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p-Hydroxybenzyl alcohol-containing biodegradable nanoparticle improves functional blood flow through angiogenesis in a mouse model of hindlimb ischemia

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ABSTRACT

Therapeutic angiogenesis has achieved promising results for ischemic diseases or peripheral artery disease in preclinical and early-phase clinical studies. We examined the therapeutic angiogenic effects of HPOX, which is biodegradable polymer composing the antioxidant *p*-hydroxybenzyl alcohol (HBA), in a mouse model of hindlimb ischemia. HPOX effectively stimulated blood flow recovery, compared with its degraded compounds HBA and 1,4-cyclohexendimethanol, via promotion of capillary vessel density in the ischemic hindlimb. These effects were highly correlated with levels of angiogenic inducers, vascular endothelial cell growth factor (VEGF), heme oxygenase-1 (HO-1), and Akt/AMPK/endothelial nitric oxide synthase (eNOS) in ischemic mouse hindlimb muscle. Blood perfusion and neovascularization induced by HPOX were reduced in eNOS$^{-/-}$ and HO-1$^{+/-}$ mice. HPOX also elevated the endothelial cell markers VEGF receptor-2, CD31, and eNOS mRNAs in the ischemic hindlimb, indicating that HPOX increases endothelial cell population and angiogenesis in the ischemic muscle. However, this nanoparticle suppressed expression levels of several inflammatory genes in ischemic tissues. These results suggest that HPOX significantly promotes angiogenesis and blood flow perfusion in the ischemic mouse hindlimb via increased angiogenic inducers, along with suppression of inflammatory gene expression. Thus, HPOX can be used potentially as a noninvasive drug intervention to facilitate therapeutic angiogenesis.

1. Introduction

Peripheral arterial disease (PAD) is a common disease that is caused by atherosclerosis, thrombosis, diabetes, and decreased blood flow perfusion to the legs. Patients with PAD are at increased risk of myocardial infarction, stroke, and death [1]. PAD is generally treated with medical management, minimally invasive endovascular procedures, such as percutaneous transluminal angioplasty or open bypass surgery [2,3]. However, 20–30% of patients with critical limb ischemia of PAD are not suitable candidates for these treatments and may require amputation. In this situation, functional and clinical improvements that relieve ischemic pain and prevent amputation can be critical options. Among them, therapeutic angiogenesis using angiogenic inducers or growth factors may be one of the best treatment options for critical limb ischemia of PAD that are not amenable to surgical treatments [4,5].

Angiogenesis is an important homeostatic process contributing to the improvement of blood flow and circulation in ischemic organs or tissues. Although numerous clinical trials of therapeutic angiogenesis for PAD have reported promising results [4,5], there

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are concerns with regard to safety and feasibility due to issues such as hypotension, acceleration of atherosclerosis, and spread of malignancy [6–8]. Indeed, delivery of the well-known angiogenic factors, vascular endothelial cell growth factor (VEGF) and basic fibroblast growth factor (bFGF), promote blood flow through neovascularization in ischemic regions. However, they can evoke harmful or undesirable side effects, such as vascular permeability, inflammation, tumor angiogenesis, and hypertension [7,8]. Therefore, there is a great need for novel approaches to therapeutic angiogenesis for PAD, without the harmful effects.

p-Hydroxybenzyl alcohol (HBA)-incorporated copolyoxalate (HPOX) is a biodegradable polymer synthesized from a condensation reaction of oxalyl chloride, 1,4-cyclohexendimethanol (CHDM), and HBA [9]. HPOX has a hydrolytic half-life of ~12 h into its initial components under aqueous solution. HPOX is rapidly degraded by the direct reaction of its polyoxalate backbone with H_2O_2, resulting in its antioxidant activity [10]. Interestingly, HBA released from the polymer backbone can also exert therapeutic effects in the treatment of inflammatory disorders and ischemic diseases [11,12]. Therefore, this nanoparticle may be an useful therapeutic agent for ischemia/reperfusion injury caused by overproducing reactive oxygen species [13]. HBA also exhibited beneficial effects in cerebral ischemic injury by up-regulating the transcription factor, nuclear factor E2-related factor 2 (Nrf2)-dependent phase II enzymes [14]. The major phase II enzyme hemeoxygenase-1 (HO-1) is critically involved in improvement of blood flow in ischemic regions through VEGF expression and angiogenesis [15,16]. Although the anti-inflammatory effects of HPOX have been also extensively studied [13], its angiogenic activity has not been elucidated.

In this study, we investigated the pharmacological and therapeutic effects of HPOX on neovascularization and blood flow in a mouse model of hindlimb ischemia. The animal studies demonstrated that HPOX nanoparticles improve blood flow perfusion and neovascularization via VEGF expression, HO-1 induction, and endothelial nitric oxide synthase (eNOS) activation, but suppress inflammatory cytokine gene expression, in the ischemic mouse hindlimb. We therefore anticipate that the HPOX nanoparticles are a potential therapeutic agent for functional improvements of ischemic diseases including PAD.

2. Materials and methods

2.1. Reagents and antibodies

HPOX was synthesized as previously reported [9]. In brief, CHDM (21.96 mmol) and HBA (5.49 mmol) were dissolved in 20 ml of dry tetrahydrofuran (THF), and triethylamine (60 mmol) was added dropwise to the solution under nitrogen at 4 °C. Polymerization was initiated by adding oxalyl chloride (27.45 mmol) in 25 ml of dry THF to the reaction solution at 4 °C, and the reaction mixture was kept under nitrogen atmosphere at room temperature for 6 h. Polymers were obtained through the extraction using dichloromethane and isolation by precipitating in cold hexane. The chemical structure of polymers was identified with a 400 MHz ^{1}H NMR spectrometer (JNM-EX400 JEOL). We used antibodies specific for HO-1 (Stressgen, Ann Arbor, Michigan), Akt and p-Akt (Cell signaling, Danvers, MA), AMPK and p-AMPK (Cell signaling, Danvers, MA), eNOS and p-eNOS (Abcam, Cambridge, UK), VEGF (Thermo Scientific or Santa Cruz Biotechnology, Santa Cruz, CA), Texas Red-conjugated mouse CD31 antibody (DB Pharmingen, San Diego, CA), and actin (Sigma–Aldrich, St Louis, MO).

2.2. Animals

Seven-week-old male mice (C57BL/6J and eNOS$^{-/-}$) were purchased from Jackson Laboratory (Bar Harbor, ME, USA) and maintained on a standard (normal) chow diet ad libitum in a laminar airflow cabinet under specific pathogen-free conditions. HO-1$^{+/-}$ mice (Balb/c) were obtained from Jackson Laboratory and backcrossed with C57BL/6J for 14 generations. Animal experiments were performed in accordance with the guidelines of the Institutional Animal Care and Use Ethics Committee of Kangwon National University. Moreover, this investigation was conformed to the Guide for the Care and Use of Laboratory Animals published by the United States National Institutes of Health (NIH Publication, 8th Edition, 2011).

2.3. Mouse model of unilateral hindlimb ischemia

Male mice (8 weeks) underwent surgical ligature of the proximal part of the right femoral artery after anesthetization with ketamine (100 mg/kg) und xylazine (2 mg/kg) as described previously [17]. For each mouse, total 50 μl of HBA, CHDM or HPOX solution containing the indicated concentration was intramuscularly injected equally at three sites on the thigh muscle of the ischemic hindlimb to guarantee a homogenous distribution. Saline was injected as a control. Sham-operated control animals were subjected to the same surgical protocol, but the femoral artery wass not ligated. Pain caused by the surgical procedures was managed pre-operatively and on days 1 and 2 after surgery by intramuscular injection with buprenorphine (0.1 mg/kg). On days 2, 5, 7, and 14 post-surgery, mice were anaesthetized with inhaled isoflurane (2%), and blood flow in both hindlimbs was determined by laser-Doppler perfusion imaging (Moor Instruments, Axminster, UK). Flow ratio of the occluded/non-occluded leg was compared between experimental groups. Following sacrifice by overdose of isoflurane via inhalation, the gastrocnemius muscles were surgically removed to determine vascular density.

2.4. Vascular density

On postoperative day 14, the distal gastrocnemius muscles were dissected from the ischemic mouse hindlimbs, fixated in 4% paraformaldehyde solution, and cut into 10 μm sections. Each section was stained for blood vessels with Texas Red-conjugated mouse CD31 antibody. Capillaries were counted in 5 random microscopic fields from 4 independent cross-sections of the gastrocnemius muscle in each animal. Capillary density is expressed as the number of capillaries per field.

2.5. Western blotting

Skeletal muscle tissues were homogenized in ten volumes of RIPA lysis buffer (20 mM Tris–HCl (pH 8.0), 137 mM NaCl, 1% Triton X-100, 10% glycerol, 2 mM EDTA, 1 mM sodium vanadate, 1 M β-glycerophosphate, 0.5 M sodium fluoride, 1 mM phenylmethylsulfonyl fluoride), followed by three cycles of freeze/thaw. Tissue lysates were obtained by centrifuging at 120,000 × *g* for 20 min, and supernatants were obtained. Equal amounts of total protein (60 μg) were resolved on 10% SDS-PAGE and transferred to polyvinyldifluoride membranes. The membranes were used for detecting target proteins using antibodies for VEGF, HO-1, p-Akt, p-AMPK, and p-eNOS as described previously [15]. Immunoreactive protein bands were detected with enhanced chemiluminescence reagents (Thermo Scientific, Rockford, IL). Relative protein levels were quantified using ImageJ software (National Institutes of Health, Bethesda, MD).

2.6. Determination of mRNA levels

Total RNA was extracted from mouse hindlimb skeletal muscle using the TRIzol reagent (Invitrogen). After cDNA was synthesized using 5 μg of RNA, the mRNA levels of several genes were determined by either reverse transcriptase-polymerase chain reaction (RT-PCR) or quantitative real-time PCR (qRT-PCR). RT-PCR was performed to determine levels of VEGF, HO-1, VEGF receptor 2 (VEGF-R2), eNOS, and CD31 mRNA using specific primers as described previously [18]. Primers used were: 5′-CTGCCGTCCGATTGAGACC-3′ (sense) and 5′- CCCCTCCTTGTACCACTGTC-3′ (antisense) for VEGF, 5′-GGCCCTGGAAGAGGAGATA-3′ (sense) and 5′-GCTGGATGTG CTTTTGGTG-3′ (antisense) for HO-1, 5′-GCAAAACACTCACCATTCCCA-3′ (sense) and 5′- GAGGTTTGAAATCGACCCTCG-3′ (antisense) for VEGF-R2, 5′-TGTGACCCTCA CCGCTACAA-3′ (sense) and 5′-GCACAATCCAGGCCCAATC-3′ (antisense) for eNOS and 5′-CTGCCAGTCCGAAAATGGAAC-3′ (sense) and 5′-CTTCATCCACCGGGGCTATC-3′ (antisense) for CD31, and 5′-CAGCCACCCGAGATTGAGCA-3′ (sense) and 5′-TAGTA GCGACGGGCGGTGTG-3′ (antisense) for 18S rRNA. Ribosomal 18S was used as an internal control. The mRNA levels were quantified using ImageJ software. qRT-PCR was also performed to determine the levels of inflammation-associated gene mRNA by using iTaqTM SYBR Green Supermix with ROX (BioRad, Hercules, CA) with ABI PRISM 7000 Sequence Detection System (Applied Biosystem) as described previously [17], except for monocyte chemotactic protein-1 (MCP-1) primers as follows: 5′-ATCCCAATGAGTAGGCTGGAGAGC-3′ (sense) and 5′-CAGAAGTGCTT-GAGGTGGTTGTG-3′ (antisense). The fold-change of inflammatory gene mRNA levels was calculated using the $2^{-\Delta\Delta Ct}$ method.

2.7. Statistical analysis

Quantitative data are expressed as the mean ± SD of at least three separate experiments. Statistical significance was determined using either one-way ANOVA or unpaired Student's *t* test, depending on the number of experimental groups analyzed. Significance was established at a p value <0.05.

3. Results

3.1. HPOX improves local blood flow perfusion in a mouse mode of hindlimb ischemia

Since HPOX is synthesized from the major compounds oxalyl chloride, CHDM, and HBA [9], we first comparatively examined