H. pylori induced autophagy is mediated by eIF2α-ATF4 pathway in which HP0175 plays a key role.
5. Results

5.1. HP0175 regulate expression of autophagy pathway-linked genes in AGS

We have formerly established that the secreted antigen HP0175 induces apoptosis in the gastric epithelial cell line AGS (Basak et al., 2005). Taking into account the reported crosstalk between of apoptosis and autophagy (Huang et al., 2013; Shimizu et al., 2004;) we asked the question whether HP0175 could regulate autophagy in AGS cells. With a specific end goal to investigate whether HP0175 manages autophagy through transcriptional regulation of autophagy-related genes, we investigated the expression of a cluster of autophagy pathway genes in cells treated with HP0175.

5.2. HP0175 induces autophagy in AGS cells

To further affirm the role of HP0175 as an inducer of autophagy, HP0175 treated AGS cells were immunostained for LC3B. The stain was equally distributed in untreated AGS cells, though in HP0175-treated cells, clear punctate structures were discovered, proposing the activation and localization of LC3 in autophagosomes. Cells having more than 5 puncta were tallied and a huge increment was found in HP0175-treated cells contrasted with untreated cells (Fig. 5.1).

![Figure 5.1. HP0175 stimulates autophagy.](image)

**Figure 5.1. HP0175 stimulates autophagy.** AGS cells were left untreated (U) or treated (with HP0175) for 5 h, washed and stained with LC3 antibody followed by Alexa -546 conjugated secondary antibody (red). The number of cells showing more than five puncta were counted and expressed as percentage of total number (>200) of cells. Results represent means ±SD of three determinations. **p < 0.01.
5.3. HP0175 induces autophagy linked genes

Interestingly, HP0175 impelled expression of various genes (MAP1LC3B, ULK1, ATG5, BECLIN1) related with the autophagy pathway (Figure. 5.2A-D). Consequently, these results contended for a feasible part of HP0175 in modulating autophagy.

![Figure 5.2A. HP0175 upregulates MAP1LC3B. AGS cells were left untreated (U) or treated with HP0175 (1μg/ml) for different periods of time and RNA was isolated. Transcription of MAP1LC3B was analysed by qRT-PCR. Gene expression was normalized with respect to GAPDH. Results represent mean ± SD of three determinations. Comparisons have been made against the untreated samples (U). ** p < 0.01; *** p < 0.001; NS, not significant.](image-url)
Figure 5.2B. HP0175 upregulates ULK1. AGS cells were left untreated (U) or treated with HP0175 (1μg/ml) for different periods of time and RNA was isolated. Transcription of ULK1 was analysed by qRT-PCR. Gene expression was normalized with respect to GAPDH. Results represent mean ± SD of three determinations. Comparisons have been made against the untreated samples (U). * p < 0.05; *** p < 0.001.

Figure 5.2C. HP0175 upregulates ATG5. AGS cells were left untreated (U) or treated with HP0175 (1μg/ml) for different periods of time and RNA was isolated. Transcription of ATG5 was analysed by qRT-PCR. Gene expression was normalized with respect to GAPDH. Results represent mean ± SD of three determinations. Comparisons have been made against the untreated samples (U). * p < 0.05; *** p < 0.001.
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Figure 5.2D. HP0175 upregulates *BECLIN1*. AGS cells were left untreated (U) or treated with HP0175 (1μg/ml) for different periods of time and RNA was isolated. Transcription of *BECLIN1* was analysed by qRT-PCR. Gene expression was normalized with respect to GAPDH. Results represent mean ± SD of three determinations. Comparisons have been made against the untreated samples (U). **p < 0.01; ***p < 0.001

5.4. Generation of a *vacA* knockout mutant

*VacA* is known to trigger autophagy in gastric epithelial cells. In order to understand the contribution of factors other than *VacA* in triggering *H. pylori*-mediated autophagy in AGS cells, we generated a *vacA* knockout of *H. pylori*. Vacuolating activity of this mutant was severely compromised compared to the wild type *H. pylori* (Fig. 5.3). The *hp0175* knockout was not compromised in terms of vacuolating activity (Fig. 5.3).
Figure 5.3. Vacuolating activity of *H. pylori*. AGS cells were left untreated (U) or treated with soluble extract of *H. pylori* (WT) or its isogenic *hp0175* mutant (KO) or *VacA* mutant (VacA-KO) and vacuolating activity was measured by neutral red uptake assay. Data represent means ± SD (n=3). Comparisons were made as indicated. ***p < 0.001; NS, not significant.

5.5. *H. pylori* regulates expression of autophagy-linked genes in an HP0175 dependent manner

We analyzed whether the upregulation of autophagy-linked genes by HP0175 was relevant to the effects of *H. pylori* on AGS cells by studying the expression of the above genes induced by wild type *H. pylori* or its mutants in AGS cells. *H. pylori* induced the expression of *MAP1LC3B*, *ULK1*, *ATG5* and *BECLIN1*. This induction was evident even when the gene was knocked out, but abrogated in the *hp0175* knockout (Fig. 5.4A-D).
Figure 5.4A. *H. pylori*-mediated induction of **MAP1LC3B** depends on HP0175. AGS cells were infected with *H. pylori* (WT or KO) or VacA- KO at an MOI of 50 or 100 for 6 h. Transcription of **MAP1LC3B** was analysed by qRT-PCR as described in Fig 1. Results represent means ± S.D. of three determinations. NS, not significant; *** p < 0.001. In all cases, comparison was made with untreated cells (U).

Figure 5.4B. *H. pylori*-mediated induction of **ULK1** depends on HP0175. AGS cells were infected with *H. pylori* (WT or KO) or VacA- KO at an MOI of 50 or 100 for 6 h. Transcription of **ULK1** was analysed by qRT-PCR as described. Results represent means ± S.D. of three determinations. NS, not significant; ** p < 0.01; *** p < 0.001. In all cases, comparison was made with untreated cells (U).
Figure 5.4C. *H. pylori*-mediated induction of ATG5 depends on HP0175. AGS cells were infected with *H. pylori* (WT or KO) or VacA- KO at an MOI of 50 or 100 for 6 h. Transcription of ATG5 was analysed by qRT-PCR as described. Results represent means ± S.D. of three determinations. NS, not significant; ** *p* < 0.01; *** *p* < 0.001. In all cases, comparison was made with untreated cells (U).

Figure 5.4D. *H. pylori*-mediated induction of BECLIN1 depends on HP0175. AGS cells were infected with *H. pylori* (WT or KO) or VacA- KO at an MOI of 50 or 100 for 6 h. Transcription of BECLIN1 was analysed by qRT-PCR as described. Results represent means ± S.D. of three determinations. NS, not significant, *** *p* < 0.001. In all cases, comparison was made with untreated cells (U).
5.6. Regulation of genes connected with the unfolded protein reaction (UPR) in gastric epithelial cells by HP0175

The unfolded protein response (UPR) is initiated by the ER stress sensors PERK otherwise called EIF2AK3, IRE-1 and activating transcription factor 6 (ATF6) (Rouschop et al., 2010). PERK is a kinase that phosphorylates eukaryotic initiation factor 2 α (eIF-2 α) to hinder the start of translation. However, a subset of transcripts is translated even under these conditions, including the transcription factor ATF4. ATF4 and CHOP both regulate autophagy (Rouschop et al., 2010). We demonstrate upregulation of the transcription of UPR-linked genes **PERK**, **ATF4**, and **CHOP** in cells treated with HP0175 (Figure 5.5A-F).

![Figure 5.5A. HP0175 upregulates PERK. AGS cells were left untreated (U) or treated with HP0175 (1μg/ml) for different periods of time and RNA was isolated. Transcription of PERK was analysed by qRT-PCR. Gene expression was normalized with respect to GAPDH. Results represent mean ± SD of three determinations. Comparisons have been made against the untreated samples (U). * p < 0.05; ** p < 0.01.](image-url)
**Figure 5.5B.** HP0175 upregulates CHOP. AGS cells were left untreated (U) or treated with HP0175 (1μg/ml) for different periods of time and RNA was isolated. Transcription of CHOP was analysed by qRT-PCR. Gene expression was normalized with respect to GAPDH. Results represent mean ± SD of three determinations. Comparisons have been made against the untreated samples (U). **p < 0.01; ***p < 0.001.

**Figure 5.5C.** HP0175 upregulates ATF4. AGS cells were left untreated (U) or treated with HP0175 (1μg/ml) for different periods of time and RNA was isolated. Transcription of ATF4 was analysed by qRT-PCR. Gene expression was normalized with respect to GAPDH. Results represent mean ± SD of three determinations. Comparisons have been made against the untreated samples (U). *p < 0.05.
Wild type \textit{H. pylori} but not the KO strain also induced expression of these UPR related genes (Fig. 5.5D-F)). Taken together, these results recommended that HP0175 assumes an essential part in UPR-connected autophagy in \textit{H. pylori}-treated gastric epithelial cells.

\textbf{Figure 5.5D.} \textit{H. pylori}-mediated induction of \textit{PERK}. AGS cells were infected with \textit{H.pylori} (WT or KO) at different MOIs for 6 h. Transcription of \textit{PERK} was quantified by qRT-PCR as described in Figure 4.2.1. Results represent means ± S.D. of three determinations. NS, not significant, ** \( p < 0.01 \). In all cases, comparison was made with untreated cells (U).
Figure 5.5E. *H. pylori* -mediated induction of CHOP. AGS cells were infected with *H.pylori* (WT or KO) at different MOIs for 6 h. Transcription of CHOP was quantified by qRT-PCR as described in Figure 4.2.1. Results represent means ± S.D. of three determinations. * p < 0.05; ** p < 0.01; *** p < 0.001. In all cases, comparison was made with untreated cells (U).

Figure 5.5F. *H. pylori*-mediated induction of ATF4. AGS cells were infected with *H.pylori* (WT or KO) at different MOIs for 6 h. Transcription of ATF4 was quantified by qRT-PCR as described in Figure 4.2.1. Results represent means ± S.D. of three determinations. NS, not significant, *** p < 0.001. In all cases, comparison was made with untreated cells (U).
5.7. Contribution of ATF4 and CHOP in modulating the expression of autophagy-related genes during *H. pylori* infection of AGS

Taking into account that ATF4 and CHOP are transcriptionally enhanced in *H. pylori* or HP0175-challenged AGS cells, we examined the role of these transcription factors in controlling the expression of autophagy-related genes amid *H. pylori* disease. qrt-PCR demonstrated that ATF4 knockdown (Fig. 5.6A) attenuated the expression of MAP1LC3B, ULK1, CHOP and ATG5 (Figure 5.6B-E), whereas CHOP knockdown (Fig. 5.6F) attenuated the expression of ATG5 (Fig. 5.6G). These results affirmed that both ATF4 and CHOP are crucial for the expression of key players in canonical autophagy.

![Figure 5.6A. Knockdown of ATF4.](image)

AGS cells were transfected without (control) or with ATF4 siRNA. Silencing of ATF4 was confirmed by qRT-PCR. Fold induction was expressed with respect to untreated control. Results of qRT-PCR are means ± S.D. (n= 3). **p < 0.01.
Figure 5.6B. **ATF4** modulates expression of **MAP1LC3B** in *H. pylori* infected AGS cells. AGS cells transfected with either scrambled (SCR) or with **ATF4** siRNA were left uninfected (-) or infected (+) with *H.pylori* (Hp) at an MOI of 50 for 4 h. Transcription of **MAP1LC3B** was quantitated by qRT-PCR. Fold induction was expressed with respect to untreated control. Results of qRT-PCR are means ± S.D. (n= 3). NS, not significant; ** p < 0.01.

Figure 5.6C. **ATF4** modulates expression of **ULK1** in *H. pylori* infected AGS cells. AGS cells transfected with either scrambled (SCR) or with **ATF4** siRNA were left uninfected (-) or infected (+) with *H.pylori* (Hp) at an MOI of 50 for 4 h. Transcription of **ULK1** was quantitated by qRT-PCR. Fold induction was expressed with respect to untreated control. Results of qRT-PCR are means ± S.D. (n= 3). NS, not significant; *** p < 0.001.
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Figure 5.6D. **ATF4 modulates expression of CHOP in *H. pylori* infected AGS cells.** AGS cells transfected with either scrambled (SCR) or with ATF4 siRNA were left uninfected (-) or infected (+) with *H. pylori* (Hp) at an MOI of 50 for 4 h. Transcription of **CHOP** was quantitated by qRT-PCR. Fold induction was expressed with respect to untreated control. Results of qRT-PCR are means ± S.D. (n= 3). NS, not significant; *** p < 0.001.

Figure 5.6E. **ATF4 modulates expression of ATG5 in *H. pylori* infected AGS cells.** AGS cells transfected with either scrambled (SCR) or with ATF4 siRNA were left uninfected (-) or infected (+) with *H. pylori* (Hp) at an MOI of 50 for 4 h. Transcription of **ATG5** was quantitated by qRT-PCR. Fold induction was expressed with respect to untreated control. Results of qRT-PCR are means ± S.D. (n= 3). NS, not significant; *** p < 0.001.
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Figure 5.6F. Knockdown of CHOP. AGS cells were transfected without (control) or with CHOP siRNA. Silencing of CHOP was confirmed by qRT-PCR. Fold induction was expressed with respect to untreated control. Results of qRT-PCR are means ± S.D. (n= 3). *** p < 0.001.

Figure 5.6G. CHOP modulates expression of ATG5 in H. pylori infected AGS cells. AGS cells transfected with either scrambled (SCR) or with CHOP siRNA were left for 48 h. The transfected cells were left uninfected (-) or infected (+) with H. pylori (Hp) at an MOI of 50 for 4 h. Transcription of ATG5 was quantitated by qRT-PCR. Fold induction was expressed with respect to untreated control. Results of qRT-PCR are means ± S.D. (n= 3). NS, not significant; ** p < 0.01.
5.8. Crosstalk between autophagy and apoptosis in H. pylori-challenged cells

The connection between autophagy and apoptosis has been in a variety of settings. We investigated the role of autophagy in regulating apoptosis by analyzing Annexin-Fluos staining (an assay of apoptosis) after infection of AGS cells in which Beclin1 had been knocked down. Annexing V-fluos staining of these cells was enhanced after Beclin1 knockdown suggesting suppression of autophagy enhances apoptotic cell death (Fig. 5.7).

![Figure 5.7. Suppression of autophagy enhances apoptotic cell death in AGS cells. AGS cells transfected without or with BECLIN 1 siRNA, were infected with H. pylori at different MOIs for 16 h. Cells were washed 856 and apoptosis was quantified by staining with Annexin V-Fluos followed by flow cytometry. Results shown are Means ± S.D. (n=3). * p < 0.05; ** p < 0.01.](image)

5.9. Discussion

H. pylori has been reported to enter the gastric adenocarcinoma cell line AGS (Kwok et al., 2002; Amieva et al., 2002). In this scenario it is plausible that autophagy could either help in clearance of the bacterium by the host, or help in survival of the bacterium inside the host. It is therefore important to understand H. pylori regulates autophagy in gastric epithelial cells.
Our late studies have brought to light that the peptidyl, prolyl cis, trans isomerase (PPIase), HP0175 induces apoptosis in gastric epithelial cells (Basak et al., 2005). In view of the recently demonstrated reports of crosstalk between autophagy and apoptosis, we asked the question whether HP0175 evokes autophagy in the gastric cell line, AGS. The present study brings to light the autophagy-inducing capacity of HP0175. We observed that HP0175 induces the formation of LC3-II puncta in AGS cells. We demonstrate that HP0175 induces transcriptional upregulation of autophagy-related genes MAP1LC3B, ULK1, ATG5 and BECLIN1. Knockout of hp0175, blunts the capacity of H. pylori to transcriptionally upregulate the same set of genes. VacA has been reported to induce autophagy in AZ-521 cells (Yahiro et al., 2012).

The unfolded protein response (UPR) has been connected to autophagy in living organisms ranging from Drosophila (Nagy et al., 2013) to mammals (Kouroku et al., 2007). PERK is one of the protein kinases that is activated by misfolded proteins in the endoplasmic reticulum (Harding et al., 1999). It triggers phosphorylation on serine 51 of the α subunit of eIF2α (Wek et al., 2006). Phosphorylation of eIF2-α brings about global tuning down of translation, except for a subset of mRNAs, including a set of bZIP transcription factors, ATF4 and CHOP which interact with C/EBP-ATF Response Element (CARE) sequences of a subset of genes. Here we demonstrate surprisingly that H. pylori-induced autophagy is connected to the UPR. HP0175 and H. pylori induce the expression of PERK. At the same time ATF4 and CHOP are also induced in an HP0175-dependent manner. Downstream of ATF4 and CHOP, the canonical autophagy pathway genes ULK1, MAP1LC3B and ATG5 were transcriptionally regulated. Using a vacA knockout, we have also demonstrated that autophagy induced by H. pylori depends on factors other than VacA, such as HP0175. Schematic diagram of the pathway demonstrated by us is given below (Fig. 5.8).
We have endeavored to unravel whether there is any crosstalk between autophagy and apoptosis. Tanaka et al. (2012) have demonstrated that limiting of autophagy by silencing of LC3B enhances hypoxia-prompted cell death in epithelial cells. Our past studies have demonstrated that *H. pylori*-induced apoptosis occurs at later time points (around 18 and 24 h after infection) (Basak et al., 2005). We observed that silencing of Beclin 1 increases apoptosis indicating a reciprocal relationship between autophagy and apoptosis. By breaking down *H. pylori* replication in AGS cells and in gastric tissues, Tang et al. have recommended that autophagy is a host-beneficial procedure which confines *H. pylori* replication.

Epithelial cell turnover likely influences the outcome of *H. pylori* infection, which is associated with pathologies ranging from ulcers to gastric adenocarcinoma. Atrophic gastritis and gastric dysplasia are connected with accelerated apoptosis in gastric epithelium after *H. pylori* infection (Xia and Tally, 2001). Enhanced reportedly helps epithelial cell proliferation advancing gastric malignancy (Cotter et al., 2009). In a recent report (Bimczok et al., 2013), it has been suggested that *H pylori* hinders clearance of apoptotic gastric epithelial cells leading to adenocarcinoma. Our studies provide insights into the regulation of autophagy and apoptosis induced by *H. pylori* in gastric epithelial cells.