Nuclear localization status of propiece interleukin (IL)-1α in HeLa cells

Yoshihiro Kudo

Nihon University Graduate School of Dentistry,

Major in Oral and Maxillofacial Surgery

（Directors: Profs. Yoshiyuki Yonehara, Masatake Asano,

Tadayoshi Kaneko, and Assist. Prof. Mariko Tsunoda）

Index

This thesis was prepared using the original article “Nuclear localization of propiece IL-1α in HeLa cells” (Journal of Oral Science, 2022 in press) with new unpublished data (Figure 5C).

Abstract

Purpose: The study aimed to examine the nuclear localization of propiece interleukin (IL)-1α (ppIL-1α) and extracellular release rates of ppIL-1α, precursor IL-1α (pIL-1α), and mature IL-1α (mIL-1α).

Methods: The subcellular localization of IL-1α molecules was observed in HeLa cells transfected with green fluorescence protein (GFP)-tagged IL-1α. Extracellular release efficiency was examined using N-terminal HiBiT-tagged IL-1α. The nuclear localization status of ppIL-1α was examined by incubating ppIL-1α transfectants with 0.1% Triton X-100 solution or with complete medium on ice.

Results: The results indicated the diffuse cytoplasmic and nuclear localization for m and p and ppIL-1, respectively. All IL-1α forms were released from the cells even in the steady state, and the release efficiency was 25%, 13%, and 8% for mIL-1α, pIL-1α, and ppIL-1α, respectively. Under oxidative stress condition, GFP-mIL-1α was totally diminished, but weak fluorescence of GFP-pIL-1α and GFP-ppIL-1α was detected; nuclear localization of GFP-ppIL-1α was completely abolished by 0.1% Triton X-100 treatment, however, it remained in the nucleus after culture in complete medium on ice.

Conclusion: The results of this study showed that ppIL-1α was localized in the nucleus and released extracellularly even in the steady state. Moreover, its nuclear localization is not firm, and it is presumed to be floating in the nucleoplasm.

Introduction

Alarmin, a molecule released by damaged cells [1], functions as a "warning signal" that induces an inflammatory response in the surrounding tissues and activates the innate and acquired immune systems in the body. Interleukin (IL)-1α is a representative alarmin released under hypoxic, oxidative, and heat conditions [2], and is involved in various activities, such as angiogenesis and wound healing, among others. IL-1α is produced in the cytoplasm as a 34 kDa precursor (pIL-1α), and is, subsequently, cleaved by enzymes such as calpain (Ca^{2+}-dependent proteolytic enzyme) [3-5], granzyme B (GzmB) [6], and thrombin [7]. The enzymatic cleavage results in the generation of N-terminal propiece IL-1α (ppIL-1α) and C-terminal mature IL-1α (mIL-1α) [8,9]. pIL-1α and mIL-1α are extracellularly secreted and exert their functions through the same IL-1 receptor type I [10]. Furthermore, pIL-1α and ppIL-1α are translocated to the nucleus due to the existence of the nuclear localization sequence (NLS) [11] and contribute to the transcriptional control of their target genes [8,12]. Additionally, pIL-1α is reported to bind to nuclear chromatin [13,14] and other nuclear proteins [15]. However, studies on the existence of intracellular ppIL-1α and the extracellular functions of ppIL-1α are lacking. Therefore, the aim of this study was to examine the nuclear localization of ppIL-1α and the extracellular release rates of ppIL-1α, pIL-1α, and mIL-1α.

Materials and Methods

Cells

The experiments were performed using HeLa cells (fibroblasts derived from human uterine cancer cells) obtained from the Health Science Research Resources Bank in Osaka, Japan. The cells were maintained in Dulbecco's minimum essential medium (DMEM) supplemented with 10% fetal calf serum (FCS), 50 μg/mL streptomycin, and 50 U/mL penicillin (Sigma-Aldrich, St. Louis, MO, USA) at 37ºC and 5% CO_2.

Plasmid construction

Expression vectors (HiBiT-pIL-1α, HiBiT-mIL-1α, and HiBiT-ppIL-1α) carrying the N-terminal HiBiT-tag (11 amino acids [VSGWRLFKKIS]) were constructed using the Quick-Change site-directed mutagenesis kit (Invitrogen, Carlsbad, CA, USA) and the pcDNA-pIL-1α vector [16] as a template. Each IL-1α was inserted into the pEGF-vector and fused to the N-terminal green fluorescence protein (GFP) tag in frame (GFP-pIL-1α, GFP-mIL-1α, and GFP-ppIL-1α vectors) (Fig.1). H2BmCherry expression vector carrying histone H2B tagged with mCherry was purchased from Addgene (Watertown, MA, USA). The NLS-deletion mutant of ppIL-1α (ΔNLS) was generated using the Quick-Change site-directed mutagenesis kit (Invitrogen) and the GFP-ppIL-1α vector as a template. The primer sequence used was as follows: forward- 5'-GTAGCAACCAACGGGAGTTTAAGCCAATCC-3', reverse- 5'-GGATTGGCTTAAACTCCCGTTGGTTGCTAC-3'. For transfection, each plasmid (125 ng) was dissolved in 25 μL of OPTI-MEM (GE Healthcare, Chicago, IL, USA) and

mixed with Plus reagent (0.75 μL) (Thermo Fisher Scientific, Waltham, MA, USA). Alternatively, lipofectamine LTX (0.75 μL) (Thermo Fisher Scientific) was dissolved in 25 μL of OPTI-MEM (GE Healthcare). Both solutions were mixed and allowed to react at room temperature for 10 min following which, the transfection mixture was added to the culture medium and cultured for 18 h.

Coverslip experiments

HeLa cells (5×10^4/well) were seeded on coverslips (diameter, 10 mm; Matsunami, Tokyo, Japan) plated in a 24-well plate. After 18 h, the cells were transfected with GFP-pIL-1α, GFP-mIL-1α, and GFP-ppIL-1α. Another 18 h later, the coverslips were washed with phosphate buffer saline (PBS) and fixed with 2% paraformaldehyde. GFP-pIL-1α, ppIL-1α, and mCherry were co-transfected into the HeLa cells to examine the nuclear localization of IL-1α; the unfixed transfectants were treated with 0.1% Triton X-100/ PBS solution for 5 min on ice. After the treatment, the cells were washed with PBS (warmed to 37°C) and fixed with 2% paraformaldehyde. After further washing with PBS, the coverslips were mounted with DAPI-Fluoromount-G (Southern Biotech, AL, USA). An all-in-one fluorescence microscope (BZ-X810, KEYENCE, Osaka, Japan) was used to observe and capture the images. For oxidative stress experiment, the transfectants were cultured in the presence or absence of 10 mM hydrogen peroxide solution (H_2O_2; Fujifilm Wako Pure Chemical Industries, Osaka, Japan) for 6 h. The transfectants were also cultured with or without 10 nM importazole (Ann Arbor, MI, USA) for 6 h to investigate energy-dependent ppIL-1α nuclear import.

The HiBiT system and enzyme-linked immunosorbent assay

HeLa cells (1×10^4/well) were seeded on a 48-well plate and transfected with the HiBiT-tagged IL-1α vectors. The cells were cultured for 3 h in the presence or absence of 10 mM H_2O_2 (Fujifilm Wako). The culture supernatant and cell lysate (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.5% Triton X-100) were collected and the expression levels of HiBiT were measured. The secretion efficiency was calculated using the following formula: [culture supernatant/ (culture supernatant + cytolysis) × 100 (%)]. HiBiT expression level was quantified using the Nano-Glo HiBiT Lytic Detection System (Promega, WI, USA). Nano-Glo HiBiT Lytic Substrate and LgBiT protein were diluted with Nano-Glo HiBiT Lytic Buffer (× 50, × 100). Subsequently, the HiBiT/LgBiT buffer and the samples were mixed well and allowed to stand at room temperature for 10 min. The GloMax 20/20 Luminometer (Promega) was used to measure luciferase activity. The cell lysates and the culture supernatants were harvested and subjected to IL-1α enzyme-linked immunosorbent assay (ELISA) using the DuoSet ELISA Development System (R & D Systems, Tokyo, Japan). A microplate reader (model 3550; Bio-Rad, Tokyo, Japan) was used for the measurements.

Statistical analysis

The expression level of HiBiT (Fig. 3) and ELISA (Fig. 4) data were set to N = 6 for both samples. The normality test was performed using the Shapiro-Wilk test. The results showed a non-normal distribution (P = 0.498 (Fig. 3), P = 0.116 (Fig. 4)). In addition, homoscedasticity check was performed by Levene test. (P = 0.490 (Fig. 3), P = 0.129 (Fig. 4)). Then, parametric analysis was conducted using t-test and one-way analysis of variance (ANOVA) followed by Tukey's test. The results are expressed as mean ± standard deviation. P values less than 0.05 were considered statistically significant. All

of the analyses are conducted using R version 4.0.1 (R Core Team (2020). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria, http://www.R-project.org/).

Results

Intracellular localization of IL-1α

The intracellular localization of the three types of IL-1α molecules was examined using the GFP-pIL-1α, GFP-ppIL-1α, and GFP-mIL-1α transfectants. mIL-1α was diffusely distributed in the cytoplasm, whereas pIL-1α and ppIL-1α were mainly localized in the nucleus (Fig. 2A). The culture of transfectants in the presence of H_2O_2 for 3 h resulted in the complete disappearance of fluorescence in the case of GFP-mIL-1α. In the cases of GFP-pIL-1α and GFP-ppIL-1α, most of the fluorescence was diminished, but some nuclear fluorescence was observed even after the oxidative stress (Fig. 2B). NLS-deletion mutant (ΔNLS) was not localized in the nucleus; hence, NLS was confirmed to be an indispensable motif for nuclear localization of ppIL-1α (Fig. 2C).

Extracellular release of IL-1α

The HiBiT-tagged vectors were transfected into the HeLa cells, and the luciferase activity in the culture supernatants and lysates was measured after 18 h of culture to examine the possible spontaneous secretions of pIL-1α, mIL-1α, and ppIL-1α. Consequently, 12.9% ± 4.6%, 25.5% ± 2.6% and, 7.6% ± 4.6% of pIL-1α, mIL-1α, and ppIL-1α were released extracellularly; mIL-1α presented with the highest percentage of extracellular release. Surprisingly, both pIL-1α and ppIL-1α were constitutively secreted into the culture supernatant (Fig. 3). To examine whether IL-1α release was augmented under oxidative stress conditions, the concentrations of pIL-1α and mIL-1α were measured by ELISA. The amount of IL-1α released under non-oxidative stress was 337.9

± 51.3 pg/mL in the culture supernatant and 488.1 ± 98.1 pg/mL in the cell lysate; on the other hand, the corresponding values under oxidative stress were 358.9 ± 51.1 pg/mL and 103.9 ± 30.9 pg/mL, respectively (Fig. 4).

Nuclear localization of ppIL-1α

The results obtained by the HiBiT-tagged vector transfectants indicated that nuclear localization of both pIL-1α and ppIL-1α might be weak. The treatment of the cells in the presence of 0.1% Triton X-100 resulted in the release of weakly associated nuclear proteins from the nucleus. GFP-ppIL-1α or GFP-pIL-1α was co-transfected with H2B-mCherry into the HeLa cells and treated with or without 0.1% triton X-100 solution. The localization of both GFP-ppIL-1α and GFP-pIL-1α in the nucleus disappeared completely within 5 min of the treatment. On the other hand, H2B-mCherry remained in the nucleus (Fig. 5A). Furthermore, in order to investigate whether the nuclear translocation of ppIL-1α was energy-dependent, the transfectants incubated in the culture medium were placed on ice for 10 min. The nuclear localization of GFP-ppIL-1α and H2B-mCherry were maintained even after the treatment (Fig. 5B). To further examine energy-dependent nuclear import of ppIL-1α, the transfectants were pre-incubated with importin β-inhibitor, importazole. As shown in Figure 5C, the treatment did not affect the nuclear localization of GFP-ppIL-1α (Fig. 5C).

Discussion

The use of GFP-tagged IL-1α expression plasmids in this study revealed that mIL-1α was diffusely distributed in the cytoplasm, whereas pIL-1α and ppIL-1α were localized in the nucleus. These results were consistent with that in a previous report, where N- and C-terminal tagging to the IL-1α molecules did not alter the intracellular localization and functions of IL-1α molecules [12]. The nuclear localization was abolished by deleting the NLS, thus demonstrating the indispensable role of NLS for nuclear localization in the current study (Fig. 2C). Although the mechanisms underlying the nuclear translocation of IL-1α have not been elucidated so far, Luheshi et al. postulated a Ran (small G protein)-dependent IL-1α translocation to the nucleus [17]. If that is the case, IL-1α must bind to the importin family protein. Attempts to elucidate the partner molecule that binds to IL-1α are currently being made.

Besides the nucleus, pIL-1α is known to localize in the cell membrane. This notion is originated from the discovery that IL-1α activity is present in the cell membrane fraction [18]; however, the mechanism involved has not been clarified yet. IL-1α can be modified by phosphorylation or myristoylation [19-22], but the relationship between these modifications and cell membrane localization remains unclear.

HiBiT-tagged IL-1α molecules were forcefully expressed in HeLa cells to determine the rate of secretion of IL-1α. Interestingly, ppIL-1α, in addition to mIL-1α and pIL-1α, was secreted spontaneously in the culture medium (Fig. 3). The GFP-tagged IL-1α molecules were diminished in the presence of oxidative stress; therefore, attempts were made to measure the rate of IL-1α secretion after H_2O_2 treatment. However, the attempt

proved unsuccessful, probably due to the presence of H_2O_2 in the culture medium. The addition of H_2O_2 to IL-1α did not seem to affect the measurements. Using the commercially available ELISA kit, which can measure only the pIL-1α and mIL-1α concentrations, a drastic reduction in intracellular IL-1α concentration was observed in response to H_2O_2. In contrast, the concentration of IL-1α in the culture media was not increased. The presence of H_2O_2 in the culture media did not seem to affect the measurement. The reason for the inability to detect an increase in IL- 1α in the culture supernatants in the current study is not clear.

pIL-1α and mIL-1α can transmit signals via the same IL-1 receptor (IL-1R) expressed on the cell surface of target cells [10]. The results obtained in the current study demonstrated the spontaneous, albeit very low, efficiency in ppIL-1α secretion (Fig. 3). IL-1α is produced as pIL-1α inside the cells and cleaved to generate both ppIL-1α and mIL-1α. Theoretically, the molecular ratio of ppIL-1α produced is equal to that of mIL-1α. Moreover, pIL-1α is cleaved by various enzymes, such as granzyme B and thrombin [6]. Thus, ppIL-1α might be generated if the secreted pIL-1α comes in contact with these enzymes extracellularly. Nonetheless, there are no reports on the existence or functions of ppIL-1α in vivo. Based on these backgrounds, the attempt was made to detect the presence of ppIL-1α in the body using recombinant ppIL-1α in *E. coli* by others. Furthermore, a polyclonal antibody was generated and the sandwich ELISA system was established [16]. However, the existence of ppIL-1α in the body fluids could not be demonstrated due to the low sensitivity of the method used. Further investigations are needed to demonstrate the presence of ppIL-1α in the body.

Spontaneous secretion of ppIL-1α was observed in the present study; therefore, the nuclear localization status of ppIL-1α was expected to be weak. The experimental method

developed by Imamoto et al. was used to explore the nuclear localization of ppIL-1α [23, 24]. The cells were consecutively treated with detergent or high salt-containing solutions. The protein of interest is released from its original location based on the mode of localization in the organelle. In the present study, the ppIL-1α transfectants were incubated with Triton X-100 solution. The nuclear localization of ppIL-1α was completely abolished within 5 min of treatment (Fig. 5A), whereas that of co-transfected H2B-mCherry was not affected. These findings indicated that ppIL-1α was localized in the nucleus in a relatively weak manner. To further investigate the energy-dependent nuclear import of ppIL-1α, the transfectant was cultured in the medium on ice for 10 min (Fig. 5B). Generally, the shuttling of proteins between the nucleus and the cytoplasm, which occurs in an energy-independent manner, is diminished in the nucleus after on ice treatment. However, it did not seem to affect the nuclear localization of ppIL-1α. Although the mechanisms underlying the nuclear translocation of IL-1α have not been elucidated so far, Luheshi et al. postulated a Ran (small G protein)-dependent IL-1α translocation to the nucleus [17]. If that is the case, IL-1α must bind to the importin family protein, especially importin β. Based on these assumptions, the transfectants were further incubated with importin β-inhibitor, however, the nuclear localization was not affected (Fig. 5C). Attempts to elucidate the partner molecule that binds to IL-1α are currently being made. ppIL-1α has been shown to contribute to the gene expression of various proteins [12]. Moreover, IL-1α interact with its partner proteins in the nucleus [14,25,26]. The results of present study suggested that ppIL-1α might be floating in the nucleoplasm. Thus, it could be speculated that ppIL-1α forms a complex with some proteins in response to the extracellular stimuli. These results of this study suggest that ppIL-1α might exert its extracellular functions. If this is the case, the receptor for ppIL-1α should be

determined. Identification of ppIL-1α receptor might further understanding about the role of IL-1α in adaptive immunity.

In the present study, ppIL-1α was found to have a weak nuclear localization due to which, it was spontaneously secreted. Additional studies are required to evaluate the existence and function of ppIL-1α in the extracellular space.

Conclusion

Based on these results, it was concluded that ppIL-1α was localized in the nucleus and released extracellularly even in the steady state. ppIL-1α nuclear localization is not firm, and it could be floating in the nucleoplasm.

Acknowledgements

I would like to thank Professors Yoshiyuki Yonehara, Masatake Asano, Tadayoshi Kaneko, and Assistant professor Mariko Tsunoda for their appropriate advice and careful guidance throughout the preparation of this thesis. I also would like to express sincere appreciation to the members of Department of Oral and Maxillofacial Surgery II, Nihon University School of Dentistry for the support to accomplish this study.

References

1. Oppenheim JJ, Yang D (2005) Alarmins: chemotactic activators of immune responses. Curr Opin Immunol 17, 359-365.
2. Rider P, Carmi Y, Guttman O, Braiman A, Cohen I, Voronov E et al. (2011) IL-1α and IL-1β recruit different myeloid cells and promote different stages of sterile inflammation. J Immunol 187, 4835-4843.
3. Kobayashi Y, Yamamoto K, Saido T, Kawasaki H, Oppenheim JJ, Matsushima K (1990) Identification of calcium-activated neutral protease as a processing enzyme of human interleukin 1 alpha. Proc Natl Acad Sci U S A 87, 5548-5552.
4. Carruth LM, Demczuk S, Mizel SB (1991) Involvement of a calpain-like protease in the processing of the murine interleukin 1 alpha precursor. J Biol Chem 266, 12162-12167.
5. England H, Summersgill HR, Edye ME, Rothwell NJ, Brough D (2014) Release of interleukin-1α or interleukin-1β depends on mechanism of cell death. J Biol Chem 289, 15942-15950.
6. Afonina IS, Tynan GA, Logue SE, Cullen SP, Bots M, Lüthi AU et al. (2011) Granzyme B-dependent proteolysis acts as a switch to enhance the proinflammatory activity of IL-1α. Mol Cell 44, 265-278.
7. Burzynski LC, Humphry M, Pyrillou K, Wiggins KA, Chan JNE, Figg N et al. (2019) The coagulation and immune systems are directly linked through the activation of interleukin-1α by thrombin. Immunity 50, 1033-1042.e6.
8. Stevenson FT, Turck J, Locksley RM, Lovett DH (1997) The N-terminal propiece of

interleukin 1 alpha is a transforming nuclear oncoprotein. Proc Natl Acad Sci U S A 94, 508-513.

9. Afonina IS, Müller C, Martin SJ, Beyaert R (2015) Proteolytic processing of interleukin-1 family cytokines: variations on a common theme. Immunity 42, 991-1004.
10. Kim B, Lee Y, Kim E, Kwak A, Ryoo S, Bae SH et al. (2013) The interleukin-1α precursor is biologically active and is likely a key alarmin in the IL-1 family of cytokines. Front Immunol 4, 391.
11. Di Paolo NC, Shayakhmetov DM (2016) Interleukin 1α and the inflammatory process. Nat Immunol 17, 906-913.
12. Werman A, Werman-Venkert R, White R, Lee JK, Werman B, Krelin Y et al. (2004) The precursor form of IL-1 alpha is an intracrine proinflammatory activator of transcription. Proc Natl Acad Sci U S A 101, 2434-2439.
13. Hu B, Wang S, Zhang Y, Feghali CA, Dingman JR, Wright TM (2003) A nuclear target for interleukin-1α: interaction with the growth suppressor necdin modulates proliferation and collagen expression. Proc Natl Acad Sci U S A 100, 10008-10013.
14. Lamacchia C, Rodriguez E, Palmer G, Gabay C (2013) Endogenous IL-1α is a chromatin-associated protein in mouse macrophages. Cytokine 63, 135-144.
15. Luheshi NM, McColl BW, Brough D (2009) Nuclear retention of IL-1 alpha by necrotic cells: a mechanism to dampen sterile inflammation. Eur J Immunol 39, 2973-2980.
16. Sata E, Takada L, Kaetsu R, Fukasawa M, Ohtsu M, Motoyoshi M et al. (2020) A new enzyme-linked immunosorbent assay system against the N-terminal propiece of interleukin-1α. J Oral Sci 62, 340-343.

17. Luheshi NM, Rothwell NJ, Brough D (2009) The dynamics and mechanisms of interleukin-1 alpha and beta nuclear import. Traffic 10, 16-25.
18. Kurt-Jones EA, Beller DI, Mizel SB, Unanue ER (1985) Identification of a membrane-associated interleukin 1 in macrophages. Proc Natl Acad Sci U S A 82, 1204-1208.
19. Beuscher HU, Nickells MW, Colten HR (1988) The precursor of interleukin-1 alpha is phosphorylated at residue serine 90. J Bio Chem 263, 4023-4028.
20. Kobayashi Y, Appella E, Yamada M, Copeland TD, Oppenheim JJ, Matsushima K (1988) Phosphorylation of intracellular precursors of human IL-1. J Immunol 140, 2279-2287.
21. Stevenson FT, Bursten SL, Fanton C, Locksley RM, Lovett DH (1993) The 31-kDa precursor of interleukin 1 alpha is myristoylated on specific lysines within the 16-kDa N-terminal propiece. Proc Natl Acad Sci U S A 90, 7245-7249.
22. Cohen I, Rider P, Vornov E, Tomas M, Tudor C, Wegner M et al. (2015) IL-1α is a DNA damage sensor linking genotoxic stress signaling to sterile inflammation and innate immunity. Sci Rep 5, 14756.
23. Imamoto N, Tachibana T, Matsubae M, Yoneda Y (1995) A karyophilic protein forms a stable complex with cytoplasmic components prior to nuclear pore binding. J Biol Chem 270, 8559-8565.
24. Kose S, Funakoshi T, Imamoto N (2015) Reconstitution of nucleocytoplasmic transport using digitonin-permeabilized cells. Methods Mol Biol 1262, 291-303.
25. Suzuki Y, Demoliere C, Kitamura D, Takeshita H, Deuschle U, Watanabe T (1997) HAX-1, a novel intracellular protein, localized on mitochondria, directly associates with HS1, a substrate of Src family tyrosine kinases. J Immunol 158, 2736-2744.

26. Pollock AS, Turck J, Lovett DH (2003) The prodomain of interleukin 1 alpha interacts with elements of the RNA processing apparatus and induces apoptosis in malignant cells. FASEB J 17, 203-213.

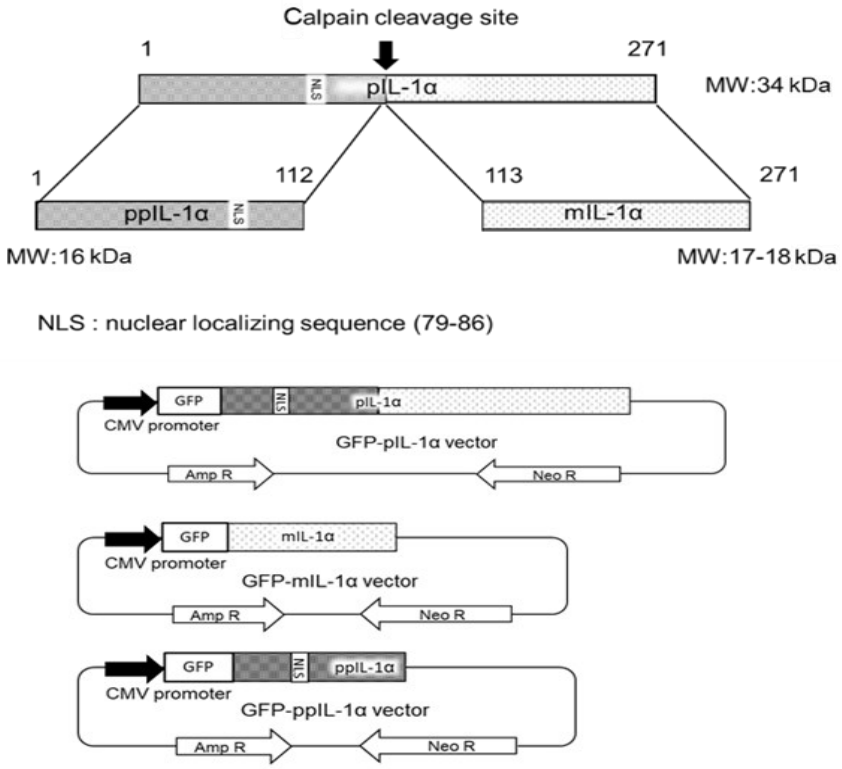

Fig. 1 Structure of IL-1α

IL-1α is produced intracellularly as precursor IL-1α (pIL-1α) (1-271). pIL-1α is cleaved to generate ppIL-1α (N-terminal 1-112) and mIL-1α (C-terminal 113-271). The nuclear localization sequence (NLS) is located at 79-86. The position of the amino acids were indicated by the numbers.

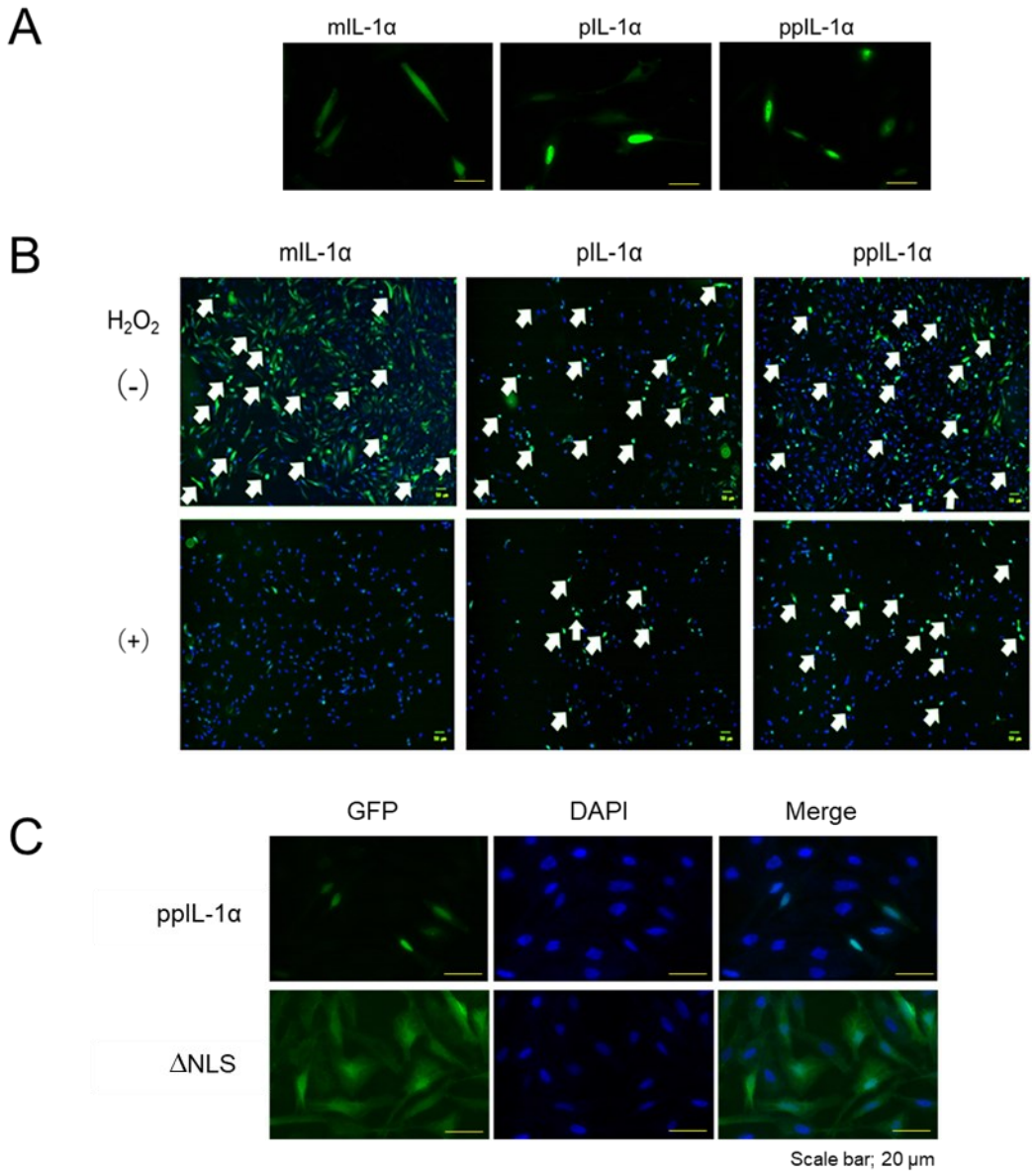

Fig. 2 Intracellular localization of IL-1α

HeLa cells were transfected with GFP-pIL-1α, GFP-mIL-1α, and GFP-ppIL-1α. (A) After transfection, the cells were observed with a fluorescence microscope (Scale bar; 20 μm). (B) The transfectants were cultured in the presence (+; lower panel) or absence (-; upper panel) of 10 mM H_2O_2 for 3 h and images were obtained. Arrows indicate the GFP fluorescence positive cells. (C) NLS-deficient mutant (ΔNLS) plasmid was transfected and the intracellular localization was observed. DAPI was used for the nuclear staining (Scale bar; 20 μm).

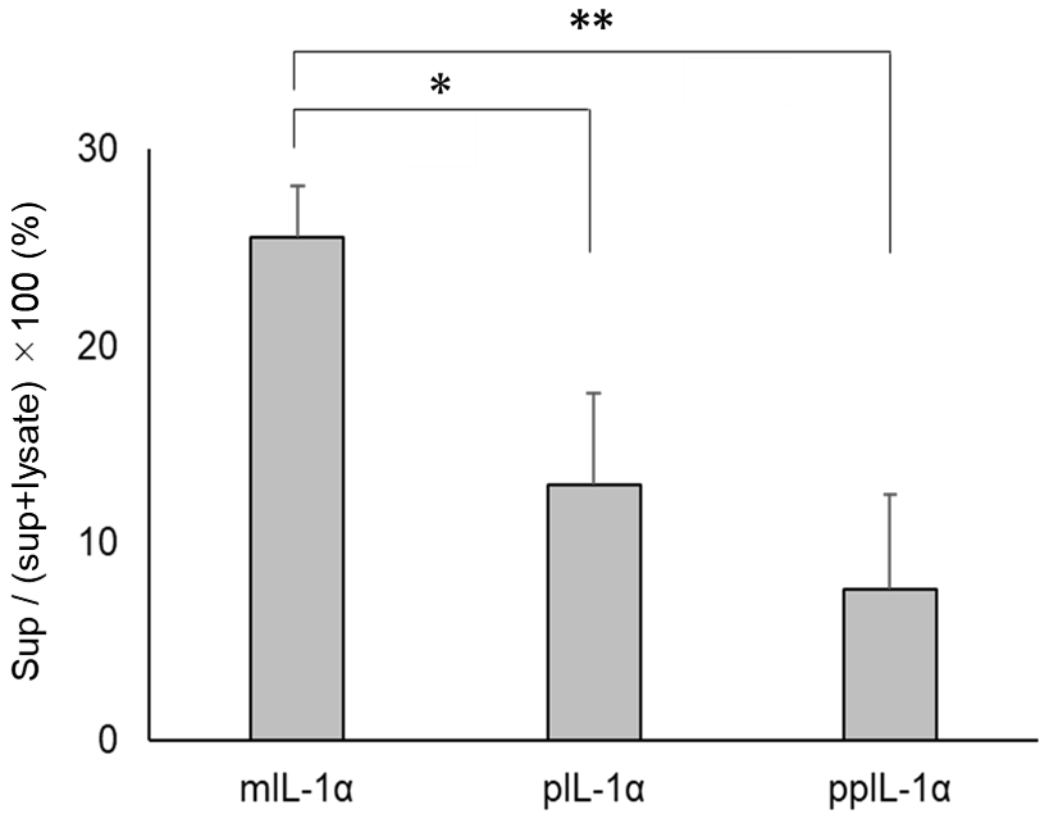

Fig. 3 Bar graph showing the release rate of IL-1α

HeLa cells were transfected with HiBiT-pIL-1α, HiBiT-mIL-1α, and HiBiT-ppIL-1α. After transfection, the cells were further cultured for 18 h and the culture supernatants and cell lysates were collected, and the amount of HiBiT released was measured. The vertical axis of the graph represents (%), and the horizontal axis represents the sample name. The secretion efficiency was calculated by the following formula. [Culture supernatant / (culture supernatant + cytolysis)] × 100 (%) (n = 6). *P = 0.012, **P = 0.001.

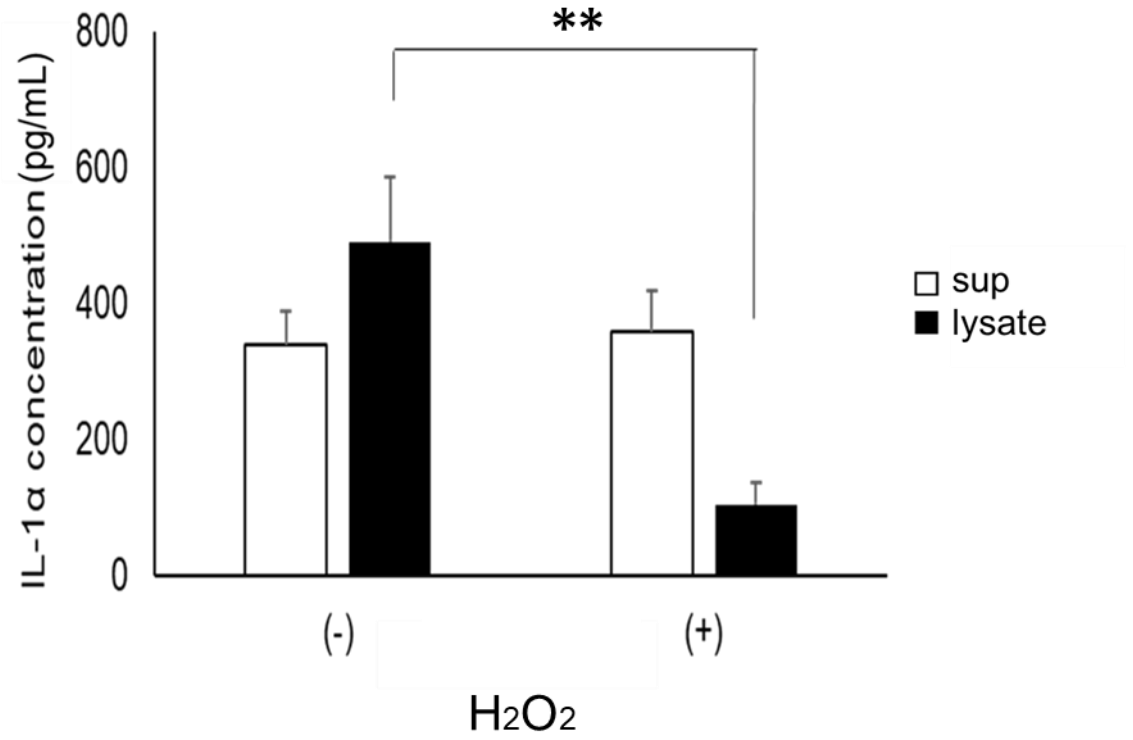

Fig. 4 Bar graph showing the effect of oxidative stress

HeLa cells were transfected with HiBiT-pIL-1α, cultured with or without H_2O_2 for 3 h. The culture supernatants and cell lysates were collected. IL-1α concentration was measured by the ELISA ($n = 6$). The amount of IL-1α concentration in the cell lysate was drastically reduced. On the other hand, IL-1α concentration in the culture supernatant was not increased with H_2O_2 treatment. $^{**}P = 0.002$.

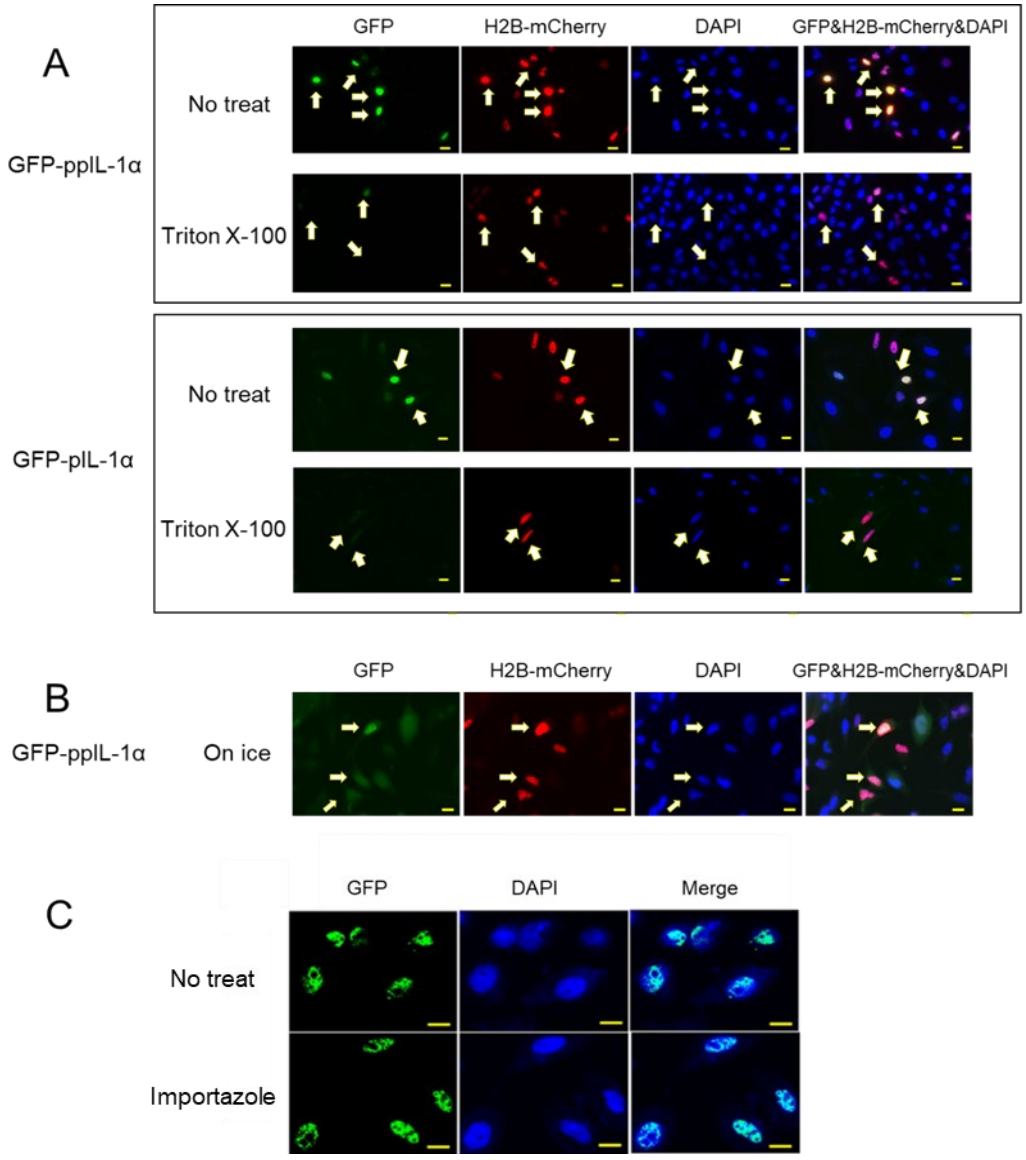

Fig. 5 Nuclear localization of ppIL-1α

HeLa cells were co-transfected with GFP-ppIL-1α or GFP-pIL-1α along with H2B-mCherry. The transfectants were treated with 0.1% Triton X-100/PBS solution on ice for 5 min and washed with PBS. (A) Arrows indicate the GFP-ppIL-1α or GFP-pIL-1α positive cells. (B) The GFP-ppIL-1α transfectant was incubated with 10% FCS-DMEM on ice for 10 min (Scale bar; 20 μm). Arrows indicate the cells positive for both GFP-ppIL-1α and H2B-mCherry overlapped with DAPI. (C) GFP-ppIL-1α transfectants were treated with (lower) or without (upper) importin β-inhibitor, importazole (10 nM) for 6 h. (Scale bar; 10 μm). The representative images of at least three independent experiments were shown for (A), (B) and (C).