Increased arginase II activity contributes to endothelial dysfunction through endothelial nitric oxide synthase uncoupling in aged mice

Woosung Shin¹, Dan E. Berkowitz²,³ and Sungwoo Ryoo¹,⁴

¹Department of Biology
College of Natural Sciences
Kangwon National University
Chuncheon 200-701, Korea
²Department of Anesthesiology and Critical Care Medicine
Johns Hopkins Medical Institutions
Baltimore, MD 21287, USA
³Corresponding author: Tel, 82-33-250-8534; Fax, 82-33-251-3990; E-mail, ryoosw08@kangwon.ac.kr
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Abbreviations: ABH, 2 (S)-amino-6-boronohexanoic acid; Ach, acetylcholine; Arg I, arginase I; ArgII, arginase II; DAF, 4-amino-5-methylamino-2',7'-difluorescein; DHE, dihydroethidium; l-arg, l-arginine; l-NAME, l-NG-nitroarginine methyl ester; MnTBAP, Mn (III)tetrakis (4-benzoic acid) porphyrin chloride; ns, not significant; ROS, reactive oxygen species; siArgII, siRNA to arginase II

Abstract

The incidence of cardiovascular disease is predicted to increase as the population ages. There is accumulating evidence that arginase upregulation is associated with impaired endothelial function. Here, we demonstrate that arginase II (ArgII) is upregulated in aortic vessels of aged mice and contributes to decreased nitric oxide (NO) generation and increased reactive oxygen species (ROS) production via endothelial nitric oxide synthase (eNOS) uncoupling. Inhibiting ArgII with small interfering RNA technique restored eNOS coupling to that observed in young mice and increased NO generation and decreased ROS production. Furthermore, enhanced vasoconstrictor responses to U46619 and attenuated vasorelaxation responses to acetylcholine in aged vasculature were markedly improved following siRNA treatment against ArgII. These results might be associated with increased L-arginine bioavailability. Collectively, these results suggest that ArgII may be a valuable target in age-dependent vascular diseases.

Keywords: aging; arginase II; endothelial nitric oxide synthase uncoupling; small interfering RNA; vascular diseases

Introduction

Cardiovascular disease is the leading cause of morbidity and mortality in both industrialized and developing countries. Despite effective treatments for several established cardiovascular risk factors, such as hypertension and hypercholesterolemia, the incidence of cardiovascular disease is predicted to increases as the population ages. Characteristic events in the aging cardiovascular system include vascular stiffness (Meaume et al., 2001), enhanced reactive oxygen species (ROS) production (Finkel and Holbrook, 2000; Brandes et al., 2005), and decreased nitric oxide (NO) bioavailability (Adler et al., 2003; Csiszar et al., 2008). Superoxide may react with NO to produce peroxynitrite (ONOO⁻), which is potentially detrimental to vascular cell function and viability. This nitroso-redox imbalance contributes to age-related endothelial dysfunction and vascular stiffness (Berkowitz et al., 2003).

Arginase is well demonstrated as an important enzyme in the last step of the urea cycle, which hydrolyzes L-arginine to L-ornithine and urea. There are two isoforms: arginase I (ArgI), also known as the hepatic isoform, and arginase II (ArgII), which is an extrahepatic enzyme. These proteins are encoded by different genes (Haraguchi et al., 1987; Morris et al., 1997) and are induced by a variety of factors (Meurs et al., 2003). Arginase isoforms are found in a wide variety of cells and tissues, and current reports suggest that they may be expressed in species- and tissue-specific manners (Abe and Berk, 1998; Morris et al., 1998; Chicoine et al., 2004; Ryoo et al., 2006; Nelin et al., 2007). There is increasing evidence that arginase upregulation...
functionally inhibits nitric oxide synthase (NOS) activity and contributes to age-related vascular dysfunction (Berkowitz et al., 2003; Ryoo et al., 2006). Pharmacological inhibition and antisense knockdown of ArgI restores endothelial NO production and endothelial function ex vivo (Ryoo et al., 2006). Arginase-mediated reciprocal regulation of NOS has been demonstrated in the majority of cell types and organs in which NO is an important signaling molecule, including cardiomyocytes (Steppan et al., 2006), the penis (Bivalacqua et al., 2007), the airway (Meurs et al., 2003), skin (Holowatz and Kenney, 2007), inflammatory mediator cells (e.g., macrophages), and endothelial cells (Berkowitz et al., 2003).

Although vascular changes associated with aging have been investigated in humans and a number of other species, it is thought that the relative contributions of dysregulated mechanisms to age-related vascular pathology are species-dependent (Santhanam et al., 2008). Moreover, the contribution of vascular control mechanisms in health, aging, and disease conditions is influenced by regional vascular beds and vessel type and size (Santhanam et al., 2008). Therefore, we investigated which arginase isoform contributes to age-related endothelial dysfunction in mice and determined whether specific inhibition of arginase isoforms with small interfering RNA (siRNA) was able to restore vascular function. We also analyzed L-arginine (L-arg) concentrations in isolated aortic vessels to elucidate the underlying mechanism of endothelial nitric oxide synthase (eNOS) activation.

**Results**

**ArgII is the key isoform regulating arginase activity in the aorta of aged mice**

We first analyzed arginase expression in young and aged groups (Figures 1A and 1B). ArgII was strongly expressed, but we did not detect ArgI. Neither the expression level of ArgII protein (A, young vs. aged, 1.0 ± 0.07 vs. 1.05 ± 0.06, ns) nor mRNA (B, young vs. aged, 1.0 ± 0.08 vs. 1.01 ± 0.04, ns) was significantly different between groups. Next, we measured arginase activity with or without preincubation of siRNAs to specific arginase isoforms (siArgI and siArgII). siArgII incubation with young aortic vessels resulted in decrease of arginase activity (Figure 1C, # vs. young, 88.6 ± 3.0 vs. 100.0 ± 7.1, P < 0.05) that was associated with decreased protein levels (Figure 1D, # vs. young, 0.81 ± 0.07 vs. 1.0 ± 0.01, P < 0.05). Furthermore, increased arginase activity in the aged group (Figure 1C, * vs. young, 137.52 ± 6.1 vs. 100.0 ± 7.1, P < 0.01) was markedly attenuated by siArgII (Figure 1C, ** vs. aged, 137.52 ± 6.1 vs. 94.8 ± 3.2%, P < 0.01) that resulted from downregulated ArgII protein levels (Figure 1D, * vs. aged, 0.77 ± 0.04 vs. 1.0 ± 0.01, P < 0.01). However, siArgI had no effect on either protein level or enzyme activity (Figures 1C and 1D, ## vs. aged, ns). These results imply that the ArgII isoform was the dominantly expressed enzyme in the vasculature of aged mice.

**eNOS uncoupling in aged aorta was restored with siArgII**

We next assessed eNOS expression in both groups. Interestingly, eNOS protein expression was significantly
increased in the aged group (* vs. young, 1.18 ± 0.05 vs. 1.00 ± 0.02, P < 0.01). This observation was not affected by incubation with siArgI or siArgII (Figure 2A, ** vs. aged, ns). Therefore, we tested whether eNOS expression levels were associated with its activity by measuring eNOS dimers and found that the dimer to monomer ratio was markedly reduced in the aged group (Figure 2B, * vs. young, 0.87 ± 0.02 vs. 1.00 ± 0.02, P < 0.05). The decreased ratio of eNOS dimer/monomer was recovered by inhibition of arginase activity using either siRNA (** vs. aged, 1.04 ± 0.02 vs. 0.87 ± 0.02, P < 0.05) or a known arginase inhibitor, ABH (** vs. aged, 1.00 ± 0.02 vs. 0.87 ± 0.02, P < 0.05). The effect of siArgII on eNOS coupling was also found in young aorta (* vs. young, 1.21 ± 0.02 vs. 1.00 ± 0.02, P < 0.05). These results suggest that endothelial function in aged mice is impaired despite increased eNOS expression.

siArgII affected NO production and ROS generation in aged mice

Based on the eNOS coupling data, we next measured NO production and ROS generation in endothelium from young and aged mice. As demonstrated in Figure 3A, NO production was significantly attenuated in aged mice (* vs. young, 0.58 ± 0.16 vs. 0.90 ± 0.19 change of DAF fluorescence/second, P < 0.01). It was restored following siArgII and ABH treatment (** siArgII vs. aged, 0.87 ± 0.11 vs. 0.58 ± 0.16, P < 0.01; ** ABH vs. aged, 0.81 ± 0.14 vs. 0.58 ± 0.16 change of DAF fluorescence/second, P < 0.01), but siArgI had no effect. Consistent with the data, siArgII induced increase in the slope of DAF fluorescence in young mice (* vs. young, 1.13 ± 0.10 vs. 0.90 ± 0.19 change of DAF fluorescence/second, P < 0.05). On the other hand, ROS generation was markedly augmented in aged mice (Figure 3B, * vs. young, 0.52 ± 0.04 vs. 0.31 ± 0.03 change of DHE fluorescence/second, P < 0.01), but this was reduced to the level of young endothelium after siArgII and ABH incubation (**, siArgII vs. aged, 0.28 ± 0.07 vs. 0.52 ± 0.04, P < 0.01; **, ABH vs. aged, 0.21 ± 0.06 vs. 0.52 ± 0.04 change of DHE fluorescence/second, P < 0.01). siArgII incubation also reduced ROS generation in young mice (* vs. young, 0.24 ± 0.04 vs. 0.31 ± 0.03 change of DHE fluorescence/second, P < 0.05). Interestingly, L-NG-
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Figure 4. siArgII improved impaired vascular reactivity in aged mice. (A) Aged aortic vessels had augmented contractile responses to U46619 compared to young mice (*, young vs. aged, \( P < 0.01 \)). siArgII restored the U46619-mediated pressor response of aged aortic vessels to levels similar to that of young aortas (*, young vs. aged, \( P < 0.01 \); **, aged vs. aged + siArgII, \( P < 0.01 \)). \( n = 4 \) mice per each group. (B) siArgII attenuated contractile responses to U46619 in young aorta (*, young vs. young + siArgII, \( P < 0.01 \)) and ABH incubation with old aorta significantly retarded U46619-dependent constriction (**, aged vs. aged + ABH, \( P < 0.01 \)). (C) Endothelium-dependent relaxation responses to Ach were impaired in aged aortas (*, young vs. aged, \( P < 0.01 \)). Impaired relaxation was recovered with siArgII pre-treatment (**, aged vs. aged + siArgII, \( P < 0.05 \)). \( n = 4 \) mice per each group. (D) ABH incubation augmented Ach-dependent vessel relaxation in aged aorta (*, aged vs. aged + ABH, \( P < 0.01 \)).

Figure 5. siArgII preincubation increased intracellular L-arg concentrations. Aortic vessels were incubated with or without siArgI, siArgII, and ABH and intracellular L-arg concentrations were measured with HPLC (see methods section). Treatment of siArgII to young vessels resulted in increase of L-arg amount (* vs. young, \( P < 0.01 \)). The reduced L-arg level in aortas from aged mice was restored to that of young aortas by incubation of siArgII and ABH (* vs. young, \( P < 0.01 \); ** vs. aged, \( P < 0.01 \); \( n = 6 \)).

siArgII restored impaired endothelial function in aged vasculature

Because upregulated arginase activity increases ROS production in aged mice (* vs. aged, \( 0.32 \pm 0.06 \) vs. \( 0.52 \pm 0.04 \) change of DHE fluorescence/second, \( P < 0.01 \)). These data imply that increased eNOS uncoupling in aged mice leads to decreased NO production and increased ROS generation.

Siarginase inhibition with ABH augmented vasorelaxation responses in both E_max (**, aged vs. aged + ABH, \( 7.32 \pm 0.05 \) M vs. \( 7.35 \pm 0.07 \) M, NS). Next, to determine the effects of siRNA-mediated arginase inhibition on endothelium-dependent vasorelaxation, mouse aorta were preconstricted with U46619 (10^{-6} M), and dose-response curves to the endothelium-dependent vasodilator acetylcholine (Ach) were constructed. The vasorelaxation responses in aged mice were significantly attenuated compared with those from young (Figure 4C, *, young vs. aged, \( 88.31 \pm 4.89 \% \) vs. \( 72.87 \pm 2.82 \% \) (E_max), \( P < 0.01 \); \( 7.23 \pm 0.13 \) M vs. \( 6.81 \pm 0.09 \) M (-logEC50), \( P < 0.01 \)). Consistent with the above data, incubation of aged aorta with siArgII enhanced vasorelaxation responses (Figure 4C, ** aged vs. aged + siArgII, \( 72.87 \pm 2.82 \% \) vs. \( 94.36 \pm 1.93 \% \) (E_max), \( P < 0.01 \); \( 6.81 \pm 0.09 \) M vs. \( 7.28 \pm 0.06 \) M (-logEC50), \( P < 0.01 \)). Also, inhibition of arginase activity with ABH augmented vasorelaxation responses in both E_max (Figure 4D, *, aged vs. aged + ABH, \( 72.87 \pm 2.82 \% \) vs. \( 94.36 \pm 1.93 \% \) (E_max), \( P < 0.01 \); \( 6.81 \pm 0.09 \) M vs. \( 7.28 \pm 0.06 \) M (-logEC50), \( P < 0.01 \)).
ArgII inhibition increased L-arg bioavailability

Given data suggesting that arginase reciprocally regulates NOS activity by limiting L-arg bioavailability, we used high-performance liquid chromatography (HPLC) to determine whether intracellular L-arg concentration was affected by altered argi

Expression can be induced by a variety of vascular insults, including oxidized low-density lipoprotein (oxLDL), peroxynitrite, lipopolysaccharide, tumor necrosis factor-α, interferon-γ, 8-bromo-cGMP, thrombin, and hypoxia (Abe and Berk, 1998; Morris et al., 1998; Chicoine et al., 2004; Nelin et al., 2007). oxLDL (Ryoo et al., 2011), thrombin (Ming et al., 2004), and peroxynitrite (Chandra et al., 2012) stimulate ArgII activation via RhoA-dependent ROCK activation without a change in mRNA and protein levels. One study reported that peroxynitrite can react with the redox-active cysteine (Cys\(^{319}\)) of RhoA, which enhances GDP release from RhoA and thus modulates their activity (Brandes et al., 2005). Indeed, we found peroxynitrite formation as measured by the thiobarbituric acid reactive substances assay, was significantly increased in blood from aged mice (young vs. aged, 0.28 ± 0.01 vs. 0.33 ± 0.01 μM MDA, \(P < 0.05\)), which is consistent with a previous publication (van der Loo et al., 2000). Taken together, ArgII upregulation in aged aorta may depend on the increase of peroxynitrite formation and RhoA activation. Although ArgI activity was upregulated by translational modification such as S-nitrosylation, ArgII was not modified by such modification (data not shown). Therefore, further study needs to find a mechanism associated with ArgII upregulation.

Vascular ROS production is enhanced in aged blood vessels (Abe and Berk, 1998; van der Loo et al., 2000; Ungvari et al., 2004; Brandes et al., 2005). There are four enzymatic systems that contribute to increased ROS production in various pathophysiological states: xanthine oxidase, NADPH oxidase, eNOS, and the mitochondrial electron transport chain (Cai and Harrison, 2000). Although eNOS normally produces the vasoprotectant molecule NO, it can also produce O\(_2^-\) in the absence of either L-arginine or BH\(_4\). This phenomenon has been referred to as eNOS uncoupling (Stuehr et al., 2001; Landmesser et al., 2003). During aging, eNOS uncoupling plays a key role in increased ROS production. Our data that increased ROS production in the endothelium of aging mice was prevented with preincubation of the eNOS inhibitor L-NAME is consistent with previous observations (Kim et al., 2009). Furthermore, the ratio of eNOS dimer to monomer in aged mice was significantly decreased compared with that in young. Together, these data suggest that eNOS uncoupling is an important contributor to age-related endothelial dysfunction.

Increased expression of eNOS may depend on shear stress and hemodynamic forces (Sessa et al., 1994; Ranjan et al., 1995) because of the presence of a shear stress-responsive element in the promoter region of the eNOS gene (Nishida et al., 1992). Consistent with the demonstration, eNOS...
expression was increased in aorta (Cernadas et al., 1998; van der Loo et al., 2000) and not changed in artery (Yang et al., 2009) and decreased in arteriole (Csizsar et al., 2002). Furthermore, increased expression of eNOS protein in aged aorta may be one of the compensatory mechanisms to counterbalance endothelial dysfunction by increased arginase activity. There are a number of potential reasons why eNOS might be uncoupled and lead to increased ROS production in aging (Stuehr et al., 2001). Oxidative stress may damage and deplete BH4 and contribute to eNOS uncoupling (Milstien et al., 1999). Limited L-arg availability also promotes eNOS uncoupling (Boger et al., 1995). Consistent with this concept, increased ArgII activity was associated with decreased intracellular L-arg and ArgII inhibition restored L-arg concentration in aortic vessels (Figure 5). We found that L-arg concentration was 66.6 μmol/mg protein in young mice and 51.9 μmol/mg proteins in aged. Closs and colleagues (Closs et al., 2000; Simon et al., 2003) have demonstrated that endothelial cells contain two pools of L-arg. Pool I is regulated by the cationic transporter and can be depleted by cationic amino acid L-lysine and pool II, which is further subdivided into IIA and IIB, is accessible to eNOS but is not freely exchangeable with extracellular L-lysine or L-arg. Mitochondrial ArgII utilizes pool IIB, which might be influenced by arginase and could modulate the local concentration of L-arg available to eNOS. Although our study did not distinguish among the L-arg pools, we demonstrated that arginase activity is involved in regulating intracellular L-arg concentration. This relationship between arginase activity and L-arg concentration was previously described in mouse plasma (Erdely et al., 2010).

In summary, we demonstrated that ArgII is upregulated in aortic vessels of aged mice and plays a role in regulating NO production through eNOS uncoupling. Incubation with siArgII inhibits arginase activity, enhances NO generation, attenuates ROS production, and improves endothelial function by augmenting eNOS coupling via increased bioavailability of L-arg. Thus ArgII may be a valuable target in age-related vascular diseases.

Methods

Materials
MnTBAP (Mn (III)tetrakis (4-benzoic acid) porphyrin Chloride) and L-NAME (N²-nitro-L-arginine methyl ester), were obtained from Calbiochem. All other reagents were purchased from Sigma unless otherwise stated.

Animals
Young (10 ± 3 weeks) and aged (45 ± 5 weeks) mice (C57BL/6J) were used for all experiments. Mice were housed at 23°C under a 12-h light/12-h dark cycle. The dark period was from 10:00 to 22:00. All animals had access ad libitum to water and food (Nara Biotech.). The study protocols were in accordance with the Guide for the Care and Use of Laboratory Animals (Institutional Review Board, Kangwon National University).

siRNA incubation
Thoracic aortas were dissected from euthanized mice, cut into 1.5-mm rings, and incubated overnight in Dulbecco’s modified Eagle’s medium (DMEM) containing siArgI and/or siArgII (100 nM each, Santa Cruz Biotechnology, sc-29727 and sc-29730, respectively).

RT-PCR
Total RNA from aortic vessels was prepared with TRIzol reagent according to the manufacturer’s protocol (Gibco). RT-PCR was performed as described previously (Ryoo et al., 2006). PCR-primer sequences of ArgI, ArgII, and GAPDH (Bioneer) are as follows: ArgI forward 5'-gga aga gac ctt cag cta cct gct gg-3', reverse 5'-ccc agt tca cag tac tct tca cc-3', 251-bp PCR-product; ArgII forward 5'-ggc caa tcc acc tga gtt ttg-3', reverse 5'-ctg acc aaa act tga aag cac cac atc c-3', 251-bp PCR product; GAPDH forward 5'-agg ccc gtc gtt cag agt tc-3', reverse 5'-ttg ctc ctt cac cacc ctt ct-3', 530-bp PCR-product.

Arginase activity assay
Aortic lysates were prepared by homogenization in lysis buffer (50 mM Tris-HCl, pH7.5, 0.1 mM ethylenediaminetetraacetic acid [EDTA] and protease inhibitors) at 4°C followed by centrifugation for 20 min at 14,000 × g at 4°C. The supernatants were used to assay arginase activity as previously described (Ryoo et al., 2006).

Western blotting and eNOS dimerization analysis
Aortic segments were homogenized in the buffer (50 mM Tris-HCl, 150 mM NaCl, 1% Nonidet P-40, 1 mM EDTA, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 μg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, and 1 mM sodium fluoride) and centrifuged for 30 min at 14,000 × g. Supernatant protein concentrations were analyzed by the Bradford method. Protein samples (100 μg) were separated using sodium dodecyl sulfate polyacrylamide gel electrophoresis (10% gels) and transferred to nitrocellulose membranes (Bio-Rad). The blots were incubated with primary antibodies: monoclonal anti-ArgI (Santa Cruz Biotechnology), anti-ArgII (Santa Cruz Biotechnology), anti-eNOS (BD Biosciences), or anti-β-tubulin (BD Biosciences) antibodies, followed by an appropriate secondary antibody (Amersham). The signals were detected using an enhanced chemiluminescence detection reagent and X-ray films. eNOS dimers and monomers were separated using low-temperature SDS-PAGE as previously described (Woo et al., 2010).
Estimation of NO or ROS generation using DAF-FM or DHE in isolated mice aorta

Mice aortic rings were isolated and incubated overnight at 37°C under 5% CO₂ in DMEM containing 2% fetal bovine serum and antibiotics (1×) in the presence or absence of siRNA (Ryoo et al., 2006). The fluorescence from the aortic endothelium was measured at different time intervals under microscopy (Woo et al., 2010).

Aortic vascular tension assay

Mice were anesthetized using isoflurane, and their thoracic aortas were rapidly removed, placed on ice-caged Krebs-Ringer bicarbonate solution (in mM: NaCl 118.3, KCl 4.7, MgSO₄ 1.2, KH₂PO₄ 1.2, CaCl₂ 1.6, NaHCO₃ 25, glucose 11.1), and cleared of adherent connective tissues. Individual mouse aortas were cut into 2.5-mm rings and suspended between two wire stirrups (150 μm) in a myograph (Multi myograph system DMT-620) in 10 ml Krebs-ringer (95% O₂ - 5% CO₂, pH 7.4, 37°C). One stirrup was connected to a three-dimensional micromanipulator, and the other was attached to a force transducer. The rings were passively stretched at 10-min intervals in increments of 200 mg to reach optimal tone (600 mg). After the arterial rings had been stretched to their optimal resting tone, the contractile response to 100 mM KCl was used to normalize the responses to agonist across vessel rings. Dose responses to the vasoconstrictor U46619 (10⁻¹⁰ - 10⁻⁶ M) were performed, followed by responses to the vasodilators acetylcholine (Ach, 10⁻⁹ - 10⁻⁵ M) and sodium nitroprusside (SNP, 10⁻⁹ - 10⁻⁵ M) were performed after preconstriction with U46619 (10⁻⁸ M). At the end of the experiments, NO-dependent vasorelaxation activity was confirmed by adding the guanylate cyclase inhibitor ODQ (1H-[1,2,4]oxadizolo[4,3-a]quinoxalin-1-one, 10⁻⁵ M). Data were collected online using a MacLab system and analyzed using Dose Response Software (AD Instruments).

Determination of intracellular L-arginine concentrations

Intracellular L-arg concentration was determined by HPLC using pre-column derivatization with o-phthalaldehyde (OPA) according to a modified version of a previously published method (Boger et al., 1995). L-arginine (100 μmol/L) was added to cells lysate (0.5 ml) as an internal standard. The samples were extracted on solid-phase extraction cartridges (CBA Bond Elut, Varian). Recovery rates were 87.5 ± 3.9%. Eluates were dried over nitrogen and resuspended in double-distilled water for HPLC analysis. HPLC was performed on a computer-controlled Waters chromatography system (M600E) consisting of an automatic injector (M7725i, Waters Co.) and a fluorescence detector (FP-1520, JASCO) in the Central Laboratory of Kangwon National University. Samples were incubated for exactly 1 min with OPA reagent (5.4 mg/ml OPA in borate buffer, pH 8.4, containing 0.4% 2-mercaptoethanol) before automatic injection into the HPLC. The OPA derivative of L-arginine was separated on a 150 × 4.6 mm - 3.5 μm Zorbax Eclipse XDB-C18 column with the fluorescence detector set at 340 nm (excitation) and 450 nm (emission). Samples were eluted from the column with 0.96% citric acid/methanol (70:30), pH 6.8, at a flow rate of 1.5 ml/min.

Statistics

All data are represented as mean ± standard deviation of at least four independent experiments. Unpaired Student’s t-tests were used to determine significant differences. A value of P < 0.05 was accepted as significant.

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