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Title Page

COX-2 mRNA IS INCREASED IN OESOPHAGEAL MUCOSAL CELLS BY A PROTON PUMP INHIBITOR

Short Title: COX-2 is increased by esomeprazole

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ABSTRACT:

Introduction: Barrett's oesophagus develops in some individuals with gastro-oesophageal reflux, and is the precursor to oesophageal adenocarcinoma. Proton pump inhibitors (PPIs) suppress gastric acid production and are used to treat reflux. Clinical trials suggest that COX inhibitors might prevent oesophageal cancer, although PPIs could offset this by increasing COX-2 expression in Barrett's oesophagus. To investigate this, we evaluated the impact of a PPI on COX expression in oesophageal mucosal cells.

Methods: The effect of the PPI esomeprazole on COX-1 and COX-2 mRNA levels in oesophageal cells was determined. Oesophageal cell lines OE33 (adenocarcinoma derived) and HET-1A (immortalized squamous cells), and a control intestinal cell line - HT29 (colon carcinoma), were treated for 24 hours with increasing concentrations of the esomeprazole.

Results: COX-2, but not COX-1, mRNA levels, dose dependently increased in OE33 and HET-1A cells vs. esomeprazole concentration. COX-2 mRNA levels did not increase in HT29 cells.

Conclusions: Exposure to esomeprazole increases COX-2 mRNA in oesophageal cells. This might contribute to the lack of benefit for COX inhibitors for oesophageal cancer prevention in recent clinical studies.

Key words: cyclooxygenase, oesophageal epithelium, proton pump inhibitor, Barrett's oesophagus.

Abbreviations: COX, cyclooxygenase; PPI, proton pump inhibitor; NSAIDs, non steroidal anti-inflammatory drugs; CYP-1A1, Cytochrome P450-1A1; PGE-2, prostaglandin-E2.

INTRODUCTION

Barrett's oesophagus arises in a subset of patients with gastro-oesophageal reflux disease, and can progress to oesophageal adenocarcinoma [1]. Reflux symptoms associated with Barrett's oesophagus are often treated with proton pump inhibitor (PPI) medication, and sometimes surgery. Some previous studies have suggested that PPI use in patients with Barrett's oesophagus may be associated with a reduced risk of developing oesophageal cancer [2-4], although a recent meta analysis identified no definitive evidence that either PPI treatment or anti-reflux surgery decreases the risk of cancer progression [5].

PPI-induced acid suppression in patients with Barrett's oesophagus has been reported to correlate with increased cyclooxygenase-2 (COX-2/PTGS2) protein expression in the columnar epithelium [6, 7], and it has been suggested that this may be the result of increased gastrin levels [8]. Other studies have shown a step-wise increase in COX-2 mRNA expression across the progression from normal squamous epithelium to Barrett's oesophagus, and then to adenocarcinoma [9-11], with higher levels of COX-2 mRNA and protein expression also associated with poorer cancer survival [12],[13, 14]. Furthermore, long-term consumption of non-steroidal anti-inflammatory agents (NSAIDs), which inhibit cyclooxygenase-1 (COX-1/PTGS1) and COX-2, has been shown to be associated with a reduced risk of developing oesophageal adenocarcinoma in some studies [15, 16], and the COX-2 inhibitor celecoxib prevents the development of oesophageal adenocarcinoma in a rat model [17]. These observations suggest a role for COX-2 in the development of oesophageal adenocarcinoma.

However, a recent multi-centre clinical study showed no difference in oesophageal cancer risk in patients with reflux managed with PPIs, between those taking vs. those not taking aspirin [18], whereas in studies undertaken before the introduction of PPIs long-term consumption of aspirin appeared to be associated with a significant reduction in oesophageal adenocarcinoma [15, 16]. Furthermore, in a study

which investigated the COX-2 inhibitor rofecoxib, there was no significant effect on the development of dysplasia in Barrett's oesophagus in patients taking a PPI, and there was evidence that the PPI may have antagonized the effect of rofecoxib [7].

The potential ability of PPIs to override any beneficial effect of COX inhibitors on oesophageal cancer development has received little attention. This is an important question as there is currently no definitive evidence that PPI treatment decreases the risk of dysplasia or cancer [5], and increased use of PPIs has not prevented the 8% per year increase in the incidence of oesophageal adenocarcinoma over recent decades [19]. No studies have investigated whether there is a direct affect of PPIs on COX-2 expression in oesophageal epithelial cells. This is an important question as for chemo-prevention with NSAIDs or COX-2 specific inhibitors to be effective, the inhibition of COX-2 within oesophageal epithelial cells is probably required [20-23]. To investigate this further, we determined the effect of the PPI esomeprazole on both COX-1 and COX-2 expression in cell lines derived from oesophageal epithelium, and compared this to a colonic epithelium control. Cytochrome P450-1A1 (CYP-1A1) mRNA levels were also determined to investigate whether the effects of esomeprazole on COX-1 and COX-2 mRNA levels were related to differential responsiveness of the cell lines to esomeprazole.

MATERIALS AND METHODS

Cell culture and esomeprazole treatments

Three cell lines were used in this study; OE33, HET-1A, and HT29. OE33 is a cell line derived from a human oesophageal adenocarcinoma [24] . HET-1A is a keratinocyte cell line derived from the oesophagus of a human male and then immortalized with SV-40 large T antigen [25]. HT29 is a human colon adenocarcinoma derived cell line [26]. These cells were grown in culture and exposed to varying concentrations of the PPI – esomeprozole, following which COX-1 and COX-2 mRNA levels were determined. Cytochrome P450-1A1 (CYP-1A1) mRNA levels were also measured to determine differential responsiveness of the cell lines to esomeprazole, and whether this might impact on the effect of esomeprazole on COX-1 and COX-2 mRNA levels.

OE33 cells and HT29 cells were grown in DMEM media supplemented with 10% foetal bovine serum, 100 U/mL penicillin and 100 μg/mL streptomycin (CSL Biosciences, Australia) at 37^{o}C with 5% CO_2. HET-1A cells [25] were grown in LHC-9 medium (Biosource, Biofluids™ Cell Culture Products) supplemented with 100 U/mL penicillin and 100 μg/mL streptomycin (CSL Biosciences, Australia) at 37^{o}C with 5% CO_2. For HET-1A cell culture tissue-culture flasks and plates were coated with 0.03mg/mL rats-tail collagen, 0.01mg/mL fibronectin and 0.01mg/mL BSA (all from Sigma) in LHC-9 media . Cells were passaged by using 0.05 %(w/v) Trypsin/ 0.53mM EDTA solution in DMEM to release the cells from the coated plastic. After the cells were released the trypsin was inactivated with 0.1% trypsin soybean inhibitor in LHC-9 media.

Esomeprazole was a gift from Astra Zeneca Australia (North Ryde, New South Wales, Australia). Two mls of phosphate buffered saline (PBS) was added to a vial containing 20 mg of esomeprazole (intravenous preparation for clinical use). Dilutions of esomeprazole were prepared in media (RPMI 140 or LHC-9) by serial dilution from a 7.5 mM stock solution in PBS. Control media were prepared

in the same way, by adding PBS instead of esomeprazole. The concentration range was chosen to cover the manufacturers estimated steady state apparent volume of distribution of 0.22 L/kg, which equates to 6 µM for an 80 kg person with 40 mg esomeprazole (from Astra Zeneca, for intravenous use), and to cover the concentrations of PPIs used in previous in vitro studies [27, 28].

OE33 cells, HET-1A, and HT29 cells were seeded at a density of 6 x 10^4 cells/well (in 300 µl medium) in 24-well plates. The cells were cultured for 24 hours before treatment so that they were adhered and at approximately 40–50% confluence. Cells were then treated by the addition of 20 µl of esomeprazole or media for a further 24 hours. Media was then removed and 500 µl Trizol reagent added for extraction of RNA. Experiments were performed 3 times, each on different days.

RNA extraction and reverse transcription

RNA was extracted from cultured cells using Trizol (Invitrogen). The RNA was dissolved in 50 ul of Ultra Pure water (Fisher Biotech, Australia), and a 25 ul aliquot was treated with DNAse I (Ambion DNA-Free kit, Ambion, Austin, USA) according to the manufacturer's instructions. RNA concentration was estimated using a Biophotometer (Eppendorf, Hamburg, Germany**).** One µg of RNA was reverse transcribed with SuperscriptIII reverse transcriptase (Invitrogen, Carlsbad, USA). In brief, 10 uL of DNAse-treated RNA was combined with 250ng of random hexamers (GE Healthcare Bio-Sciences AB, Uppsala, Sweden), and the total volume was brought to 12uL using ultra pure water (Fisher Biotech, West Perth, Australia). The RNA/ hexamer mix was heated to 70^0C for 3 minutes, and then snap chilled on ice. To each tube, 4uL of 5x first strand buffer, 4uL of 0.1M dithiothreitol, 1uL of deoxynucleotide triphosphates (10mM each, Promega, Madison, WI, USA), and 1uL of SuperscriptIII was added. Reactions were incubated at 25^0C for 10 minutes before being heated to 50^0C for 50 minutes. Reverse transcription was stopped by incubation at 70^0C for 15 minutes. Each reaction was diluted 1/30 using ultra pure water, and 6uL of this cDNA dilution was used for a PCR reaction.

Real-time PCR analysis of COX-1, COX-2, β-actin and CYP-1A1 expression

The house keeping gene β-actin was used to normalize the levels of the genes of interest (COX-1, COX-2 and CYP-1A1) for differences in RNA estimates, and for differences in reverse transcription efficiency. β-actin was chosen because it was not affected by esomeprazole treatment. CYP-1A1 mRNA levels were determined for quality control, to determine whether any effects of esomeprazole on COX-1 and COX-2 mRNA levels were related to differential responsiveness of cell lines to esomeprazole.

PCR was performed in 20uL reactions using a Rotorgene 3000 real time PCR machine (Corbett Research, Sydney, Australia). For COX-1, β-actin and CYP-1A1 each reaction consisted of 10uL of 2x Quantitect Sybr Green real time PCR mix (Qiagen, Hilden, Germany), 2uL of each primer (5uM stock), and 6uL of cDNA. For COX-2 each reaction consisted of 10uL of 2x Quantitect real time PCR mix, 0.2uL of each primer (50 uM stock), 1.2uL of 25mM $MgCl_2$, 2.4uL of ultra pure water and 6uL of cDNA.

Reactions were heated to 95^0C for 15 minutes, then subjected to 45 cycles of 95^0C for 20 seconds, a gene-specific annealing temperature for 20 seconds (see primer details below), and 72^0C for 20 seconds (acquisition at this step). After a final incubation at 72^0C for 4 minutes, the melting profile was obtained by heating the reactions from 60^0C to 99^0C and acquiring the fluorescence at 0.5^0 C increments. The identity of the PCR products were confirmed by sequencing, agarose gel electrophoresis, and melt profile. Variations in the amounts of RNA used in the reverse transcription reactions and differences in the efficiency of the reverse transcriptions were normalised by using the expression of ß-actin.

Cycle-thresholds were determined using the Relative Quantitation module in the Rotorgene Software (v 1.7). This module uses the second derivative method [29]. The cycle-thresholds were exported from the Rotorgene software to Microsoft Excel, and relative quantification was performed using the method of Muller et al (2002; qGene) [30], which use dilution-series derived amplification efficiency estimates for each gene to calculate efficiency-adjusted normalized relative levels. PCR reactions were performed in triplicate. Primers and PCR conditions were optimised to maintain precision errors below a coefficient of variation of 20%.

Primer sequences and annealing temperatures were as follows, 5' to 3' –

COX-1 F GGGGTTCTTATTTTGCATTCC

COX-1 R ATTTGGGATACGAGCCACTGT

Annealing temp: 60° C

COX-2 F TTCTTTTCCACATCTCATTGTCACTG

COX-2 R AGGCTTAAACACAGTTTATAACCATAG

Annealing temp: 50° C

CYP-1A1 F GGCTGAGCAATCTGACCCTA

CYP-1A1 R GGGCAGAGGAATGTGATGTT

Annealing temp: 55° C

β-actin F TTGCCGACAGGATGCAGAAG

β-actin R GCCGATCCACACGGAGTACT

Annealing temp: 60° C

Statistical analysis

mRNA expression data were analysed using SPSS v17 for Windows. Differences in mRNA expression between vehicle control and esomeprazole treatments were assessed for statistical significance using a one way ANOVA test.

RESULTS

COX-2 mRNA levels increased dose-dependently in oesophageal adenocarcinoma-derived OE33 cells with increasing esomeprazole concentrations, up to 3.7 fold at 20 µM esomeprazole compared with the vehicle control (figure 1.A; $p > 0.0001$), whereas COX-1 mRNA levels remained unchanged (figure 1.B). In oesophageal squamous HET-1A cells there was a smaller 1.9 fold increase in COX-2 mRNA at 20 µM esomeprazole (figure 1.A; $p = 0.037$) with no corresponding change in COX-1 mRNA levels (figure 1.B). COX-2 mRNA levels were unchanged in colon carcinoma-derived HT29 cells by esomeprazole treatment (figure 1.A), however, COX-1 mRNA levels increased by 1.7 fold at 5 µM esomeprazole (figure 1.B; $p = 0.005$).

CYP-1A1 mRNA levels were determined to assess the responsiveness of each cell line to esomeprazole, and are summarised in figure 1.C. In OE33 cells CYP-1A1 levels increased dose dependently with increasing esomeprazole concentrations up to 8 fold at 10 µM (figure 1.C; $p = 0.005$). In HT29 cells esomeprazole induced a dose dependent increase in CYP-1A1 mRNA ($p = 0.000$), with a 25 fold increase at 20 µM esomeprazole. The effect in HET-1A cells was less pronounced, with a 2.5 fold increase in CYP-1A1 mRNA at 20 µM esomeprazole (figure 1.C; $p = 0.053$).

DISCUSSION

Studies reported in the early to mid 1990's suggested that aspirin might prevent the development of oesophageal adenocarcinoma [15, 16]. However, since the introduction of PPIs, 7 out of 8 studies evaluating this have failed to replicate these earlier findings [18, 31]. Further, none of the 5 studies evaluating non-aspirin NSAIDs have found any benefit [31]. From these observations it is reasonable to hypothesise that PPIs might be counteracting a chemo-preventative effect of COX inhibitors. A potential mechanism for this concept is highlighted by evidence from *in vivo* studies in humans and rats which demonstrate that PPI use is associated with increased levels of COX-2 in the gastrointestinal tract [6-8]. It is therefore possible that PPIs might reduce any beneficial effect of COX inhibitors by increasing the level of COX-2 in oesophageal mucosa.

Some insights into the regulation of COX-2 by PPIs have come from animal studies. In rat stomach, PPI treatment resulted in increased COX-2, prostaglandin-E2 (PGE-2), and mucosal protection against ethanol. This protection was blocked, as expected, by specific inhibition of COX-2. The up-regulation of COX-2 was blocked by the gastrin receptor antagonist AG-041R [8], suggesting that PPI-induced up-regulation of gastrin may be a mechanism of COX-2 activation in the stomach of rats. However, increased gastrin levels result from decreased gastric acid secretion. Therefore these findings do not explain the ability of PPIs to heal ethanol-induced gastric ulcers at doses too low to affect gastric acid secretion. At low doses, PPI-induced ulcer healing is associated with increased mucous secretion [32], and this is likely to be the result of increased COX-2 expression and synthesis of PGE-2.

Chemo-prevention of oesophageal adenocarcinoma using NSAIDs or COX-2 inhibitors appears to require the inhibition of COX-2 within oesophageal epithelial cells [20-23]. Therefore a critical question is whether increased COX-2 expression results from a direct affect of PPIs on oesophageal epithelial cells. In our study, COX-2 mRNA levels, but not COX-1, increased dose-dependently in oesophageal

adenocarcinoma derived OE33 cells exposed to the PPI esomeprazole. COX-2 mRNA levels also increased in HET-1A immortalized oesophageal squamous cells treated with esomeprazole, although to a lesser extent than in the OE-33 cells. While we have not investigated the effect of esomeprazole on COX protein levels in these cell lines, COX-2 is primarily regulated at the level of transcription and mRNA stability [33], so it is likely that these observed changes in COX-2 mRNA levels are biologically significant. A further limitation of this study is that we only investigated the effect of esomeprazole in two oesophageal cell lines. These observations will need to be confirmed in other human cell lines.

The direct effects of esomeprazole on COX-2 mRNA levels in cultured oesophageal cells could be due to inhibition of vacuolar H+ K+ ATPases. In support of this, it has been reported that PPIs appear to inhibit vacuolar H+-ATPases[34], and specific inhibition of vacuolar H+-ATPases has been shown to increase the level of COX-2 mRNA and protein in macrophages [35] . Esomeprazole might also increase COX-2 mRNA expression via activation of the aryl hydrocarbon receptor as there is evidence for this mechanism in lung cancer cells [36], and chromatin immunoprecipitation (ChIP) studies have shown an association of the aryl hydrocarbon receptor with xenobiotic response elements (XRE) harbored in the COX-2 promoter and CYP1A1 promoter oligonucleotides [37].

In contrast to the OE33 and HET-1A oesophageal cells, COX-2 mRNA levels were not increased by esomeprazole in HT29 colon adenocarcinoma derived cells, suggesting the possibility that the effect of esomeprazole on COX-2 mRNA might be limited to oesophageal cell lines. We did, however, observe a small (1.7 fold) but significant increase in COX-1 mRNA in HT29 cells. This is in agreement with a previous report showing that COX-1 can be up-regulated in HT29 cells by a physiological stimulus [38], and suggests that these cells are responsive to esomeprazole. To explore this further we investigated the response of the cell lines to esomeprazole via the mRNA expression of the drug metabolising enzyme CYP1A1.

CYP1A1 is a cytochrome p450 enzyme which has been reported to be up-regulated by PPI's in cultured cells [39, 40]. Esomeprazole causes transcriptional activation of the CYP1A1 gene via activation of the human aryl hydrocarbon receptor [41], which has been implicated in the regulation of COX-2 mRNA expression [36]. In our study we evaluated its expression to further evaluate whether the effects of esomeprazole on COX-1 and COX-2 mRNA levels were related to the responsiveness of the cell lines to esomeprazole. In our study 20 μM esomeprazole was associated with a 7.7 fold increase in CYP1A1 mRNA levels in OE33 cells, a 2.5 fold in HET-1A cells, and a 25 fold in HT29 cells. The large increase in CYP1A1 mRNA in response to esomeprazole in HT29 cells suggests the lack of effect on COX-2 mRNA levels is unlikely to be due to a relative unresponsiveness to esomeprazole. These results also suggest that HET-1A cells may be less responsive to esomeprazole than OE33 cells, and this correlates with the lower level of COX-2 induction in the HET-1A cells.

Our finding that esomeprazole treatment was associated with an increased level of COX-2 in cell lines derived from oesophageal epithelium raises the possibility that PPI's might counteract the previously suggested chemo-preventative effect of NSAIDs on the development of oesophageal adenocarcinoma. Our study, however, is a cell-line based in vitro study, and as such, its findings should be used to draw new hypotheses for testing in the clinical setting. We have shown a negative effect for the use of esomeprazole in this context. In the clinical setting, investigations are underway, although benefits are yet to be demonstrated. Aspirin is the main NSAID COX-inhibitor under investigation in the context of oesophageal adenocarcinoma. The main side-effect of aspirin is peptic ulcers; therefore co-administration of aspirin with a PPI is an attractive treatment option. This combination is being evaluated in a large randomised trial of high and low dose esomeprazole with vs. without aspirin [42], although there is no control group in this trial which is not taking a PPI, and therefore testing the potential negative impact of esomeprazole on aspirin based chemo-prevention. Further investigation

may therefore be needed to determine whether COX-inhibition, or COX-2-specific inhibition, is beneficial for patients with Barrett's oesophagus whose reflux symptoms are managed without the use of PPIs, such as following effective antireflux surgery.

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FIGURE LEGENDS

Figure 1 Effects of esomeprazole on COX-2, COX-1, and CYP1A1 mRNA levels.

A. COX-2 relative mRNA levels in OE33, HET-1A, and HT29 cells after treatment for 24 h with increasing concentrations of esomeprazole.

B. COX-1 relative mRNA levels in OE33, HET-1A, and HT29 cells after treatment for 24 h with increasing concentrations of esomeprazole.

C. CYP-1A1 relative mRNA levels in OE33, HET-1A, and HT29 cells after treatment for 24 h with increasing concentrations of esomeprazole.