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Intermedilysin induces EGR-1 expression through calcineurin/NFAT pathway in human cholangiocellular carcinoma cells

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ABSTRACT

Intermedilysin (ILY) is a cholesterol-dependent cytolysin produced by *Streptococcus intermedius*, which is associated with human brain and liver abscesses. Although intrahepatic bile duct cells play a valuable role in the pathogenesis of liver abscess, the molecular mechanism of ILY-treated intrahepatic bile duct cells remains unknown. In this study, we report that ILY induced a nuclear accumulation of intracellular calcium ($[Ca^{2+}]i$) in human cholangiocellular cells HuCCT1. We also demonstrate that 10 ng/ml ILY induced NFAT1 dephosphorylation and its nuclear translocation in HuCCT1 cells. In contrast to the result that ILY induced NF-κB translocation in human hepatic HepG2 cells, ILY did not affect NF-κB localization in HuCCT1 cells. Dephosphorylation and nuclear translocation of NFAT1 caused by ILY were prevented by $[Ca^{2+}]i$ calcium chelator, BAPTA/AM, and calcineurin inhibitors, cyclosporine A and tacrolimus. ILY induced early growth response-1 (EGR-1) expression and it was inhibited by the pre-treatment with cyclosporine A, indicating that the calcineurin/NFAT pathway was involved in EGR-1 expression in response to ILY. ILY-induced calcineurin/NFAT1 activation and sequential EGR-1 expression might be related to the pathogenesis of *S. intermedius* in human bile duct cells.

1. Introduction

Streptococcus intermedius, a commensal bacterium in the mouth and both gastrointestinal and genitourinary tracts [1], has been shown to strongly associate with liver and brain abscesses, particularly its strains expressing a high level of intermedilysin (ILY) [2,3]. ILY, initially purified from human liver abscess-derived *S. intermedius* UNS46 [4], is a cholesterol-dependent cytolysin (CDC) which would bind the host cells via human glycosyl-phosphatidylinositol-linked membrane protein CD59 [5,6]. This cytotoxin is also known as a critical virulence factor for the invasion of *S. intermedius* in human liver cells [7]. Since intrahepatic bile duct cells highly express CD59 [8], ILY is aggregated with their cell surface selectively and causes a critical damage to these cells, which play a crucial role in the pathogenesis of liver abscess [9]; however, the molecular mechanism followed by ILY-CD59 binding in intrahepatic bile duct cells remains unclear.

The alteration in intracellular calcium ($[Ca^{2+}]i$) controls diverse cellular processes [10], including the activity of proinflammatory transcription factors such as nuclear factor of activated T cells (NFAT) and nuclear factor-κB (NF-κB) [11]. Recently, it was reported that gram-positive bacterium *Streptococcus pneumoniae* activates NFAT signalling pathway and subsequent up-regulation of inflammatory mediators via pneumococcal virulence factor, pneumolysin [12]. This result suggested that NFAT activation by a cytolysin from bacteria has an important role for its pathological capacity. Despite the critical function of calcineurin/NFAT in broad spectrum of biological processes including immune responses [13,14], its molecular signalling and implication in ILY-exposed intrahepatic bile duct cells are still unclear. Early growth response-1 (EGR-1) functions as a positive regulatory factor for B and T cells mediated by transcriptional regulation of key cytokines and co-stimulatory molecules, and its interaction with NFAT [15]. It was reported that EGR-1–NFAT interaction and complex formation is essential for human cytokine expression such as IL-2 and TNF-α [16]. However, there is no information about EGR-1 expres-

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sion and its relationship with calcineurin/NFAT pathway in ILY-treated HuCCT1 cells. Therefore, we investigated whether ILY affect $[Ca^{2+}]i$ and the calcineurin/NFAT pathway and EGR-1 expression in the human cholangiocellular cell line, HuCCT1.

2. Materials and methods

2.1. Cell culture

Human cholangiocellular cell line HuCCT1 and human hepatic cell line HepG2 were obtained from Japanese Health Science Research Resources Bank (Osaka, Japan). HuCCT1 and HepG2 cells were cultured in RPMI 1640 (Sigma–Aldrich, St. Louis, MO, USA) and DMEM (GIBCO BRL, Gaithersburg, MD, USA), respectively, with 10% fetal bovine serum, 100 IU/ml penicillin, and 100 μg/ml streptomycin at 37 °C in the presence of 5% CO_2.

2.2. Intracellular Ca^{2+} monitoring of HuCCT1 cells

In order to measure $[Ca^{+2}]i$ levels, HuCCT1 cells were incubated with 4.5 μM Ca^{+2}-sensitive probe Fluo-4 acetoxymethyl (AM) ester (Fluo-4/AM, Molecular Probes, Eugene, OR, USA) for 30 min and washed twice with HEPES buffer solution. Fura-4/AM-loaded cells showed under detectable level of $[Ca^{2+}]i$ in untreated control cells (data not shown). After treatment of cells with 10 ng/ml ILY for 5 min, the increase level of $[Ca^{+2}]i$ was measured by the intensity of green fluorescence under a real-time confocal microscope (Model 510, Carl Zeiss, Jena, Germany) using blue-beam (488 nm).

2.3. Immunocytochemistry

HuCCT1 and HepG2 cells were grown on 15 mm diameter cover slips. The cells were exposed to ILY, and tumor necrosis factor alpha (TNF-α) was used as a positive control for NF-κB nuclear translocation. To invesitigate whether Ca^{2+} and calcineurin are involved in cell response, 1, 2-bis (*o*-aminophenoxy) ethane-*N*,*N*,*N*′,*N*′-tetra-acetic acid tetra (acetoxy-methyl) ester (BAPTA/AM) (Calbiochem, La Jolla, CA, USA), cyclosporine A (CsA) (Tocris Cookson, Ellisville, MO, USA), or tacrolimus (FK506) (Cayman Chemical, Ann Arbor, MI, USA) were applied for 30 min before ILY treatment in HuCCT1 cells. After stimulation, the cells were fixed in 10% formalin and followed by methanol permeabilization. Blocking with 4% BSA was followed by exposure to mouse anti-NFAT1 antibody (×1000, BD Transduction Laboratories, Lexington, KY, USA) or rabbit anti-NF-κB antibody (×1000, Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 45 min at RT. Alexa Fluor 488 anti-mouse antibody or Alexa Fluor 564 anti-rabbit antibody (Invitrogen, Carlsbad, CA, USA) as secondary antibody was applied for 40 min and followed by nuclear staining using 10 μg/ml Hoechst 33342. After PBS washing (three times for 5 min each), the cover slips were then examined under a fluorescence microscope (BX-50, Olympus Optical Co. Ltd., Tokyo, Japan).

2.4. Western blot analysis

HuCCT1 cells were stimulated by 10 ng/ml ILY for 10 min with or without the pre-treatment of BAPTA/AM, CsA, or FK506 for 30 min. Cell lysates were fractionated by SDS–PAGE and transferred to PVDF membranes (Millipore, Medford, MA, USA). After blocking with 5% skim milk, the membranes were probed with anti-NFAT1 (1:2500) or anti-β-actin (1:1000) antibody for 2 h at 4 °C, followed by the incubation with HRP anti-mouse IgG (1:5000) for 1 h at room temperature. The membranes were treated with an ECL system (Amersham Pharmacia Biotech, Uppsala, Sweden) to analyze the expression of NFAT1 protein.

2.5. Real-time PCR analysis

Total RNA was extracted by RNeasy Plus Mini Kit (QIAGEN, Maryland, MD, USA) as recommended by the manufacturer. Two microgram of RNA was subjected to reverse-transcribe to generate cDNA by PrimeScript RT reagent kit (Takara BIO, Shiga, Japan). Real-time PCR was performed with a 7300 Real-time PCR system (Applied Bio-systems, Carlsbad, CA, USA) using SYBR Premix Taq II (Takara BIO). The sequences of primers are as follows: human GAPDH; forward 5′-GCACCGTCAAGGCTGAGAA-3′, reverse 5′-TGG TGAAGACGCCAGTGGA and human EGR-1; forward 5′-CTGCGAC ATCTGTGGAAGAA, reverse 5′-TGTCCTGGGAGAAAAGGTTG-3′. Data are presented as the relative mRNA variation in treated cells versus control cells.

3. Results

3.1. ILY induced a nuclear accumulation of $[Ca^{2+}]i$

We examined free $[Ca^{2+}]i$ level and its localization in ILY-treated HuCCT1 cells (Fig. 1). Free $[Ca^{2+}]i$ levels and its subcellular localization were showed as green fluorescence by Fura-4/AM. In untreated cells, the green fluorescence was under detectable level (Fig. 1, left panel). The green fluorescence was clearly observed in nuclear region in HuCCT1 cells treated with 10 ng/ml ILY for 5 min (Fig. 1, right panel), indicating that the free $[Ca2^{+}]i$ levels was increased in nuclear region by ILY treatment.

3.2. ILY induced NFAT1 activation

NFAT has been known to be driven by Ca^{2+} signalling for its activation, and expressed widely in broad spectrum of cell type. NFAT1 was translocated into the nuclei when the cells were treated with 10 ng/ml ILY (Fig. 2A, upper panel). NF-κB nuclear translocation was not observed in HuCCT1 cells treated with 10–40 ng/ml ILY (Fig. 2A, lower panel or data not shown), although 10 ng/ml TNF-α induced NF-κB nuclear translocation in HuCCT1 cells (Fig. 2B). In contrast to the case of HuCCT1 cells, NF-κB nuclear translocation was observed in ILY-treated human hepatic HepG2 cells (Fig. 2C). These results indicate that NFAT1, rather than NF-κB, may be more sensitive in HuCCT1 cells in response to ILY.

Fig. 2D shows that ILY induced dephosphorylation of NFAT1 proteins in HuCCT1 cells. Phosphorylated NFAT1 (p-NFAT1) was detected as a ~120 kDa band in the lysate of untreated control

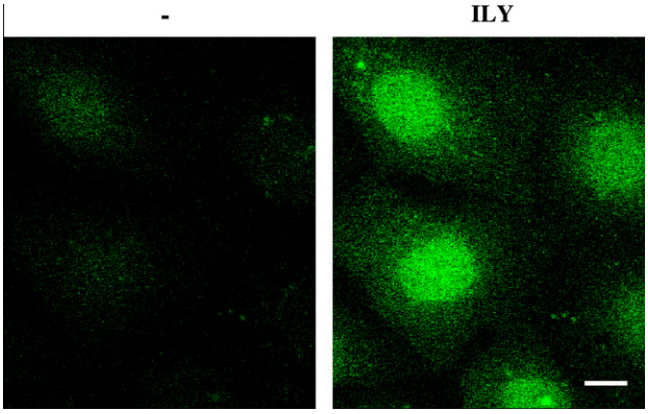

Fig. 1. ILY-induced $[Ca^{2+}]i$ movement in HuCCT1 cells. The movement of $[Ca^{2+}]i$ was detected as green fluorescence under a confocal microscope using Fluo-4/AM reagent. Increase of $[Ca^{2+}]i$ in nuclear region were observed in the cells exposed to 10 ng/ml ILY for 5 min, but not in the untreated cells. Bar indicates 10 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

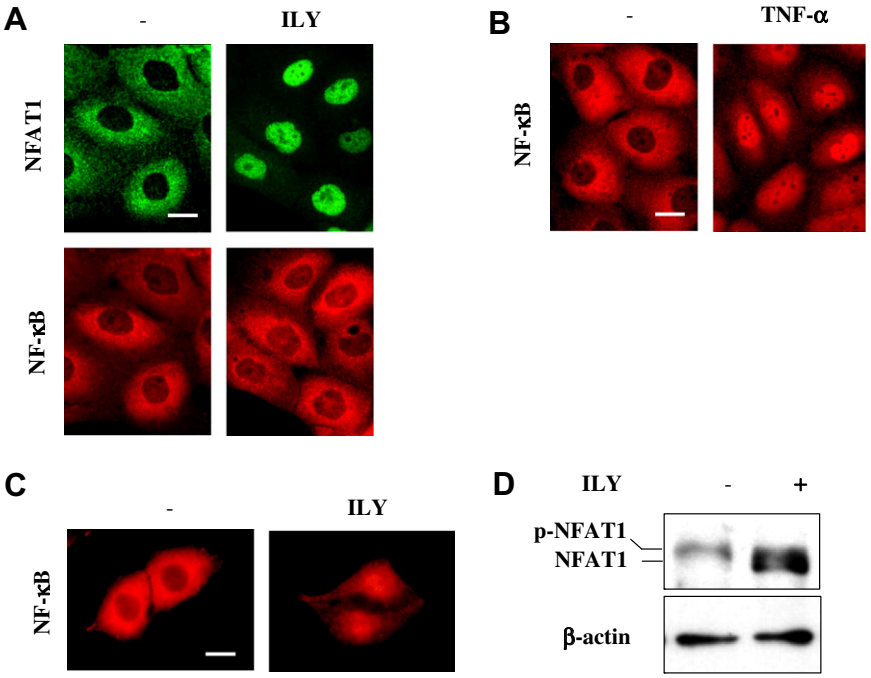

Fig. 2. ILY-induced NFAT1 activation. NFAT1 (green) and NF-κB (red) were located in the cytoplasm of untreated HuCCT1 cells. NFAT1 nuclear translocation was observed in HuCCT1 cells treated with 10 ng/ml ILY (A, upper panel), whereas NF-κB was not (A, lower panel). NF-κB was translocated into the nuclei of HuCCT1 cells treated with 10 ng/ml TNF-α (B), and in the ILY-treated HepG2 cells (C). NFAT1 was dephosphorylated by ILY treatment (D), determined by Western blot. Bars indicate 10 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

HuCCT1 cells, which can be dephosphorylated followed by nuclear translocation [17]. ILY induced a downward shift of NFAT1 protein, indicating dephosphorylation (Fig. 2D), followed by its nuclear translocation (Fig. 2A, upper panel).

3.3. ILY-induced NFAT1 activation is regulated by Ca^{2+}/calcineurin pathway

To investigate whether the Ca^{2+}/calcineurin pathway is involved in NFAT activation by ILY, we use $[Ca^{2+}]$i chelator BAPTA/AM and calcineurin inhibitors, CsA and FK506. BAPTA/AM suppressed ILY-induced nuclear translocation and dephosphorylation of NFAT1 (Fig. 3 A and B, respectively), indicating that increased $[Ca^{2+}]$i plays a crucial role in ILY-induced NFAT1 activation. The fact that BAPTA/AM could not inhibit NFAT1 activation completely, may be caused by incomplete diminish of $[Ca^{2+}]$i level in ILY-treated HuCCT1 cells (unpublished results). On the other hand, calcineurin inhibitors, CsA and FK506, were completely inhibited NFAT1 nuclear translocation in ILY-stimulated HuCCT1 cells (Fig. 3C). Indeed, further assessments indicated that NFAT1 dephosphorylation in ILY-stimulated HuCCT1 cells was inhibited by these calcineurin inhibitors (Fig. 3D).

3.4. ILY induced EGR-1 expression through the calcineurin/NFAT pathway

From the microarray analysis, EGR-1 was identified as a factor, which is up-regulated by ILY treatment in HuCCT1 cells (data not shown). To examine whether the calcineurin/NFAT pathway is involved in EGR-1 expression induced by ILY, the cells were pre-treated with or without CsA, followed by 10 ng/ml ILY treatment for 30 min. RNA were extracted from the cells and detected EGR-1 expression using real-time PCR (Fig. 4). In corespondence with microarray analysis, EGR-1 expression was augmented in the ILY-treated cells compared with that in the untreated cells. CsA pre-treatment significantly inhibited EGR-1 expression induced by ILY in HuCCT1 cells.

4. Discussion

In the present study, we provided evidence for the first time to demonstrate that ILY induces nuclear accumulation in $[Ca^{2+}]$i, leading to NFAT1 activation and EGR-1 expression in HuCCT1 cells through calcineurin-dependent pathway. Increase in intracellular-free Ca^{2+} is general process used by eukaryotic cells to regulate many cellular functions. Several studies have shown that this ubiquitous signalling pathway can be hijacked by bacterial pathogens to promote the activity of transcription factor and cytokine expression [18–20]. In recent years, calcium flux induction has emerged as a widespread mechanism by which pathogenic bacteria influence host cells [21].

It is clearly defined that the intracellular Ca^{2+} levels increase enabling Ca^{2+} to bind to calcineurin, resulting in a conformational change and activation of calcineurin as protein phosphatase [22]. Subsequently, activated calcineurin binds to and dephosphorylates NFAT, which leads to nuclear translocation of NFAT where it potentiates NFAT-dependent gene expression [22]. NFAT1 investigated in this study is one of four calcium-regulated members of the NFAT family of transcription factors and was well studied in the field of inflammation and immune responses [23]. In the present study, the increased $[Ca^{2+}]$i triggered by ILY were accumulated into nucleus in HuCCT1 cells, which in turn leads to NFAT1 dephosphorylation and translocation. This is the first report demonstrating that ILY-induced $[Ca^{2+}]$i was moved into nucleus in HuCCT1 cells, determined visually by confocal microscope. This phenomenon strongly suggest that ILY-induced accumulation of $[Ca^{2+}]$i into nucleus is involved in activation of the calcineurin/NFAT pathway. We further showed in this study that both $[Ca^{2+}]$i chelator (BAPTA/AM) and calcineurin inhibitors (FK506 and cyclosporine A) inhibit dephosphorylation and nuclear translocation of NFAT, indicating that

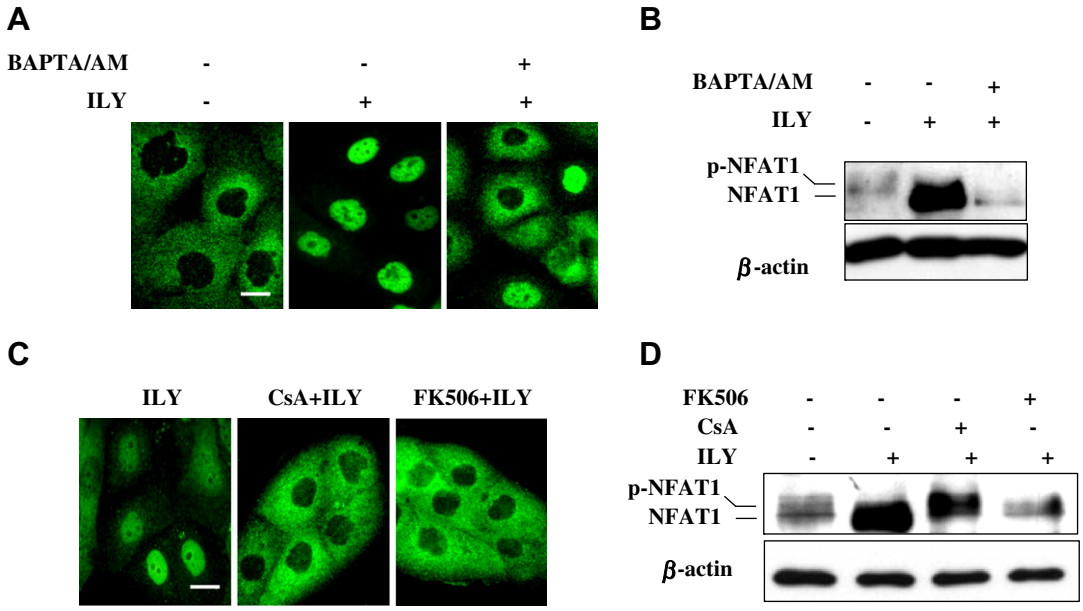

Fig. 3. The increase in $[Ca^{2+}]i$ and calcineurin is essential for ILY-induced NFAT1 activation. BAPTA/AM (10 μM) suppressed ILY-induced NFAT1 nuclear translocation in HuCCT1 cells (A). NFAT1 dephosphorylation was reduced by BAPTA/AM (B). CsA (100 μM) and FK506 (6 μM) also prevented ILY-induced NFAT1 nuclear translocation in HuCCT1 cells (C). NFAT1 dephosphorylation was prevented by CsA or FK506 (D). Bar indicates 10 μm.

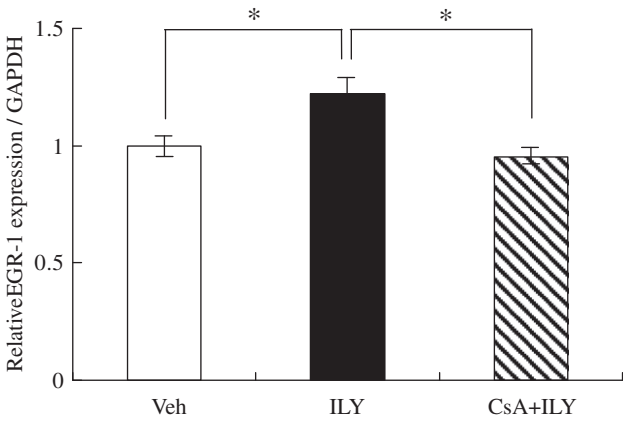

Fig. 4. The calcineurin/NFAT pathway is involved in EGR-1 expression induced by ILY. Total RNA was extracted after ILY (10 ng/ml) treatment for 30 min with or without pre-treatment of CsA (100 μM) for 30 min. EGR-1 mRNA expression was determined by Real-time PCR and normalized on the basis of GAPDH expression. Data are presented as the relative mRNA expression level of cells treated with ILY to control cells. Data represent the mean ± SD of three independent experiments (*$p < 0.01$).

ILY-induced NFAT1 activation in HuCCT1 cells is a calcium/calcineurin-dependent mechanism.

NF-κB is also Ca^{2+}-dependent transcription factor that responsible for the activation of immune and inflammatory response especially in T lymphocytes [24]. Another interesting result in our study is that ILY did not induce NF-κB translocation in HuCCT1 cells although it caused its activation in HepG2 cells. It was also reported that Ca^{2+} influx caused by pneumolysin resulted in the activity of NF-κB in the immune response of neutrophils [25]. These findings suggest that NF-κB can be activated by ILY in another type of cell. Selective activation of NFAT1, but not NF-κB, in HuCCT1 cells by ILY seems to be based on cellular specificity, which might be related with selective damage observed in bile duct cells in liver abscess caused by oral commensal *S. intermedius*. However, further studies are needed to clarify which factors regulated by activated NFAT1 in bile duct cells.

Finally, we demonstrated in this report that ILY induced EGR-1 expression and the calcineurin/NFAT pathway is essential for EGR-1 expression in HuCCT1 cells. EGR-1 has been shown to have great impact on growth, proliferation and immune response in a wide variety of cells [15,26]. NFAT1 physically interacts and functionally cooperates with EGR-1, which induces expression of cytokines, including IL-2, TNF-α, and tissue factor [27–30]. It was recently reported that EGR-1 cooperatively binds to an NFAT/NF-κB-overlapping IL-4 enhancer element and activate the IL-4 promoter synergistically in T cells [31]. It was also demonstrated that EGR-1 plays an important role in acceleration of hepatic inflammation, apoptosis, and subsequent mortality in galactosamine/lipopolysaccharide-induced acute liver injury [32]. These reports and our present result raise the possibility that activated NFAT1 and upregulated EGR-1 cooperatively function in immune response in ILY-treated HuCCT1 cells although its target genes remains to be clarified.

Bacterial toxins function as crucial virulence factor for infection and immune responses. Several recent studies focused on Ca^{2+} signalling induced by bacterial cytotoxin. Induction of Ca^{2+} signals by listeriolysin O in target cells is extremely important in context of bacteria survival in the host [33]. The $[Ca^{2+}]i$ oscillation and subsequent NFAT translocation were triggered by listeriolysin O, which is involved in cellular responses such as degradation and cytokine synthesis in a Ca^{2+}-dependent manner [33]. Pneumolysin plays as a key mediator of cell cytotoxicity in infection by *S. pneumoniae* by changing $[Ca^{2+}]i$ concentration, which lead to NFAT activation [34]. In addition, listeriolysin induces transcription of TNF-α gene by activation of NFAT following $[Ca^{2+}]i$ increase and membrane pore formation [33]. Based on these observations and our results in this study, the increase in $[Ca^{2+}]i$ and NFAT activation induced by ILY, which leads to EGR-1 expression, is thought to play a role in immune or pathological responses in HuCCT1 cells against infection by *S. intermedius*.

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