



Original article

Oleanolic acid modulates the immune-inflammatory response in mice with experimental autoimmune myocarditis and protects from cardiac injury. Therapeutic implications for the human disease

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ABSTRACT

Myocarditis and dilated cardiomyopathy (DCM) are inflammatory diseases of the myocardium, for which appropriate treatment remains a major clinical challenge. Oleanolic acid (OA), a natural triterpene widely distributed in food and medicinal plants, possesses a large range of biological effects with beneficial properties for health and disease prevention. Several experimental approaches have shown its cardioprotective actions, and OA has recently been proven effective for treating Th1 cell-mediated inflammatory diseases; however, its effect on inflammatory heart disorders, including myocarditis, has not yet been addressed. Therefore, the present study was undertaken to determine the effectiveness of OA in prevention and treatment of experimental autoimmune myocarditis (EAM). The utility of OA was evaluated *in vivo* through their administration to cardiac α -myosin (MyHc- $\alpha_{614-629}$)-immunized BALB/c mice from day 0 or day 21 post-immunization to the end of the experiment, and *in vitro* through their addition to stimulated-cardiac cells. Prophylactic and therapeutic administration of OA dramatically decreased disease severity: the heart weight/body weight ratio as well as plasma levels of brain natriuretic peptide and myosin-specific autoantibodies production were significantly reduced in OA-treated EAM animals, compared with untreated ones. Histological heart analysis showed that OA-treatment diminished cell infiltration, fibrosis and dystrophic calcifications. OA also decreased proliferation of cardiac fibroblast *in vitro* and attenuated calcium and collagen deposition induced by relevant cytokines of active myocarditis. Furthermore, in OA-treated EAM mice the number of Treg cells and the production of IL-10 and IL-35 were markedly increased, while proinflammatory and profibrotic cytokines were significantly reduced. We demonstrate that OA ameliorates both developing and established EAM by promoting an antiinflammatory cytokine profile and by interfering with the generation of cardiac-specific autoantibodies, as well as through direct protective effects on cardiac cells. Therefore, we envision this natural product as novel helpful tool for intervention in inflammatory cardiomyopathies including myocarditis.

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1. Introduction

Myocarditis, an inflammatory disease of the heart, is a precursor of dilated cardiomyopathy (DCM) and represents the most common cause of chronic heart failure, or even sudden death, in people younger than 40 years of age [1–3]. The pathogenesis of myocarditis is not fully understood but there is substantial evidence suggesting that the

autoimmune response to heart antigens, particularly cardiac myosin, after viral infection may contribute to the disease process [4,5]. Interestingly, patients with heart-specific autoantibodies but no evidence of viral genome persistence show improvement in their cardiac function on immunosuppressive therapy, thus pointing to autoimmunity as a key factor in the pathogenesis of postinflammatory cardiomyopathy [6,7].

Experimental autoimmune myocarditis (EAM) is an animal model for human fulminant myocarditis in the acute phase, which develops into postmyocarditic DCM in the chronic phase. EAM has been produced in susceptible rodent animals by immunization with cardiac specific peptides [8,9]. In the myosin-induced myocarditis model, pathogenic roles for macrophages, CD4⁺ T cells and autoantibodies have been described, and proinflammatory cytokines and chemokines are upregulated during the course of the autoimmune response [10].

Abbreviations: DCM, dilated cardiomyopathy; OA, oleanolic acid; EAM, experimental autoimmune myocarditis; E&H, Eosin–Hematoxylin; AR, Alizarin Red; pSR, picro-Sirius Red; CK-MB, Creatine kinase MB; BNP, brain natriuretic peptide; CF, cardiac fibroblast.

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At present, therapeutic strategies in myocarditis have focussed on the different stages of the disease, targeting the elimination of the infecting agent, inhibition of the heart-specific autoimmune response, as well as reduction of inflammation and inflammatory-associated tissue remodeling. Toxicity, limited efficacy and expense, however, are serious disadvantages of the new anti-inflammatory and/or immunosuppressive agents. Hence, the identification of novel molecules with low toxicity is a very useful challenge for the treatment of myocarditis and DCM.

Along these lines, there is considerable interest in bioactive compounds present in edible plants and thus in “letting your food be your medicine”, as recommended by Hippocrates. Triterpenoids stand out amongst these bioactive plant constituents that are being actively studied for their beneficial effects on human health. Oleanolic acid (OA) is a naturally occurring triterpene that possesses numerous biological activities and displays many pharmacological properties with therapeutic potential [11]. OA is widely present in edible and medicinal plants, being an integral part of the human diet, and is extensively used in Asian medicine, usually in the form of herbal extracts [12]. Among other plant species, OA can be extracted from olive leaves and roots (*Olea Europaea*), a plant that has been widely utilized in folk medicine of African and European Mediterranean countries. In addition, it is present in grapes and olives, a substantial component of the Mediterranean diet, and particularly considerable amounts are found in grape- and olive pomace. So, to fully characterize their health-related properties, it could be both interesting for drug development and clinical studies, as well as for nutraceutical studies. At present, OA, as well as herbal extracts containing OA, are well known for their anti-inflammatory, immunomodulatory and antioxidative properties, and its effectiveness in the treatment of some pathological conditions has been extensively documented [12–18]. However, the potential beneficial actions of OA on cardiac damage, in the context of inflammatory heart disease, remain speculative and have not been addressed. In the present study, we tested the hypothesis that OA can act as a potent anti-inflammatory/immunoregulatory drug in myocarditis.

2. Materials and methods

2.1. Animals and immunization

BALB/c mice from Charles River Laboratories were housed in the animal care facility at the Medical School of the University of Valladolid (UVA) and were provided food and water *ad lib*, under standard conditions. All experimental protocols were reviewed and approved by the Animal Ethics Committee of the UVA and were in accordance with the European legislation.

Disease was induced in 6–8 week-old male and female mice by immunization at days 0 and 7 with 50 µg of the murine specific α -myosin-heavy chain-derived acetylated peptide (MyHC $\alpha_{614-629}$) [Ac-SLKLMATLFSTYASAD-OH] in a 1:1 emulsion with Complete Freund's Adjuvant containing *Mycobacterium tuberculosis* H37RA (5 mg/ml) [19]. MyHC $\alpha_{614-629}$ was generated in the peptide synthesis laboratory of Dr F. Barahona (CBM, Madrid, Spain). After terminal anesthesia with xylazine/ketamine, mice were sacrificed either on day 21 or on day 65. The heart was removed from the chest, weighed and viewed using a NIKON SMZ800 stereo microscope with objective lenses 1×. Body weight was also recorded.

2.2. Treatment regimen

OA, from Extrasynthese, was first dissolved in 2% w/v dimethyl sulfoxide (DMSO) and then diluted with phosphate buffered saline (PBS) for each experiment (the final concentration of DMSO is 0.2%, w/v). The drug solution was freshly prepared each time. EAM mice were treated, at random, daily with 0.2% w/v DMSO or 50 mg/kg body weight/day of OA with intraperitoneal injection from day 0 (immunization day, OA₀) to day 21 or 65. In addition, another

group of EAM mice was treated with 50 mg/kg body weight/day of OA from day 21 after immunization (late treatment group, OA₂₁) to day 65. Control mice (without EAM induction) were also injected daily with OA for an equivalent period of time. Each experimental group included 15 animals, which were used to evaluate the different parameters mentioned below.

2.3. Histological studies

Hearts were obtained from all animals. One half was fixed in 4% paraformaldehyde and embedded in paraffin; the other half was frozen at –80 °C for protein studies. Embedded tissues were cut on a microtome (3 µm thickness) and stained with Hematoxylin–Eosin (H&E), Alizarin Red (AR) and picro-Sirius Red (pSR) (Sigma-Aldrich, St Louis, MO, USA), and examined by light microscopy to detect inflammatory infiltrates, calcium deposits, and fibrotic areas, respectively. A qualitative evaluation was performed in each specimen in order to control the morphological changes that occur along the treatment. Histological examination was performed with a Nikon Eclipse 90i microscope connected to a DS-Ri1 digital camera (Nikon Instruments Inc) (magnification ×200 and ×400). Sections from 4 to 10 segments per mouse were examined blindly by two investigators.

2.4. Creatine kinase MB (CK-MB) isoenzyme assay

The activity of the CK-MB was measured in serum with a commercial kit (Bioloab SA, France) according to the manufacturer's recommendations. Incubation of sera samples with the substrate led to an increase in the concentration of the reduced form of β -nicotinamide adenine dinucleotide phosphate (NADPH) directly proportional to the enzyme activity in the samples. Data are expressed as a rate of NADPH increase (Δ Abs/min) in seven sequential readings in a spectrophotometer (VERSAmix Microplate Reader. Molecular Devices LLC, CA, USA) at 340 nm.

2.5. Measurements of cytokines and BNP by an enzyme-linked immunosorbent assay (ELISA)

BNP levels were determined in both serum samples and culture medium of HL-1 cardiomyocytes by using a mouse BNP-specific ELISA kit (RayBiotech, Norcross, GA). For Galectin-3, IL-6, IL-10, IL-17, TNF α (eBioscience, San Diego, CA) and IL-35 (Cusabio Biotech Co, Wuhan, China) quantification, serum and supernatant from heart tissue were analyzed according to the manufacturer's protocols. Heart tissue homogenates were prepared with the apical part of the heart, homogenized in 1 ml of ice-cold PBS, supplemented with a protease inhibitor cocktail (Sigma-Aldrich, St Louis, MO). Samples were centrifuged at 800 ×g for 15 min at 4 °C. Aliquots of supernatant were stored at –80 °C until cytokine assays were performed. Total protein concentration in the supernatants was determined by using the Bradford method with bovine serum albumin (BSA) as standard. Data were processed and expressed as concentration of cytokine/mg of tissue or concentration of cytokine/ml for serum samples.

2.6. Detection of auto-antibodies

Anti-MyHC α -specific IgM and IgG isotypes were detected in serum samples using the ELISA technique. In brief, 96-well microtiter plates were coated with 0.5 mg/well of MyHC $\alpha_{614-629}$ peptide diluted in PBS overnight, followed by washing with PBS and blocking for 2 h with 5% BSA in PBS. The wells were then incubated in duplicate with the serum samples diluted 1:60 in PBS for 3 h at room temperature. After washing, HRP-labeled rat anti-mouse IgM (1:1000), anti-mouse IgG, IgG1 and IgG2a (1:2000) from Serotec (Sigma-Aldrich, St Louis, MO) were added for 1 h. After another washing, adding the substrate, and

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