Supplementary Information

Methods

Sensitivity and Specificity of Terephthalic Acid for Hydroxyl Radical Detection

The reaction principle of the hydroxylation of terephthalic acid is shown in the figure below. The resulting 2-hydroxy terephthalic acid has fluorescent activity (excitation 326 nm, emission 432 nm).

![Diagram of Terephthalic Acid Reaction](adapted from Linxiang Fig.1)

Figure 1: Reaction of terephthalic acid with hydroxyl radical. (adapted from Linxiang Fig.1)

The detection of \( \text{•OH} \) by TPA was used as a highly sensitive and specific \( \text{•OH} \) detection method and to the best of our knowledge the present study is the first to use TPA for quantification of \( \text{•OH} \) formation from intact murine vascular specimens. Our experiments were performed based on previous studies demonstrating the specificity of TPA as a probe of \( \text{•OH} \) (1-3, 6, 7, 11). The concentration of TPA (2.5 mM) used in the experiments shown in Figure 1 of the manuscript is in the middle of a concentration range as indicated in previous studies ensuring an excess of the \( \text{•OH} \) probe (TPA) (3, 6, 11). Accordingly, Mishin et al. used 1 mM TPA to detect hydroxyl radicals in liver microsomal enzymes (3) and in a study investigating \( \text{•OH} \) formation after melanin irradiation 2 mM TPA was used to probe for hydroxyl radicals (6). In fetal sheep brain in utero, Yan and Co-workers used a single TPA
concentration of 5 mM (11). A study by Linxiang et al., compared detection of hydroxyl radicals by electron spin resonance (ESR) technique with a HPLC-based method using TPA hydroxylation (2). These investigators demonstrated that TPA hydroxylation measured by HPLC was as specific and sensitive as the ESR technique using 5,5 dimethyl-1-pyrroline N-oxide (DMPO) as a spin trap in trifluoroacetate solution (2).

![Figure 2: Detection of •OH by TPA and DMPO using Fenton reaction. The concentration of Fe(II) was 50 μmol/L, and the final concentration of DMPO or TPA was 4.25 mmol/L. Data adapted from Linxiang et al, 2004 (2).](image)

Interestingly, in phosphate buffer, TPA was found to be even more sensitive than DMPO in terms of concentrations to obtain similar results (TPA: 4.25 mM; DMPO: 42.5 mM) (2). Linxiang et al., reported that hydroxylated TPA (TPH-OH) formed by the reaction with superoxide was suppressed in the presence of mannitol, a •OH scavenger (2) suggesting the possibility that the formation of TPH-OH was due to the reaction of •OH derived from superoxide. These investigators also reported TPH-OH cannot be formed by the reaction with H$_2$O$_2$ (2). Thus, TPA can be considered a specific and sensitive probe to detect •OH.

**Chemical Structure and Antioxidant Properties of EPC-K1**

EPC-K1 or α-tocopheryl-L-ascorbate-2-O-phosphate diester is a phosphate ester derivative of vitamin C (ascorbic acid) and vitamin E (α-tocopherol), which is soluble in both water and
lipid (9, 10). Vitamin C contains two enolic hydroxyl groups and Vitamin E has a phenolic hydroxyl group. These groups act as the antioxidant sites. In EPC-K1, one enolic hydroxyl group of vitamin C and the phenolic hydroxyl group of vitamin E are converted to ester bonds. The remaining enolic hydroxyl group of vitamin C thus acts as the active antioxidant site for EPC-K1 (10). The molecular weight of EPC-K1 (C\textsubscript{35}H\textsubscript{56}O\textsubscript{10}PK) is 706.90, thus 0.1 mg/ml as used in our study is equivalent to a concentration of 141.46µM. EPC-K1 is a white powder which is water-soluble (4).

![Figure 3: Chemical structure of EPC-K1.](image)

**Characterization of the Antioxidant Activities of EPC-K1**

**A. H\textsubscript{2}O\textsubscript{2} Detection Using Amplex Red Assay**

The amplex red assay is a highly sensitive method for the fluorometric detection of H\textsubscript{2}O\textsubscript{2}. In the presence of horseradish peroxidise (HRP), amplex red reacts with H\textsubscript{2}O\textsubscript{2} to produce a fluorescent product resorufin which is measured fluorometrically. In our experiments, H\textsubscript{2}O\textsubscript{2} (0.15 µM – 10 µM) was pre-incubated in a concentration-dependent manner in the presence or absence of EPC-K1 (0.1 mg/ml) for 10 min in the dark and amplex red reagent (containing amplex red and HRP in sodium phosphate buffer) was added. As negative control EPC-K1 (0.1 mg/ml) was added with only amplex red reagent in the absence of H\textsubscript{2}O\textsubscript{2}. After 30 min of incubation in the dark at room temperature, the fluorescent measurements were performed. No fluorescence was detected in the negative control samples indicating no auto-fluorescence.
properties of EPC-K1. In the absence of EPC-K1, H$_2$O$_2$ increased the fluorescence in a concentration-dependent manner (Figure 4A). In the presence of EPC-K1, the H$_2$O$_2$-mediated fluorescence was reduced. Thus, EPC-K1 interacts with H$_2$O$_2$ and reduces H$_2$O$_2$-mediated fluorescence. The most potent effects were observed at lower H$_2$O$_2$ concentrations (0.313µM and 0.156µM) compared to higher concentrations (10µM), as represented below (Figure 4B).

![Figure 4: Detection of H$_2$O$_2$ (0.15-10 µM) using the amplex red method (A). Measurements were done flurometrically (excitation: 542 nm; emission: 590 nm). Effect of EPC-K1 on H$_2$O$_2$–mediated fluorescence (B). EPC-K1 more significantly scavenges H$_2$O$_2$ at lower concentration (0.3 and 0.15 µM). The data is calculated after normalization of 10 µM H$_2$O$_2$ to 1. * $P<0.05$ vs. 10 µM H$_2$O$_2$.](image)

**B. Detection of Superoxide Using the L012-Assay**

The effect of EPC-K1 on superoxide anion formation in vitro was determined using an enzyme-based, cell-free system using xanthine and xanthine oxidase in aerated Krebs-Ringer solution. Superoxide anion formation was quantified, as described, using 8-amino-5-chloro-7-phenylpyridol[3,4-d]pyridazine-1,4(2H,3H)dione sodium salt (L-012), a highly sensitive chemiluminescence probe, using a Lumat LB 9507 (Berthold, Bad Wildbad, Germany). Briefly, in 5 ml polystyrene tube (12 x 75 mm; BD Biosciences, Franklin Lakes, NJ USA) the required concentrations of xanthine (20 µM; 90 µl) and L-012 (100 µM; 30 µl) was injected into Krebs-Ringer solution (37°C, pH 7.4). The final reaction volume including drugs and/or enzymes was set to 600 µl. Time between the injections was 1 sec. As defined by the
experimental set-up, the required concentrations of xanthine oxidase (5 mU), EPC-K1 (0.1 mg/ml) or superoxide dismutase (200 U/ml) were added to the Krebs-Ringer solution before starting the injections. Superoxide dismutase was used to confirm the specificity of the assay. After adding the reaction substances the measurement program was started. Twenty four measurements were performed over a period of 6 min and duration of each measurement was 10 sec with a delay measurement time of 5 sec. The emitted chemiluminescence corresponding to the superoxide formation was calculated after subtracting the background. Total superoxide anion formation was determined by cumulative measurements of chemiluminescence signals from 1-6 minute.
Results

Reactive Oxygen Species-Mediated Concentration-Dependent Vascular Relaxation

To examine the vascular effect of exogenous ROS, predominantly consisting of \( \cdot \text{OH} \) (ROS/\( \cdot \text{OH} \)), equal concentrations of ascorbic acid and iron (10 \( \mu \text{mol/L} \) – 5 mmol/L) were added simultaneously to the bath to generate ROS/\( \cdot \text{OH} \) (Figure 5)(5). While no dilatory effect was observed at 10 \( \mu \)M of ascorbic acid and 10 \( \mu \)M iron, the EC\(_{50}\) of the response was at approximately 100 \( \mu \)M (EC\(_{50}\) = 125 \( \mu \)M, Figure 5). We therefore decided to use concentration of 100 \( \mu \)M of each compound for all experiments.

![Dilation (%) Precontraction vs. Concentration of Vitamin C + Fe\(^{2+}\)](image)

**Figure 5:** Reactive Oxygen Species/\( \cdot \text{OH} \)-mediated concentration-dependent vascular relaxation. Pre-contraction aortic rings were treated with vitamin C and Fe\(^{2+}\) (0.01 mM - 5 mM) as indicated. Data are expressed as percentage of precontraction.

In a previous study by Takayama *et al.* concentration-response curves of EPC-K1 were performed which revealed a maximal effect at a concentration of \( 10^{-1} \) mg/ml in canine coronary arteries (8). Due to the limitation of vascular tissue EPC-K1 was used only at a single concentration (\( 10^{-1} \) mg/ml).
References


