行政院國家科學委員會專題研究計畫 成果報告

提昇私校研發能量專案計畫-自體免疫疾病中心之建立--子計畫五:內質網病變所引發之細胞凋亡在自體免疫疾病的角色(3/3)

研究成果報告(完整版)

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處 理 方 式 ： 本計畫涉及專利或其他智慧財產權，2 年後可公開查詢

中 華 民 國 96 年 10 月 02 日

行政院國家科學委員會補助專題研究計畫 ■期中進度報告

（計畫名稱）

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研究計畫中文摘要

關鍵字：內質網，細胞凋亡、細胞週期、訊息傳遞

- 簡介

內質網是細胞合成及修飾分泌性蛋白例如 β細胞之胰島素、免疫細胞之細胞激素、趨化因子或細胞膜蛋白例如接受器、MHC 分子，以及調控鈣離子的重要胞器。當這些功能調控失常就會引發內質網病變反應（ER stress responses）包括（a）引發 chaperon 基因表現，（b）蛋白質合成降低，（c）加速異常蛋白的分解，及（d）細胞凋亡（ER stress-mediated apoptosis）。 內質網病變引起的細胞凋亡主要靠 caspase 12 及 CHOP 所調控。

之前本實驗室在利用內質網壓力的藥物 thapsigargin （抑制內質網鈣離子幫浦）、ionomycin（在細胞膜形成鈣離子通道）、tunicamycin （抑制蛋白質醣基化）、brefeldin A （抑制分泌性蛋白質運送）、DTT（抑制雙硫鍵形成）刺激 HL-60 細胞能引發 ER stress 相關的基因之表現，經過比較之後，發現其誘導的基因表現不完全相同，顯示出有不同的活化路徑及基因表現調控機制。而 以低劑量之 tunicamycin 處理血管內皮細胞之後，造成細胞對 cytokine 的發炎反應降低，因而促使本實驗室進一步研究內質網壓力與抗發炎反應之間的關係。

- 實驗目的:

1．比較不同 ER stress 藥物對基因之表現之影響，以及基因表現之訊息調控路徑
2．內質網壓力與抗發炎反應

- 實驗方法:

利用 ER stress 藥物、磷酸激活酶之抑制劑及 RT-PCR 技術分析 HL-60 細胞基因之表現。利用 glucosamine 研究其對 Hela 細胞造成的影響以及對 cytokine 的反應。

- 實驗結果:

（1） Thapsigargin、tunicamycin 可引發 ER stress 等基因之表現，ionomycin 卻無此作用。Thapsigargin、ionomycin 二者可能藉由鈣離子的效應誘發 p21、IL-8 之表現，tunicamycin 卻無此作用，而 Thapsigargin 單獨誘發和 ER stress 相關的蛋白酶的表現，顯示其誘發三群基因：ER stress 調控基因，鈣離子調控基因，以及受此二者協同調控之基因。
（2）以低劑量 tunicamycin 或 glucosamine 刺激 Hela 細胞 6 小時之後 IL-8 表現下降，對細胞激素 IFN-γ所誘發 IP-10 的表現（NFκB 所調控）明顯下降，而其他基因則較不受影響，使用訊息路徑抑制劑發現，細胞在 ER stress 的情形下，可能誘發新生蛋白，分別活化 GSK-3β導致 IL-8 表現下降，以及抑制 NFκB 路徑的活化使 IFN-γ所誘發 IP-10 的作用無法進行。

- 下階段目標:

1. 利用 siRNA 干擾技術對 CHOP/GADD153 做專一性抑制，並探討 glucosamine 的抗發炎作用是否透過 CHOP 的下游產物來抑制 NFκB 的活化
2. 內質網壓力與僵直性脊椎炎細胞分子病理學的研究，利用 HLA-B27 細胞研究是否因爲內質網壓力導致免疫活化反應較差，是否可以用 CHOP/GADD153 siRNA 加以改善。
3. 研究具抗發炎效用之中草藥是否藉由產生內質網壓力而產生藥效。

1. Background and aims of study

1.1. ER stress-mediated apoptosis

Endoplasmic reticulum (ER) is an important site for biosynthesis and modification of secretory and membrane proteins. It is also the site for Ca^{2+} storage and regulation of intracellular Ca^{2+} levels in response to extracellular signals. Therefore, proper function of the ER is essential to cell survival. Defective folding and rapid degradation of mutant proteins in ER is one of the causes of diseases like amyloidosis, cystic fibrosis, diabetes [1], neurodegenerative disorders such as Alzheimer's disease and Parkinson's disease.

Accumulation of unfold proteins in ER stimulates unfolded protein responses (UPRs), including (a) increased expression of chaperons GRP78 [2], PDI to assist folding (b) increased proteasome proteins for degradation of malfolded proteins (d) attenuation of protein synthesis: phosphorylation of eIF-2α by PERK [3] (c) induction of apopotosis: via caspase 12 gene expression [4] and CHOP [5].

In this study we tried to compare the gene expression profiles induced by different ER stress drugs. The drugs include A23187 (Ca^{2+} ionophore), tunicamycin (inhibits *N*-linked glycosylation), brefeldin A (inhibits protein folding), thapsigargin (inhibits sarco-ER Ca^{2+} ATPase, SERCA), dithiothreitol (DTT, prevents disulfide bond formation).

In the previous study we have found that in ECV-304 cells after long-termed treatment with low dose (2.5 μg/ml) tunicamycin, cells survived and became resistance to cytokine-induced gene expression. In this study we tried to find possible mechanisms for this phenomenon, and to study the relationship of ER stress responses and anti-inflammation.

1.2. Specific aims:

- Comparison of gene expression induced by different ER stress inducer drugs, and signal transduction pathways involved in the gene induction
- Study the relationship of ER stress responses and anti-inflammation, and the possible mechanisms involved.

2. Materials and methods

Cell culture

Human leukemia HL-60 (1x 10^6/per condition) were cultured in RPMI (containing10%FCS medium. Cells were then treated 6~24h with ER stress stimuli: thapsigargin (0.5~2.5 μM), tunicamycin (2.5~10 μg/ml) or brefeldin (2.5~10μM), ionomycin (1.25~5μM), DTT (1.25~5 mM).

Hela cells were treated with two concentrations (2.5 and 10 μg/ml) of tunicamycin or (20 and 40 mM) glucosamine-HCl (a drug used to treat osteoarthritis and has inflammatory effects [6, 7]) for

6h, cells were then left with or without IFN-γ (1000 U/ml) for 6h. Gene expression affected by IFN-γ was analyzed by RT-PCR for their gene expression. Inhibitors for protein synthesis (cyclohexasamide), NFkB (MG-132), and GSK-3β (LiCl) were used to study the mechanisms involved in the suppressive effect of glucosamine on the IFN-induced IP-10 gene expression.

mRNA isolation and RT-PCR

Poly(A) RNA was isolated from cells using oligo$(dT)_{25}$-coated polystyrene Dynabeads (Dynal, Oslo, Norway). RT-PCR was performed to study gene expression. mRNAs of GRP78, CHOP, p21, IL-8 were analyzed in HL-60 responded to different ER stress inducers. In Hela cells, the effects of glucosamine on IFN-γ-induced genes IP-10, STAT-1, IRF-1 were studied. Polymerase chain reactions included 5 min pre-denaturation (hot start PCR) at 95°C and then cycles of: 94°C for 45s, 58°C for 45s and 72°C for 80s.

3. Results

3.1. Differential gene expression of HL-60 cells in response to ER stress inducers

Tunicamycin and thapsigargin induced ER stress-related gene expression, while ionomycin (ion) did not have such effec. Both thapsigargin and ionomycin increased expression of p21 and IL-8 mRNA, which was not observed in the cells treated with tunicamycin. Interestingly, thapsigargin induced expression of ER stress related protease 1 (assigned by ERP1 in our lab, new finding), suggesting its induction is probably regulated via cooperation of ER stress and intracellular Ca^{2+} (Figure 1).

3.2. Anti-inflammatory effects of ER stress drug and glucosamine

In Hela cells both tunicamycin and glucosamine (a drug used to treat osteoarthritis) induced ER stress genes GRP78 and CHOP, decreased basal IL-8, and suppressed IP-10 that was induced by IFN-γ. STAT-1 and IRF-1mRNA which were increased by IFN-γ was not affected by ER stress drugs. (Figure 2).

IFN-γ-induced IP-10 was suppressed by MG132, an inhibitor of NFκB pathway, in a dose-dependent way. The other two IFN-induced genes IRF-1 and STAT-1 were not significantly affected by MG-132. (Figure 3). The result suggested that anti-inflammatory effects of ER stress drugs may act specifically via suppression of NFκB-dependent pathway.

LiCl, a selective inhibitor for GSK-3β, reverted the suppressive effect of glucosamine on basal IL-8 expression, and enhance the suppressive effect of glucosamine on IFN-γ-induced IP-10 (Figure 4). The results indicated that GSK-3β activation during ER stress is involved in the glucosamine-caused suppression of IL-8 [8]. GSK-3β is probably not involved in the glucosamine-caused suppression of IFN-γ-induced NFκB activation and IP-10 gene expression.

Cyclohexamide, an inhibitor for protein synthesis, reverted the suppressive effect of glucosamine on basal IL-8 expression and on IFN-γ-induced IP-10 (Figure 5). The results indicated that *de novo* synthesized proteins induced during ER stress are required for the suppressive effect of glucosamine on basal IL-8 and IFN-induced IP-10, which were respectively via activation of GSK-3β and via suppression of activation of NFκB [9].

4. Conclusions

In this study have found that ER stresses induced 3 groups of genes: classical ER stress genes, Ca^{2+} mediated genes, and genes which are regulated by cooperation of ER stresses and Ca^{2+}. ER stresses may cause activation of GSK-3β and inactivation of NFκB, the later is probably the mechanism for the anti-inflammatory drugs.

5. Future works

1. We will use CHOP siRNA to identify the pathways and molecules that are involved in the glucosamine-caused activation of GSK-3β and inactivation of NFκB.
2. We will study the molecular mechanisms of ER stress induced cellular inactivation to cytokines in HLA-B27 cells, which may be involved in the development of of ankylosing spondylitis.
3. We will study the molecular mechanisms of Chinese herbs with anti-inflammatory effect and study the relationship of ER stresses and anti-inflammation.

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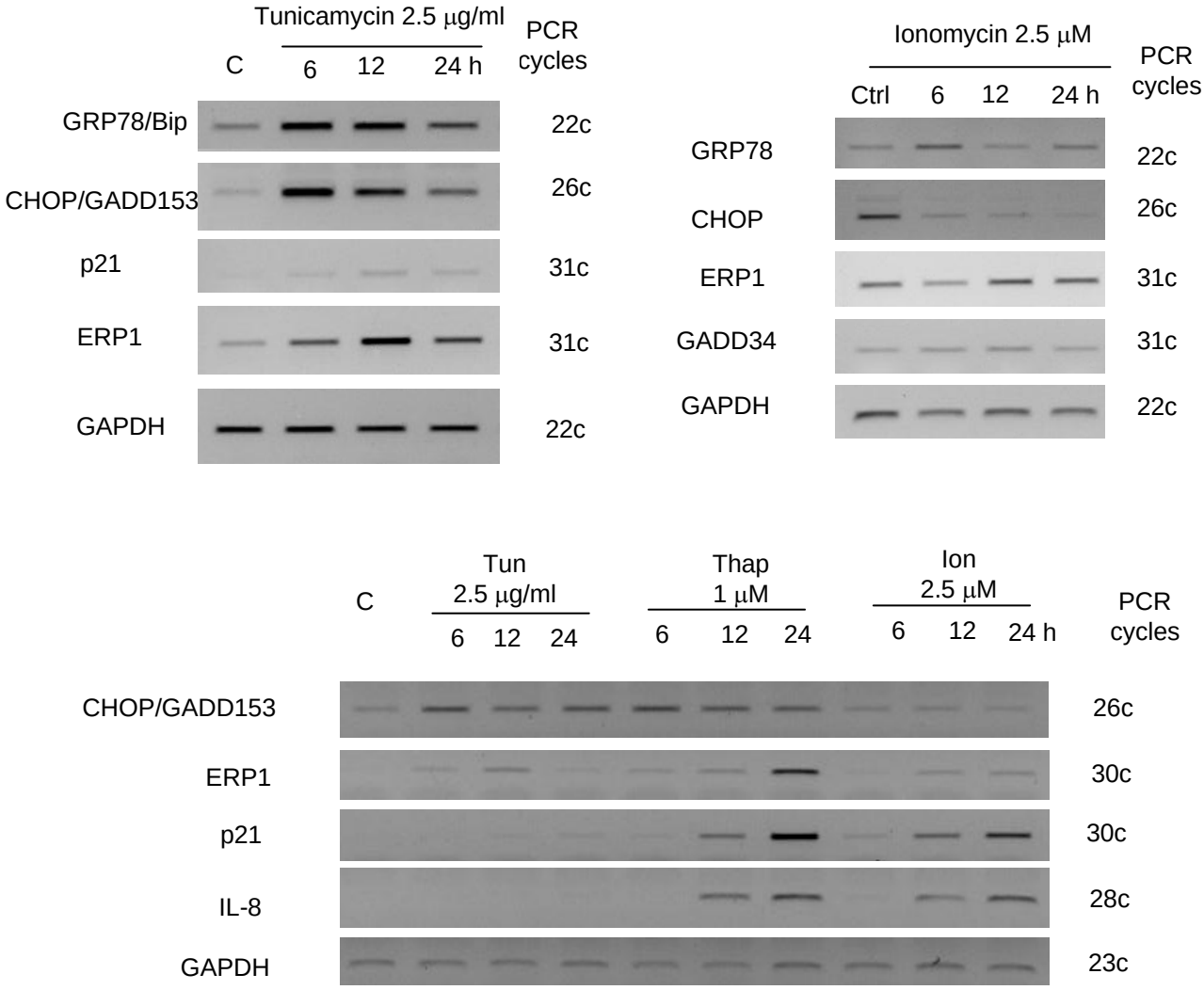

Figure 1. Differential gene expression of HL-60 cells in response to ER stress inducers. Tunicamycin (Tun) and thapsigargin (Thap) induced ER stress-related gene expression, while ionomycin (ion) did not have such effec. Both thapsigargin and ionomycin increased expression of p21 and IL-8 mRNA, which was not observed in the cells treated with tunicamycin. Interestingly, thapsigargin induced expression of ER stress related protease 1 (ERP1), suggesting its induction is probably regulated via cooperation of ER stress and intracellular Ca^{2+}.

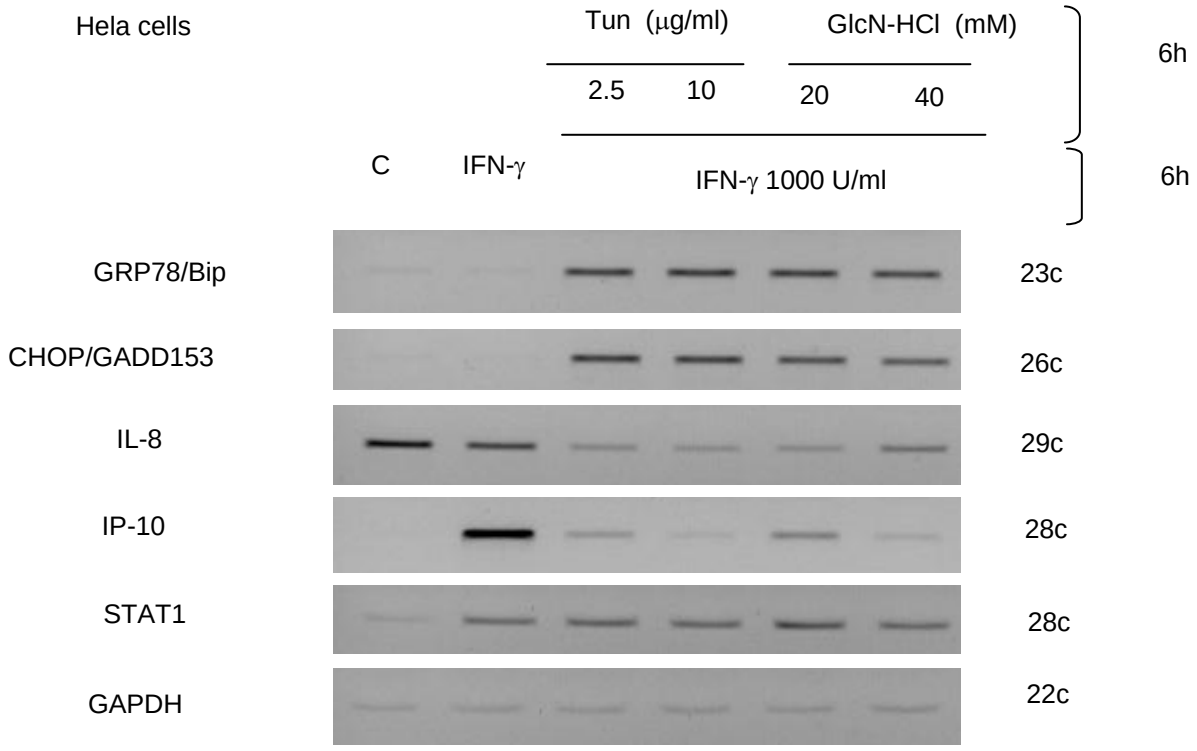

Figure 2. Effects of tunicamycin and glucosamine on IFN-γ-induced gene expression. Hela cells were pretreated with two concentrations of tunicamycin (Tun) and glucosamine (GlcN) for 6h, IFN-γ 1000 U/ml was then added to the medium and treated for 6h. mRNA levels were analyzed by RT-PCR. Both drugs induced ER stress genes GRP78 and CHOP, decreased basal IL-8, and suppressed IP-10 that was induced by IFN-γ. STAT-1 mRNA which was increased by IFN-γ was not affected by ER stress drugs.

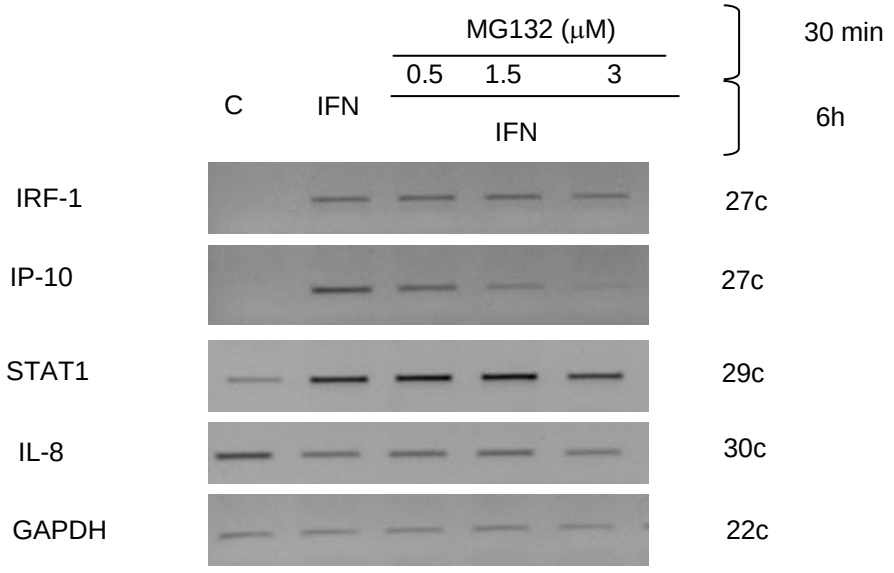

Figure 3. Effects of NFκB inhibitor MG-132 on IFN-γ-induced gene expression. Hela cells were pretreated with different concentrations of MG-132 for 30 min, IFN-γ 1000 U/ml was then added to the medium for 6h. mRNA levels were analyzed by RT-PCR. The results showed that IFN-γ-induced IP-10 was suppressed by MG132 in a dose-dependent way. The other two IFN-induced genes IRF-1 and STAT-1 were not significantly affected by MG-132.

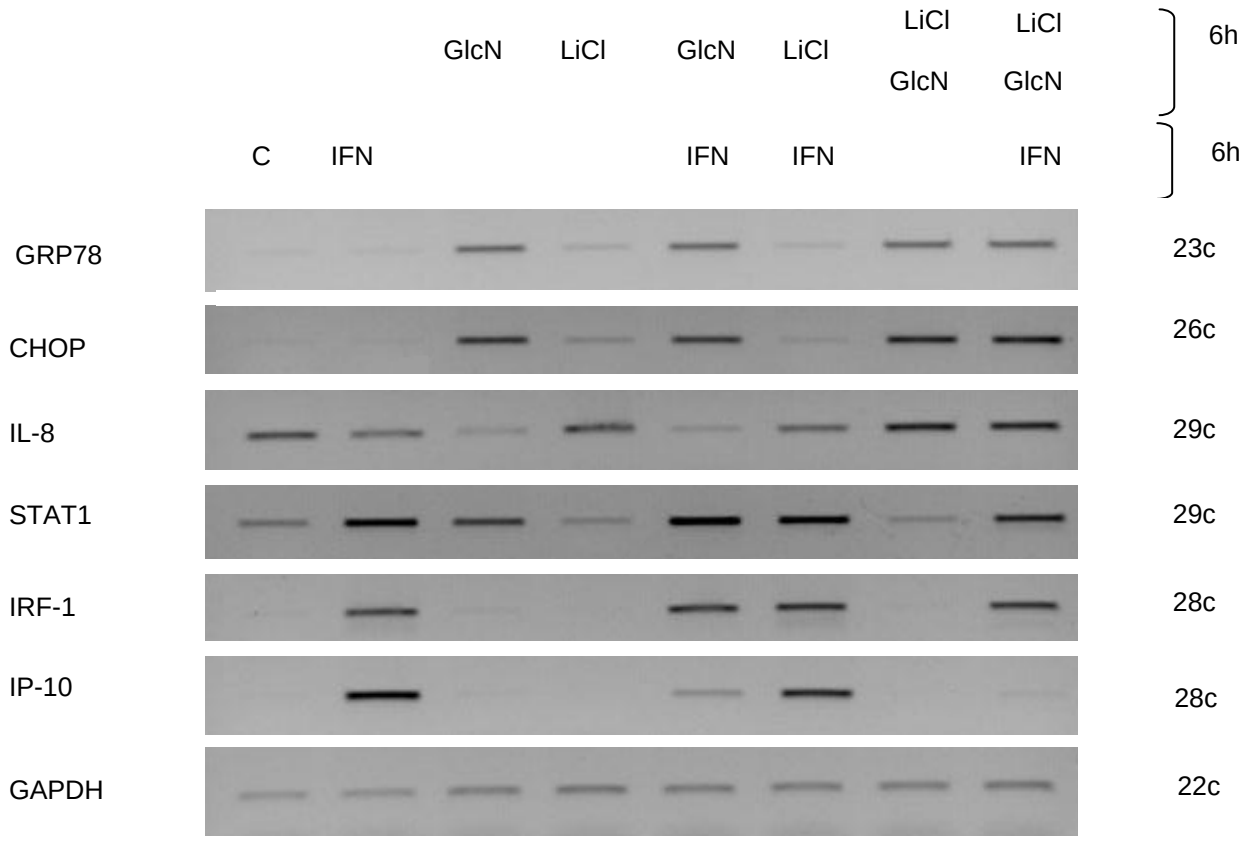

Figure 4. Effects of GSK-3β inhibitor LiCl on the suppressive effects of glucosamine in IFN-γ-induced gene expression. Hela cells were pretreated with two concentrations of glucosamine (GlcN, 30 mM) alone or together with LiCK (40 mM) for 6h, IFN-γ 1000 U/ml was then added to the medium for another 6h. mRNA levels were analyzed by RT-PCR. LiCl reverted the suppressive effect of glucosamine on basal IL-8 expression

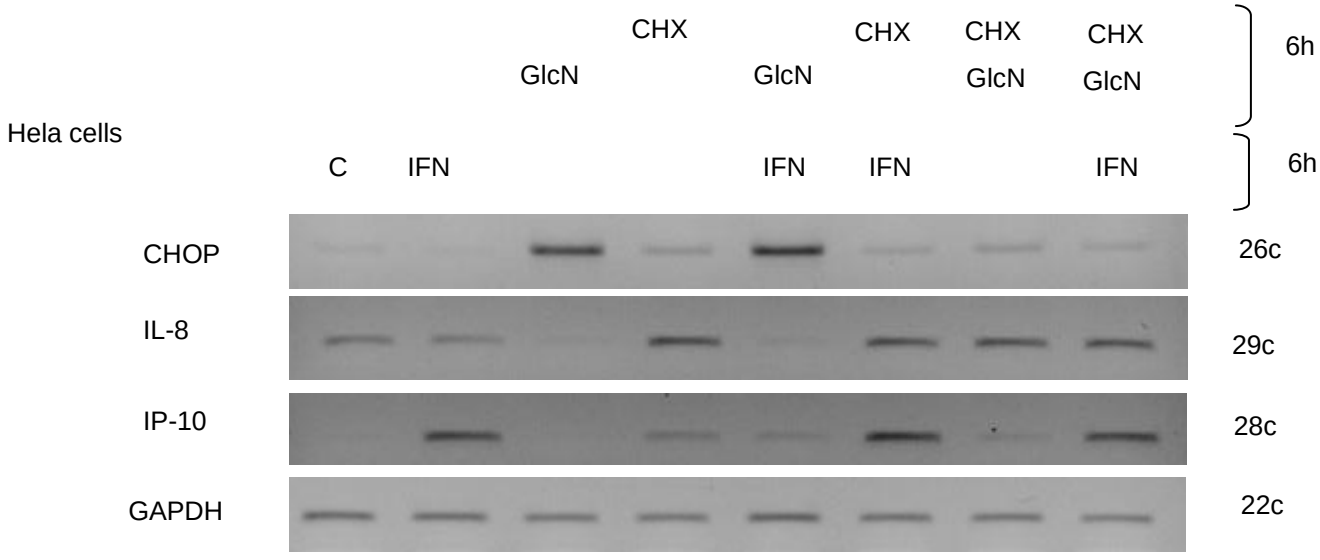

Figure 5. Effects of protein synthesis inhibitor on the suppressive effects of glucosamine in IFN-γ-induced gene expression. Hela cells were pretreated with two concentrations of glucosamine (GlcN, 30 mM) alone or together with cyclohexamide (CHX, 10 μg/ml) for 6h, IFN-γ 1000 U/ml was then added to the medium for another 6h. mRNA levels were analyzed by RT-PCR. Cyclohexamide reverted the suppressive effect of glucosamine on basal IL-8 expression and on IFN-γ-induced IP-10.