



Perspective

Palmitoylethanolamide and Polydatin combination reduces inflammation and oxidative stress in vascular injury



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ABSTRACT

Acute and chronic inflammation responses are important risk factors for vascular remodeling processes such as in atherosclerosis, arteriosclerosis and restenosis. Inflammation and oxidative stress in the intimal region after vascular damage are a key event in the development of neointimal hyperplasia. In this study, we used this model of vascular damage, which involves the complete ligation of the left carotid artery for 14 days, to observe the role of N-palmitoylethanolamine in combination with Polydatin at the dose of 30 mg/kg, on regulation of inflammatory process, and oxidative stress. Palmitoylethanolamide (PEA), an endogenous fatty acid amide belonging to the N-acylethanolamine family, has anti-inflammatory and neuroprotective effects. However, PEA lacks direct capacity to prevent formation of free radicals. Polydatin (PLD) that is a natural precursor of resveratrol has antioxidant activity. Thus, the combination of PEA and PLD could have beneficial effects on inflammatory process and oxidative stress. This model shows that 14 days after carotid artery ligation there is a significant structural change within the vessel, and that there is an important involvement of the inflammatory pathway in the progression of this disease. In this study we demonstrated that PEA/PLD combination treatment reduces vessel damage, adhesion molecules expression such as intercellular adhesion molecules-1 (ICAM-1) and vascular cell adhesion molecules-1 (V-CAM), proinflammatory cytokines production (Tumor Necrosis Factor alpha (TNF- α) and Interleukin 1 beta (IL-1 β), the inducible nitric oxide synthase (iNOS) and Poly (ADP-ribose) polymerase (PAR), formation, Nuclear factor kappa-B expression and apoptosis (BAX, Fas-Ligand) activation. Our results clearly demonstrated that treatment with PEA/PLD 30 mg/Kg is able to reduce vascular damage and attenuates the inflammatory process.

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Abbreviations: VSMCs, vascular smooth muscle cells; ROS, reactive oxygen species; I-CAM, intercellular adhesion molecules-1; V-CAM, vascular cell adhesion molecules-1; TNF- α , tumor necrosis factor alpha; IL-1 β , Interleukin 1 beta; iNOS, inducible nitric oxide synthase; PAR, poly (ADP-ribose) polymerase; NF-kB, nuclear factor kappa-B; I κ B- α , kappa-B inhibitor alpha; α -sma, alpha smooth muscle actin; MCP-1, monocyte chemoattractant protein-1; BrdU, bromodeoxyuridine; Fas-L, fas-ligand; PEA, palmitoylethanolamide; PLD, polydatin; SHAM, sham-operated animals served as controls.

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1. Introduction

Vascular remodeling diseases are responsible for several important adverse vascular events, such as restenosis, hypertension, arteriosclerosis and atherosclerosis, consist of the growth and migration of vascular smooth muscle cells (VSMCs), proliferation of endothelial cells, and activation of macrophages, that are rapidly attracted to sites of disturbed flow, characterized by low-grade inflammation [1]. This histological remodeling causes an increased in the media wall thickness and neointimal formation. In addition to the events described above, inflammation may play a critical role in the development of vascular remodeling as in the case of, restenosis after angioplasty, and bypass graft failure [2]. The vascular wall is an integrated functional component that is continually remodeling or response to hemodynamic or biomechanical stress. The complete

ligation of the vessel near the carotid bifurcation, and the consequent cessation of blood flow, can produce flow-induced vascular re-modeling with neointimal formation in the mouse carotid artery [3]. The reduction in shear stress induces a proinflammatory phenotype and increases endothelial cell apoptosis and proliferation [4]. Moreover, oxidative stress has also been shown to play a key role in vascular damage. Reactive oxygen species (ROS) have been recognized as intracellular signaling molecules that are involved in physiological repair processes after a vascular injury [5]. In VSMC, excessive and/or persistent stimulation by ROS inflicts oxidative stress and can alter the functions of VSMC, enhance proliferation and migration. These changes can contribute to the development of cardiovascular diseases, including arteriosclerosis [6].

In the present study, we used this model to observe the role of N-palmitoylethanolamine (PEA) in combination with Polydatin (PLD), on regulation of inflammatory process, and oxidative stress. N-palmitoylethanolamine is an endogenous fatty acid amide which is part of the family of the N-acylethanolamines (NAEs) [7]. PEA has an important analgesic, anti-inflammatory [8] and neuroprotective action, acting at different molecular targets such as immune cells [9]. However, PEA lacks direct antioxidant capacity to prevent formation of free radicals and to counteract damage to DNA, lipids and proteins. For this purpose, we tested the effect of PEA in combination with an important antioxidant, as Polydatin (3,4',5-trihydroxystilbene-3- β -mono-D'Glucoside) is a natural glucoside of resveratrol. PLD has been proved to have numerous biological effects, such as protective effect on some organs [10], anti-aggregation of platelet and improvement of micro-circulation [11,12], anti-inflammatory effect [13], anti-shock effect [14], and anti-oxidation effect [15]. Inhibits migration and proliferation of vascular endothelial cells and inhibits the angiogenic process via inhibition of proangiogenic factors [16]. In this study, we investigated whether and how PEA/PLD combination exerts a protective effect on carotid artery ligation-induced vascular inflammation and remodeling

2. Materials and methods

2.1. Animals

Adult mice (C57Bl/6; 25–30 g, Envigo, Italy) were placed in a controlled location with standard rodent chow and water. Animals were maintained at $22 \pm 1^\circ\text{C}$ with a 12-h light, 12-h dark cycle. The study was permitted by the University of Messina Review Board for the care of animals. All animal experiments were performed following the regulations in Italy (D.M. 116192), Europe (O.J. of E.C. L358/1 12/18/1986), USA (Animal Welfare Assurance No A5594-01, Department of Health and Human Services, USA)

2.2. Mouse carotid ligation model: surgical procedures

The carotid artery ligation model was performed as previously described [17]. The animals were anesthetized by sevoflurane inhalation, the left common carotid artery was exposed through a small midline incision in the neck and completely ligated near the carotid bifurcation. All animals recovered and showed no symptoms of a stroke. Immediately after ligation to simulate a clinical situation PEA/PLD at a dose of 30 mg/Kg was administered orally by gavage. The treatment was repeated daily, for 14 days after surgery. At the end of 14 days, the left common carotid artery entire ligation was excised and the samples treated for histological and biochemical studies. The PEA/PLD (30 mg/kg) dose was chosen on the basis of previous *in vivo* studies [18,19].

2.3. Experimental groups

Mice were casually divided into the following experimental groups (n = 10 for each group)

Group 1: vehicle group; mice were subjected to carotid artery occlusion as described above, and vehicle carboxymethylcellulose (1.5% w/v in saline) was administered orally by gavage, for 14 days.

Group 2: PEA/PLD (30 mg/kg); mice were subjected to carotid artery occlusion, and PEA/PLD was administered by gavage, one hour after surgery and daily for 14 days.

Group 3: sham + vehicle group; sham-operated animals served as controls is abbreviated as Sham, mice were subjected to identical surgical procedures except for carotid artery occlusion and were administered with vehicle solution carboxymethyl cellulose, by gavage for 14 days.

Group 4: sham + PEA/PLD (30 mg/kg); mice were subjected to identical surgical procedures except for carotid artery occlusion and were administered with PEA/PLD in carboxymethyl cellulose, by gavage for 14 days.

Mice were sacrificed at 14 days after surgical procedures. The PEA/PLD (30 mg/kg) dose was chosen on the basis of previous *in vivo* studies, and the ratio between PEA/PLD in the formulation is 10:1 [18,19].

2.4. Histological analysis

At the end of the 14 days, histological examination and toluidine blue staining, was performed as indicated in our previous studies [18]. Afterwards, special stains were applied to highlight the presence of collagen into tissue, with Mallory trichrome stain. Explant tissue sections were stained with Mallory trichrome according to the manufacturer's protocol (Bio-Optica Italy, Milan).

2.5. Immunohistochemical analysis

Immunohistochemical analysis was performed as described previously [18]. Subsequently, the sections were incubated overnight with: anti-TNF- α antibody (1/100, sc-52746), or anti-IL-1 β antibody (1/100, sc-7884), anti-ICAM-1 antibody (1/100, sc-8439), anti-PAR antibody (1/100, sc-7150), anti-i-NOS antibody (1/100, sc-8310), anti Bromodeoxyuridine (BrdU) antibody (1/100, sc-32323), anti Bax antibody (1/100, sc-526), anti Fas-Ligand (Fas-L) antibody (1/100, sc-834), anti VCAM antibody (1/100, sc-1504). Sections were rinsed with PBS and incubated with peroxidase-conjugated bovine anti-mouse IgG, secondary antibody or peroxidase-conjugated goat anti-rabbit IgG (1:2000 Jackson Immuno Research, West Grove, USA). Specific labeling was detected with a biotin-conjugated goat anti-rabbit IgG or biotin conjugated goat anti-mouse IgG and avidin-biotin peroxidase complex (Vector Laboratories, USA). Immunohistochemical images were collected using a Zeiss microscope and Axio Vision software. For graphic display of densitometric analyses, the intensity of positive staining (brown staining) was measured by computer-assisted color image analysis (Leica QWin V3, UK). The percentage area of immunoreactivity (determined by the number of positive pixels) was expressed as percent of total tissue area (red staining).

2.6. Western blot analysis

Western blots were performed as described from our previous studies [18]. Specific primary antibody: anti-Ik β - α (1:1000; sc-371), anti-MCP-1 (1:500, sc-28879), anti- α -sma (1:1000, sc-53015), or anti-NF- κ B p65 (1:1000; sc-109) were mixed in 1x PBS, 5% w/v nonfat dried milk, 0.1% Tween-20, and incubated at 4°C , overnight. After, membranes were incubated with peroxidase-conjugated bovine anti-mouse IgG secondary antibody

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