JPP Journal of Pharmacy and Pharmacology
ROYAL PHARMACEUTICAL SOCIETY

JPP Journal of Pharmacy and Pharmacology

JPP 2011, 63: 1572–1580

Received March 28, 2011
Accepted October 23, 2011
DOI
10.1111/j.2042-7158.2011.01358.x
ISSN 0022-3573

Research Paper

Analysing the role of COX-2 in acute oesophagitis and in melatonin-exerted protection against experimental reflux oesophagitis in rats

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Abstract

Objectives Cyclooxygenase(COX)-2 is implicated in variety of pathophysiological processes, although its role in acute reflux oesophagitis is debatable. This study was designed to evaluate the role of COX-2 during oesophagitis and in melatonin-elicited protection in rats.

Methods Reflux oesophagitis was induced in rats by ligating the pyloric end and the limiting ridge of the stomach for 5 h. Celecoxib (COX-2 blocker; 10 mg/kg), 16,16-dimethyl prostaglandinE_2 (dmPGE_2; a synthetic analogue of PGE_2; 10 μg/kg), melatonin (20 and 40 mg/kg) and omeprazole (10 mg/kg) were given intra-peritoneally 45 min before induction of oesophagitis in rats. Alterations in COX-1 and 2 gene expression and protein levels level were analysed via RT-PCR and Western blotting, respectively. Mucosal PGE_2 level and myeloperoxidase (MPO) activity were measured using an enzyme immunoassay (EIA) kit and spectrophotometrically, respectively.

Key findings COX-2 over-expression during reflux oesophagitis promotes inflammation of the oesophagus as celecoxib pretreatment significantly reduced tissue damage and MPO activity in rats with reflux oesophagitis (RE-rats). By contrast, dmPGE_2 pretreatment significantly exacerbated tissue injury and simultaneously increased COX-2 expression, PGE_2 levels and MPO activity in RE-rats. Further, melatonin pretreatment significantly reduced the tissue injury, COX-2 over-expression, PGE_2 level and MPO activity in RE-rats. Melatonin offered more potent suppression of COX-2, PGE_2 and MPO activity than the proton-pump inhibitor omeprazole; however, both reduced the lesion injury to a similar extent. Melatonin at a dose of 20 mg/kg failed to inhibit significantly the dmPGE_2-induced tissue damage, COX-2 expression, PGE_2 level and MPO activity in RE-rats while at a higher dose of 40 mg/kg it significantly attenuated these changes.

Conclusion Our results suggest that COX-2 plays an important pro-inflammatory role during acute reflux oesophagitis in rats and its inhibition contributes significantly to melatonin-exerted protection against reflux oesophagitis.

Keywords acid reflux oesophagitis; COX-2; melatonin; prostaglandin E_2

Introduction

Gastro-oesophageal reflux disease (GERD), a chronic gastrointestinal tract disorder, arises from the effortless movement of the gastric contents from the stomach to the oesophagus due to weakened sphincter motility in the cardiac region, leading to symptoms of mucosal damage and inflammation. Recent strategies to treat this disorder include acid suppressing agents, H_2 receptor antagonists and proton pump inhibitors (PPIs); however, they are often followed by high relapses and impaired mucosal healing,[1] thus indicating the involvement of multiple factors in the development and progression of GERD.

Inflammation in the oesophagus or reflux oesophagitis, commonly occurs as a benign complication of GERD. In recent years, plenty of studies have shown cytokines and/or chemokine-stimulated neutrophils to be the main inflammatory mediators involved in the pathogenesis of reflux oesophagitis.[2] Interestingly, cytokines are also implicated in the enhanced expression of cyclooxygenase-2 (COX-2) and prostaglandins in the oesophagitis mucosa.[3] However, the role of COX-2 in reflux oesophagitis is debatable. There are conflicting data on COX-2 expression in oesophagitis compared with non-inflamed oesophageal mucosa. COX-2 and prostaglandins were found to be increased in human and animal models of reflux oesophagitis.[4,5] In addition, COX-2 inhibition reduced mucosal damage

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CDRI communication no. – 7845.

in a reflux oesophagitis rabbit model.[6] In contrast, Mantry *et al*., showed that COX-2 inhibitors actually increase the oesophagitis-induced damage.[7] More recently, studies have shown that COX-2 over-expression in the inflamed oesophagus may contribute to the cascade of sequence progression from metaplasia to dysplasia and finally to adenocarcinoma.[8] Recently, Tekeuchi *et al*., showed that prostaglandinE_2 (PGE_2) has a biphasic role in oesophagitis in that it protects the oesophagus at pharmacologically low levels but at higher concentration it damages the mucosa via increasing the peptic activity.[9] Thus, these contradictory reports offer the opportunity to re-examine the role of COX-2 in reflux oesophagitis-associated damage and inflammation. If COX-2 promotes inflammation during reflux oesophagitis one would expect to find an increase in its level in association with oesophageal inflammation and that its inhibition could reverse these changes.

Melatonin is normally produced mainly in the gastrointestinal tract besides the pineal gland and possesses a number of beneficial properties including those of antioxidation, anti-inflammation and immunoregulation,[10–12] and could alleviate oesophagitis symptoms in patients with GERD.[13] Moreover, previously we have shown that melatonin protects against experimental reflux oesophagitis-induced damage in rats.[14] It is reasonable to extrapolate that the protective role of melatonin might be related to its effect on the expression and activity of COX-2 in local tissue. We therefore performed this study in an attempt to confirm this hypothesis.

Our findings for the first time demonstrate that COX-2 activation during acute reflux oesophagitis adds to oesophageal injury via increasing the PGE_2 production and inflammatory cell accumulation in oesophagtis mucosa. Further, melatonin exerts a protective function against reflux oesophagitis most likely by inhibiting COX-2 over-expression and activity.

Materials and Methods

Materials

All the chemicals, unless otherwise stated, were obtained from M/s. Sigma Chemicals (St Louis, MO, USA). Antibodies were procured from Santa Cruz Biotechnology Inc (Santa Cruz, CA, USA). Celecoxib was a gift from Dr Reddy's Laboratories (Hyderabad, India). 16,16-Dimethyl prostaglandinE_2 ($dmPGE_2$) was purchased from Cayman Chemical Co. (Ann Arbor, MI, USA).

Experimental animals

Experimental protocols were approved by the Institutional Ethical and Usage Committee of Central Drug Research Institute (CDRI), Lucknow, following the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA). Adult Sprague–Dawley rats, 180–220 g, procured from National Laboratory Animal Centre, CDRI, were used in the study. Rats were housed three or four per cage, at room with temperature regulated at 22 ± 2°C, with a 12-h light–dark cycle (lights on 0700 h, lights off 1900 h). Standard chow pellets and water were given freely, except during the period when food deprivation was applied.

Induction of reflux oesophagitis

Rats were deprived of food for 18 h before induction of oesophagitis but water provided freely. Reflux oesophagitis was induced in rats according to a method described earlier,[15] with minor modifications. Briefly, under pentobarbital anaesthesia (30 mg/kg, i.p.) the abdomen was incised along the midline and then both the pyloric end of the stomach and limiting ridge (transitional region between the fore stomach and corpus) were simultaneously ligated tightly, resulting in the reflux of gastric juice into the oesophagus. After 5 h of ligation rats were killed and the oesophagus was removed and incised lengthwise. Haemorrhagic lesions were observed under Trinocular zoom microscope (SZ-CTV Olympus) and the area of lesions (mm^2) developed were measured using Biovis image analyzer software (Expert Vision Lab Private Ltd, Mumbai, India).

Experimental protocol

Rats were randomized into various groups, each consisting of six rats. The respective groups were as follows. Normal control group: rats underwent sham operation. Reflux oesophagitis group: reflux oesophagitis was produced in rats following surgical procedure described above. Treatment group: rats were pretreated with melatonin (20 mg/kg, i.p), selective COX-2 inhibitor celecoxib (10 mg/kg, i.p.) or omeprazole (10 mg/kg, i.p.) 45 min before induction of reflux oesophagitis. Additional groups of melatonin and celecoxib treated normal control rats were set up to study their per-se effect.

In a separate set of experiments two more groups were included: one group received $dmPGE_2$ at a non-antisecretory dose (i.e. 10 µg/kg, i.p.)[16] 45 min before induction of reflux oesophagitis and the other group received $dmPGE_2$ for 45 min followed by melatonin (20 mg/kg) treatment for subsequent 45 min, before infliction of reflux oesophagitis in rats.

Additionally, to examine the effect of melatonin on PGE_2 inhibition, another group of six rats were included in which RE-rats were pretreated intraperitoneally with 40 mg/kg dose of melatonin before $dmPGE_2$ administration.

The doses of the various drugs used in the study were selected on the basis of our pilot studies and available literature.[14,17] Melatonin, celecoxib and omeprazole were suspended in aqueous solution of 0.5% carboxymethyl cellulose (CMC). $dmPGE_2$ was first diluted in a few drops of ethanol and then diluted in water to a final concentration of 5% ethanol. All the drugs were prepared freshly before administration.

Semi-quantitative reverse transcriptase polymerase chain reaction analysis of cyclooxygenase-1 and cyclooxygenase-2 gene expression

Total RNA was extracted from oesophageal samples using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA). cDNA was generated from 5 µg of total RNA using ReverAid H Minus First strand cDNA synthesis kit (Fermentas life sciences, Burlington, Canada) following the manufacturer's instructions. Genes for COX-1, COX-2 and β-actin were amplified with specific primer sets as previously described.[18] cDNA samples were annealed at 94°C (5 min) and amplified for 28 cycles

with the following cycling conditions: 94°C for 1 min, respective annealing temperature for COX-1 (70°C), COX-2 (63°C) and β-actin (55°C) for 1 min and extension at 72°C for 1 min followed by a final extension at 72°C for 10 min, and was run on Bioer XP Cycler. Polymerase chain reaction (PCR) products were electrophoresed on a 1.0% agarose gel using O'RangeRuler 100-bp DNA Ladder (Fermentas life sciences, Burlington, Canada) and intensity was measured using Biovis gel documentation software and expressed as relative intensity of PCR-product/β-actin ratio.

Immunoblot analysis of COX-1 and COX-2

Oesophageal tissues were homogenized in a proteinase inhibitor buffer (50 mM Tris HCl, pH 7.5, 150 mM NaCl, 0.5% sodium deoxycholate, 0.1% SDS, 2 mM EDTA, 1% Triton X-100, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride (PMSF) and aprotinin) and then centrifuged at 10 000*g* (Sigma centrifuge, model 3 K30; Sigma, St Louis, MO, USA) for 15 min at 4°C. An equal amount of protein (40 μg) was loaded onto an SDS polyacrylamide gel and electroblotted onto a Hybond-ECL nitrocellulose membrane (Amersham Biosciences, Amersham, UK) for Western blot analysis. The membrane was incubated overnight at 4°C with anti-COX-1 and COX-2 goat polyclonal primary antibodies at a dilution of 1 : 500. Further, the membrane was probed with Goat ExtrAvidin Peroxidase Staining Kit (Sigma, St Louis, MO, USA) according to the manufacturer's instructions. Subsequently, chemiluminescence was detected using SuperSignal West Pico Chemiluminescent substrate (Thermo Fisher Scientific Inc., Rockford, IL, USA).

To quantify COX-1 and COX-2 protein levels in the oesophageal samples, the signal for both proteins were corrected with the levels of non-inducible β-actin protein on the same blots using a mouse monoclonal antibody against it (1 : 6000; Sigma, St Louis, MO, USA). The immunoreactivity was determined by densitometric analysis using Biovis gel documentation software (Expert Vision Lab Private Ltd, Mumbai, India).

ProstaglandinE_2 enzyme immunoassay

PGE_2 enzyme immunoassay was performed following the manufacturer's instruction. Briefly, the oesophagus was homogenized in an ice-cold Tris/HCl buffer containing 50 mM Tris/HCl (pH 7.4), 100 mM sodium chloride, 1 mM calcium chloride, 1 mg/ml D-glucose and 28 μM indometacin. PGE_2 recovery and purification was conducted according to protocols provided with the Amersham Prostaglandin E_2 Enzyme-immunoassay Biotrak (EIA) system (GE Healthcare UK Ltd, Amersham, UK). Purified PGE_2 samples were stored at −80°C. Samples were dissolved in 0.5–1.0 ml of sample buffer and assayed in 96-well plates. The quantities of PGE_2 were determined using standard protocols with PGE_2 standards ranging from 2.5 to 320 pg/ml. The results were represented as pg PGE_2/mg protein.

Assessment of esophageal myeloperoxidase activity

Tissue-associated myeloperoxidase (MPO) activity was assessed as a marker of oesophagitis-associated inflammation according to the method described by Grisham *et al*.,[19] with slight modification. Briefly, oesophageal tissue was homogenized in 0.1 M phosphate buffer and centrifuged at 20 000*g* at 4°C. The pellet thus obtained was re-homogenized in 100 mM phosphate buffer containing 0.5% hexadecyltrimethylammonium bromide (HETAB, pH 5.4) and 5 mM EDTA and subjected to three freeze–thaw cycles before centrifugation to yield clear supernatant. The reaction mixture consisted of 200 μl of 100 mM phosphate buffer, pH 5.4, 20 μl of 20 mM 3,3′,5,5′-tetramethylbenzidine dihydrochloride (TMB) and 30 μl of the supernatant. The reaction was started by adding 50 μl of 0.01% H_2O_2 and the conversion of the substrate noted at 655 nm using a spectrophotometer. MPO activity was determined from a standard curve and expressed as mU/mg protein.

Protein assay

Total protein in the oesophageal homogenates was quantified using Bradford assay[20] and bovine serum albumin (BSA) as standard.

Statistical analysis

Data were evaluated by one-way analysis of variance followed by Newman–Keuls post-hoc test. Significance was ascertained at $P < 0.05$. All the data are presented as means ± standard error of the means (SEM).

Results

Area of oesophageal haemorrhagic lesions

As illustrated in Figure 1, induction of reflux oesophagitis in rats caused development of severe haemorrhagic lesions along the entire length of the oesophagus in 5 h. Celecoxib pretreatment significantly ($P < 0.001$) lessened the area of lesions compared with RE-rats. Also, melatonin significantly ($P < 0.001$) reduced the area of tissue damage compared with RE-rats. Moreover, the proton pump inhibitor omeprazole showed significant ($P < 0.001$) protection against oesophageal lesions compared with RE-rats.

Meanwhile, as shown in Figure 2, supplementation with $dmPGE_2$ significantly ($P < 0.05$) worsened the area of oesophageal lesions in the RE-rat group. Melatonin used at 20 mg/kg did not significantly reduce the area of lesion compared with the $dmPGE_2$-treated RE-group while at 40 mg/kg it significantly ($P < 0.001$) improved the lesion injury compared with the RE-rat group pretreated with $dmPGE_2$. Melatonin at this higher dose also significantly ($P < 0.001$) decreased the area of lesion compared with the RE-rat group.

COX-1 and COX-2 expression and protein level

As represented in Figure 3, onset of reflux oesophagitis resulted in significant ($P < 0.001$) up-regulation of COX-2 gene as compared with normal control rats. This increase in COX-2 expression was significantly ($P < 0.001$) reversed by melatonin pretreatment with respect to the RE-rat group. Also, omeprazole pretreatment significantly ($P < 0.05$) suppressed the COX-2 expression compared with the RE-rat group. The suppression of the COX-2 gene was more pronounced ($P < 0.01$) in the melatonin-treated group compared with omeprazole-treated RE-rats. Melatonin *per se*

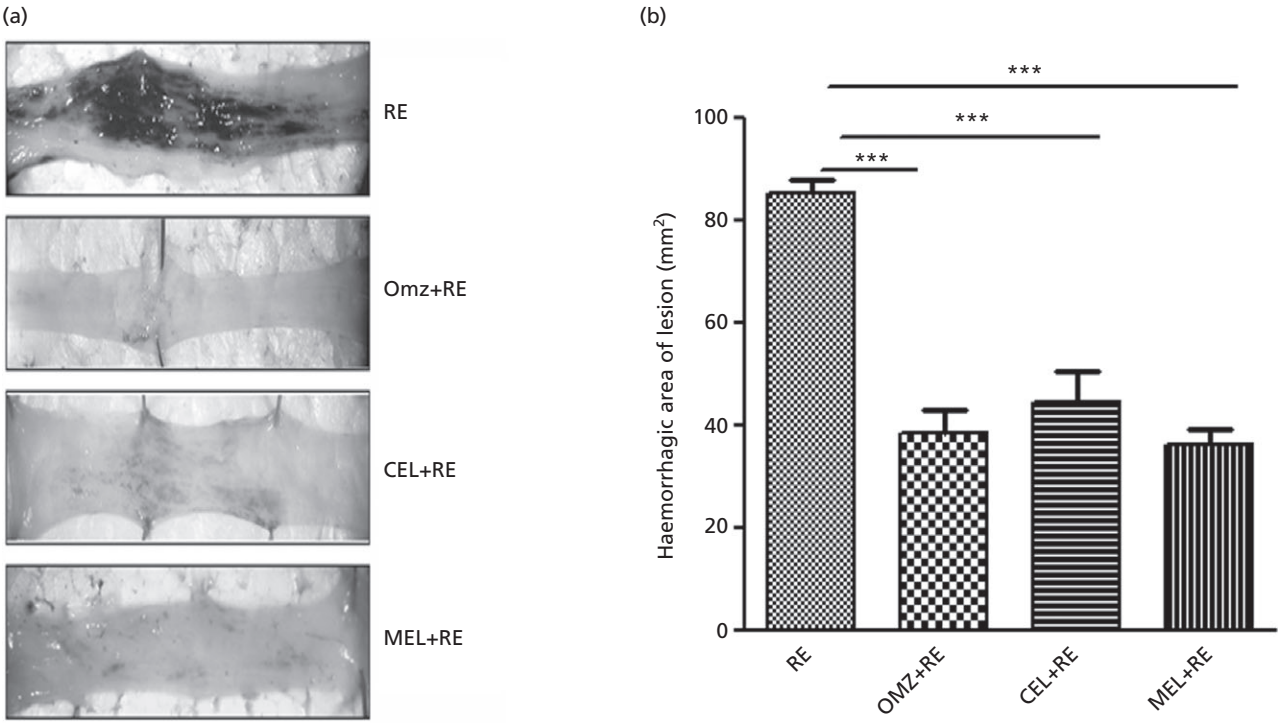

Figure 1 (a) Pictomicrograph representative of the gross appearance of whole oesophagus in different studied rat groups. (b) Bar diagram representing the changes in the area of haemorrhagic lesions in reflux oesophagitis (RE) group and RE group pretreated with omeprazole (OMZ+RE), celecoxib (CEL+RE) and melatonin (MEL+RE). Results are expressed as mean ± SEM, with six rats ($n = 6$) in each group. ***$P < 0.001$, respectively (*d.f.* = 3, 20 and *F*-value = 27.69).

significantly ($P < 0.05$) reduced the expression of COX-2 compared with its expression in normal control rats. Furthermore, expression of COX-1 remained essentially the same between the studied groups.

As shown in Figure 4, $dmPGE_2$ pretreatment in RE-rats significantly ($P < 0.05$) up-regulated the COX-2 expression with respect to untreated RE-rat group. Melatonin at 20 mg/kg was unable to significantly reverse this $dmPGE_2$-induced increase in COX-2 expression, whereas at the higher dose of 40 mg/kg, it significantly ($P < 0.001$) suppressed the COX-2 expression induced by $dmPGE_2$ intake in RE-rats. The expression of COX-1 remained essentially similar among the above-discussed groups.

The Western blot study of COX-1 and COX-2 proteins supported our findings with COX-1 and COX-2 gene expression. As shown in Figure 5, induction of reflux oesophagitis significantly ($P < 0.001$) up-regulated the COX-2 protein whereas melatonin and omeprazole pretreatment significantly ($P < 0.001$ and $P < 0.01$, respectively) reduced the level of COX-2 compared with the RE-rats. Melatonin more significantly ($P < 0.01$) repressed the COX-2 level compared with the omeprazole-pretreated RE-rat group. Melatonin *per se* significantly ($P < 0.05$) suppressed the COX-2 level over normal control values. Meanwhile, COX-1 protein expression remained unchanged between the studied groups.

Oesophageal prostaglandin (PGE_2) level

As summarized in Figure 6a, It was observed that infliction of reflux oesophagitis caused significant ($P < 0.001$) elevation of mucosal PGE_2 level with respect to normal control rats. Celecoxib pretreatment significantly ($P < 0.001$) lowered the PGE_2 concentration compared with RE-rats. Also, melatonin significantly ($P < 0.001$) lowered the level of PGE_2 over RE-rats. Moreover, administration of omeprazole significantly ($P < 0.05$) decreased the oesophagitis-induced level of PGE_2. Melatonin offered more significant ($P < 0.01$) lowering of oesophagitis-induced PGE_2 level compared with omeprazole-treated RE-rats. As represented in Figure 6b, the per se effect of omeprazole was insignificant on PGE_2 level with respect to normal control values while melatonin *per se* caused a significant ($P < 0.05$) reduction of PGE_2 level compared with the normal control group. Also, celecoxib *per se* caused significant ($P < 0.05$) reduction of oesophageal PGE_2 level compared with the normal control group.

In addition, as represented in Figure 7, administration of $dmPGE_2$ in RE-rats significantly ($P < 0.05$) augmented the mucosal PGE_2 levels compared with the untreated RE-rat group. Melatonin pretreatment at a dose of 20 mg/kg insignificantly lowered the PGE_2 levels in the RE-rats co-administered with $dmPGE_2$, while at a dose of 40 mg/kg it offered significant ($P < 0.001$) lowering of $dmPGE_2$-induced increase in PGE_2 levels. Melatonin used at this high dose also significantly reduced the PGE_2 level compared with the untreated RE-rats.

Oesophageal myeloperoxidase activity

As shown in Figure 8, onset of reflux oesophagitis significantly ($P < 0.001$) increased the oesophageal MPO activity

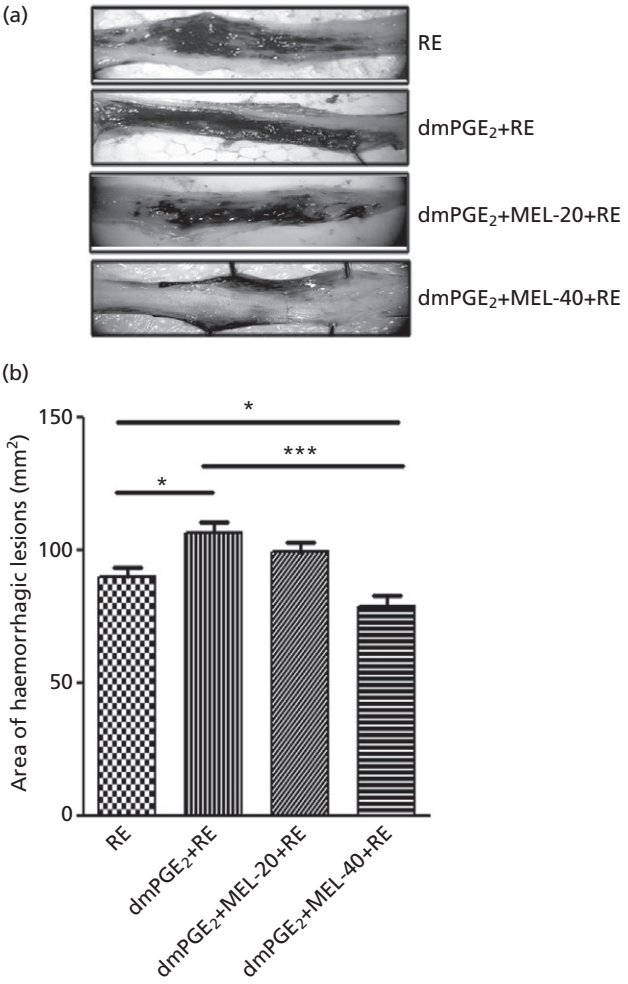

Figure 2 (a) Pictomicrograph representative of the gross appearance of whole oesophagus in different studied rat groups. (b) Bar diagram representing the changes in the area of haemorrhagic lesions in reflux oesophagitis (RE) group, RE group pretreated with $dmPGE_2$ ($dmPGE_2$+RE) and RE-group receiving $dmPGE_2$ and melatonin simultaneous pretreatment at doses of 20 mg/kg ($dmPGE_2$+MEL-20 + RE) and 40 mg/kg ($dmPGE_2$+MEL-40 + RE). Results are expressed as mean ± SEM, with six rats ($n = 6$) in each group. $^*P < 0.05$ and $^{***}P < 0.001$, respectively. (*d.f.* = 3, 20 and *F*-value = 9.64).

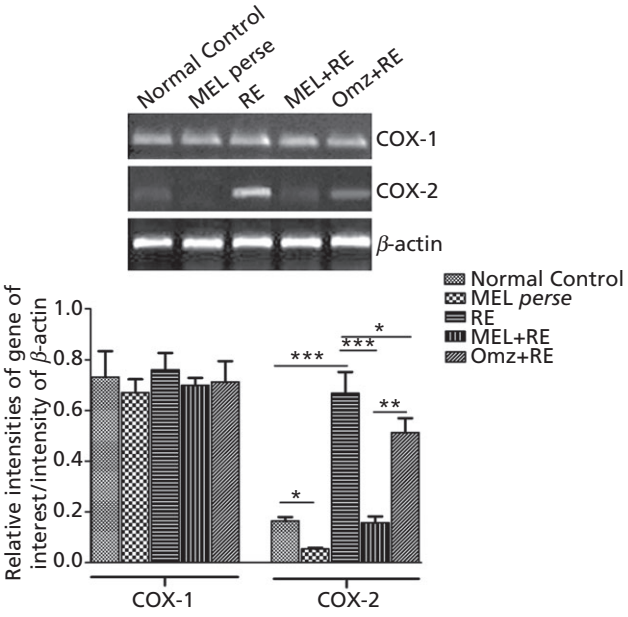

Figure 3 Histogram representing the changes in the gene expression of COX-1 and COX-2 in normal control rats, melatonin *per se* (MEL *perse*) group, reflux oesophagitis (RE) group and RE group pretreated with melatonin (MEL+RE) and omeprazole (Omz+RE). Results are expressed as mean ± SEM with three rats ($n = 3$) in each group. The gel image is representative of three separate experiments. $^*P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$, respectively (*d.f.* = 4, 10 and *F*-value = 0.91 and d.f. = 4, 10 and *F*-value = 31.28 for COX-1 and COX-2 respectively).

compared with normal control rats. Celecoxib significantly ($P < 0.01$) decreased the MPO activity in RE-rats compared with the untreated RE-rat group. Also, melatonin and omeprazole significantly ($P < 0.001$ and $P < 0.05$, respectively) reversed the RE-induced elevation in MPO activity. The reduction of MPO activity in melatonin-treated group was slightly, though insignificantly, more than in the omeprazole-treated group. $dmPGE_2$ administration in RE-rats significantly ($P < 0.05$) augmented the mucosal MPO activity compared with the RE-group. However melatonin failed to produce significant reversal of $dmPGE_2$-induced MPO activity in RE-rats.

Discussion

This study demonstrated that reflux of gastric juice induces oesophageal injury and subsequently causes over-expression of COX-2 in rats with reflux oesophagitis. In addition, PGE_2 production and MPO activity were also accelerated in the oesophageal mucosa of RE-rats. A specific COX-2 inhibitor, celecoxib, suppressed the MPO activity and prevented the development of lesions in RE-rats. Melatonin mimics the action of celecoxib and significantly suppresses the COX-2 expression and lowers the PGE_2 levels and MPO activity of oesophagitis mucosa. In contrast, exogenously administered PGE_2, exacerbates the tissue injury and increases the inflammation in the oesophagus of rats with reflux oesophagitis.

An over-expression of COX-2 has been demonstrated in the oesophageal squamous mucosa of humans, as well as in the rat, in response to acid and duodenal reflux, respectively.[4,21] We also observed that COX-2 expression increased significantly in the oesophageal mucosa of RE-rats. The increased COX-2 level was also noted to be accompanied by a high PGE_2 content and MPO activity in the oesophagitis mucosa of RE-rats. Interestingly, inhibition of COX-2 by celecoxib reversed this reflux oesophagitis-induced increase in both PGE_2 level and MPO activity, suggesting its pro-inflammatory character. In agreement with our findings, an investigation revealed that the effect of COX-2 activation differs depending on the phase of rat oesophagitis, and includes inflammatory responses to acid in the acute phase and an increase in epithelial proliferation of the basal layer and persistent inflammatory cell infiltration in the chronic phase.[17] However, there are studies showing contrasting results: Murphy *et al*., showed that COX-2 inhibition failed to ameliorate severe inflammation in the oesophagojejunostomy

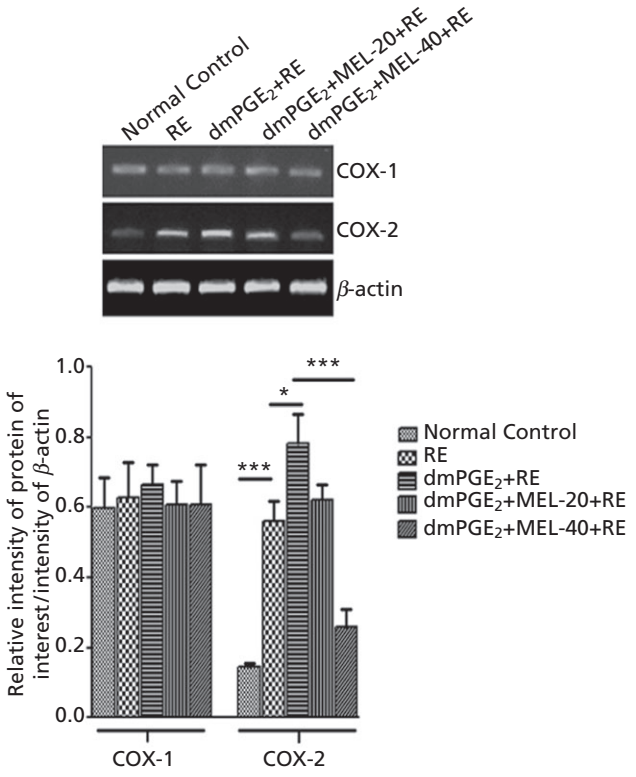

Figure 4 Histogram representing the changes in the gene expression of COX-1 and COX-2 in normal control rats, reflux oesophagitis (RE) group, RE group pretreated with $dmPGE_2$ alone ($dmPGE_2$+RE), RE-group receiving both $dmPGE_2$ and melatonin at the doses of 20 mg/kg ($dmPGE_2$+MEL-20 + RE) and 40 mg/kg ($dmPGE_2$+MEL-40 + RE). Results are expressed as mean ± SEM with three rats ($n = 3$) in each group. The gel image is representative of three separate experiments. $^*P < 0.05$ and $^{***}P < 0.001$, respectively (*d.f.* = 4, 10 and *F*-value = 0.98 and d.f. = 4, 10 and *F*-value = 23.42 for COX-1 and COX-2 respectively).

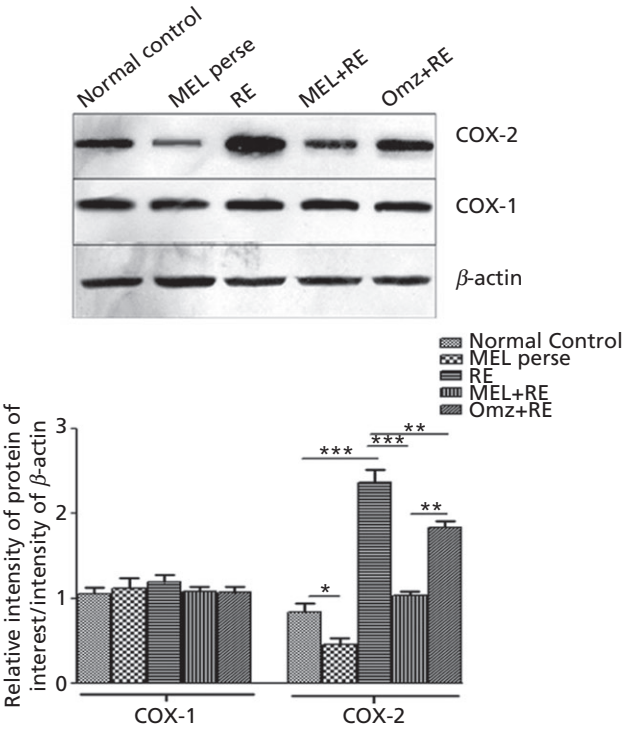

Figure 5 Bar diagram showing alterations in the protein level of COX-1 and COX-2 in the normal control rats, melatonin *per se* (MEL *perse*) group, reflux oesophagitis (RE) group, melatonin (MEL+RE) and omeprazole (Omz+RE) pretreated RE group. Results are expressed as mean ± SEM with three rats ($n = 3$) in each group. The image presented is representative of three separately studied experimental groups. $^*P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$, respectively (*d.f.* = 4, 10 and *F*-value = 0.49 and d.f. = 4, 10 and *F*-value = 65.85 for COX-1 and COX-2 respectively).

rat model of oesophagitis.[22] The differences with respect to our results may be explained based on the use of a different experimental model and the exposure time to the etiological agent.

To support, our postulation that COX-2 is the pro-inflammatory mediator during reflux oesophagitis, we next examined the role of the PGE_2 analogue $dmPGE_2$ in reflux oesophagitis-induced injury and MPO activity. It was noted that $dmPGE_2$ intake in RE-rats amplified the MPO activity and tissue damage compared with untreated RE-rats, which complements our interpretations that COX-2 activation resulting in increased PGE_2 production, in fact, augments oesophageal inflammation and adds to tissue injury. In agreement with our findings, Northway *et al*., demonstrated in the opossum model, that administration of a prostaglandin analogue before oesophageal insult accelerated the resultant inflammation.[23] Further, estimation of PGE_2 levels after $dmPGE_2$ systemic administration in RE-rats revealed that $dmPGE_2$ actually increased its own synthesizing inducible COX-2 expression and thereby PGE_2 level in the oesophagus, thus signifying that COX-2 activation may be the underlying mechanism via which it aggravated tissue damage in RE-rats. $dmPGE_2$ has also been shown to increase the mRNA levels of COX-2 enzyme in certain in-vitro setups,[24] which also supports our findings. Thus our results with $dmPGE_2$ allowed the assumption that endogenous PGE_2 is not the mediator of oesophageal mucosal protection; in fact it may be detrimental.

Acute and chronic inflammation, characterized by microvascular injury, oedema and infiltration by polymorphonuclear leukocytes, which release vasoactive substances, are the important hallmarks in reflux oesophagitis.[25,26] Prostaglandins may be one category of substances that mediate inflammation in the oesophagus. Prostaglandins and other vasoactive substances produce vasodilatation and increased vascular permeability leading to oedema, stasis and migration of neutrophils and eosinophils into the area. $dmPGE_2$ has also been shown to aggravate gastric ulcers by increasing the vascular permeability of the gastric epithelium challenged by histamine.[27] As oesophagitis mucosa also has compromised vascular permeability, it might be possible that $dmPGE_2$ administration may increase the vascular permeability and thereby caused more migration of inflammatory cells to the damaged area, which adds to injury.

Although the mechanism of COX-2-induced increase in MPO activity is unclear from current results, the relationship can be explained considering the findings of Yamato *et al*., who reported that PGE_2 had a biphasic role in rat oesophagi-

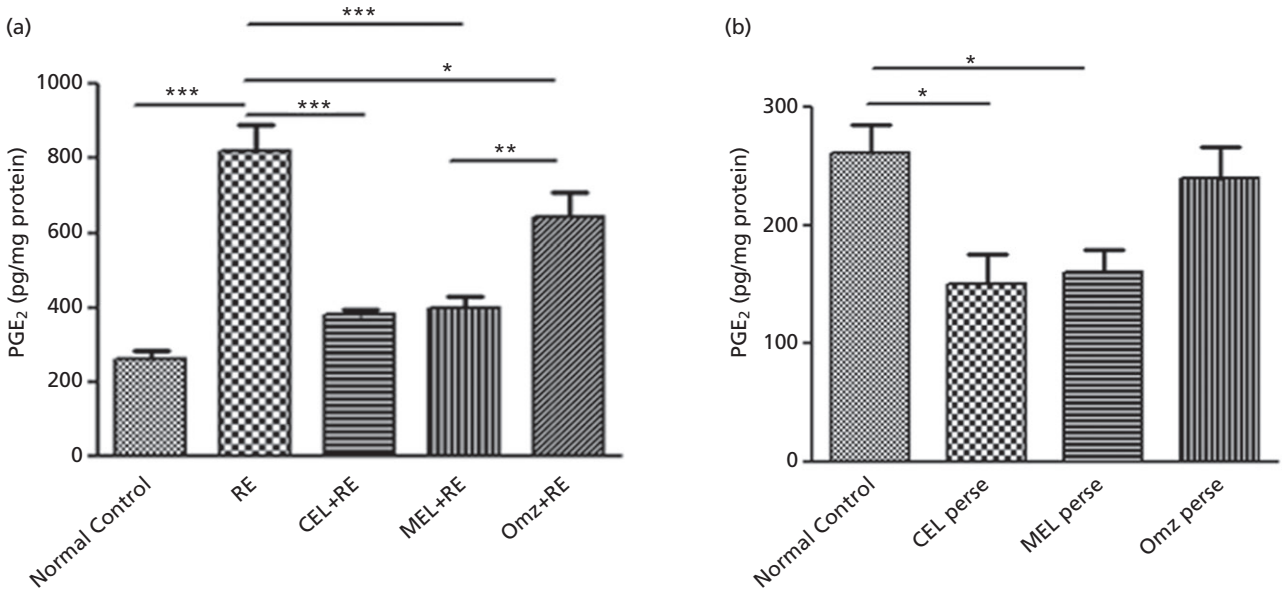

Figure 6 (a) Alterations in the oesophageal prostaglandin (PG)E_2 levels in normal control, reflux oesophagitis (RE), celecoxib (CEL+RE), melatonin (MEL+RE) and omeprazole (Omz+RE) pretreated RE rats. Results represent means ± SEM, with six rats ($n = 6$) in each group. *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$, respectively (*d.f.* = 4, 25 and *F*-value = 23.25). (b) Changes in the oesophageal PGE_2 content in normal control rats, celecoxib (CEL perse), melatonin (MEL perse) and omeprazole (Omz perse) *per se* groups. Results represent means ± SEM, with six rats ($n = 6$) in each group. *$P < 0.05$ (*d.f.* = 3, 20 and *F*-value = 5.37).

tis. It can prevent the damage at pharmacologically low doses, although at high doses it harms the oesophageal mucosa by increasing peptic activity in gastric juice.[28] Thus it may be possible that overproduction of PGE_2 due to COX-2 activation in reflux oesophagitis, increases the peptic activity in refluxed material, which in turn causes damage and, further, since injured epithelia have a high propensity for inflammatory cells,[29] it may also result in increased MPO activity.

Our results thus implicate COX-2-derived PGE_2 in the pro-inflammatory reactions arising in reflux oesophagitis, reflecting the importance of its inhibition in therapeutic approaches against reflux oesophagitis. In this regard, melatonin represents a promising therapeutic agent having a well-established modulatory role on COX-2.[30–32] Moreover, the protective role of melatonin against GERD has also been proposed.[33] We thus advanced our study to elucidate whether melatonin exerts protection against reflux oesophagitis-induced inflammation relative to its modulatory actions on the COX/PG system.

We observed that melatonin profoundly repressed the over-expression of COX-2 level during reflux oesophagitis that coincides with the decreased PGE_2 level, MPO activity and tissue injury. Since COX-2 appeared to be responsible for the induced PGE_2 production during reflux oesophagitis, it was likely that the repression of COX-2 induction via melatonin reduces the inflammatory PGE_2 synthesis and thus dramatically reduces the MPO activity. Melatonin did not modify the COX-1 levels in RE-rats, which suggested its constitutive occurrence in oesophagus. Interestingly, melatonin *per se* apparently reduced the COX-2 expression, protein and PGE_2, supporting our notion of its suppressive role on COX-2 expression in the oesophagus. Meanwhile, melatonin failed to reproduce its protective effect in RE-rats when administered

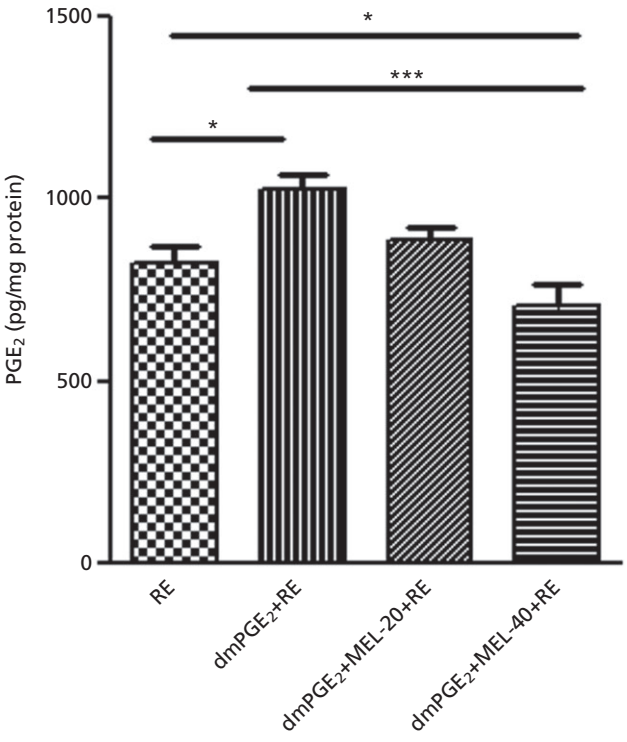

Figure 7 Alterations in the oesophageal prostaglandin (PG)E_2 levels in reflux oesophagitis (RE) rats, RE-group pretreated with $dmPGE_2$ alone ($dmPGE_2$+RE) and along with melatonin at doses of 20 mg/kg ($dmPGE_2$+MEL-20+RE) and 40 mg/kg ($dmPGE_2$+MEL-40+RE). Results represent means ± SEM, with six rats ($n = 6$) in each group. *$P < 0.05$ and ***$P < 0.001$, respectively (*d.f.* = 3, 20 and *F*-value = 8.17).

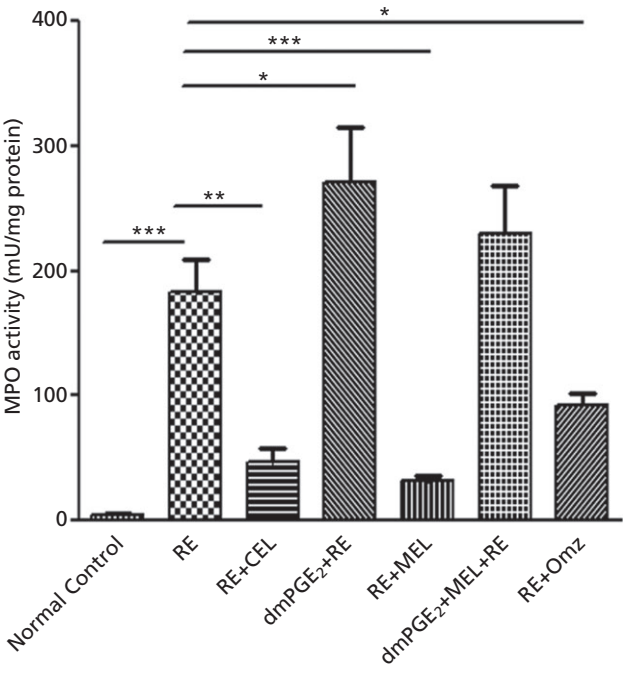

Figure 8 Bar diagram showing changes in the mucosal myeloperoxidase (MPO) activity in normal control rats, reflux oesophagitis (RE) group, RE group pretreated with celecoxib (CEL+RE), $dmPGE_2$ ($dmPGE_2$+RE), melatonin (MEL+RE), $dmPGE_2$+melatonin ($dmPGE_2$+MEL+RE) and omeprazole (Omz+RE). Results are expressed as mean ± SEM with six rats ($n = 6$) in each group. $^*P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$, respectively (*d.f.* = 6, 35 and *F*-value = 17.62).

along with $dmPGE_2$. The possible rationale might be the inadequacy of the selected dose of melatonin to buffer entirely the augmented PGE_2 level resulting from additional supplementation with its synthetic analogue during reflux oesophagitis. To further address this issue we examined the effect of melatonin at the higher dose of 40 mg/kg on COX-2 expression and PGE_2 levels after $dmPGE_2$ intake in RE-rats. We found that melatonin used at this dose significantly normalized the $dmPGE_2$-augmented COX-2 expression and PGE_2 level in RE-rats. Also, melatonin at this higher dose significantly protected against the damage caused by systemic administration of $dmPGE_2$ in RE-rats. Thus our results suggested that the effect of melatonin on COX-2 expression and PGE_2 is dose related and their inhibition underlies the protective functions of melatonin against reflux oesophagitis-induced damage.

These results are, however, contradictory to those of a previous investigation, which showed that the protective effects of melatonin on reflux oesophagitis-induced mucosal lesions is mediated by increased expression of the COX-2-PGE_2 system.[34] Although a possible explanation for the difference in the respective outcome of both studies is unclear, it may be based on the use of different methods for the reflux oesophagitis model preparation in rats, which in turn can influences the drug's actions.

A large body of evidence suggests that the acid content of gastric juice is the main casual factor that induces expression of COX-2 during reflux oesophagitis.[4,21,35] Our data demonstrated that suppression of acid secretions via proton pump inhibition was not adequate to bring effective reversal of reflux oesophagitis-induced COX-2 expression and a consequent PGE_2 level. In contrast, melatonin more efficiently suppressed COX-2 expression and protein, bringing down its level to normal control values. These data suggested that while suppression of acid can reverse the oesophageal inflammation to some extent, complete reversal may not be achieved, which may underlie the failure of proton pump inhibitors to offer complete symptomatic relief in GERD patients. The more pronounced action of melatonin on COX-2 expression can be explained in terms of its inhibiting actions on gastric acid[36] as well as nuclear factor kappa–B (NF-κB), which tanscriptionally regulate COX-2 expression.[37] Therefore, there is a possibility that melatonin elicits more potent suppression of COX-2 than omeprazole due to its collective influence on both COX-2 regulating factors (i.e. gastric acid secretion and NF-κB).

To summarize, our study for the first time showed that COX-2 is pro-inflammatory during acute oesophagitis in rats and its inhibition has a therapeutic implication against reflux oesophagitis. Moreover, we showed that melatonin protects against reflux oesophagitis-induced damage in rats possibly via inhibiting COX-2 activity.

Conclusions

Conclusively, our study gives a clear impression that COX-2 activation during acute oesophagitis in rats contributes to oesophageal damage by producing high PGE_2 levels, which facilitates inflammatory reactions in oesophagitis and adds to injury. Moreover we presented data that supported melatonin as a promising therapy against reflux oesophagitis, in that its supplementation brings successful reversal of reflux oesophagitis-induced increase in COX-2 expression, PGE_2 content and MPO activity. Although care must be paid in extrapolating data from rodents to a complex human gastro-oesophageal reflux disease (GERD), the findings reported herein lend support to the concept of the clinical utility of melatonin against this oesophageal pathology.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

Funding

The Authors deeply acknowledge Indian Council of Medical Research (ICMR), New Delhi, India for providing financial support.

Acknowledgements

The Authors thank Mrs Shibani Sen Gupta for her technical help.

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