Sirtuin 1 suppresses nuclear factor κB induced transactivation and pro-inflammatory cytokine expression in cat fibroblast cells

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ABSTRACT. Nuclear factor κB (NF-κB) is a key factor in the development of chronic inflammation and is deeply involved in age-related and metabolic diseases development. These diseases have become a serious problem in cats. Sirtuin 1 (SIRT1) is associated with aging and metabolism through maintaining inflammation via NF-κB. In addition, fibroblasts are considered an important factor in the development of chronic inflammation. Therefore, we aimed to examine the effect of cat SIRT1 (cSIRT1) on NF-κB in cat fibroblast cells. The up-regulation of NF-κB transcriptional activity and pro-inflammatory cytokine mRNA expression by p65 subunit of NF-κB and lipopolysaccharide was suppressed by cSIRT1 in cat fibroblast cells. Our findings show that cSIRT1 is involved in the suppression of inflammation in cat fibroblast cells.

KEYWORDS: feline, fibroblast, inflammation, NF-κB, sirtuin

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Aging and obesity are associated with immune function failure, which leads to chronic inflammation and causes age-related and metabolic diseases in humans [7, 12]. These diseases have recently also become a serious problem in cats due to an increase in their indoor lifestyle and lifespan [10]. Nuclear factor κB (NF-κB) is a key factor in the development of chronic inflammation and is deeply involved in age-related and metabolic disease development by driving pro-inflammatory cytokines [1, 5]. The p65 subunit of NF-κB is extremely important in the NF-κB family, because it is the only ubiquitously expressed NF-κB protein containing a transcriptional activation domain [4]. Because the cat p65 subunit of NF-κB (cp65) is expressed in a wide range of tissue and up-regulates NF-κB transcriptional activity and pro-inflammatory cytokine expression [15], regulation of cp65 may be a potential target for protection from age-related and metabolic diseases.

The mammalian homologs of the yeast silent information regulator 2 (Sir2), sirtuin 1 (SIRT1), mediate a wide array of cellular responses that maintain metabolic and immune functions through nicotinamide adenine dinucleotide (NAD+)-dependent reactions [21]. Because of its NAD+-dependent activities, SIRT1 is deeply associated with aging and metabolism [13]. SIRT1 has many substrates, and among these substrates, NF-κB plays a central role in regulating immune function [18]. It was initially shown that SIRT1 regulates NF-κB transcriptional activity via direct deacetylation of the p65 subunit of NF-κB [28]. Subsequently, the regulatory role of SIRT1 in the NF-κB pathway involves various mechanisms, such as pathway through the deacetylation of coactivator and activities, that do not depend on the deacetylation in mice and humans [2, 11]. We have previously reported that cat SIRT1 (cSIRT1) may be involved in inflammation in vivo [14], but there is little information on inflammation regulatory activity of cSIRT1 via the NF-κB pathway in vitro.

Fibroblasts have the ability to produce structural connective tissue proteins, such as collagen, and to modify the extracellular matrix and play a role in maintaining homeostasis in damaged tissue [17]. In addition, fibroblasts produce pro-inflammatory cytokines, such as IL-1β, IL-6 and TNF-α, and modify the level of inflammation [23]. For these reasons, fibroblasts are considered an important factor in the formation of chronic inflammation and have attracted attention as therapeutic targets [6]. However, the underlying mechanism of chronic inflammation in cat fibroblasts remains elusive. In the present study, we aimed to examine the effect of cSIRT1 on NF-κB transactivation and pro-inflammatory cytokine expression in cat fibroblast cells.

Cat fibroblast cells were prepared from uterine tissues obtained from 5- to 8-month-old healthy client-owned cats (n=3) that were spayed and cultured as previously described [15]. Written informed consent for the experiment was obtained from the owners. Full coding regions were obtained by polymerase chain reaction (PCR) with specific primers of cp65 (primers 1, 2) and cSIRT1 (primers 3, 4) (Table 1). PCR products were cloned into mammalian-based expression vectors pcDNA3.1 V5-His B (Invitrogen, Carlsbad, CA, U.S.A.). Cloned cp65 and cSIRT1 expression vectors (pcDNA3.1-cp65 and pcDNA3.1-cSIRT1) were sequenced, and they confirmed the mRNA expression in cultured cells.
in 96-well plates at a density of 2 × 10^4 cells/well. Each vector was transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. All cells were transfected with 40 ng/well of pGL 4.32 [Luc2P/NF-κB-RE/Hygro] reporter vector, with the luciferase coding sequence under the control of a minimal promoter containing five NF-κB-binding sites (Promega Corporation, Madison, WI, U.S.A.). The pGL 4.73 [hRluc/SV40] vector (Promega) was included in all transfections at 10 ng/well to allow normalization for transfection efficiency. In the co-expression analysis, 40 ng/well of expression vectors (mock plasmid pcDNA3.1, pcDNA3.1-cp65 and pcDNA3.1-cSIRT1) were transfected in each cell. Furthermore, fibroblast cells transfected with mock or SIRT1 were incubated with lipopolysaccharide (LPS, Escherichia coli 011:B4, Sigma, St. Louis, MO, U.S.A.) 5 µg/ml for 48 hr. LPS is a large molecular constituting the outer wall of gram-negative bacteria. LPS acts as the endotoxin and induces NF-κB activation and pro-inflammatory cytokine production [3]. The uterine fibroblast cells express Toll-like receptor 4 and LPS induced inflammatory reaction in human and cats [8, 16]. In this study, the luciferase activity of the pGL 4.32 [Luc2P/NF-κB-RE/Hygro] reporter vector was up-regulated by transient transfection of pc65, and up-regulation of NF-κB transcriptional activity was considerably suppressed by co-transfection with cSIRT1 in cat fibroblast cells (Fig. 1a). In addition, LPS treatment of fibroblast up-regulated NF-κB transcriptional activity and up-regulation of NF-κB transcriptional activity was considerably suppressed by transient transfection of SIRT1 (Fig 1b). Increased transcriptional activity of NF-κB triggers various intracellular events and leads to multiple cell responses, such as inflammation and apoptosis [20]. In human cells, SIRT1 overexpression suppresses NF-κB transcriptional activity and apoptosis [27]. Furthermore, in several mouse experimental case models, SIRT1 overexpression protected cells and suppressed symptom severity by suppressing NF-κB activity [19, 22]. Therefore, cSIRT1 can suppress NF-κB transcriptional activity, which may indicate that cSIRT1 protects cells and biological objects from a variety of cellular responses caused by NF-κB in cats. Further investigation is required to understand the underlying molecular mechanisms and cellular responses involved in the NF-κB suppression ability of cSIRT1.

To investigate the ability of SIRT1 anti-inflammatory reaction, we determined the mRNA expression levels of pro-inflammatory cytokines (IL-1β, IL-6 and TNF-α) in fibroblast cells by quantitative real-time PCR. Cat fibroblast cells were plated in 12-well plates at densities of 1 × 10^5 cells/well and transfected with 400 ng/well of expression vectors (mock plasmid pcDNA3.1, pcDNA3.1-cp65 and pcDNA3.1-cSIRT1) using lipofectamine 2000. Furthermore, fibroblast cells transfected with mock or SIRT1 were incubated with LPS 5 µg/ml for 48 hr. Total RNA was extracted using TriPure Isolation Reagent (Boehringer, Mannheim, Germany), and reverse transcription was performed using the QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany). Real-time PCR was performed with Real Time PCR System 7300 (Applied Biosystems, Foster City, CA, U.S.A.) using SYBR Premix Ex Taq II (Takara, Otsu, Japan). The beta-actin (primers 5, 6), IL-1β (primers 7, 8), IL-6 (primers 9, 10) and TNF-α (primers 11, 12) amplification primers are listed in Table 1. In the present study, all pro-inflammatory cytokine expression levels were up-regulated by transiently transfection of cp65, and up-regulation of expression levels was considerably suppressed by co-transfection with cSIRT1 in cat fibroblast cells (Fig. 2a). Furthermore, LPS treatment of fibroblast up-regulated pro-inflammatory cytokine expression levels, and up-regulation of expression levels was considerably suppressed by transiently transfection.

### Table 1. Sequences of primers used for PCR

| Primer | Sequence (5′–3′)          | Position | Accession Number |
|--------|---------------------------|----------|------------------|
| cp65   | CTGGCTAGTTAAGCTGACGACTGCTGTCCTGTTTCC | 115–132 | AB930130.1 |
|        | CTGGACATGAGGATTACCTGATGCTGAGTCTGACTC | 1784–1765 | AB930130.1 |
| cSIRT1 | CTGGACATGAAATGCTAGGACAGAGGACGGGAGGGA | 21–38 | NM_001290246.1 |
|        | CTGGACATGAGGATTACCTGATGCTGACTC | 2321–2299 | NM_001290246.1 |
| Beta-actin | GCCAAAGCTGAGGATGACCTGTC | 152–172 | AB051104.1 |
|        | CCCAGAGCTCATGACATACCAG | 280–257 | AB051104.1 |
| IL-1β  | TGGCACCCAGTACCTGACCTC | 46–65 | NM_001077414.1 |
|        | GCACTGGATGCGGTCATCTC | 195–175 | NM_001077414.1 |
| IL-6   | GGGGTAGCTTTCCTCTCTCACCC | 69–88 | NM_001009211.1 |
|        | GGTGGGTCTTGTGTAGCTGCC | 259–240 | NM_001009211.1 |
| TNF-α  | CCCACACTTCCTGTGGCCTGCT | 134–152 | NM_001009835.1 |
|        | GAGTTGCCCTTCAGCTTCCG | 305–287 | NM_001009835.1 |
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...tion of SIRT1 (Fig. 2b). An increase in pro-inflammatory cytokines is found in chronic inflammatory conditions [9, 25], and fibroblast cells play a central role in producing pro-inflammatory cytokines in chronic inflammatory milieu [6]. Furthermore, pro-inflammatory cytokines produced in local cells by LPS stimulation were dispersed in the blood, and it causes also increase of pro-inflammatory cytokines levels and forming chronic inflammation in other tissues in mouse [26]. A recent study indicated that SIRT1 suppresses the production of pro-inflammatory cytokines and apoptosis caused by TNF-α or LPS treatment in human fibroblast cells [24, 27]. Taken together, these results suggest that cSIRT1 suppresses the expression of pro-inflammatory cytokines in fibroblasts, indicating that cSIRT1 may be a useful target for chronic inflammation treatment. Further studies on

the relationship between chronic inflammation and cSIRT1 are needed for understanding pathogenesis and developing treatment modalities for age-related and metabolic diseases in cats.

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REFERENCES

1. Baker, R. G., Hayden, M. S. and Ghosh, S. 2011. NF-κB, inflammation, and metabolic disease. Cell Metab. 13: 11–22. [Medline] [CrossRef]

2. Bouras, T., Fu, M., Sauve, A. A., Wang, F., Quong, A. A., Perkins, N. D., Hay, R. T., Gu, W. and Pestell, R. G. 2005. SIRT1 deacetylation and repression of p300 involves lysine residues...
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1020/1024 within the cell cycle regulatory domain 1. J. Biol. Chem. 280: 10264–10276. [Medline] [CrossRef]

3. Burke, J. P., Cunningham, M. F., Watson, R. W., Docherty, N. G., Coffey, J. C. and O’Connell, P. R. 2010. Bacterial lipopolysaccharide promotes probiotic activation of intestinal fibroblasts. Br. J. Surg. 97: 1126–1134. [Medline] [CrossRef]

4. Chen, F. E., Kempka, S., Huang, D. B., Phelps, C. and Ghosh, G. 1999. Construction, expression, purification and functional analysis of recombinant NFkappaB p50/p65 heterodimer. Protein Eng. 12: 423–428. [Medline] [CrossRef]

5. Chung, H. Y., Cesari, M., Anton, S., Marzetti, E., Giovannini, S., Seo, A. Y., Carter, C., Yu, B. P. and Leeuwenburgh, C. 2009. Molecular inflammation: underpinnings of aging and age-related diseases. Ageing Res. Rev. 8: 18–30. [Medline] [CrossRef]

6. Flavell, S. J., Hou, T. Z., Lax, S., Filer, A. D., Salmon, M. and Buckley, C. D. 2008. Fibroblasts as novel therapeutic targets in chronic inflammation. Br. J. Pharmacol. 153 Suppl 1: S241–S246. [Medline] [CrossRef]

7. Franceschi, C. and Campisi, J. 2014. Chronic inflammation (inflamming) and its potential contribution to age-associated diseases. J. Gerontol. A Biol. Sci. Med. Sci. 69 Suppl 1: S4–S9. [Medline] [CrossRef]

8. Fukuyama, A., Tanaka, K., Kakizaki, I., Kasai, K., Chiba, M., Nakamura, T. and Mizumura, H. 2012. Anti-inflammatory effect of proteoglycan and progesterone on human uterine cervical fibroblasts. Life Sci. 90: 484–488. [Medline] [CrossRef]

9. Gabay, C. 2006. Interleukin-6 and chronic inflammation. Arthritis Res. Ther. 8 Suppl 2: S3. [Medline] [CrossRef]

10. German, A. J. 2006. The growing problem of obesity in dogs and cats. J. Nutr. 136 Suppl: 1940S–1946S. [Medline] [CrossRef]

11. Ghosh, H. S., Spencer, J. V., Ng, B., McBurney, M. W. and Robbins, P. D. 2007. Sirt1 interacts with transducin-like enhancer of split-1 to inhibit nuclear factor kappaB-mediated transcription. Biochem. J. 408: 105–111. [Medline] [CrossRef]

12. Hotamisligil, G. S. 2006. Inflammation and metabolic disorders. Nature 440: 860–867. [Medline] [CrossRef]

13. Imai, S. and Yoshino, J. 2013. The importance of NAMPT/NAD/ SIRT1 in the systemic regulation of metabolism and ageing. Diabetes Obes. Metab. 15 Suppl 3: 26–33. [Medline] [CrossRef]

14. Ishikawa, S., Li, G., Takemitsu, H., Fujimura, M., Mori, N., Yamamoto, I. and Arai, T. 2013. Change in mRNA expression of sirtuin 1 and sirtuin 3 in cats fed on high fat diet. BMC Vet. Res. 9: 187. [Medline] [CrossRef]

15. Ishikawa, S., Takemitsu, H., Li, G., Mori, N., Yamamoto, I. and Arai, T. 2015. Short communication: molecular characterization of dog and cat p65 subunits of NF-kappaB. Res. Vet. Sci. 99: 145–148. [Medline] [CrossRef]

16. Jursza, E., Szostek, A. Z., Kowalewski, M. P., Boos, A., Okuda, K. and Siemieniuch, M. J. 2014. LPS-challenged TNFα production, prostaglandin secretion, and TNFα/TNFα receptors expression in the endometrium of domestic cats in estrus or diestrus, and in cats with pyometra or receiving medroxyprogesterone acetate. Mediators Inflamm. 2014: 689280. [CrossRef] [Medline]

17. Kalluri, R. and Zeisberg, M. 2006. Fibroblasts in cancer. Nat. Rev. Cancer 6: 392–401. [Medline] [CrossRef]

18. Kong, S., McBurney, M. W. and Fang, D. 2012. Sirtuin 1 in immune regulation and autoimmunity. Immunol. Cell Biol. 90: 6–13. [Medline] [CrossRef]

19. Lee, J. H., Song, M. Y., Song, E. K., Kim, E. K., Moon, W. S., Han, M. K., Park, J. W., Kwon, K. B. and Park, B. H. 2009. Overexpression of SIRT1 protects pancreatic beta-cells against cytokine toxicity by suppressing the nuclear factor-kappaB signaling pathway. Diabetes 58: 344–351. [Medline] [CrossRef]

20. Li, Q. and Verma, I. M. 2002. NF-kappaB regulation in the immune system. Nat. Rev. Immunol. 2: 725–734. [Medline] [CrossRef]

21. Michishita, E., Park, J. Y., Burneskis, J. M., Barrett, J. C. and Horikawa, I. 2005. Evolutionarily conserved and nonconserved cellular localizations and functions of human SIRT proteins. Mol. Biol. Cell 16: 4623–4635. [Medline] [CrossRef]

22. Nimmagadda, V. K., Bever, C. T., Vartiukuta, N. R., Talat, S., Ahmad, V., Nagalla, N. K., Trisler, D., Judge, S. I., Royal, W. 3rd., Chandrasekaran, K., Russell, J. W. and Makar, T. K. 2013. Overexpression of SIRT1 protein in neurons protects against experimental autoimmune encephalomyelitis through activation of multiple SIRT1 targets. J. Immunol. 190: 4595–4607. [Medline] [CrossRef]

23. Parsonage, G., Filer, A. D., Haworth, O., Nash, G. B., Rainger, G. E., Salmon, M. and Buckley, C. D. 2005. A stromal address code defined by fibroblasts. Trends Immunol. 26: 150–156. [Medline] [CrossRef]

24. Park, G. J., Kim, Y. S., Kang, K. L., Bae, S. J., Baek, H. S., Auh, Q. S., Chun, Y. H., Park, B. H. and Kim, E. C. 2013. Effects of sirtuin 1 activation on nicotine and lipopolysaccharide-induced cytotoxicity and inflammatory cytokine production in human gingival fibroblasts. J. Periodontal Res. 48: 483–492. [Medline] [CrossRef]

25. Popa, C., Netea, M. G., van Riel, P. L., van der Meer, J. W. and Stalenhoef, A. F. 2007. The role of TNF-alpha in chronic inflammatory conditions, intermediary metabolism, and cardiovascular risk. J. Lipid Res. 48: 751–762. [Medline] [CrossRef]

26. Qin, L., Wu, X., Block, M. L., Liu, Y., Breese, G. R., Hong, J. S., Knapp, D. J. and Crews, F. T. 2007. Systemic LPS causes chronic neuroinflammation and progressive neurodegeneration. Glia 55: 453–462. [Medline] [CrossRef]

27. Wang, W., Yan, C., Zhang, J., Lin, R., Lin, Q., Yang, L., Ren, F., Zhang, J., Ji, M. and Li, Y. 2013. SIRT1 inhibits TNF-α-induced apoptosis of vascular adventitial fibroblasts partly through the deacetylation of FoxO1. Apoptosis 18: 689–701. [Medline] [CrossRef]

28. Yeung, F., Hoberg, J. E., Ramsey, C. S., Keller, M. D., Jones, D. R., Frye, R. A. and Mayo, M. W. 2004. Modulation of NF-kappaB-dependent transcription and cell survival by the SIRT1 deacetylase. EMBO J. 23: 2369–2380. [Medline] [CrossRef]