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Induction mechanism of lipocalin-2 expression by co-stimulation with interleukin-1β and interferon-γ in RINm5F beta-cells

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ABSTRACT

Lipocalin-2 (LCN-2) was known to play a role in obesity and insulin resistance, however, little is known about the expression of LCN-2 in pancreatic islet β-cells. We examined the molecular mechanisms by which proinflammatory cytokines interleukin-1β (IL-1β) and interferon-γ (IFN-γ) induce LCN-2 expression in RINm5F β-cells. IL-1β significantly induced LCN-2 expression while IFN-γ alone did not induce it. IFN-γ significantly potentiated IL-1β-induced LCN-2 protein and mRNA expression. However, promoter study and EMSA showed that IFN-γ failed to potentiate IL-1β-induced LCN-2 promoter activity and binding activity of transcription factors on LCN-2 promoter. Furthermore, LCN-2 mRNA stability and transcription factors NF-κB and STAT-1 were not involved in the stimulatory effect of IFN-γ on IL-1β-induced LCN-2 expression. Meanwhile, Western Blot and promoter analyses showed that NF-κB was a key factor in IL-1β-induced LCN-2 expression. Collectively, IL-1β induces LCN-2 expression via NF-κB activation in RINm5F β-cells. IFN-γ potentiates IL-1β-induced LCN-2 expression at mRNA and protein levels, but not at promoter level and the stimulatory effect of IFN-γ is independent of NF-κB and STAT-1 activation. These data suggest that LCN-2 may play a role in β-cell function under an inflammatory condition.

1. Introduction

Lipocalin-2 (LCN-2, known as NGAL or 24p3) is a 25-kDa glycoprotein and it was originally characterized from human neutrophil granules [1]. LCN-2 belongs to the large family of lipocalins which possess common structure with a hydrophobic pocket for binding lipophilic molecules [1]. LCN-2 has diverse biological actions including apoptosis and inflammation, and it is expressed in several tissues such as liver, adipose tissue, and macrophages [2,3]. In these tissues, LCN-2 is highly expressed by several inflammatory stimuli including interleukin-1β (IL-1β) and lipopolysaccharide [4,5], which suggests the involvement of LCN-2 in inflammatory responses.

LCN-2 has been recognized as a novel adipokine that links obesity and insulin resistance [6]. LCN-2 expression in adipose tissue, liver, and serum was highly increased in animal obesity model. LCN-2 reduced insulin sensitivity in human omental adipose tissue, 3T3-L1 adipocytes, and hepatocytes [6,7]. Especially, LCN-2 expression in adipocytes was increased by several stimulants of insulin resistance such as TNF-α and high glucose, and this phenomenon was relieved by an insulin sensitizer thiazolidinedione [6,8].

However, LCN-2 knockout mice studies showed discrepant findings about the role of LCN-2 in regulation of insulin sensitivity as follows: one group addressed the role of LCN-2 more insulin sensitive [9], another group more insulin resistant [10], and the other group irrelevant [11]. Meanwhile, the role and expression mechanism of LCN-2 have not been revealed in islet β-cells, which play a key role in glucose and lipid metabolism. There is only one report showing leptin, one of adipokine, induced LCN-2 mRNA transcript in RINm5F β-cells [12]. Therefore, this study was aimed to explore molecular mechanisms by which IL-1β and interferon-γ (IFN-γ) regulate LCN-2 expression in RINm5F β-cells.

2. Materials and methods

2.1. Materials

RINm5F cell was from American Type Tissue Collection (Rockville, MD). FBS and RPMI 1640 were from Gibco BRL (Grand Island, NY). Recombinant human IL-1β, mouse IFN-γ, and anti-goat LCN-2 antibody were from R&D systems (Minneapolis, MN). Lipofect-

Abbreviations: IL-1β, interleukin-1β; IFN-γ, interferon-γ; LCN-2, lipocalin-2; NF-κB, nuclear factor-κB; iNOS, inducible nitric oxide synthase; EMSA, electrophoretic mobility shift assay.

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amine™ 2000 reagent was from Invitrogen (Carlsbad, CA). pGL3-Basic Vector and pRL-TK were from Promega (Madison, WI). Anti-rabbit IκBα antibody and anti-mouse phospho-IκBα antibody were from Cell Signaling Technologies (Danvers, MA). Anti-rabbit p65 antibody, anti-rabbit p50 antibody, anti-rabbit STAT-1 antibody, NF-κB consensus, mutant NF-κB, and STAT-1 consensus oligonucleotides were from Santa Cruz Biotechnology (Santa Cruz, CA).

2.2. Cell culture and drugs treatment

RINm5F cells were cultured in RPMI 1640 medium supplemented with 10% FBS, 2 mM L-glutamine, and antibiotics (100 U/mL penicillin and 100 U/mL streptomycin), and maintained at 37 °C in a 5% CO_2 incubator. We fixed the concentration (100 pg/mL) of IL-1β based on our previous study [13].

2.3. Western Blot analysis

The cells were solubilized with RIPA buffer. Thirty microgram of protein was separated on an 10–12% SDS–PAGE and transferred onto nitrocellulose membrane. After blocking with skimmed milk, the membrane was incubated with primary antibody (0.5 μg/mL in TTBS). Then, the membrane was probed with peroxidase-conjugated IgG (0.5 μg/mL in TTBS). The immunoreactive band intensity was determined by densitometry using Scion Image program (Scion Corporation, Frederick, MD). Equal loading of sample was verified by probing the same blot for β-tubulin.

2.4. Real time-polymerase chain reaction (RT-PCR)

Total RNA isolation and cDNA synthesis were conducted as described previously [14]. RT-PCR was performed with a KAPA SYBR FAST qPCR kit (Kapa Biosystems, Inc., Woburn, MA) and Stratagene Mx3000P QPCR System (Stratagene, La Jolla, CA). The β-actin, GAPDH, or 18S rRNA were amplified in the same reaction to serve as an internal control. All data were expressed as the ratio of the each gene to internal control. The specific primers are listed in Table 1.

2.5. Plasmids

The pGL3 basic-pLCN2 promoter construct was provided by Dr. S. Jauliac (Université Paris Diderot, Institut d'Hématologie, France). Using pLCN-2 promoter as a template, serial deletion constructs were prepared on the basis of transcription factor binding sites (Fig. 2A). The forward primers with *KpnI* site (underlined) were as follows: pLCN2-1230, 5′-TCAGGTACCTCCAGTGGGTGCCTCTCTAG-3′; pLCN2-640, 5′-TCAGGTACCTCACAGGAGAGGAAGGGTCC-3′; pLCN2-300, 5′-TCAGGTACCAGAGATGACAGCTCTTCCGGC-3′; pLCN2-210, 5′-TCAGGTACCTCGTTGTCCCTGCCAGAGG-3′ pLCN2-183, 5′-TCAGGTACCTCCGGGAATGTCCCTCACTC-3′; pLCN2-153, 5′-TCAGGTACCTCTGTCTTGCCCAATCCTGACC-3′; pLCN2-119, 5′-TCAGGTACCCTTGCCAAGTGTTTCCGCAGG-3′. The reverse primer with *XhoI* site (underlined) was as follows: 5′-TCACTCGAGAAGCAGGCGCTGTGGTG-3′. To examine the effect of 3′-untranslated region (3′-UTR) on LCN-2 mRNA stability, cDNA for 3′-UTR of LCN-2 mRNA was amplified with the primers based on the previous study (forward primer: 5′- TCATCTAGAGTGCACAGTGAGTGTGGCTG-3′ with *XbaI* site (underlined), reverse primer: 5′-TCAGGATCCCCACAAAGCTGCTGGTGATGAC-3′ with *BamHI* site (underlined) [2]). This resultant cDNA was inserted into pLCN2-1230 to generate pLCN2-1230-3′UTR. The integrity of all constructs was confirmed by DNA sequencing (Macrogen, Seoul, Korea).

2.6. Transient transfection and luciferase reporter assay

Transient transfection was performed by lipofection as described previously [15]. RINm5 cells were plated at a density of 3×10^5 cells/mL in a 12-well plate. The cells were cotransfected with LCN-2 promoter constructs (0.5 μg) and pRL-TK (50 ng, as an internal control). After 8 h, the cells were treated with IL-1β (100 pg/mL) and/or IFN-γ (100 ng/mL) for 16 h, and then harvested for determination of the firefly and Renilla luciferase activities. All data were normalized by the ratio of firefly to Renilla luciferase activities.

2.7. Electrophoretic mobility shift assay

RINm5F cells were treated with either IL-1β (100 pg/mL) and/or IFN-γ (100 ng/mL) for 30 min. Nuclear extracts were isolated as described previously [15]. The oligonucleotide (5′-GTGCAGCACTCCGGGAATGTCCCTCACTCTCC-3′) covering putative NF-κB and STAT-1 binding sites was used (Fig. 2A). Two complementary oligonucleotides were end-labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase. Nuclear extracts (10 μg) were incubated with labeled probe (30,000 cpm). The reaction product was resolved on a 6% non-denaturing polyacrylamide gel. The gel was dried and exposed to X-ray film. For competition assay, samples were preincubated with 100-fold molar excess of unlabeled NF-κB consensus, mutant NF-κB, and STAT-1 consensus oligonucleotides for 30 min before the addition of probe. For immune-supershift assay, samples were preincubated with specific antibodies (1 μg) against p65, p50, and STAT-1 for 30 min before the addition of probe.

2.8. LCN-2 mRNA stability assay

RINm5F cells were treated with either IL-1β (100 pg/mL) and/or IFN-γ (100 ng/mL) for 24 h, and then exposed to actinomycin D (0.5 μg/mL) to block RNA synthesis for the indicated time. The cells were harvested and then total RNA was extracted and RT-PCR for LCN-2 mRNA was performed, and the expression was normalized by different housekeeping genes β-actin, GAPDH, and 18S rRNA mRNA expression.

2.9. Statistical analysis

All data from four independent experiments were expressed as mean ± SD and were analyzed using one-way ANOVA with Origin

Table 1
Primers used in real time-polymerase chain reaction.

Gene (GenBank No.)	Forward primer	Reverse primer	Product size (bp)
LCN-2 (BC089053.1)	5′-CCAGGGCAGGTGGTTCGTTG-3′	5′-GCCCCTGACCAGGATGGAAG-3′	139
iNOS (U03699.1)	5′-CTGATCTTGTGCTGGAGGTGACC-3′	5′-TTGAAGGGGCAGGCTGGG-3′	142
COX-2 (U03389.1)	5′-TCAGGTCATCGGTGGAGAGGTG-3′	5′-CACCAGACCAAAGACTTCCTGCC-3′	124
β-Actin (NM_031144.2)	5′-CACCCGCGAGTACAACCTTC-3′	5′-CATGCCGGAGCCGTTGTC-3′	111
GAPDH (BC059110.1)	5′-GGCAAGTTCAACGGCACAGTC-3′	5′-GCGGAGATGATGACCCTTTTGG-3′	149
18S rRNA (M11188.1)	5′-TTAGTGAGGCCCTCGGATCG-3′	5′-ATGATCCTTCCGCAGGTTCACC-3′	137

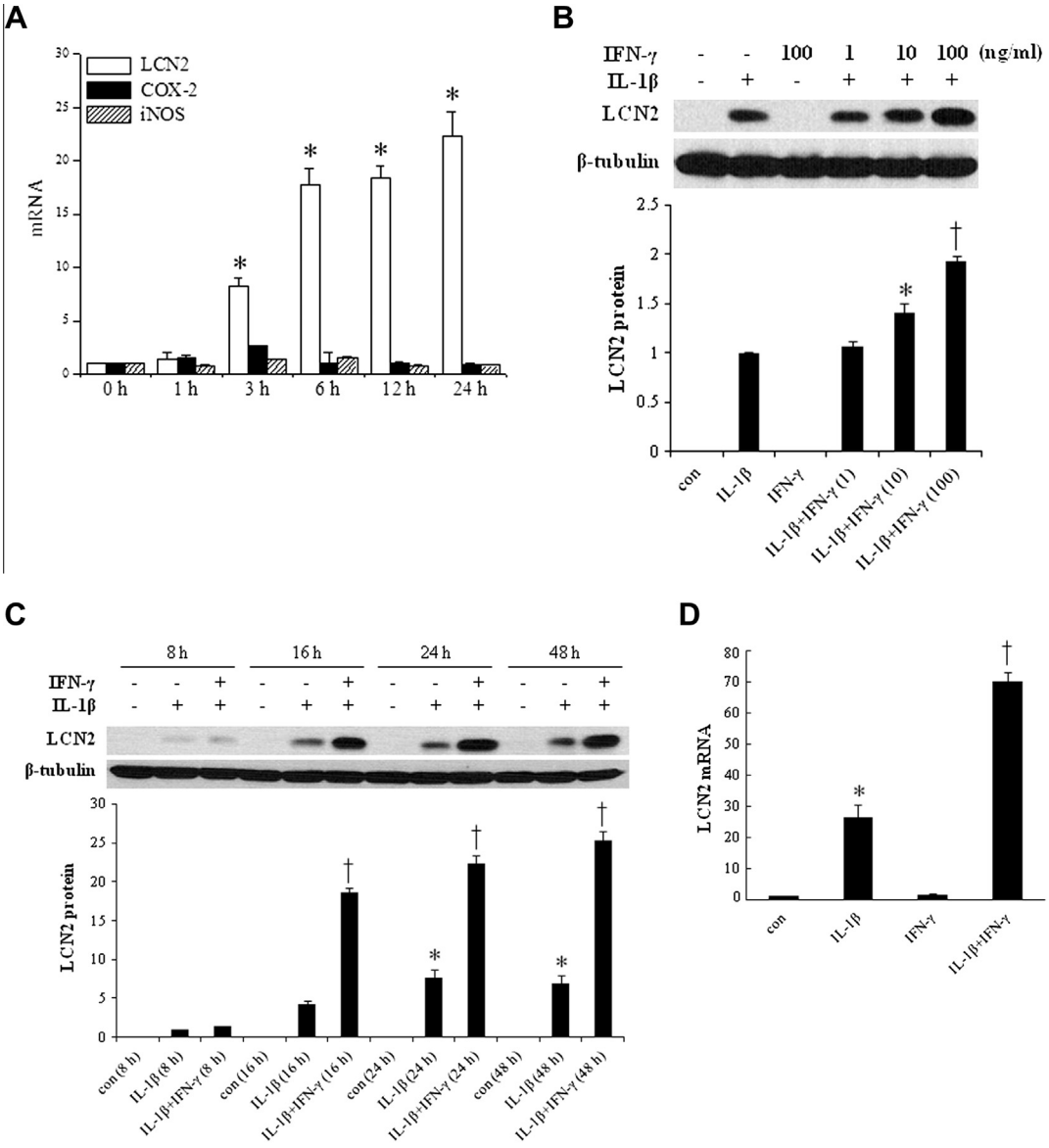

Fig. 1. The effect of IL-1β and IFN-γ on LCN-2 expression. (A) The expression of LCN-2 by IL-1β. RINm5F cells were treated with IL-1β for the indicated time points. RT-PCR was conducted for the expressions of LCN-2, iNOS, and COX-2, and the expression was normalized by β-actin. Data are expressed as mean ± SD and each value is expressed relative to LCN-2 value at 0 h. $^{*}P < 0.05$ vs. COX-2 and iNOS value of respective group. (B) The effect of IL-1β and IFN-γ on LCN-2 protein expression. RINm5F cells were treated with IL-1β and/or IFN-γ. Western blot for LCN-2 was performed. Equal loading was verified by probing the same blot for β-tubulin. Data are expressed as mean ± SD and each value is expressed relative to IL-1β value; $^{*}P < 0.05$ vs. IL-1β + IFN-γ (1); $^{\dagger}P < 0.05$ vs. IL-1β + IFN-γ (10). (C) Time-dependent expression of LCN-2 protein. RINm5F cells were treated with IL-1β and/or IFN-γ for the indicated time points. Data are expressed as mean ± SD and each value is expressed relative to IL-1β value at 8 h. $^{*}P < 0.05$ vs. IL-1β at 16 h; $^{\dagger}P < 0.05$ vs. IL-1β of respective group. (D) The effect of IL-1β and IFN-γ on LCN-2 mRNA expression. RINm5F cells were treated with IL-1β and/or IFN-γ. RT-PCR was conducted for LCN-2 expression, and the expression was normalized by β-actin. Data are expressed as mean ± SD and each value is expressed relative to control value. $^{*}P < 0.05$ vs. con; $^{\dagger}P < 0.05$ vs. IL-1β.

7.0 software (Microcal Software, Northampton, MA). Statistical comparisons among the groups were done by Bonferroni's multiple range *t*-test after the ANOVA. $P < 0.05$ was accepted as statistically significant.

3. Results

3.1. IFN-γ potentiated IL-1β-induced LCN-2 expression

To surmise the functional importance of LCN-2 in an inflammatory condition, we compared the mRNA expression of LCN-2 and that of major proinflammatory mediators iNOS and COX-2 after IL-1β treatment. There was no difference in the expression of these molecules at 1 h. However, LCN-2 expression was significantly increased from 3 to 24 h compared with both iNOS and COX-2 (Fig. 1A). Next, we examined LCN-2 protein expression following IL-1β and IFN-γ treatment. IL-1β induced LCN-2 protein expression, however, IFN-γ alone did not induce LCN-2 protein. IFN-γ potentiated IL-1β-induced LCN-2 expression in a dose-dependent manner (Fig. 1B). Based on this finding, we set the concentration of IFN-γ at 100 ng/mL in subsequent experiments. Next, we observed a time-dependent expression of LCN-2 protein. LCN-2 protein was significantly expressed at 16 h after IL-1β and IFN-γ treatment, and its expression was increased until 48 h (Fig. 1C). Based on this finding, the treatment time of IL-1β and IFN-γ was set at 24 h. We examined LCN-2 mRNA expression following IL-1β and IFN-γ treatment. Consistent with the result of LCN-2

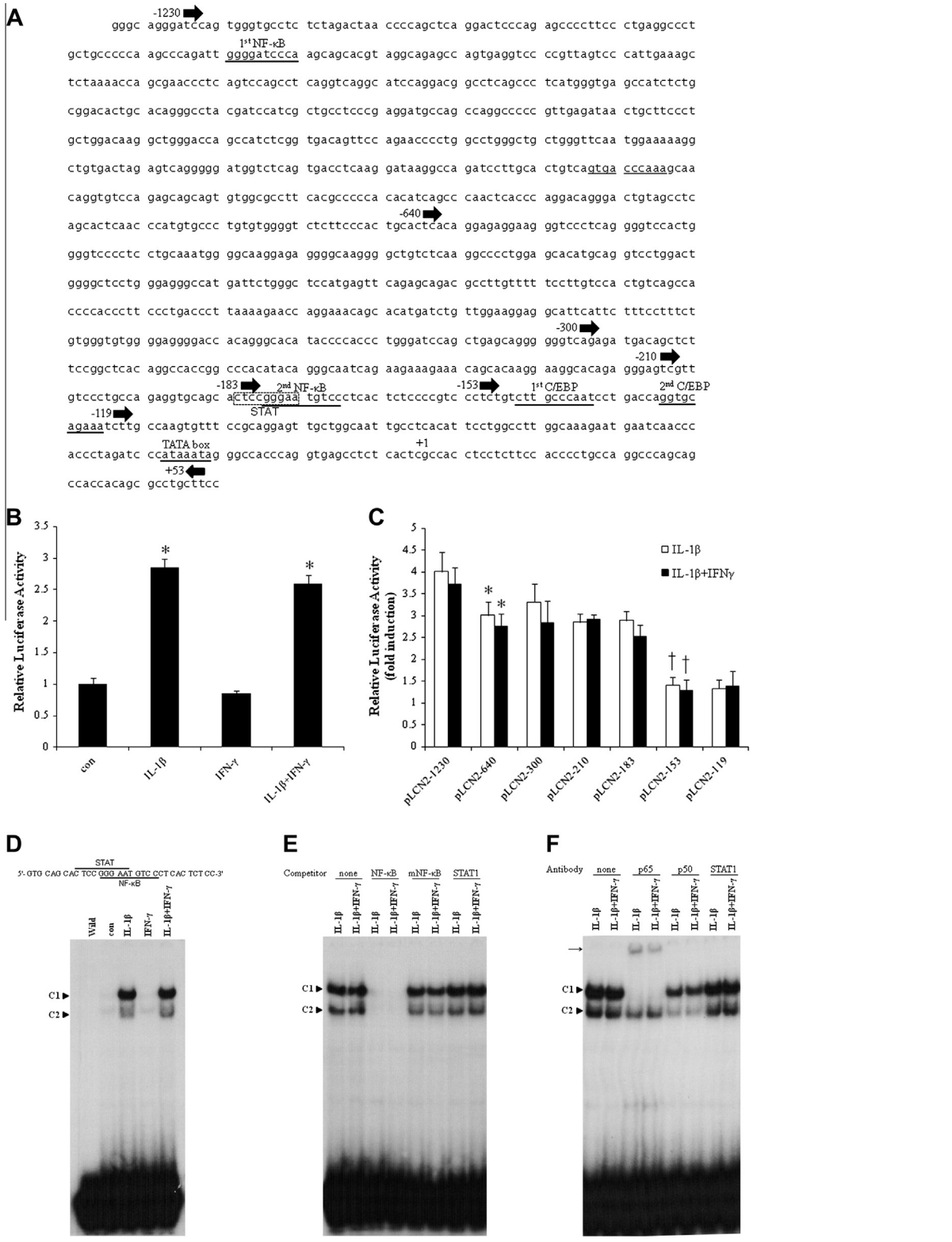

Fig. 2. The effects of IL-1β and IFN-γ on LCN-2 promoter activity and on binding activity of transcription factors to LCN-2 promoter. (A) Schematic distribution of LCN-2 promoter. The start sites of seven LCN-2 promoter constructs were marked as arrows and putative transcription factor binding sites were marked as line (NF-κB and C/EBP) or dotted box (STAT), which were analyzed by TFsearch program http://www.cbrc.jp/research/db/TFSEARCH.html (Parallel Application TRC Laboratory, Japan). (B) RINm5F cells were transiently cotransfected with pLCN2-1230 and pRL-TK. The cells were treated with IL-1β and/or IFN-γ. Data are expressed as mean ± SD and each value is expressed relative to control value. $^{*}P < 0.05$ vs. con. (C) Promoter activities of each construct by IL-1β and IFN-γ. RINm5F cells were transiently cotransfected with each construct and pRL-TK. The cells were treated with IL-1β and/or IFN-γ. Data are expressed as mean ± SD and each value represents fold induction of luciferase activity by IL-1β and IFN-γ divided by the corresponding untreated value. $^{*}P < 0.05$ vs. pLCN2-1230; $^{\dagger}P < 0.05$ vs. pLCN2-183. (D) RINm5F cells were treated with IL-1β and/or IFN-γ. Nuclear extracts were analyzed on EMSA. C1, C2: DNA-protein complexes. (E) For competition assay, samples were preincubated with unlabeled NF-κB consensus, mutant NF-κB, and STAT-1 consensus oligonucleotide before the addition of probe. (F) For supershift assay, an antibody against p65, p50, and STAT-1 was incubated with samples before the addition of probe.

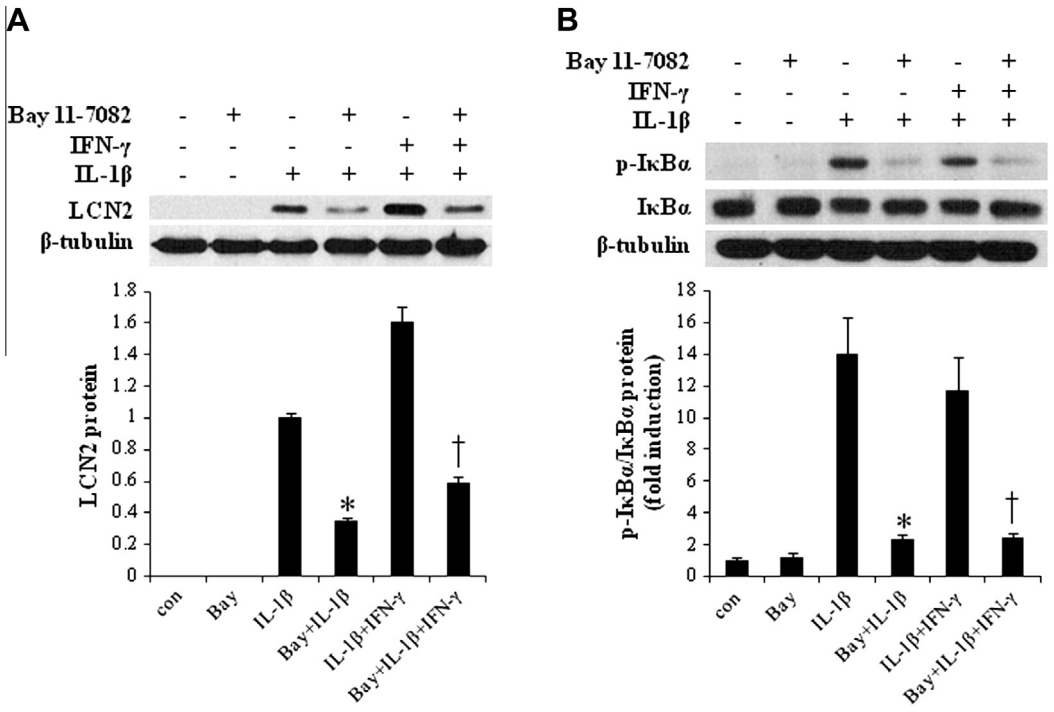

Fig. 3. The effect of IFN-γ on IL-1β-induced LCN-2 expression was independent of NF-κB. (A) RINm5F cells were pretreated with Bay 11-7082 (5 μM) for 30 min, and then the cells were incubated with IL-1β and/or IFN-γ. Western blot for LCN-2 was performed. Equal loading was verified by probing the same blot for β-tubulin. Data are expressed as mean ± SD and each value is expressed relative to IL-1β value. $^{*}P < 0.05$ vs. IL-1β; $^{\dagger}P < 0.05$ vs. IL-1β + IFN-γ. (B) RINm5F cells were pretreated with Bay 11-7082 for 30 min, and then the cells were treated with IL-1β and/or IFN-γ for 30 min. Western Blot for phospho-IκBα and IκBα were performed. Data are expressed as mean ± SD and each value is expressed relative to control value. $^{*}P < 0.05$ vs. IL-1β; $^{\dagger}P < 0.05$ vs. IL-1β + IFN-γ.

protein expression, IFN-γ alone did not induce LCN-2 mRNA expression whereas IFN-γ significantly potentiated IL-1β-induced LCN-2 mRNA expression (Fig. 1D).

3.2. IFN-γ did not potentiate IL-1β-induced LCN-2 promoter activity and NF-κB binding sites was essential for IL-1β-induced LCN-2 promoter activity

We evaluated the effect of IL-1β and IFN-γ on transactivation activities of LCN-2 promoter. LCN-2 promoter sequences are illustrated based on the transcription start site (GenBank accession number: X99133.1) and putative transcription factor binding sites are marked (Fig. 2A). IL-1β strongly induced LCN-2 promoter activity while IFN-γ alone did not increase it. Contrary to the results of LCN-2 protein and mRNA expression, IFN-γ did not potentiate IL-1β-induced LCN-2 promoter activity (Fig. 2B). LCN-2 promoter contains several *cis*-elements such as putative NF-κB, STAT, and C/EBP binding sites. Therefore, to investigate the role of *cis*-elements on LCN-2 promoter activity, serially-deleted LCN-2 promoter constructs were employed. As shown in Fig. 2C, there was no difference in each promoter activity between IL-1β-treated and IL-1β plus IFN-γ-treated groups. The responses to IL-1β or IL-1β plus IFN-γ in pLCN2-640 (without 1st NF-κB site) and pLCN2-153 (without 2nd NF-κB site) were significantly decreased compared with pLCN2-1230 (with 1st NF-κB site) and pLCN2-183 (with 2nd NF-κB site), respectively. Meanwhile, there was no difference in promoter activity between pLCN2-210 (with STAT site) and pLCN2-183 (without STAT site). Also, there was no difference in promoter activity between pLCN2-153 (with C/EBP site) and pLCN2-119 (without C/EBP site) (Fig. 2C). Next, we evaluated the effect of IFN-γ on binding activity of transcription factors to LCN-2 promoter. Two main DNA-protein complex bands (C1 and C2) were detected in IL-1β-treated group (Fig. 2D). Consistent with the result of LCN-2 promoter activity, these band intensities were not further increased by IFN-γ (Fig. 2D). To examine the binding specificities of these bands, a competition assay was conducted. The complex bands were completely competed away by NF-κB consensus but not by mutant NF-κB and STAT-1 consensus oligonucleotides (Fig. 2E). Next, transcription factors binding to this site was identified. As shown in Fig. 2F, C1 band was supershifted by preincubation with anti-p65 antibody (arrow) and C2 band was considerably abolished by anti-p50 antibody; however, there was no difference in band intensity between IL-1β-treated and IL-1β plus IFN-γ-treated groups. Meanwhile, the preincubation with anti-STAT-1 antibody did not affect the band intensity or mobility.

3.3. The stimulatory effect of IFN-γ on IL-1β-induced LCN-2 protein expression was independent of NF-κB

We examined whether NF-κB was involved in the stimulatory effect of IFN-γ on IL-1β-induced LCN-2 protein expression. Bay 11-7082, an NF-κB inhibitor significantly inhibited LCN-2 protein expression in IL-1β-treated and IL-1β plus IFN-γ-treated groups, and the inhibitory effect of Bay 11-7082 was not different in both groups (Fig. 3A). Since IκB phosphorylation is requisite for NF-κB activation, the effect of IL-1β and IFN-γ on IκBα phosphorylation was examined. IL-1β significantly induced IκBα phosphorylation, however, IFN-γ did not potentiate IL-1β-induced IκBα phosphorylation. Bay 11-7082 significantly blocked IκBα phosphorylation in IL-1β-treated and IL-1β plus IFN-γ-treated groups, and the inhibitory effect of Bay 11-7082 was similar in both groups (Fig. 3B). These findings suggest that the effect of IFN-γ on LCN-2 protein expression was independent of NF-κB.

3.4. IFN-γ did not affect LCN-2 mRNA stability

Since IFN-γ failed to potentiate IL-1β-induced promoter activity and NF-κB activation, we evaluated the effect of IFN-γ on LCN-2

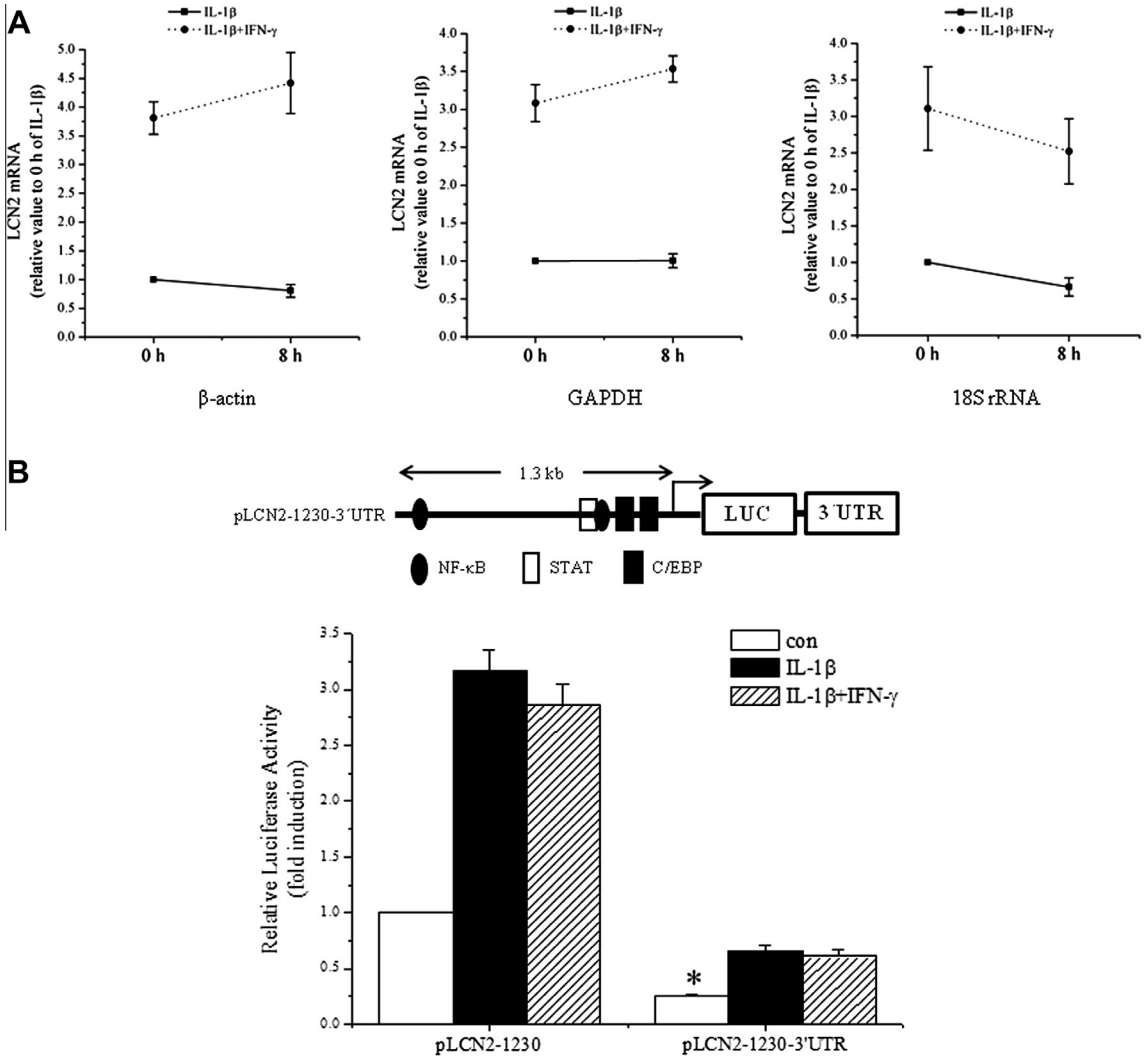

Fig. 4. Effect of IFN-γ on LCN-2 mRNA stability. (A) RINm5F cells were incubated with IL-1β and/or IFN-γ for 24 h, then the cells were treated with actinomycin D for 1 h, and then further incubated for 8 h. RT-PCR was conducted for LCN-2 expression, and the expression was normalized by β-actin, GAPDH, and 18S rRNA. Each value is expressed relative to IL-1β at 0 h. Data are plotted on amount of remaining mRNA relative to 0 h and represent the mean ± SD. (B) Schematic representation of pLCN2-1230-3′UTR and the effect of IFN-γ on the luciferase activity of pLCN2-1230-3′UTR. RINm5F cells were transiently cotransfected with pLCN2-1230-3′UTR and pRL-TK. The cells were treated with IL-1β and/or IFN-γ. Data are expressed as mean ± SD and each value is expressed relative to control value. $^{*}P < 0.05$ vs. con of pLCN2-1230.

mRNA degradation using actinomycin D chase study. We normalized the LCN-2 mRNA expression by comparing expressions of different housekeeping genes β-actin, GAPDH, and 18S rRNA. IFN-γ slightly tended to increase LCN-2 mRNA content when normalized by β-actin and GAPDH while IFN-γ slightly tended to decrease LCN-2 mRNA content when normalized by 18S rRNA (Fig. 4A). Taken together, IFN-γ did not affect LCN-2 mRNA degradation. To further examine the effect of IFN-γ on LCN-2 mRNA stability, we analyzed the luciferase activity using pLCN2-1230-3′UTR. Under basal condition, the presence of 3′-UTR reduced luciferase activity by 70% compared with the absence of 3′-UTR (Fig. 4B), which means that 3′-UTR affects LCN-2 mRNA stability. Consistent with the result of actinomycin D chase study, IFN-γ did not affect the luciferase activity by IL-1β (Fig. 4B, right graph).

4. Discussion

LCN-2 was characterized about two decades ago, however, most studies were confined to inflammation and cancer [16]. Recently, LCN-2 was known to play an important role in lipid metabolism, obesity, and insulin resistance [6,7]. However, the expression mechanism and the role of LCN-2 were never evaluated in pancreatic β-cells which synthesize and secrete insulin, a key regulator in metabolism. Therefore, we investigated the expression mechanism of LCN-2 under exposure to IL-1β and IFN-γ in RINm5F β-cells.

Our RT-PCR study showed that the expression of LCN-2 mRNA by IL-1β was significantly higher and more prolonged than those of iNOS and COX-2 which are well known to be involved in pancreatic β-cell dysfunction. This finding suggests that LCN-2, one of adipokine, may play a role as a proinflammatory or an anti-inflammatory molecule in β-cells under an inflammatory condition. This hypothesis is supported by the fact that various adipokines play a positive (e.g. adiponectin, visfatin) or negative role (e.g. resistin, TNF-α) in β-cell function [17].

We observed that IL-1β significantly induced LCN-2 expression at protein and mRNA levels. IFN-γ alone did not induce LCN-2 expression while IFN-γ potentiated IL-1β-induced LCN-2 expression. This finding is consistent with previous studies in which IL-1β significantly induced LCN-2 expression, however, IFN-γ alone did not increase LCN-2 expression in rat ventricular cardiomyocytes and HepG2 cells [18,19].

Next, we examined the effects of IL-1β and IFN-γ on LCN-2 promoter activity. IL-1β significantly induced LCN-2 promoter activity while IFN-γ alone did not increase it. Unlike LCN-2 mRNA and protein expression, IFN-γ did not potentiate IL-1β-induced LCN-2 promoter activity, which means that IFN-γ does not affect LCN-2 expression at a transcriptional level.

LCN-2 promoter possesses putative *cis*-elements for binding transcription factors NF-κB, STAT, and C/EBP [2]. To characterize the *cis*-elements responsible for LCN-2 induction by IL-1β and IFN-γ, luciferase activities of serially-deleted LCN-2 promoter constructs were evaluated. Our findings showed that NF-κB binding sites, not C/EBP binding site, are involved in IL-1β-mediated LCN-2 promoter activity. This finding is supported by other's study in which C/EBP binding sites were not necessary for IL-1β-induced LCN-2 up-regulation in lung epithelial cells [20]. Also, EMSA and NF-κB inhibitor study showed that NF-κB is a positive regulator in IL-1β-induced LCN-2 expression. This finding is supported by other's studies in which IL-1β increased LCN-2 expression via NF-κB activation in lung epithelial cells, vascular smooth muscle cells, and 3T3-L1 adipocytes [20–22]. Accordingly, NF-κB is a key transcription factor in IL-1β-mediated LCN-2 expression in various cell types including β-cells.

Meanwhile, growing evidence suggests that IFN-γ is by itself not harmful on β-cells, however, together with IL-1β and tumor necrosis factor-α, it evokes β-cell damage [23,24]. In this study, IFN-γ itself did not induce LCN-2 expression. This finding was shown in previous study in which IFN-γ failed to induce LCN-2 expression in human keratinocytes [25]. However, IFN-γ potentiated IL-1β-induced LCN-2 expression at the protein and mRNA level. Our promoter study, EMSA and NF-κB inhibitor study showed that this stimulatory effect of IFN-γ on LCN-2 expression was independent of NF-κB activation. IFN-γ exerts its effects mainly via Janus kinase/STAT-1 signaling pathway in β-cells [26]. Of course, there is putative *cis*-element for STAT-1 on LCN-2 promoter, which is overlapped with 2nd NF-κB binding site (Fig. 2A). Our promoter and EMSA studies showed that STAT-1 was not involved in the induction of LCN-2 expression. Accordingly, the stimulatory effect of IFN-γ on IL-1β-induced LCN-2 expression seems to be exerted via another pathway instead of STAT-1 activation.

In this study, IFN-γ potentiated IL-1β-induced LCN-2 mRNA and protein expression, but failed to potentiate LCN-2 promoter activity. To solve this discrepancy between promoter activity and mRNA expression, we examined the effect of IFN-γ on LCN-2 mRNA stability since the 3′-UTR of mRNA transcript contains important *cis*-elements affecting the fate of mRNA and thus protein synthesis [27]. Our actinomycin D chase study and luciferase assay using pLCN2-1230-3′UTR demonstrated that IFN-γ did not affect LCN-2 mRNA stability. Accordingly, further studies will be needed to reveal the inconsistent findings between LCN-2 promoter activity and mRNA expression.

In summary, this study shows that IL-1β stimulates LCN-2 expression in β-cells and this induction is mediated by NF-κB. Additionally, IFN-γ potentiates IL-1β-induced LCN-2 expression at mRNA and protein levels, but not at the promoter level and this stimulatory effect of IFN-γ is independent of NF-κB and STAT-1 activation.

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