Beneficial Effects of Caffeic Acid Phenethyl Ester on Wound Healing in a Diabetic Mouse: Role of VEGF and NO

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Abstract: Cutaneous wound healing is delayed in patients with diabetes. Caffeic acid phenethyl ester (CAPE) has been identified as an effective constituent of propolis with improved wound healing abilities via an oxidative stress decrease. However, its impact on wound healing in diabetic models and its underlying mechanisms remain unclear. Determining the vascular endothelial growth factor (VEGF) contents in a human vascular smooth muscle cell (VSMC)-conditioned medium was assessed using human VEGF immunoassay and vascular reactivity using porcine coronary artery rings. Later, C57BL/6 or db/db mice were anesthetized, after which a 6-mm biopsy punch was manipulated for perforation via the back skin. Subsequently, CAPE was applied to the wound and changed daily. Furthermore, the injury in each mouse was digitally photographed, and the wound area was quantified. We observed that CAPE increased VEGF levels in human VSMC-conditioned medium, improved endothelium-dependent nitric oxide (NO)-mediated vasorelaxation, inhibited U46619-induced vasoconstriction porcine coronary artery, and enhanced cutaneous wound healing in the diabetic mouse model. Hence, we propose that CAPE improves wound healing in diabetic mice, which is aided by increased VEGF and NO expression.

Keywords: caffeic acid phenethyl ester; wound healing; vascular endothelial growth factor; nitric oxide

1. Introduction

Delayed cutaneous wound healing is a common feature in patients with diabetes caused by continuous inflammation, which is commonly observed during diabetic ulceration [1]. Specifically, foot ulceration is a major complication in patients with diabetes [2]. Thus, patients with diabetes have a 25% risk of foot ulcers for their whole life [3], resulting in 50–70% of whole, nontraumatic amputations [4–6]. Recently, the rate of amputation has increased, which has led to increased morbidity and mortality [7–10]. Therefore, the cure for diabetic foot ulcers remains a significant issue, including developing therapies that enhance wound healing in these patients [11].

Propolis, a substance gathered by honeybees to protect their hive from fungal and bacterial infections [12,13], has been used as a medicine from time immemorial [14]. In humans, propolis has been suggested as an effective topical therapy for ulcers and its use for treatment has been approved, especially in Australasia [15]. Moreover, it has been recently reported that the topical application of propolis is well tolerated and facilitates the healing of human diabetic foot ulcers [16]. However, despite the rising use of propolis, the active components in this substance and the exact underlying mechanism of diabetic wound healing have not been explored.
Caffeic acid phenethyl ester (CAPE) is a principally active component of propolis [17]. It is proposed that CAPE has immunomodulatory, anti-inflammatory, and antioxidant effects, and inhibits lipid peroxidation [17–20]. However, even though CAPE accelerates cutaneous wound healing via antioxidant effects [21], and enhances wound contraction and re-epithelialization by decreasing oxidative stress [22], it has not been reported whether it improves cutaneous wound healing in a diabetic model. Therefore, in this study, we investigated the effect of CAPE on cutaneous wound healing in a diabetic mouse model, after which we elucidated its possible underlying mechanisms.

2. Materials and Methods

2.1. Chemicals

CAPE was purchased from Bachem (catalog no. Q-2305). However, unless otherwise specified, all other materials were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

2.2. Determination of Vascular Endothelial Growth Factor (VEGF)

Human aortic vascular smooth muscle cells (VSMCs) were obtained from Clonetics and cultured as recommended. VSMCs from passages 5–15 were then exposed to a serum-free culture medium containing 0.1% bovine serum albumin (QBiogene) for 24 h before treatment. Subsequently, the VEGF content was estimated in human VSMC-conditioned medium (24 h) using human VEGF immunoassay (R&D system).

2.3. Vascular Reactivity

As demonstrated previously, the vascular reactivity study was conducted using left anterior descending coronary arteries from pig hearts (acquired from a local slaughterhouse in Mokpo, Republic of Korea) [23]. In brief, after removing the connective tissue, the dissected arteries were cut into rings (4–5 mm). Then, to evaluate the alterations in isometric tension, the rings were placed in organ baths containing 10 mL oxygenated (95% O$_2$ and 5% CO$_2$) Krebs bicarbonate solution (composition in mM: NaCl 119, KCl 4.7, KH$_2$PO$_4$ 1.18, MgSO$_4$ 1.18, CaCl$_2$ 1.25, NaHCO$_3$ 25, and D-glucose 11, pH 7.4) at 37°C. After equilibration for 90 min at a resting tension of 5 g, the rings were contracted with KCl-containing Krebs solution (80 mM). Following a 30-min washout, the rings were contracted with U46619 (thromboxane mimetic, 1–60 nM) to ~80% of the maximal contraction produced by the KCl-containing Krebs solution before adding bradykinin (0.3 µM) to verify the endothelial function. After washout and 30-min equilibration, the rings were subsequently contracted with U46619 once more before constructing a concentration–relaxation curve for CAPE. In some examinations, the rings were pretreated with pharmacological inhibitors for 30 min before adding the U46619. However, in others, the rings were incubated with CAPE (10 µM, 100 µM) for 5 min before constructing the concentration–contraction curve for U46619.

2.4. Wound Biopsy and Assessment of Wound Closure

This study was conducted on C57BLKS/J m+/m+ or db/db male mice (10 weeks old) obtained from the National Taiwan University Animal Center. All animal experiments were performed according to National Institutes of Health guidelines and were approved by the Laboratory Animal Committee of the College of Medicine, Catholic University (IRB no. CUMC-2012-0077-01). Subsequently, the mice were anesthetized using a 2% injection comprising Rompun solution (0.1 mL/20 g body wt). Then, the back of the mouse was trimmed, and an alcohol swab was used to accomplish sterilization. Subsequently, a 6-mm biopsy punch was used to punch the mouse’s back skin. The mouse would be unable to touch a wound in this area to avoid self-licking. Afterward, to determine whether CAPE improved cutaneous wound healing in a diabetic condition, 35% EtOH saline alone or that containing 20 µL of 5 mM CAPE was applied to the wound at the back of C57BLKS/J db/db mice, twice daily for 21 d. Furthermore, 35% EtOH saline, used as a vehicle for CAPE administration, was applied to the wound on the back of C57BLKS/J m+/m+ mice to examine whether the vehicle affected wound healing compared with the untreated mice.
Finally, injuries in individual mice were digitally photographed on days 0, 3, 7, 14, and 21 post wounding, after which the wound area was estimated using Scion software (Scion Corporation, Frederick, MD, USA).

2.5. Statistical Evaluation

The values were expressed as mean ± standard error of mean (SEM). Statistical assessment was conducted with 2-way analysis of variance, followed by Fischer’s protected least significant difference test wherever appropriate. Values of $p < 0.05$ were regarded statistically significant.

3. Results

3.1. CAPE Increases the Release of VEGF from VSMCs

Wound angiogenesis plays a critical role in the proliferative phase of healing [2,24]. Moreover, studies have reported that VEGF is the main factor in angiogenesis processes, and is particularly associated with wound repair [24]. Therefore, the effect of CAPE on VEGF’s dose-dependent release in human VSMCs was assessed. The exposure of VSMCs to CAPE for 24 h raised the basal release of VEGF, with significantly increased levels observed at doses >3 μM, reaching approximately 441% at 30 μM, which was comparable to the effect of platelet-derived growth factors (PDGF) at 30 ng/mL (Figure 1).

![Figure 1](image1.png)

**Figure 1.** The dose-dependent effect of CAPE on the expression of VEGF released from VSMCs. The level of VEGF was expressed as the percentage of VEGF levels at the control. The values were the mean ± SEM of $n = 3$. *$p < 0.05$ vs. control.

3.2. CAPE Causes Endothelium-Dependent NO-Mediated Vasorelaxation and Inhibits U46619-Induced Vasoconstriction in Porcine Coronary Arteries

It has previously been suggested that blood supply to foot ulcers in patients with diabetes is diminished owing to the decline in NO formation by vascular endothelial cells (ECs) [25]. Moreover, improved endothelium-dependent vasodilation has been shown to be associated with vascular repair and re-endothelialization in diabetic animal models [26]. Based on such reports, we conducted a vascular reactivity study to investigate the effect of CAPE on vascular function and subsequently elucidate the underlying mechanisms. The results showed that CAPE induced the relaxation of isolated porcine coronary artery rings with endothelium (but not of those without endothelium) in a concentration-dependent manner (Figure 2a). The relaxation responses were 12.9 ± 4.6%, 34.7 ± 8.2%, and 75.6 ± 8.3% at concentrations of 3, 10, and 30 μM, respectively. CAPE relaxation was also significantly reduced by $N^ω$-nitro-L-arginine (L-NA, a NO synthase inhibitor) and 1H-[1,2,4]oxadiazolo[4,3-α]quinoxalin-1-one (ODQ), a guanylyl cyclase inhibitor. Alternatively, indomethacin (INDO), a cyclooxygenase inhibitor, and tetraethylammonium (TEA), a potassium channel blocker, did not inhibit the relaxation induced by CAPE (Figure 2b).
These findings indicate that CAPE-induced vasorelaxation of the porcine coronary artery rings depended on vascular endothelium, and was mainly mediated by the NO/cGMP pathway.

Figure 2. CAPE-induced vasorelaxation in porcine coronary artery rings. The relaxation response was expressed as the percentage relaxation of the U46619-induced contraction (100% represents complete relaxation). (a) CAPE’s concentration-dependent relaxation curves (0.3, 1, 3, 10, 30, and 100 μM) with and without endothelium. (b) CAPE’s concentration-dependent vasorelaxation curves (0.1, 0.3, 1, 3, 10, 30, and 100 μM) on U46619-induced vasoconstriction with pharmacological inhibitors. The pharmacological inhibitors L-NA (300 μM), indomethacin (10 μM), TEA (10 mM), and ODQ (10 μM) were treated for 30 min before U46619-induced contraction. Values were presented as the mean ± SEM (n = 4). *p < 0.05 vs. (a) without endothelium or (b) control.

Additionally, this study showed that CAPE inhibited U46619-induced contraction. As observed, the treatment of porcine coronary artery rings with 10 μM and 100 μM CAPE for 5 min before adding the increasing concentrations of U46619 attenuated the contractions significantly (Figure 3).

Figure 3. The effect of CAPE on contractile responses. Intact rings were incubated with CAPE (10 and 100 μM) for 5 min before adding U46619 at increasing concentrations. The contraction response was expressed as the percentage of the U46619-induced contraction. Values were expressed as the mean ± SEM (n = 3). *p < 0.05 vs. control.
3.3. CAPE Enhances Cutaneous Wound Healing in a Diabetic Mouse Model

The wound area in the diabetic mice treated with CAPE was smaller than that in the diabetic mice treated only with 35% EtOH saline (Figure 4a). The areas of the wound expressed as percentages of the initial wound area treated with CAPE were 38.7% and 16.1% on days 7 and 14, respectively, whereas the areas of the wound treated only with 35% EtOH saline were 76.1% and 65.1% on the same days (Figure 4b). Therefore, to examine whether 35% EtOH saline affected wound healing in nondiabetic mice, it was applied on the wound in nondiabetic mice. We observed that the areas of the injuries treated with 35% EtOH saline were 58.0% and 20.7% on days 3 and 7, respectively, whereas the areas of the untreated wounds were 90.1% and 52.2% (Figure 4b). Although 35% EtOH saline improved wound healing in nondiabetic mice to a certain degree, the results suggest that CAPE significantly enhanced cutaneous wound healing in diabetic mice.

Figure 4. The effect of a single and simultaneous application of CAPE on cutaneous wound areas in diabetic and nondiabetic mice treated with either saline containing 35% EtOH as control or 5 mM CAPE or 20 μL in saline containing 35% EtOH. (a) Representative photos of wounds in diabetic mice. (b) Group data of wound area expressed as a percentage of the initial wound area. Although the injury in one nondiabetic mice group was untreated, another group was treated with saline containing 35% EtOH. Alternatively, while the wounds in one group of diabetic mice were treated with saline containing 35% EtOH as control, another group was treated with CAPE in saline containing 35% EtOH. The values are presented as the mean ± SEM (n = 3) per group. * p < 0.05 vs. 35% EtOH of the m+/m+ group and # p < 0.05 vs. 35% EtOH of the db/db group.

4. Discussion

Propolis has been suggested to exert immunomodulatory, antiulcer, antidiabetic [27], and tissue healing/improvement effects [28–31]. The constituent compounds of propolis are apigenin, galangin, kaempferol, myricetin, pinocembrin, quercetin, and CAPE [15]. Among these, CAPE has been identified as the principal compound of propolis and has been reported to have anti-inflammatory, antibacterial, and tissue-repair properties [17–20]. CAPE has also been proposed to improve diabetic wound healing. Furthermore, several studies have suggested that CAPE attenuated the inflammatory response to periodontal pathogens via NF-κB inhibition [32], and that it exerted positive effects on gingival wound healing in nondiabetic Wistar albino rats [33]. In diabetic rats induced by streptozotocin, CAPE also diminished pancreatic oxidative stress by acting as a heme oxygenase-1 inducer [34] and had beneficial effects on the brain by decreasing oxidative damage and inflammation [35].
Foot ulceration is a major complication in patients with diabetes [4–6]. The problem occurs in at least 15% of these patients, making it the reason for 50–70% of nontraumatic amputations. Foot ulceration has also been associated with increased morbidity, mortality, and decreased quality of patients’ lives [7,8]. Thus, developing therapies for foot ulcers in patients with diabetes is imperative [11]. This investigation is the first to identify the effects of CAPE on cutaneous wound healing in a diabetic mouse model. In this study, we observed that CAPE accelerated cutaneous diabetic wound healing. This protective effect was associated with, at least in part, the induction of VEGF release in VSMCs and the relaxation of the artery via the NO/cGMP pathway.

Furthermore, angiogenesis is essential for wound healing as it helps in the delivery of nutrients, oxygen, and other molecules, thereby participating in the wound healing process [36]. In patients with diabetes, ECs are seriously defective in angiogenesis and cause an ischemic limb, which impairs wound healing [37]. Therefore, inducing angiogenesis in an ischemic limb can be key to improving the blood flow to the limb, consequently leading to healed limb wounds, which are delayed by diabetes-induced ischemia [38]. Additionally, VEGF is an important component that induces angiogenesis [39], produced by different cell types, such as VSMCs, fibroblasts, tumor cells, macrophages, and ECs [40–47]. VEGF is involved in the angiogenic process, enhances microvascular permeability, and promotes EC survival, migration, proliferation, and matrix metalloproteinases secretion [48]. However, CAPE has a double aspect regarding VEGF. It has considerably been reported that CAPE inhibits the expression of VEGF and VEGF-induced angiogenesis [49]. Nevertheless, these findings have not been completely assessed in cancer cells. Our study showed that VEGF in cultured human VSMC-conditioned medium increased when CAPE was administered to the cells. According to our result, in the cells that are not cancerous or under insufficient VEGF or ischemia conditions, CAPE rather induces VEGF expression. Therefore, it is assumed that it acts as a regulator of homeostasis.

NO has also been reported to play crucial roles in wound healing [50]. NO induces angiogenesis [51,52] and the proliferation and migration of ECs [53]. Furthermore, CAPE has been proposed to enhance NO activity and prevent reactive oxygen species from degrading NO [54]. Moreover, investigations have suggested that sufficient NO production is needed for efficient wound healing and that topical NO treatment advances the closure of the excisional wounds in diabetic rats [55]. Additionally, worsened angiogenesis was observed in eNOS-deficient mice [36]. Besides, VEGF has been induced to induce the upregulation of eNOS expression and endothelial NO production [57,58]. Similarly, NO influences vasorelaxation, which symbolizes increased blood flow. Increased blood flow to the limb accelerates the wound healing rate in the limb delayed by diabetes-derived ischemia [38]. Likewise, this study demonstrated that CAPE induced endothelium-dependent vasorelaxation in isolated porcine coronary artery rings. The inhibitor of NO synthase and guanylyl cyclase, but not the potassium channel blocker and inhibitor of cyclooxygenase, strongly inhibited this vasorelaxant effect of CAPE. This result suggests that CAPE caused an endothelium-dependent vasorelaxation mainly by mediating the NO-sGC-cGMP pathway. Taken together, CAPE can contribute to improved wound healing, at least in part, via the blood flow increase caused by NO-mediated vasorelaxation.

Nevertheless, further studies are needed to demonstrate how CAPE induces the expression of VEGF. Follow-up studies on the detailed mechanism are also required.

5. Conclusions

CAPE had a potent wound healing effect on the diabetic mouse model, which we speculate to have been caused by an increase in the expressions of NO and VEGF. Therefore, CAPE is suggested as a potential candidate for the treatment of foot ulceration in patients with diabetes.

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and editing, supervision and project administration, K.C. and M.-H.O. All authors have read and agreed to the published version of the manuscript.

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**Data Availability Statement:** All the data generated and analyzed during this study are included in this article.

**Conflicts of Interest:** The authors declare no conflict of interest.

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