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Gene Expression of Peroxisome Proliferator-Activated Receptor Is Upregulated by Nonsteroidal Anti-Inflammatory Drugs and Correlates with Cyclooxygenase-2 Suppression In Inflamed-Rat Muscle

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Abstract: *The peroxisome proliferator-activated receptors (PPARs) have been implicated in the regulation of endothelial cell inflammatory response. The purpose of the present study was to clarify the molecular mechanism of NSAIDs in controlling inflammation regarding the gene expression of PPARα and PPARγ1 in a rat model of chronic inflammation. Wistar rats were classified into 5 experimental groups; 9 rats each. Group (1) normal control; group (2) injected s.c. with 0.3 % carrageenan in muscle on days 1, 4 and 7. Groups (3, 4 and 5) were injected s.c. with carrageenan and at the same time given orally 10 mg/Kg Celecoxib, 12.5 mg/Kg Nimesulide or 10 mg/Kg sulindac, respectively. On day 7, edema was measured before scarification. Gene expression PPARγ1 and PPARα was measured in rat muscle by RT-PCR. COX-2 was analyzed in rat muscle by ELISA. Celecoxib produced the greatest % inhibition of carrageenan-induced edema. PPARγ1 and PPARα gene expression were significantly increased by NSAIDs treatment compared with carrageenan-untreated group. The inhibition of COX-2 together with upregulation of PPARα and PPARγ1 nominate NSAIDs to be promising candidates for pharmacologic treatment of tumorgenesis.*

Key words: *Celecoxib, Nimesulide, Sulindac, PPARα, PPARγ1, carrageenan.*

I. Introduction

Chronic inflammation is defined as inflammation of prolonged duration in which active inflammation, tissue destruction and attempts at healing are proceeding simultaneously **(1).** Chronic inflammation results in either repair by fibrosis or granuloma formation **(2)**. Nonsteroidal anti-inflammatory drugs (NSAIDs) are cyclooxygenase (COX) inhibitors frequently used in the treatment of acute and chronic inflammation. Side effects of NSAIDs are often due to their ability to induce apoptosis, gastric and duodenal ulcers and cytotoxicity in liver cells **(3).** The inhibition of COX-1 is responsible for the adverse effects of traditional NSAIDs (as sulindac) on the gastrointestinal mucosa, while their therapeutic benefits depend on the inhibition of COX-2. Therefore, COX-2 selective inhibitors (as Celecoxib and Nimesulide) were developed to reduce the adverse effects produced from the inhibition of COX-1 by conventional NSAIDs **(4).**

The peroxisome proliferator-activated receptors (PPARs) belong to the group of nuclear receptor superfamily. There are three subtypes of these receptors, PPARα, PPARβ/δ, and PPARγ. PPARs mainly regulate lipid and carbohydrate metabolism (**5)**. These receptors are also involved in inflammatory process, reproduction, carcinogenesis and other physiological processes in the body. PPAR activation inhibits inflammatory response genes and decreases the production of inflammatory mediators like IL-6, IL-2, TNFα, and COX-2, and also suppresses cells like T cell and macrophages. PPARα and PPARγ have been implicated in the regulation of endothelial cell inflammatory response and induction of apoptosis **(6).**

A wide variety of natural and synthetic compounds was identified as PPAR ligands. Among the synthetic ligands, the lipid lowering drugs; fibrates, and the insulin sensitizers; thiazolidinediones are PPARα and PPARγ agonists, respectively, which underscores the important role of PPARs as therapeutic targets **(7).** Interestingly, indomethacin and other NSAIDs that inhibit the production of prostaglandins are also able to activate PPARα and PPARγ **(8).**

The purpose of the present study was to clarify the molecular mechanism of NSAIDs in controlling inflammation regarding the gene expression of PPARα and PPARγ1 in a rat model of chronic inflammation. The relation between COX-2 inhibition and PPARs expression in rat muscle was also investigated.

II. Materials and Methods

Forty five Wistar rats (male and female), weighing 120-160 g, were utilized in the present study. The rats were obtained from the animal house of Faculty of Pharmacy and Drug Manufacturing, Pharos University, Alexandria. The animals were maintained in plastic cages at 25°C in animal house for two weeks for acclimatization and were allowed free access to water and food. The rats were fed bread and milk.

Rats were classified into 5 experimental groups; 9 rats each. Group 1: normal control group given the vehicle (polyethyleneglycol 400/saline 2:1 v/v, El-Amria and El-Nasr Companies). Group 2: inflammation control group injected s.c. with carrageenan (Sigma-Aldrich Inc. USA) 0.3 % in saline **(9)** on days 1, 4 and 7 **(10).** Group 3: Celecoxib group administered Celecoxib (El-Amria Company) 10 mg/Kg bw orally daily **(11).** Group 4: Nimesulide group administered Nimesulide (Cayman Chemical Co. USA) 12.5 mg/Kg bw orally daily **(12).** Group 5: Sulindac group administered Sulindac (Cayman Chemical Co. USA) 10 mg/Kg bw orally daily **(13).** Rats of groups 3, 4, 5 were subjected to carrageenan injection as in group 2 on days 1, 4 and 7, whereas the administration of drugs continued from day 1 to day 7.

Four hours after the 3rd injection of carrageenan, the edema was measured by caliber around the rat muscle of the carrageenan-treated leg. Twenty four hours after last treatment, the rats were sacrificed by cervical dislocation and then dissected. The gastrocnemius muscle was divided into three portions and kept at -80°C. The first portion was used for measurement of gene expression of PPARα **(14)** and PPARγ1 **(15)** by reverse transcriptase polymerase chain reaction (RT-PCR). The second portion was used to measure COX-2 by enzyme-linked immunosobent assay (ELISA) **(16)**. The third portion was embedded in 10% formaline (El-Gomhoria Chemical Company, Egypt) and utilized for histopathological examination.

2.1 Reverse transcriptase PCR

Total RNA was extracted from frozen muscle using Total RNA Extraction Kit (Bioer Technology, China). RNA (1µg) was reverse transcribed to give complementary DNA (cDNA) according to the manufacturer's instructions (The ProtoScript® AMV, First Strand cDNA Synthesis Kit, New England Biolabs, Inc.). cDNA was PCR amplified using 0.05 U/µL Taq DNA polymerase in a thermal cycler (Little Genius, Bioer, Germany). The Primers for amplification of PPAR-γ1 gene: (Forward): 5′-TGCTGGTGATCAGAAGGCTG3′. (Reverse): 5′ACGCAGGCTCTACTTTGAT CG-3′. The Primers for amplification of PPAR-α gene: (Forward): 5′-TGCATGTCCGTGGAGACCGTCAC-3′. (Reverse): 5′-ACTCGGTCTTCTTGAT GACC-3′. Initial pre-denaturation temperature was 94ºC for 1 min for one cycle. After that 35 cycles of the following program were carried out: denaturation step was at 94 ºC, for 1 min for the two genes. Annealing step was 55 ºC (1 min) for PPARγ1 and was 51°C (1 min) for PPARα. Extension step was 72°C (1 min) for the two genes, and a final extension step was carried out at 72°C (5-7 min).

The PCR product was then loaded onto 3 % agarose (Sigma-Aldrich Inc. USA) gel stained with ethidium bromide (Biobasic Inc. Canada) and the bands on the gel were visualized using UV transilluminator (Uvitec, EEC). The intensity of DNA bands were measured by photoshop version 7.

2.2 Measurement of COX-2 by ELISA

Citrate buffer (pH 5.5) was added to the muscle tissue (2:1) (v/w), which was then homogenized, then centrifuged for 10 min at 13,000 rpm (Baujahr centrifuge, Germany). The supernatant was used for estimation of COX-2 by ELISA using Rat COX-2 assay kit-IBL (Immuno-Biological Laboratories Co., Ltd.). The concentration of COX-2 in rat muscle was obtained from a preconstructed standard curve and was expressed as ng/g tissue.

2.3 Histopathological examination of rat muscle

The gastocnemius muscles were fixed in 10% formaline overnight. The tissues were dehydrated with alcohol then cleared in xylene. The tissues were embedded in warm paraffin wax, after cooling; the tissue hardens (blocks), and can be used to cut slices (sectioned). 4 µm sections were stained with hematoxylin & eosin (H&E). Then sections were investigated under light microscope (Olympus PX-41, Tokyo, Japan) using image analysis system under magnification X400. The tissues were investigated by a pathologist for the number of inflammatory cells present. The inflammation was evaluated as mild inflammation (+), moderate inflammation (++) or severe inflammation (+++).

2.4 Statistical analysis:

Data were fed to the computer using the Predictive Analytics Software (PASW Statistics 18). Quantitative data were described using mean and standard error. The comparison between two independent populations was done using independent t-test. Correlations between two quantitative variables were assessed using Pearson coefficient. Significance test results are quoted as two-tailed probabilities. Significance of the obtained results was judged at the 5% level.

III. Results

3.1 Effect of NSAIDs on carrageenan-induced edema in rat muscle

Subcutaneous injection of 100 µL of 0.3% carrageenan solution into gastocnemius muscle of rats resulted in edema formation. Treatment with Celecoxib, Nimesulide and Sulindac inhibited edema. The percent inhibition was 64.32 ± 5.13 % for Celecoxib, 61.43 ± 8.55 % for Nimesulide and 48.67 ± 3.97 % for Sulindac.

Celecoxib treatment significantly inhibited edema compared with Sulindac group. The anti-inflammatory activity of NSAIDs in the descending order was as follows: Celecoxib > Nimesulide > Sulindac (Fig. 1).

3.2 Effect of NSAIDs on gene expression of PPARγ1 and PPARα in rat muscle

The RT-PCR products of amplified PPARγ1 gene and PPARα gene were separated by gel electrophoresis where the bands of PPARγ1 appeared at 373 bp (Figure 2) and the bands of PPARα gene appeared at 523bp (Fig. 3). PPARγ1 gene expression showed significant decrease in carrageenan untreated group ($p < 0.05$) compared with normal control group (Fig. 4). The studied NSAIDs exhibited a significant increase in PPARγ1 gene expression ($\simeq$ 26.45 % ↑, $\simeq$ 18.9 % ↑ and $\simeq$ 62.35 % ↑, $p < 0.05$) in Celecoxib, Nimesulide and Sulindac group, respectively, compared with carrageenan untreated group (Fig. 4).
Treatment with Sulindac resulted in a significant increase in PPARγ1 gene expression *versus* each of Celecoxib group, Nimesulide group, and control group (Fig. 4).
PPARα gene expression significantly decreased in carrageenan untreated rats ($p < 0.05$) compared with normal control rats. The selected NSAIDs exhibited a significant increase in PPARα gene expression ($\simeq$ 26.6 % ↑, 18.5 % ↑ and $\simeq$ 21.5 % ↑, $p < 0.05$) in Celecoxib, Nimesulide and Sulindac group, respectively, compared with carrageenan untreated group (Fig. 5).

3.3 Effect of NSAIDs on COX-2 level in rat muscle

Carrageenan untreated group showed significant increase in COX-2 level ($p < 0.05$) compared with normal control group. Each of Celecoxib and Nimesulide produced a significant decrease ($p < 0.05$) in COX-2 level compared with carrageenan group. Sulindac treatment exhibited a significant increase in COX-2 level *versus* each of Celecoxib and Nimesulide groups ($p < 0.05$). COX-2 level in the NSAID-treated rats did not return to its level in the normal control group (Table 1).

3.4 Correlation study:

The correlation study revealed that there was a significant positive correlation between gene expression of muscle PPARα and gene expression of muscle PPARγ1 ($p = 0.012$, Figure 6). A significant negative correlation was observed between gene expression of muscle PPARα and each of muscle COX-2 ($p = 0.01$, Figure 7) and edema ($p = 0.005$, Figure 8). A significant negative correlation was found between gene expression of muscle PPARγ1 and edema ($p = 0.24$, Fig. 9).

3.5 Histopathological examination of rat muscle

Normal control group showed normal muscle fibers without inflammation (Figure 10A). Carrageenan-inflamed muscle showed moderate (++) to severe (+++) inflammation in between muscle fibers in the form of leukocytes, also there is dilatation and thickening of blood vessels (Fig. 10B).

Celecoxib treated rats showed mild (+) inflammation as the number of leukocytes was less than its number in carrageenan untreated group. The muscle fibers are normal without necrosis (Fig. 10C). Nimesulide treated rats showed mild (+) to moderate (++) inflammation with tissue granulation; the muscle is intact without necrosis (Fig. 10D). Sulindac treated rats showed moderate (++) to severe (+++) inflammation. Inflammatory cells present in between muscle fibers with thickening and proliferation of blood vessels (Fig. 10E).

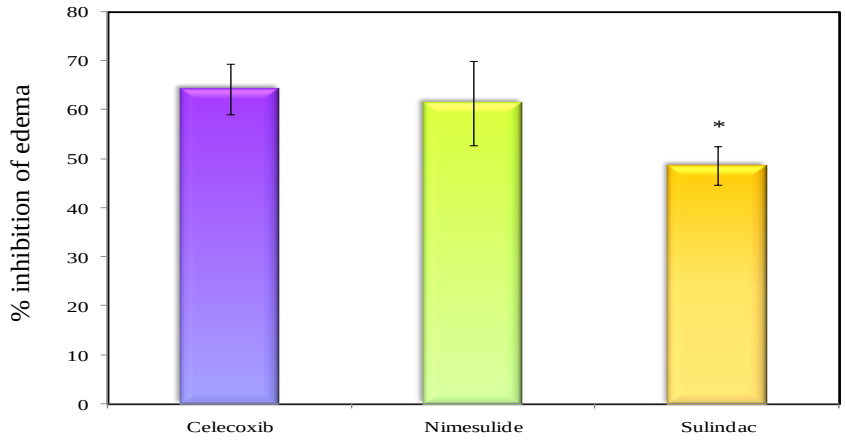


Figure (1): Effect of NSAIDs on carrageenan-induced edema in rat muscle
Data are presented as mean ± SEM, n= 9 for each group, *: significant versus Celecoxib

Table (1): Effect of NSAIDs on COX-2 level in rat muscle

Groups	Normal control	Carrageenan	Celecoxib	Nimesulide	Sulindac
COX-2 (ng/g)	10.67 ± 2.40	23.53[a] ± 1.83	16.32 [ab] ± 0.66	18.25[ab] ± 0.48	23.20[acd] ± 1.14

Data are presented as mean ± SEM; n= 9 for each group
a: Significant *versus* control group
b: Significant *versus* carrageenan group
c: Significant *versus* celecoxib group
d: Significant *versus* nimesulide group

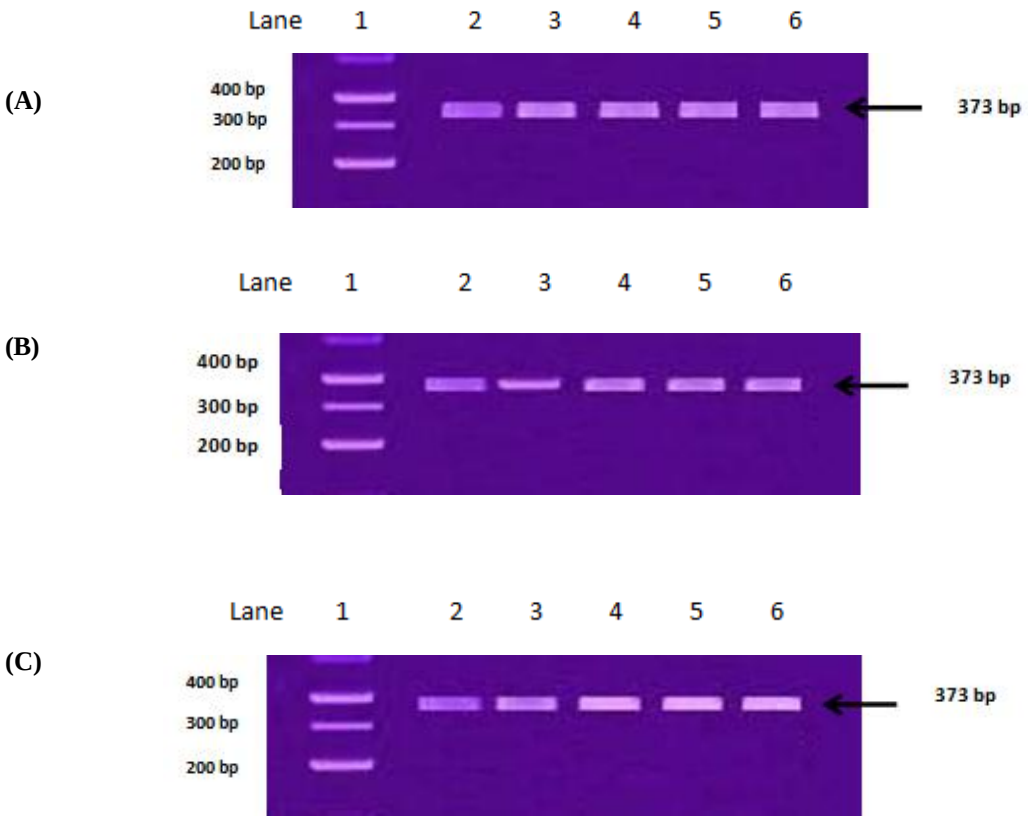


Figure (2): Ethidium bromide stained agarose gel showing bands of amplified PCR products of **PPAR □1 gene** of rat muscle. Lane 1: DNA marker, lane 2: carrageenan group, lane 3: normal control group, lanes 4, 5 and 6: NSAIDs-treated groups: **(A) Celecoxib** group, **(B) Nimesulide** group and **(C) Sulindac** group. Each lane represents pooled sample of muscles of three different rats.

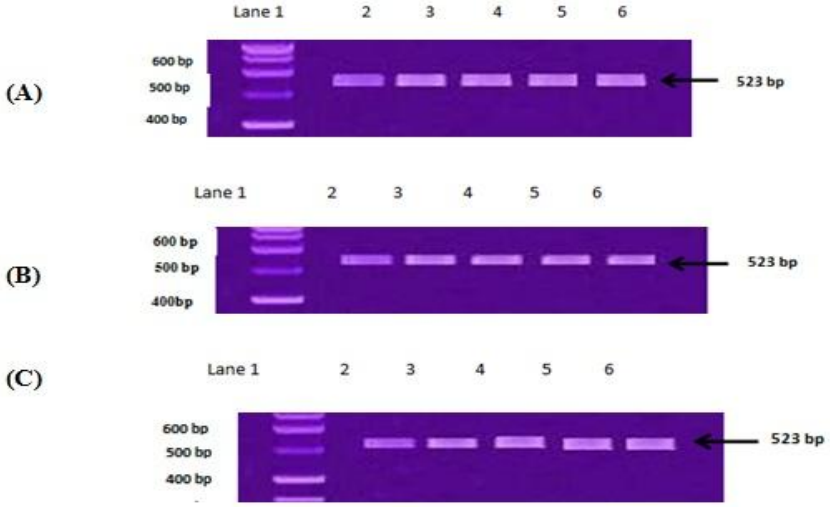


Figure (3): Ethidium bromide stained agarose gel showing bands of amplified PCR products of **PPAR gene** of rat muscle. Lane 1: DNA marker, lane 2: carrageenan group, lane 3: normal control group, lanes 4, 5 and 6: NSAIDs-treated groups: **(A) Celecoxib** group, **(B) Nimesulide** group and **(C) Sulindac** group. Each lane represents pooled sample of muscles of three different rats.

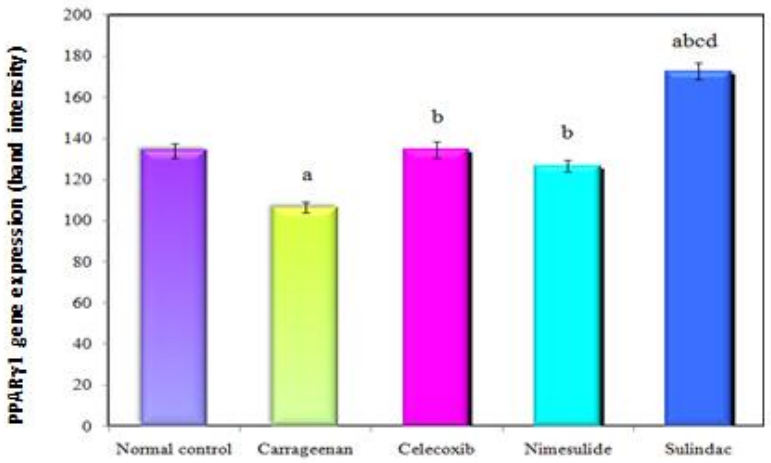


Figure (4): Effect of NSAIDs on PPARγ1 gene expression in rat muscle

Data are presented as mean ± SEM; n= 9 rats for each group
a: Significant *versus* normal control group
b: Significant *versus* carrageenan group
c: Significant *versus* celecoxib group
d: Significant *versus* nimesulide group

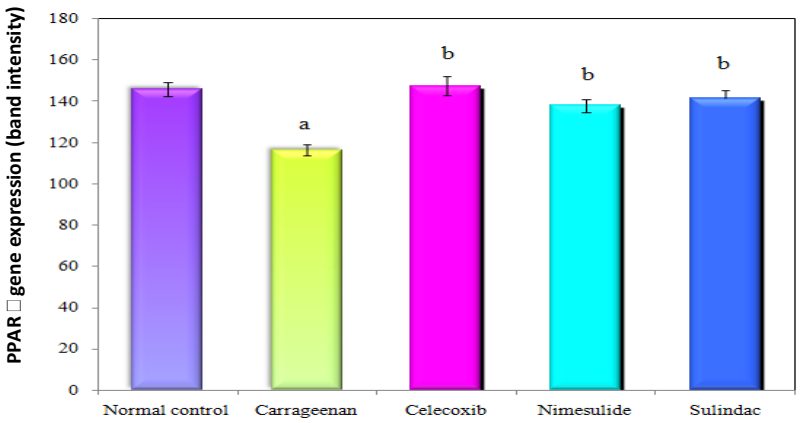


Figure (5): Effect of NSAIDs on PPAR*α* gene expression in rat muscle

Data are presented as mean ± SEM; n= 9 rats for each group
a: Significant *versus* normal control group
b: Significant *versus* carrageenan group

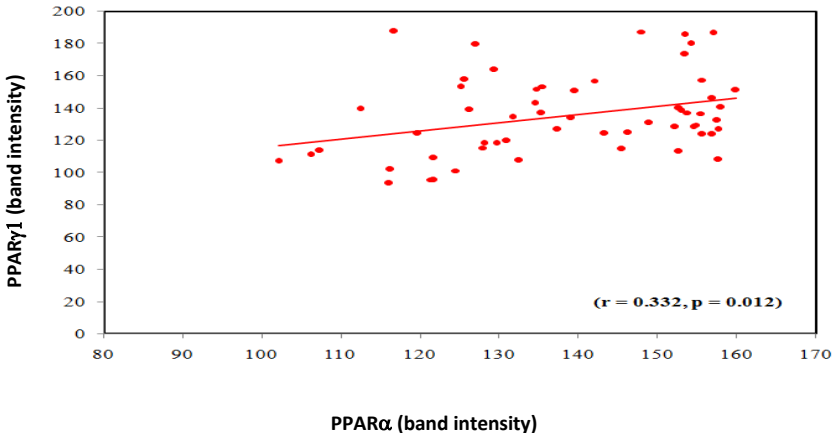


Figure (6): Correlation between gene expression of muscle PPAR □ and gene expression of muscle PPAR □1

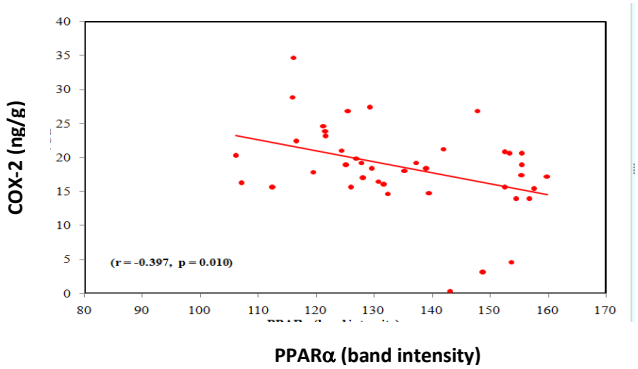


Figure (7): Correlation between gene expression of muscle PPAR andCOX-2

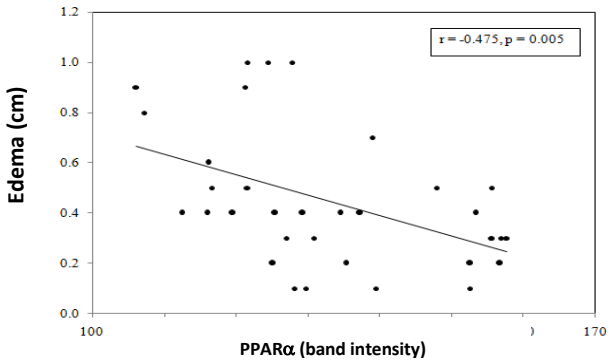


Figure (8): Correlation between gene expressions of muscle PPARα and edema

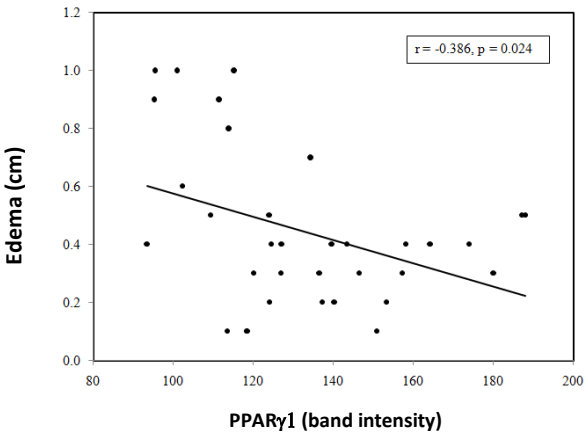
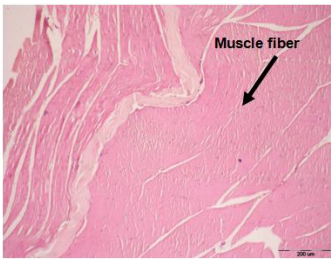
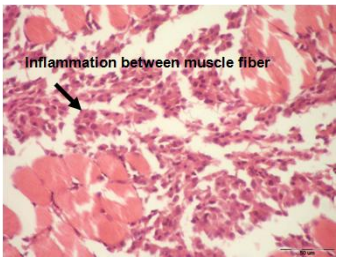


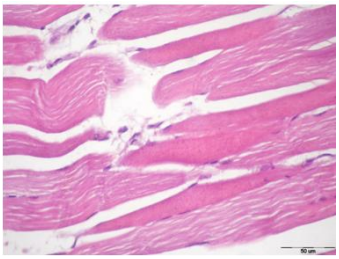
Figure (9): Correlation between gene expressions of muscle PPARγ1 and edema



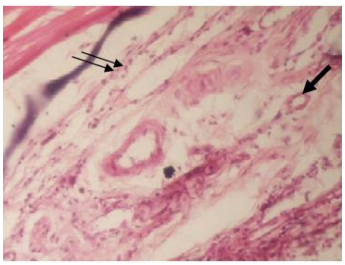
A. Rat muscle of control group showing normal muscle fibers (single arrow) and no inflammation (H&E X400).



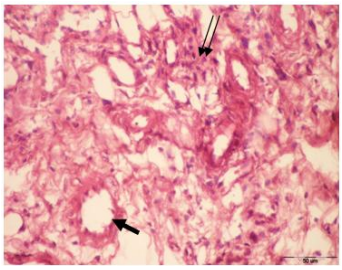
B. Rat muscle of carrageenan group showing moderate (++) to severe (+++) inflammation in between the muscle fibers in the form of leukocytes and dilatation in blood vessels (H&E X400).



C. Rat muscle of Celecoxib group showing mild (+) inflammation in between the muscle fibers. The muscle fibers are normal without necrosis (H&E X400).



D. Rat muscle of Nimesulide group showing mild (+) to moderate (++) inflammation with granulation tissue formation (double arrows), and newly formed blood vessels (single arrow). The muscle is intact without necrosis (H&E X400).



E. Rat muscle of Sulindac group showing moderate (++) to severe (+++) inflammation, infiltrate of inflammatory cells (double arrows) in between muscle fibers, thickening in blood vessels and vascular proliferation (single arrow) (H&E X400).

Figure (10): Photomicrographs showing histopathological changes of rat muscle

IV. Discussion

PPARα and PPARγ activators have been shown to induce differentiation, inhibit proliferation and regulate apoptosis in cancer cells **(17).** PPARα had been proven to have anti-inflammatory and anticarcinogenic action **(18).** More recent evidence implied an important role for the nuclear hormone receptor PPARγ in controlling various diseases based on its anti-inflammatory, cell cycle arresting, and proapoptotic properties **(19).**

The present study was conducted to elucidate the molecular mechanism of some NSAIDs, as selective and nonselective COX inhibitors, on gene expression of PPARα and PPARγ1 as well as COX-2 level in a rat model of chronic inflammation.

The present results showed that injection of carrageenan into the gastrocnemius muscle of rats three times per week produced edema with severe inflammation and histological changes in muscle fibers. Moderate to severe inflammation was observed in the form of leukocyte infiltration and dilatation of blood vessels. These

findings were in agreement with **(9),** who reported that carrageenan can be used as a model of chronic inflammatory hyperalgesia after 1–2 weeks. The carrageenan-induced inflammatory response has been linked to neutrophil infiltration and the production of neutrophil-derived free radicals, such as superoxide, hydroxyl radicals and hydrogen peroxide **(20).**

Administration of NSAIDs to carrageenan-treated rats decreased the severity of inflammation and reduced edema formation. The anti-inflammatory activity of the selected NSAIDs was arranged in the following descending order; Celecoxib > Nimesulide > Sulindac. The histological results supported these results. The NSAIDs exerted an anti-inflammatory effect where the muscles of rats showed mild inflammation in Celecoxib group, mild to moderate inflammation in Nimesulide group, and moderate to severe inflammation in Sulindac group.

The present results were in agreement with **(21),** who reported that Celecoxib, a selective COX-2 inhibitor, in a dose of 10 mg/kg was effective in reducing paw edema. **(22)** stated that Nimesulide is a multi-factorial drug in controlling inflammation and pain. The mechanism of anti-inflammatory activity of Nimesulide is related to the preferential inhibition of the production of COX-2 and other inflammatory mediators whose production is controlled by stimulation of cyclic-3, 5'-adenosine monophosphate (cAMP). **(23)** reported that Sulindac decreased rat paw carrageenan-induced edema formation to some extent but it was not the most effective NSAID tested for this purpose.

Measurement of COX-2 in rat muscle in the present work provided additional support. COX-2 was greatly increased in carrageenan group compared to normal controls. The percent decrease of COX-2 in Celecoxib group was greater than in the Nimesulide group than in the Sulindac group. These results may attribute to that Celecoxib is a selective COX-2 inhibitor, Nimesulide is a preferential selective COX-2 inhibitor and Sulindac is a nonselective COX inhibitor. Although COX-2 level in NSAIDs treated rats was significantly lower than in the carrageenan-untreated group, it remained higher than the normal control values**.**

(24) reported that injection of carrageenan increased both edema and COX-2 mRNA level. COX-2 is a major contributor to the inflammatory response and cancer progression and is an attractive target for molecular imaging **(25).** The higher expression of COX-2 in malignant tissues is also related to nuclear factor-kB (NF-kB), which positively regulates the COX-2 gene. Sulindac and Celecoxib efficiently suppressed the activation and the transcriptional activity of NF-kB, suggesting an anti-inflammatory role for NSAIDs in colorectal cancer **(26).** COX-2 expression has often been associated with the poor response to chemotherapy. The induction of proliferation arrest, alteration in cell cycle profile, and cell death by Nimesulide could be related to the downregulating effect of blocking COX-2 on cell survival proteins such as VEGF and IL-10 **(27).**

In the present work, PPARγ1 gene expression in muscle was significantly decreased in carrageenan group compared with control group. Treatment with NSAIDs upregulated PPARγ1, which was significantly increased compared with carrageenan untreated group. Nimesulide and Celecoxib treated rats showed gene expression of PPARγ1 as in the normal controls, whereas in the Sulindac treated group, PPARγ1 gene expression was about 1.4 fold as that in the normal control group. A significant negative correlation was found between PPARγ1 and edema. The expression levels of both PPAR and RXR mRNA have been found to be decreased in animal model with liver inflammation, indicating that PPARγ and RXR agonists may play an important role in response to inflammation and fibrosis (**28).**

The present results were in line with the reports that the protein expression of PPARγ was upregulated but COX-2 protein expression was downregulated in the Lewis lung carcinoma cells exposed to Celecoxib **(29).** Another evidence was provided by the findings that Celecoxib and a PPARγ agonist, separately, inhibited COX-2 and upregulated PPARγ expression. These effects were paralleled by inhibition of PGE_2 synthesis **(30).** Thus interference of the arachidonic acid pathway and upregulation of PPARγ simultaneously by Celecoxib have demonstrated great promise in cancer chemoprevention and treatment. In contrast, other studies showed that Celecoxib had no significant effect on PPARγ expression in hepatic stellate cells **(31).**

The upregulation of PPARγ1 by Nimesulide in the present work was confirmed by the work of **(32),** who found an intense immunohistochemical staining for PPAR-γ in tumor tissue sections from Nimesulide-treated group as compared with the negligible expression in control tumor. Our findings also revealed that Sulindac was a potent inducer of PPARγ1 gene expression in carrageenan-treated rat muscle. These results could be explained by the work of **(33)**, who demonstrated that Sulindac sulfide as well as its 2′-des-methyl derivatives are potent inducers of PPARγ, as the carboxylic side chain is required for activity; also it was found that non polar and aromatic substituents on the benzylidene ring in Sulindac structure lead to potent PPARγ agonists.

It is generally assumed that inflammatory bowel disease-related carcinogenesis occurs as a result of chronic inflammation. Thus immunomodulation by the PPARs ligands might contribute to inhibition of colitis and colon carcinogenesis. In addition, PPARα could suppress COX-2 induction **(34).** Several NSAIDs can bind to PPARα and PPARγ and are identified as PPAR ligands; thus activation of PPARs could contribute to anti-inflammatory effect of NSAIDs (**35)**.

Our results showed that PPARα gene expression in muscle was significantly decreased in carrageenan-untreated rats compared with control rat group. Treatment with NSAIDs resulted in significant increase in

PPARα gene expression compared with carrageenan-untreated group. In Celecoxib, Nimesulide and Sulindac treated rats, the gene expression level of PPARα was returned to near its normal level in the control group. In addition, PPARα gene expression showed a significant positive correlation with PPARγ1 gene expression and a significant negative correlation with each of edema and COX-2 level in muscle.

These results were in agreement with **(36),** who stated that expression of PPARα was found to be significantly higher in cells treated with higher doses of NSAIDs as Celecoxib, Nimesulide, Sulindac and indomethacin. Thus, PPARα mediates the cell growth modulatory effects and contributes to the mechanisms underlying the chemopreventive effects of NSAIDs.

The research conducted by **(37)** revealed that the pro-inflammatory cytokines IL-6, TNFα and IL-1 cause a reduction in the expression of PPARα, and that the decrease in PPARα expression and function may contribute to the excessive host inflammatory response. It has been documented that treatment with appropriate doses of PPARα agonists can inhibit inflammatory diseases development **(38).** The antinociceptive effects of Nimesulide in carrageenan model of inflammatory hyperalgesia may be mediated by PPARα **(39).**

V. Conclusion

The anti-inflammatory effect of NSAIDs was mediated by upregulation of PPARγ1 and PPARα genes. Celecoxib showed the highest potency as anti-inflammatory and COX-2 inhibition, whereas Sulindac exhibited the greatest effect as PPARγ1 inducer. NSAIDs could be considered promising candidates for pharmacologic treatment of tumorgenesis.

Acknowledgments

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