

Gastrin induced autophagy in gastric adenocarcinoma cells

Master of Science in Molecular Medicine

Guri Solum, May 2014



Acknowledgement

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Abstract

The Gastroenterology and Cell biology research group at NTNU studies the role of gastrin in gastric cancer. Gastrin is shown to be involved in proliferation, anti-apoptosis, migration, invasion and angiogenesis. The role of gastrin in gastric adenocarcinoma is debated, but hypergastrinemia is considered to be a risk factor for gastric cancer. Autophagy is a cellular degradation process, wherein damaged cytosolic components are engulfed, and the components are degraded and recycled. The role of autophagy in cancer is complex since autophagy both may promote and inhibit malignancy. In this study it was of interest to examine the role of gastrin in autophagy. Two gastric adenocarcinoma cell lines have been used during the study.

We show for the first time that gastrin treatment increases the expression of the autophagic markers LC3-II and p62 in AGS-Gr and MKN45 cells. Blocking of the CCK2 receptor inhibited the gastrin-mediated increase of LC3-II and p62. Gastrin treatment increased the expression of phosphorylated LKB1 (Ser428), AMPK (Thr172) and ULK1 (Ser317, 5), a pathway known to induce autophagy. Moreover, gastrin was shown to reduce the apoptotic effect mediated by cisplatin in AGS-Gr cells. This survival effect of gastrin was reduced when blocking autophagy. This indicates that gastrin-mediated autophagy likely induces protective properties in response to cisplatin, and autophagy may be a mechanism of the survival effects of gastrin.

Abbreviations

AICAR	5-amino-4-imidazolecarboxamide riboside
AMPK	Adenosine-activated protein kinase
ATF6	Activating transcription factor 6
Atg	Autophagy-related genes
Baf	Bafilomycin A1
BSA	Bovine serum albumine
CCK2	Cholecystokinin 2
ECL	Enterochromaffin-like
ER	Endoplasmic reticulum
FCCP	Carbonyl cyanide p-trifluoromethoxyphenylhydrazone
FCS	Fetal calf serum
GRP	Gastrin-releasing peptide
HCQ	Hydroxychloroquine
IRE1	Inositol-requiring enzyme 1
Jak/STAT	Janus Kinase/Signal Transducer and Activator of Transcription
LC3	Microtubule-associated protein 1 light chain 3
LKB1	Liver kinase B1
MAPK	Mitogen-activated protein kinase
mTOR	Mammalian target of rapamycin
PBS	Phosphate buffered saline
PE	Phosphatidylethanolamine
PERK	Pancreatic ER kinase (PKR)-like ER kinase
PI3K	Phosphatidylinositol 3-kinase
PLC	Phospholipase C
ROS	Reactive oxygen species
TBST	Tris-buffered saline Tween
ULK1	UNC-51-like kinase 1
UPR	Unfolded protein response
ZES	Zollinger-Ellinson syndrome

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1. Introduction

1.1 Gastrin

The peptide hormone gastrin exerts its function as a regulator of gastric acid secretion to maintain an appropriate pH value for digestive enzymes. Gastric acid also makes an unfavourable environment for microorganisms. Gastrin is shown to have a general trophic effect on the gastric mucosa, and regulates growth and differentiation of the stomach [1-3]. The stomach is lined by an epithelium that is folded into glands, and contains a diversity of cells. Among them are G-cells, D-cells, enterochromaffin-like (ECL) cells, parietal cells, mucous-producing pit and neck cells, and stem cells. Stem cells differentiate into the specialized cells of the gastric mucosa, and the gastric epithelium is under constant renewal [1].

Gastric acid is regulated by the interplay between G cells, ECL cell, D cells and parietal cells (figure 1.1). Endocrine G-cells have microvilli on their surface that detects dietary amino acids and peptides after a meal. In response to a meal, gastrin is synthesized from the G-cells and released by exocytosis into the circulation. Gastrin binds to its receptor on the neuroendocrine ECL cells in the gastric epithelium, and this binding stimulate release of histamine from the ECL cells. Histamine interacts with parietal cells and leads to the release of gastric acid by the parietal cells into the lumen of the stomach [1, 4]. Gastric acid secretion is regulated by somatostatin and gastrin-releasing peptide (GRP). GRP is a positive regulator of gastrin and induces the production of gastrin from the G-cells. However, GRP may also induce the release of somatostatin from the D-cell in a negative feedback loop. Somatostatin is released by D-cells in response to gastric acid and histamine secretion, and inhibits the transcription of gastrin and thus its release from the G cells [1, 2, 4].

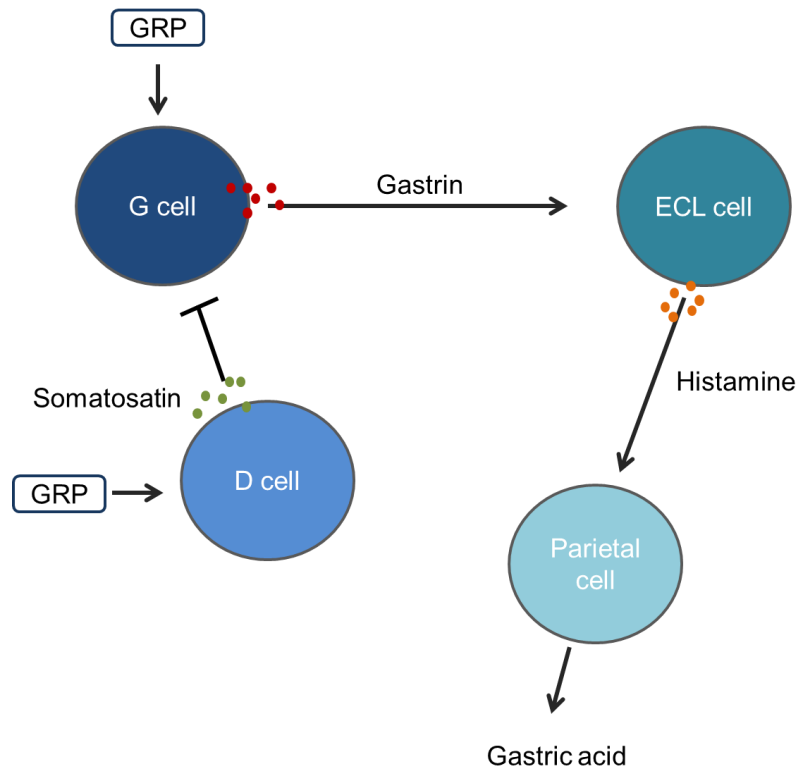


Figure 1.1: Regulation of gastrin secretion. Gastrin-releasing peptide (GRP) stimulates gastrin-secretion from the G cell, and gastrin binds to its receptor on the ECL cell and induces secretion of histamine. Histamine stimulates the parietal cell to secrete gastric acid. Somatostatin inhibits the release of gastrin in response to histamine and acid secretion.

1.2 Gastric cancer

Gastric cancer is the second most common cause of cancer-related death worldwide. Although the incidence has decreased over the decades, mortality is still high. Adenocarcinomas account for most of malignant gastric cancers [5-7]. Gastric adenocarcinomas are associated with chronic *Helicobacter pylori* (*H. pylori*) infection, which cause inflammatory cascades that may further result in malignancy [8]. *H. pylori* colonize the mucus layer of the stomach and produces urease enzymes. Infection with *H. pylori* causes reduced secretion of gastric acid (achlorhydria) and inhibits the somatostatin negative feedback regulation, leading to increased pH. This gives a basic environment that stimulates more production of gastrin, leading to hypergastrinemia. Achlorhydria gives a more favourable environment for microorganisms, with subsequent infections and inflammation, and further proliferation of *H. pylori* [1, 2].

Gastric adenocarcinomas can be divided into two subgroups; a poorly differentiated diffuse type, and an intestinal type associated with *H. pylori* infection [9]. The intestinal type develops through pathological stages from chronic active gastritis, atrophic gastritis, via metaplasia and dysplasia to a malignant tumour. In the development of gastric adenocarcinoma, the epithelial cells of the gastric mucosa are transformed into an intestinal cell type [2, 10]. Studies with transgenic INS-GAS mice have shown that hypergastrinemia can induce adenocarcinoma in these mice. INS-GAS mice have elevated serum gastrin concentrations at young age, followed by increased proliferation of parietal and ECL cells and acid hypersecretion. After 20 months the number of parietal and ECL cells decreases and the mice becomes achlorhydric, and progress to dysplasia and develop adenocarcinomas [2, 11, 12]. In INS-GAS mice infected with *H. pylori*, adenocarcinomas develop after only 6-7 months, suggesting that *H. pylori* accelerates the tumorigenesis [13, 14]. In humans hypergastrinemia alone is not shown to induce adenocarcinoma, but is regarded as a risk factor in combination with *H. pylori* infection [1, 2]. Gastrin is reported to stimulate proliferation *in vitro* of the human gastric adenocarcinoma cell lines AGS-Gr [15, 16] and MKN45 [17], the rat pancreatic adenocarcinoma cell line AR42J [18-20], and in the human colon adenocarcinoma cell line HT-29 [21, 22]. In AGS-Gr cells, gastrin is also shown to induce migration and invasion [23].

Gastrin is shown to stimulate the proliferation of ECL cells in the stomach [2, 24]. Hypergastrinemia is associated with the development of ECL-omas, and hypergastrinemia has been shown to induce ECL-omas in rats (reviewed in [1, 2]). In the human disease known as Zollinger-Ellison syndrome (ZES), in which gastrin-producing tumours (gastrinomas) cause hypergastrinemia, ECL hyperplasia and thicker gastric mucosa has been observed [1, 2]. However, in a study where biopsies from 106 patients with sporadic ZES were examined, 99% had developed ECL hyperplasia, 7% dysplasia, but none had developed carcinoids [25], indicating that results from animal models are not directly applicable to humans.

1.3 Gastrin-mediated signalling and cellular processes

Cells are dependent on the communication with other cells and their environment. External signals (hormones, cytokines, and growth factors) mediate responses in cells through a signal transduction cascade. Ligand binding to a receptor causes a conformational change in the receptor, and intracellular mediators and second messengers are activated. Signals can be transduced as phosphorylation cascades mediated by kinases. The cascade eventually results in activation of transcription factors and altered gene expression [26]. G-protein coupled receptors are the most abundant plasma membrane receptors, and are involved in cellular processes like proliferation, differentiation and migration [27]. Gastrin binds to and signals through the G-protein-coupled cholecystokinin 2 receptor (CCK2 receptor) located among others on ECL and parietal cells in the stomach [4, 28].

Several signalling pathways, including MAPK, PI3K/Akt, PLC- γ and Jak/Stat, are activated by the CCK2 receptor. These signalling pathways are known to be involved in proliferation, differentiation, adhesion, migration, invasion, angiogenesis and apoptosis (figure 1.2) (reviewed in [1, 28]). Deregulation of these cellular processes is crucial in development of cancer. In gastric cancer cell model systems, gastrin is reported to stimulate expression of genes involved in proliferation and anti-apoptosis via the MEK/ERK and PI3K/Akt pathway, and both to induce and inhibit cell proliferation [15, 28-31]. Gastrin is also known to be involved in migration and invasion (reviewed in [1, 28]).

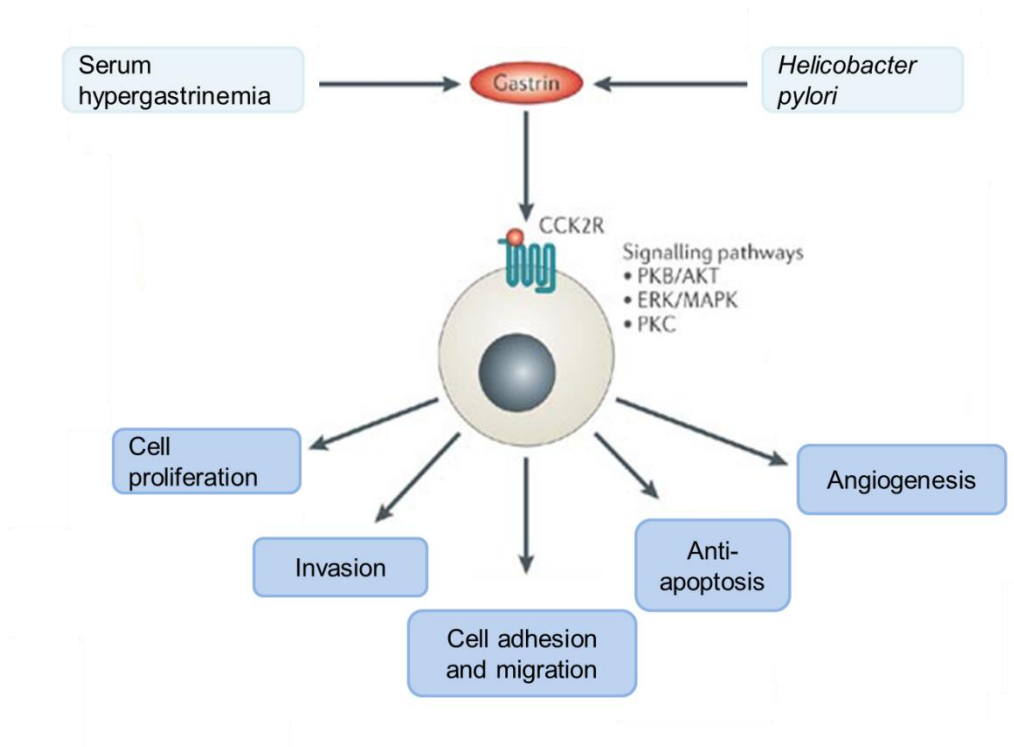


Figure 1.2: Overview of gastrin-mediated biological processes. Gastrin activates signalling pathways through the CCK2 receptor which may induce cell proliferation, migration, adhesion, invasion, anti-apoptosis and angiogenesis. Modified from Watson et al. [1].

1.4 Autophagy

Autophagy is an intracellular metabolic degradation system which maintains cellular homeostasis by engulfing damaged cytosolic components, degrading and recycling them. Some level of basal autophagy is needed for turnover of cytosolic components, but autophagy can also be induced in response to cellular stress such as nutrient deprivation, pathogen infection and hypoxia [32]. In the initial stage of autophagy, damaged components are engulfed by a double membrane called a phagophore, which elongates, encloses and matures into an autophagosome (figure 1.3). These steps are commonly referred to as initiation, nucleation, and elongation. The autophagosome eventually fuses with a lysosome, and the acidic environment of the lysosome, containing hydrolytic enzymes, degrades the cytosolic components. The understanding of autophagy on a molecular level started with the discovery of the autophagy-related genes (Atg) in yeast, and later the mammalian homologs. The finding of Atg genes in yeast and homologs in higher eukaryotes suggest that autophagy is

highly conserved through evolution. Deregulation of autophagy is linked to several pathological conditions, like infections, neurodegeneration and cancer (reviewed in [32, 33]).

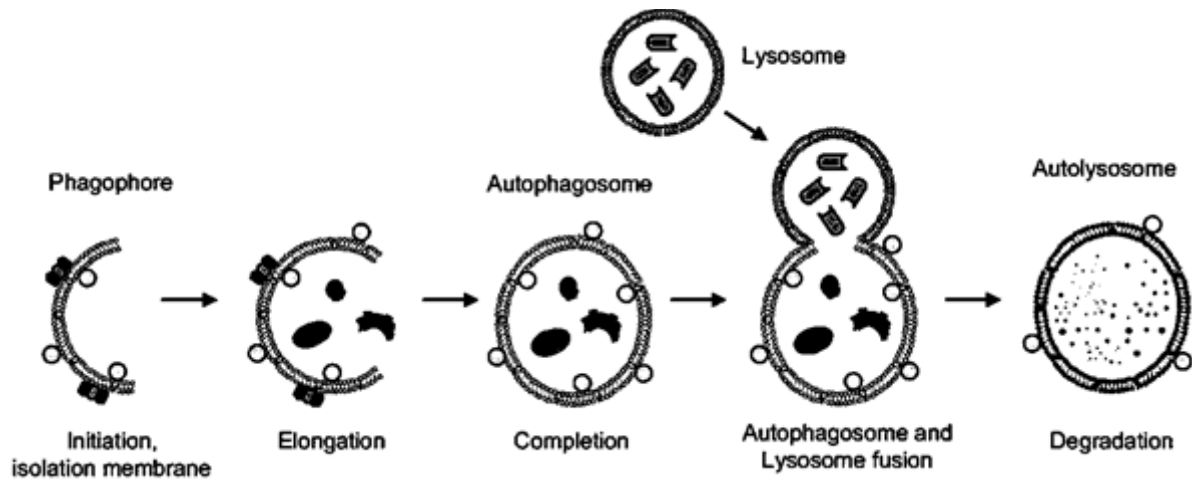


Figure 1.3: Formation of autophagosomes. Damaged cytosolic components is engulfed by a phagophore which elongates and matures into an autophagosome. The autophagosome fuses with a lysosome, which lead to the degradation and recycling of cytosolic components. Modified from Nakahira et al. [34].

1.5 Signalling pathways involved in autophagy

Autophagy is initiated under nutrient starvation, and is suppressed under nutrient-rich conditions. Autophagy is regulated by adenosine monophosphate-activated protein kinase (AMPK) and the mammalian target of rapamycin (mTOR), which serve as nutrient and energy sensors in the cells (figure 1.4). The ULK1 complex is a key modulator of autophagy and is required for initiation of autophagy and formation of the phagophore. Under nutrient deprivation, activated AMPK (phosphorylated at Thr172) activates ULK1 directly through phosphorylation at multiple residues, including Ser317 and Ser555, and indirectly by phosphorylation of raptor in the mTOR complex, which inhibits mTOR activity [35, 36]. Under nutrient-rich conditions, mTOR is activated via PI3K/Akt and MAPK/ERK pathways, and phosphorylates ULK1 (Ser757) to suppress autophagy [36, 37]. Activated LKB1 (phosphorylated at Ser428), a serine/threonine kinase upstream of AMPK, activates AMPK by phosphorylation (Thr172) (figure 1.4) [38].

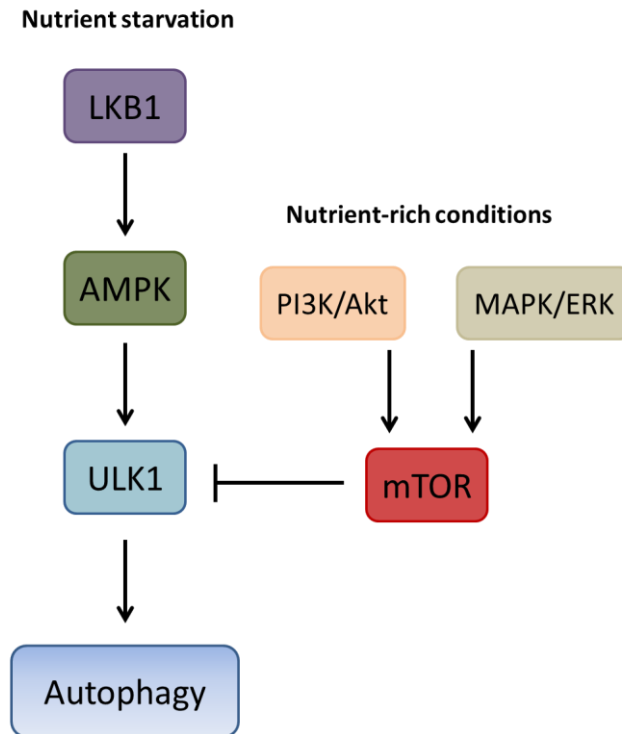


Figure 1.4: Regulation of autophagy. When deprived of nutrients, AMPK phosphorylates ULK1 at several residues to initiate autophagy. LKB1 is an activator of AMPK. mTOR phosphorylates ULK1 in nutrient-rich conditions, thus inhibiting autophagy initiation.

The class III phosphatidylinositol 3-kinase (PI3K) complex is required for the recruitment of Atg-proteins involved in nucleation, elongation and maturation of the autophagosome (figure 1.5). The PI3K complex consists of multiple proteins, including Beclin 1. Under normal nutrient-rich conditions, Beclin 1 is bound to Bcl-2, a protein known to be involved in anti-apoptosis. Under nutrient starvation, Bcl-2 dissociates from Beclin 1, which may induce autophagy [32]. For maturation into an autophagosome, the Atg12-Atg5-Atg16L complex and lipidation of LC3 is required. The Atg12-Atg5-Atg16L complex is involved in recruiting LC3-I to the phagophore membrane for lipidation. LC3 is cleaved by Atg5 to form the cytosolic form LC3-I. LC3-I is then conjugated to phosphatidylethanolamine (PE) to form the lipidated LC3-II, a reaction requiring both Atg7 and Atg3. LC3-II is found on the mature autophagosome, and when the autophagosome fuses with the lysosome, LC3-II is degraded. Since LC3-II is located on the autophagosome, it is commonly used as a marker for autophagy [32, 33, 37, 39]. When using lysosomal inhibitors (Baf and HCQ) that prevent the fusion of autophagosomes with lysosomes, it has been shown that LC3-II accumulates [40,

41]. Another protein used as an autophagic marker is p62, which is involved in degradation of ubiquitinated proteins, and linking of these proteins to the autophagosome. p62 binds to LC3-II on the autophagosome and is degraded by autophagy [33, 42, 43].

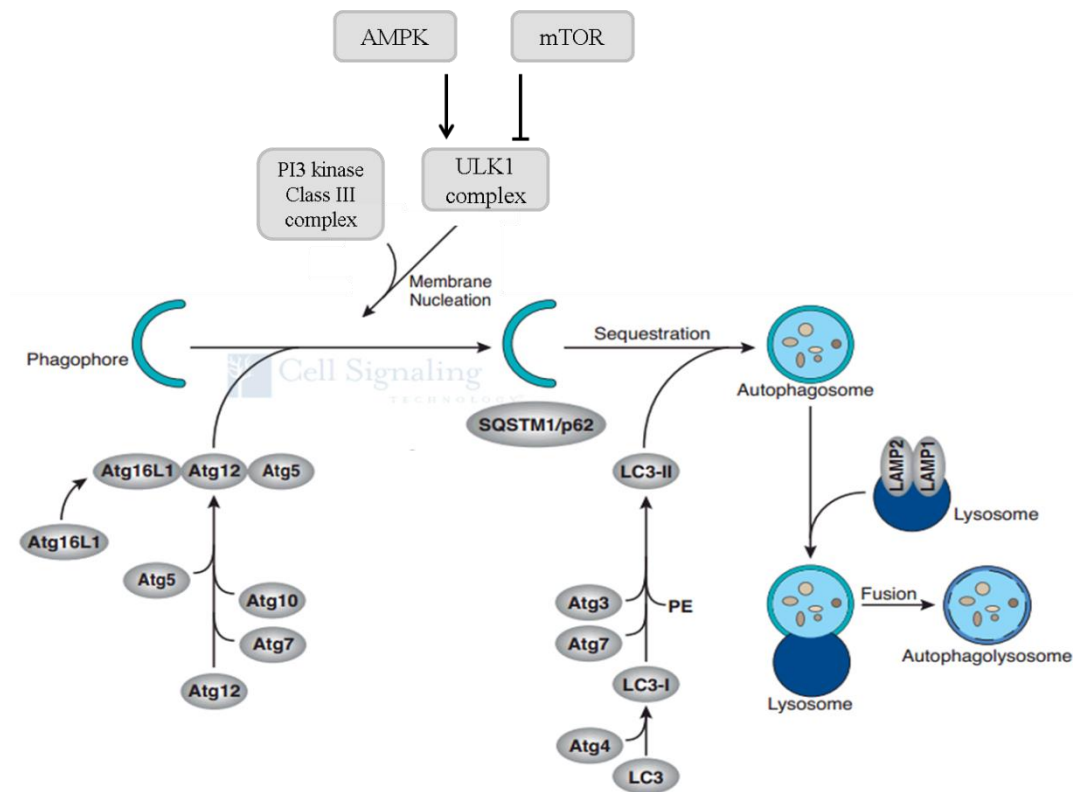


Figure 1.5: Overview of signalling and regulation of autophagy. Autophagy is shown to be regulated by AMPK and mTOR. The PI3K class III complex is required for recruiting Atg-proteins involved in forming the autophagosome. The Atg16L-Atg12-Atg5 complex and LC3-II is required for elongation and maturation of the phagophore. LC3 is cleaved by Atg4 to LC3-I which is conjugated with PE to form the lipidated form LC3-II. p62 is found on the autophagosome membrane and is degraded by autophagy. LC3-II and p62 accumulates when autophagy is inhibited. Modified from Cell signalling technology's website [44].

1.6 Autophagy and ER-stress

Physiological or pathological conditions may cause an unbalance in the protein folding capacity of the endoplasmic reticulum (ER), referred to as ER stress. If the chaperons that are in charge of correct folding of proteins exceed their capacity, misfolded, unfolded or damaged proteins may accumulate. This initiates the unfolded protein response (UPR) that aims to cope with the stress and restore the protein synthesis. The UPR is regulated by three ER stress sensors; pancreatic ER kinase (PKR)-like ER kinase (PERK), activating transcription factor 6 (ATF6), and inositol-requiring enzyme 1 (IRE1). When activated, the proteins translocate to the nucleus where they regulate the ER capacity [45-47].

Tumours may suffer from ER stress and one way to cope with this is induction of autophagy to maintain ER homeostasis and preventing cell death. It has been shown that PERK, IRE1 and ATF6 can interact with proteins in the autophagy pathway, and proteins downstream of PERK, IRE1 and ATF6 can induce autophagy (reviewed in [46, 47]). It has previously been shown by the Gastroenterology and Cell biology group at NTNU that gastrin treatment induces genes involved in ER stress and UPR in the pancreatic adenocarcinoma cell line AR42J [30].

1.7 Autophagy in cancer and apoptosis

Autophagy in cancer is often referred to as a double-edged sword – it can both suppress and promote tumour survival [48]. In early stages of cancer development, autophagy is commonly suppressed, but is up-regulated in later stages (reviewed in [49]). In the central areas of a tumour the blood supply is often inadequate, and tumour cells may suffer from hypoxia, starvation and metabolic stress. Initiation of autophagy may help tumour cells to handle this stress. Tumour cells can enter a period of dormancy in which they shrink in size, suppress their motility and stop proliferating. During this dormancy state, autophagy contributes to survival until normal conditions are re-established [48, 50]. Even though autophagy may help tumour cells to survive stressful situations, defective autophagy may also be of advantage for tumour cells. Damage in cells can lead to the production of reactive oxygen species (ROS), and thus cause DNA damage. Defective autophagy can cause accumulation of DNA damage, and further genetic instability for the tumour cells [50, 51]. Defective autophagy may also

induce necrotic cell death, tissue damage and an inflammation response that favours further tumour progression [50].

Autophagy may also act in a tumour-suppressing manner. Over-stimulation of autophagy can cause a rate of self-digestion that exceeds the capacity of the cells, resulting in apoptosis. Induction of DNA damage can be counteracted by autophagy and thus prevent genetic instability in tumour cells [48, 52]. Autophagy can suppress tumour development by limiting necrotic cell death and thus limit inflammation [48].

Autophagic cell death is morphologically characterized by autophagic vacuoles in the cytosol of the dying cell. In apoptotic cell death the end product is cellular destruction mediated by caspases, and is morphologically characterized by fragmentation, shrinkage, and nuclear condensation [53, 54]. The interplay between apoptosis and autophagy in cancer is complex. Autophagy can help tumour cells to escape apoptotic cell death under stress, and this have been further established by pharmacological inhibition of autophagy, which induced apoptosis [50, 51, 55, 56]. The stage of autophagy inhibition affects the phenotype of the cell death. With inhibition at an early stage the morphology resembles to an apoptotic cell death. By inhibiting the fusion of autophagosomes with lysosomes, accumulation of autophagosomes is seen prior to apoptotic cell death, giving a mixed phenotype [51, 55].

Several proteins, including Beclin 1, are known to interact with both apoptosis and autophagy (reviewed in [57]). Anti-apoptotic proteins of the BCL-2 family interact with Beclin 1, and this interaction inhibits initiation of autophagy. Pro-apoptotic BH3-only proteins can disrupt this interaction, and allows Beclin 1 to induce autophagy by interacting with the PI3K class III complex [57, 58]. Accumulation of p62 due to defective autophagy is shown to produce ROS and DNA damage, which can lead to apoptosis [59]. Induction of apoptosis leads to activation of caspases which can inhibit autophagy by cleavage of proteins essential for autophagy. Cleaved fragments from Beclin 1 can gain pro-apoptotic properties by inducing the release of cytochrome c from the mitochondria [57, 60].

1.8 Aim of study

The Gastroenterology and Cell biology group at NTNU is interested in the role of gastrin in adenocarcinoma cell systems, and have previously shown that gastrin is involved in endoplasmic reticulum (ER) stress responses. The possible role of gastrin in regulation of autophagy was therefore of interest. The understanding of mechanisms regulating autophagy is of great impact in cancer therapy strategies. Today, little is known about autophagy in gastric cancer and the possible role of gastrin. The aim of the study was:

- Characterize the role of gastrin in autophagy in gastric adenocarcinoma cell lines
 - Examine whether gastrin regulate autophagy in gastric adenocarcinoma cells
 - Characterize signalling pathways that are involved in gastrin mediated-autophagy
 - Optimizing Western blot for detection of phosphorylated kinases involved in autophagy
 - Characterize the cellular consequence of gastrin-mediated autophagy

2. Materials and method

2.1 Cell culture and experimental setup

The human gastric adenocarcinoma cell lines AGS-Gr and MKN45 have been used during the course of this study. AGS-Gr cells are transfected AGS cells (ATTC), which originate from a tumour in the epithelium of the stomach. AGS-Gr cells are stably transfected with the CCK2 receptor, and was given as a gift from Professor Andrea Varro at the University of Liverpool, England. MKN45 cells expressing the CCK2 receptor endogenously, were given as a gift from Professor Sue Watson at the Department of Surgery, Queens Medical Centre at the University Hospital, Nottingham.

The AGS-Gr cells were cultivated in HAM's F12 medium (Gibco, Carlsbad, CA) and MKN45 cells were cultivated in Dulbecco's modified Eagle's medium (DMEM) (Gibco). Both cell lines were supplemented with 10% FCS (Sigma, St. Louis, MO), 100 U/ml penicillin, 100 µg/ml streptomycin (Gibco) and 1 µg/ml fungizone (Gibco). The cells were incubated at 37°C in a humidified environment containing 5% CO₂. During splitting, cells were detached from the surface by trypsin with 0.25% EDTA (Gibco).

For Western blot analysis, 0.3×10^6 AGS-Gr and MKN45 cells were seeded into 6 well plates or 1.5×10^6 cells were seeded into 100 x 20 mm petri dishes for detection of LC3-II and p62. For the detection of phosphorylated proteins, 1.5×10^6 cells were seeded into 100 x 20 mm petri dishes. Cells were allowed to grow for one day. The cells were then treated with gastrin (10 nM) (G17, Sigma) in the presence of Bafolimylin A1 (Baf) (100 nM) (Sigma) for 2, 4 and 8 hours, both in serum-free F12/DMEM and HANKS balanced salt solution (Sigma). In the experiments where the CCK2 receptor was blocked, cells were treated with YM022 (Sigma) (100 nM) overnight before gastrin was added for 4 hours. For phosphorylated proteins, the cells were serum-starved for 14-20 hours and treated with 10 nM gastrin for 5, 10, 15, 30 and 60 minutes. As a positive control for phosphorylation of LKB1 and AMPK, cells were treated two hours with AICAR (0.5 nM). As a positive control for ULK1 cells were treated with FCCP (100 nM) for two hours.

2.2 Western blot

Cells were harvested after the stimulation, centrifuged (430 x g) for 8 minutes, and the cell pellet was re-suspended in PBS (Thermo Scientific, Rockford, IL) with 10% FCS. The cell suspension was centrifuged (430 x g) for 8 minutes, and 2x volume of the cell pellet of urea lysis buffer (Sigma) was added (Appendix 1). The samples were mixed several times, before a final centrifugation (16 000 x g) for 16 minutes. The supernatant was transferred into new Eppendorf tubes, and stored at -80°C.

For protein estimation, the Bio-Rad protein assay dye reagent (BIORAD Laboratories, Hercules, CA) was used (diluted 1:5 in H₂O). Protein lysate (1 µl) was added the diluted Bio-Rad solution (1 ml). The absorbance in the samples was measured at 595 nm. Each sample was measured in triplicates. A standard curve was generated by use of BSA, and used for calculating the protein concentrations.

The samples were mixed with loading dye (LDS) (5 µl) (Invitrogen, Carlsbad, CA) and sterile water to add up the same volume for each sample. The proteins were then denatured by heating the samples (70°C) for 10 minutes. For detection of LC3-II and p62, 35 µg protein was loaded, and for the CCK2 receptor, phosphorylated LKB1, AMPK and ULK1, 60 µg protein was loaded onto the gel. For further specifications, see table 2.1. The prepared protein sample, MagicMark XP Western Protein standard (2µl) (Invitrogen) and Kaleidoscope precision plus protein standard (4µl) (BIORAD) was separated on a Bis-Tris NuPAGE gel (Invitrogen) with NuPAGE MOPS SDS Running buffer (Novex, Carlsbad, CA). The membrane was activated in methanol and washed in 1X (Appendix 1). The proteins were transferred to a PVDF Immobilon-p membrane (Millipore, Billerica, MA) with NuPAGE MOPS Transfer buffer (Novex) (Appendix 1). To control that the transfer had been successful the membranes was activated in methanol and stained with Ponceau (Sigma) (Appendix 1) to visualize the bands. The membranes were blocked in 5 % milk (Nestlé, Vevey, Switzerland), Odyssey blocking buffer (LI-COR, Nebraska, US)) or 5 % BSA (Sigma). For specification of the antibodies, see table 2.2. Primary antibody was added and incubated in room temperature or overnight at 4°C. The membrane was washed in TBST after incubation, before secondary antibody was added and incubated in room temperature. The membrane was washed in TBST when using horse-radish peroxidase (HRP)-probed antibodies, and in TBS when using fluorescent secondary antibodies. SuperSignal (Thermo Scientific) was applied for 40 seconds to 5 minutes to detect HRP activity from HRP-probed antibodies, and the blot was imaged by

Odyssey FC (LI-COR). When the membrane was probed with fluorescent secondary antibody, the incubation and wash was performed in the dark, and the membrane was dried in darkness for 2-3 hours before developing. The membrane was imaged with Odyssey (LI-COR). Bands were normalised to β -Actin or GAPDH, and blots were quantified by use of Image Studio 3.1 software.

Table 2.1: Western blot specifications.

Protein detected	Running	Transfer	Blocking
CCK2 receptor	200 V, 75 minutes, room temperature, 10 % gel	30 V, 80 minutes, room temperature	Overnight in 5 % milk
LC3-II and p62	200 V, 75 minutes, room temperature, 12 % gel	30 V, 80 minutes, room temperature	2 hours in 5 % milk
Phosphorylated proteins	200 V, 40 minutes and 120 V, 80 minutes on ice 10 or 4-12 % gel.	30 V, 80 minutes, room temperature or 30 V, 130 minutes on ice	5% milk, 5 % BSA or Odyssey blocking buffer

Table 2.2: Antibodies used in Western blot.

Protein detected	Antibody	Company	Dilution	Molecular weight
CCK2 receptor	Anti-CCK2R Mouse polyclonal	Abcam	1:1000	48 kDa
LC3-II	LC3B antibody Rabbit	Cell signalling technology	1:1000	14 kDa
LC3-II	LC3B (D11) XP Rabbit IgG	Cell signalling technology	1:1000	14 kDa
p62	p62 Guinea pig polyclonal	Progen Biotechnik	1:1000	62 kDa
ULK1 (total)	ULK1 Rabbit IgG	Cell signalling technology	1:1000	150 kDa
pULK1 (Ser317)	Phospho-ULK1 (Ser317) Rabbit IgG	Cell signalling technology	1:1000/ 1:500	140 kDa
pULK1 (Ser555)	Phospho-ULK1 (Ser555) Rabbit	Cell signalling technology	1:1000	140 kDa
AMPKα	AMPK α Rabbit	Cell signalling technology	1:1000	62 kDa
pAMPK	Phospho-AMPK α (Thr172) Rabbit mAb	Cell signalling technology	1:1000	62 kDa
LKB1	LKB1 Rabbit IgG	Cell signalling technology	1:1000	54 kDa
pLKB1 (Ser428)	Phospho-LKB1 (Ser428) Rabbit IgG	Cell signalling technology	1:1000	54 kDa
β-Actin (Loading ctrl)	Anti-beta Actin Mouse monoclonal	Abcam	1:5000	42 kDa
GAPDH (Loading ctrl)	Anti-GAPDH mouse monoclonal	Abcam	1:5000	40 kDa
Anti-Rabbit	Secondary antibody Goat anti-rabbit	Thermo Scientific	1:5000	
Anti-mouse	Secondary antibody Polyclonal Goat anti- mouse	DAKO	1:5000	
Anti-Guinea pig (Fluorescent)	Secondary antibody Donkey anti-guinea pig	LI-COR	1:5000	
Anti-mouse (Fluorescent)	Secondary antibody Goat anti-mouse	LI-COR	1:20 000	
Anti-Rabbit (Fluorescent)	Secondary antibody Goat anti-rabbit	LI-COR	1:5000	

2.3 Confocal microscopy

10 000 cells per well was seeded in a chambered coverglass, and the cells were left overnight. The cells were then fixed with 4 % paraformaldehyde (Merck, Darmstadt, Germany), and left for 10 minutes. The liquid was removed and cells were washed once with PBS. Cold methanol (-20°C) was added for 10 minutes, and the wells were placed on ice. The cells were washed once with PBS. For blocking, 3 % goat serum (Gibco) in PBS was added, and the cells were left in blocking overnight at 4°C. The blocking solution was removed, and primary antibody, diluted in 1 % goat serum, was added and left for one hour on a shaker. The cells were then washed with PBS for 3x10 minutes in room temperature. Secondary antibody was diluted in 1% goat serum, and was left for one hour on a shaker. The cells were washed in PBS, 4x10 minutes. The cells were protected from light and stored in PBS at 4°C until images were taken. Zeiss LSM 510 META microscope was used to capture the images. The 488 channel (495 nm, green) was used for detection of LC3-II and the 647 channel (650 nm, red) for p62. Specifications about the antibodies used are shown in table 2.3:

Table 2.3: Antibodies used for immunocytochemistry.

Protein detected	Primary antibody	Secondary antibody
p62	Guinea pig polyclonal (1:2000) Progen Biotechnik	Goat Anti- guinea pig (1:5000) Progen Biotechnik
LC3-II	Rabbit (1:2000) Cell signalling technology	Goat Anti-rabbit (1:5000) Cell signalling technology

2.4 XTT assay

XTT assay was performed by using TACS XTT Cell proliferation assay kit (Trevigen, Gaithersburg, MD). XTT working solution was made by mixing XTT activator (100 μ l) and XTT reagent (5 ml) from the kit. 10 000 cells per well were seeded into flat bottom 96 well plates and left overnight. The cells were then serum-starved and stimulated with gastrin (10nM) for 2 hours, before cisplatin (Merck) was added. Following cisplatin treatment, the cells were left for different time points and the XTT working solution (50 μ l) was added and left for 2 hours before the absorbance was read with iMark Microplate Reader (BIORAD) at dual wavelength; 490 nm and 620 nm. In experiments with hydroxy-chloroquine (HCQ) (Sigma), HCQ (10 μ M) was added 12 and 24 hours after cisplatin and the absorbance was measured after 36 and 48 hours of cisplatin stimulation.

3. Results

3.1 CCK2 receptor expression

The model system used in this study includes the gastric adenocarcinoma cell lines AGS-Gr and MKN45. Gastrin has been shown to induce the expression of the CCK2 receptor in AGS-Gr cells [61], AR42J [62] and the gastric KATOIII cells [63]. There are only few, and relatively old studies about the expression of the CCK2 receptor in MKN45 [64, 65], and examining the expression of the CCK2 receptor in these cells was therefore of interest. MKN45 cells were serum-starved and treated with gastrin for 2, 4, 8 and 16 hours. The results show that the CCK2 receptor is expressed in MKN45 cells, and further show a small increase in protein levels after gastrin-stimulation (figure 3.1B). HEK293 cells were included as a negative control in this experiment. AGS-Gr is stably transfected with the CCK2 receptor [66], and expression of the CCK2 receptor in AGS-Gr have previously been shown by using immunocytochemistry (figure 3.1A). However, AGS-Gr cells showed low expression of the CCK2 receptor (figure 3.1B). This may be caused by the antibody used for immunoblotting.

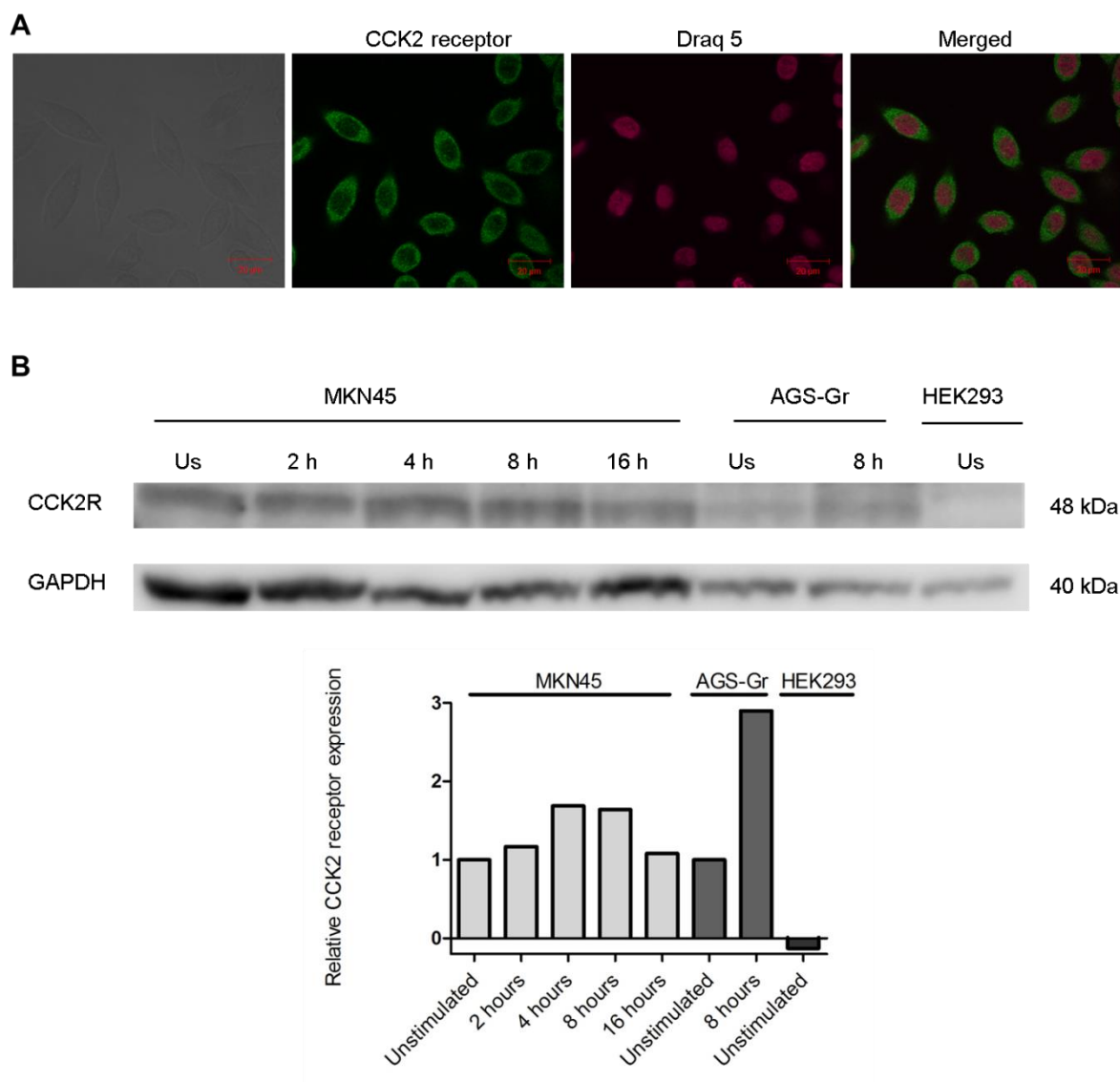


Figure 3.1: CCK2 receptor expression. **A)** Cells were fixed and stained for the CCK2 receptor with an antibody from Abbiotech. The cells were co-stained using DRAQ5 (experiment performed by Ph.D fellow Shalini Rao). **B)** Cells were treated with gastrin (10 nM). Protein levels of the CCK2 receptor was detected by Western blot, using an antibody from Abcam. The results in B) are representative of two biological replicates.

3.2 Gastrin mediated induction of the autophagic markers LC3-II and p62

To determine whether gastrin affects autophagy in gastric adenocarcinoma cells lines, the levels of the autophagic markers LC3-II and p62 were examined by Western blot. Cells were treated with gastrin in the presence of the lysosomal inhibitor Bafilomycin A1 (Baf), and simultaneously serum-starved in F12/DMEM or amino acid-starved in HANKS. Since

HANKS media contain no amino acids we expected starvation in HANKS to induce higher levels of autophagy. Baf prevents the fusion of the autophagosome and the lysosome, resulting in accumulation of LC3-II and p62. If gastrin induces autophagy, we expected that treatment with gastrin in the presence of Baf would result in higher increase in protein levels of LC3-II and p62 than treatment with Baf alone. The results show that LC3-II levels increase after 4 and 8 hours of gastrin treatment in the presence of Baf compared to Baf alone in AGS-Gr cells cultivated in F12 (figure 3.2). The protein level of p62 also increases after 4 and 8 hours of treatment, but the increase is not as pronounced as for LC3-II (figure 3.2). The level of LC3-II increased after 2 and 8 hours of gastrin treatment in the presence of Baf, while the level of p62 increased after 2 and 4 hours in cells cultivated in HANKS (figure 3.3). These results indicate that gastrin increases the protein levels of the autophagic markers LC3-II and p62 in AGS-Gr cells.

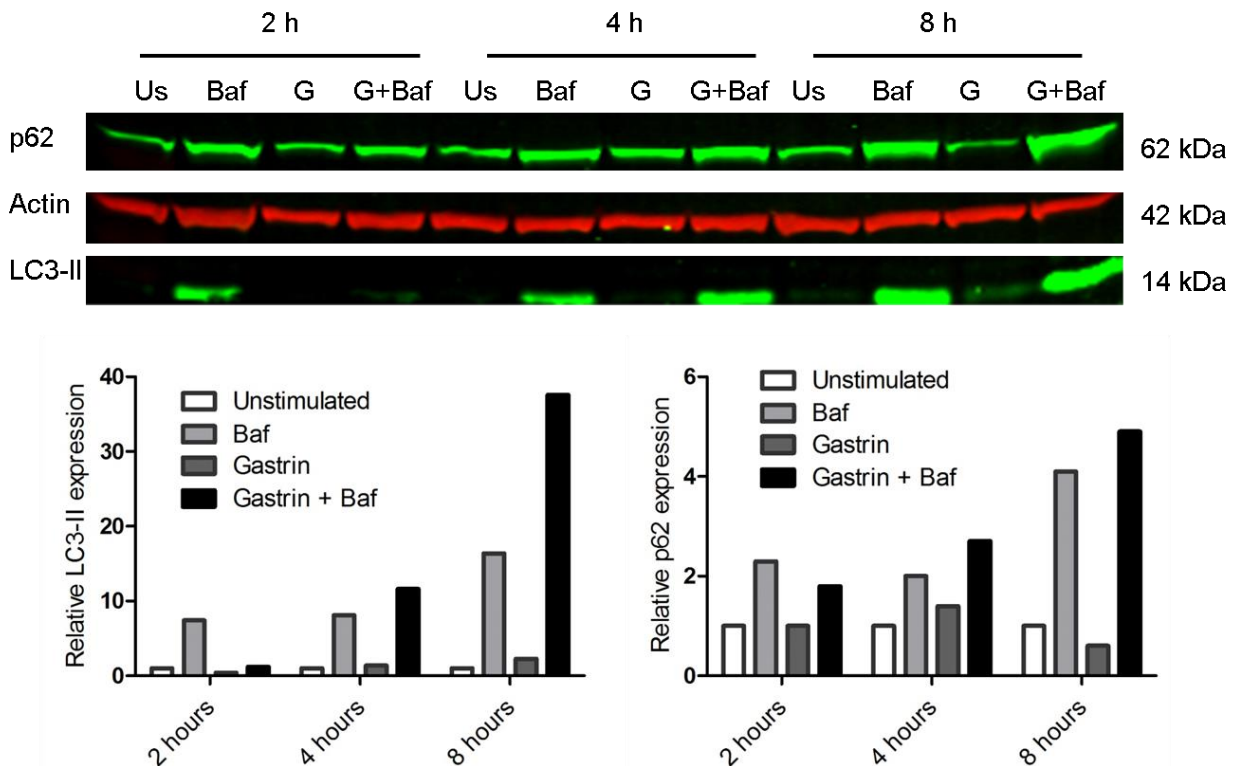


Figure 3.2: Expression of LC3-II and p62 in AGS-Gr cells cultivated in F12. Cells were serum-starved and simultaneously treated with gastrin (10 nM). The results shown are representative from one of three independent experiments.

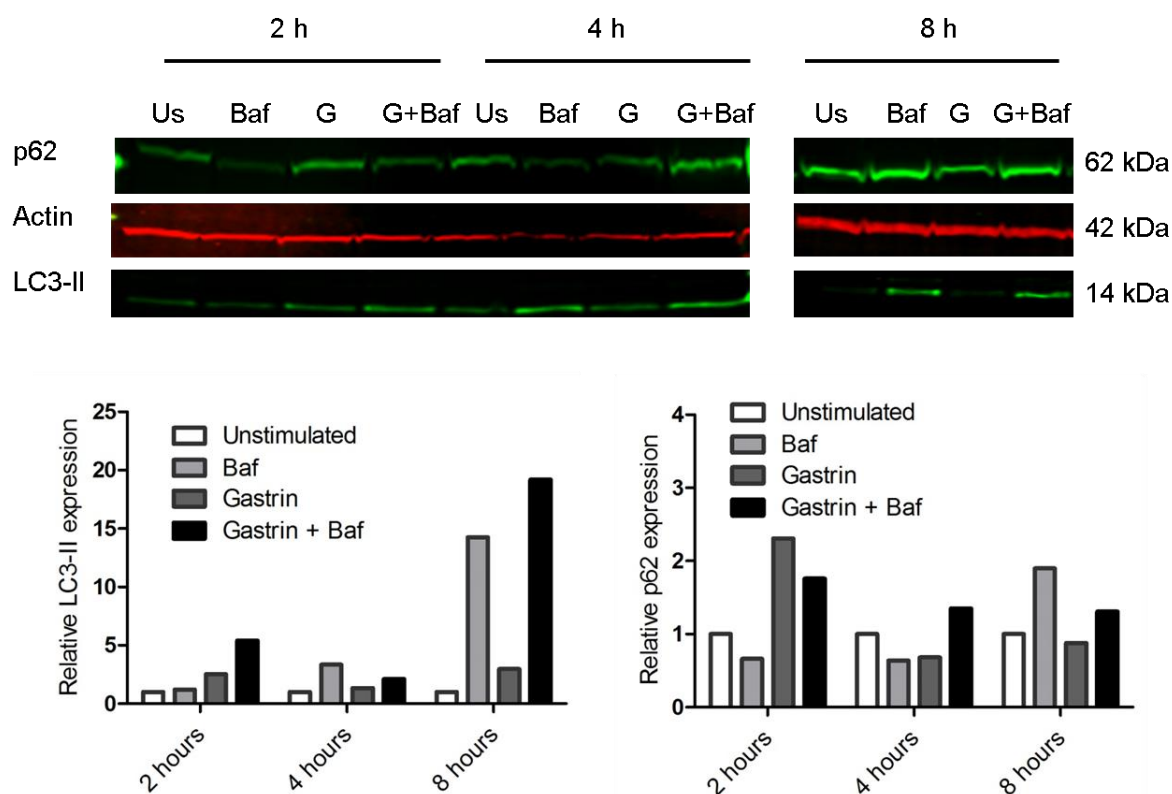


Figure 3.3: Expression of LC3-II and p62 in AGS-Gr cells cultivated in HANKS. Cells were amino acid-starved and simultaneously treated with gastrin (10 nM). The results shown are from one experiment.

Further, we examined the expression of LC3-II and p62 in MKN45 cells. We found that cultivation in DMEM increased the expression of LC3-II after 4 hours of gastrin treatment in presence of Baf compared to Baf alone (figure 3.4). Increase in p62 levels were observed after 2 and 4 hours of treatment. When cultivating cells in HANKS, the gastrin-induced increase of LC3-II was not very pronounced (figure 3.5). Gastrin did not enhance p62 protein levels in cells starved in HANKS. Taken together, these results show that gastrin increases protein levels of LC3-II and p62 in MKN45 cells cultivated in DMEM, indicating a role of gastrin in autophagy. However, gastrin did not increase autophagy further when cultivating cells in HANKS.

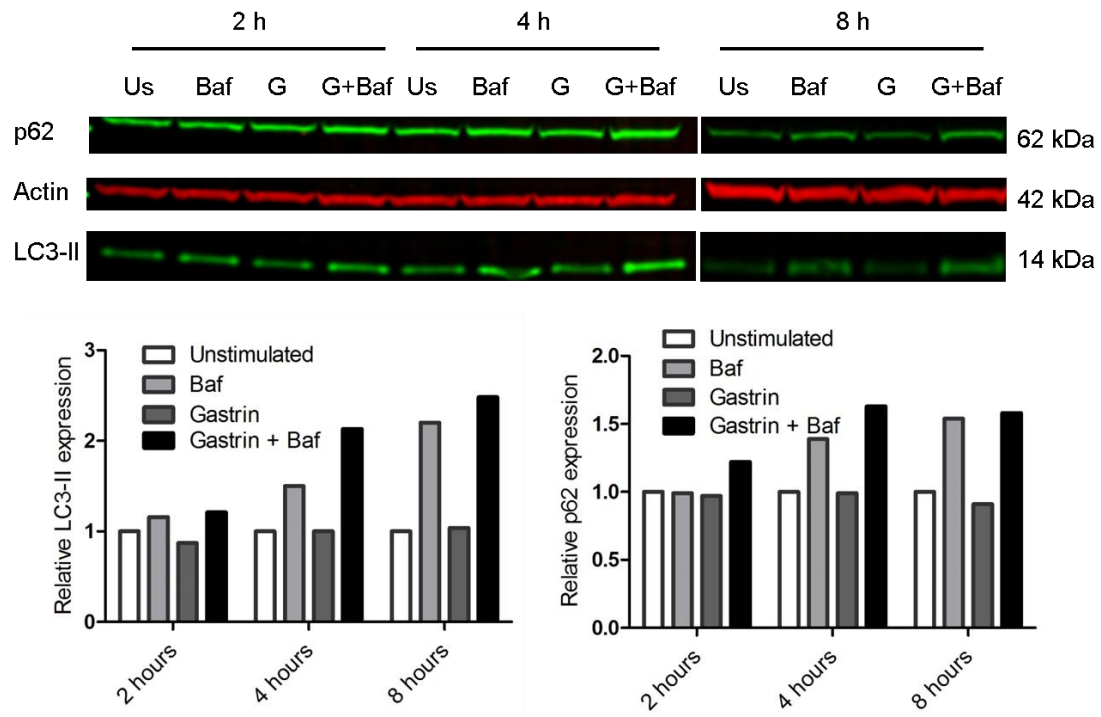


Figure 3.4: Expression of LC3-II and p62 in MKN45 cells cultivated in DMEM. Cells were serum-starved and simultaneously treated with gastrin (10 nM). The results shown are representative from one of three independent experiments.

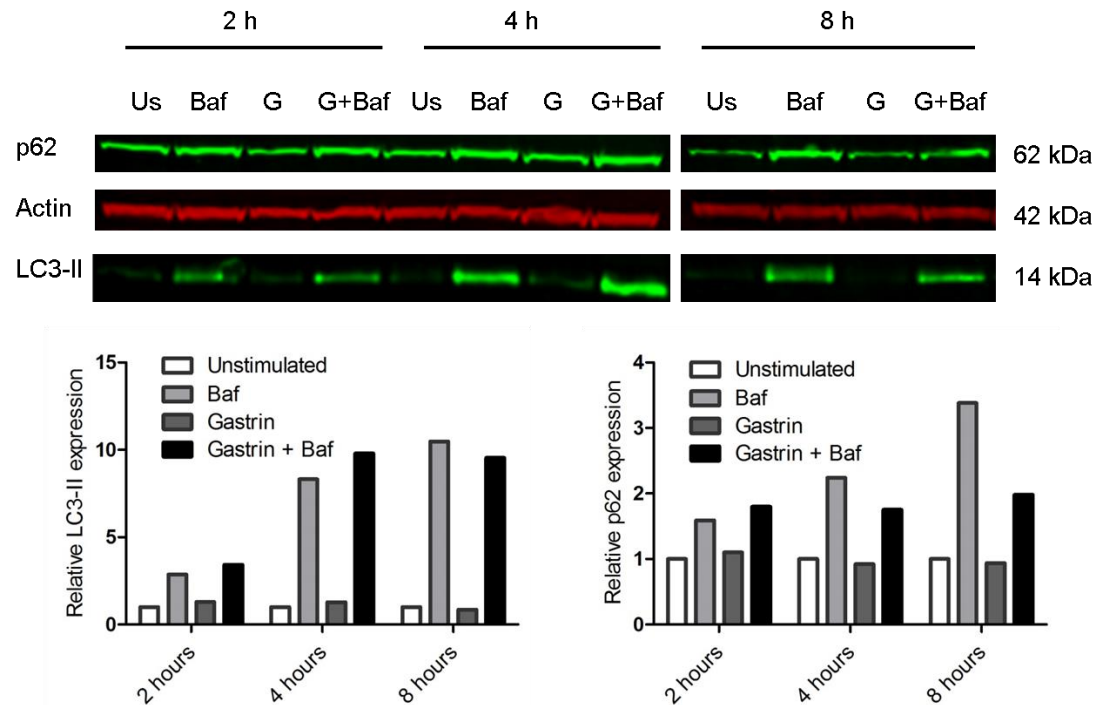


Figure 3.5: Expression of LC3-II and p62 in MKN45 cells cultivated in HANKS. Cells were amino acid-starved and simultaneously treated with gastrin (10 nM). The results shown are from one experiment.

3.3 Detection of gastrin induced LC3-II and p62 by use of immunocytochemistry

It was further of interest to assess LC3-II and p62 by immunocytochemistry. MKN45 were treated with gastrin in the presence of Baf for 8 hours, and cultivated in both DMEM and HANKS media. The staining was visualized using the imaging technique confocal microscopy. Gastrin treatment resulted in increased expression of LC3-II and p62 when cells were cultivated in DMEM (figure 3.6A). In cells cultivated in HANKS media, no clear enhancement of LC3-II and p62 levels were observed (figure 3.6B). Taken together, these results are congruent with the Western blot results, and suggest a role of gastrin induced autophagy in gastric adenocarcinoma cells.

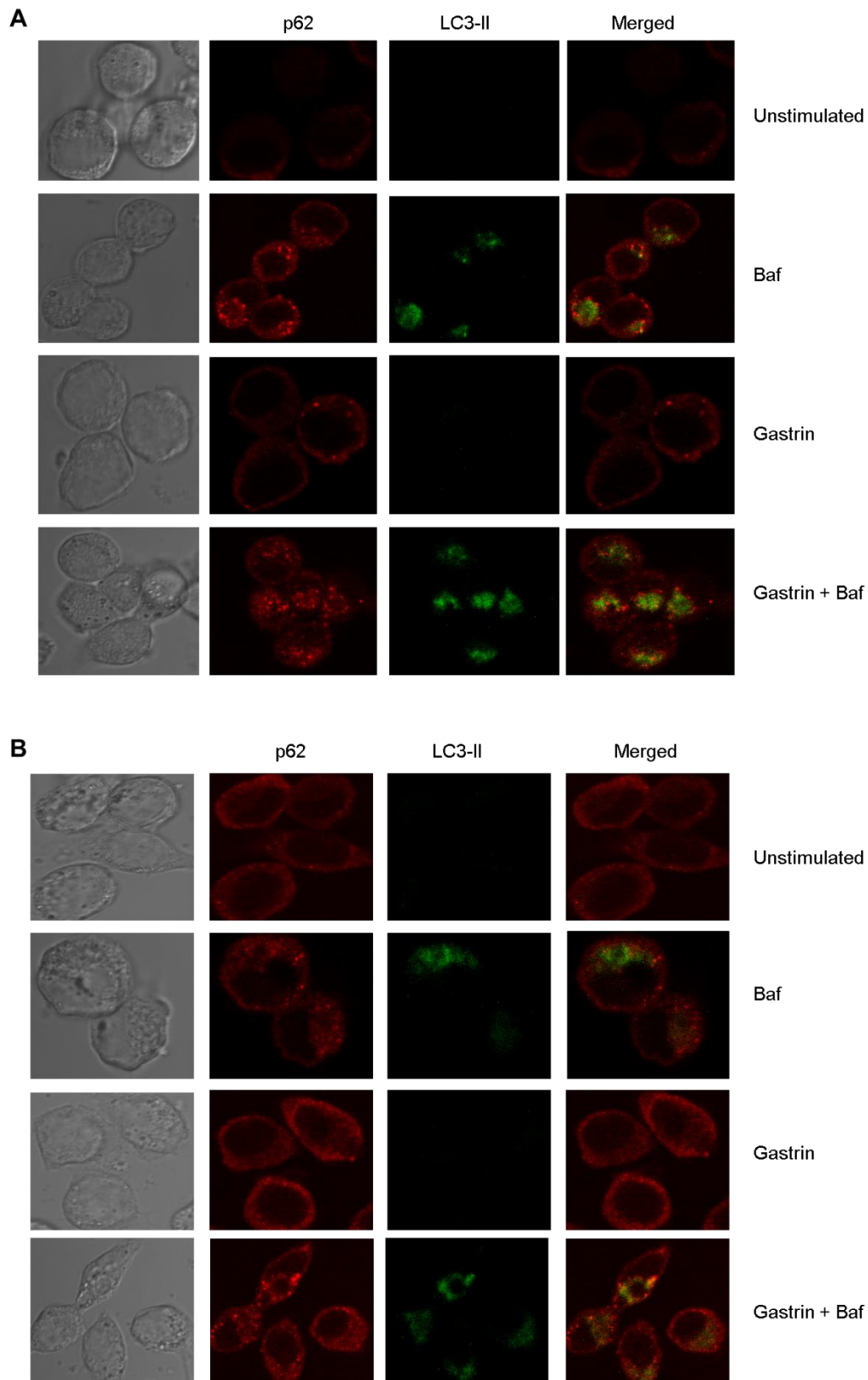


Figure 3.6: Staining of LC3-II and p62 in MKN45. Cells were starved and simultaneously treated with gastrin (10nM) for 8 hours and then stained for p62 and LC3-II. **A)** Cells cultivated in DMEM. **B)** Cells cultivated in HANKS.

3.4 Inhibition of gastrin mediated autophagy

The results so far showed that gastrin induces the autophagic markers LC3-II and p62 in AGS-Gr and MKN45 cells. Next we wanted to confirm whether this induction actually was mediated through the CCK2 receptor. We found recently that the CCK2 receptor antagonist YM022 reduced gastrin-mediated migration in AGS-Gr and MKN45 cells (manuscript in progress). YM022 was therefore added to see if blocking the CCK2 receptor would influence gastrin-induced LC3-II and p62 levels.

We observed that the presence of CCK2 receptor antagonist reduced the levels of LC3-II and p62 in AGS-Gr cells (figure 3.7A). Similar results were observed in MKN45 cells (figure 3.7B), indicating that the effect of gastrin was mediated via the CCK2 receptor.

Notably, pre-treatment with YM022 showed an increase in LC3-II and p62 levels in unstimulated MKN45 cells, indicating an off-target effect of YM022. Thus, the role of the CCK2 receptor was examined with siRNA, which showed similar results as with the YM022 inhibitor experiments (data not shown). Taken together, these results indicate that gastrin treatment causes an increase in protein levels of LC3-II and p62, and that blocking or knock down of the CCK2 receptor inhibits gastrin mediated increase of LC3-II and p62 in AGS-Gr and MKN45 cells.

3.5 Gastrin mediated phosphorylation of LKB1, AMPK and ULK1

Since our results suggest that gastrin increases autophagy in AGS-Gr and MKN45 cells, we wanted to unravel signalling pathways involved in gastrin induced autophagy. The kinase LKB1 is known to phosphorylate and activate the kinase. AMPK is further shown to phosphorylate and activate ULK1 to initiate autophagy. Therefore we wanted to examine whether gastrin mediated activation of the LKB1 – AMPK – ULK1 pathway. MKN45 and AGS-Gr cells were serum-starved and treated with gastrin for 5-60 minutes. The phosphorylation of LKB1 (Ser428), AMPK (Thr172), and ULK1 (Ser317, Ser555) was then examined by Western blot. AICAR is shown to induce phosphorylation of both LKB1 and AMPK [38], and was included as a positive control for phosphorylated LKB1 and AMPK. As a positive control for ULK1, cells were treated with FCCP, shown to induce ULK1 [67].

Our results showed that the level of phosphorylated LKB1 increased upon gastrin treatment with a decline after 60 minutes (figure 3.8). Phosphorylated AMPK seemed to peak after 10 minutes of gastrin treatment in AGS-Gr cells (figure 3.8) and the levels were still elevated after 60 minutes compared to the unstimulated cells. These results indicate that gastrin stimulation increases the phosphorylation of LKB1 (Ser428) and AMPK (Thr172) in AGS-Gr cells.

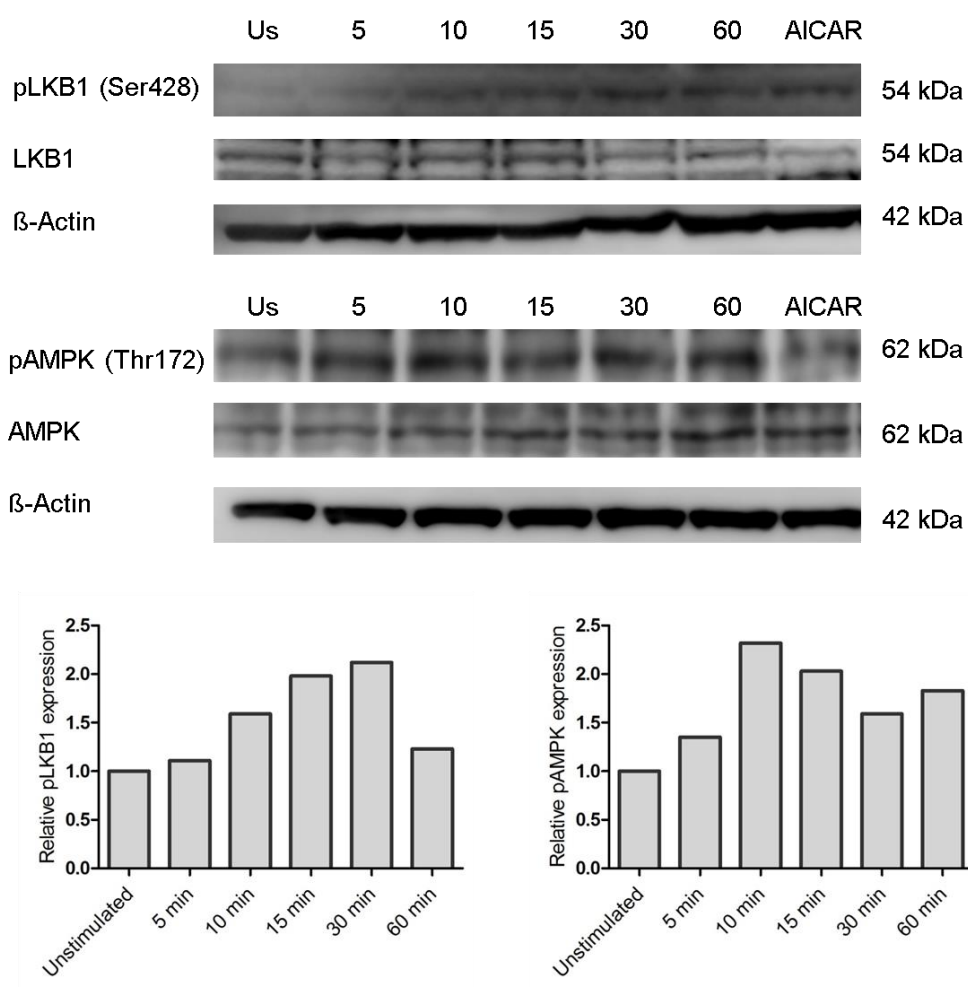


Figure 3.8: Gastrin-induced phosphorylation of LKB1 and AMPK in AGS-Gr. Cells were serum-starved and treated with gastrin (10 nM). Phosphorylated LKB1 (Ser428) and AMPK (Thr172) were detected by Western blot. Results are representative of two (pLKB1) and three (pAMPK) independent experiments.

Next we examined whether gastrin phosphorylated the same kinases in MKN45 cells. We found that the level of phosphorylated LKB1 seems to be increased after 10 minutes (figure 3.9). The level of phosphorylated AMPK increased after 10 minutes and was still increased compared to unstimulated cells after 60 minutes (figure 3.9). These results indicate that gastrin treatment induces phosphorylation of LKB1 (Ser428) and AMPK (Thr172) also in MKN45 cells, but the induction is not as pronounced as in AGS-Gr cells.

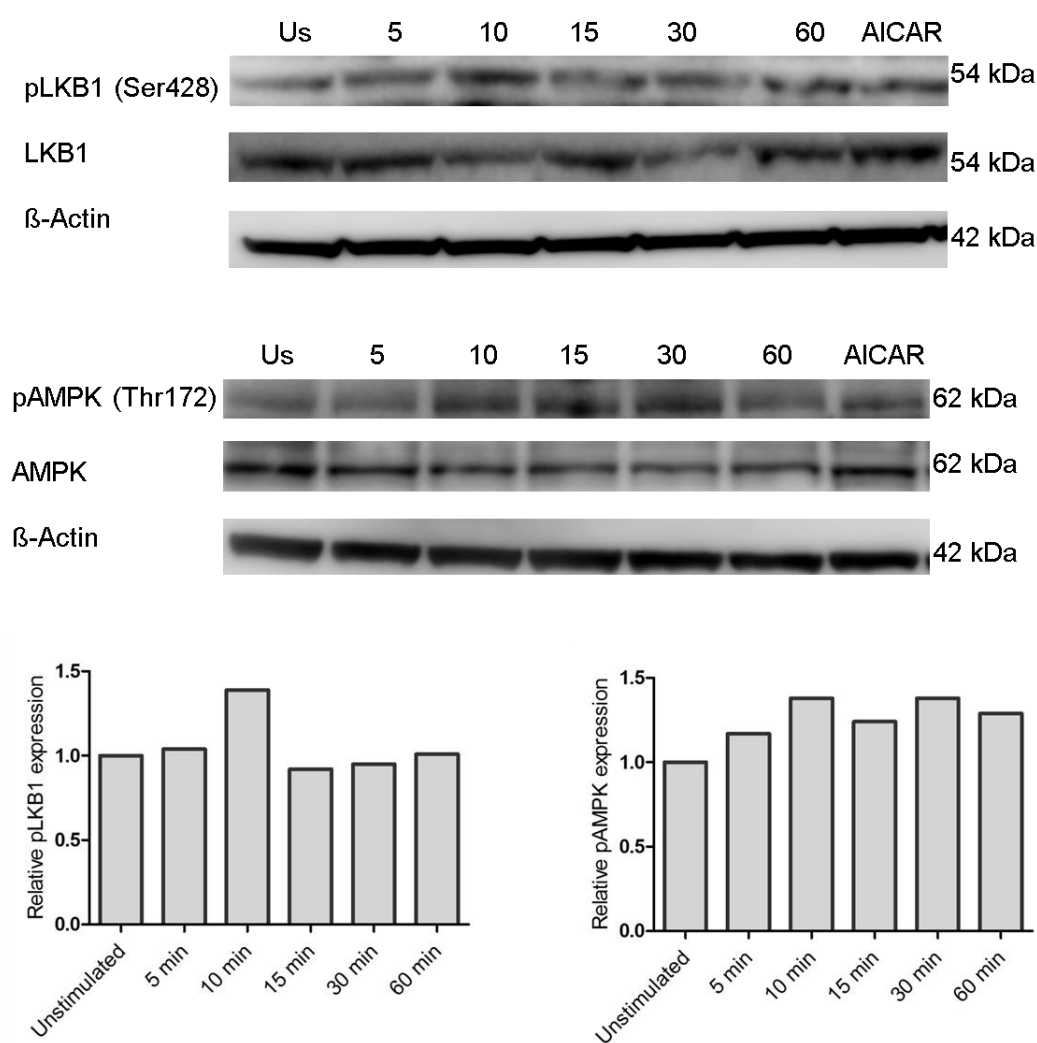


Figure 3.9: Detection of phosphorylated LKB1 and AMPK in MKN45. Cells were serum-starved and stimulated with gastrin (10 nM). Phosphorylated LKB1 (Ser428) and AMPK (Thr172) were detected by Western blot. Results are representative of two (LKB1) and three (AMPK) independent experiments.

3.5.1 Optimizing the method for detection of phosphorylated ULK1

When detecting phosphorylated ULK1, several problems were encountered in AGS-Gr and MKN45 cells. Initially, gastrin induced phosphorylation of ULK1 (Ser317) was examined in both cell lines. The membrane was blocked 2 hours in 5% BSA. This experimental setup was not very successful as unspecific bindings occurred and the bands were poorly separated (data not shown). To improve the method, the incubation with blocking buffer (5% BSA) was performed overnight at 4°C, but this did not mitigate the problem of unspecific bindings. The

next step was therefore to change the blocking solution. Two new blocking solutions were used; 5% milk and Odyssey blocking buffer, but neither reduced the unspecific bindings.

When detecting phosphorylated ULK1 (Ser317), two bands between 120 and 220 kDa was detected (figure 3.10A). Phosphorylated ULK1 has a molecular weight of 140 kDa, thus the lower band was considered phosphorylated ULK1 (Ser317). The molecular weight of total ULK1 is according to the manufacturer 150 kDa. Additional effort was made to improve the separation of the proteins. The conditions of separating the proteins was changed from 75 minutes in room temperature (200 V) to 40 minutes in room temperature (200 V), and then placed on ice for 75 minutes (180 V). This method gave better separation of the bands (figure 3.10B), even though unspecific bindings still persisted.

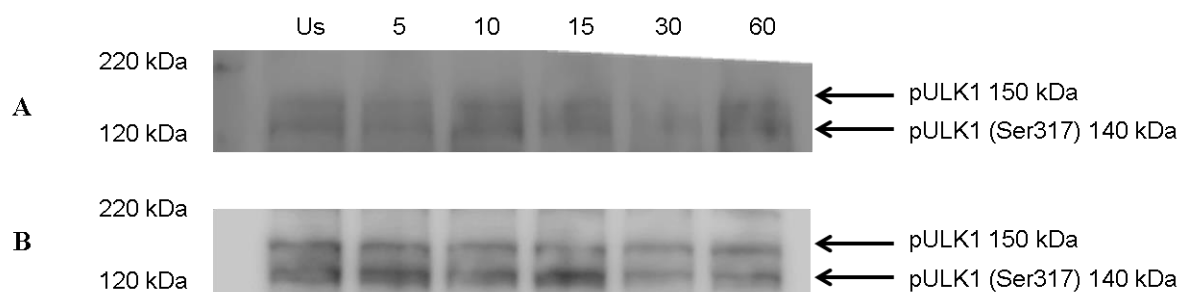


Figure 3.10: Detection of phosphorylated ULK1 (Ser317). **A)** Two bands between 120-220 kDa were detected. Separation of the proteins was performed in room temperature for 75 minutes (200 V). **B)** Separation of the proteins was performed 40 minutes in room temperature (200 V) and 75 minutes placed cold (180 V).

As three different blocking methods did not reduce the unspecific bindings, we speculated whether the antibody dilution could be improved. The primary antibody towards phosphorylated ULK1 (Ser317) was diluted 1:1000 in the initial experiments according to the manufacturers recommendation, but was subsequently changed to 1:500. To check if the secondary antibody was causing unspecific bindings, a blot was re-probed with a fluorescent secondary antibody. Unspecific bindings still occurred with a fluorescent secondary antibody, and two bands between 120 and 220 kDa were detected with both HRP-probed and fluorescent antibody (figure 3.11).

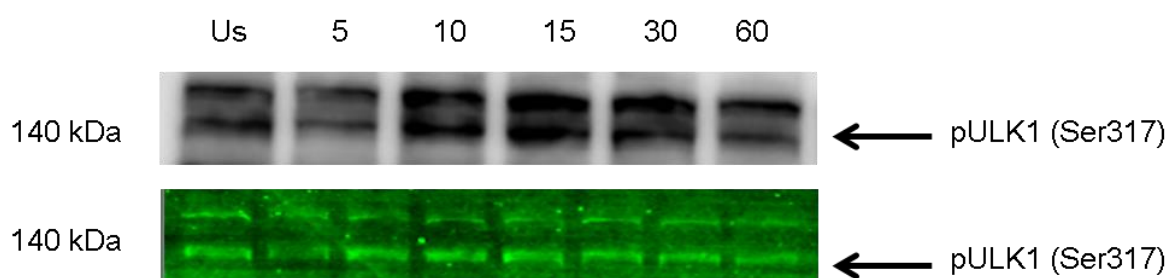


Figure 3.11: Detection of phosphorylated ULK1 with HRP (upper panel) and fluorescent (lower panel) secondary antibody.

Another modification which was done to improve the method was to change the lysis buffer. Phosphorylated proteins are dephosphorylated by phosphatases, and the concentration of phosphatase inhibitors (PIC2 and PIC3) was thus increased.

AMPK may phosphorylate ULK1 at several residues, including Ser317 and Ser555. An antibody specific for ULK1 phosphorylated at Ser555 was used in addition to the antibody specific for Ser317. A slight increase in the level of phosphorylated ULK1 (Ser317) was observed in AGS-Gr cells (figure 3.12A). Examination of ULK1 with the antibody specific for Ser555 also detected a band at 140 kDa. The increase of phosphorylated ULK1 seen by use of this antibody peaked after 15 minutes, and then decreased (figure 3.12A). In MKN45, the level of phosphorylated ULK1 (Ser317) peaked after 10 minutes and then slowly decreased. A pronounced increase of phosphorylated ULK1 (Ser555) was not observed. These results indicate that gastrin treatment induces phosphorylation of ULK1 in AGS-Gr and MKN45 cells. All together, gastrin mediated phosphorylation of the signalling cascade LKB1-AMPK-ULK1 suggest a role of gastrin in regulation of autophagy.

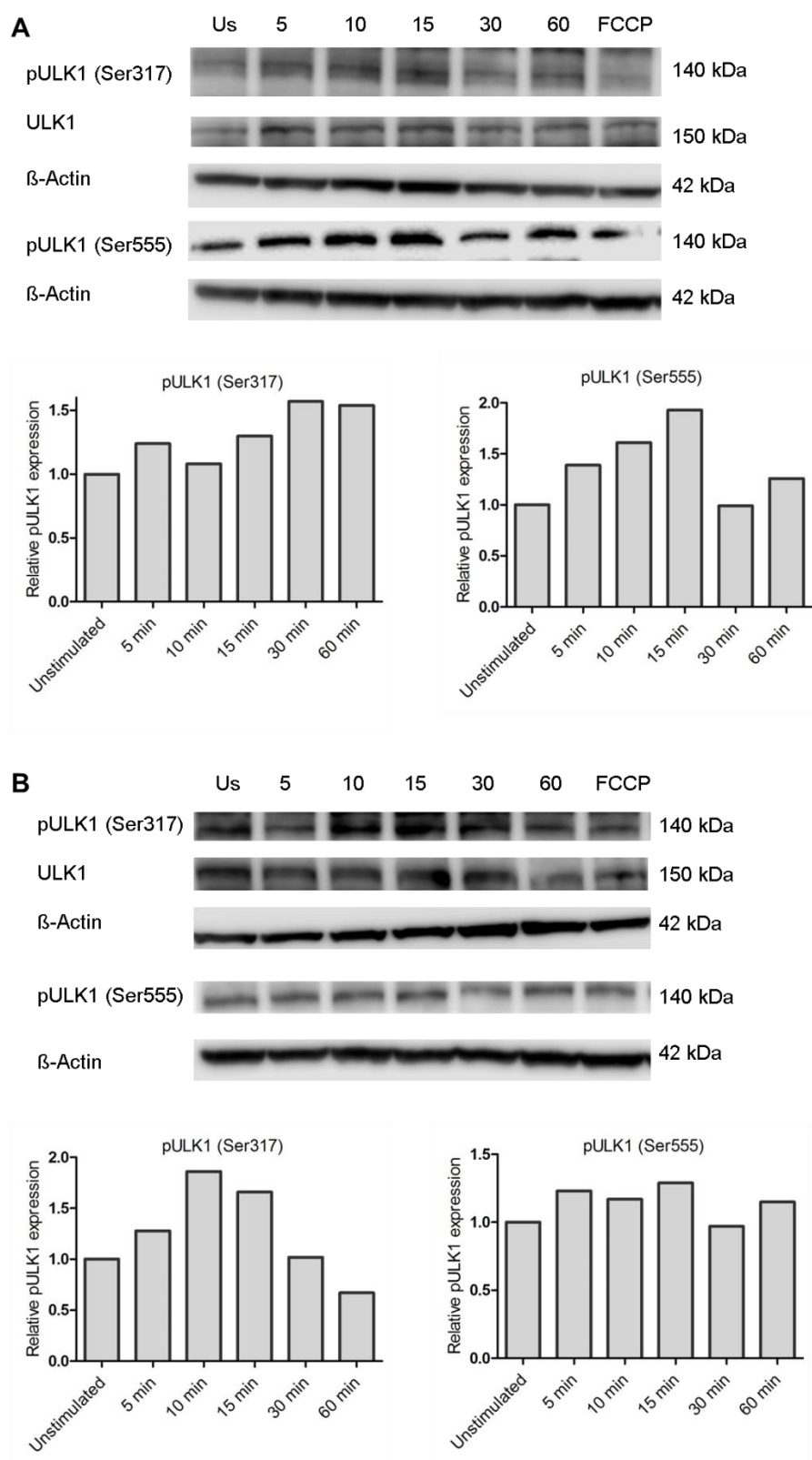


Figure 3.12: Gastrin mediated phosphorylation of ULK1. Serum-starved cells were treated with gastrin (10 nM). Phosphorylated ULK1 (Ser317, Ser555) were detected by Western blot. **A)** AGS-Gr cells. **B)** MKN45 cells.

3.6 Inhibition of autophagy reduces the survival effect of gastrin

Gastrin has previously been shown to induce proliferation via anti-apoptotic/survival signalling pathways *in vitro* [30, 31] and we speculated whether the gastrin mediated increase of autophagy was part of this survival effect. To investigate this, we induced cellular stress by cisplatin treatment, and investigated whether gastrin induced autophagy would influence the survival of the cells. XTT experiments were performed with AGS-Gr cells treated with cisplatin or cisplatin together with gastrin. Addition of cisplatin causes DNA damage and induction of reactive oxygen species (ROS) in the cells, resulting in cellular stress and inhibition of cell division.

Initially, cisplatin was added in various concentrations (7.5, 15, 30, 60 and 90 μM), and the experiment was terminated after 6, 24 and 48 hours of cisplatin stimulation. In unstimulated and gastrin treated cells, the anti-apoptotic effect of gastrin was significant after 48 hours of treatment (figure 3.13A, 3.14A, 3.15A,C). After 6 hours there was no significant difference in cell viability between cells treated with cisplatin alone or cells treated with cisplatin together with gastrin (figure 3.13B). Cells treated with 15 μM cisplatin together with gastrin showed better viability compared to cisplatin-treated cells alone after 24 hours (figure 3.13C). After 48 hours, cells treated with gastrin and cisplatin showed significantly higher viability than cells treated with cisplatin alone (figure 3.13D). Taken together, these results indicate that the apoptotic effect of cisplatin is reduced with gastrin treatment.

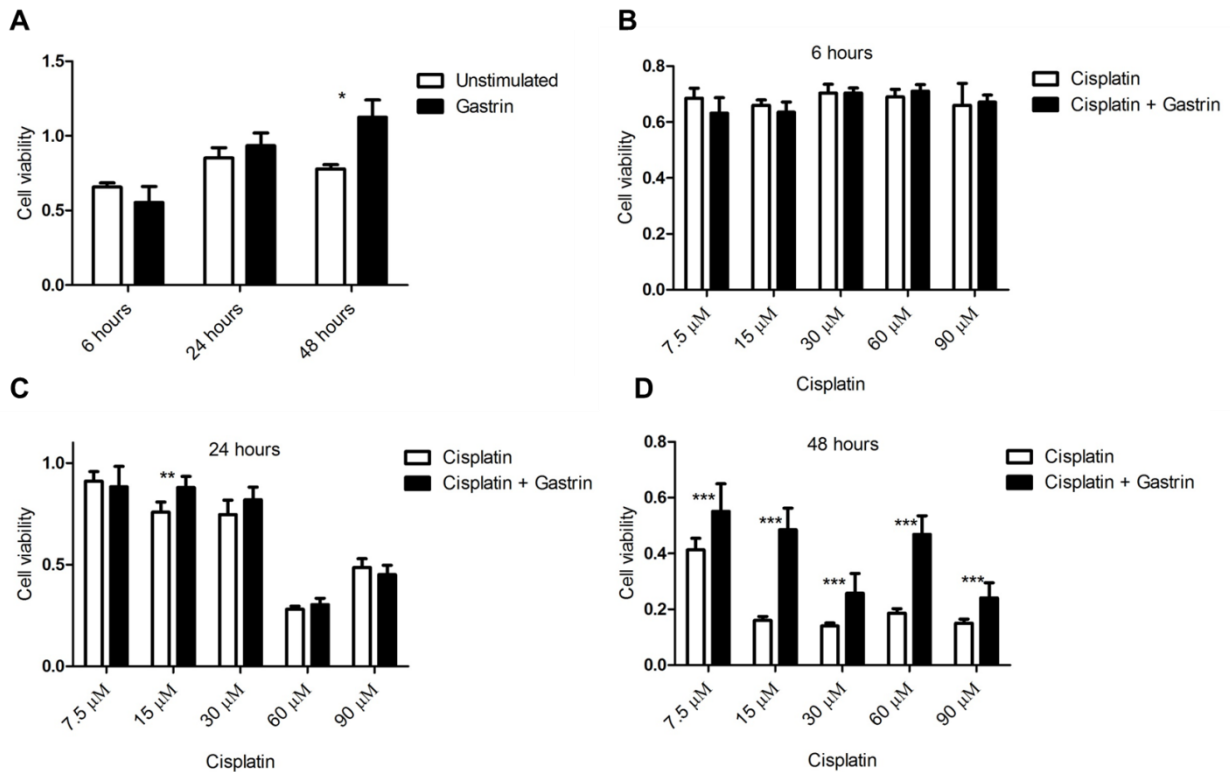


Figure 3.13: Gastrin reduces the apoptotic effect of cisplatin in AGS-Gr cells. Cells were treated with gastrin (10 nM) for 2 hours, and then treated with cisplatin. **A)** Unstimulated and gastrin-treated cells. **B-D)** Gastrin and cisplatin treated cells. The statistical significance between unstimulated vs. gastrin treated cells, and cisplatin and gastrin treatment vs cisplatin treatment was found by two-tailed student T-test. *: P-value ≤ 0.05 . **: P-value ≤ 0.01 . ***: P-value ≤ 0.001 . Graphs show mean value and standard deviation.

In the initial experiment, high doses (15-90 μM) of cisplatin showed relatively low cell viability after 48 hours, and it was of interest to examine whether the effect of gastrin was still significant with lower concentrations of cisplatin. Cells were therefore treated with 1.0, 4.0, 7.5 and 15 μM of cisplatin and the experiment was terminated after 24, 48 and 72 hours (figure 3.14B-D). The cell viability was also significantly higher in cells treated with 15 μM cisplatin together with gastrin, compared to cisplatin alone after 24 hours (figure 3.14B). The cell viability increased significantly when stimulating cisplatin together with gastrin after 48 and 72 hours treatment (figure 3.14C,D). These results indicate that the anti-apoptotic effect of gastrin is best shown after 48 hours treatment and that the apoptotic effect caused by cisplatin is partly reduced with gastrin treatment.

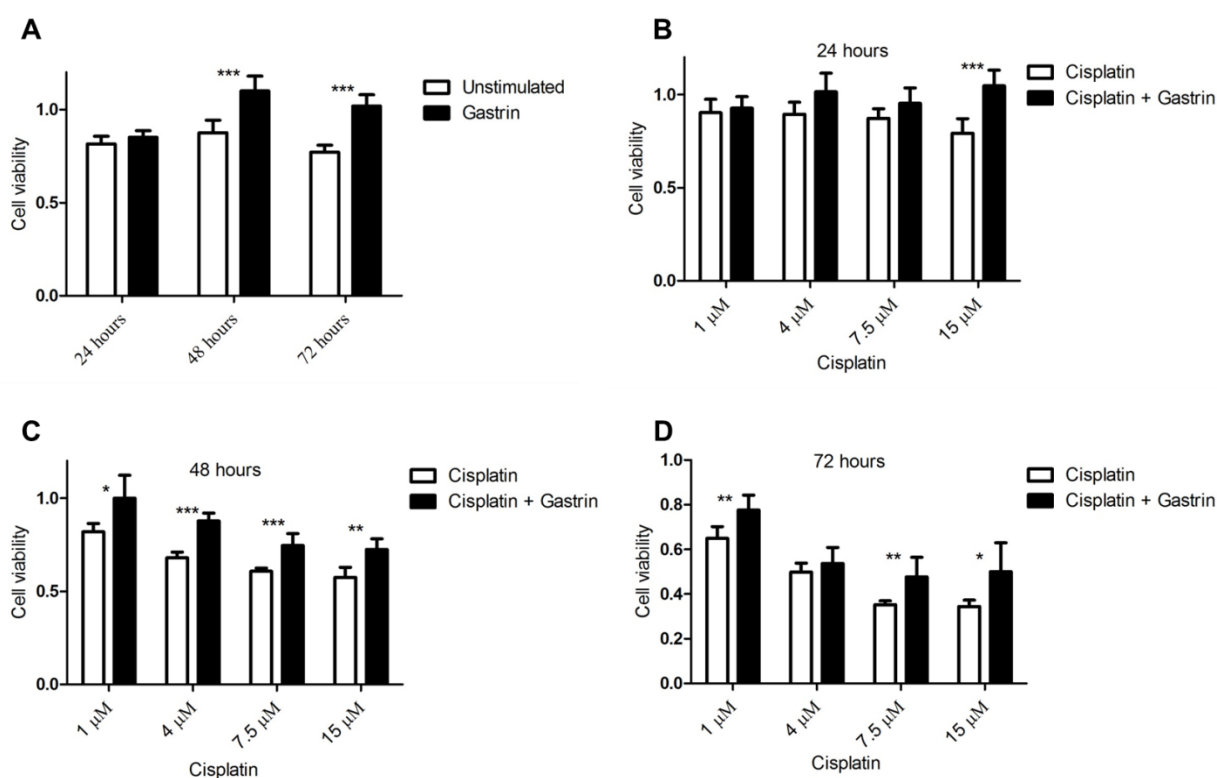


Figure 3.14: Gastrin reduces the apoptotic effect of cisplatin in AGS-Gr cells. Cells were pre-treated with 10 nM gastrin for 2 hours, and treated with the cisplatin. **A)** Unstimulated and gastrin-treated cells. **B-D)** Gastrin and cisplatin treated cells. The statistical significance between unstimulated vs. gastrin stimulated, and Cisplatin and Gastrin vs Cisplatin was found by two-tailed student T-test. *: P-value ≤ 0.05 . **: P-value ≤ 0.01 . ***: P-value ≤ 0.001 . Graphs show mean value and standard deviation.

Further, we hypothesized that induction of autophagy could be one of the mechanisms of the survival effect of gastrin. Therefore we blocked autophagy by hydroxy-chloroquine (HCQ) (10 μ M) in cells treated with gastrin and cisplatin for 24 hours (figure 3.15B) and 12 hours (figure 3.15D) and terminated the experiments after 48 and 36 hours, respectively. HCQ inhibits the acidification of lysosomes and prevents the fusion of lysosomes and autophagosomes. The results showed that blocking of autophagy in cells treated with gastrin displayed decreased viability compared to cells treated with gastrin alone (figure 3.15A,C). The viability in cells treated with HCQ and cisplatin was also examined (Appendix 2).

Results showed that cells treated with cisplatin and gastrin showed decreased viability when autophagy was blocked for 12 hours (figure 3.15B). To check if blocking of autophagy at an earlier time-point would reduce the viability further, HCQ was added for 24 hours and the experiment was terminated after 36 hours of cisplatin treatment. The cell viability was

significant decreased when comparing gastrin treated cells to unstimulated cells, and the reduction in viability was also significant when blocking autophagy (figure 3.15C). Blocking of autophagy for 24 hours resulted in further significant reduction in cell viability (figure 3.15D). Taken together, these results suggest that blocking of autophagy with HCQ partly reduces the anti-apoptotic effect of gastrin, and sensitized cells to cisplatin-induced cell death.

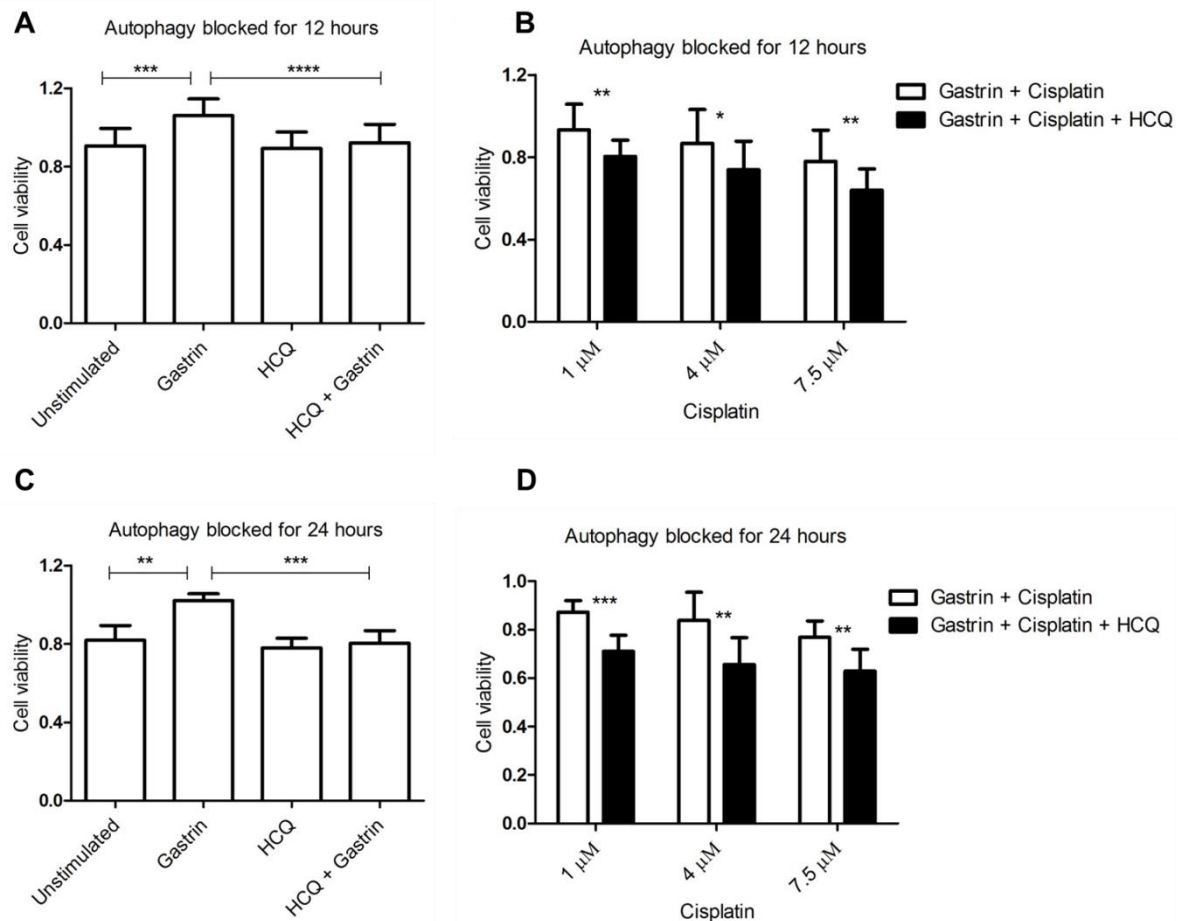


Figure 3.15: Blocking of autophagy reduces the survival effect of gastrin in AGS-Gr cells. Cells were treated with 10 nM gastrin for 2 hours, and then treated with cisplatin for 48 hours (A-B) and 36 hours (C-D). **A, B**) Autophagy was blocked for 12 hours. Results are representative of two independent experiments. **C, D**) Autophagy was blocked for 24 hours. Results are from one experiment. The statistical significance between unstimulated cells vs. gastrin treated cells, and cisplatin, gastrin and HCQ treatment vs cisplatin and gastrin treatment was found by two-tailed student T-test. *: P-value ≤ 0.05 . **: P-value ≤ 0.01 . ***: P-value ≤ 0.001 . Graphs show mean value and standard deviation.

4. Discussion

Understanding the molecular mechanisms of gastrin may contribute to a better understanding of gastrin in pathological conditions. In this thesis, gastrin-mediated autophagy in two human gastric adenocarcinoma cell lines has been examined; AGS-Gr, which overexpresses the CCK2 receptor and MKN45 which expresses the receptor endogenously. The levels of the autophagy markers LC3-II and p62 were shown to increase by gastrin treatment, and the signaling pathway LKB1 – AMPK – ULK1, known to induce autophagy, was activated by gastrin. Taken together, our results suggest a role for gastrin in regulating autophagy in AGS-Gr and MKN45 cells. Gastrin showed a survival effect in cisplatin-treated cells, and blocking of autophagy reduced the survival effect of gastrin.

In this study we show for the first time that gastrin, which exerts growth-promoting effects on AGS-Gr and MKN45 cells, induces autophagy in these gastric adenocarcinoma cell lines. Transforming growth factor beta (TGF- β) has been shown to induce autophagy in renal tubular epithelial cells [68], hepatocellular carcinoma cells and in mammary carcinoma cells [69]. To date, few studies have uncovered hormone or growth factor regulation of autophagy.

Since gastrin is shown to have survival effects, we speculated that gastrin-mediated autophagy could have a cytoprotective role in our cell line system. By performing XTT experiments, we showed that cells treated with cisplatin and gastrin showed better viability than cells treated with cisplatin alone. Cisplatin is an anti-cancer drug used in a variety of malignancies, including gastric cancer [70]. Its cytotoxic mode of action is induction of DNA damage wherein DNA adducts induce signaling cascades that result in apoptosis. Cisplatin commonly works well initially, but treatment with cisplatin often results in resistance [71, 72]. In our experiments we show that gastrin partially counteracts the apoptotic effect of cisplatin, and that blocking of autophagy reduced the survival effect of gastrin. Harhaji-Trajkovic et al. [73] showed that cisplatin induced both phosphorylation of AMPK and autophagy in glioma cell lines. Inhibition of autophagy at both early and late stages increased cisplatin-induced apoptosis, and siRNA towards AMPK impaired autophagy and also sensitized cells to apoptosis [73]. Liang et al. [74] found that 5-Fluorouracil (5-FU) induced autophagy in a gallbladder carcinoma cell line, and that blocking of autophagy sensitized the cells to 5-FU-induced apoptosis. They also showed that siRNA towards Atg5 and Atg7 induced 5-FU-mediated apoptosis. Similar results were found in a lung cancer cell line where Atg5 was knocked down with siRNA and blocking of autophagy enhanced the 5-FU induced apoptosis

[75]. Furthermore, blocking of autophagy has been shown to sensitize gastric cancer cells to oxaliplatin-induced apoptosis in a study by Xu et al. [76]. Kim et al. [77] showed that cisplatin treatment induced phosphorylation of AMPK in AGS cells, and that pharmacological inhibition of AMPK and siRNA towards AMPK sensitized the cells to cisplatin-induced apoptosis [77]. These results suggest that AMPK and autophagy contribute to general chemotherapy resistance and act in a cytoprotective manner in response to several anti-cancer drugs. A study by Li et al. [78] showed that blocking of autophagy induced 5-FU-mediated apoptosis in human colon cancer, both *in vitro* and in an *in vivo* xenograft model. The studies are consistent with our results showing that blocking of autophagy enhanced cisplatin-mediated apoptosis in AGS-Gr cells.

In a study by Yu et al. [79], a cisplatin-resistant ovarian cancer cell line showed increased expression of p62, and knock-down of p62 in the cisplatin-resistant cells re-sensitized the cells to cisplatin-induced apoptosis [79]. These findings also indicate a protective role for autophagy in cisplatin treatment. However, conflicting results have been reported. Yang et al. found that cisplatin-induced autophagy enhanced apoptosis in bladder cancer cells [80]. In cisplatin-resistant ovarian cancer cells Feng et al. [81] reported that a combined treatment of cisplatin and Dihydroartemisinin (DHA) sensitized cells to apoptosis, and that autophagy was induced by DHA [81]. However, blocking of autophagy in the resistant cells did not affect cell viability.

In an *in vivo* study by Kang et al. [82], immunohistochemistry showed that patients with low expression of phosphorylated AMPK in the tumor had higher relapse-free survival than patients with high expression of phosphorylated AMPK. The results were from 74 patients suffering from gastric adenocarcinoma who had undergone resection and adjuvant cisplatin treatment [82]. Zheng et al. [83], on the other hand, reported that phosphorylated AMPK was down-regulated in patients with hepatocellular carcinoma, and that low expression of phosphorylated AMPK was associated with poor prognosis. Consistent with these results, William et al. [84] found high expression of AMPK in non-small cell lung cancer (NSCLC) to be associated with better prognosis. Choi et al. found that negative expression of LC3-II *in vivo* was associated with poor survival in patients with colorectal adenocarcinoma [85]. LC3-II was shown to have high expression in gastrointestinal cancers, including gastric adenocarcinoma in addition to esophageal and colorectal cancer, compared to noncancerous epithelial cells [86]. Altogether, these conflicting results emphasise the dual and complex role of autophagy in cancer and apoptosis. Whether autophagy acts in a cytotoxic or cytoprotective

manner is likely context dependent, and may also be dependent on stimuli. In our cell line system, gastrin induced both phosphorylation of AMPK and autophagy. In addition, gastrin reduced cisplatin-mediated apoptosis in AGS-Gr, while blocking of autophagy sensitized cells to cisplatin-induced apoptosis (figure 3.15). Our results suggest a role of gastrin-mediated autophagy as a protective factor in cisplatin resistance in AGS-Gr.

When cultivating MKN45 cells in HANKS no increase of LC3-II and p62 was observed. HANKS medium lacks amino acids, and cultivation in HANKS should thus be a potent inducer of autophagy and, in turn, increase the autophagic flux. The term autophagic flux denotes the whole process of autophagy, wherein autophagosomes are formed, fuses with the lysosome and is degraded [41]. Kim et al. [36] showed that amino acid-starvation induced activation of ULK1 in MEF cells, but ULK1 was not phosphorylated at the Ser317 residue known to be phosphorylated by AMPK. Glucose starvation, on the other hand, resulted in phosphorylation of ULK1 (Ser317). In cells with AMPK mutant, amino acid starvation could still stimulate ULK1 activation, even though the overall ULK1 activation was reduced in the cells. ULK1 was not activated in glucose-starved cells expressing the AMPK mutant [36]. These results indicate that amino acid-starvation may activate autophagy in an AMPK-independent manner. In contrast, Sato et al. [87] demonstrated that amino acid-starvation in colorectal cancer cell lines induced the expression of LC3-II and increased phosphorylation of AMPK. These results indicate that it is likely cell-specific whether amino acid-starvation activates AMPK and thus initiates autophagy. In our study we have shown that AMPK is activated in serum-starved cells upon gastrin treatment, but whether cultivation in HANKS activates AMPK was not examined. The basal autophagic flux induced by amino acid starvation might have been so high that there was no additional effect of gastrin in MKN45 cultivated in HANKS.

After finishing the laboratory work for this thesis, the gastrin-mediated increase in autophagy was further demonstrated by combined knock-down of Atg5 and Atg7 by siRNA in MKN45 cells. Some more experiments will be conducted to further show the gastrin-mediated increase of autophagy. Since ULK1 is a direct activator of autophagy, knock-down of ULK1 could be performed to further show that gastrin mediates autophagy in the cell lines. We could also examine ULK1 phosphorylated by mTOR (Ser757) upon gastrin treatment.

To further establish whether gastrin activates the LKB1 – AMPK – ULK1 pathway to initiate autophagy, additional experiments could be performed. Knock-down of LKB1 by siRNA

could have been used to examine whether LKB1 knock-down would reduce or cancel the gastrin-mediated increase in LC3-II and p62. LKB1 resides in the nucleus, but translocates to the cytoplasm when phosphorylated (Ser428) and activated, and LKB1 may then activate AMPK [38]. With this in mind, it would be interesting to examine whether gastrin treatment would mediate the translocation of LKB1 from the nucleus to the cytoplasm.

Hypergastrinemia is considered to be a risk factor for gastric malignancy, but the role of gastrin in gastric cancer has been debated for decades. We have shown for the first time that gastrin induces autophagy, likely through activation of LKB1, AMPK and ULK1. Conflicting results regarding the role of autophagy in cancer suggest that whether autophagy acts in a cytoprotective or cytotoxic manner is likely context dependent. In this study we have shown that gastrin-induced autophagy in gastric adenocarcinoma cells likely exerts a protective effect in response to chemotherapy. These results may contribute to a better understanding of the role of gastrin in gastric cancer. Blocking of autophagy may be a potential target for sensitizing gastric cancer cells to apoptosis in response to chemotherapy.

5. References

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Appendix 1

Urea lysis buffer

The extraction buffer was produced by dissolving urea (2.4 g) in dH₂O (3 ml, 25°C), and then adding additional dH₂O until the total volume was 5 ml. 25 µl Triton x100 (VWR) was added. Aliquots (410 µl) were stored at -20°C. When the lysis buffer was to be used, DL-Dithiothreitol solution (DTT), protease (PI) and phosphatase (PIC) inhibitors were added to the extraction buffer. For details, see table A1.

Table A1: Urea lysis buffer

	Concentration	Concentration in buffer	Volume
Extraction buffer			410 µl
DTT (Sigma)	1 M	0.1 M	50 µl
PI (Roche)	25X	1X	20 µl
PIC2 (Sigma)	250X	5X	10 µl
PIC3(Sigma)	250X	5X	10 µl

TBST

Table A2: 20X TBS Stock solution

1 M Trizma Base (Sigma)	122 g
18% NaCl (Merck)	180 g
Tween 20 (VWR)	1 ml
dH ₂ O	Add until 1000 ml

The pH of the buffer was adjusted to 7.6. TBST working solution was made by diluting the 20X TBS stock solution 1:20 in dH₂O and adding 0.1% Tween 20.

Transfer buffer

Table A3: Transfer buffer

20X NuPAGE Transfer buffer (Novex)	50 ml
NuPAGE Antioxidant (Invitrogen)	1 ml
10% Methanol	100 ml
dH ₂ O	849 ml

Ponceau staining

Table A4: Ponceau staining

Ponceau	1 g
Acetic acid	50 ml
dH ₂ O	Add until 1000 ml

Appendix 2

When blocking autophagy in cisplatin-treated cells, the viability significantly decreased compared to cisplatin treatment alone (figure A1). Results shown are from cells treated with 7.5 μ M cisplatin for 48 hours. Similar results were observed in cells treated with other concentrations (1.0 and 4.0 μ M) of cisplatin. In cells treated with cisplatin in presence of gastrin, the viability was higher than in cells treated with cisplatin in absence of gastrin.

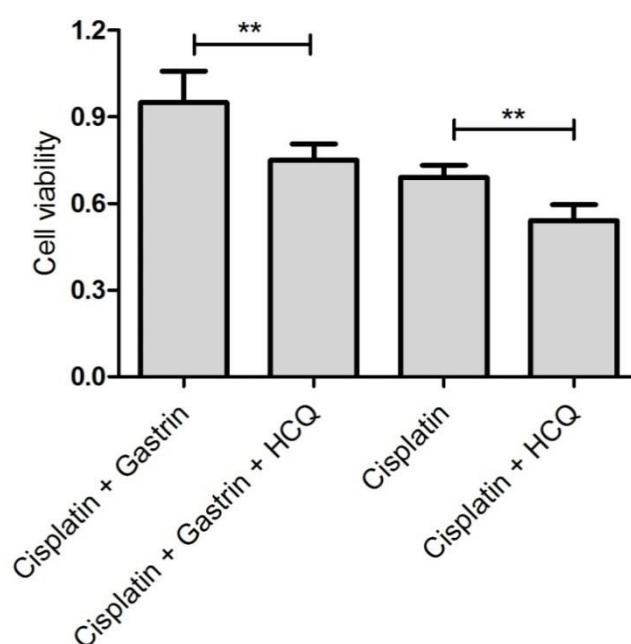


Figure A1: Cells were pre-treated with 10 nM gastrin for 2 hours, and treated with cisplatin (7.5 μ M) for 48 hours. HCQ (10 μ M) was added after 36 hours. The statistical significance was found by student T-test. *: P-value \leq 0.05. **: P-value \leq 0.01. ***: P-value \leq 0.001. Graphs show mean value and standard deviation.