Oleuropein Aglycone, an Olive Oil Compound, Ameliorates Development of Arthritis Caused by Injection of Collagen Type II in Mice

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ABSTRACT

The aim of this study was to investigate the effect of oleuropein aglycone, an olive oil compound, on the modulation of the inflammatory response in mice subjected to collagen-induced arthritis (CIA). CIA was induced in mice by an intradermal injection of 100 μl of an emulsion containing 100 μg of bovine type II collagen (CII) and complete Freund’s adjuvant (CFA) at the base of the tail. On day 21, a second injection of CII in CFA was administered. Mice developed erosive hind paw arthritis when immunized with CII in CFA. Macroscopic clinical evidence of CIA first appeared as periarticular erythema and edema in the hind paws. The incidence of CIA was 100% by day 28 in the CII-challenged mice and the severity of CIA progressed over a 35-day period with resorption of bone. The histopathology of CIA included erosion of the cartilage at the joint. Treatment with oleuropein aglycone starting at the onset of arthritis (day 25) ameliorated the clinical signs at days 26 to 35 and improved histological status in the joint and paw. The degree of oxidative and nitrosative damage was also significantly reduced in oleuropein aglycone-treated mice. Plasma levels of the proinflammatory cytokines were also significantly reduced by oleuropein aglycone. In addition, we have confirmed the beneficial effects of oleuropein aglycone on an experimental model of CIA in a therapeutic regimen of post-treatment, with treatment started at day 28, demonstrating that oleuropein aglycone exerts an anti-inflammatory effect during chronic inflammation and ameliorates the tissue damage associated with CIA.

Introduction

Reactive oxygen species (ROS) have been considered as risk and enhancer factors for autoimmune diseases (Filippin et al., 2008) because there is a significant relation between oxidative stress and such diseases (Avalos et al., 2007; Filippin et al., 2008). Rheumatoid arthritis (RA) is an autoimmune disease characterized by the sequestration of various leukocyte subpopulations within both the developing pannus and synovial space. The chronic nature of this disease results in multiple joint inflammation with subsequent destruction of joint cartilage and erosion of bone. Although distribution of this disease is worldwide, its pathogenesis is not clearly understood (Harris, 1990). Type II collagen-induced arthritis (CIA) in the mouse has proven to be a useful model of RA, because it possesses many of the cell and humoral immunity characteristics found in human RA (Holmdahl et al., 1990). The pathogenesis of CIA is dependent on the host’s response to type II collagen challenge and the subsequent generation of antibodies that recognize collagen-rich joint tissue (Holmdahl et al., 1990). The chronic activities initiated by immune...
complexes trigger a variety of cell-mediated and humoral events. Moreover, the recruitment and activation of neutrophils, macrophages, and lymphocytes in joint tissues and the formation of the pannus are hallmarks of the pathogenesis of both CIA and human RA. It has been demonstrated that interleukin (IL)-8, macrophage inflammatory protein (MIP)-1α, MIP-1β, and regulated on activation normal T cell expressed and secreted are differentially chemotactic for lymphocyte subsets (Taub et al., 1993). Chemokines may play prominent roles in RA, because neutrophil and mononuclear cell stimulation and activation are prevalent in this disease. Concomitant with inflammation is the generation of ROS (Trichopoulou et al., 2003), which increase oxidation of proteins and lipids, resulting in signals that trigger more inflammation. Olive oil is an integral ingredient of the traditional Mediterranean diet, and several studies attribute many of the healthy advantages of this diet to olive oil’s unique characteristics (Menendez et al., 2007). Different studies have also shown that the consumption of olive oil has a potential protective effect against several malignancies, especially cancers (Menendez et al., 2007). The major constituent of the leaves and unprocessed olive drupes of *Olea europaea* is oleuropein and the majority of polyphenols found in olive oil or table olives are derived from its hydrolysis. Oleuropein has high antioxidant activity in vitro, comparable to that of a hydrosoluble analog of tocopherol (Kremastinos, 2008). Oleuropein scavenges superoxide anions and hydroxyl radicals and inhibits the respiratory burst of neutrophils and hypochlorous acid-derived radicals (Visioli et al., 1998). In addition to their antioxidant properties, polyphenolic compounds have been shown to exhibit a range of indirect actions that may be beneficial to health, including the inhibition of enzymes involved in the inflammatory process (Kohyama et al., 1997), the inhibition of platelet aggregation (Petroni et al., 1995), and the inhibition of the metabolic activation of procarcinogens (Stavric, 1994). The present study demonstrated that oleuropein aglycone, a hydrolysis product obtained from oleuropein by the action of β-glucosidase on the parent glucoside (Walter et al., 1973) ameliorates development of arthritis caused by injection of collagen type II (CII) in mice. We have evaluated the following endpoints of the inflammatory process: 1) clinical score, 2) body weight, 3) inducible nitric-oxide synthase (iNOS) and cyclooxygenase (COX)-2 expression and the serum levels of prostaglandin E₂ (PGE₂), 4) nitrotyrosine formation and poly(ADP-ribose) (PAR) expression in the joint tissues, 5) cytokine and chemokine production, 6) neutrophil infiltration in the joint tissues, and 7) joint histopathology.

**Materials and Methods**

**Animals.** Male DBA/1J mice (9 weeks; Harlan Nossan, Milan, Italy) were used for these studies. Mice were housed in individual cages (two for each group) and maintained under a 12-h light/dark cycle at 21 ± 1°C and 50 ± 5% humidity. The animals were acclimated to their environment for 1 week and had ad libitum access to tap water and a standard rodent diet. All animal experiments complied with regulations in Italy (Ministerial Decree 116192), Europe (Official Journal of Ethical Committee L 358/1 12/19/1986), and the United States (Animal Welfare Assurance No. A5594-01, U.S. Department of Health and Human Services). All behavioral testing was conducted in compliance with the National Institutes of Health laboratory animal care guidelines (Institute of Laboratory Animal Research, 1996) and with protocols approved by the institutional animal care and use committee (Council Directive No. 87-848, October 19, 1987, Ministère de l’Agriculture et de la Forêt, Service Vétérinaire de la Santé et de la Protection Animale, permission no. 92-256 to S.C.). The study was approved by the University of Messina review board for the care of animals (PRIN ID 1042).

**Experimental Groups.** Mice were divided into the following six experimental groups:

1. **CIA-control.** Mice were subjected to collagen-induced arthritis (as described below) and administered 200 μl of 10% ethanol solution (intraperitoneally, vehicle for oleuropein aglycone) every 24 h, starting from day 25 to day 35 (n = 20).
2. **CIA-oleuropein aglycone (pretreatment I).** Mice subjected to collagen-induced arthritis (as described below) were administered oleuropein aglycone (molecular mass 394 kDa; 40 μg/kg, 10% ethanol i.p.) every 24 h, starting from day 25 to day 35 (n = 20).
3. **CIA-oleuropein aglycone (pretreatment II).** Mice subjected to collagen-induced arthritis (as described below) were administered oleuropein aglycone (molecular mass 394 kDa; 20 μg/kg, 10% ethanol i.p.) every 24 h, starting from day 25 to day 35 (n = 20).
4. **CIA-oleuropein aglycone (post-treatment).** Mice subjected to collagen-induced arthritis (as described below) were administered oleuropein aglycone (molecular mass 394 kDa; 40 μg/kg, 10% ethanol i.p.) every 24 h, starting from day 28 to day 35 (n = 20).
5. **Sham-control.** Mice subjected to an intradermal injection at the base of the tail of 10 μl of 0.01 M acetic acid instead of the emulsion containing 100 μg of CII were treated with 200 μl of 10% ethanol solution (i.p., vehicle for oleuropein aglycone), every 24 h starting from day 25 to day 35 (n = 20).
6. **Sham-oleuropein aglycone.** Mice subjected to an intradermal injection at the base of the tail of 100 μl of 0.01 M acetic acid instead of the emulsion containing 100 μg of CII were administered oleuropein aglycone (40 μg/kg, 10% ethanol i.p.), every 24 h starting from day 25 to day 35 (n = 20).

The dose and the route of oleuropein aglycone administration used here to reduce joint injury were chosen on the basis of previous studies (Procopio et al., 2009; Impellizzeri et al., 2011).

**Induction of CIA.** Chicken type II collagen was dissolved in 0.01 M acetic acid at a concentration of 2 mg/ml by stirring overnight at 4°C. Dissolved CII was frozen at −70°C until use. Complete Freund’s adjuvant (CFA) was prepared by the addition of *Mycobacterium tuberculosis* H37Ra at a concentration of 2 mg/ml. Before injection, dissolved CII was reconstituted in 0.01 M acetic acid (as described below) were administered oleuropein aglycone (molecular mass 394 kDa; 40 μg/kg, 10% ethanol i.p.) every 24 h starting from day 25 to day 35 (n = 20).

**Clinical Assessment of CIA.** The development of arthritis in mice in all experimental groups was evaluated daily starting from day 20 after the first intradermal injection by using a macroscopic scoring system: 0, no signs of arthritis; 1, swelling and/or redness of the paw or one digit; 2, two joints involved; 3, more than two joints involved; and 4, severe arthritis of the entire paw and digits (Szabó et al., 1998). The arthritic index for each mouse was calculated by adding the four scores of individual paws. Clinical severity was also determined by quantitating the change in the paw volume using plethysmometry (model 7140; Ugo Basile, Comerio, Italy).

**Histological Examination.** On day 35, animals were sacrificed while they were under anesthesia (sodium pentobarbital, 45 mg/kg i.p.), and paws and knees were removed and fixed in 10% formalin. The paws were trimmed and decalcified in EDTA for 7 to 8 days as described previously (Cuzzocrea et al., 2003a) and, after being dehydrated in different graded alcohol concentrations, were embedded in paraffin, sectioned at 5 μm, stained with hematoxylin and eosin and...
Masson’s trichrome stain, and studied using light microscopy (Leitz Dialux 22). Arthritis damage (histological damage score) was evaluated and scored by an investigator blinded for the treatment regimen. The following morphological criteria were considered: score 0, no damage; score 1, edema; score 2, inflammatory cell presence; and score 3, bone resorption.

**Immunohistochemical Localization of Nitrotyrosine, PAR, iNOS, and COX-2.** On day 35, the joints were trimmed and placed in a decalcifying solution of EDTA for 7 to 8 days. Then 8-μm sections were prepared from paraffin-embedded tissues. After deparaffinization, endogenous peroxidase was quenched with 0.3% H₂O₂ in 60% methanol for 30 min. The sections were permeabilized with 0.1% Triton X-100 in PBS for 20 min. Nonspecific adsorption was minimized by incubating the section in 2% normal goat serum in phosphate-buffered saline for 20 min. Endogenous biotin- or avidin-binding sites were blocked by sequential incubation for 15 min with avidin and biotin. Sections were incubated overnight with 1) anti-rabbit polyclonal antibody directed at iNOS (1:1000 in PBS, v/v) (DBA, Milan, Italy) or 2) anti-COX-2 goat polyclonal antibody (1:500 in PBS, v/v) or 3) anti-nitrotyrosine rabbit polyclonal antibody (1:1000 in PBS, v/v) or 4) with anti-PAR goat polyclonal antibody rat (1:500 in PBS, v/v). Controls included buffer alone or nonspecific purified rabbit IgG. Specific labeling was detected with a biotin-conjugated goat anti-rabbit IgG (for nitrotyrosine, iNOS, PAR, and COX-2) and avidin-biotin peroxidase complex. To confirm that the immunoreaction for the nitrotyrosine was specific, some sections were also incubated with the primary antibody (anti-nitrotyrosine) in the presence of excess nitrotyrosine (10 mM) to verify the binding specificity. To verify the binding specificity for PAR, COX-2, and iNOS, some sections were also incubated with only the primary antibody (no secondary antibody) or with only the secondary antibody (no primary antibody). In these situations, no positive staining was found in the sections, indicating that the immunoreaction was positive in all the experiments performed. Immunocytochemistry photographs (n = 5) were assessed by densitometry by using Optilab Graffek software on a Macintosh personal computer (Cuzzocrea et al., 2003b).

**Measurement of PGE₂ in the Serum.** The amount of PGE₂ present in the serum was measured with an ELISA kit (R&D Systems, Milan, Italy). Blood serum samples were diluted 5 to 10 times in assay diluents (BioFX, Milan, Italy), and 100 μl of the dilution were added in 96-well plates. No special treatment was necessary for standard series. The rest of the process followed the kit provider’s protocol.

![Fig. 1. Effect of oleuropein aglycone (OLE AGLYCONE) on the clinical expression of CIA and on body weight. A, no clinical signs were observed in sham mice. CIA developed rapidly in mice immunized with CII and clinical signs such as periarticular erythema and edema (B) were seen with a 100% incidence of CIA at day 28 (D). E, hind paw erythema and swelling increased in frequency and severity in a time-dependent mode. CIA-oleuropein aglycone mice demonstrated a significant reduction in the clinical signs of CIA (C), leading to a decrease in the incidence of arthritis in a dose-dependent manner (D). Swelling of hind paws (F) over time was measured at 2-day intervals. G, beginning on day 25, the CII-challenged mice gained significantly less weight and this trend continued through day 35. CIA-oleuropein aglycone mice demonstrated a significant reduced incidence of weight loss (G) as well as less paw edema in a dose-dependent manner (F). The figure is representative of all the animals in each group. Values are means ± S.E.M. of 20 animals for each group. **, P < 0.01 versus sham-control; *, P < 0.01 versus CIA.**
Measurement of Cytokines. TNF-α, IL-1β, and IL-6 levels were evaluated in the plasma from CIA and sham mice as described previously (Cuzzocrea et al., 2003b). The assay was performed using a colorimetric commercial ELISA kit (Calbiochem-Novabiochem Corporation, Milan, Italy) with a lower detection limit of 10 pg/ml.

Measurement of Chemokines. Levels of chemokines MIP-1α and MIP-2 were measured in the aqueous joint extracts. In brief, joint tissues were prepared by first removing the skin and separating the limb below the ankle joint. Joint tissues were homogenized on ice in 3 ml of lysis buffer (PBS containing 2 mM phenylmethylsulfonyl fluoride and 0.1 mg/ml final concentration) each of aprotinin, antipain, leupeptin, and pepstatin A) using a Polytron homogenizer (Brinkmann Instruments, Westbury, NY). The homogenized tissues were then centrifuged at 2000g for 10 min. Supernatants were sterilized with a Millipore filter (0.2 μm) and stored at −80°C until analyzed. The extracts usually contained 0.2 to 1.5 mg of protein/ml, as measured by a protein assay kit (Thermo Fisher Scientific, Waltham, MA). The levels of MIP-1α and MIP-2 were quantified using a modification of a double ligand method, as described previously (Kasama et al., 1994). In brief, flat-bottomed 96-well microtiter plates were coated with 50 μl/well of rabbit anti-cytokine antibodies (R&D Systems, Milan, Italy) (1 μg/ml in 0.6 M NaCl, 0.26 M H3BO4, and 0.08 N NaOH, pH 9.6) for 16 h at 4°C and then were washed with PBS, pH 7.5, and 0.05% Tween 20 (wash buffer). Nonspecific binding sites on microtiter plates were blocked with 2% bovine serum albumin in PBS and incubated for 90 min at 37°C. Plates were rinsed four times with wash buffer, and diluted aqueous joint samples (50 μl) were added, followed by incubation for 1 h at 37°C. After washing of plates, chromogen substrate was added. The plates were incubated at room temperature to the desired extinction, after which the reaction was terminated with 50 μl/well of 3 M H2SO4 solution. The plates were then read at 490 nm in an ELISA reader. This ELISA method consistently had a sensitivity limit of ~30 pg/ml.

Myeloperoxidase Assay. Neutrophil infiltration to the inflamed joints was indirectly quantitated using an MPO assay, as described previously for neutrophil elicitation (Mullane et al., 1985). Tissue was prepared as described above and placed in 50 mM phosphate buffer (pH = 6.0) with 5% hexadecyltrimethyl ammonium bromide (Sigma-Aldrich, Milan, Italy). Joint tissues were homogenized, sonicated, and centrifuged at 12,000g for 15 min at 4°C. Supernatants were assayed for MPO activity using a spectrophotometric reaction with O-dianisidine hydrochloride (Sigma-Aldrich) at 460 nm.

Materials. Oleuropein aglycone was obtained from the controlled hydrolysis of oleuropein extracted from olive leaves by means of the patented method reported by Procopio et al., 2009. Unless otherwise stated, other compounds were obtained from Sigma-Aldrich. All chemicals were of the highest commercial grade available. All stock solutions were prepared in nonpyrogenic saline (0.9% NaCl; Baxter Healthcare Ltd., Thetford, Norfolk, UK) or 10% ethanol (Sigma-Aldrich).

Data Analysis. All values in the figures and text are expressed as the mean ± S.E.M. of n observations. For the in vivo studies, n represents the number of animals studied. In the experiments involving histology or immunohistochemistry, the figures shown are representative of at least three experiments (histological or immunohistochemistry coloration) performed on different experimental days on the tissue sections collected from all the animals in each group. Data sets were examined by one- or two-way analysis of variance, and individual group means were then compared with a Student’s unpaired t test. For the arthritis studies, a Mann-Whitney U test (two-tailed, independent) was used to compare medians of the arthritic indices (Szabo et al., 1998). P < 0.05 was considered significant.

Results

Effect of Oleuropein Aglycone on Joint Injury during Experimental Arthritis. To imitate the clinical scenario of RA, mice were subjected to CIA. CIA developed rapidly in mice immunized with CII, and clinical signs (periarticular erythema and edema) (Fig. 1B) of the disease first

![Fig. 2. Morphological changes of CIA. Representative hematoxylin and eosin-stained section of joint was examined by light microscopy. The histological evaluation (on day 35) of joint from CIA-control mice (B and G) revealed signs of severe arthritis, with inflammatory cell infiltration and bone erosion. The histological alterations of the joint were significantly reduced in the tissues from CIA-oleuropein aglycone (OLE AGLYCONE) (40 μg/kg)-treated mice (C and G). Masson’s trichrome stain reveals decreased collagen in bone and cartilage of arthritic joint due to bone erosion and cartilage degradation in CIA-control mice (E and G). The alterations of joint were significantly reduced in oleuropein aglycone (40 μg/kg)-treated mice (F and G). There was no evidence of pathology in the sham-control mice (A, D, and G). The histological score (G) was made by an independent observer. The figure is representative of at least three experiments performed on different experimental days. Values are means ± S.E.M. of 20 animals for each group. **, P < 0.01 versus sham-control; †, P < 0.01 versus CIA.](https://jpet.aspetjournals.org/doi/10.1090/jpet/16526)
appeared in hind paws between 24 and 26 days postchallenge (Fig. 1E), leading to a 100% incidence of CIA at day 28 (Fig. 1D). Hind paw erythema and swelling increased in frequency and severity in a time-dependent mode with maximal score values of approximately 12 that can be seen in Fig. 1E from days 32 to 35 after immunization in CIA-control mice. Oleuropein aglycone treatment (40 μg/kg, 10% ethanol and 20 μg/kg, 10% ethanol) demonstrated a significant reduction of joint inflammation, as identified by a significant reduction in the incidence of arthritis in a dose-dependent manner (Fig. 1D). CIA-oleuropein aglycone mice showed a 40% reduction in the development of arthritis and a significantly lower arthritis index compared with CIA-control mice (Fig. 1E). There was no macroscopic evidence of either hind paw erythema or edema in the sham-control group mice (Fig. 1A).

The data in Fig. 1F demonstrate a time-dependent increase in hind paw volume (each value represents the mean of both hind paws). The CIA-oleuropein aglycone mice showed a significant reduction of paw edema formation in a dose-dependent manner compared with the CIA-control mice (Fig. 1F). No increase in hind paw volume over time was observed in the sham-control mice (data not shown).

The rate and the absolute gain in body weight were comparable in sham-control and in CIA-control mice in the 1st week (data not shown). From day 25, the CII-challenged mice gained significantly less weight than the sham-control mice (data not shown), and this trend continued through to day 35. Oleuropein aglycone treatment determined a significant increase of the weight gain compared with the vehicle treatment in CIA-control mice (Fig. 1G).

The histological evaluation (on day 35) of the tibiotarsal joint from CIA-control mice (Fig. 2B) revealed signs of severe arthritis, with inflammatory cell infiltration and bone erosion. The histological alterations of the tibiotarsal joint were significantly reduced in oleuropein aglycone-treated mice (40 μg/kg) (Fig. 2C). Moreover, Masson's trichrome stain reveals decreased collagen in bone and cartilage of arthritic joints due to bone erosion and cartilage degradation in CIA-control mice (Fig. 2E). The alterations of the tibiotarsal joint were significantly reduced in oleuropein aglycone-treated mice (40 μg/kg) (Fig. 2F).
There was no evidence of pathology in the sham-control mice (Fig. 2, A and D). The histological score (Fig. 2G) was determined by an independent observer.

**Effect of Oleuropein Aglycone on Chemokine and Cytokine Expression and Neutrophil Infiltration.** We initiated studies to assess the effect of oleuropein aglycone on the expression of chemokines in the aqueous joint extracts during the development of CIA. As shown in Fig. 3, A and B, the expression of MIP-1α and MIP-2, measured by ELISA, was significantly increased in the joint 35 days after CII immunization. MIP-1α and MIP-2 levels in CIA-oleuropein aglycone mice on day 35 were significantly reduced in a dose-dependent manner in comparison with those in vehicle-treated CIA-control mice. Assessment of neutrophil infiltration into the inflamed joint tissue was performed by measuring the activity of MPO. It was significantly elevated 35 days after CII immunization in vehicle-treated CIA-control mice (Fig. 3F), whereas in the CIA-oleuropein aglycone group, MPO activity was markedly reduced in a dose-dependent manner (Fig. 3F). To test whether oleuropein aglycone modulates the inflammatory process through the regulation of cytokine secretion, we analyzed the plasma levels of the proinflammatory cytokines TNF-α, IL-1β, and IL-6. A substantial increase in TNF-α (Fig. 3C), IL-1β (Fig. 3D), and IL-6 (Fig. 3E) production was found in CIA-control mice 35 days after CII immunization. Levels of TNF-α (Fig. 3C), IL-1β (Fig. 3D), and IL-6 (Fig. 3E) were significantly reduced in a dose-dependent manner in CIA-oleuropein aglycone mice in comparison to CIA-control mice.

**Effect of Oleuropein Aglycone Treatment on iNOS, COX-2, PGE₂, Nitrotyrosine, and PAR Formation.** Immunohistochemical analysis of the tibiotarsal joint sections obtained from CIA-control mice revealed positive staining for iNOS (Fig. 5, A and A1) and COX-2 (Fig. 4, A and A1), which were primarily localized in inflammatory cells. In contrast, staining for iNOS (Fig. 5B) and COX-2 (Fig. 4B) was mark-

![Fig. 4. Effect of oleuropein aglycone (OLE AGLYCONE) treatment on COX-2 immunostaining and on serum PGE₂ levels. A marked increase in COX-2 (A and in particular A1) staining was evident in the paw 35 days after initiation of CIA. There was a marked reduction in the immunostaining for COX-2 (B) in the paw of CIA-oleuropein aglycone (40 μg/kg) mice. To verify the binding specificity for COX-2, some sections were also incubated with only the secondary antibody (no primary antibody). No positive staining for COX-2 was found in the sections indicating that the immunoreaction was positive (see negative control C). In addition, a marked increase of PGE₂ levels was found in the serum of CIA control mice 35 days after CII immunization (E). The treatment with oleuropein aglycone also caused a significant reduction in a dose-dependent manner of the serum levels of the metabolite of COX-2 (E). The figure is representative of at least three experiments performed on different experimental days. Densitometry analysis of immunocytochemistry photographs (n = 5) for COX-2 from paw section was assessed (D). The assay was performed by using Optilab Grafeck software on a Macintosh personal computer (CPU G3-266). Data are expressed as a percentage of total tissue area. *, P < 0.01 versus sham-control; **, P < 0.01 versus CIA. ND, not detectable.
edly reduced in the tibiotarsal joints of CIA-oleuropein aglycone (40 μg/kg) mice. No staining for either iNOS or COX-2 was detected in the tibiotarsal joints obtained from sham-control mice (data not shown). Moreover, we also evaluated the levels of PGE₂, the metabolite of COX-2, in the serum during the development of CIA. A substantial increase in PGE-2 production was found in CIA-control mice 35 days after CII immunization (Fig. 4E). Levels of PGE-2 were significantly reduced in CIA-oleuropein aglycone mice in a dose-dependent manner compared with those in CIA-control mice (Fig. 4E).

The release of free radicals and oxidant molecules during chronic inflammation has been suggested to contribute significantly to the tissue injury (Cuzzocrea et al., 2001). On day 35, positive staining for nitrotyrosine, a marker of nitrosative injury, was found in the tibiotarsal joints of vehicle-treated CIA-control mice (Fig. 6, A and A1). Oleuropein aglycone (40 μg/kg) treatment significantly reduced the formation of nitrotyrosine (Fig. 6B).

Immunohistochemical analysis of joint sections obtained from CIA-challenged mice revealed positive staining for PAR (Fig. 7, A and A1). In contrast, no positive PAR was found in the tibiotarsal joints of CIA-challenged mice treated with oleuropein aglycone (40 μg/kg) (Fig. 7B). There was no staining for either nitrotyrosine or PAR in the tibiotarsal joints obtained from sham-control mice (data not shown).

Oleuropein Aglycone Inhibits the Progression of Established Arthritis. To confirm that oleuropein aglycone exerts beneficial effects in the experimental model of collagen-induced arthritis, we have also evaluated its effect in a therapeutic regimen of post-treatment (40 μg/kg), starting the treatment at day 28. CIA-oleuropein aglycone post-treatment mice also showed a reduction in the devel-

![Image of immunostaining results](image-url)

**Fig. 5.** Effect of oleuropein aglycone (OLE AGLYCONE) treatment on iNOS immunostaining. A marked increase in iNOS (A and in particular A1) staining was evident in the paw 35 days after initiation of CIA. There was a marked reduction in the immunostaining for iNOS (B) in the paw of CIA-oleuropein aglycone (40 μg/kg) mice. To verify the binding specificity for iNOS, some sections were also incubated with only the secondary antibody (no primary antibody). No positive staining for iNOS was found in the sections, indicating that the immunoreaction was positive (see negative control C). The figure is representative of at least three experiments performed on different experimental days. Densitometry analysis of immunocytochemistry photographs (n = 5) for iNOS from paw section was assessed (D). The assay was performed by using Optilab Graftek software on a Macintosh personal computer (CPU G3-266). Data are expressed as a percentage of total tissue area. ***, P < 0.01** versus sham-control; *, **P < 0.01** versus CIA. ND, not detectable.
opment of arthritis and a significantly lower arthritis score compared with those in CIA-control mice as shown in Fig. 8A. Oleuropein aglycone post-treatment also significantly reduced paw edema formation (Fig. 8B). In addition, oleuropein aglycone post-treated mice showed significantly reduced histological alterations of the tibiotarsal joint as shown in the histological score (Fig. 8C) and increased body weight (Fig. 8D).

Discussion

Rheumatoid arthritis is an inflammatory disease characterized by chronic inflammation of the synovial joints associated with proliferation of synovial cells and infiltration of activated immunoinflammatory cells, including memory T cells, macrophages, and plasma cells, leading to progressive destruction of cartilage and bone (Hitchon and El-Gabalawy, 2004). Another central feature of RA synovitis is the transformation of fibroblast-like synovial cells into autonomously proliferating cells with a tissue-infiltrating nature, forming hyperplastic tissue with the potential for bone erosion and cartilage degradation known as pannus (Filippin et al., 2008). Therefore, it is necessary to establish and characterize experimental animal models to assess cellular and molecular events that contribute to the pathogenesis of joint inflammation. Of interest, CII-induced arthritis in the mouse has proven to be a useful model, because it possesses many of the cellular and humoral immune events found in human rheumatoid arthritis.

Oxidative stress describes an imbalance between ROS synthesis and antioxidants. Many studies have demonstrated a role of ROS in the pathogenesis of inflammatory chronic arthropathies, such as rheumatoid arthritis (Filippin et al., 2008).

Epidemiological studies have shown that populations...
consuming a predominantly olive oil-based Mediterranean-style diet exhibit lower incidences of breast cancer and other chronic diseases (Menendez et al., 2007). A fundamental characteristic of olive oil is the presence of a large number of phenolic compounds such as oleuropein, a glucoside with hydroxyaromatic functionality, which has attracted considerable attention because of its antidiabetic, antiatherosclerotic (Miles et al., 2005), and anti-inflammatory (Covas, 2008) properties. Several studies have also shown that olive oil phenols possess potent antioxidant activity (Vissers et al., 2004) and prevent reactive oxygen species-mediated cell injury (Salvini et al., 1998). A more efficient anti-inflammatory role of the aglycone compared with the glycosidic form of oleuropein possibly derives from the greater lipophilicity of the former, a property that should allow better cell membrane incorporation and/or interaction with other lipids (Saija et al., 1998). In this regard, we investigate here the effects of oleuropein aglycone, a hydrolysis product of oleuropein, in a mouse model of CII-induced arthritis.

Although T-cell and antibody responses against CII are a crucial event for the initiation of CIA (Holmdahl et al., 1989), it has been demonstrated that several cytokines also appear to direct cell-to-cell communication in a cascade fashion during the progression of CIA such as IL-1 (Hom et al., 1992), TNF-α (Dong et al., 2010), and IL-6 (Ferraccioli et al., 2010). In addition, it has been demonstrated that monocyte chemotactic protein-1, MIP-1α, MIP-1β, and regulated on activation normal T cell expressed and secreted are differentially chemotactic for lymphocyte subsets and are expressed in tissue from the inflamed joints of patients with rheumatoid arthritis (Koch et al., 1994). In this study, we have confirmed that the cytokines (TNF-α, IL-1β, and IL-6) as well as the chemokines (MIP-1α and MIP-2) are expressed at sites of inflamed joints and probably contribute in different capac-

**Fig. 7.** Effect of oleuropein aglycone (OLE AGLYCONE) treatment on PARP immunostaining. A marked increase in PARP (A and in particular A1), staining was evident in the paw 35 days after initiation of CIA. There was a marked reduction in the immunostaining for PARP (B) in the paw of CIA-oleuropein aglycone (40 μg/kg)-treated mice. To verify the binding specificity for PARP, some sections were also incubated with only the secondary antibody (no primary antibody). No positive staining for PARP was found in the sections, indicating that the immunoreaction was positive (see negative control C). The figure is representative of at least three experiments performed on different experimental days. Densitometry analysis of immunocytochemistry photographs (n = 5) for PARP from paw section was assessed (D). The assay was performed by using Optilab Graftek software on a Macintosh personal computer (CPU G3-266). Data are expressed as a percentage of total tissue area. **P < 0.01 versus sham-control; °, P < 0.01 versus CIA. ND, not detectable.
ities to the progression of chronic joint inflammation. Several cytokines, including TNF-α and IL-1β, are known initiators of the nuclear factor (NF-κB) activation cascade (Filippin et al., 2008) and are under its transcriptional control, constituting a positive feedback loop. Recent studies have observed that the acute consumption of olive oil decreased the activation of the NF-κB system on mononuclear cells from healthy men (Perez-Martinez et al., 2007) and that oleuropein aglycone, trans-resveratrol, and hydroxytyrosol incubated with human umbilical vein endothelial cells inhibit lipopolysaccharide-triggered NF-κB and activator protein-1 activation (Carluccio et al., 2003). Of interest, using oleuropein aglycone, we have demonstrated an inhibition of the release of proinflammatory cytokines and chemokines and a reduction of leukocyte infiltration measured by MPO activity. Several studies also showed that the potential cardioprotective activity of oleuropein in acute cardiotoxicity induced by doxorubicin treatment was determined in vivo in rats (Andreadou et al., 2007) by inhibiting lipid peroxidation products, decreasing oxidative stress, and reducing iNOS in cardiomyocytes and that the olive oil polyphenols are capable of down-regulating COX-2 expression in colon cancer cells by a mechanism involving the early inhibition of p38 mitogen-activated protein kinase and downstream inhibition of the transcription factor cAMP response element-binding protein (Corona et al., 2007). We show here that oleuropein aglycone decreased iNOS and COX-2 expression by immunohistochemical staining and also reduced the levels of the metabolite of COX-2, PGE₂, in the serum of oleuropein aglycone-treated mice.

Reactive nitrogen species, such as the peroxynitrite radical (ONOO⁻) generated by the reaction between O₂⁻ and nitric oxide, can also cause oxidative damage (Soneja et al., 2005). The addition of ONOO⁻ to body cells, tissues, and fluids leads to fast protonation, which may result in the depletion of −SH groups and other antioxidants, oxidation and nitration of lipids, DNA disruption, and nitration and deamination of DNA bases (Filippin et al., 2008). In this report, an intense immunostaining of nitrotyrosine formation also suggested that a structural alteration of joint had occurred, most probably due to the formation of highly reactive nitrogen derivatives.

ROS produce strand breaks in DNA, which triggers energy-consuming DNA repair mechanisms and activates the nuclear enzyme poly(ADP-ribosyl) polymerase (PARP). There is various evidence that the activation of PARP may also play an important role in inflammation (Genovese et al., 2005). Continuous or excessive activation of PARP produces extended chains of ADP-ribose (PAR) on nuclear proteins and results in a substantial depletion of intracellular NAD⁺ and subsequently, ATP, leading to cellular dysfunction and, ultimately, cell death (Chiarugi, 2002). We demonstrate here that oleuropein aglycone treatment reduced the activation of PARP with a decrease in PAR expression in the joint during CIA. In this regard, several studies demonstrated that hydroxytyrosol, a hydrolysis product of oleuropein aglycone, also exerts an inhibitory effect on peroxynitrite-dependent DNA base modifications and tyrosine nitration (Deiana et al., 1999). Likewise, Salvin et al. (2006) showed a 30% reduction of oxidative DNA damage in peripheral blood lymphocytes during intervention in postmenopausal women with virgin olive oil containing high amounts of phenols. Thus, oleuropein aglycone, given at the onset of the disease, reduced paw swelling, clinical score, and the histological severity of the disease when injected after the onset of clinical arthritis. Amelioration of joint disease was associated with near to full inhibition of cytokines as well as inhibition of neutrophil infiltration, which is a key player in RA.

Therefore, oleuropein aglycone was also administered from day 28 after collagen immunization, targeting this early initiation phase of CIA. Then, with treatment starting at day 28, oleuropein aglycone post-treatment caused a significant reduction of inflamed joints collected at day 35.

In conclusion, RA is a complex chronic inflammatory disease dependent on multiple interacting environmental and genetic factors, making it difficult to understand its pathogenesis and thereby to find effective therapies. Taken together, the results of the present study enhance our understanding of the role of ROS generation in the pathophysiology of CIA-induced arthritis, implying that olive oil compounds such as oleuropein aglycone may be useful in the therapy of inflammation.
rheumatoid arthritis with involvement of both DTH and immune complex medi- 

the development of type II collagen-induced arthritis only in susceptible and not 

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