A Novel Antioxidant, EPC-K1, Stimulates Endothelial Nitric Oxide Production and Scavenges Hydroxyl Radicals

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EPC-K1, a hydroxyl radical scavenger synthesized by phosphate linkage of vitamin E and vitamin C, prevents myocardial reperfusion injury in vivo; however, the direct effects of EPC-K1 on coronary arteries are unknown. These experiments were undertaken to define possible mechanisms through which EPC-K1 imparts its protective action on the coronary vasculature. EPC-K1 (10⁻⁵ to 10⁻¹ mg/ml) induced concentration-dependent relaxation in contracted canine coronary artery segments with endothelium, but no change in tension of arterial segments without endothelium (p<0.05, ANOVA). Endothelium-dependent relaxation to EPC-K1 was inhibited by NG-monomethyl-L-arginine (l-NMMA) (10⁻⁵ mol/L). Inhibition of relaxation by l-NMMA was reversed by the addition of L-arginine (10⁻⁴ mol/L), but not by d-arginine (10⁻⁴ mol/L). Subsequent exposure of canine coronary artery segments with intact endothelium to hydroxyl radicals for 30 min (generated by FeSO₄ [0.56 mmol/L] + H₂O₂ [0.56 mmol/L]) impaired endothelium-dependent relaxation. However, pretreating the vascular segments with EPC-K1 (10⁻⁴ mg/ml) prevented hydroxyl radical-mediated endothelial cell injury and maintained endothelium-dependent relaxation. These experiments indicate that EPC-K1 stimulates the release of endothelium-derived nitric oxide, an endogenous vasodilator and inhibitor of platelet and leukocyte activation and adhesion, from the coronary artery endothelium. Additionally, EPC-K1 scavenges hydroxyl radicals that mediate endothelial cell injury. These 2 independent and important actions are possible mechanisms by which EPC-K1 prevents reperfusion injury in the ischemic heart. (Circ J 2003; 67: 1046–1052)

Key Words: Endothelium; Free radicals; Ischemia; Nitric oxide; Reperfusion

The vascular consequences of ischemia–reperfusion injury remain a significant concern in cardiac surgery, particularly during coronary revascularization and heart transplantation. Ischemia–reperfusion injury can produce endothelial dysfunction in the perioperative period with a subsequent impaired release of nitric oxide (NO), a potent vasodilator and inhibitor of platelet aggregation and neutrophil activation. After coronary artery bypass grafting, impaired endothelial NO production may lead to early coronary vasospasm and reduced long-term patency of vascular grafts or native coronary arteries. Endothelial dysfunction following cardiac transplantation may contribute to acute graft failure and post-transplant coronary artery disease.

Oxygen-derived free radicals, including the superoxide and hydroxyl species, are key mediators of ischemia–reperfusion injury that inactivate endothelial NO release and stimulate vascular smooth muscle. In physiologic conditions, oxygen-derived free radicals are continuously scavenged by endogenous antioxidant systems, including superoxide dismutase, glutathione peroxidase, and catalase; however, overproduction of these free radicals following ischemia–reperfusion can overwhelm these defense systems. Furthermore, no known endogenous scavenging mechanism exists for the hydroxyl radical, which is the most reactive of the species.

EPC-K1 is a novel antioxidant synthesized by phosphate linkage of L-ascorbic acid (vitamin C) and -tocopherol (vitamin E), 2 endogenous compounds that independently protect against oxidative stress-induced injury. In vitro, EPC-K1 scavenges hydroxyl radicals and inhibits phospholipase A₂ activity. Also, it has demonstrated protective effects against ischemia–reperfusion injury, particularly in animal transplantation in vivo models. These in vivo results may likely be explained by its hydroxyl radical scavenging activity; however, the direct effects of EPC-K1 on endothelial function have not been examined. The present study was designed to determine in vitro the direct effect of EPC-K1 on vascular function and confirm its protective benefit on endothelial function in the presence of exogenously generated hydroxyl radicals.

Methods

All procedures and handling of animals were in compliance with the ‘Guide for the Care and Use of Laboratory Animals’ formulated by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). In addition, this protocol was reviewed and approved by the Institutional Animal Care and Use Committee of the Mayo Foundation.

Adult heartworm-free mongrel dogs (25–30kg) of either sex were anesthetized with pentobarbital sodium (30 mg/kg bolus by intravenous injection; Fort Dodge Laboratories, Fort Dodge, IA, USA) and exsanguinated.
Hearts were excised and immersed in cool, oxygenated physiologic salt solution (Kreb's) of the following millimolar composition: NaCl, 118.3; KCl, 4.7; MgSO₄, 1.2; KH₂PO₄, 1.22; CaCl₂, 2.5; NaHCO₃, 25.0; and glucose, 11.1. Proximal coronary artery segments were dissected from hearts under magnification and placed in Kreb's solution, with care taken to remove as much of the connective tissue as possible.

Vessel segments were sectioned into rings 5–6 mm in length. In some of the rings, the endothelium was mechanically removed by inserting the tip of a watchmaker's forceps and scraping gently, a technique that has been previously described. Rings with and without endothelium were then suspended in 25-ml organ chambers filled with Kreb's solution maintained at 37°C and aerated with 95% oxygen and 5% carbon dioxide (pH 7.4). Each ring was suspended by 2 stainless steel clips passed through the vessel lumen. One clip was attached to a stationary post, and the other was attached to a strain gauge (Statham Gould UC 2, Viggo Spectamed Inc., Critical Care Division, Oxnard, CA, USA) for the measurement of isometric force. Rings were placed at the optimal point of their length–tension relationship by progressively stretching them until the contraction to potassium chloride (20 mM/L) was maximal.

In all experiments, the presence of functioning endothelium was confirmed by the response to acetylcholine (1×10⁻⁶ mol/L) following precontraction with potassium ions (20 mM/L). After optimal tension was determined and the presence or absence of endothelium confirmed, the rings were allowed to equilibrate 30 min before performance of any further experiments. All experiments were performed in the presence of indomethacin (1×10⁻⁵ mol/L) to inhibit cyclooxygenase activity.

**Experiment 1**

The focus of Experiment 1 was to study the direct effects of EPC-K1 on vascular reactivity. Following the above preparation, vessel segments with and without endothelium from 6 dogs were divided into 5 groups and treated with (1) no drugs (Control), (2) NG-monomethyl-L-arginine (L-NMMA) (1×10⁻⁵ mol/L), (3) L-NMMA and L-arginine (1×10⁻⁴ mol/L), (4) L-NMMA and D-arginine (1×10⁻⁴ mol/L), and (5) L-NMMA and tetraethylammonium chloride (TEA) (25 mM/L). Vessel rings were contracted with prostaglandin E₂ (PGF₂α) (2×10⁻⁶ mol/L) and exposed to increasing concentrations of EPC-K1 (10⁻⁵ to 10⁻¹ mg/ml) as isometric tension changes were continuously recorded.

The independent effect of vitamin C on vascular reactivity was also examined. Here, arterial segments from 6 dogs, with and without endothelium, were treated either with no drugs or with L-NMMA (1×10⁻⁵ mol/L). Rings were similarly treated with PGF₂α (2×10⁻⁶ mol/L) and exposed to increasing concentrations of vitamin C (10⁻⁴ to 1 mg/ml). Again, isometric tension changes were continuously recorded throughout the experiments.

**Experiment 2**

Experiment 2 was designed to confirm the ability of EPC-K1 to preserve the stimulated release of endothelial NO following endothelial exposure to damaging concentrations of hydroxyl radicals. Again, after preparations as detailed above, vessel rings with and without endothelium from 6 dogs were divided into 5 groups. Four study groups were pretreated with (2) no drug (·OH), (3) EPC-K1 (10⁻⁴ mg/ml), (4) EPC-K1 (10⁻⁵ mg/ml), or (5) superoxide dismutase (SOD) (150 U/L) for 2 min and then exposed to hydroxyl radicals for 30 min. The remaining group, (1) control, was incubated in organ chambers for 30 min without the addition of EPC-K1 or SOD, or exposure to exogenous hydroxyl radicals.

Hydroxyl radicals were generated by Fenton's reagent, created by adding 0.56 mM/L FeSO₄ plus 0.56 mM/L H₂O₂ to each organ chamber. Following the 30-min incubation, vessel rings were serially washed with fresh Kreb's solution and allowed to equilibrate for 30 min. Precontraction was accomplished with the addition of PGF₂α (2×10⁻⁶ mol/L), and a cumulative dose-response curve of acetylcholine-induced relaxation was obtained.

**Drugs**

The following drugs were used in conducting all experiments: acetylcholine chloride, vitamin C, iron sulfate (FeSO₄), hydrogen peroxide (H₂O₂), indomethacin, PGF₂α, SOD, and TEA (Sigma Chemical Company, St Louis, MO, USA); L-arginine, D-arginine, and L-NMMA (Calbiochem, San Diego, CA, USA); and EPC-K1 (Senju Pharmaceutical, Osaka, Japan). All powdered drugs were prepared with distilled water except vitamin C and indomethacin, which were dissolved in NaClO₃. Because of the buffering effect of the Kreb's solution and aeration with 5% CO₂, addition of EPC-K1, vitamin C, L-NMMA, L-arginine, or D-arginine did not alter the organ bath pH. All drug concentrations are expressed as final concentration in organ chambers.

**Data Analysis**

Vascular tensions in response to EPC-K1, vitamin C, or acetylcholine were expressed as the percent change from the maximal contraction level of each coronary arterial ring following precontraction with PGF₂α. The results were expressed as means± standard error of the mean (SEM). In all experiments, 'n' referred to the number of animals from which vessel rings were taken. Statistical evaluation of the data was performed with Student's t test for either paired or unpaired observations. Analysis of paired results also utilized ANOVA with Neuman-Keuls posthoc comparison where appropriate. Values were considered statistically significant when the p value was less than 0.05.

**Results**

**Experiment 1**

In the canine coronary arteries contracted with PGF₂α (2×10⁻⁶ mol/L), addition of EPC-K1 (10⁻⁵ to 10⁻¹ mg/ml) produced concentration-dependent relaxation in segments with an intact endothelium; in contrast, coronary arteries without endothelium manifested only a modest decrease in precontraction tension (Figs 1,2). Maximal vasorelaxation induced by EPC-K1 (10⁻¹ mg/ml) was 94±5% in endothelium-intact segments and 14±5% in endothelium-denuded segments (p<0.05).

Pretreatment of arterial segments with L-NMMA (10⁻⁵ mol/L), the competitive inhibitor of NO production, for 20 min before contraction with PGF₂α did not induce any consistent change in tension in arterial segments with or without endothelium. However, pretreatment of arterial segments with L-NMMA attenuated EPC-K1-induced...
Fig 1. Representative tracings from canine coronary artery segments with (+) and without (−) endothelium in response to increasing concentrations of EPC-K1 (10⁻⁵ to 10⁻¹ mg/ml). Segments were precontracted with PGF₂α (2×10⁻⁶ mol/L) (arrow). EPC-K1 produced relaxation at concentrations above 10⁻² mol/L in arterial segments with endothelium while segments without endothelium showed little response to EPC-K1.

Fig 2. Percent relaxation in canine coronary artery segments with (+) and without (−) endothelium exposed to increasing concentrations of EPC-K1 (10⁻⁵ to 10⁻¹ mg/ml) following no treatment (control) or treatment with L-NMMA (10⁻⁵ mol/L). Relaxation is given as percent decrease in maximal tension produced by PGF₂α (2×10⁻⁶ mol/L) precontraction. Vessel segments without endothelium (○, □) showed little response to EPC-K1 regardless of L-NMMA treatment. Control vessels with endothelium (●) demonstrated dose-related relaxation above an EPC-K1 concentration of 10⁻² mg/ml, achieving maximum relaxation at 1 mg/ml. Pretreatment with L-NMMA (▲) significantly decreased relaxation responses to EPC-K1 in segments with endothelium. *p<0.05 compared to control vessels with endothelium at each EPC-K1 concentration. Results given as mean ± SEM.

Fig 3. Comparison of maximal percent relaxation in canine coronary artery segments with (+) and without (−) endothelium exposed to EPC-K1 (10⁻² mg/ml). Relaxation is given as percent decrease in maximal tension produced by PGF₂α (2×10⁻⁶ mol/L) precontraction. EPC-K1 produced the greatest relaxation in control vessel segments with endothelium, which was significantly inhibited by L-NMMA (10⁻⁵ mol/L) (p<0.05). Addition of l-arginine (l-Arg) (10⁻⁴ mol/L) to segments treated with L-NMMA restored relaxation in response to EPC-K1; addition of d-arginine (d-Arg) (10⁻⁴ mol/L) had no effect. Segments without endothelium showed little reactivity regardless of treatment regimen. *p<0.05 compared to control vessels with endothelium. Results given as mean ± SEM.
relaxations in rings with endothelium, but had no effect on the response of rings without endothelium (Figs 2, 3). The inhibitory effect of L-NMMA on vasorelaxation was reversed by the addition of excess L-arginine (10^{-4} mol/L) but was unaffected by the addition of d-arginine (10^{-4} mol/L) (Fig 3), confirming the specificity of L-NMMA for L-arginine metabolism.

Inhibition of endothelium-dependent relaxation in response to EPC-K1 by combined use of L-NMMA (10^{-5} mol/L) and TEA (25 mmol/L), a nonselective potassium channel inhibitor, was not significantly different from that produced by L-NMMA alone (Fig 4).

Progressive addition of vitamin C (10^{-4} to 1 mg/ml) to canine coronary arterial segments resulted in endothelium-dependent vasodilatation (Fig 5). Endothelium intact segments relaxed to 48±8% of the maximum tension generated by precontraction with PGE_{2} (2×10^{-6} mol/L) compared to 11±6% relaxation by endothelium-denuded segments (p<0.05). Addition of L-NMMA (10^{-5} mol/L) did not alter the degree of relaxation in either group.

**Experiment 2**

Precontracted coronary arterial segments with endothelium relaxed maximally (approaching 100% of precontraction tension) in response to increasing concentrations of acetylcholine (10^{-9} to 10^{-5} mol/L). Exposure of similar arterial segments to FeSO_{4} and H_{2}O_{2} for 30 min prior to precontraction significantly impaired this vasodilatation response to acetylcholine (80±5%, p<0.05) (Fig 6). Pretreatment with EPC-K1 (10^{-4} mg/ml) preserved relaxation in response to acetylcholine in arterial segments exposed to this hydroxyl radical-generating system.
However, a lower concentration of EPC-K1 (10^{-5} \text{mg/ml}) did not preserve relaxation in response to acetylcholine (Fig 7).

Pretreatment with SOD (150 U/L) failed to preserve the maximum relaxation response to acetylcholine of precontracted coronary arterial segments exposed to the hydroxyl radical-generating system (Fig 7).

**Discussion**

During ischemia–reperfusion, the equilibrium between oxygen-derived free radical production and scavenging systems is dramatically disrupted, increasing the local concentrations of free radicals within reperfused vessels. Indeed, post-ischemic contractile dysfunction, reperfusion-induced arrhythmias, and arterial spasm and thrombotic complications associated with endothelial damage have all been linked with release of free radicals in animal models\cite{18-20}. Likewise, new evidence from human studies suggests that free radical activity is important during post-ischemic myocardial reperfusion\cite{21}.

It appears that hypoxia impairs the physiologic activity of endogenous antioxidant systems while reperfusion activates mononuclear and polymorphonuclear lymphocytes, leading to increased local synthesis and release of free radicals\cite{22,23}. Endothelial cells themselves can provide additional sources of free radicals during oxidative stress by the actions of intracellular xanthine oxidase\cite{24} and auto-oxidation of catecholamines\cite{25}. These substances, especially the hydroxyl radical, produce tissue damage through lipid peroxidation, oxidation of protein sulfhydryl groups, and disruption of deoxyribonucleic acid strands\cite{26}.

EPC-K1 is a novel antioxidant synthesized from phosphate linkage of vitamin C and vitamin E. Clinically, both of these vitamins have demonstrated some protective benefit against development of coronary artery disease\cite{27} and graft dysfunction following organ transplantation\cite{28}. The water-soluble vitamin C can inactivate aqueous peroxyl radicals as well as other species, while the liposoluble vitamin E can react with lipid peroxyl radicals, interrupting radical chain reactions\cite{9}. EPC-K1 may be advantageous because it maintains both hydrophilic and lipophilic properties. In previous in vitro studies, EPC-
EPC-K1 has been observed to scavenge hydroxyl radicals and inhibit phospholipase A2 activity. Furthermore, in vivo animal transplantation models have demonstrated protective effects of EPC-K1 against ischemia–reperfusion injury. The direct effects of EPC-K1 on endothelial function have not been examined. The present study was designed to determine in vitro the direct effect of EPC-K1 on coronary arterial function and confirm its protective benefit on endothelial function in the presence of exogenously generated hydroxyl radicals.

The key findings in this study are: (1) EPC-K1 stimulates NO release from canine coronary arteries, (2) endothelium-dependent vasodilatation in response to EPC-K1 is mediated not only by NO but also one or more factors independent of prostaglandin–K channel-mediated endothelial derived hyperpolarizing factor(s) (EDHF) pathways, (3) vitamin C causes a partial endothelium-dependent, NO-independent vasodilatation in canine coronary arteries, and (4) EPC-K1 protects against endothelial dysfunction resulting from exogenously produced hydroxyl radicals.

A major observation from this study is that EPC-K1 stimulates release of NO from canine coronary endothelium. Indeed, coronary segments with intact endothelium demonstrated significant vasodilatation in response to increasing concentrations of EPC-K1 (Fig 1), and this vasodilatation was partially inhibited by l-NMMA, a competitive inhibitor of endothelial nitric oxide synthase l-arginine metabolism (Fig 2). Previously, NO has been observed to stimulate dilatation of vascular smooth muscle, suppress production of endothelin-1, resist platelet and neutrophil adhesion and aggregation, and promote dissolution of platelet aggregates. Clinically, these beneficial effects of endothelial NO production may play a critical role in maintenance of vascular patency and preservation of cardiac function. Furthermore, NO replacement therapies such as exogenous NO, l-arginine, and NO donor compounds during reperfusion have provided protection against ischemia–reperfusion injury. Therefore, the ability of EPC-K1 to stimulate endothelial NO production may increase the pharmacologic utility of the compound.

Previous studies have shown NO-dependent vasodilation in response to vitamin C administration in both animal models as well as clinical studies of chronic heart failure. In our model, vitamin C administration did result in endothelium-dependent vasodilatation of canine coronary artery segments; however, this effect appeared to be independent of the NO pathway (Fig 5). These results conflict with those of Chang et al and may be a result of interspecies variation in vascular biology. It is interesting that the vasoactive effects of vitamin C and EPC-K1 differed substantially in our study: EPC-K1 produced vasorelaxation at lower concentrations and in part through an NO-dependent pathway. It may be that the vitamin E moiety is more influential on EPC-K1 vasoactivity than vitamin C. Unfortunately, vitamin E is not available in aqueous form and its direct individual effects are unable to be studied in our model.

We hypothesized that EDHF may be responsible for the small amount of NO-independent vasodilatation that occurred in response to EPC-K1 in this model since pretreatment of all segments with indomethacin eliminated the possibility of cyclooxygenase (COX) mediated vasodilation, as evidenced by the lack of any relaxation in control segments (Fig 2). Previous studies have shown EDHF to produce vasodilatation through the activation of potassium channels, which can be inhibited by TEA, a nonselective potassium channel blocker. However, addition of high doses of TEA to l-NMMA failed to affect the remaining relaxation produced by EPC-K1 in this study (Fig 4). Thus, another endothelial system other than the NO, COX, and EDHF pathways must be responsive to EPC-K1 to account for this remaining endothelium-dependent vasodilatation. Further investigation is needed to determine the exact nature of this additional system.

Mori et al has previously demonstrated that EPC-K1 can scavenge hydroxyl radicals in vitro. To assess the ability of EPC-K1 to protect endothelium from hydroxyl radical-induced injury, we chose a well established model of hydroxyl radical generation which consistently produces endothelial damage on vascular rings in organ chambers. Exposure of canine coronary artery segments to hydroxyl radicals produced endothelial dysfunction demonstrated by a blunted vasodilatory response to increasing concentrations of acetylcholine (Fig 6). While initial studies without the presence of hydroxyl radicals failed to show any influence of a higher concentration of EPC-K1 (5×10^{-4}) on acetylcholine-induced coronary artery vasodilatation (data not shown), the addition of EPC-K1 (10^{-4}mg/ml) to the preparation preserved maximum vasodilatation in response to acetylcholine. This provides strong evidence that EPC-K1 can protect endothelial function by effectively scavenging hydroxyl radicals in this system. Interestingly, pretreatment with high concentrations of SOD did not show the same protective effects on the coronary endothelium as did EPC-K1, indicating that endogenous free radical production, specifically superoxide, did not have an influence on our results. Furthermore, it confirms the inability of SOD to scavenge hydroxyl radicals.

The influence of EPC-K1 on NO release may supplement its ability to scavenge hydroxyl radicals as NO has been known to inhibit lipid peroxidation and reduce free radical generation in some studies. However, the concentration at which EPC-K1 induced NO release was 100-fold higher than that needed to maintain endothelial-dependent relaxation in the presence of hydroxyl radicals. Thus, preservation of endothelial function by EPC-K1 in the presence of hydroxyl radicals was not influenced by NO activity in our model.

In conclusion, high concentrations of EPC-K1 stimulate release of NO by canine coronary artery endothelium. However, endothelial-dependent vasodilatation in response to EPC-K1 is only partially NO mediated and is independent of the COX and EDHF systems. EPC-K1 also preserves endothelial function in the presence of exogenous hydroxyl radicals, but this ability to scavenge hydroxyl radicals appears to be independent of its ability to stimulate NO release and endothelium-dependent coronary artery vasodilatation. Further study is warranted to investigate the potential benefits of EPC therapy against ischemia–reperfusion injury and preservation of vascular bypass graft patency and cardiac performance following cardiac surgery.
References


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