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Glycine-extended gastrin stimulates proliferation via JAK2- and Akt-dependent NF-κB activation in Barrett's oesophageal adenocarcinoma cells

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Running title

Mechanisms of G-Gly induced oesophageal cell proliferation

Key words

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Abbreviations used; OAC, oeosophagal adenocarcinoma: ERK, extra-cellular signal related kinase; p38 MAP kinase, p38 mitogen activated protein kinase; JNK, c-jun-NH2-terminal kinase; COX-2, cyclo-oxygenase-2; G-Gly, glycine-extended gastrin; G-17, amidated gastrin-17; JAK2, janus kinase 2; NF-κB, nuclear factor-κB; IL-1β, interleukin-1 beta; MTT, 3-[4, 5-dimethylthiazol-2-y-l]-2, 5-diphenyltetrazolium bromide; FBS, foetal bovine serum.

Summary

Glycine-extended gastrin (G-Gly) is a mitogen for several gastrointestinal tissues although the mechanisms responsible are ill-defined and it is unknown if G-Gly can influence signalling in Barrett's oesophagus. G-Gly stimulated proliferation in OE19 and OE33 cells in a dose-dependant manner. This was unaffected by a CCK2 receptor antagonist but abolished by COX-2 inhibitors. G-Gly induced proliferation, COX-2 mRNA abundance, and PGE2 secretion, were all abolished by inhibition of JAK2, PI3-kinase, Akt or NF-κB. G-Gly stimulated phosphorylation of JAK2 and increased PI3-kinase activity in JAK2 immunoprecipitates. G-Gly increased Akt phosphorylation and kinase activity and NF-κB reporter activity in a JAK2-, PI3-kinase- and Akt-sensitive manner. G-Gly increased COX-2 promoter transcription in an Akt and NF-κB-dependent manner and also reduced COX-2 mRNA degradation in an Akt-insensitive manner. We conclude that G-Gly induced signalling involves a JAK2/PI3-kinase/Akt/NF-κB sequence leading to COX-2 transcription. G-Gly also seems to stabilise COX-2 mRNA via a separate pathway.

Introduction

Peptide hormones products of gastrin gene are increasingly being implicated as growth factors important in the promotion of various cancers. Amidated gastrin, the classical end product of processing of preprogastrin, secreted by G-cells in the gastric antrum and duodenum, plays a central role in the physiological and pathophysiological regulation of gastric acid (Dockray et al. 2001). Using a variety of cell culture models, as well as transgenic over-expression or exogenous administration, it has been shown that amidated gastrin is a growth factor for gastric, pancreatic and colon cancer cells (Todisco et al. 1997; Stepan et al. 2004; Dockray et al. 2001). Similarly the 80 amino acid progastrin peptide has been shown to stimulate the growth of colonic and pancreatic cancer cells (Rengifo-Cam et al. 2007; Singh et al. 2007). Glycine-extended gastrin (G-Gly) is an alternative end production of progastrin processing both physiologically in G-cells as well as within some tumour cells (Dockray et al. 2001). Previously believed to be biologically inactive, recent studies have confirmed that G-Gly can act as a growth factor for cultured gastric, colonic and pancreatic cancer cells (Iwase et al. 1997; Beales and Ogunwobi, 2006; Stepan et al. 1999; Seva et al. 1994) as well as non-transformed gastric and colonic cells (Hollande et al. 1997; He et al. 2005) and human embryonic kidney cells (Stepan et al. 1999a). Transgenic overexpression of G-Gly leads to colonic hyperproliferation and increases colonic tumorgenesis in the APC (min -/+) mouse model (Koh et al. 2000; Aly et al. 2001; Ferrand et al. 2005) and acts as a growth factor for lung cancer (Koh et al. 2003).

Despite these studies, the cellular signalling events mediating the effects of G-Gly are not defined. The majority of the effects of G-Gly appear to be mediated by a cellular

receptor distinct from the amidated gastrin (CCK_2) receptor (Todisco et al. 1997; Stepan et al. 2004; Dockray et al. 2001). Recently a progastrin receptor (annexin II) has been described and although G-Gly has been shown to bind to this, there are, as yet, no data showing biological activity upon such binding (Singh et al. 2007). Although a specific G-Gly receptor has not yet been characterised, G-Gly has been reported to activate separate and sometimes complimentary signalling pathways to those activated by gastrin, suggesting that G-Gly is biologically relevant.

We, and others, have shown that G-Gly can stimulate cancer cell growth by activating a variety of intracellular kinases including janus kinase 2 (JAK2), Akt and all 3 mitogen activated protein kinase cascades (extra-cellular signal related kinase (ERK), p38 mitogen activated protein kinase (p38 MAP kinase) and c-jun-NH2-terminal kinase (JNK)), however there are as yet no clear data showing how these pathways interact with each other and other signalling cascades (Stepan et al. 1999b; Beales and Ogunwobi, 2006; Ogunwobi and Beales, 2006; Ferrand et al. 2005). Because of the potential importance of G-Gly in promoting the growth of cancer cells understanding these areas would enable us to usefully interfere with these pathways for therapeutic gain.

Oesophageal adenocarcinoma (OAC) is another cancer that may potentially be regulated by G-Gly. The incidence and death rate from OAC has increased rapidly in the developed world: increasing 6 fold over the last 30 years (Buttar and Wang, 2004). It is believed that the increasing incidence of both obesity and gastro-oesophageal reflux disease contribute to these changes. It is widely accepted that most cases of OAC develop from metaplastic oesophageal glandular epithelium (Barrett's

oesophagus) and as such agents that promote the growth of Barrett's and OAC cells are of great interest in understanding the pathophysiology. Drugs which suppress gastric acid production, particularly the proton pump inhibitors (PPIs) are widely and increasingly used to treat symptomatic acid reflux and Barrett's oesophagus. Proton pump inhibitors bind covalently to the H^+/K^+/ATPase (the proton pump) in gastric parietal cells. They not only potently suppress gastric acid secretion and raise intragastric pH but initiate a negative feedback mechanism: gastric G-cells secrete increased amounts of gastrin in response to the elevated gastric pH. Thus a degree of relative hypergastrinaemia is commonly seen in patients exposed to PPIs (Cats et al. 2000; Nemeth et al. 1992). This hypergastrinaemia could be trophic for various tissues and contribute to the growth of OAC cells. In light of this, it should be noted that the use of potent acid suppressive agents has increased at rapid rate similar to that of OAC. This does not of course prove a causal association but does provide some food for thought. Limited previous studies have began to explore this hypothesis and initial data have shown that amidated gastrin stimulated proliferation of oesophageal cancer cells via the CCK_2 receptor, suggesting that this feedback mechanism deserves further exploration in the aetiology of OAC (Abdalla et al. 2004; Harris et al. 2004; Haigh et al. 2003).

Although serum G-Gly levels have never been systematically studied in OAC, Barrett's oesophagus or indeed any human disease state, it is known that G-cells co-process and co-secrete G-Gly with amidated gastrin. In some studies, secreted and serum G-Gly levels have been shown to be equivalent to amidated gastrin levels (Ciccotosto and Shulkes, 1992; Del Valle et al. 1987; Siddheshwar et al. 2001; Sugano et al. 1987; DelValle et al. 1989). It is also worth noting that most *in vitro*

studies have shown that G-Gly effects can be produced at concentrations at least an order of magnitude less than typically seen with gastrin, suggesting that even minor degrees of hypersecretion of glycine-extended gastrinaemia could be relevant to the growth of OAC (Beales and Ogunwobi, 2006; Stepan et al. 1999; Seva et al. 1994). Although CCK_2 receptor expression has been documented in Barrett's oesophagus and oesophageal adenocarcinoma, this is not always found. Haigh *et al* (2003) only demonstrated CCK_2 receptor mRNA expression in 7/12 cases of adenocarcinoma and 20-40 % of non-dysplastic and dysplastic Barrett's oesophageal tissue samples failed to show demonstrable CCK_2 receptor expression by RT-PCR (Abdalla et al. 2004). Therefore G-Gly acting via non-CCK_2R pathways could mediate mitogenic effects of hypergastrinaemia.

Therefore we have examined the ability of G-Gly to stimulate the growth of 2 well-defined OAC cell lines (OE33 and OE19). These have previously been used to examine the cellular mechanisms of leptin-, acid- and gastrin-induced proliferation in Barrett's oesophagus and OAC (Tselepis et al. 2003; Beales and Ogunwobi, 2007; Ogunwobi and Beales, 2008; Haigh et al. 2003). We have examined the sequence of intracellular events involved in these effects of G-Gly. Previous studies have separately suggested that the activation of both the protein kinase Akt and nuclear factor-κB (NF-κB) are central to the progression of OAC (O'Riordan et al. 2005; Sagatys et al. 2007; Beales et al. 2007) and several studies have shown that Akt may be an upstream activator NF-κB signalling (Gustin et al. 2004). We, and others, have previously shown that G-Gly can activate Akt signalling and in this study we have specifically examined the upstream and downstream mediators of Akt/NF-κB signalling.

Materials and Methods

Materials

Human glycine-extended gastrin-17 (G-Gly) was purchased from Neosystem (Strasbourg, France) and amidated gastrin-17 (G-17) was from Bachem (St.Helens, UK). SB 203580, GF 109203X, SP 600125, PP2, AG 490, Tyrene CR4 ((*E*,*E*)-2-(Benzylaminocarbonyl)-3-(3,4-dihydroxystyryl)acrylonitrile), Akt inhibitor VIII (1,3-Dihydro-1-(1-((4-(6-phenyl-1H-imidazo[4,5-g]quinoxalin-7-yl)phenyl)methyl)-4-piperidinyl)-2H-benzimidazol-2-one) and BAY 11-7082 were purchased from Merck (Nottingham, UK). Indomethacin, LY 294002, celecoxib, NS-398, AG 1478 and PD 98059 were from Alexis Biochemicals (Nottingham, UK). 740 Y-P was purchased from Tocris Bioscience (Bristol, UK). 3-[4, 5-dimethylthiazol-2-y-l]-2, 5-diphenyltetrazolium bromide (MTT), actinomycin D and CR 2945 were from Sigma (Poole, UK). Recombinant human interleukin-1 beta (IL-1β) was from R&D Systems (Abingdon, UK). When appropriate, stock solutions of inhibitors were dissolved in dimethyl sulfoxide (DMSO) (Sigma, UK). The final concentration of DMSO in experiments was always < 0.1 % and control wells contained an equivalent concentration. Inhibitors were added 60 minutes before the addition of G-Gly in all experiments and all inhibitor concentrations were chosen based on published data from our own and others' laboratories (Jijon et al. 2002; Grunberger et al. 2003; DeFeo-Jones et al. 2005; Langmesser et al. 2007; Souza et al. 2000; Ogunwobi and Beales, 2007; Ogunwobi et al. 2006).

Cell culture

The OE33 and OE19 human oesophageal adenocarcinoma cell lines were obtained from the European Collection of Cell Cultures (Salisbury, UK) and grown in

Dulbecco's modified Eagle medium (DMEM) containing 4500mg/l glucose, 100 mg/l penicillin, 100 mg/l streptomycin and 2mM L-glutamine and supplemented with 10% foetal bovine serum (FBS) as previously described (Ogunwobi et al. 2006). All cell culture media and supplements were from Invitrogen (Paisley, UK).

3-[4, 5-dimethylthiazol-2-y-l]-2, 5 diphenyltetrazolium bromide (MTT) assay

Cells were seeded at 5 x 10^4 cells per well in 48 well plates in complete medium for 24 hours and subsequently serum-starved for 24 hours before being treated with peptides for 48 hours. Relative cells numbers were determined using the modified MTT colorimetric assay as previously described (Beales, 2004).

Bromodeoxyuridine (BrdU) incorporation assay

2 x10^4 cells per well were cultured in 96 well plates in serum-containing medium until 60-70% confluent and subsequently serum-starved for 24 hours. Cells were then treated with 1nM G-Gly in serum-free medium and incubated for a further 48 hours. DNA synthesis and cell proliferation as previously described were measured using the BrdU incorporation assay (Roche, Burgess Hill, UK)(Ogunwobi et al. 2006).

COX-2 mRNA assay and measurement of PGE2 release

1 x 10^6 cells per well were seeded into 12 well plates and cultured in complete culture medium for 48 hours. After serum-starving for 24 hours, cells were treated with inhibitors and G-Gly. Four hours after stimulation the medium was aspirated and cells lysed and COX-2 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels in the cell lysates were measured using Quantikine mRNA enzyme-linked immunosorbent assay (ELISA) (R and D Systems) according to the manufacturer's

instructions as previously described (Ogunwobi et al. 2006). To investigate COX mRNA decay, serum-starved OE33 cells were stimulated with IL-1β for 1 hour to stimulate new mRNA synthesis. Cells were then treated with the mRNA synthesis inhibitor actinomycin D (5 μg/ml)(Jijon et al. 2002) and G-Gly (1 nM), the Akt inhibitor (5 μM) (DeFeo-Jones et al. 2005) or the PI3-kinase activator peptide 740 Y-P (50 μg/ml)(Derossi et al. 1998). Total RNA was isolated at 60-min intervals following mRNA synthesis inhibition for up to 4 h and analysed as described above. G-Gly stimulated PGE2 release was measured as previously described (Ogunwobi et al. 2006) using a specific PGE2 ELISA (R & D Systems).

Detection of phosphorylated JAK2, Akt and NF-κB

Tyrosine (1007/1008) phosphorylated JAK2 and total JAK2 were quantified using a cell based ELISA in formaldehyde-fixed cells as described previously (Beales and Ogunwobi, 2006). Total Akt and active serine-473 phosphorylated Akt were measured using a cell-based ELISA (Active Motif, Rixensart, Belgium) as previously described (Ogunwobi and Beales, 2007). Serine-536 phosphorylated and total NF-κB were quantified using a commercially available cell-based ELISA (NFκB p65 S536 CASE) according to the manufacturer's instructions (SuperArray, Peterborough, UK).

Detection of PI3-kinase and Akt kinase activities

Akt was immunoprecipitated from G-Gly stimulated OE33 cells essentially as described previously (Todisco et al. 2001). Similarly PI3-kinase activity associated with JAK2 immunoprecipitates was quantified with a modification on previous methods (Ferrand et al. 2006). Serum-starved OE33 cells were treated with G-Gly for

3-10 minutes and then lysed with cell lysis buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 10% glycerol, 1% triton X-100, 1 mM EDTA, 1.5 mM $MgCl_2$, 1mM Na_3VO_4, 10 mM NaF, 10 mM $Na_4P_2O_7$, 1mM PMSF, 20 μM leupeptin, 10 μg/ml aprotonin). Protein concentrations were measured with the Bio-Rad protein assay (Bio-Rad, Hemel Hempstead, UK). Equal amounts of protein were incubated with a rabbit polyclonal antibodies to Akt (1:50, Cell Signalling Technology, Hitchin, UK) or JAK2 (1:100, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and immunoprecipitation was performed by adding 20 μL of protein A sepharose.(Santa Cruz) and centrifuging. Pellets were washed four times with PBS and then Akt activity in equal aliquots was quantified using the Akt K-LISA™ activity assay (Merck) following the manufacturer's instructions. In brief, this assay detects the kinase activity in cell lysates or immunoprecipitates by incubating the test aliquots with a biotinylated peptide substrate in the presence of ATP in the wells of a streptavidin-coated 96-well plate. This allows for phosphorylation and substrate capture in a single step. Quantitation is performed using a HRP-conjugated anti-phospho-serine antibody followed by colour development with TMB detection solution and reading on an ELISA plates reader at 450/600 nm. PI3-kinase activity was measured using the PI3-kinase ELISA kit (Echeleon, Salt Lake City, UT, USA) according to the manufacturer's instructions. In brief, this is a competitive ELISA, with the products of the kinase reaction incubated with a $PI(3,4,5)P_3$ detector protein and the transferred to a $PI(3,4,5)P_3$-coated microplate. Binding of the detector is quantified against a $PI(3,4,5)P_3$ standard curve, using a peroxidise-labelled secondary detector and the colormetric signal is inversely proportional to the amount of $PI(3,4,5)P_3$ produced.

G-Gly induced transcriptional activity

NF-κB transcriptional activity in OE33 cells was measured using the RapidReporter® pRR-High-NF-κB *Gaussia* luciferase reporter system (Active Motif) according to the manufacturer's instructions. The plasmid vector contains the NF-κB tandem response element upstream of the *Gaussia* luciferase gene; 60-70% confluent OE33 cells in 10 cm dishes were transfected with the RapidReporter pRR-High-NF-κB plasmid using FuGENE 6 transfection reagent (Roche) according to the manufacturer's protocol and after 6 hours were seeded into a 96 well plate at 30 000 cells per well and cultured in complete medium for 24 hours. After a subsequent 24 hour period of serum-starvation the cells treated with inhibitors and G-Gly. Three hours after the addition of G-Gly, the media was removed and replaced with lysis buffer and luminescence was measured after the addition of the *Gaussia* assay buffer and substrate.

The specific effects of G-Gly on transcriptional activity of the COX-2 promoter were examined using the pDrive-hCOX2 reporter plasmid (Autogen Bioclear, Calne, UK). This contains the full length human COX-2 promoter (1568 bp) upstream of a β-galactosidase reporter enzyme. OE33 cells in 96-well plates were transfected with the reporter plasmid and the pRL-SV40 *Renilla* luciferase control plasmid (Promega, Southampton, UK) with FuGENE 6 according to the manufacturer's instructions and cultured for 24 hours, after which cells were serum-starved or 24 hours and then treated with inhibitors and G-Gly. COX-2 promoter transcriptional activity was measured after 6 hours using the BetaRed β-galactosidase assay kit (Merck) as directed and normalised with respect to luciferase activity measured using the *Renilla* luciferase assay kit (Promega).

Statistical analysis

Proliferation studies were done in triplicate or quadruplicate wells. COX-2 and mRNA studies were performed in duplicates. Experiments for direct detection of other signalling intermediates were performed in triplicate wells. Each experiment was repeated 3-8 times. Results are expressed as mean ± standard error of the mean. One way analysis of variance was used for dose response curves and paired t-tests were used to analyse the effect of inhibitors. A P value < 0.05 was considered significant.

Results

G-Gly stimulates proliferation of OE33 and OE19 oesophageal adenocarcinoma cells

G-Gly stimulation led to a dose dependent increase in cell number in both the OE33 and OE19 cell lines. The dose-response effects were similar with significant stimulation over the dose range 0.01 – 10 nM (fig 1A). Maximal effects were seen with 1nM and this was used for further characterisation. OE33 cells appeared to be more responsive that OE19 cells to G-Gly. To confirm that the effect on cell number observed was due to an effect on DNA synthesis and hence cell proliferation, the effect of 1nM G-Gly on BrdU incorporation was examined. 1nM G-Gly increased BrdU incorporation by 61 ± 15% above untreated control in OE33 cells and by 34 ± 9 % in OE19 cells. (fig 1B).

G-Gly-induced proliferation is independent of the CCK_2 (gastrin) receptor

To examine whether the proliferative effect of G-Gly is mediated via the CCK_2 (gastrin) receptor, we examined the effect of pre-treatment of cells with CR2945 (a specific CCK_2 receptor antagonist) (Langmesser et al. 2007) on G-Gly-induced proliferation. The proliferative effect of G-Gly was unaffected by pre-treatment of OE33 or OE19 cells with CR2945 (fig 1C). Gastrin-17 (10 nM) significantly stimulated OE33 cell proliferation, although less potently that G-Gly and this was inhibited by CR2945. Gastrin-17 did not stimulate OE19 proliferation.

G-Gly-induced proliferation is dependent on COX-2

Pre-treatment of OE33 and OE19 cells with selective COX-2 inhibitors (celecoxib and NS-398) as well as the non-selective COX inhibitor indomethacin (all in concentrations previously shown to block COX-2 activity in OE33 cells (Ogunwobi et al. 2006)) abolished the proliferative effect of G-Gly in both cell lines (fig 2A) as well as the effect of gastrin-17 in OE33 cells (fig 2B).

G-Gly-induced COX-2 mRNA expression and PGE2 release are dependent on PI3K/Akt, JAK2 and NF-κB

Having determined that G-Gly induced proliferation was dependent on COX-2 activity we next examined the effect of G-Gly on COX-2 mRNA and PGE2 production. G-Gly (1 nM) increased mRNA expression of COX-2 in OE33 cells by 240 ± 18% above untreated control (Fig 2C). This effect was abolished by pre-treatment of cells with specific inhibitors of PI3K/Akt (LY294002), JAK2 (AG490) and NF-κB (BAY 11-7082 (fig 2C). We further examined the effect on PGE2 as a further biomarker of COX-2 activity: untreated OE33 control cells secreted 302 ± 14 pg/ml of PGE2 and G-Gly stimulated cells secreted of 752 ± 20 pg/ml of PGE2 (fig 2D). The COX-2 inhibitor NS-398 blocked G-Gly stimulated PGE2 production and G-Gly stimulated PGE2 production was also blocked by LY294002, AG490 and BAY 11-7082.

G-Gly-induced proliferation is dependent on JAK2, PI3K/Akt and NF-κB

Consistent with the data showing that JAK2, PI3K/Akt and NF-κB were all acting upstream of COX-2 we confirmed that inhibition of these pathways also blocked G-Gly-driven proliferation in both OE33 (fig 3A) and OE19 (fig 3B) cell lines.

Inhibition of JAK2 kinase with both AG490 and a separate distinct specific inhibitor, tyrene CR4 (Grunberger et al. 2003), blocked the effect of G-Gly, as did inhibition of PI3-kinase with LY294002 as well as inhibition of Akt with a separate inhibitor (Akt inhibitor VIII)(DeFeo-Jones et al. 2005). The NF-κB inhibitor BAY 11-7082 was similarly effective. The pattern of responses was identical in the two oesophageal cancer cell lines.

G-Gly activates JAK2 upstream of Akt

G-Gly increased phosphorylation of JAK2 by 221 ± 14% above basal. This effect was significantly inhibited by pre-treatment of cells with AG 490 and tyrene CR4, the JAK2 inhibitors (Fig 4A). In contrast LY294002, Akt inhibitor VIII or BAY 11-7082 had no effect on G-Gly induced JAK phosphorylation. G-Gly also increased phosphorylation of Akt by 249 ± 39% above basal (fig 4B); this effect was abolished by pharmacological inhibition with LY294002 and the Akt inhibitor. It was also abolished by both of the JAK2 inhibitors. The NF-κB inhibitor Bay 11-7082 failed to block G-Gly induced Akt phosphorylation. Pretreatment of OE33 cells with inhibitors of Src (PP2), epidermal growth factor receptor kinase (AG1478), ERK (PD98059), p38 MAP kinase (SB 203580), protein kinase C (GF 109203X) or JNK (SP 600125), all in concentrations shown to be effective in OE33 and OE19 cells failed to affect G-Gly induced Akt phosphorylation (data not shown).

To further characterise the regulation of Akt by G-Gly we examined the effect on Akt kinase activity utilising a commercially available assay measuring phosphorylation of a synthetic peptide. Consistent with the Akt phosphorylation data, G-Gly increased Akt activity by 482% and this was blocked by both inhibition of JAK2 with AG490

and tyrene CR4 as well as the PI3-kinase inhibitor LY294002 and the specific Akt inhibitor but was unaffected by BAY 11-7082 (fig 4C).

We next further examined the linkage of upstream G-Gly-induced signalling to Akt activation by quantifying PI3-kinase activity associated with JAK2 immunoprecipitates. As shown in figure 4D, 5 minutes after G-Gly stimulation PI3-kinase activity had significantly increased (by 144%) and this was inhibited by both of the JAK2 inhibitors as well as LY294002 the specific PI3-kinase inhibitor but was insensitive to the Akt inhibitor and BAY 11-7082 the NF-κB inhibitor.

G-Gly induced Akt activation leads to NF-kB activation

G-Gly increased serine-526 NF-κB phosphorylation by 278 % and this was abolished by pre-treating the cells with inhibitors of JAK2, PI3-kinase and Akt (fig 5A). As expected, Bay 11-7082 blocked G-Gly induced NF-κB phosphorylation. To confirm these results we then assessed the effects of G-Gly and the inhibitors on the transcriptional activity of NF-κB. G-Gly significantly enhanced NF-κB driven transcription by 7.4 fold, in a JAK2-, PI3-kinase- and Akt-dependent manner (fig 5B). However the MAP kinase inhibitors PD98059, SB213580 and SP600125 failed to reduce G-Gly induced NF-κB transcriptional regulation (data not shown). A similar pattern was seen when the core native human COX-2 promoter was studied using a different reporter plasmid: G-Gly induced transcriptional activity by 4.2 fold and this was also blocked by JAK2, Akt, PI3-kinase and NF-κB inhibitors (fig 5C).

G-Gly induced Akt activity does not increased COX-2 mRNA stability

We finally investigated if G-Gly stimulated Akt activity increased COX-2 mRNA levels by a post-transcriptional mechanism. In the presence of actinomycin D which prevents further transcription, activating PI3-kinase and hence Akt with 740 Y-P, the cell permeable activator peptide, had no effect of the rate of decay of COX-2 mRNA. Interestingly Glycine-extended gastrin itself significantly reduced COX-2 mRNA degradation; however this effect was not affected by pre-treatment with the Akt inhibitor (fig 5D).

Discussion

In the current study we have shown that G-Gly stimulates proliferation of OAC cells via a specific JAK2/PI3-kinase/Akt/NF-κB/COX-2 pathway. This is the first demonstration of a proliferative effect in oesophageal cells and also extends our understanding of the signalling mechanisms initiated by G-Gly in promoting cell growth. This is the first study confirming that G-Gly stimulates proliferation via NF-κB-dependant pathways. These data also support the hypothesis that gastrin peptides released from the gastrin antrum may have a role in promoting the proliferation of metaplastic Barrett's epithelium and oesophageal adenocarcinoma.

Consistent with previous studies (Stepan et al. 1999b; Seva et al. 1994), these effects of G-Gly were not mediated via the classical gastrin (CCK_2) receptor. We showed that amidated gastrin stimulated OE33 cell proliferation via the CCK_2 receptor and COX-2 induction consistent with previous studies (Haigh et al. 2003): yet the CCK_2 receptor antagonist failed to affect G-Gly induced proliferation in either of the OAC cell lines. Again consistent with previous studies examining the membrane binding and functional effects of G-17 and G-Gly: effects of G-Gly were characteristically seen at lower concentrations (at least 10 fold) than G-17, showing that even mild hypersecretion of G-Gly may be important in stimulating the oesophageal epithelium (Beales and Ogunwobi, 2006; Stepan et al. 1999; Seva et al. 1994). Thus it is possible that G-Gly acts as growth factor for a substantial portion of OAC cells. Recent studies have demonstrated expression of gastrin mRNA and peptides in the epithelium of Barrett's oeosophagus, although the specific cell of origin, and products of peptide processing are as yet unclear (Buchan et al. 1985; Konturek et al. 2004; Harris et al. 2004). Thus in addition to endocrine effects of circulating G-Gly released from the

gastrin antrum, it is conceivable that G-Gly or other gastrin-related peptides act as autocrine or paracrine stimulators of growth in Barrett's oesophagus and adenocarcinoma.

In this study we concentrated on those pathways which have been demonstrated to be most involved in the pathogenesis of OAC. Increased activation of Akt has been demonstrated using immunohistochemistry with progression along the metaplasia/dysplasia/carcinoma sequence (Beales et al. 2007; Sagatys et al. 2007) and inhibitors of Akt activation reduce proliferation and induce apoptosis in OAC cells (Vona-Davis et al. 2005; Ogunwobi et al. 2006). Similarly activation of NF-κB has been shown to increase with progression of OAC (O'Riordan et al. 2005). In some models Akt acts as an upstream activator of NF-κB, although the specific mechanisms are unclear (Gustin et al. 2004). Akt has been reported to activate IKKα and tissues with a high ratio of IKKα to IKKβ seem to be more reliant on NF-κB activation via Akt (Gustin et al. 2004). Akt may also activate NF-κB in an IKK-independent manner by directly phosphorylating p65 (Ghosh and Karin, 2002). Our studies clearly showed that G-Gly activation of NF-κB in both OAC cells lines is mediated via Akt. Activation of Akt was confirmed by both a kinase assay and assessment of Akt phosphorylation and inhibitor studies demonstrated that Akt was upstream of NF-κB. At present, our studies do not allow us to determine the precise mechanism of activation of NF-κB but Akt enhanced both serine-536 phosphorylation of p65 and NF-κB transcriptional activity. These could be direct effects of Akt or dependent on the activation of another downstream kinase such as mTOR.

We showed that NF-κB-mediated increased transcription of COX-2 was an essential downstream mediated effect of the G-Gly stimulated pathway. Increased COX-2 expression is seen in Barrett's oesophagus and OAC (Cheong et al. 2003), and COX-2 inhibitors reduce proliferation in cell culture models (Souza et al. 2000) and human Barrett's oesophagus (Kaur et al. 2002) as well as prevent progression of Barrett's oesophagus in animal models (Buttar et al. 2002). We have previously shown that activation of the prostaglandin EP-4 receptor by the released PGE2 seems to be the essential downstream signalling event in stimulating proliferation in OE33 OAC cells, but in addition a variety of pro-carcinogenic actions of prostaglandins including immune evasion, invasion and angiogenesis have been described and in many cases the cellular mechanisms are unclear (Ogunwobi et al. 2006).

In addition to the effects on transcription, G-Gly also seemed to have separate action in increasing COX-2 mRNA stability. This effect was independent of Akt, as demonstrated by the failure of the Akt inhibitor to reverse the G-Gly effect and the lack of effect of the cell-permeable PI3-kinase activator peptide against the rate of COX-2 mRNA degradation, and suggests that other pathways are important in these effects. We did not find any evidence that MAP kinase or protein kinase C inhibitors affected activation of the Akt/NF-κB pathway but ERK has been shown to stabilise COX-2 mRNA in SEG-1 OAC cells (Souza et al. 2004) and it is quite possible that other pathways activated by G-Gly integrate at this point.

Here we have shown that PI3-kinase enzymic activity is increased in JAK2 immunoprecipitates strongly suggesting that JAK2 directly associates with, and activates, PI3-kinase subsequent upon G-Gly stimulation and seems to be an essential

intermediate in signalling from the putative membrane receptor to the intracellular kinase cascades. Ferrand *et al* showed that in HC116 colon cancer cells G-Gly activated PI3-kinase via separate JAK2 and Src pathways (Ferrand et al. 2006), whereas in HT-29 colon cancer cells we showed that G-Gly induced Akt phosphorylation was completely blocked solely by the PI3-kinase inhibitor LY294002 (Beales and Ogunwobi, 2006). In the current study, we failed to demonstrate any effect of the same specific Src inhibitor in 2 oesophageal cell lines. Whether this difference reflects variability in cell lines or tissues remains to be determined.

In the current study we have used pharmacological inhibition as the primary means of dissecting the pathways. Such approaches can be criticised as some inhibitors may not be as specific as often claimed. However we feel that our results are both reliable and generalisable; we have used widely used inhibitors and in most cases also utilised different separate inhibitors to confirm the effects on each point in the pathway. We also demonstrated activation of the specific pathways using both specific kinase assays and protein phosphorylation.

Previous studies have concentrated on the effects of G-Gly in colonic and pancreatic cell lines, but our data clearly show that oesophageal adenocarcinoma cells should be added to the list of G-Gly responsive cells. We have utilised two different well defined and studied OAC cell lines and although the Akt and NF-κB pathways are known to be activated in BO and OAC, we cannot yet extrapolate directly from our experimental data to the *in vivo* situation: further studies examining the possible role of G-Gy in the progression of BO and OAC are required. It will be important to

determine if the specific signalling pathway we have outlined is also activated in other cell lines and tissues as well as premalignant Barrett's oesophageal cells.

Therefore in conclusion we have shown that glycine-extending gastrin stimulates the proliferation of OAC cells via a signalling pathway involving activation of JAK2, leading to PI3-kinase activation and subsequent Akt activation. This then leads to increased phosphorylation and transcriptional activity of NF-κB and increased transcription of COX-2 and PGE2 production. Glycine-extended gastrin also stabilised COX-2 mRNA via a separate pathway. These effects were seen with picomolar and low nanomolar G-Gly concentrations. These results demonstrate that physiological release of G-Gly from antral G-cells may be pathologically important in the progression of OAC and that more attention should be paid to the potential adverse effects of acid suppression in these patients. Our study also confirms that a JAK2/Akt/NF-κB/COX-2 sequence is central to the proliferative response of OAC cells and links together the sometimes disparate observations implicating these pathways in OAC and providing impetus for therapeutic interventions.

Declaration of Interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Figure legends

Figure 1: Glycine-extended gastrin stimulates oesophageal adenocarcinoma cell proliferation via a CCK_2 receptor independent mechanism.

Serum-starved OE33 **(A, B and C)** and OE19 cells **(A,B and C)** were stimulated with either increasing concentrations of G-Gly **(A)**, 1 nM G-Gly **(B and C)** or G-17 (10 nM) **(C);** where appropriate cells were pre-treated as indicated with the CCK_2 receptor antagonist CR2945 (1 μM) **(C)**. After 48 hours, relative cell numbers were quantified with the MTT assay **(A and C)** or new DNA synthesis quantified with a BrdU incorporation ELISA **(B**). Results expressed as relative cell numbers or BrdU incorporation compared to untreated control cells, mean ± SEM, N = 3-8, * $P < 0.05$ vs untreated control cells, ** $P < 0.05$ vs untreated control cells, *** $P < 0.05$ vs G-17 treated cells.

Figure 2: Glycine-extended gastrin stimulates oesophageal adenocarcinoma cell proliferation via cyclo-oxygenase-2 dependant pathways.

Serum-starved OE33 cells **(A-D)** and OE19 cells **(A)** were pre-treated with the specific inhibitors indomethacin (indo) (10 μM), celecoxib (1 μM), NS 398 (5 μM), AG490 (25 μM), LY294002 (10 μM), Akt inhibitor VIII (5 μM) for 60 minutes and the stimulated with either G-Gly (1 nM) or G-17 (10 nM). After 48 hours relative cell numbers were quantified with the MTT assay **(A and B)**. Results expressed as cell numbers compared to untreated control cells, mean ± SEM, N = 3-5, * $P < 0.05$ vs corresponding peptide treatment, ** $P < 0.05$ vs untreated control.

After 4 hours COX-2 and GAPDH mRNA levels were quantified **(C)**. Results expressed as COX-2 relative to GAPDH compared to untreated control cells. Mean ± SEM, N = 3, * $P < 0.05$ vs G-Gly treatment, ** $P < 0.05$ vs untreated control.

After 18 hours cells PGE2 secretion measured using a specific ELISA **(D)**. Results expressed as PGE2 secreted / 30 minutes, mean ± SEM, N = 3, * $P < 0.05$ vs G-Gly treatment, ** $P < 0.05$ vs untreated control.

Figure 3: Glycine-extended gastrin stimulated oesophageal adenocarcinoma proliferation requires JAK2, PI3-kinase, Akt and NF-κB.

Serum starved OE33 **(A)** and OE19 **(B)** cells were pre-treated with inhibitors for 60 minutes; AG490 (25 μM), tyrene CR4 (1 μM), LY294002 (10 μM), Akt inhibitor VIII (Akt inh)(5 μM) or BAY 11-7082 (10 μM). Cells were them stimulated with G-Gly (1 nM). After 48 hours relative cell numbers were quantified with a MTT assay. Results expressed as cell numbers relative to untreated control cells, mean ± SEM, N = 3, * $P < 0.05$ vs G-Gly treatment, ** $P < 0.05$ vs control.

Figure 4: Effect of Glycine-extended gastrin on the activation of JAK2. PI3-kinase and Akt.

Serum-starved OE33 cells were pre-treated with inhibitors for 60 minutes; AG490 (25 μM), tyrene CR4 (1 μM), LY294002 (10 μM), Akt inhibitor VIII (Akt inh) (5 μM) or BAY 11-7082 (10 μM) and then stimulated with 1 nM G-Gly.

A: After 3 minutes cells were formalin-fixed and JAK2 tyrosine-phosphorylation quantified using a specific cell-based ELISA. Results are expressed as ratio of phospho-JAK2/total JAK2 as percentage of the ratio in untreated control cells.

B: After 5 minutes cells were formalin fixed and Akt phosphorylation was quantified using a specific cell-based ELISA. Results are expressed as ratio of phospho-Akt/total Akt as percentage of the ratio in untreated control cells.

C: After 5 minutes cells were lysed and Akt kinase activity quantified in Akt immunoprecipitates using a specific kinase (K-LISA) kit. Results expressed as phosphorylating activity compared to untreated controls.

D: After 3 minutes cells were lysed and PI3-kinase activity in JAK2 immunoprecipitates measured using a specific PI3-kinase assay. Results expressed as PI3-kinase activity compared to untreated control cells. All results expressed as mean ± SEM, N = 3, * $P < 0.05$ vs G-Gly treatment, ** $P < 0.05$ vs control.

Figure 5: Glycine-extended gastrin activates NF-κB, increases COX-2 transcription and reduces COX-2 mRNA degradation.

A: Serum-starved OE33 cells were pre-treated with AG490 25 μM, LY294002 10 μM, Akt inhibitor (Akt inh) 5 μM or BAY 11-7082 10 μM for 60 minutes and then stimulated with 1 nM G-Gly and formalin-fixed after 30 minutes. Serine-536 phosphorylation of NF-kB p65 was quantified using a cell-based ELISA. Results are expressed as ratio of phospho-p65/total p65 as percentage of the ratio in untreated control cells.

B: OE33 cells were transfected with NF-κB reporter luciferase plasmid and serum starved. After pre-treatment with inhibitors as in 5A, cells were stimulated with G-Gly 1 nM and luciferase activity measured after 3 hours.

C: OE33 cells transfected with a COX-2 promoter/β-galactosidase reporter plasmid were serum starved and treated with inhibitors and stimulated with G-Gly 1 nM. After 6 hours galactosidase activity was quantified.

D: Serum-starved OE33 cells were treated with IL-1β 1 ng/ml to induce COX-2 expression. After 1 hour (time 0) cells were treated with actinomycin D (5 μg/ml) to arrest new mRNA synthesis and G-Gly (1 nM), Akt inhibitor (5 μM) or the PI3-kinase activator peptide 740 Y-P (50 μg/ml). Cells were subsequently lysed and COX-2 mRNA quantified using a specific ELISA. COX-2 levels were normalised to GAPDH and expressed as % of untreated control cells. All results expressed as mean ± SEM, N = 3-4, * $P < 0.05$ vs G-Gly treatment, ** $P < 0.05$ vs control, *** $P <$ vs 0.05 vs control cells at the same time point.