

Eicosapentaenoic acid attenuates arthritis-induced muscle wasting acting on atrogen-1 and on myogenic regulatory factors.

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Keywords: adjuvant-induced arthritis, ubiquitin-proteasome system, MyoD, myogenin, PCNA.

ABSTRACT

Eicosapentaenoic acid (EPA) is an omega-3 polyunsaturated fatty acid that has anti-inflammatory and anti-cachectic actions. The aim of this work was to elucidate whether EPA administration is able to prevent arthritis-induced decrease in body weight and muscle wasting in rats. Arthritis was induced by intradermal injection of Freund's adjuvant; 3 days later 9 rats received 1g/kg EPA or coconut-oil daily. All rats were killed 15 days after adjuvant injection. EPA administration decreased the external signs of arthritis, paw volume as well as liver TNF- α mRNA. EPA did not modify arthritis-induced decrease in food intake or body weight gain. However, EPA treatment prevented arthritis-induced increase in muscle TNF- α and atrogen-1, whereas it attenuated the decrease in gastrocnemius weight and the increase in MuRF1 mRNA. Arthritis did not only decrease myogenic regulatory factors, but it increased PCNA, MyoD and myogenin mRNA in the gastrocnemius. Western blot analysis showed that changes in protein content followed the pattern seen with mRNA. In the control rats EPA administration increased PCNA and MyoD mRNA and protein. In arthritic rats, EPA did not modify the stimulatory effect of arthritis on these myogenic regulatory factors. The results suggest that in experimental arthritis, in addition to its anti-inflammatory effect, EPA treatment attenuates muscle wasting by decreasing atrogen-1 and MuRF1 gene expression and increasing the transcription factors that regulate myogenesis.

INTRODUCTION

Chronic inflammatory diseases such as cancer, sepsis and rheumatoid arthritis are associated with a decrease in body weight, skeletal muscle atrophy and cachexia. Cachexia is a complex metabolic syndrome associated with underlying illness and characterized by loss of muscle with or without loss of fat mass (12). Adjuvant-induced arthritis is a widely used experimental because in many respects it mimics rheumatoid arthritis in humans (45). Arthritis can be induced in rats by an intradermal injection of Freund's adjuvant (heat killed *Mycobacterium butyricum*). On days 10-11 after adjuvant injection rats develop chronic inflammation and polyarthritis that lead to a marked decrease in body weight and cachexia (37) by a dramatic loss of adipose and skeletal muscle mass (8, 31). Cachexia has also been reported in rheumatoid arthritis (RA) patients, adversely affecting morbidity and mortality (36). In RA patients, weight is lost equally from adipose tissue and muscle, and is not secondary to a decrease in caloric intake, but rather to an increase in resting energy expenditure (2). Similarly, a decrease in the relative skeletal muscle mass is observed in arthritic rats but not in pair-fed rats (8).

Muscle wasting in arthritic rats is associated with an increase in the gene expression of two genes of the ubiquitin-proteasome system, MuRF1 (Muscle Ring Finger 1) and atrogin-1 (14). These genes are known as "atrogenes", since they are upregulated in several conditions that induce muscle wasting such as cancer, sepsis, diabetes and fasting (27). In addition, mice lacking these genes are resistant to denervation-induced muscle wasting (5). For that reason, these genes serve as early markers of skeletal muscle atrophy, aiding in the diagnosis of muscle disease. In arthritic rats the upregulation of atrogenes is specific to

the skeletal muscle, since it does not occur in the cardiac muscle; accordingly there is no wasting in the cardiac muscle in chronic arthritis (14).

It is well known that omega-3 polyunsaturated fatty acids (PUFA) have beneficial effects on cardiovascular health and on inflammatory diseases. Eicosapentaenoic acid (EPA) is a PUFA that is essential for normal growth and development, since it is part of the cellular membranes. EPA has anti-inflammatory actions in both human and experimental animals. A diet rich in fish, in which EPA is the major component, is able to ameliorate autoimmune diseases (33). There are several clinical studies that show benefits from fish oil in patients with rheumatoid arthritis (23). Among the lipid mediators of the fish oil, EPA has been shown to reduce joint stiffness in RA patients (40).

EPA competes with arachidonic acid for incorporation in the cell membrane phospholipid, as substrate of cyclooxygenase-2, leading to a decrease in PGE₂ synthesis. We have recently reported that COX-2 inhibition by meloxicam administration has an important anti-cachectic effect in arthritic rats (15) by preventing arthritis-induced increase in MuRF1 and atrogenin-1 gene expression in the skeletal muscle. However, anti-inflammatory treatment with COX-2 inhibitors has been demonstrated to have several side effects on the cardiovascular system (16).

In addition to its anti-inflammatory effect, the anti-cachectic effect of fish oil treatment in cancer cachexia is well known. EPA administration is able to prevent cancer-induced decrease in body weight gain and skeletal muscle wasting in both human (13) and experimental animals (49). The beneficial effect of EPA on skeletal muscle wasting is secondary to a decrease in muscle protein degradation by preventing the activation of the ubiquitin-proteasome pathway.

This mechanism has been reported in cancer (41), hyperthermia (42) and fasting (48). Furthermore, only one day administration of EPA is able to prevent sepsis-induced muscle proteolysis in mice (25).

Taking into account the EPA anti-inflammatory effect in arthritis and the observed muscle atrophy in chronic arthritis, the purpose of this study was to examine whether one of the beneficial effects of EPA on chronic arthritis can be a reduction in skeletal muscle atrophy. For this purpose, expression of atrogen-1 and MuRF1 in the gastrocnemius muscle of arthritic rats treated with EPA was analyzed. Proliferation and differentiation of muscular precursor cells, or satellite cells, into mature muscular cells depends on hormones and growth factors such as PCNA, MyoD and myogenin. As adjuvant-induced arthritis also increases the expression of the myogenic regulatory factors PCNA, MyoD and myogenin (8), their response to EPA administration was also analyzed.

MATERIAL AND METHODS

Animals

Arthritic and control male Wistar rats (100-125 g/5 weeks old) were purchased from Charles River Laboratories (Barcelona, Spain). Arthritis was induced in the rats by an intradermal injection of 4 mg heat-inactivated *Mycobacterium butyricum* in the right paw, under isoflurane anaesthesia. Control animals were injected with vehicle (0.1 ml of paraffin oil). After arriving (day 3 after adjuvant injection), rats were housed 3-4 per cage, and maintained under standardized conditions of temperature (20-22 °C) and light (lights on from 7:30 to 19:30 h). Water and standard chow (A=04; Panlab, Barcelona, Spain) were provided *ad libitum*. The procedures followed the guidelines

recommended by the EU for the care and use of laboratory animals, and were approved by the Complutense University Animal Care Committee.

Experimental design

On day 3 after adjuvant injection, both control and rats injected with adjuvant were randomly divided in two groups, being 9 the number of animals for each treatment group. The first group received, at a dosage of 1g/kg bw daily by oral gavage, highly purified ethyl ester of eicosapentaenoic acid (E-EPA) containing 90% EPA and 0.02% vitamin D3 (Oy Bio-Vita Ab, Espoo, Finland). The other group received 1 g/kg bw of coconut oil to ensure isocaloric intake. A pair-fed group was also included, as arthritis decreases food intake. Pair-fed rats received the same amount of food (g/100g bw) eaten by arthritic rats treated with coconut oil on the previous day, and were gavaged daily with 1 g/kg bw coconut oil.

Body weight and arthritis severity were assessed daily. Evaluation of arthritis severity was performed by measuring the arthritis index of each animal, which was clinically scored by grading each paw from 0 to 4, since inflammation of the paw is associated with radiological and histological alterations of the joints (17). Grading was determined as previously reported (43): 0- no erythema or swelling. 1- slight erythema or swelling of one or more digits. 2- swelling of paw. 3- swelling of entire paw and the ankle. 4- ankylosis, incapacity to bend the ankle. The severity score was the sum of the clinical scores of the four limbs, the maximum value being 16.

After 12 days of EPA treatment, and 15 days after adjuvant or vehicle injection, all rats were killed by decapitation between 12:00 and 13:00 h, in a separate room, within 30 seconds after being removed from their cages.

Although the maximal external sings have the maximum value around day 21 after adjuvant injection (31), rats were killed on day 15, since we have previously observed a high increase in atrogen-1 and MuRF1 mRNA, and well as in myogenic regulatory factors PCNA, MyoD and myogenin on this day in the gastrocnemius of the arthritic rats (8). Immediately after decapitation, gastrocnemius muscle was removed and weighed. The left hind paw was amputated at the ankle level, and its volume was measured by water displacement. Gastrocnemius and liver were dissected, frozen in liquid nitrogen and stored at -80 °C until ribonucleic acid (RNA) or protein extraction. Isolation and manipulation of tissues were always performed under sterile conditions.

RNA extraction and real-time PCR

Gastrocnemius or liver (100 mg) were homogenized, and total RNA was extracted using Ultraspec™ (Biotech Laboratories Inc. Houston, Texas, USA), following the manufacturer's protocol. The final concentration of RNA was determined with a BioPhotometer (Eppendorf, Germany), and the integrity of the RNA was confirmed by agarose gel electrophoresis. First-strand cDNA synthesis was performed using 1 µg of total RNA with a Quantiscript Reverse Transcription kit (Qiagen Combh Hilden, Valencia, CA, USA).

Real-time PCR for quantification of mRNA was performed on a SmartCycler® (Cepheid, Sunnyvale, CA, USA) using a SYBR-Green protocol on the fluorescence temperature cycler. Each real-time PCR reaction consisted of 10 ng total RNA equivalents, 1x Takara SYBR Green Premix Ex Taq (Takara BIO INC, Otsu, Shiga, Japan), and 300 nM forward and reverse primers in a reaction volume of 25.5 µl. Primers for real-time PCR (Table 1) were obtained

from Roche (Madrid, Spain) by using the EXIQON Universal Probe Library (atrogin-1, myostatin, PCNA and myogenin) or from previously published sequences of MuRF-1 (11), MyoD (19) and 18S (6). The thermal cycling profile consisted of a preincubation step at 95°C for 10s followed by 40 cycles of 95°C denaturation steps for 15 s, 60°C annealing steps for 30 s, and 72°C extension steps for 30 s. Results were expressed relatively to the control animals treated with coconut oil, where the relative mRNA abundance has been arbitrarily set to 1, using cycle threshold 2($\Delta\Delta$ CT) method (29) with 18S as reference gene. PCR products were separated using agarose gel electrophoresis to confirm the product presence and size.

Immunoblot

Muscle samples were homogenized in lysis buffer (10 μ l/mg) with protease inhibitor cocktail (Sigma-Aldrich, Madrid, Spain). The homogenate was later centrifuged at 13000 rpm at 4°C for 30 min to remove tissue debris. Protein concentration was determined using the Bradford protein assay with bovine serum albumin as standard. The protein extract was boiled for 5 min with a 1:1 volume of Laemmli loading buffer. Proteins (50 μ g) were resolved by electrophoresis on 14% polyacrylamide gels under reducing conditions, and then transferred onto nitrocellulose membranes that were blocked by incubation in 5 % non-fat dry milk, 0.1 % Tween (Sigma-Aldrich, Madrid, Spain), in Tris-buffered saline. Membranes were probed overnight at 4 °C sequentially with antibodies against myogenin, PCNA, myostatin, MyoD (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and α -tubulin (Sigma-Aldrich, Madrid, Spain) with stripping of membranes before each new antibody. Membranes

were then incubated for 90 min in the appropriate horse-radish peroxidase-conjugated secondary antibody (anti-mouse IgG Amersham Biosciences, Little Chalfont, UK; anti-rabbit IgG Biorad, Madrid, Spain), and peroxidase activity was detected using enhanced chemiluminescent reagent (Amersham Biosciences, Little Chalfont, UK). Band intensities were quantified by densitometry using Gene Tools Analysis software. The density of the protein band in each lane was expressed as the percentage of the mean density of control rats, after loading normalization using α -tubulin.

Statistical analysis

Results were compared using the statistics program STATGRAPHICS plus for Windows. Normal distribution of data was assessed by a Shapiro-Wilks W test. Continuous variables are presented as mean \pm standard error of the mean and were tested with analysis of variance (ANOVA); post hoc comparisons were made using the LSD multiple range test. Data that were not normally distributed (TNF, atrogin-1 and MuRF1 mRNA in the gastrocnemius) were analyzed by Mann-Whitney (Wilcoxon) W test, those data are presented as scatter plots with median lines. Arthritis score index was analyzed by impaired Student's t test. Statistical significance was set at $P < 0.05$.

RESULTS

As shown in Fig.1A, on day 10 after adjuvant injection the arthritis score increased in the arthritic rats fed with coconut oil, reaching its highest value on day 15. In the arthritic rats that received EPA the increase in the arthritis scores was lower than in the rats treated with coconut oil ($P < 0.01$). In the group treated with coconut oil all rats had arthritis in other than the right hind paw. In contrast,

in the group of rats treated with EPA, two rats had arthritis only in the right hind paw, two rats had arthritis in just other than the injected paw (but the arthritis score decreased from day 12 to 15), and the other 5 had arthritis in the four limbs. The anti-inflammatory effect of EPA was also evident in the volume of the left hind paw (Fig. 1B). The arthritic rats treated with coconut oil had an increased paw volume ($P < 0.01$), whereas the arthritic rats treated with EPA had lower paw volume than the arthritic rats treated with coconut oil ($P < 0.01$), but this was higher than that observed in the control or pair-fed rats ($p < 0.05$).

In the rats treated with coconut oil, arthritis increased TNF- α gene expression in the liver ($P < 0.01$, Fig. 1C and D) and in the gastrocnemius muscle ($P < 0.01$). However, the arthritic rats treated with EPA had similar TNF- α mRNA values to the control or pair-fed rats, both in the liver and in the gastrocnemius.

The evolution of body weight is shown in Fig. 2A. Arthritis decreased body weight gain, where this difference was statistically significant from day 6 after adjuvant injection ($P < 0.01$). From day 10 to 15, arthritic rats did not gain body weight. The decrease in body weight gain is not only due to lower food intake, but also due to inflammation, since pair-fed rats had higher body weight gain than the arthritic rats. Arthritis decreased food intake ($P < 0.01$), whereas EPA administration did not modify food intake in either control or arthritic rats (Fig 2B). There was a decrease in the relative gastrocnemius weight ($P < 0.01$) in the arthritic rats treated with coconut oil, but not in pair-fed rats. EPA administration increased the relative gastrocnemius weight in arthritic rats ($P < 0.01$, Fig. 2C).

As expected, the arthritic rats treated with coconut oil increased the expression of both atrogenes MuRF1 and atrogen-1 in the gastrocnemius muscle ($P < 0.01$, Fig. 3A and B). EPA administration prevented the effect of arthritis on atrogen-1 mRNA, and attenuated the effect of arthritis on MuRF1 mRNA in the gastrocnemius. The expression of MuRF1 and atrogen-1 in the gastrocnemius of the pair-fed rats was similar to that of the control rats.

Fig 4. shows myostatin in gastrocnemius muscle of the five experimental groups. EPA administration tended to decrease myostatin in the gastrocnemius of the arthritic rats, but there was no significant difference in myostatin mRNA or myostatin protein between the groups.

As previously reported (8), arthritis induced an increase in PCNA mRNA ($P < 0.01$) and PCNA protein ($P < 0.05$) in the rats that received coconut oil (Fig. 5A and B). EPA administration tended to elevate both mRNA and protein of PCNA in control rats to values similar to those observed in arthritic rats, although this increase was not significant. The arthritic rats treated with EPA have similar PCNA mRNA and protein compared to the arthritic rats treated with coconut oil.

The effect of EPA administration on MyoD in the gastrocnemius is shown in Fig. 6 A and B. EPA administration increased MyoD mRNA and protein in control rats, but only the increase in mRNA was significant ($P < 0.05$). Arthritis increased MyoD mRNA and protein ($P < 0.05$), having values similar to those of the arthritic rats treated with coconut oil or with EPA (Fig 6A and B).

EPA administration did not modify myogenin mRNA or protein in the gastrocnemius of the control rats (Fig. 7A and B). Arthritis increased myogenin

mRNA and protein ($P < 0.01$) in both groups of arthritic rats treated with coconut oil or with EPA.

The effect of arthritis on gene expression and the protein of the different muscle regulatory factors (myostatin, PCNA, MyoD and myogenin) were not secondary to the decrease in food intake, since pair-fed rats had values similar to those observed in control rats treated with coconut oil (Fig. 4, 5, 6 and 7).

DISCUSSION

Our data show that EPA administration to arthritic rats has an anti-inflammatory effect and attenuates skeletal muscle wasting. The protective effect of EPA on the gastrocnemius muscle is not only due to a decrease in atrogene expression, but also to a stimulatory effect on myogenic regulatory factors.

The effects of EPA administration on the development of arthritis are in agreement with previous reports showing, in several models of arthritis, that fish oil administration has an anti-inflammatory effect (28, 46). A beneficial effect of fish oil supplements in rheumatoid arthritis patients has also been reported (22). Furthermore, EPA suppresses the “in vitro” proliferation of synoviocytes from rheumatoid arthritis patients (18). EPA is also able to reduce the expression of COX-2, inflammatory cytokines as well as cartilage-degrading protein, in chondrocyte cultures (51). The anti-inflammatory effect of EPA in the arthritic rats can be related to its inhibitory action on TNF- α expression. The inhibitory effect of EPA on TNF- α synthesis and release, as well as on TNF- α -induced activation of the NF- κ B pathway, has been observed in several cell types after inflammatory stimuli (26, 52). It has been proposed that the anti-inflammatory

effect of EPA is exerted through membrane phospholipids, becoming incorporated into the cell membrane, instead of arachidonic acid and modifying eicosanoids synthesis, by reducing the formation of pro-inflammatory eicosanoids (e.g. PGE₂) (3). On the other hand, EPA can form several potent anti-inflammatory lipid mediators such as resolving E1 (RvE1), which counteracts the effect of TNF- α , and inhibits NF- κ B activation (for review see 39). RvE1 plays a role in the resolution of the inflammatory response, and reduces inflammation to a lower extent in several animal models of inflammatory diseases (20, 50).

As previously reported in arthritic rats (46), EPA treatment decreased footpad inflammation without modifying food intake or body weight in arthritic rats. In contrast, in cancer cachexia EPA preserves body weight (38). This difference can be due to the fact that, although cancer and arthritis induce muscle wasting and fat mass loss, these mechanisms do not seem to be identical in both illnesses. Muscle wasting is associated with an increase in myostatin in cancer (10), whereas no modification in myostatin was observed in the skeletal muscle of arthritic rats. Furthermore, fat mass loss in arthritis is associated with a decrease in adipogenesis rather than to an increase in lipolysis (31). On the other hand, in cancer lipolysis is induced by an increase in zinc- α ₂-glycoprotein (ZAG), which plays an important role in loss of fat mass (4), whereas ZAG expression is not modified in arthritic rats (31). In cancer cachexia, EPA prevents adipose tissue loss by downregulation of ZAG expression through interference with glucocorticoid signalling (38).

Despite the fact that EPA does not have an effect on body weight gain, EPA treatment increased gastrocnemius weight in arthritic rats. This effect can

be explained by the fact that EPA administration prevented atrogen-1 and attenuated MuRF1 arthritis-induced increase in the gastrocnemius. To our knowledge, the effect of EPA on atrogen-1 or MuRF1 expression has not been previously reported. However, it has been reported that EPA is able to prevent the upregulation of other components of the ubiquitin-proteasome system. In this sense, EPA attenuates muscle protein degradation in cancer and sepsis by preventing the increase in both gene expression and protein of the α and β subunit of the 20S proteasome, as well as the functional activity of the proteasome (25, 49). Taking into account that the ubiquitin-proteasome proteolytic system is the main contributor to muscle wasting in cachexia (27), the beneficial action of EPA on muscle loss can be due to its action on the ubiquitin-proteasome pathway. Together these data suggest that EPA decreased the activity of the ubiquitin-proteasome pathways induced by chronic inflammation. The inhibitory effect of EPA on skeletal muscle proteolysis can be exerted directly on the muscular cell, since EPA is able to prevent hyperthermia-induced proteolysis by the ubiquitin-proteasome in myotube cultures (42).

The muscular wasting observed in the arthritic rats and the beneficial effect of EPA are independent of food intake, since as previously reported (8) the decrease in the relative gastrocnemius weight is not observed in pair-fed rats, and EPA administration increased gastrocnemius weight without modifying food intake in arthritic rats.

Myostatin is a negative regulator of muscle growth that increases in situations of muscle atrophy such as cancer cachexia (10). However, myostatin is not modified by chronic arthritis or by EPA administration. Similarly, myostatin

was not modified 15 days after immobilization, although a reduction in quadriceps lean mass was observed (24).

As we have previously reported (8), in spite of gastrocnemius wasting, there was an increase in PCNA, MyoD and myogenin in arthritic rats. These data indicate that in arthritic rats, muscle repair/regeneration coexists with the activation of the ubiquitin-proteasome pathway. Similarly, short bouts of passive stretching are able to increase the gene expression of factors associated with muscle growth (MyoD), and atrophy (atrogin-1) (35). The factors responsible for the upregulation of these myogenic regulatory factors in the gastrocnemius of arthritic rats are unknown. However, there is evidence that suggests a relationship between inflammation and muscle regeneration (32, 44). The pro-inflammatory cytokines TNF and IL-6 have been reported to promote myogenesis (7, 9). Furthermore, we have observed that administration of the non-steroid anti-inflammatory drug meloxicam to arthritic rats prevents the increased expression of TNF, atrogen-1 and MuRF1 in the gastrocnemius (15). It has recently been reported that another COX-2 inhibitor, NS-398, decreases muscle hypertrophy after synergist ablation (34).

EPA administration prevents arthritis-induced increase in atrogen-1 and MuRF1, whereas PCNA, MyoD and myogenin expression remained elevated in arthritic rats treated with EPA. Furthermore, in control rats EPA treatment increased PCNA and MyoD. These data suggest that, in addition to the inhibitory effect on atrogen-1 and MuRF1, part of the beneficial effect of EPA on the gastrocnemius mass can be mediated by stimulating myogenic regulatory factors. To our knowledge, the effect of EPA on the regulatory myogenic factor “in vivo” has not been previously described. It has been reported that EPA is

able to prevent the inhibitory effect of high levels of TNF- α on C2C12 myotube myogenesis, by increasing myotube diameter and myoglobin high chain expression (30).

In the muscle cell “in vitro” EPA directly modulates lipid and glucose metabolism, promoting increased glucose uptake and metabolism (1). A similar effect of EPA has recently been described in diabetic myotubes, where EPA also improved insulin resistance and fatty acid handling in type 2 diabetes skeletal muscle (47). Proteolysis-inducing factor (PIF), secreted by tumours that induce cachexia, decreases glucose uptake by myoblast and induces proteolysis, being the two effects attenuated by EPA (21).

PERSPECTIVES AND SIGNIFICANCE

Rheumatoid cachexia is an important contributor in increasing morbidity and premature mortality in rheumatoid arthritis patients. Adjuvant-induced arthritis is a well-established model of rheumatoid arthritis that is associated with cachexia and muscle wasting secondary to an increase in the activity of the ubiquitin-proteasome proteolytic pathway. The data presented in this manuscript demonstrate that administration of the omega-3 polyunsaturated fatty acid EPA to arthritic rats decreases the external signs of inflammation and TNF- α expression in the gastrocnemius muscle, whereas it increases gastrocnemius weight. EPA treatment ameliorates skeletal muscle wasting through preventing arthritis-induced increase in the expression of the E3 ubiquitin-ligating enzymes atrogin-1 and MuRF1 in the gastrocnemius. In addition, EPA treatment is also able to increase the transcription factors that regulate myogenesis such as PCNA and MyoD. Our observations suggest that

EPA treatment could be used therapeutically to reduce symptoms of arthritis and to preserve muscle mass in rheumatoid arthritis.

ACKNOWLEDGMENTS

The authors are indebted to Dr Matti Tolonen for the supply of EPA, to Antonio Carmona for technical assistance and to Cristina Brickart for the English correction of the manuscript.

GRANTS

This work was supported by a grant from the Ministerio de Educación Ciencia (ABFU2006-11899/BFI), a Fellowship from the Gobierno Vasco to E Castillero (BFI06.31), and from the Ministerio de Educación y Ciencia to M López-Menduiña (BES-2007-16001).

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LEGEND OF FIGURES

Fig.1. Effect of EPA administration on arthritis score evolution (A), left paw volume (B), TNF- α mRNA in the liver (C) and in the gastrocnemius muscle (D)

on day 15 after adjuvant injection. EPA (1 g/kg) or coconut oil (1 g/kg) was administered p.o. daily, from day 3 to day 15. AA= arthritic rats, PF= pair-fed rats. TNF- α mRNA was measured by real time PCR by analyzing the CT numbers corrected by CT readings of corresponding internal 18S mRNA controls. Results are expressed relatively to the control animals treated with coconut oil of the control rats treated with coconut oil. The increase in the arthritis scores was higher in the rats treated with coconut oil than in the rats treated with EPA ($P<0.05$). EPA administration decreased the paw volume in arthritic ($P<0.01$), but not in control rats. Arthritis increased liver TNF- α mRNA in the rats treated with coconut oil ($P<0.01$), whereas it had no effect on rats treated with EPA. Results represent means \pm SEM. Similarly, arthritis increased TNF- α mRNA ($P<0.01$) in the gastrocnemius of the rats treated with coconut oil but not in the rats treated with EPA. Data are presented as scatter plots with medians. ** $P<0.01$, * $P<0.05$ vs. control rats treated with coconut oil, ## $P<0.01$, vs. arthritic rats treated with coconut oil. + $P<0.05$ vs. control rats treated with EPA.

Fig.2. Evolution of body weight gain (A), food intake between days 4 and 15 (B) and relative gastrocnemius weight (C) in control, arthritic (AA) or pair-fed rats (PF) treated with 1 g/kg of coconut oil or with 1 g/kg of EPA. Arthritis decreased food intake ($P<0.01$), and the relative gastrocnemius weight ($P<0.01$). EPA administration did not modify food intake, but increased gastrocnemius weight in arthritic rats ($P<0.01$). Results represent means \pm SEM for $n=3$ cages and $n=7-9$ rats per group. ** $P<0.01$, * $P<0.05$ vs. control rats treated with coconut

oil, ###P<0.01, #P<0.05 vs. arthritic rats treated with coconut oil. ++P<0.01 vs. control rats treated with EPA.

Fig.3. EPA administration prevented arthritis-induced increase of atrogen-1 and attenuated the increase in MuRF1 gene expression in the gastrocnemius muscle. Atrogen-1 (A) and MuRF1 mRNA (B) were quantified from gastrocnemius muscles of control, arthritic (AA) or pair-fed (PF) rats treated with 1 g/kg coconut oil or 1 g/kg EPA, from day 3 to day 15 after adjuvant injection. Atrogen-1 and MuRF1 mRNA were measured by real time PCR by analyzing the CT numbers corrected by CT readings of corresponding internal 18S mRNA controls. Results are expressed relatively to the control animals treated with coconut oil. Arthritis increased atrogen-1 mRNA in rats treated with coconut oil (P<0.01), but did not increase it in the rats treated with EPA. Arthritis increased MuRF1 mRNA in both groups of rats, but the arthritic rats treated with EPA had lower (P<0.05) MuRF1 mRNA than the rats treated with coconut oil. Data are presented as scatter plots with medians. ** P<0.01 vs. control rats treated with coconut oil, #P<0.05 vs. arthritic rats treated with coconut oil. +P<0.05 vs. control rats treated with EPA.

Fig.4. Myostatin mRNA (A), myostatin protein (B) and representative immunoblots (C) in the gastrocnemius of control, arthritic (AA) and pair-fed rats treated with 1 g/kg coconut oil or 1g /kg EPA, from day 3 to day 15 after adjuvant injection. Myostatin mRNA was measured by real-time PCR and results are expressed relatively to the control animals treated with coconut oil, by analyzing the CT numbers corrected by CT readings of corresponding

internal 18S mRNA controls. Myostatin protein was measured by Western blotting, quantified, normalized against the α -tubulin and expressed as percentage of the control rats treated with coconut oil. Data are expressed as mean \pm S.E.M. (n= 7-9 rats).

Fig.5. PCNA mRNA (A), PCNA protein (B) and representative immunoblots (C) in the gastrocnemius of control, arthritic (AA) and pair-fed rats treated with 1 g/kg coconut oil or 1g /kg EPA, from day 3 to day 15 after adjuvant injection. PCNA mRNA was measured by real-time PCR and results are expressed relatively to the control animals treated with coconut oil, by analyzing the CT numbers corrected by CT readings of corresponding internal 18S mRNA controls. PCNA protein was measured by Western blotting, quantified, normalized against the α -tubulin and expressed as percentage of the control rats treated with coconut oil. Arthritis increased PCNA mRNA ($P<0.01$) and PCNA ($P<0.05$) in the rats treated with coconut oil. Data are expressed as mean \pm S.E.M. (n= 7-9 rats). ** $P<0.01$, * $P<0.05$ vs. control rats treated with coconut oil.

Fig.6. MyoD mRNA (A), MyoD protein (B) and representative immunoblots (C) in the gastrocnemius of control, arthritic (AA) and pair-fed rats treated with 1 g/kg coconut oil or 1 g/kg EPA, from day 3 to day 15 after adjuvant injection. PCNA mRNA was measured by real-time PCR and results are expressed relatively to the control animals treated with coconut oil, by analyzing the CT numbers corrected by CT readings of corresponding internal 18S mRNA controls. MyoD protein was measured by Western blotting, quantified,

normalized against the α -tubulin and expressed as percentage of the control rats treated with coconut oil. Arthritis increased MyoD mRNA ($P<0.05$) and MyoD ($P<0.05$) in the rats treated with coconut oil. EPA increased MyoD mRNA ($P<0.05$). Data are expressed as mean \pm S.E.M. ($n= 7-9$ rats). * $P<0.05$ vs. control rats treated with coconut oil.

Fig.7. Myogenin mRNA (A), myogenin protein (B) and representative immunoblots (C) in the gastrocnemius of control, arthritic (AA) and pair-fed rats treated with 1 g/kg coconut oil or 1 g/kg EPA, from day 3 to day 15 after adjuvant injection. Myogenin mRNA was measured by real-time PCR and results are expressed relatively to the control animals treated with coconut oil, by analyzing the CT numbers corrected by CT readings of corresponding internal 18S mRNA controls. Myogenin protein was measured by Western blotting, quantified, normalized against the α -tubulin and expressed as percentage of the control rats treated with coconut oil. Arthritis increased myogenin mRNA ($P<0.01$) and myogenin ($P<0.01$) in the rats treated with coconut oil or EPA. Data are expressed as mean \pm S.E.M. ($n= 7-9$ rats). ** $P<0.01$ vs. control rats treated with coconut oil. ++ $P<0.01$, + $P<0.05$ vs. control rats treated with EPA.

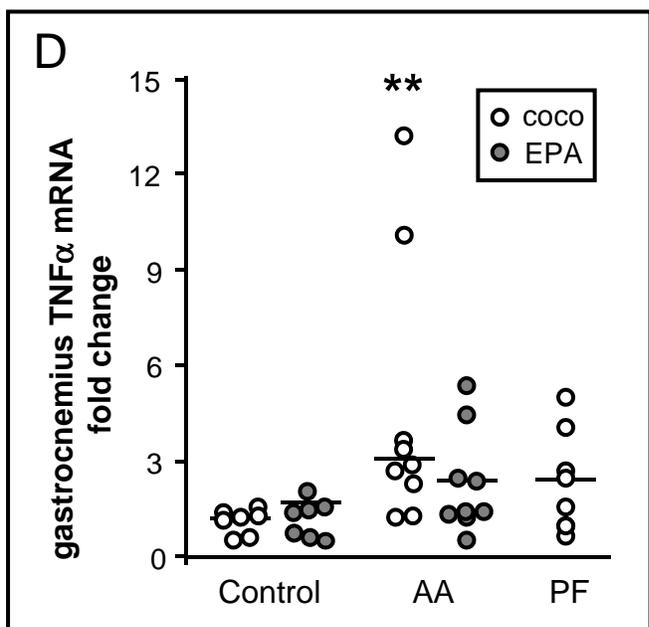
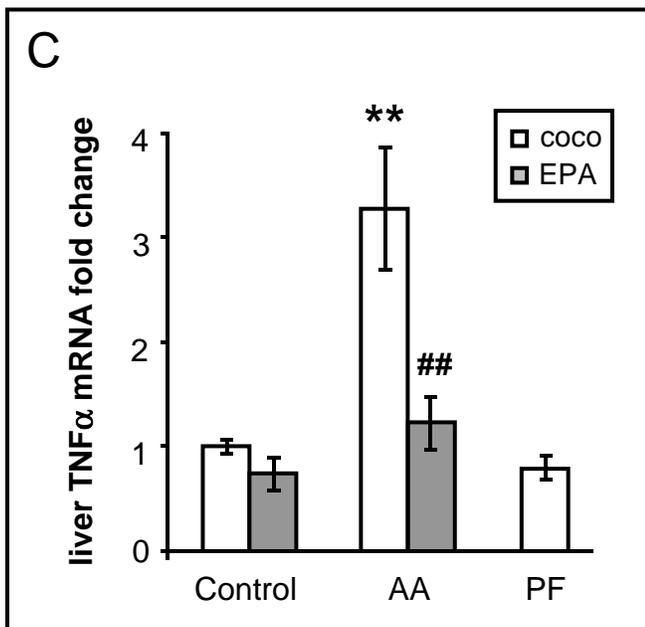
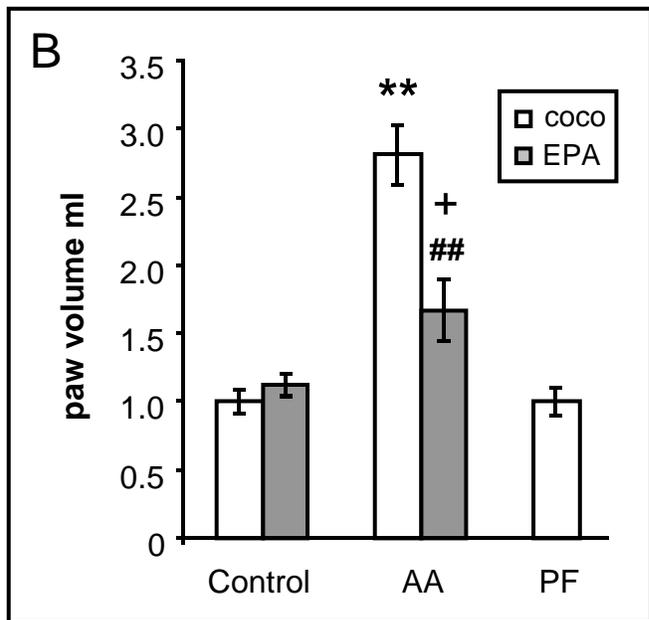
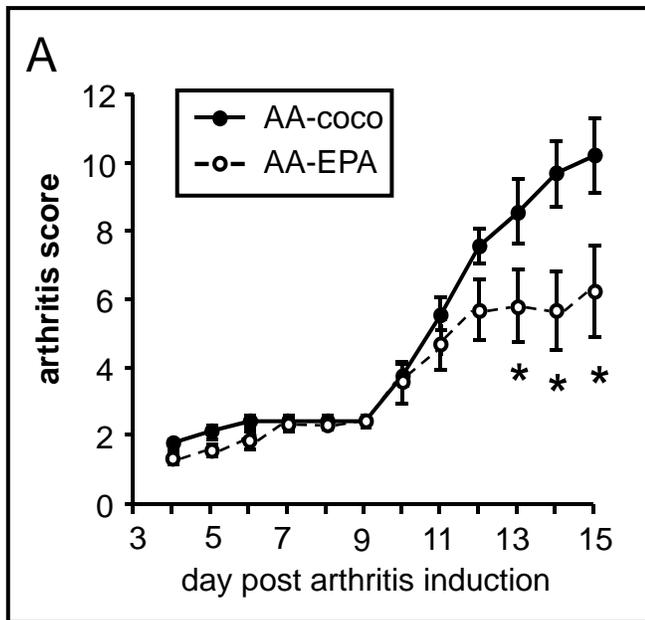


Fig. 1

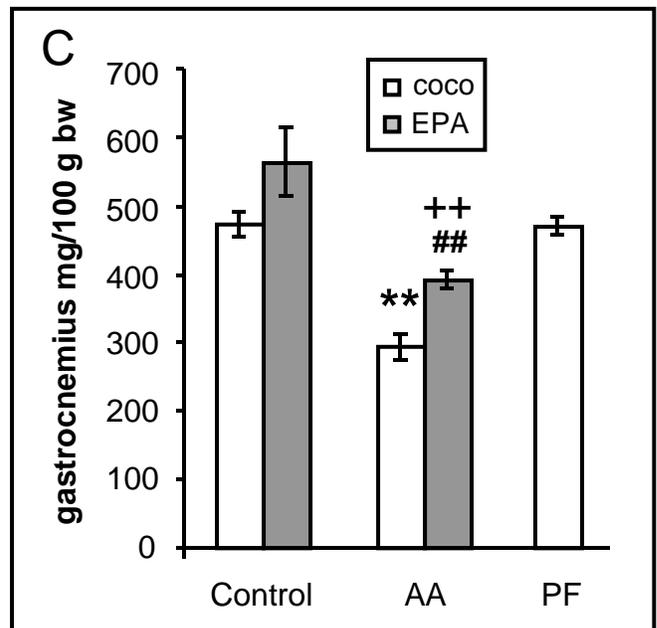
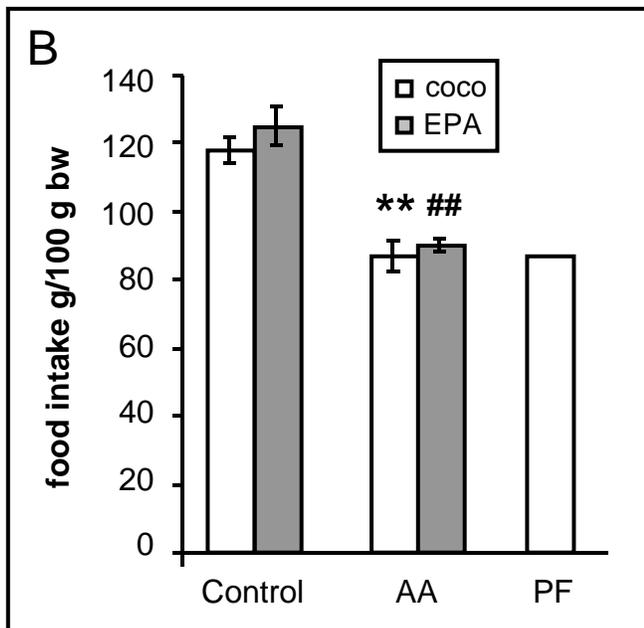
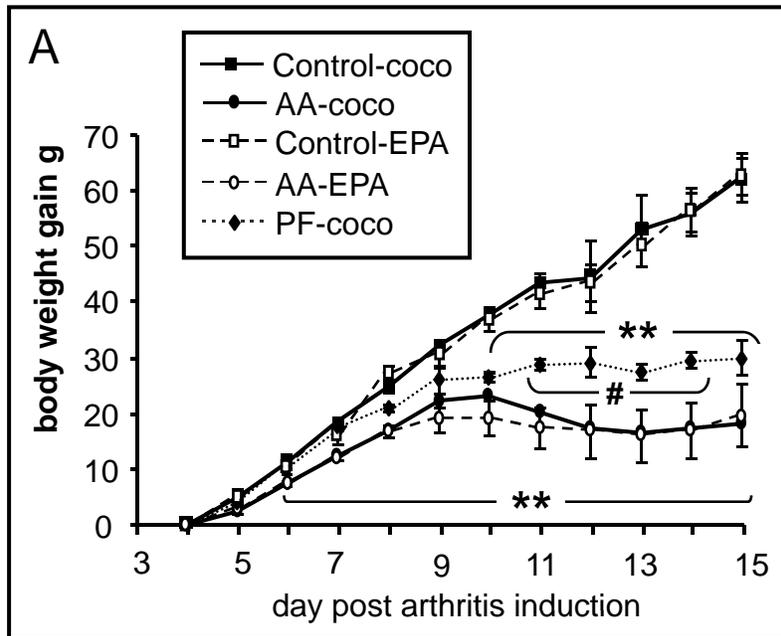


Fig. 2

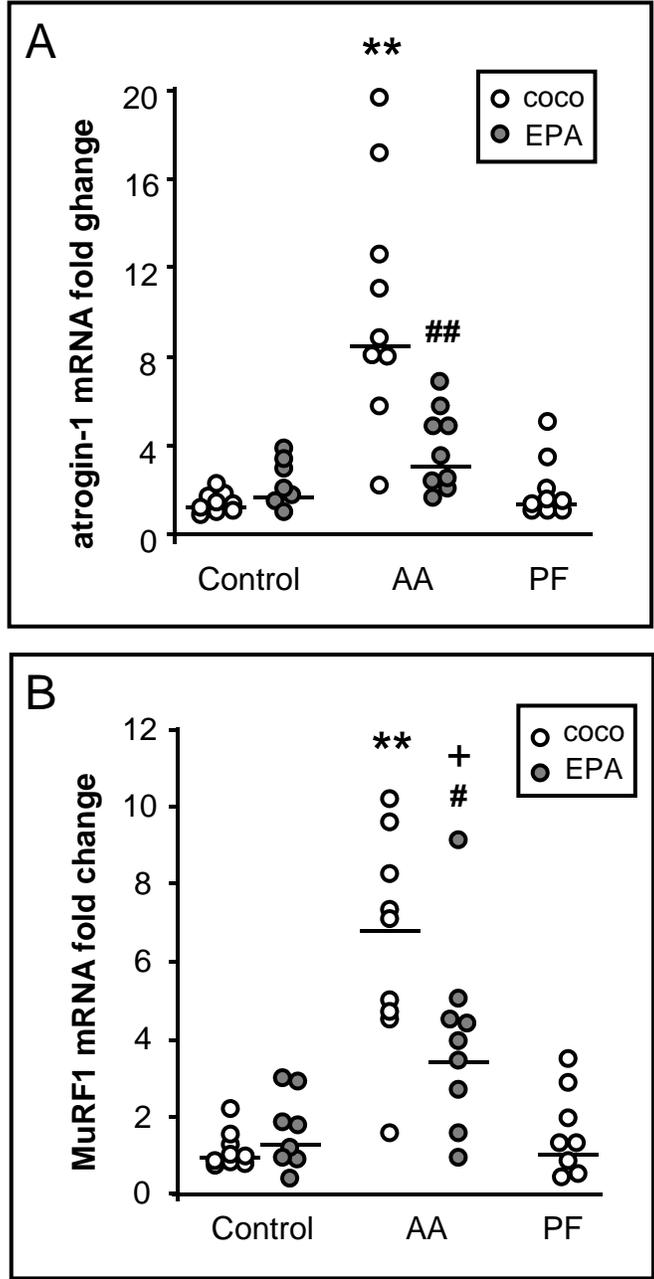


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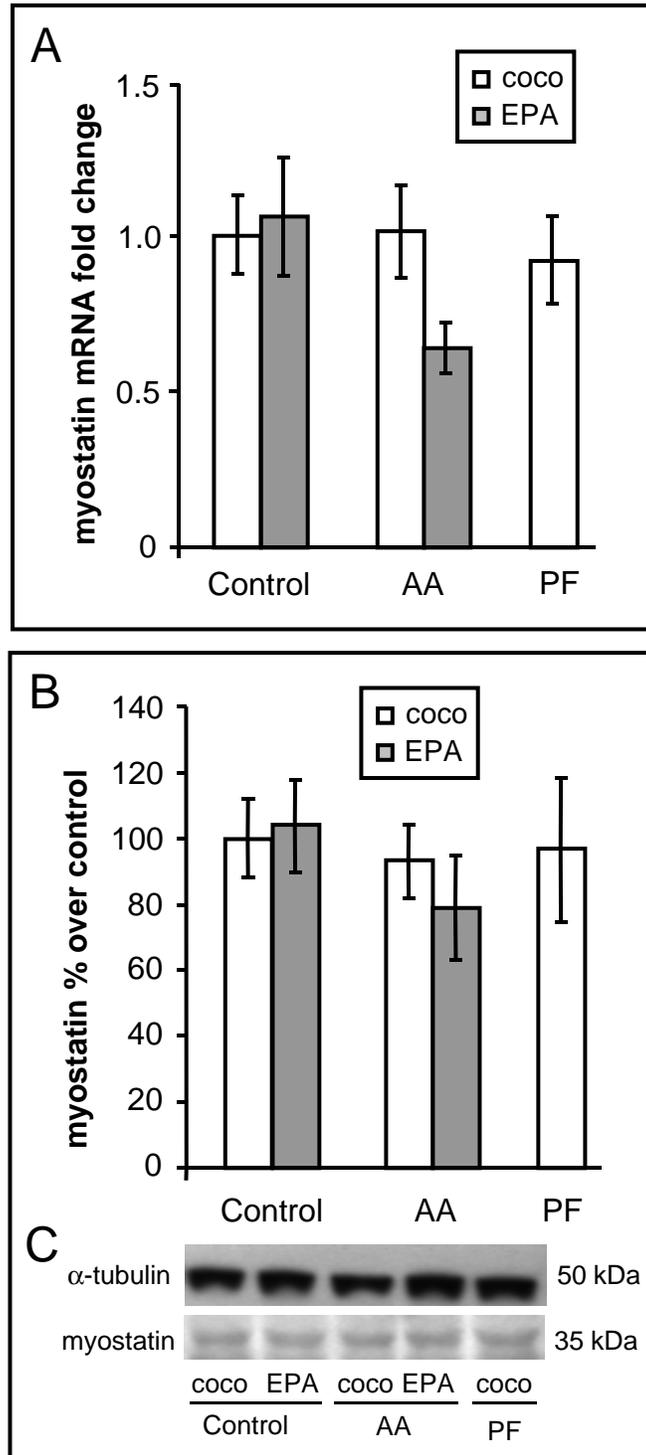


Fig. 4

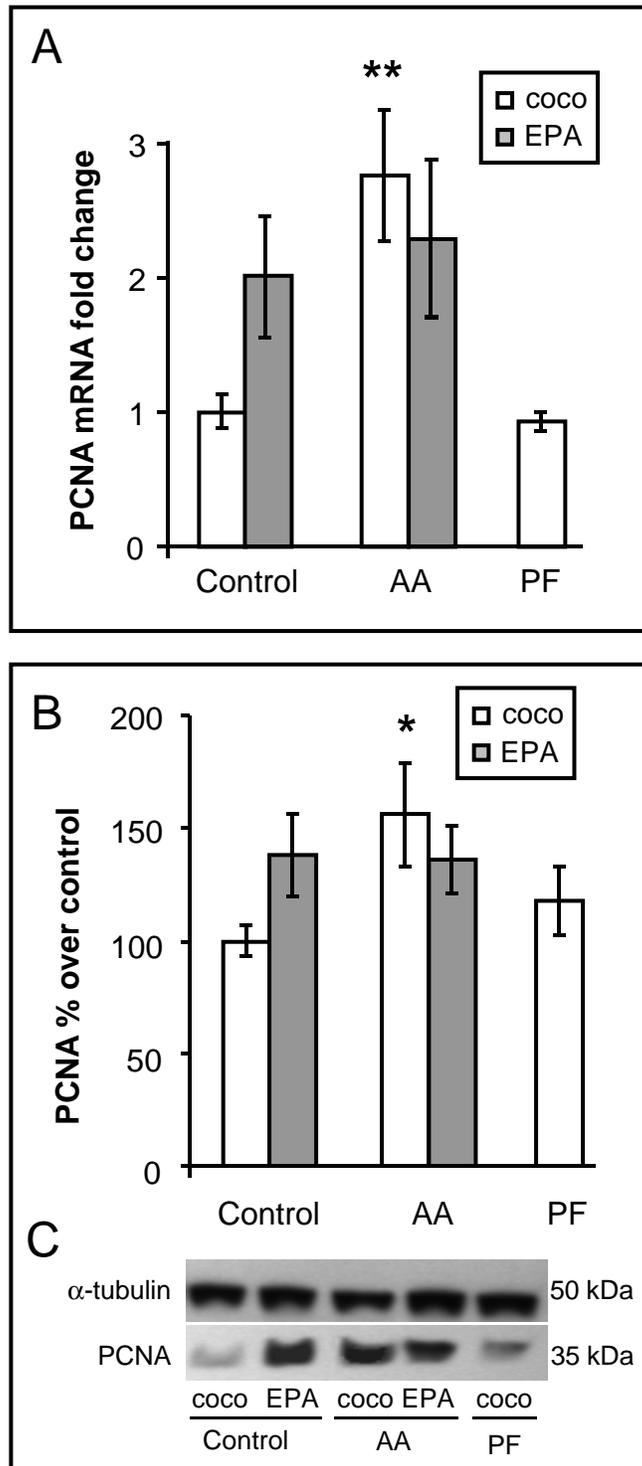


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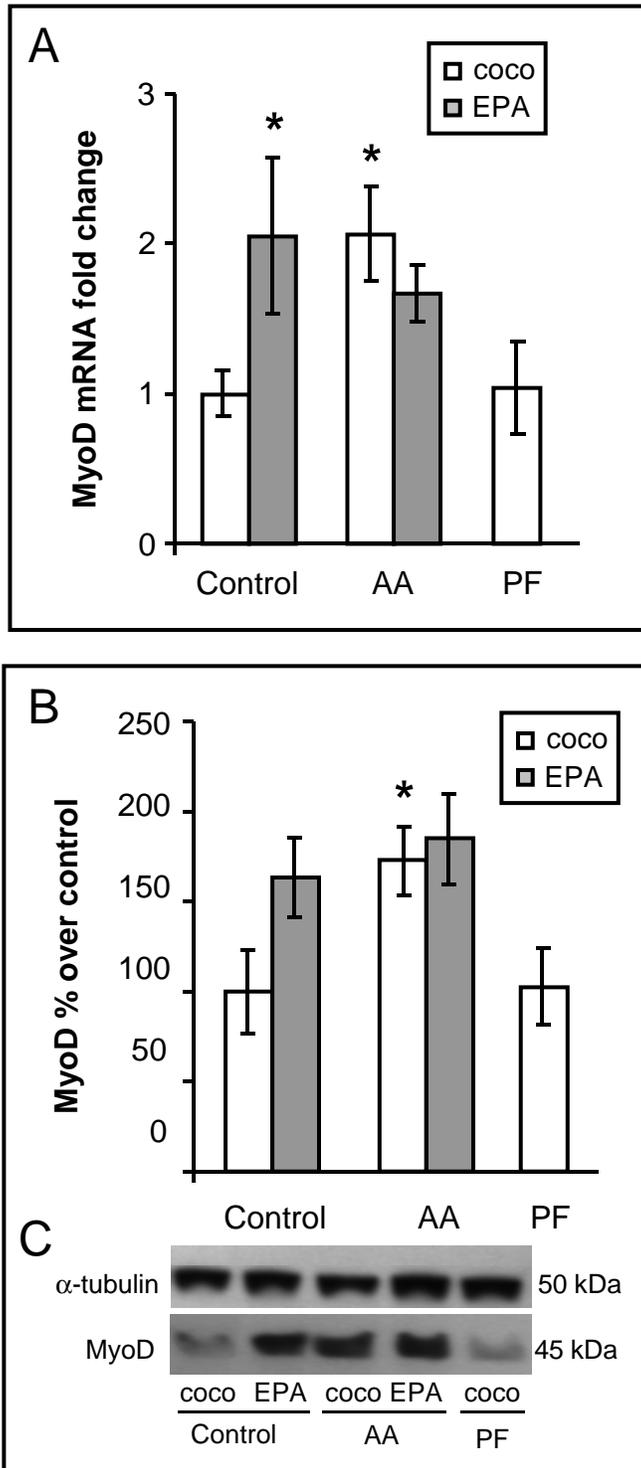


Fig. 6

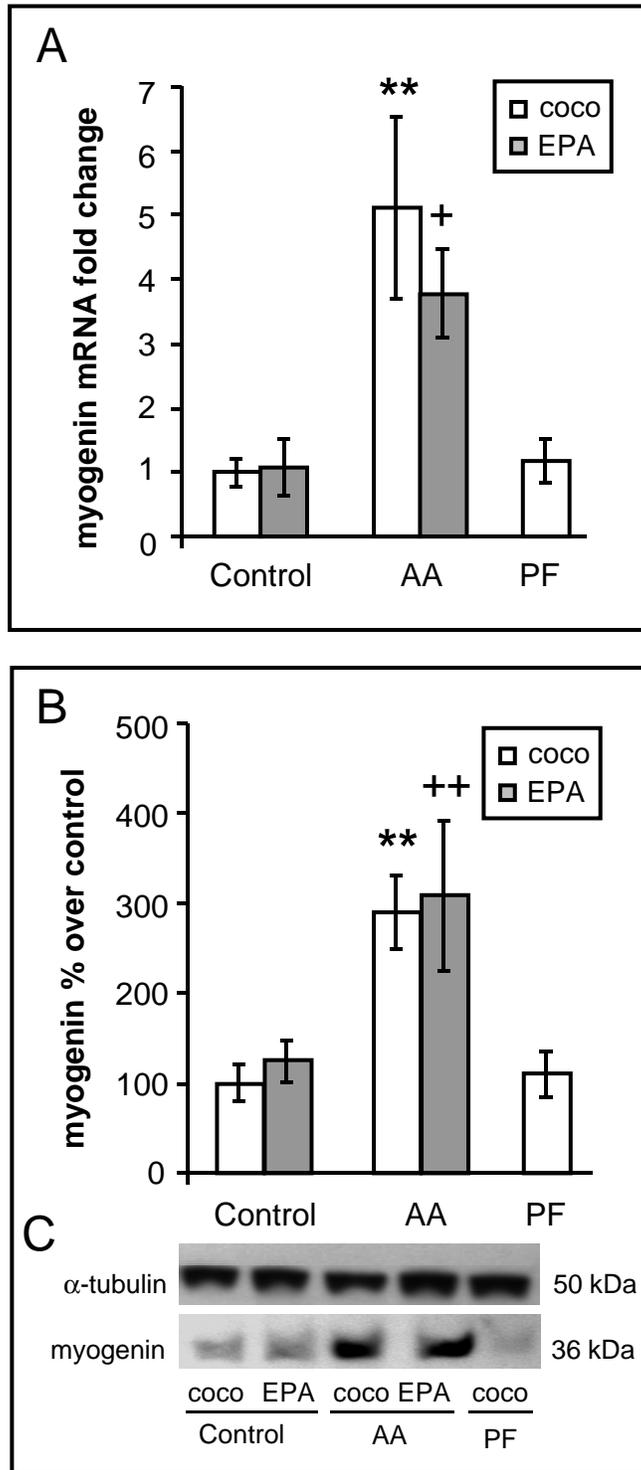


Fig. 7