

## **Effects of immunosuppressive treatment on microsomal PGE synthase 1 and cyclooxygenases expression in muscle tissue of patients with polymyositis or dermatomyositis**

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## **Abstract**

*Objectives:* To investigate the expression of microsomal PGE synthase 1 (mPGES-1) and cyclooxygenase (COX) in muscle biopsies from patients with polymyositis or dermatomyositis before and after conventional immunosuppressive treatment.

*Methods:* mPGES-1 and COX expression was evaluated by immunohistochemistry in muscle tissue from healthy individuals and from patients with polymyositis or dermatomyositis before and after conventional immunosuppressive treatment. The number of inflammatory cell infiltrates, T lymphocytes and macrophages was estimated before and after treatment. To localize the mPGES-1 expression double immunofluorescence was performed with antibodies against mPGES-1, CD3, CD68, CD163 and a fibroblast marker. Functional index was used to assess muscle function.

*Results:* In myositis patients, mPGES-1, COX-2 and COX-1 expression was significantly higher compared to healthy individuals and associated with inflammatory cells. Double immunofluorescence demonstrated a predominant expression of mPGES-1 in macrophages. Conventional immunosuppressive treatment resulted in improved but still lower muscle function than normal. A decreased number of CD68-positive macrophages and reduced COX-2 expression in muscle tissue was also seen. In contrast, following the same treatment no significant changes were observed in muscle tissue regarding number of infiltrates, T lymphocytes, CD163-positive macrophages or mPGES-1 protein levels.

*Conclusions:* Increased expression of mPGES-1, COX-1 and COX-2 at protein level was observed in muscle tissue from myositis patients compared to healthy individuals. Conventional immunosuppressive treatment led to a significant down-regulation of COX-2 in myositis muscle tissue. However, the expression of mPGES-1 and COX-1 remained unchanged indicating a role of these enzymes in the chronicity of these diseases.

Polymyositis and dermatomyositis are chronic muscle disorders characterized by muscle weakness and fatigue and by skin involvement in the case of dermatomyositis. [1] These diseases are also characterized by infiltration of inflammatory cells in skeletal muscle tissue, muscle fiber degeneration and regeneration. The pathogenesis of myositis has not been well characterized yet, but pro-inflammatory cytokines have been consistently found in the inflamed muscle and are implicated in the pathogenesis. [2-4]

Arachidonic acid metabolites such as prostaglandins (PG) might also contribute to the pathogenesis of inflammatory myositis. Human skeletal muscles have a considerable capacity to produce PGE<sub>2</sub>, PGD<sub>2</sub>, PGF<sub>2α</sub> and PGI<sub>2</sub>. [5] PGE<sub>2</sub> appears to be involved in a number of biological processes, including protein turnover and myogenesis and is a potent mediator of muscular pain and inflammation. [6-10] Interleukin (IL)-1β and tumor necrosis factor (TNF), which are markedly expressed in myositis muscle tissue, stimulate PGE<sub>2</sub> production in skeletal muscles. [2, 3, 11, 12] In the PGE<sub>2</sub> biosynthetic pathway, cyclooxygenase (COX)-1 and COX-2 catalyze the conversion of arachidonic acid into PGH<sub>2</sub> (fig 1). Recently, enhanced expression of COX-1 and COX-2 mRNA was demonstrated in inflamed muscle tissue from myositis patients, suggesting a role for them in this disease. [13] Three terminal PGE synthases (PGES) catalyze the formation of PGE<sub>2</sub> from PGH<sub>2</sub>. Microsomal PGE synthase 1 (mPGES-1) is strongly induced by proinflammatory stimuli in various cells and preferentially couples with COX-2 contributing to significant PGE<sub>2</sub> release. [14-16] Cytosolic PGES (cPGES) and mPGES-2 are both constitutively expressed and likely to function in the basal production of PGE<sub>2</sub>. [17, 18] Studies of mPGES-1 *-/-* knock-out mice have demonstrated a critical role for mPGES-1 in the development of pain, fever and inflammation. [19, 20] mPGES-1 is up-regulated in a range of inflammatory diseases and considered a new target for therapeutic strategies to control induced PGE<sub>2</sub> synthesis. [21-23] However, the expression of mPGES-1 in muscle tissue from myositis patients has not been studied.

Despite clinical improvement with conventional immunosuppressive treatment including high doses of glucocorticoids (GC) many myositis patients experience persistent muscle weakness. There are also reports of persisting inflammatory cells and increased expression of IL-1 in muscle tissue despite long-time treatment with high doses of GC. [3, 24] Intra-articular treatment with GC significantly reduces mPGES-1, COX-1 and COX-2 expression in the synovial tissue from patients with rheumatoid arthritis and is associated with clinical improvement. [25] Whether mPGES-1 and COX expression in skeletal muscle from myositis patients is affected by immunosuppressive treatment has not been investigated.

In the present study we examined the expression and localization of mPGES-1 and COX in skeletal muscle tissue from patients with polymyositis or dermatomyositis and from healthy subjects. In addition we studied the effects of immunosuppressive treatment on mPGES-1 and COX expression in skeletal muscle tissue from myositis patients in relation to the effects on clinical function.

## PATIENTS AND METHODS

### Patients and muscle biopsies

In a first cohort, nine patients with recently diagnosed polymyositis or dermatomyositis and three with treatment-resistant myositis (disease duration 3-6 yr) meeting the Bohan and Peter criteria were included (median age 54 yr, range 44-76 yr). [26, 27] Clinical data of the patients are presented in Table 1. Detailed clinical data for these patients have been reported previously. [28]

Table 1. Clinical data on the patients at the time of biopsies.

I cohort : patients with recently diagnosed and with treatment-resistant myositis (marked with *)					
Patient	age	sex	Bohan and Peter diagnostic criteria	Biopsy site	Treatment at time of biopsy, mg/day
1	53	f	probable polymyositis	M vastus lat	none
2	59	f	probable polymyositis	M deltoideus	Pred 5, NSAID
3	76	f	definite polymyositis	M vastus lat	none
4	44	f	definite polymyositis	M vastus lat	none
5	65	f	definite dermatomyositis	M vastus lat	Pred 15
6	72	f	definite polymyositis	M vastus lat	Pred 15-40, AZA
7	60	m	probable polymyositis	M vastus lat	NSAID
8	56	m	definite polymyositis	M vastus lat	NSAID
9	55	m	probable dermatomyositis	M vastus lat	none
10*	67	m	definite dermatomyositis	M vastus lat	AZA,MTX,Cs
11*	54	f	probable polymyositis	M tib ant	AZA,MTX,Cs
12*	61	f	definite polymyositis	M vastus lat	AZA,MTX,Cs

II cohort : myositis patients before and after conventional immunosuppressive treatment					
Patient	age	sex	Bohan and Peter diagnostic criteria	Treatment at time of I biopsy, mg/day	Treatment at time of II biopsy, mg/day
1	60	f	definite polymyositis	none	Pred 12.5, MTX
2	23	f	probable dermatomyositis	none	Pred 5, AZA
3	49	f	definite dermatomyositis	NSAID	Pred 15, NSAID
4	53	f	definite polymyositis	none	Pred 20, AZA
5	41	f	probable dermatomyositis	none	Pred 30
6	44	f	probable dermatomyositis	none	Pred 7.5
7	67	m	probable polymyositis	none	Pred 10
8	88	f	definite dermatomyositis	none	Pred 7.5
9	60	m	probable polymyositis	NSAID	Pred 30
10	62	m	definite dermatomyositis	none	Pred 10, AZA

Ant, anterior; AZA, azathioprine; Cs, cyclosporine; f, female; lat, lateralis; m, male; M, musculus; MTX, methotrexate; NSAID, nonsteroidal anti-inflammatory drugs; tib, tibialis; Pred, prednisolone.

To investigate the effects of immunosuppressive treatment a second cohort of ten patients with recently diagnosed polymyositis or dermatomyositis with available follow-up biopsies was included (median age 56.5 yr, range 23-88 yr). The patients were initially treated with oral prednisolone (40-60 mg/day) with slowly tapering doses and all patients (except two) received an additional immunosuppressive agent (Table 1). Two patients were treated with nonsteroidal anti-inflammatory drugs (NSAID) at the time of the first biopsy. Muscle tissue biopsies were taken from *m. vastus lateralis* before and after a median of 8.5 months (range 4-11 months) with immunosuppressive treatment. Muscle tissue biopsies were obtained under local anaesthesia using the semi-open biopsy technique. [28, 29]

Muscle biopsies from seven healthy individuals (four women and three men, median age 46 yr, range 38-50 yr) without clinical or histopathological signs of muscle disease were included as controls. Muscle biopsies were obtained from *m. vastus lateralis* in six individuals and from *m. tibialis anterior* in one individual.

The approval was granted by the Ethic's Committee at the Karolinska University Hospital, Stockholm and all patients and controls gave their informed consent to participate in the study.

### **Clinical assessment of patients**

Muscle performance (Functional Index (FI) of myositis) was assessed by the number of repetitions performed in defined muscle groups before treatment and at the time of the second biopsy. [30] The individual total score is presented as percentage of the maximal score 64 (mean values of left and right side). The responder criterion was set to 20% improvement.

### **Immunohistochemical analysis**

Staining of serial cryostat sections with mouse monoclonal anti-CD3 (BD Biosciences, San José, CA, USA), anti-CD68 (marker of monocyte/macrophage lineage, KP-1 clone, Dako Cytomation, Glostrup, Denmark) and anti-CD163 (resident tissue macrophage marker, BerMac3 clone, Dako Cytomation) antibodies was performed using a standard protocol. [31] Staining with rabbit polyclonal anti-human mPGES-1 antiserum [22], polyclonal anti-cPGES, anti-COX-2 and anti-COX-1 (Cayman Chemicals, Ann Arbor, MI, USA) and mouse monoclonal anti-COX-1 antibodies (Wako Chemicals, Neuss, Germany) was performed as previously described. [32] Staining in skeletal muscle tissue was abolished by pre-incubation of anti-mPGES-1 serum with mPGES-1 protein and by pre-incubation of commercial antibodies with respective blocking peptides (Cayman Chemical). Isotype-matched irrelevant antibodies were used as negative controls. The first and the last sections from each series of consecutive sections were stained with hematoxylin and eosin to evaluate the number of inflammatory infiltrates.

Stained tissue sections were examined using a Polyvar II microscope (Reichert-Jung, Vienna, Austria) and photographed with a digital Leica camera 300F (Leica, Cambridge, UK). The number of PGES and COX positive cells was assessed by conventional microscopy measurements of the entire tissue section (2-9 mm<sup>2</sup>) using semi-quantitative scale: (0) no staining; (1) few stained scattered cells; (2) many stained scattered cells; (3) many stained scattered cells and cells in one infiltrate; (4) strong staining in many scattered cells and several infiltrates. Evaluation of coded sections was performed by two independent observers. The mean scores from the two assessments were used for statistical analysis.

Expression of CD3, CD68 and CD163 was assessed quantitatively using computer-assisted image analysis. The images were analyzed with a Quantimet 600 image analyzer (Leica) and positive staining was expressed as percentage of total counterstained tissue area.

Double immunofluorescence was performed using anti-human mPGES-1 antiserum and mouse monoclonal anti-CD3, anti-CD68, anti-CD163 and anti-prolyl-4-

hydroxylase (fibroblast marker, 5B5 clone, Dako Cytomation) antibodies as previously published. [22]

### **Statistical analysis**

Data were analysed using Mann-Whitney's U test and Wilcoxon's signed rank test and Bonferroni corrections for multiple comparisons. P values <0.05 were considered statistically significant. Correlation between muscle FI and enzyme expression in muscle tissue was analysed using Spearman rank correlation test.

## **RESULTS**

### **Expression of PGES and COX**

A marked mPGES-1 staining localized in many scattered mononuclear cells and in mononuclear cells in infiltrates was observed in all patients with some inter-individual variations (fig 2A). For 6 patients mPGES-1 staining was also localized to smooth muscle cells in large vessels and for 10 patients to capillaries. In muscle tissue from healthy individuals, weak mPGES-1 staining was detected in a few scattered mononuclear cells and capillaries (fig 2B), as well as in smooth muscle cells in large vessels in two individuals. Using conventional microscopic assessment, we found that mPGES-1 expression in muscle tissue from myositis patients (n=12) was significantly higher ( $p<0.01$ ) when compared to healthy individual tissue (n=7) (fig 3).

Staining of cPGES was observed in scattered mononuclear cells, cells surrounding large vessels and muscle fibers both in myositis and healthy muscle tissues with a similar distribution pattern (data not shown). In two patient biopsies, cPGES positive cells were detected in inflammatory cells in infiltrates. The staining for cPGES in the myositis muscle was not significantly different when compared to healthy controls (fig 3).

COX-1 expression was detected in muscle fibers, blood vessels and scattered mononuclear cells both in patients and healthy subjects. In patients a strong COX-1 staining was additionally observed in mononuclear cells within the inflammatory infiltrates (fig 2C and 2D). COX-2 was expressed in macrophage-like cells within inflammatory infiltrates, in scattered mononuclear cells and in some large vessels in myositis muscle tissue. On the other hand, in healthy muscle tissue COX-2 staining was only detected in few scattered mononuclear cells and in some vessels (fig 2E and 2F). Both COX-1 and COX-2 expression was significantly enhanced ( $p<0.01$ ) in myositis muscle tissue when compared to healthy control (fig 3).

Double immunofluorescence revealed the expression of mPGES-1 in CD163-positive (fig 4A-C) and of CD68-positive macrophages (data not shown). However, we could not detect any mPGES-1 staining in T lymphocytes or fibroblasts (data not shown).

### **Effects of immunosuppressive treatment on PGES and COX expression**

The expression of mPGES-1 in myositis muscle tissue was not altered by the treatment (n=10) (fig 5A). In patients without infiltrates after treatment (4 out of 10), mPGES-1 staining was still apparent in scattered mononuclear cells. Likewise, the distribution pattern or score for cPGES and COX-1 positive cells remained unchanged (fig 5A). In contrast, the score for COX-2 positive cells in muscle tissue was significantly reduced ( $p<0.01$ ) after treatment (fig 5A and B). There was no

significant difference between polymyositis and dermatomyositis regarding the expression pattern of these enzymes before or after treatment.

### **Effects of immunosuppressive treatment on muscle histopathology**

Before treatment, eight out of ten patients presented infiltrates of mononuclear cells. After 4-11 months of immunosuppressive treatment the number of inflammatory infiltrates tended to be lower (median 0.52, range 0-2.6 infiltrates/mm<sup>2</sup> *versus* median 0.22, range 0-2.4 infiltrates/mm<sup>2</sup> before and after treatment, respectively), but this difference did not reach statistical significance.

Positive staining for CD3, CD68 and CD163 markers was detected in infiltrates, in scattered cells and in cells surrounding large vessels. The total number of monocytes/macrophages (CD68 positive cells) was significantly reduced after the treatment ( $p < 0.01$ ), while the positively stained area was not altered for T lymphocytes or CD163-positive resident macrophages (fig 5C).

### **Effects of immunosuppressive treatment on muscle function.**

Before treatment, muscle weakness in patients was confirmed by a reduced FI (median 38.4 %, range 22-65 %), performed in all but one patient (n=9). After 4-11 months of immunosuppressive treatment, a significantly increased FI was recorded (median 86.3 %, range 37.5-100 %,  $p < 0.05$ ), reflecting improved muscle function (fig 5D). On an individual basis, eight out of ten patients had improved by more than 20% in FI score. For one patient a decreased FI score was noted and for another the results of the first FI was not available. Despite the marked improvement in muscle function during the treatment period, a majority of the patients still had FI scores below the maximal value. Only one patient improved up to maximal score (100%).

There was no correlation between enzyme expression in muscle tissue and FI before or after treatment and the changes in enzyme expression did not correlate with changes in FI after treatment. In the patient who deteriorated clinically, the score for COX-2 expression was decreased after the treatment (from 2.5 to 1.5), in similar fashion to other patients who clinically improved.

## **DISCUSSION**

The present study demonstrates significantly increased expression of mPGES-1, COX-2 and COX-1 in skeletal muscle tissue from patients with polymyositis or dermatomyositis compared to that from healthy individuals. In healthy muscle tissue both COX-1 and cPGES proteins were detected in scattered mononuclear cells, in vessels and in muscle fibres, suggesting that these enzymes account for the basal production of PGE<sub>2</sub>. In healthy muscle tissue we also observed mPGES-1 and COX-2 staining in few scattered mononuclear cells and blood vessels, indicating constitutive expression of these enzymes in muscle tissue. Constitutive expression of mPGES-1 and COX-2 has been reported in other tissues e.g. kidney, suggesting their possible role for basal PGE<sub>2</sub> production under non-pathological conditions. [33, 34] Moreover, increased expression of COX-2 and mPGES-1 has been demonstrated in response to non-inflammatory stimuli, such as mechanical stretch and mechanical stress in certain cells. [8, 35, 36]

The observed enhanced expression of COX-1 and COX-2 in muscle tissue from myositis patients is in accordance with previously published data demonstrating

increased levels of COX-1 and COX-2 mRNA in inflammatory cells and vessels in muscle tissue from myositis patients. [13] Notably there have been no clinical trials addressing specifically the impact of selective or non selective NSAIDs in myositis patients. In addition, mPGES-1 expression was enhanced in myositis muscle tissue, both as an increased number of positively stained scattered mononuclear cells and as positively stained cells within inflammatory infiltrates compared to low constitutive expression in healthy muscle tissue. Using double staining, we identified CD68 and CD163-positive macrophages as the major cell types expressing mPGES-1. IL-1 $\beta$  and TNF are both strongly expressed in myositis skeletal muscle tissue [2, 3] and known to maintain mPGES-1 expression in macrophages, consequently contributing to the enhanced release of PGE<sub>2</sub> and inflammation in the muscle tissue.

We also examined the effects of conventional immunosuppressive treatment on muscle functional activity, muscle histopathology and expression of mPGES-1/COX in muscle tissues. Treatment resulted in significant improved muscle function in the majority of the patients. However, most patients still had impaired muscle function at the time of the second biopsy. Significant reduction in the total number of macrophages (CD68 positive cells) confirmed anti-inflammatory effects of the treatment. After treatment the COX-2 positive cells in muscle tissue were significantly decreased, while the expression of mPGES-1, cPGES or COX-1 was not suppressed. Down-regulation of COX-2 expression is one of the expected anti-inflammatory effects of GC and is associated with suppression of PGE<sub>2</sub> biosynthesis. However, there was no correlation between COX-2 expression in muscle tissue and FI before or after treatment, probably due to small number of observations.

The reduced number of COX-2 positive cells could be explained by GC dependent down-regulation of COX-2 expression and/or a significant reduction in total number of macrophages (CD68 positive cells). In contrast, resident tissue macrophages (CD163-positive cells) did not decrease significantly after treatment. It is tempting to speculate that the population of CD68 cells that decreased by the treatment constitutes cells that did not express CD163 or mPGES-1, as these molecules were not changed after treatment. A similar relative persistence of synovial CD163-positive resident tissue macrophages compared to infiltrating macrophages during anti-TNF treatment has been demonstrated in chronic autoimmune arthritis. [37] In addition, immunosuppressive treatment did not affect the number of T lymphocytes in myositis muscle providing a basis for persisting immune reaction that targets muscle fibers.

Interestingly, in some conditions mPGES-1 functionally utilizes PGH<sub>2</sub> generated by COX-1 [34, 38, 39]. The persisting expression of mPGES-1 and COX-1 in inflamed muscle tissue despite treatment might preserve PGE<sub>2</sub> production and contribute to chronic muscle inflammation. However, the role of the mPGES-1/COX-1 pathway in the overall PGE<sub>2</sub> production in muscle tissue remains to be elucidated.

Recent data suggest that COX-2-dependent PG synthesis is important for skeletal muscle regeneration. [40-42] While COX-2 inhibition reduces inflammation to a large extent due to the suppression of PGE<sub>2</sub> formation; it might impede the functional recovery of muscles via the suppression of other PGs. In this context mPGES-1 may constitute a more selective and safe therapeutic target than COX-2. Selective inhibition of mPGES-1 will allow for intact baseline PGE<sub>2</sub> production as well as intact production of other PG important for muscle regeneration and at the end



constitute a more preferable anti-inflammatory treatment than the currently used systemic GC.

In conclusion, we have demonstrated a significantly enhanced expression of mPGES-1, COX-2 and COX-1 in patients with polymyositis or dermatomyositis compared to healthy controls, suggesting its role in the pathogenesis of these diseases. Moreover, we have shown for the first time that conventional immunosuppressive treatment led to a significant down-regulation of COX-2 in myositis muscle tissue, while the expression of mPGES-1 and COX-1 remained unchanged. This persisting expression of mPGES-1 and COX-1 could possibly have a role in the chronicity of myositis.

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#### **COMPETING INTERESTS**

None

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## FIGURE LEGENDS

Figure 1. A schematic overview of the prostaglandin biosynthesis cascade.

Figure 2. Immunohistochemical staining (brown) for (A, B) microsomal PGES (mPGES)-1, (C, D) cyclooxygenase (COX)-1 and (E, F) COX-2 in representative muscle tissue sections counterstained with hematoxylin (A, C, E) from patients with polymyositis and (B, D, F) from healthy individuals (original magnification X500).

Figure 3. Expression of microsomal PGE synthase (mPGES)-1, cytosolic PGES (cPGES), cyclooxygenase (COX)-1 and COX-2 in muscle tissue from patients with polymyositis or dermatomyositis and from healthy individuals. Results are expressed as positive cell score (mean±SE). \*  $P < 0.05$ , patients *vs* healthy individuals.

Figure 4. Double fluorescence staining demonstrating cellular localization of microsomal PGE synthase (mPGES)-1: (A) mPGES-1 positive (green) cells, (B) CD163 positive (red) cells and (C) double stained (yellow) cells in representative muscle tissue section from the patient with polymyositis (original magnification X250).

Figure 5. (A) Expression of mPGES-1 and related enzymes in muscle tissues of patients with polymyositis or dermatomyositis before and after conventional treatment. Results are expressed as positive cell score (mean ± SE). (B) Expression of COX-2 in muscle tissues of patients with polymyositis or dermatomyositis before and after conventional treatment. Results are expressed as positive cell score. (C) Number of inflammatory cells in muscle tissue from polymyositis and dermatomyositis patients before and after conventional treatment. Results are expressed as percentage of the total area of counterstained tissue. (D) Effects of conventional treatment on muscle function in patients with polymyositis or dermatomyositis. Results are expressed as % of functional index (FI) for each patient. \*  $P < 0.05$ , before *vs* after treatment.

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Fig. 1

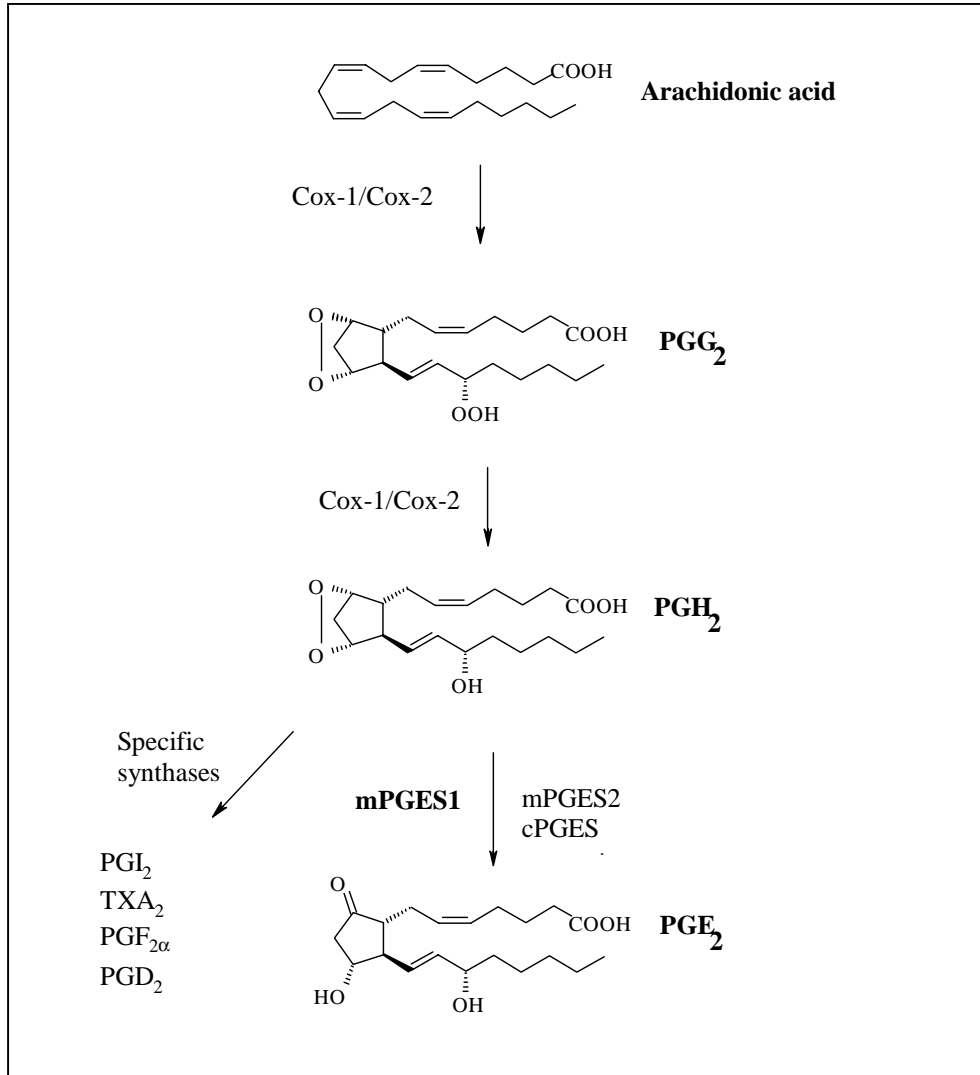


Fig.2

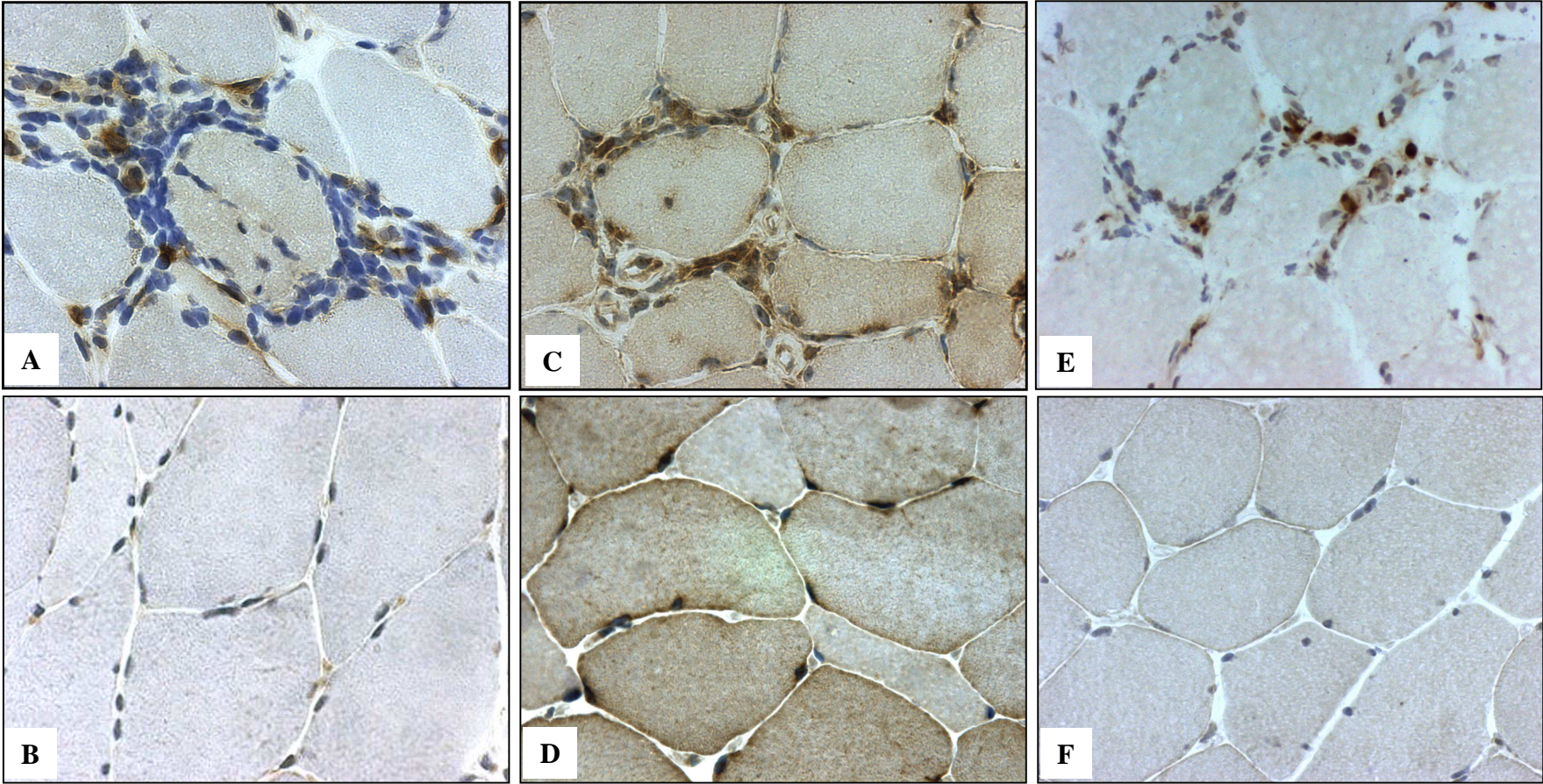


Fig.3

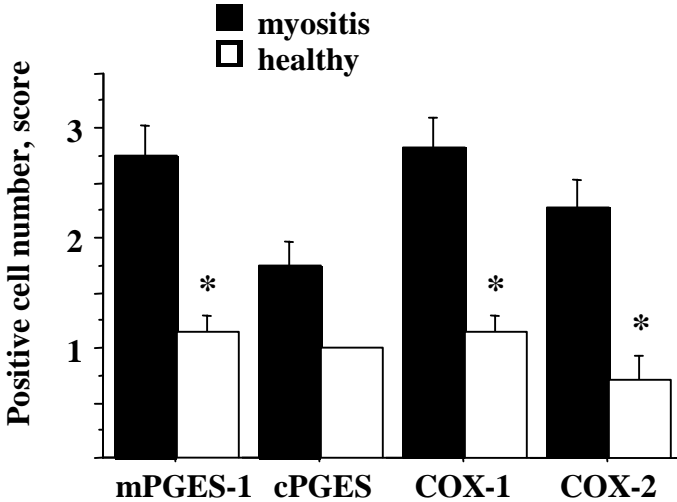




Fig.4

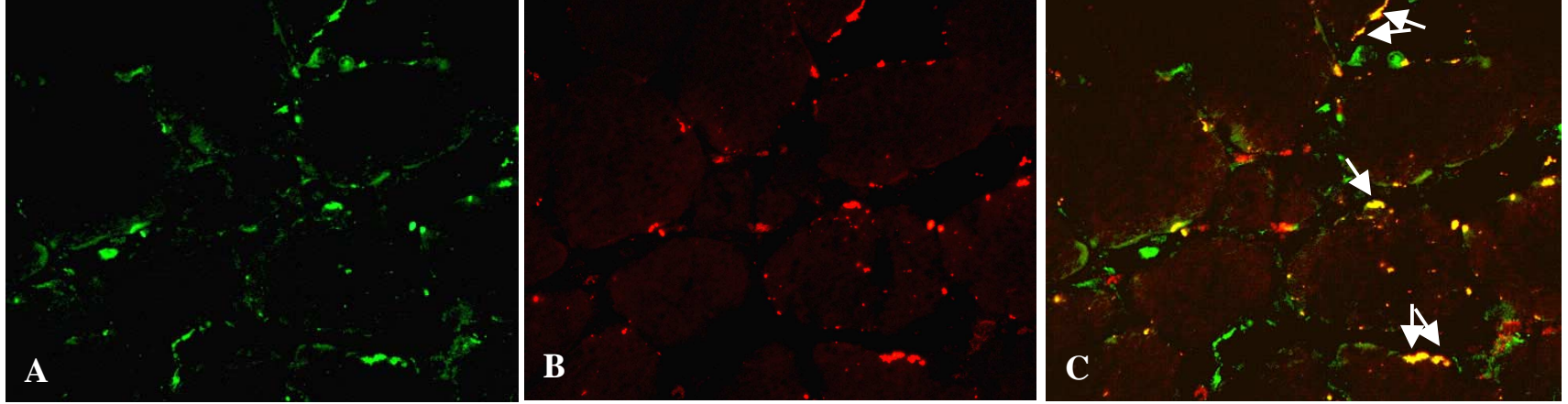


Fig.5

