Chapter 1

Ire1 activation in glucose dependent manner by gatifloxacin is independent of xbp1 splicing in pancreatic β cells
**Chapter 1**

1.1 *Introduction*

1.2 *Materials and Methods*

1.3 *Results*

1.3.1 Effect of GFLX on intracellular proinsulin and secreted insulin in medium along with effect of various glucose concentrations on RIN-5f cells.

1.3.2 Effect of GFLX induced Ire1α activation depends upon concentration of glucose in culture medium.

1.3.3 GFLX induced activation of Ern1 in correlation with reduced insulin mRNA without stimulating UPR.

1.4 *Discussion*

1.5 *Conclusion*
1.1 Introduction:

Fluoroquinolones are broad-spectrum antibacterial agents prescribed during infection of gram-positive, gram-negative and anaerobic bacteria (Bauernfeind, 1997). Fluoroquinolones were expected to secure and effectual drug but disturbances in glucose homeostasis are increasingly recognized as one of the most relevant adverse effects in reported adverse cases (Leone et al., 2003). Gatifloxacin (GFLX) as 3-methylpiperazine at position 7 of the quinolone ring and a methoxy group at position 8 is one of the members of fourth generation fluoroquinolone.

Among fluoroquinolones GFLX is reported to induce dysglycemia with maximum rate (Mohr et al., 2005) beside this pharmacoepidemiologic data also shows its utmost role in inducing hypoglycaemia (Frothingham, 2005; Park-Wyllie et al., 2006). In compared with the use of other broad-spectrum oral antibiotics, GFLX is reported to induce hypoglycaemia in 2 day whereas hyperglycaemia in 2 to 6 days of drug administration (Arce et al., 2004; Biggs, 2003; Donaldson et al., 2004; Happe et al., 2004; Park-Wyllie et al., 2006) results in glucose homeostasis disruption. Fluoroquinolones are reported to cause hypoglycaemia due to insulin release by blocking β-cell K⁺ ATP channel in isolated rat pancreatic islets (Maeda et al., 1996). This activity is also directly demonstrated by GFLX (Saraya et al., 2004; Zunkler and Wos, 2003). A hyperglycaemic incident which is more frequent than hypoglycaemia as reported by Park-Wyllie et al., 2006 in the course of GFLX treatment still to be understood. However histamine release as a mechanism of gatifloxacin-induced epinephrine secretion followed by hyperglycaemia is also advocated but at cellular level this mechanism seems less persuasive (Ishiwata and Yasuhara, 2010). GFLX treatment have shown to reduce cellular content of insulin mRNA without affecting promoter activity but exact mechanism which leads to decrease in mRNA is not known (Yamada et al., 2006).

Inositol-requiring enzyme 1α (IRE1) is an endoplasmic reticulum (ER) localised transmembrane protein with kinase and endonuclease activity. IRE1 activated by dimerisation and subsequent autophosphorylation of BiP-Ire1 complex in response to unfolded protein response (UPR). IRE1 activation may leads to activation
ER stress response gene by active X-box binding protein 1 (Xbp-1) transcription factor (Yoshida et al., 2001; Calfon et al., 2002).

Figure 10: The three pillars of unfolded protein response (UPR) stress sensors, inositol requiring protein 1α (IRE1α), protein kinase RNA-like endoplasmic reticulum (ER) kinase (PERK) and activating transcription factor 6 (ATF6), transduce information about the folding status of the ER to the cytosol and nucleus to restore protein-folding capacity (Adapted from Hetz, 2012).

Additionally IRE1 is also known to engage in specific cleavage following degradation of ER membrane localised sets of translating mRNA in Xbp-1 independent manner (Hollien and Weissman, 2006) as well as degradation of insulin mRNA in pancreatic β-cells (Lipson et al., 2008) during ER stress condition. In rat insulinoma RIN-5F cells, the two insulin gene transcripts most profusely transcribed in pancreatic β-cell line and quickly degraded upon induction of ER stress (Pirot et al., 2007). Taking evidence from these studies, we have hypothesised that GFLX might be involved in activation of ER stress components that may lead to initiation of endonucleolytic cleavage of insulin encoding mRNA in rat insulinoma RIN-5F cells. In this study, we report that GFLX induced hyper-activation of IRE1 in addition to glucose concentration upshot in mRNA reduction and allude to endoplasmic reticulum (ER) stress in the molecular mechanisms of GFLX toxicity.
Materials and Methods

Materials

Acrylamide, N, N-methylene bis acrylamide, sodium chloride, sodium hydroxide, glycine, sodium dodecyl sulfate (SDS), agarose, coomassie brilliant blue R250, Imidazole, Triton X-100, Tween-20, Proteinase K, Hybond N membrane, were purchased from Amersham Biosciences, Piscataway, NJ, USA. Bovine serum albumin, Calcium chloride, Casein, Cytidine 5’ diphosphate, Diamino benzidine (DAB), Dithiothreitol, Magnesium chloride, Manganese chloride, N-lauryl sarcosine, Ammonium persulfate, phenylmethyl sulfonyl fluoride (PMSF), were purchased from Sigma Chemical Co., St. Louis, USA. Plasmid DNA isolation and purification kit was purchased from Qiagen (GmbH, Hilden, Germany).

Drug and chemicals

Gatifloxacin (C19H22FN3O4 · 1.5H2O, Molecular Weight 402.42) and other chemicals used in experiment were procured from Sigma (St. Louis, MO, USA), unless specified.

Cell culture

All experiments were performed in Rat Insulinoma RIN-5F cells obtained from the National Centre for Cell Science, Pune, India. RIN-5F cells proffer models for the study of the biology of pancreatic islet cells, specifically the mechanisms controlling the synthesis, storage and secretion of insulin. Cells were maintained in RPMI-1640 culture medium containing 5.6 mM glucose supplemented 2mM L-glutamine and 1mM sodium pyruvate with 10% fetal bovine serum (biological industry) and without antibiotic–antimycotic at 37 °C in a humidified atmosphere of 5% CO2. Cells were preincubated for 18 hr in antibiotic free culture medium containing 3.3 mM glucose before incubation and confluent cells were washed three times by PBS without Ca2+/Mg2+, followed by culturing in fresh medium containing 5.5, 10 or 16.7 mM of glucose without antibiotic with various dosage of Gatifloxacin and incubation was continued for between 6-72 hr according to experimental protocol.
**ELISA for Insulin and Proinsulin**

2-3 x 10⁴ RIN-5F cells were seeded into 6-well tissue culture plates. At a confluence of 75% (72 hr of culture), the tissue culture medium was removed, washed three times with PBS and after preincubation the cells were incubated in fresh antibiotic free tissue culture medium containing various concentration of GFLX with 5.5 mM, 10 mM and 16.7 mM glucose for an incubation period of 6, 24 and 72 hr, tissue culture medium supernatant was harvested and frozen at -70°C until further insulin analysis using the a rat insulin ELISA as per manufacturer’s instructions (Mercodia, Uppsala, Sweden). Adherent cells were washed three times with PBS and lysed using TRIzol reagent (Invitrogen). Homogenates were then stored at -70°C until analysis. The Proinsulin content of the homogenates was measured with a rat insulin ELISA according to the manufacturer’s instructions (Mercodia, Uppsala, Sweden).

**RNA isolation and quantitative real-time PCR**

The subsequent effects of Gatifloxacin incubation prior on the transcript levels of insulin synthesis genes were examined as described by Gabbay et al. Cells were incubated either in the presence of 5.5 mM glucose for 18 h, followed by 50 µM gatifloxacin for 72 h exchanging incubation medium every 48h. Total RNA was isolated following product manual from the cells using the Trizol reagent (Invitrogen,). Reverse transcription was carried out with M-MuLV reverse transcriptase (MBI Fermentas, USA) according to the manufacturer’s protocol using 2 ug of total RNA. Genomic contamination in each sample was tested keeping a control in identical parallel PCR reaction (RT-negative) containing starting material, which had not been reverse transcribed. PCRs were performed in the presence of 0.25 mm dNTPs, 1.5 mm MgCl₂, 10 pmol of each primer and 0.5 U Taq polymerase, and consisted of 30 cycles of denaturation at 94 °C for 45 s, annealing at 58 °C for 30 s and extension at 72°C for 30 s (10 min last cycle; Verti 96 well fast thermal cycler, Applied Biosystems). PCR products were separated on a 2.0% agarose gel, and visualized by ethidium bromide staining. Integrated density of the bands was used as quantitative parameter and was calculated by the Alpha Innotech gel documentation.
system. The intensity of each band was plotted after normalization of each gene with corresponding housekeeping gene after densitometric analysis.

All reactions were performed in triplicate and expression levels were normalized to those of actin. Real-time PCR for quantification was performed as described above, according to the manufacturer’s instructions (ABI 7500, Applied Biosystems). SYBR Green PCR master mix (Applied Biosystems, Foster City, CA) were used for real-time PCR. Reactions were performed in triplicate and the expression of each transcript was calculated by a standard curve of cycle thresholds for serial dilutions of cDNA sample and normalized to the amount of beta actin. GFLX concentration zero µM for each condition was standardized to 1 and the consequent expression of mRNA was measured.

**Detection of mRNA Degradation**

Cellular mRNA transcription was attenuated by treating cells with 100 mg/mL actinomycin D (Sigma A-4262) for 1 hr followed by treatment with 20 µM of gatifloxacin for 6-72 hr with different glucose concentration exchanging incubation medium every 48h. Total RNA was isolated from the cells and subjected to real time for insulin I and insulin II as above described method.

**Western blot**

Cells were lysed in ice-cold cell lysis buffer (Sigma, St Louis, MO, USA) containing 1x protease inhibitors cocktail (Sigma) for 15 min on ice. The lysates were then cleared by centrifuging the cells at 13,000 g for 15 min at 4 °C. Lysates were normalized for total protein (60 µg per lane), separated using 8%-10% SDS-PAGE and electroblotted on to nitrocellulose membrane. The blots were blocked with 3% BSA in TBST (10 mM Tris–HCl, pH 7.4, 150 mM NaCl, 0.05% Tween-20) and probed with primary antibody for overnight. Anti-β-actin (1:2000), GAPDH (1:500), anti-body was procured from Cell Signaling (Danvers, MA, USA), anti-BiP (GRP78) 1:500, anti-IRE1α (1:500), anti-phospho-IRE1α (1:250), anti-α-tubulin (1:1000), anti-β-tubulin (1:1000) was procured from Abcam (Cambridge, MA, USA), anti-eIF2α (1:500), anti-phospho-eIF2α (1:250) antibody was purchased from Sanat Cruz Biotechnology (Santa Cruz, CA). Immunoreacted primary antibodies were further
detected with appropriate alkaline phosphatase conjugated secondary antibodies and detected with BCIP/NBT (Sigma).

**Statistical analysis**

Student's t-test was used for statistical comparison between individual variables as appropriate. Values represent as mean ± S.E.M. of three independent experiments. A probability value of \( P < 0.05 \) was taken as the criterion for statistical significance.

**Table 1: The following sets of primers were used for semiquantitative and real-time PCR**

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<th>FORWARD</th>
<th>REVERSE</th>
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<tr>
<td>1</td>
<td>RAT ACTIN</td>
<td>5'-GCAAATGCTCTAGGCGGAC-3’</td>
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<tr>
<td>2</td>
<td>RAT INSULIN 1</td>
<td>5'-GTCTCTCTGGGAGCCAAG-3’</td>
</tr>
<tr>
<td>3</td>
<td>RAT INSULIN 2</td>
<td>5'-ATCCTCTGGGAGCCAAG-3’</td>
</tr>
<tr>
<td>4</td>
<td>RAT SPLICED XBP-1</td>
<td>5'-CTGAGTCCGAATCGTGTA-3’</td>
</tr>
<tr>
<td>5</td>
<td>RAT ATF4</td>
<td>5'-GTTGGCTAGTCGCTAGACA-3’</td>
</tr>
<tr>
<td>6</td>
<td>RAT CHOP</td>
<td>5'-CCAGCAAGTCCACAAGCA-3’</td>
</tr>
<tr>
<td>7</td>
<td>RAT BIP</td>
<td>5'-CCACCGAGGATGCAGAT-3’</td>
</tr>
<tr>
<td>8</td>
<td>RAT XBP1</td>
<td>5'-GAGCAAGTGGAGTTGATTT-3’</td>
</tr>
<tr>
<td>9</td>
<td>RAT GAPDH</td>
<td>5'-GGCGTGAAACCAGAAGTGA-3’</td>
</tr>
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1.2 Results

1.2.1. Effect of GFLX on intracellular proinsulin and secreted insulin in RIN-5F cells

We first examined whether GFLX affect intracellular proinsulin content and insulin secretion from pancreatic islet cells using the insulin secreting RIN-5F cell line. The insulin concentration released into the medium was determined. Residual cells were lysed with 1 ml of 1% SDS and total protein as well as cellular content of proinsulin was determined by bicinchoninic acid (BCA) assay method and proinsulin ELISA respectively as reveal in product manual. Total insulin content secreted in media was divided by total protein present in well. Proinsulin biosynthesis and insulin secretion from RIN-5F cells was pretentious by various concentration of GFLX in time dependent manner and there was significant decline in proinsulin biosynthesis in 5.5 mM glucose containing media at 6 hr by 100 µM of GFLX (p<0.001).

However at 24 hr and 72 hr time points more significant decrease were observed at levels over 20 µM of GFLX (p<0.001) (Fig. 1.1.1-1.1.3). Insulin secretions were measured in same cell culture medium at different time pointes induced by different dosage of GFLX. A significant increase in insulin secretion was observed at 100 µM in 6 hr (Fig. 1.2.1).
We next examined whether glucose is having any additive effect on proinsulin biosynthesis and insulin secretion induced by diverse concentration of GFLX at 6 hr, 24 hr and 72 hr incubation. Accordingly, when the glucose concentration was
increased to 10 mM, proinsulin content of cell clearly reduces over 20μM of GFLX (Fig. 1.1.2).

![Image](image1.png)

**Figure 1.2.1:** Effect of GFLX on insulin secretion in culture medium from Rin5f cells in 5.5 mM glucose containing medium value are mean ± SEM of six observation (*p< 0.05, **p< 0.001 vs corresponding control(0 μM Gatifloxacin). )

Insulin measured in same culture medium elicited very significantly by 100 μM GFLX over 6 hr of incubation, whereas a considerable (p < 0.001) decrease in insulin secretion was observed at 72 hr over 20 μM of GFLX (Fig 1.2.2), further abomination in cellular proinsulin content was found on incubation with increased 16.7 mM glucose concentration (Fig. 1.1.3).

![Image](image2.png)

**Figure 1.2.2:** Effect of GFLX on insulin secretion in culture medium from Rin5f cells in 10 mM glucose containing medium value are mean ± SEM of six observation (*p<0.05, **p<0.001 vs corresponding control(0 μM Gatifloxacin))
Increased glucose has shown increased insulin secretion in culture medium at 6 hr and a major decrease at 72 hr over 20 µM of GFLX (Fig. 1.2.3). From above result, we can conclude that GFLX clearly trim down proinsulin content of the RIN-5F cells along with increased insulin secreted in culture medium at early time point and decreased in insulin secretion at later time point, these possessions further elicited by the augmented glucose concentration.

![Figure 1.2.3: Effect of GFLX on insulin secretion in culture medium from RIN5F cells in 16.7 mM glucose containing medium value are mean ± SEM of six observation (*p<0.05, **p<0.001 vs corresponding control (0 µM Gatifloxacin).)](image)

Although glucose exhibited to increase insulin secretion in culture medium and decrease in proinsulin content of cells in the different concentrations used in this experiment, further enhancement in insulin secretion and diminution in proinsulin biosynthesis was found in compare to control when they incubated over 20 mM GFLX.

1.2.2 Effect of GFLX induced Ire1α activation, which depends upon concentration of glucose in culture medium

The degree of IRE1 activation can be directly measured by IRE1 autophosphorylation thus, we measured the IRE1 autophosphorylation and subsequent activation level in RIN-5F cells by a phospho-IRE1 and total IRE1 specific antibodies. Lipson et al. have shown role of IRE1 activation in glucose homeostasis and GFLX related with abnormality in glucose homeostasis(Fonseca et al., 2005). We have observed the effect of GFLX on the autophosphorylation of IRE1 at different
time points. We observed significant increase autophosphorylation of IRE1 in RIN-5F cells after 72 hr of GFLX incubation in compare to 6 hr and 24 hr (Fig 1.3.1).

As IRE1 activation level in rat pancreatic cells primarily involve in glucose metabolism by insulin secretion and physiological glucose level fluctuates between 4 mM and 11 mM of glucose, so we have treated the cell with 5.5 mM and 10 mM glucose. We studied the additive effect of glucose on IRE1 activation by treating RIN-5F cells with two different concentrations of glucose 5.5 mM and 10 mM, we have also treated the cell with physiological high 16.7 mM of glucose concentration.

![Figure 1.3.1: Immunoblot analysis of Phospho-Ire1α (P-Ire1α) and total Ire1α using lysates from RIN 5F cells pretreated with 3.3 mM glucose for 18 hr and again incubated with 5.5 mM glucose and 20 µM GFLX for 6 hr, 24 hr and 72 hr.](image)

Treatment with 16.7 mM and 10 mM glucose along with 20 µM GFLX increase IRE1 phosphorylation than 5.5 mM glucose and 20 µM GFLX, indicating that IRE1 is activated by high physiological concentrations of glucose.

![Figure 1.3.2: Immunoblot analysis of Phospho-Ire1α (P-Ire1α) and total Ire1α using lysates from RIN 5F cells pretreated with 3.3 mM glucose for 18 hr and again incubated with 5.5 mM, 10 mM and 16.7 mM Glucose along 20 µM GFLX for 72 hr.](image)

The phosphorylation level of IRE1 at 10 mM and 16.7 mM glucose was higher than 5.5 mM glucose (Fig. 1.3.2), signifying that glucose concentration levels have an additive effect on IRE1 activation level. To further study the correlation between GFLX concentrations and IRE1α activation, we measured the phosphorylation levels of IRE1 in RIN-5F cell after 72 hr of GFLX incubation (Fig. 1.3.3).
We observed gradual increase in IRE1 phosphorylation and subsequent activation levels in RIN-5F cells. Along with IRE1 we have also measured level of the endoplasmic reticulum chaperone BiP in cells at 72 hr in which it is shown to increase up to 50 µM of GFLX and over it shows a decrease. The GFLX incubation decreased cellular proinsulin content and increased secreted insulin in medium as expected (Fig.1.1-1.2). In addition, we found that the GFLX stimulated IRE1 phosphorylation and the degree of phosphorylation correlated with the levels of glucose in medium. These results indicate that GFLX treatment activates IRE1 in RIN-5F cells in addition of glucose.

Despite the fact that glucose exhibited IRE1 phosphorylation in the concentrations used in this experiment, additional enhancement in IRE1 phosphorylation with respect to control was found when they were incubated over 20 mM GFLX.

1.1.1 GFLX induced activation of IRE1α in correlation with reduced insulin mRNA without stimulating UPR

Two homolog genes having more than 90 % sequence homology encode for insulin in rat pancreatic β cells namely Ins1 and Ins2 shared similarities with the other mammalian insulin genes having two intronic sites while insulin 1 in rats’ has arisen from the duplication by retro-transposition. On gatifloxacin introduction, reduction in the mRNA (transcription level) of Ins1 and Ins2 in rat pancreatic cells has been concluded by real time analysis. Blockage in the insulin gene’s transcription is fairly
relieved by the introduction of 2-deoxyglucose, which is known to cause glucose cytopenia in both normal and diabetes rats.

We measured mRNA expression and protein expression of a number of distinguished markers of ER stress along with Ins1 and Ins2 mRNA in rat insulinoma substantiate the activation of IRE1 by GFLX treatment. RIN-5F cells were treated with 20 μM GFLX in 10 mM of glucose containing medium for various time pointes and expression was analysed by real-time polymerase chain reaction (RT-PCR), semiquantitative PCR and western blott(Fig.1.4.1, 1.4.2 and 1.4.3).
GFLX incubation for 72 hr caused an increase in the expression of IRE1 which is further evidenced by increased expression of stress-regulated transcription factor CHOP and Wolfram syndrome 1 (Wfs1), a target gene of IRE1 signalling in pancreatic β cells (Fig. 1.4.4). mRNA expression level of ER stress marker activating transcription factor 4 (Atf4) remained same which was further evident by no change in eukaryotic translation Initiation Factor 2alpha (elf2α) phosphorylation and protein expression of CHOP, indicating it is neither inhibiting the translation nor involved in driving the cell towards apoptosis (Fig. 1.4.1 and 1.4.3). Furthermore splicing of Xbp-1 was not activated by GFLX treatment in RIN-5F cells. To confirm this observation, we measured the cellular levels of total Xbp-1 and there was decrease in cellular content of Xbp-1 mRNA over 50µM GFLX (fig.1.4.1). GFLX have shown IRE1 activation and subsequent reduction in Xbp1 in compare to control at 72 hr (Fig. 1.4.1). These results suggest that GFLX causes activation of IRE1 that is escorted by reduced XBP-1 content in cells.
To study the effect of GFLX treatment on levels of insulin mRNA, we treated RIN-5F cells with increasing concentrations of GFLX for 72 hr, followed by Ins1 and Ins2 mRNA level measurements. Seventy-two hours treatment with GFLX caused a reduction in both Ins1 and Ins2 mRNA, which was more prominently observed in Ins2 mRNA. This decrease is directly correlated with the decrease in cellular proinsulin content in compare to control (Fig. 1.4.2).

Additionally, we have observed no change in insulin mRNA of pancreatic β cells (Fig.1.4.5) that was further supported by decreased phosphorylation of IRE1 either with 5.5 mM glucose and 2-deoxyglucose (2-DG) or 10 mM glucose in the presence of 50 µM GFLX in compare to cells treated in similar condition except 2-DG (Fig. 1.4.6). IRE1 is known to be involved in activation of ER stress response.
genes. In ER stress conditions, IRE1α cleaves the mRNA of some proteins like Xbp1-1, Atf6, etc. It also causes the endonucleolytic cleavage in proinsulin mRNA leading to its degradation. In pancreatic β-cells, increase in gatifloxacin dose and glucose concentration causes hyperactivation of IRE1 but has no effect in the expression other ER related genes like Atf-4 and phospho-eIF2α. No change in the eIF2 levels indicates that no translational change has been induced on gatifloxacin introduction. Hyperactivation of IRE1 is further confirmed by evaluating the expression of its target gene, Wolfram Syndrome Gene (Wfs1). Wfs1 regulates the ER stress signalling by down-regulating Atf-6 and up-regulating an E3 ubiquitin ligase (Hrd-1). ATF6 comes under UPR genes and its function is to contribute in the secretary pathway of ER. Its cleaved N-terminus translocates into nucleus and acts by up-regulating BiP and Xbp-1.
Discussion

It has been established that upstream component of ER stress signalling IRE1 and XBP-1 has important role in exocrine pancreatic cells (Lee et al., 2005a). In this study we have shown that hyper-activation of IRE1 by gatifloxacin under different physiological glucose condition have role pancreatic β cells homeostasis commotion. Gatifloxacin has been previously reported to impart major side-effects and is found to be involved in glucose homeostasis perturbation (Arce et al., 2004; Biggs, 2003; Donaldson et al., 2004; Frothingham, 2005; Park-Wyllie et al., 2006). We determined that gatifloxacin is activator of unfolded protein response in pancreatic β cells. Since activation of IRE1 by gatifloxacin treatment does not escort Xbp-1 splicing. We have named this gatifloxacin-induced phenomenon as gatifloxacin coupling adaptation to ER folding (GCAEF).

The ephemeral activation of IRE1α by gatifloxacin treatment negatively correlates between the gatifloxacin amount used for treatment and the proinsulin levels in rat pancreatic β cells RIN-5F. This conclusion was further supported by the study of insulin secretion from cultured pancreatic β cells. PKR-like ER kinase (PERK) a upstream component of ER stress is highly expressed in pancreatic islets (Harding et al., 2001b). The α subunit of eukaryotic translation initiation factor 2 (eIF2α) is phosphorylated by PERK, leading to the attenuation of general protein translation. This protect cells from ER stress induced apoptosis (Harding et al., 2000). Increase in gatifloxacin concentration from causes decrease in the concentration of proinsulin in total cell lysate with maximum effect at 24 hours with 5.5mM of glucose concentration. This effect is additively supported by similar proinsulin concentration decrease with increasing glucose concentration in media from 5.5 mM to 10 mM or 16.7 mM. In PERK knockout mice islets, high glucose stimulates insulin biosynthesis which markedly increased as compared to that in control mice, indicating that PERK is needed to suppress insulin biosynthesis by high glucose in cells (Harding et al., 2001a). Thus, the equilibrium between PERK signalling and gatifloxacin activated IRE1α appears to be important in the maintenance of β cell homeostasis. Undeniably, a disproportion between these two pathways may cause β cell death.
IRE1α is an ER localized transmembrane protein which functions as protein kinase and endonuclease. Our results depict activation of IRE1α by gatifloxacin is different from classical ER stress inducers. The “Competitive Deprivation” model of BiP dissociation is one of the most recent IRE1 activation model (Bertolotti et al., 2000). In this model BiP binds to IRE1 luminal domain of ER under normal condition and prevent dimerisation and successive activation of IRE1. Under ER stress conditions, IRE1 dimerisation and activation achieved by binding of BiP with unfolded proteins and is released from IRE1. It is well-known that BiP dissociates from IRE1 under ER stress conditions caused by nonphysiological experimental environment. In recent reports it has been shown that BiP binding domain is not the only determinants of IRE1 activation (Kimata et al., 2004). The BiP binding to IRE1 may serve to reduce activation of downstream IRE1 signalling targets (i.e., XBP-1) under conditions of mild ER stress (Credle et al., 2005). Our result of IRE1 activation without BiP dissociation by higher concentration of gatifloxacin supports this possibility.

IRE1 activation in physiological conditions is unlikely to activate apoptosis signalling pathways. Our finding, that glucose dependent gatifloxacin activation of IRE1 does not activate JNK signalling pathways supports this conclusion. Which are proapoptotic components of the UPR. Nevertheless, we were surprised to find that XBP-1 also is not spliced by glucose dependent gatifloxacin activation either, indicating that IRE1-XBP-1 signalling is not essential for insulin folding in β cells under certain physiological conditions. These explanations are supported by current work showing that XBP-1 knockout mice have a severe exocrine pancreatic phenotype, whereas islets from these same mice are indistinguishable from wild-type islets (Lee et al., 2005b). It is promising that in endocrine pancreatic tissue, IRE1 has other target mRNAs that are subjected to its endonuclease activity. These mRNAs may compete with XBP-1 mRNA so that XBP-1 is not spliced under physiological conditions when GFLX present in higher concentration and the pancreatic β cells need to process large quantities of proinsulin. The physiological consequence of this mechanism, however, leftovers to be unwavering and requires additional investigation.
Our result shows that IRE1 has important physiological functions in pancreatic β cells. It is activated by gatifloxacin in glucose dependent manner and positively regulates proinsulin biosynthesis. However, IRE1 activation could have a pathological effect under persistent pathological conditions or in other tissues. The type 2 diabetic patients known to have peripheral resistance to insulin action as major abnormality, which leads to a long-lasting boost in insulin biosynthesis in response to elevated glucose levels. The overwhelmed capacity of ER secretion cause prolong activation of IRE1 signalling pathway. This could lead to glucose toxicity linked with hyperglycemia due to insulin resistance. Glucose toxicity is a nonphysiological and potentially irreversible β cell damage caused by chronic exposure to supraphysiological glucose concentrations (Leahy, 2004; Robertson et al., 2004). Our study regarding IRE1 hyperactivation and the suppression of insulin biosynthesis by insulin mRNA degradation suggests that chronic ER stress is a cause for gatifloxacin toxicity that is further amplified by increased glucose concentration. However, this observation may reflect that the stress response cannot keep up with the damage that chronic hyperglycemia causes. More studies are needed to determine the relationship between IRE1 hyperactivation (i.e. ER stress) and glucose dependent gatifloxacin toxicity.

The IRE1-JNK signalling in liver cells of Type 2 diabetes patients contributes in insulin resistance (Ozcan et al., 2004). In obesity induced liver ER stress that, in turn, causes serine phosphorylation of insulin receptor substrate-1 (IRS-1) and inhibits insulin action in liver cells. Consequently a high level of ER stress in liver cells could contribute to the progress of insulin resistance in patients with type 2 diabetes. These findings indicate that activation of IRE1 by pathological conditions, such as obesity, polyglutamine accumulation, drug and chronic hyperglycemia, is detrimental to cells.
Conclusion

Our results suggested that gatifloxacin plays an assiduous role in hyperactivation of IRE1α in presence of glucose, which causes decrease in cellular insulin mRNA contents in pancreatic β cells, RIN-5F. The strong glucose dependent activation of IRE1α that leads to the increased expression of Xbp-1 mRNA in combination with the loss of XBP-1 splicing through GFLX treatment. These finding guide us to speculation that GFLX have unswerving function in hyper-activation of IRE1α that subsequently leads to reduced proinsulin content of cell via reduced insulin mRNA that may leads to hyperglycaemic condition.

![Proposed mechanism of GFLX side effect](image)

**Figure 1.5.1** Pictorial representation of proposed mechanism that explains GFLX, induced hypoglycaemic and hyperglycaemic condition.

In summary, our result suggests that GFLX activates an Xbp-1 independent and glucose dependent IRE1α activation that leads to decrease in cellular insulin mRNA contents.