofilin, CI and CII) (figures 4, 5 and 6). To evidence the nature of the effects on a recognized mitochondria biogenesis related endpoint and the role played by NO, we also assessed the effects of treatments on a key regulator of mitochondria gene transcription, TFAM [38]. Results indicate that **EPI** and **PEPI** induce significant increases in TFAM and effects are blocked by L-NAME (figure 7). The complete blockade of compound effects on mitochondrial endpoints by L-NAME strongly evidence the critical role that NO production may play in regulating mitochondrial biology in endothelial cells and thus, CVD. To further explore the relative role played by NO on mitochondrial protein (CI) levels induced by the compounds, we use a dose of each that yielded a similar induction in NO levels. As shown in figure 8, EPI and PEPI led to significant increases in CI levels. A possible means by which the compounds yield variable effects may be explained by different diffusion properties and/or selective metabolism.

We provide evidence that ethyl O-alkylation of **EPI**'s B ring produces a more potent stimulant of NO production that can trigger significant increases in mitochondrial enzyme activity and protein levels. Surprisingly, the acetylation of all the EPI OH groups notably increased the potency on NO production vs. **EPI** and **Ethyl EPI** translating into significant increases in some but not all mitochondrial endpoints that we assessed. Thus, **PEPI** may represent a particular effective way of targeting NO production and mitochondrial activity at very low concentrations; however, more work is necessary to examine its *in vivo* metabolism since it could be rapidly hydrolyzed by intracellular and membrane-bound esterases, which may in turn release **EPI**. In other words, **PEPI** may represent a pro-drug form of **EPI**, which could be highly favorable for drug development. The testing of these compounds for mitochondrial targeting is warranted for diseases whose impaired cellular

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bioenergetics are an important part of the pathophysiology of endothelial dysfunction and/or CVD.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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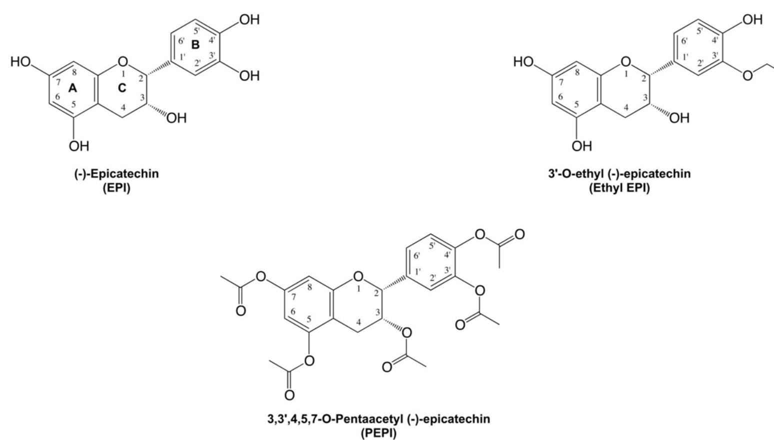

Figure 1. Chemical structures of (−)-epicatechin and derivatives

Bold letters and numbers correspond to the ring position and carbon atom numbering, respectively.

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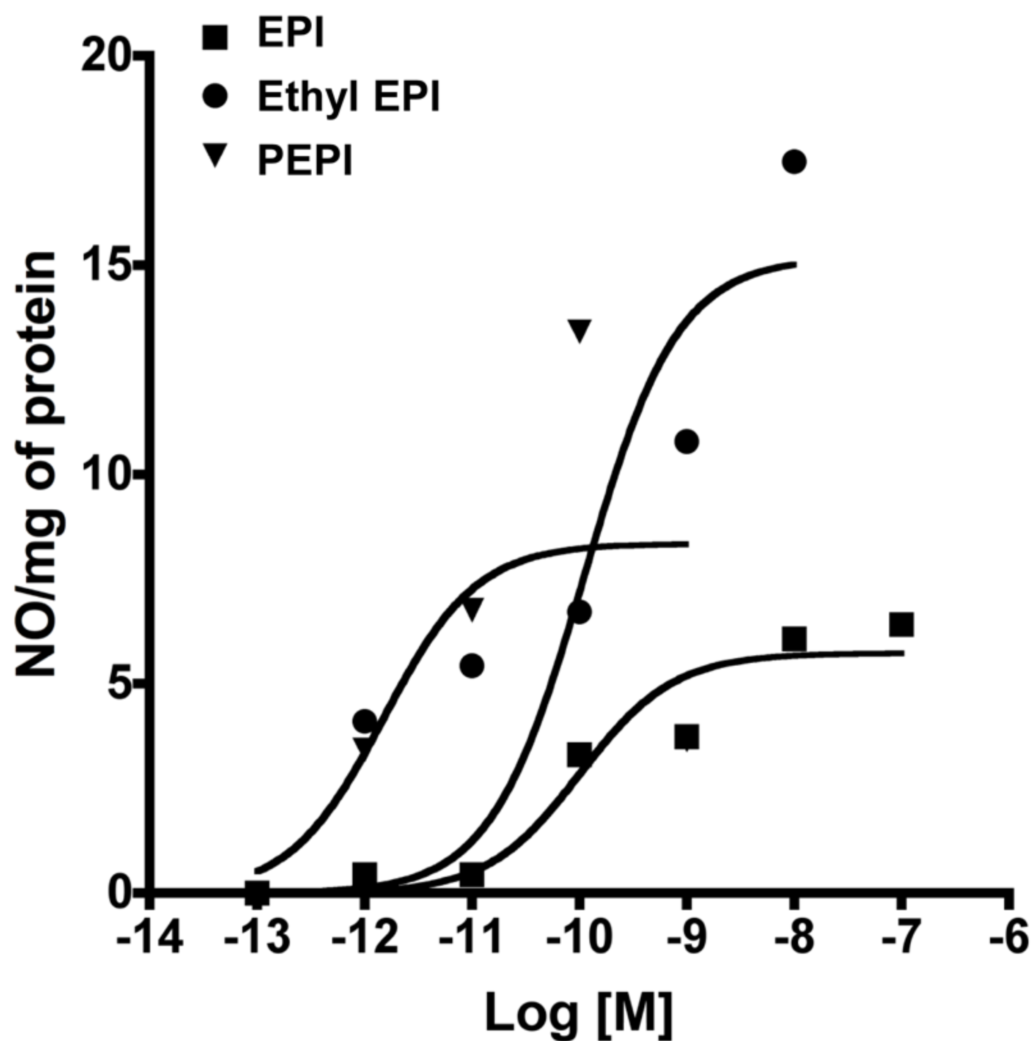

Figure 2. Dose response effects of EPI, Ethyl EPI and PEPI on nitric oxide (NO) levels
BCAEC (in triplicate) were treated at different doses with each compound and media collected 10 min after treatment. Nitric oxide (nitrite/nitrate) levels were assessed by kit (Cayman Inc.). Values represent means of triplicate assays and sigmoidal curves were generated by Prizm 5 (Graphpad Inc).

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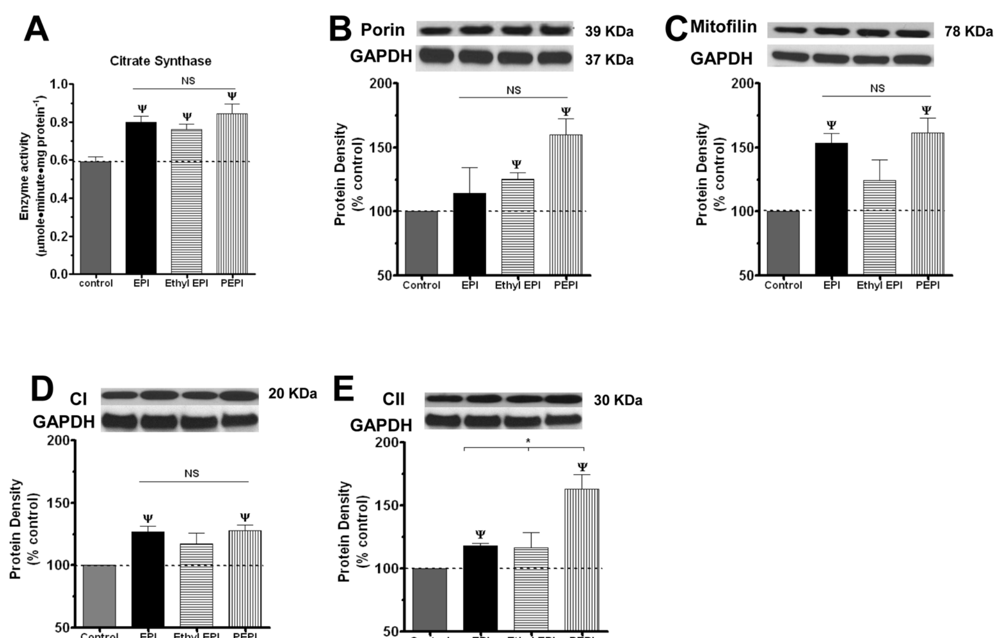

Figure 3. Effects of EPI, Ethyl EPI and PEPI on BCAEC citrate synthase activity and mitochondrial protein levels

(A) CS was evaluated in BCAEC treated with either vehicle (control), 2 nmol/L EPI, 1 nmol/L Ethyl EPI, or 0.006 nmol/L PEPI for 48 h. BCAEC; Bovine coronary artery endothelial cells, CS; citrate synthase activity, EPI; (−)-epicatechin, Ethyl EPI; 3′-O-ethyl (−)-epicatechin, PEPI; 3, 3′, 4′, 5, 7-O-penta-acetyl (−)-epicatechin. Western blots were probed with specific antibodies against (**B**), Porin; (**C**), Mitofilin; (**D**), complex I, and (**E**), complex II. Values were normalized by arbitrarily setting the densitometry of control levels to 100. Values are mean ± SEM; n=3 per treatment. *$p<0.05$ vs. control, Ψ$p<0.01$ vs. control.

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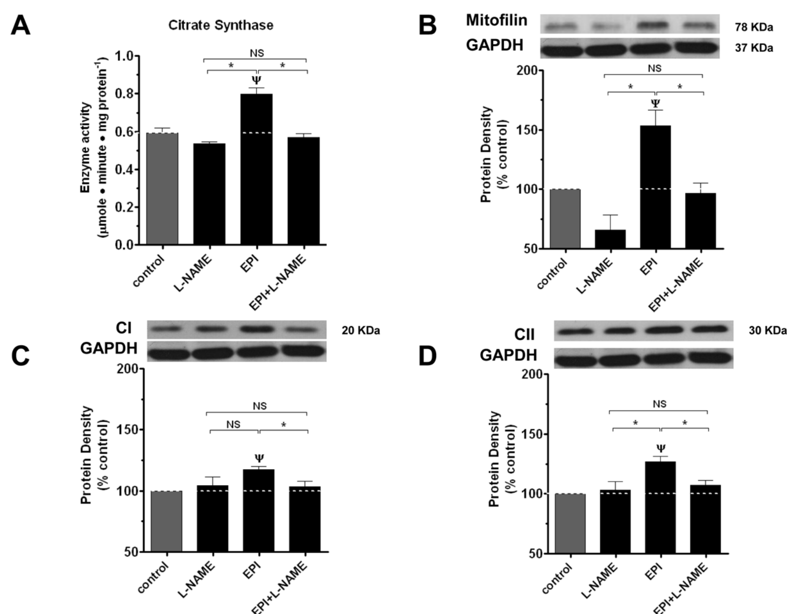

Figure 4. Effects of L-NAME on EPI-induced increases in BCAEC citrate synthase activity and mitochondrial protein levels

CS (**A**) and mitofilin (**B**), complex I (**C**), complex II (**D**) protein densities in BCAEC following exposure for 48 h to either vehicle (control), 2 nmol/L EPI, or 300 μmol/L L-NAME + 2 nmol/L EPI. BCAEC; Bovine coronary artery endothelial cells, CS; citrate synthase activity, EPI; (−)-epicatechin, L-NAME; L-N^G-Nitroarginine methyl ester. Values were normalized by arbitrarily setting the densitometry of control levels to 100. Values are mean ± SEM; n=3 per treatment. *p<0.05 vs. control, $^{\Psi}$p<0.05 EPI vs. EPI + L-NAME.

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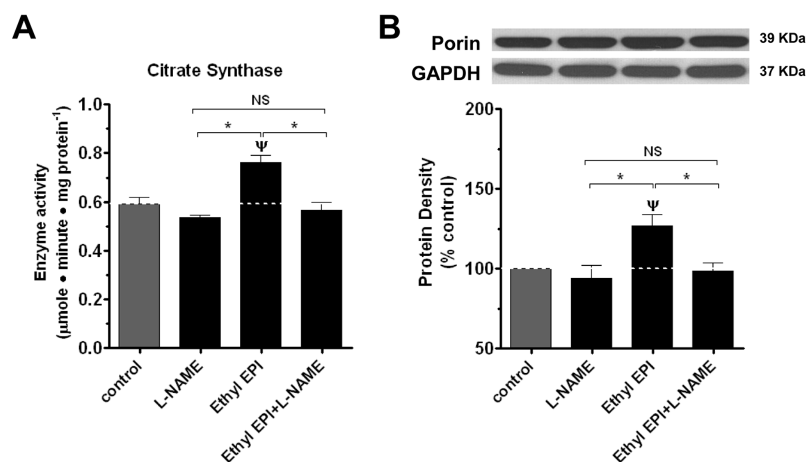

Figure 5. Effects of L-NAME on Ethyl EPI-induced changes on citrate synthase activity and mitochondrial protein levels

CS (**A**) and porin (**B**) protein densities in BCAEC following exposure for 48 h to either vehicle (control), 1 nmol/L Ethyl EPI, or 300 μmol/L L-NAME + 1 nmol/L Ethyl EPI. BCAEC; Bovine coronary artery endothelial cells, CS; citrate synthase activity, Ethyl EPI; 3′-O-ethyl epicatechin, L-NAME; L-N^G-Nitroarginine methyl ester. Values were normalized by arbitrarily setting the densitometry of control levels to 100. Values are mean ± SEM; n=3 per treatment. *$p<0.05$ vs. control, $^{\Psi}p<0.05$ Ethyl-EPI vs. Ethyl-EPI + L-NAME.

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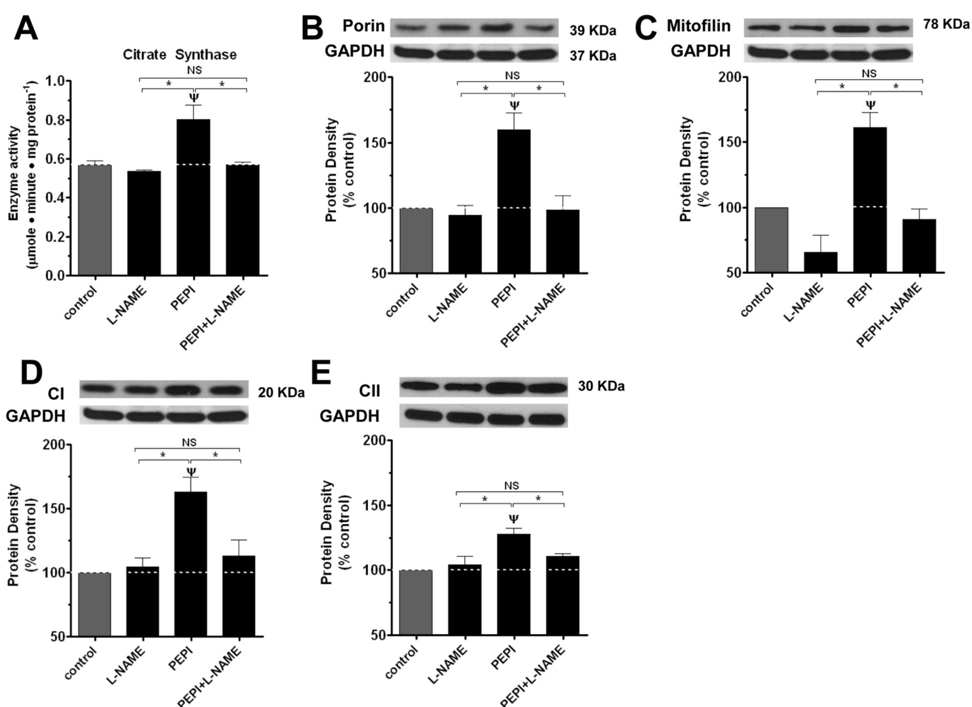

Figure 6. Effects of L-NAME on PEPI-induced changes on citrate synthase activity and mitochondrial protein levels

CS (**A**) and porin (**B**), mitofilin (**C**), complex I (**D**), complex II (**E**) protein densities in BCAEC following exposure for 48 h to either vehicle (control), 0.006 nmol/L PEPI, or 300 μmol/L L-NAME + 0.006 nmol/L PEPI. BCAEC; Bovine coronary artery endothelial cells, CS; citrate synthase activity, PEPI; 3, 3′, 4′, 5, 7-O-penta-acetyl (−)-epicatechin, L-NAME; L-N^{G}-Nitroarginine methyl ester. Values were normalized by arbitrarily setting the densitometry of control levels to 100. Values are mean ± SEM; n=3 per treatment. *$p<0.05$ vs. control, $^{\Psi}p<0.05$ EPI vs. EPI + L-NAME.

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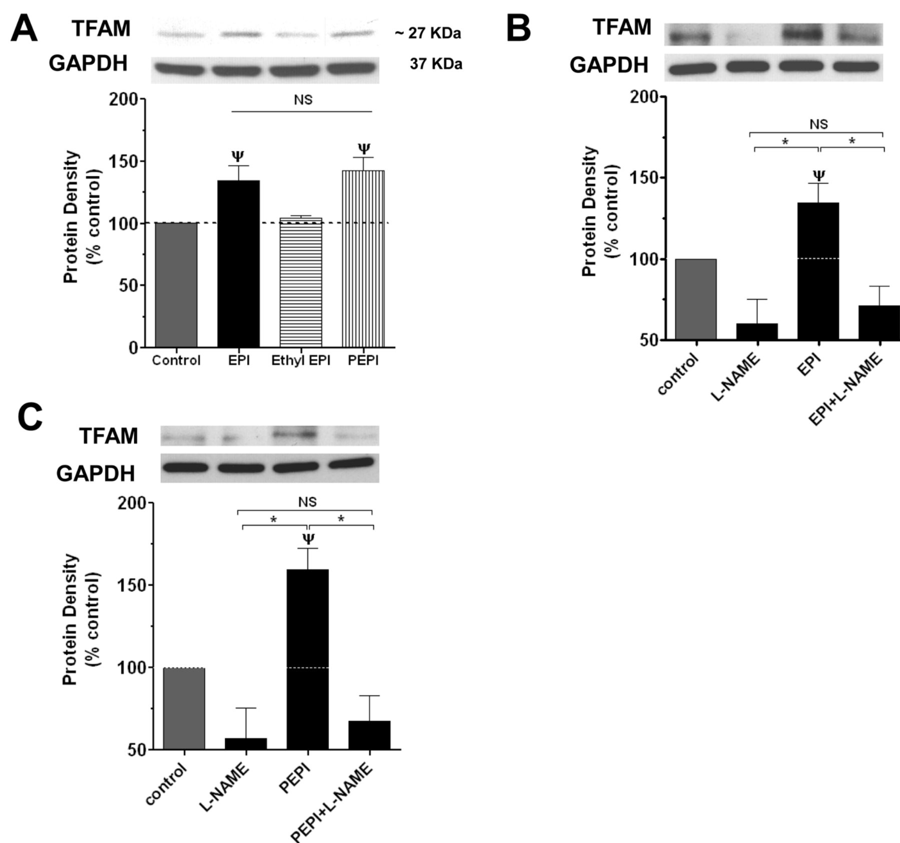

Figure 7. Effects of EPI, Ethyl EPI and PEPI and L-NAME on BCAEC TFAM levels

BCAEC were treated with either vehicle (control), 2 nmol/L EPI, 1 nmol/L Ethyl EPI or 0.006 nmol/L PEPI for 48 h ± L-NAME (300 μM). Western blots were probed with specific antibodies against TFAM. (**A**) Effects of EPI, Ethyl-EPI and PEPI, (**B**) Effects of EPI ± L-NAME and (**C**) Effects of PEPI ± L-NAME. EPI; (–)-epicatechin, Ethyl EPI; 3′-O-ethyl (–)-epicatechin, PEPI; 3, 3′, 4′, 5, 7-O-penta-acetyl (–)-epicatechin. Values were normalized by arbitrarily setting the densitometry of control levels to 100. Values are mean ± SEM; n=3 per treatment. *$p<0.01$ Ψ$p<0.01$ vs. control.

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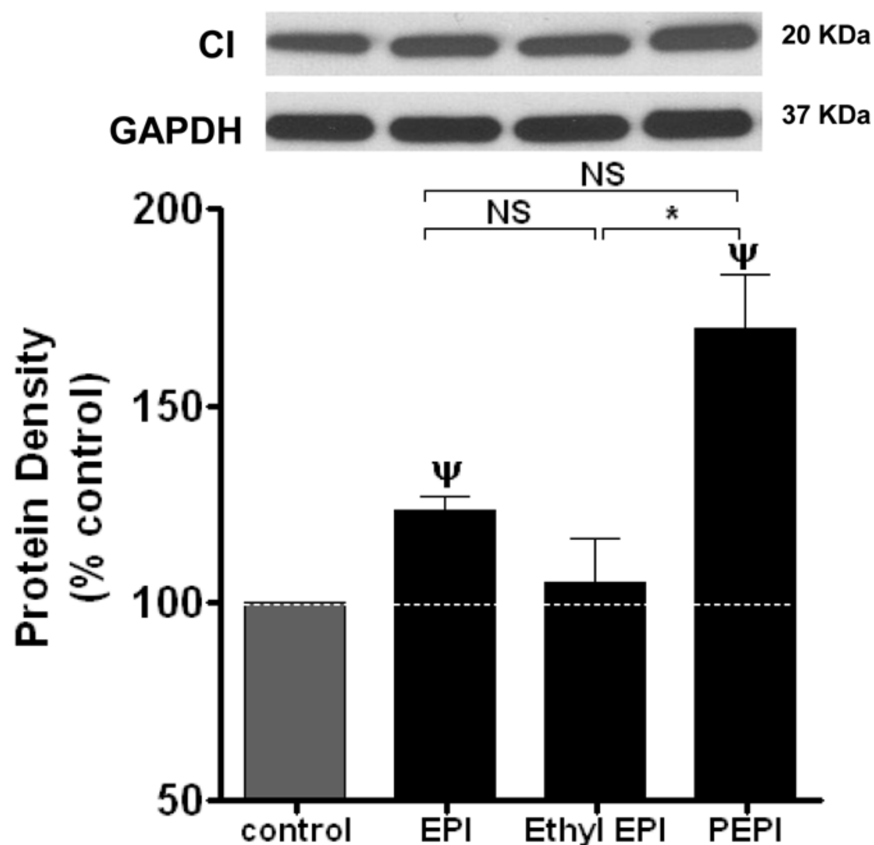

Figure 8. Effects of EPI, Ethyl EPI and PEPI on BCAEC mitochondrial complex I protein levels
BCAEC were treated with either vehicle (control), 100 nmol/L EPI, 0.01 nmol/L Ethyl EPI or 0.01 nmol/L PEPI for 48 h. Western blots were probed with specific antibodies against complex I (CI). EPI; (−)-epicatechin, Ethyl EPI; 3′-O-ethyl (−)-epicatechin, PEPI; 3, 3′, 4′, 5, 7-O-penta-acetyl (−)-epicatechin. Values were normalized by arbitrarily setting the densitometry of control levels to 100. Values are mean ± SEM; n=3 per treatment. *p<0.01, Ψp<0.01 vs. control.