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PGlyRP3 concerts with PPARγ to attenuate DSS-induced colitis in mice

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ABSTRACT

Nutrients may modulate immunity through their transcription factors that act on both metabolic and immunity genes. It has been shown that the transcription factor of lipid ligands PPARγ physically binds the gene promoter of the peptidoglycan recognition protein (PGlyRP3), which showed anti-inflammatory action *in vitro*. It is hypothesized in the present work that olive oil feeding protects against toxicity of DSS-induced colitis *via* activation of the lipid transcription factor PPARγ that stimulates the anti-inflammatory PGlyRP3. Results: PGlyRP3 is expressed in mouse colon and up-regulated by olive oil feeding. Olive oil reduced mortality and severity scores of DSS-induced colitis and down-regulated the proinflammatory IL-1b, IL-6 and TNFα genes. This protective effect was accompanied by up-regulation of both PPARγ and PGlyRP3. Inhibition of PPARγ by its antagonist BADGE down-regulated PGlyRP3 and abolished the anti-inflammatory effect of olive oil feeding in this DSS-induced colitis model, reflecting the pivotal role of PPARγ binding nutrition and inflammation. Activation of PGlyRP3 by its ligand peptidoglycan was not responsible for the inflammation caused by peptidoglycan, since neutralization of TLR2 attenuated this inflammatory response without affecting the peptidoglycan-induced PGlyRP3 level. Olive oil activated the IκBα and inhibited NF-κB and cox-2 gene expressions, and p65 nuclear translocation in DSS-colitis mice, reflecting the involvement of the inhibition of NF-κB signaling pathway in the anti-inflammatory olive oil - PPARγ - PGlyRP3 access. This pathway was reactivated by the PPARγ antagonist BADGE. Conclusions: Olive oil regulates by the same transcription factor (PPARγ) both lipid metabolic and immune gene (PGlyRP3) expressions, exerting the anti-inflammatory effect, and protecting against DSS-induced colitis in mice.

1. Introduction

The chronic inflammation of the gut epithelial surface in inflammatory bowel diseases (Crohn's disease and ulcerative colitis) was found to be modulated by lipids. Omega-3 fatty acids or increased n-3 PUFA in transgenic mice that endogenously biosynthesize n-3 PUFA effectively reduced inflammation and tissue injury in colitis [1,2]. Dietary extra virgin olive oil was many times reported [3–8] to attenuate both acute and chronic inflammatory responses in DSS-induced colitis model. On the other hand, saturated fatty acids [9], and milk fat feeding [10,11] activated proinflammatory signaling pathways, may be through dysbiosis of the intestinal microbiota [12].

Lipophilic ligands may modulate inflammation and immunity through activation of their transcription factors as the nuclear receptor superfamily named peroxisome proliferator activated receptors (PPARs). Activation of PPARγ is involved in the regulation of intestinal inflammation and reduction of the severity of inflammatory Bowel diseases and experimental colitis [13–18]. This makes PPARγ, the nuclear transcription factor of lipophilic ligands, one of the most important anti-inflammatory molecules in the intestine. However, the mechanism of its anti-inflammatory action is not fully understood.

In previous studies of our group [19–21], we hypothesized that PPARγ may bind the promoter region and activate some immunity-modulating genes, so that lipophilic ligands could modulate inflammation and immunity. *In silico* analysis has shown that the promoter of PGlyRP3 gene has some PPRE regions, and physically binds by PPARγ and RXR [20]. PGlyRP3 protein recognizes peptidoglycan and constitutes a part of the innate immune system. This binding activated the PGlyRP3 to trigger an anti-inflammatory action in the intestinal Caco2 cell line *in vitro*. This action was in response to both dietary and microbial fatty acids. Other ligands of PPARγ were also able to mediate the anti-inflammatory action of PGlyRP3. Thus, prebiotic

Abbreviations: BADGE, Bisphenol A diglycidyl ether; Cox-2, Cyclooxigenas-2; DGAT1, Diacylglycerol *O*-acyltransferase 1; DSS, Dextran sodium sulphate; FABP2, fatty acid binding protein 2; IκBα, Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor alpha; IL, Interleukin; LPS, Lipopolysaccharide; NF-κB, Nuclear factor kappa-light-chain-enhancer of activated B cells; NODs, Nucleotide-binding oligomerization domain proteins; PGlyRP3, Peptidoglycan recognition protein 3; PGN, Peptidoglycan; PPARγ, Peroxisome proliferator-activated receptor gamma; PPRE, PPAR-response element; PUFA, Poly unsaturated fatty acids; TLR2, Toll-like receptor 2; TNFα, Tumor necrosis factor alpha

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Table 1
Primer sequences for real-time RT-PCR.

Nucleotide	Accession no.	Sense primer	Antisense primer	Product size (bp)
PGlyRP3	NM_207247.4	TATCACACCACGGTCTGCTT	CCATCCTGGCCCACTAGAAA	210
PPARγ	NM_011146.3	TTTCAAGGGTGCCAGTTTCG	TGAATCCTTGGCCCTCTGAG	199
Dgat1	NM_010046.3	GTGGCCAGGACAGGAGTATT	CATACATGAGCACAGCCACC	214
FABP2	NM_007980.3	TGTTGTGTTTGAGCTCGGTG	ACCAGAAACCTCTCGGACAG	154
IL-1b	NM_008361.4	ACTCATTGTGGCTGTGGAGA	TTGTTCATCTCGGAGCCTGT	199
IL-6	NM_031168.2	CCTCTGGTCTTCTGGAGTACC	GTCCTTAGCCACTCCTTCTGT	220
TNFα	NM_013693.3	AGCACAGAAAGCATGATCCG	CTGATGAGAGGGAGGCCATT	212
IkBα	NM_010907.2	TTGGTCAGGTGAAGGGAGAC	GCGTCAAGACTGCTACACTG	213
NF-κB1	NM_008689.2	CGGGAGGGGAGGAGATTTAC	TGAACAAACACGGAAGCTGG	208
COX-2	NM_011198.4	ATCATAAGCGAGGACCTGGG	TCAGGGATGTGAGGAGGGTA	205

oligosaccharides [19] reduced inflammatory cytokines in Caco2 cells *in vitro via* PPARγ-dependent PGlyRP3 activation. Peptidoglycan (PGN) is the main constituent of the bacterial cell wall, and recognized by several pattern recognition proteins (PRPs) such as PGlyRPs, TLR2 and NODs [22]. PGN itself is an inflammatory molecule, and exerts its inflammatory action mainly by TLR2 and NODs' recognition and activation of their inflammatory signaling cascade. Neutralization of TLR2 cascade by inhibition of MyD88 reduced PGN inflammatory action in the same Caco2 cells. As well, overexpression of PGlyRP3 suppressed the expression of PGN-induced inflammatory cytokines [21]. Thus, these previous studies [19–21] revealed that PGlyRP3 is anti-inflammatory in nature and is a good model of studying how lipids modulate immunity. Other studies also confirmed the anti-inflammatory action of PGlyRP3 in DSS-induced colitis [23,24]. However, they referred this anti-inflammatory action to the promotion normal intestinal microflora and prevention of induction of interferon-gamma.

The objective of this study was to use the DSS-colitis mouse model to examine the same hypothesis *in vivo* that lipophilic ligands (contained in olive oil) exert their inflammatory-protective effect by activating their transcription factor PPARγ, which in turn activates a part of the innate immunity system (the pattern recognition protein PGlyRP3) that triggers an anti-inflammatory action.

2. Material and methods

2.1. DSS-induced colitis model

Experimental colitis was induced according to the protocol described by Chassaing et al. [25]. Mice have been administered 2.5% DSS (MP Biomedicals) in drinking water for 7 days. Disease severity was evaluated using a point score system consisting of a) stool consistency (pellets, 0; loose, 1; and watery, 2); b) rectal bleeding (no bleeding, 0; slight bleeding, 1 and bloody, 2) and c) body weight loss (± 2% of original weight, 0; < 10% loss, 1 and > 10% loss, 2). Thus the maximal severity in an individual scored 6 and this score decreased in less severe cases.

2.2. Animal handling and grouping

Fifty 8 weeks old male C57Bl/6 mice were used in this study. Animals were grouped as follows: (1) Five mice were continued on normal chow and water *ad libitum* and served as a negative control group. (2) Thirty mice were fed on ordinary chow that was previously blended with 20% w/w of extra virgin olive oil from a local supplier. Experimental colitis was induced in 20 mice of this group. Both DSS-induction of colitis and olive oil feeding started simultaneously on the same day. Five mice of the olive oil-fed group and 10 mice of the DSS + olive oil feeding group were treated with the PPARγ antagonist Bisphenol A diglycidyl ether (BADGE, Tocris Bioscience). BADGE was daily intraperitoneally administered at a dose of 30 mg/kg body weight throughout the 7 days. (3) Ten mice were administered 500 μg of *Staphylococcus aureus* PGN (Sigma), suspended in 0.5 ml water, per oral gavage three times over 7 days as described by Woolverton et al. [26]. (4) Five mice were treated with DSS to induce colitis as described above. These mice, together with 5 others of the PGN-treated group, were injected in the tail vein with 1 microgram of anti-TLR2 antibody (T2.5, ab16894, Abcam) on the third day of treatment start point. Methods and doses of different treatments were taken from the literature [26–29]. Samples for RNA and protein analyses were extracted directly on the 8th day, while survival and morphological analyses continued for another week (until the 15th day, if possible).

2.3. RNA extraction and quantitative real-time PCR

Total RNA was extracted from the colon tissue samples using GeneJET RNA extraction kit (Fermentas). For reverse transcription, 2 μg of the total RNA was reverse transcribed to first strand complementary DNA in 25 μl reactions using the High-Capacity reverse transcription kit (Applied Biosystems). Quantitative RT-PCR analysis was performed on a quantity of cDNA that corresponded to 20 ng RNA. Quantitative PCR analysis was performed using SYBR Green (Power SYBR green Mastermix, Applied Biosystems) on the real-time PCR instrument (ABI Prism 7000, Applied Biosystems). The thermal cycling program was 10 min at 95 °C for enzyme activation, denaturation for 15 s at 95 °C, 60 s annealing at 60 °C. A dissociation curve was performed for each product to assure the absence of primer dimers or unspecific products. Specific primers used in the present study for studied genes are listed in Table 1. To normalize expression data, β-actin was used as an internal control gene. Relative quantification was performed by ΔΔCt method, in which ΔCt was the difference in the cycle threshold between the target gene and internal control, and ΔΔCt was the difference between the ΔCt values of the test sample and the control. The relative expression of a target gene was calculated as $2^{-\Delta\Delta Ct}$. Data are presented as fold change from control, considering the control values as 1.

2.4. Western blot analysis

Colon samples were lysed using PhosphoSafe lysis buffer (Novagen/Merck) and centrifuged at 13000 × *g* for 15 min at 4 °C. Protein concentration was determined in the supernatant by bicinchoninic acid assay (BCA, Thermo Fisher Scientific) and equal protein quantities of different lysates were separated by SDS-PAGE electrophoresis before blotting to PVDF membranes (Immobilon P). Protein-carrying membranes were then probed with different primary antibodies and immunoreactive bands were detected by chemiluminescence (Pierce). Primary antibodies used were anti-PGlyRP3 (ARP66426_P050, Aviva), anti-β-actin (Sigma), anti-PPARγ (sc-271,392, Santa Cruz), anti-TLR2 (ab16894, Abcam) and anti-P65 (DLN-012809, Dianova). Some blots were subjected to densitometric analysis using the NIH imageJ software. In most experiments, western blot membrane has been exposed to stripping and re-staining to measure many proteins in the same

samples. For this purpose, a stripping solution (15 g/l glycine, 1 g/l SDS, 10 ml tween 20/l, pH 2.2) has been used. PVDF membranes have been incubated 2×, 10 min in stripping solution, then washed 2 × 10 min in TBST. Membranes were then processed for a new primary antibody.

2.5. Statistical analysis

Data were presented as means ± SEM (standard error of mean). Statistical differences between different groups were investigated using one or two way analysis of variance (ANOVA) followed by *t*-test for multiple comparisons as a post-hoc test. Student's *t*-test was used to compare the difference between 2 groups. The p values of ANOVA were shown in the results section or figure legends. A p values of < 0.05 was considered statistically significant in *t*-test comparisons.

3. Results

3.1. PGlyRP3 is produced in the colon and activated by olive oil

It was first sought to determine the expression of PGlyRP3 in the colon tissue. For this purpose, protein was extracted from the mice colon tissue and also from esophagus and muscle for comparison, since PGlyRP3 is known to be highly expressed in esophagus and not expressed in muscular tissue. PGlyRP3 is found to be weakly produced in the colon (Fig. 1A) as determined by Western blotting, when compared with its expression in the esophagus. Densitometric analysis using the imageJ revealed that its protein expression in the colon was < 1/10th of that in the esophagus. This weak expression was highly amplified when mice were exposed to peptidoglycan (the main ligand of PGlyRPs) or fed on a diet containing olive oil (Fig. 1B). RNA was extracted from colons of both groups and some genes of lipid metabolism (FABP2, PPARγ and DGAT1) have been determined by quantitative RT-PCR. In a separate group, it was found that PGN exposure did not affect the gene expression of all of these genes, as olive oil did (Fig. 1C). Thus, both PGN and olive oil induce the expression of PGlyRP3, but PGN has no effect on lipid metabolism genes expression.

3.2. Olive oil feeding attenuates the DSS-induced colitis

Next, the effect of olive oil on the development of DSS-induced colitis has been examined. Mice have been simultaneously exposed to both DSS in drinking water and olive oil merged with the chow for 7 days. Other groups were exposed to either DSS or olive oil only for the same period to serve as positive control groups. Mice survival, body weight, rectal bleeding and stool state have been recorded daily. Only 3 animals from each group have been sacrificed on the 8th day and colon samples have been subjected to RNA and protein extractions and examination by RT-PCR and Western blotting. The results (Fig. 2) revealed that DSS caused mortality that began on the 7th day of treatment and continued so that all mice of this group died within 13 days (Fig. 2A). From the 6th day after DSS treatment, body weight loss was significantly different from the starting value and individual day body weight values were significantly lower than the corresponding values of olive oil fed group (ANOVA $p < 0.003$, Fig. 2B). Some oily diarrhea was observed in some individuals of the olive oil-fed group, but no other signs of colitis have been observed in this group. Point-scored colitis severity was significantly observed from the 4th day in the DSS-treated group (ANOVA $p < 0.001$, Fig. 2C).

Addition of olive oil to the diet during the development of DSS-induced colitis attenuated the severity of the colitis. However, olive oil did not prevent the colitis to develop, since severity score values were still significantly higher than the olive oil group values from the 6th day. However, the individual day severity scores in this group were significantly lower than that of the DSS group (ANOVA $p < 0.01$ between different groups, Fig. 2C). Similarly, Olive oil feeding increased the survival (6/10 has survived until the end of recording at day 15, Fig. 2A), and reduced significantly the weight loss (Fig. 2B).

The attenuation of the DSS-induced colitis by olive oil was confirmed on the gene expression level of inflammatory cytokines (Fig. 2D). The mRNA level of IL-1b, IL-6 and TNFα were elevated to great extents in colon tissue after the 7-day-treatment with DSS. Simultaneous feeding with olive oil during the development of DSS-induced colitis model reduced this elevation significantly, but not to the control levels (one way ANOVA $p < 0.00001$ for the 3 tested inflammatory cytokines, Fig. 2D).

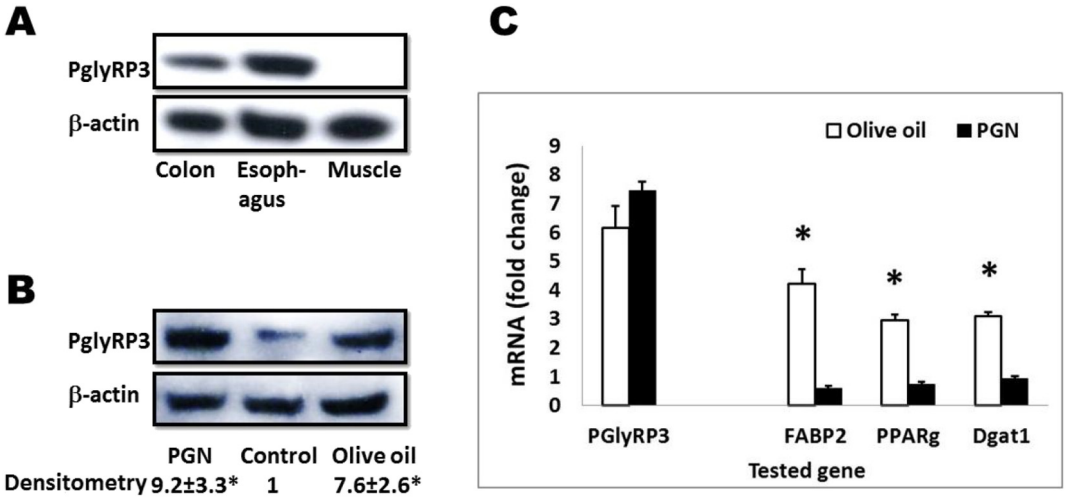

Fig. 1. PGlyRP3 expression in mouse colon is up-regulated by olive oil. Mice were treated with PGN or olive oil as described in the Material and Methods section and their colons were subjected to protein and mRNA analysis by western blotting and qRT-PCR, respectively. (A) PGlyRP3 protein expression in control mouse colon. Samples from the esophagus and abdominal muscles served as positive and negative controls, respectively. (B) Both peptidoglycan (PGN) and olive oil up-regulate PGlyRP3 expression in mouse colon. Densitometric analysis by ImageJ shows relative values (mean ± SEM) to the control, N = 3, * denotes significantly higher than the control (pair comparisons only, *t*-test). (C) Quantitative RT-PCR showing up-regulation of PGlyRP3 gene expression by both olive oil and PGN and of lipid metabolism genes FABP2, PPARγ and Dgat1 only by olive oil. Data are presented as mean ± SEM of N = 3. Statistical analyses: * denotes significantly different values of olive oil and PGN groups (*t*-test).

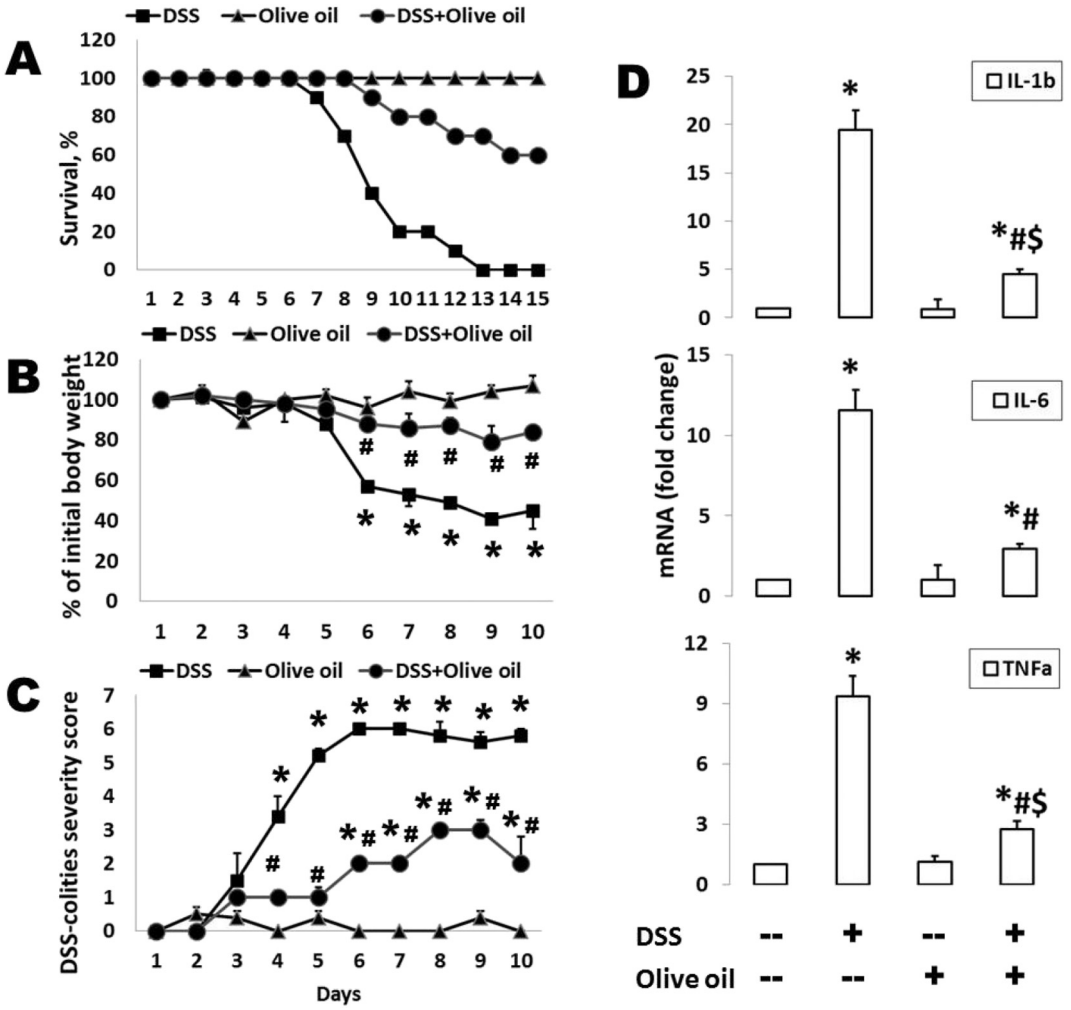

Fig. 2. Olive oil feeding attenuates the DSS-induced colitis in mice. Groups of mice were fed on DSS for 7 days to induce colitis or simultaneously on DSS and olive oil. Other 2 groups were fed on normal chow or olive oil only and served as controls. (A) Olive oil feeding decreases the mortality caused by DSS-induced colitis. (B) Reduction of body weight loss in DSS-induced colitis mice by olive oil feeding. (C) Olive oil feeding reduces the severity of DSS-induced colitis. The severity score is calculated depending on the severity of diarrhea, rectal bleeding, and body weight loss. (D) Olive oil feeding reduces the gene expression of the proinflammatory cytokines IL-1b, IL-6, and TNF-α as determined by qPCR. Data are presented as mean ± SEM. Statistical analyses: ANOVA denoted significance in all cases, * = significantly different from the basic value or the negative control value, # = significantly different from the corresponding DSS group value, $ = significantly higher than the corresponding olive oil-fed control group value (*t*-test after ANOVA).

3.3. PGlyRP3 and PPARγ are involved in the protective role of olive oil against DSS-colitis

The mRNA and protein levels of PPARγ, and PGlyRP3 were analyzed in the colon tissues of the different groups described above (Fig. 3A). PPARγ mRNA expression, which increased as expected in response to the lipophilic ligand, was found to be decreased in response to DSS. However, simultaneous feeding with olive oil and DSS kept the PPARγ level similar to that of olive oil only fed group (ANOVA $p < 0.0001$ between different groups, *t*-test $p < 0.05$ for each 2-group comparison). DSS did not affect the mRNA expression of PGlyRP3 in colon tissue, but olive oil did. The PGlyRP3 level was kept elevated in the olive oil-DSS-fed group. Identical results were obtained on the protein expression level.

Thus, both PGlyRP3 and PPARγ mRNA and protein expressions were positively responsive to olive oil. To test that the change in PGlyRP3 level is PPARγ-dependent, a group of mice was fed similarly on olive oil for the same 7 day period and injected daily with 30 mg/kg of the PPARγ antagonist BADGE. It was found that BADGE treatment decreased the protein level of both PPARγ and PGlyRP3 in the colon tissue (Fig. 3B), despite olive oil feeding. This finding indicates that PPARγ inhibition down-regulates the expression of PGlyRP3 in the mouse colon.

To study the role of this PPARγ-dependent regulation of PGlyRP3 in the development of DSS-induced colitis in mice, a group of mice was fed on DSS + olive oil and treated daily with BADGE. The results of this group were compared with that of DSS and DSS + olive oil groups. In fact, the colitis severity and mortality results of this group were worse than that of DSS group. After excluding the 3 mice samples used for RNA and protein analyses, the rest 7 mice died within 11 days, compared with 13 days for the DSS group. Body weights (Fig. 3C) and severity scores (Fig. 3D) of individuals of this group were statistically similar to that of DSS group and significantly different from DSS-olive oil-fed group (ANOVA $p < 0.01$ for both figures). These results support that PPARγ is responsible for the up regulation of PGlyRP3 and the anti-inflammatory role against DSS-induced colitis in mice.

3.4. PGlyRP3 is not responsible for the inflammation in mice colon

Neutralization of TLR2 has been previously shown to suppress the PGN-induced inflammatory response in Caco2 cells. Silencing of PGlyRP3 in that case re-activated the expression and secretion of

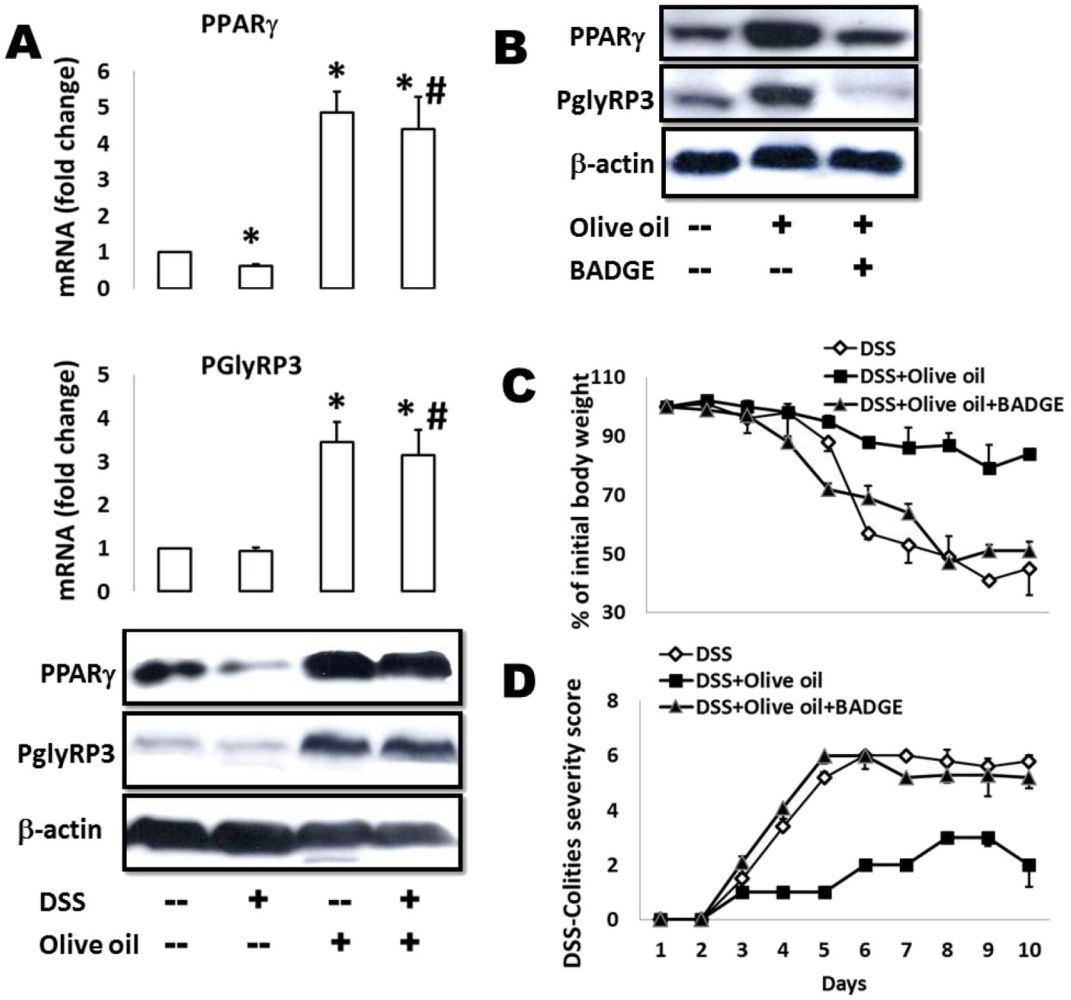

Fig. 3. PPARγ and PPARγ-dependent PGlyRP3 activation are involved in the protective role of olive oil against DSS-induced colitis. (A) Parallel activation of the gene expression of PPARγ (upper panel), PGlyRP3 (middle panel) and protein expression of both (lower panel) by olive oil feeding in DSS-induced colitis mouse model. (B) Inhibition of PPARγ and PGlyRP3 protein expression in colons of mice fed on olive oil and treated with the PPARγ inhibitor BADGE. Inhibition of PPARγ by BADGE abolished the attenuating effect of olive oil diet on body weight loss (C) and disease severity score (D) in DSS-colitis mice. Data are expressed as mean ± SEM of N = 3 for protein and PCR groups. Statistical analyses: * = significantly different from the control value, # = significantly different from the corresponding value of DSS group (*t*-test after ANOVA).

different inflammatory cytokines [21]. Knocking down of Pglyrp3, especially when combined with Nod2, resulted in higher severity to DSS-induced colitis *in vivo* [23,24]. These experiments supported the anti-inflammatory role of PGlyRP3. In the present experiment, groups of mice were treated with either PGN or PGN + anti-TLR2 (Fig. 4A and B). PGN increased the protein production of both TLR2 and PGlyRP3 in mice colon. Treatment with anti-TLR2 antibodies suppressed the production of TLR2, but did not alter the activated PGlyRP3. Neither PGN nor anti-TLR2 treatments affected the PPARγ protein level (Fig. 4A). This means that PGlyRP3 may be activated by both the inflammatory PGN and the anti-inflammatory PPARγ. Neutralization of TLR2 by treatment of PGN-fed mice with anti-TLR2 antibodies suppressed significantly the expression of the inflammatory cytokines IL-1b, IL-6 and TNFα (Fig. 4B). Although activated by PGN, PGlyRP3 was not a major player determining the expression level of inflammatory cytokines. In samples from the DSS-induced colitis model, TLR2 protein expression was found to be activated (Fig. 4C), in comparison with the untreated control, resulting in high mortality and other severity of the model as described above. In a subgroup of this mouse DSS-induced colitis model, treatment with the anti-TLR2 reduced the TLR2 protein expression in the colon tissue (Fig. 4C). This neutralization of TLR2 by treatment of DSS-treated mice with anti-TLR2 antibodies also significantly suppressed the expression of the inflammatory cytokines IL-1b, IL-6 and TNFα (Fig. 4D), which are downstream of the TLR2 pathway, and maintained the survival of all mice for the 15 days experimental duration (Fig. 4E). It also restored body weights and reduced colitis severity score (data not shown). In both cases, neither DSS nor the combined DSS-Anti-TLR2 treatments affected the protein expression of PGlyRP3. Taken together, these results suggest that TLR2 is responsible for the inflammatory response in the DSS-induced colitis. PGlyRP3 does not seem to have inflammatory-activating action. In the contrary, the previous results of our group and others [21,24] confirm the anti-inflammatory role of PGlyRP3 in intestine.

3.5. Negative regulation of NF-κB pathway is involved in the anti-DSS-induced colitis role of olive oil in mice

It was reported that ligands of PPARs exert their anti-inflammatory effects in different cellular systems by suppressing the inflammatory transcriptional activity of NF-κB *via* mechanisms involving induction of IκB and suppression of COX-2 [19,30,31]. NF-κB is found in inactivated state in the cytoplasm due to inhibition by IκB-α. As the latter is inhibited by inflammatory stimulation, the activation of NF-κB is accompanied by its translocation to the nucleus from the cytoplasm, so

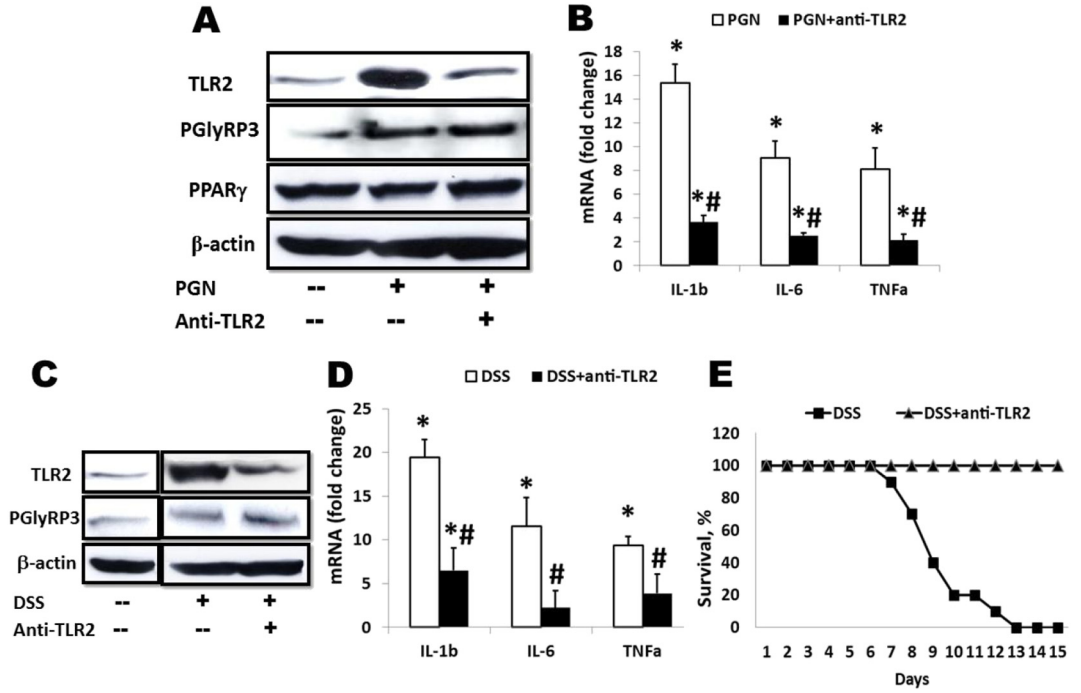

Fig. 4. PGlyRP3 is not the major player in mouse colitis. Two groups of five mice each were administered PGN (A and B) or DSS to develop colitis (C, D and E) and then iv injected with anti TLR2 on day 3 of treatment with either PGN or DSS. Anti-TLR2 reduced TLR2 but did not affect the PGN-induced PGlyRP3. PPARγ was neither affected by PGN nor anti-TLR2 (A). The anti-TLR2 treatment inhibited the PGN-induced expression of the proinflammatory cytokines (B). DSS increased TLR2 protein expression, compared to the control, while PGlyRP3 protein expression level was neither affected by DSS nor anti-TLR2 (C). Treatment with anti-TLR2 inhibited the DSS-induced expression of TLR2-downstream proinflammatory cytokines (D). Anti-TLR2 restored the survival in mice of DSS-colitis group (D). Data are presented as mean ± SEM for N = 3 in B and D. Statistical analyses: * = significantly different from the control value, # = significantly different from the corresponding value of DSS group (*t*-test after ANOVA).

that its member protein subunit P65 (RelA) decreases by activation, as appears in immunoblots. In the present work, DSS suppressed significantly the gene expression of IκB-α, stimulated significantly the expression of the NF-κB and COX-2 (Fig. 5A) and decreased the NF-κB subunit P65 (Fig. 5B). Olive oil feeding reversed these results to the anti-inflammatory direction: it increased IκB-α and P65 protein and suppressed the expression of NF-κB and COX-2. These results were significantly different from that of the DSS-colitis group. To test the role of PPARγ, samples from the group of mice that was fed on DSS + olive oil and treated with the PPARγ-antagonist (BADGE) were included. The results showed that inhibition of PPARγ reversed the anti-inflammatory action of olive oil, increasing the expression of NF-κB and COX-2 genes (Fig. 5A) and decreased the p65 protein (Fig. 5B).

Taken together, these results suggest that olive oil attenuates the development of DSS-induced colitis in mouse colon *via* activation of the anti-inflammatory PPARγ and PGlyRP3, which up regulate the IκB-α gene expression, down regulate the NF-κB and COX-2 and inhibit the nuclear relocation and activity of NF-κB (Fig. 5C).

4. Discussion

PGlyRP3 was reported to be expressed in the intestinal Caco2 cells and to have an anti-inflammatory action. Its gene promoter was found to physically bind the PPARγ and PPARγ-RXR [19] so that the reported anti-inflammatory action of PPARγ could be attributed, at least in part, to its induction of the innate immunity genes like PGlyRP3. When these Caco2 cells have been stimulated by LPS, PGN or saturated fatty acids, the inflammatory response was inhibited by PGlyRP3 overexpression or ligands of PPARγ [19–21]. This intestinal anti-inflammatory role of PGlyRP3 was confirmed *in vivo* in the present study and in other studies [23,24] in experimental mouse model of colitis. In fact, the author [23,24] attributed the anti-inflammatory action of PGlyRP3 in the intestine mainly to the decreased intestinal epithelial apoptosis, promoting normal gut microbiota and prevention of damaging production of interferon-γ by natural killer cells. On the other side, lipophilic ligands have been also reported to have anti-inflammatory effect in the intestine [1–8]. This effect has been attributed to many factors including, among others, enhancement of mucoprotection due to high expression of trefoil factor 3, Toll-interacting protein, and zonula occludens-1 [2], down regulation of p38 MAPK with consequent cytokine modulation and reduction of COX-2 and iNOS [5], PPARγ up regulation and NF-κB and MAPK signaling pathway inhibition [7]. To this end, the present study is in agreement with the results of Sánchez-Fidalgo et al. [7] and goes a step further by proposing that lipophilic ligands up regulate PPARγ, which activates the expression of the innate immunity molecule PGlyRP3. This, in turn, exerts its anti-inflammatory action by inhibiting the NF-κB signaling pathway. Results of the present study confirm the up-regulation of PGlyRP3 production by olive oil, mediated by PPARγ and resulted in the inhibition of the proinflammatory cytokines and protection against DSS-induced colitis. The up-regulation of PGlyRP3 was PPARγ-dependent, since inhibition of PPARγ by its antagonist BADGE has resulted in inhibition of PGlyRP3 and recurrence of inflammatory response, even in olive oil-fed mice. Activation of PGlyRP3 may be considered as an additional arm of the many anti-inflammatory arms of PPARγ. These arms include PPARγ inhibition of NF-κB nuclear translocation [31], modulation of lysosomal pathways and antigen presentation [32], inhibition of AP-1, either directly or indirectly through suppressing its Jun subunit, inhibiting its binding to the Fos subunit to form the AP-1 [33].

The beneficial effects of olive oil feeding in DSS-colitis mice included reduced mortality, a lower intensity of colitis inflammatory damage, and inhibition of NF-κB pathway mediating inflammatory cytokines. It is hypothesized that olive oil contains lipophilic ligands of PPARγ. Activation of the latter led to up-regulation of the immunomodulatory PGlyRP3 by direct physical binding of PPARγ to the PGlyRP3 gene, as previously proven by electrophoretic mobility shift

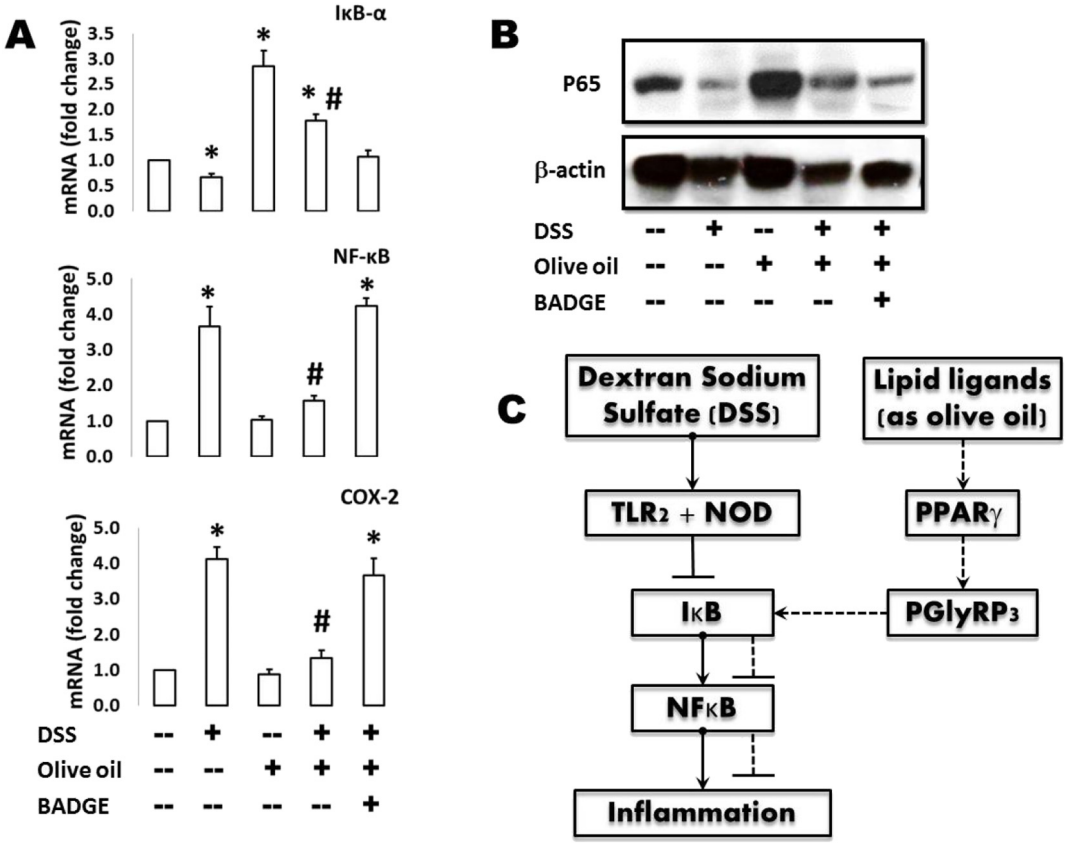

Fig. 5. Inhibition of NF-κB signaling pathway is involved in the anti-inflammatory effect of olive oil in DSS-induced colitis in mice. (A) olive oil feeding activated the gene expression of IκB-α (upper panel) and the cytoplasmic localization of p65 protein (B), and inhibited the DSS-induced gene expression of NF-κB (A, middle panel) and cox-2 (A, lower panel). All of these actions were reversed by the PPARγ inhibitor BADGE. Data in (A) are presented as mean ± SEM of N = 3. Statistical analysis: * = significantly different from the control value, # = significantly different from the value of DSS-colitis group (*t*-test after group comparisons by ANOVA). (C) a cartoon illustrating the proposed action of DSS and the sequence of protective events by PPARγ-induced PGlyRP3 and their lipid ligands.

assay (EMSA) [19]. Activation of PGlyRP3 exerted anti-inflammatory actions, and its silencing led *per se* to the increase of the proinflammatory cytokines *in vitro* [19,21] and *in vivo* [23,24]. In fact, similar to the present hypothesis, PPARγ was reported to bind and modulate other immune-modulating genes such as NF-κB, STAT, AP-1 [33] and TNIP1, which interacts with NF-κB pathway to reduce TNFα-induced signaling [40]. The promoter of all of these genes contains PPRE elements that bind directly to, and modulated by PPARγ. Thus, PPARγ is a major player in the anti-inflammatory actions in colitis through modulating of many immune genes including, and not restricted to, PGlyRP3.

Apparently, the major factor in intestinal inflammation could be Toll-like receptors. In fact, TLRs has been reported to recognize PGN, as PGlyRPs do. TLRs also sense other components of microorganisms as LPS. However, the activation of TLR2 by PGN is inflammatory in nature and includes the measureable secretion of many inflammatory cytokines and chemokines [21,34–37]. Neutralization of TLR2 in the present study inhibited the inflammatory cytokines and maintained the survival of PGN- and DSS-stimulated mice. In ulcerative colitis patients, mRNA and protein expressions of TLR2, TLR4 and TLR9 in colon epithelial cells were significantly higher than in the control group [38]. On the other side, PGlyRP3 showed an anti-inflammatory action. It was reported that the downstream signaling cascade of TLRs leads also to the production of protective factors that maintain the intestinal epithelial homeostasis [39] in response to commensal bacteria that they recognize. The anti-inflammatory action of PGlyRP3 may proceed in this direction. It recognizes PGN but exerts an anti-inflammatory action, which appears only when overexpressed [20,21] or by stimulation by ligands or in inflammation. In fact, the increase of proinflammatory cytokines by silencing of PGlyRP3 in Caco2 cells [20] and enhancement of DSS-colitis in PGlyRP3-knocked mice [23,24] may confirm the role of PGlyRP3 in maintaining this intestinal inflammatory balance.

Conflict of interest

None.

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