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Cannabinoids inhibit peptidoglycan-induced phosphorylation of NF-κB and cell growth in U87-MG human malignant glioma cells

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Abstract

Nuclear factor (NF)-κB is the key transcription factor involved in the inflammatory responses, and its activation aggravates tumors. Peptidoglycan (PGN), a main cell wall component of gram-positive bacteria, stimulates Toll-like receptor 2 and activates a number of inflammatory pathways including activation of NF-κB. Cannabinoids have been reported to exert anti-inflammatory and anti-tumoral actions. The mechanisms underlying these actions, however, are largely unknown. The purpose of this study was to investigate whether cannabinoids could suppress PGN-induced activation of NF-κB and cell growth via cannabinoid receptors in U87-MG human malignant glioma cells. PGN treatment induced the phosphorylation of NF-κB and cell proliferation in a concentration-dependent manner. The major endocannabinoid 2-arachidodonoylglycerol prevented the PGN-induced phosphorylation of NF-κB, which was reversed by the CB1 cannabinoid receptor antagonist AM281. The synthetic cannabinoid WIN55,212-2 abolished the PGN-activated cell growth, and this effect was reversed by AM281. Preferential expression of CB1 receptors rather than CB2 in these cells was confirmed by reverse transcription-mediated polymerase chain reaction experiments and the observation that WIN55,212-2-induced morphological changes were completely reversed by AM281 but not by the CB2 antagonist AM630. Our finding that cannabinoids suppress the NF-κB inflammatory pathway and cell growth via CB1 receptors in glioma cells provides evidence for therapeutic potential of targeting cannabinoid receptors in the treatment of inflammation-dependent tumor progression.

Introduction

Bacteria stimulate the innate immune system of a host and lead to the release of inflammatory molecules such as cytokines and chemokines. Lipopolysaccharide (LPS) and peptidoglycan (PGN) are the main cell wall components of gram-negative and gram-positive bacteria, respectively. LPS and PGN stimulate different Toll-like receptors (TLRs), TLR-4 and TLR-2 respectively, and activate a number of inflammatory pathways, including activation of the transcription factor nuclear factor (NF)-κB (1, 2). NF-κB is the key transcription factor involved in the inflammatory responses, and also plays a critical role in tumor cell survival, growth and migration. Many of the signal pathways implicated in tumor converge to the NF-κB signal. Its activation aggravates tumors, while its suppression inhibits the tumor cell growth (3-5). Therefore, NF-κB signaling pathway has emerged as one of the attractive targets for treatment of many types of tumors including glioblastoma (6).

The endocannabinoid system, consisting of two types of G-protein-coupled cannabinoid receptors (CB1 and CB2) and their endogenous ligands (endocannabinoids) (7), is implicated in a variety of physiological and pathological conditions including inflammation and tumor. The CB1 receptor is abundantly expressed in the CNS, while the CB2 receptor is predominantly expressed by immune cells. Endogenous and exogenous cannabinoids exert anti-inflammatory and anti-tumoral actions (8-10). However, the precise mechanisms underlying these actions are not fully understood.

In the present study, we examined effects of cannabinoids on PGN-induced NF-κB activation and cell growth using U87-MG human malignant glioma cells. We show here that cannabinoids suppress PGN-induced phosphorylation of NF-κB and cell growth in a CB1-dependent manner. Our findings provide a mechanistic basis for opening up new therapeutic approaches of treating or preventing brain tumors aggravated by the inflammation.

Materials and Methods

Chemicals

PGN, 2-AG, WIN55,212-2, and Dulbecco's modified Eagle's medium (DMEM) were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). AM281 (CB1 specific antagonist) and AM630 (CB2 specific antagonist) were obtained from Calbiochem (La Jolla, CA). Fetal bovine serum (FBS) was obtained from Invitrogen

Corporation (Carlsbad, CA). Anti-phospho-specific NF-κB p65 (Ser536), anti-β-actin, and horseradish peroxidase (HRP)-linked anti-rabbit IgG were purchased from Cell Signaling Technology, Inc. (Danvers, MA).

Cell culture

U87-MG human malignant glioma cells were provided by Dr Nakata. The cells were maintained in DMEM containing 10% FBS at 37°C in a 5% CO2 incubator.

NF-κB activity assay

To determine the effect of 2-AG on NF-κB activity, we investigated phospho-NF-κB level using western blotting methods. Increase in phospho-NF-κB level indicates the amount of NF-κB signaling activation. U87-MG human malignant glioma cells were incubated in DMEM containing serum for 24 h and pretreated with 2-AG (1 μM) for 10 min, and then treated with PGN (1-30 μg/ml) for 16 h. Western blotting analyses were performed using phospho-NF-κB p65 (Ser536) antibody and anti-β-actin antibody.

Western blotting analysis

Western blotting was performed as described previously [11].

RT-PCR analysis

To evaluate the expression patterns of CB1 and CB2 mRNA in the cells, reverse transcription-mediated polymerase chain reaction (RT-PCR) was performed as follows. Briefly, RNA was extracted from the cells and reverse transcribed by using the reverse transcriptase ReverTra Ace (TOYOBO, Tokyo, Japan). PCR-based subtype specific gene amplification for CB1, CB2 and β-actin was performed with LA Taq (TAKARA, Tokyo, Japan) using the sets of primers as follows: 5'-caggccttcctaccacttcat-3' and 5'-accccacccagtttgaacaga-3' for CB1, 5'-aagccctcgtacctgttcat-3' and 5'-acagaggctgtgaaggtcat-3' for CB2, and 5'-atggtgggtatgggtcagaag-3' and 5'-ctggggtgttgaaggtctcaa-3' for β-actin.

Microscopic analysis

U87 cells were seeded on 35 mm dishes at a density of 1×10^3 cells/dish. After 24 hr incubation, the cells in DMEM with serum were treated with 1 μM WIN55,212-2, a combination of WIN55,212-2 (1 μM) and AM281 (1 μM), or a combination of

WIN55,212-2 (1 μM) and AM630 (1 μM) for 72 hr. Morphological changes of cells were assessed by light microscopy.

Cell proliferation assay

Cell proliferation was analyzed using the Cell Counting Kit 8 (Wako, Japan). U87-MG human malignant glioma cells were seeded in 96-well plates at a density of 1×10^3 cells/well. After a 24-h incubation, the cells were treated with PGN, WIN55,212-2, or AM281 for 72 h. Next, the cells were incubated with 10 μL WST-8 for 2 h. Absorbance of the colored formazan product produced by mitochondrial dehydrogenases in metabolically active cells was recorded at 450 nm as the background value. Cell proliferation was expressed as a percentage of absorbance obtained in treated wells relative to that in untreated (control) wells.

Statistical analysis

Data are presented as means ± SEM from at least three independent experiments. Statistical analyses were performed using ANOVA followed by Dunnett's test, and results were considered statistically significant when $p < 0.05$.

Results

PGN enhances phosphorylation of NF-κB and cell growth

First, we examined effects of peptidoglycan (PGN), a major cell wall component of gram-positive bacteria, on NF-κB activation and cell growth. As shown in Fig. 1A, PGN (1-30 μg/mL) increased the level of phosphorylated NF-κB (p65) in a concentration-dependent manner, indicating that PGN activates the NF-κB signaling pathways in U87 human glioblastoma cells. PGN also accelerated cell growth of these cells in a concentration-dependent manner (Fig. 1B).

Endocannabinoid 2-AG inhibits PGN-induced phosphorylation of NF-κB

We next examined effects of cannabinoids. Anandamide and 2-arachidodonoylglycerol (2-AG) are the two major endocannabinoids, and activate both CB1 and CB2 cannabinoid receptors. Anandamide behaves as a partial agonist at these cannabinoid receptors and also activates the vanilloid receptor (TRPV1). 2-AG acts as a full agonist at cannabinoid receptors and does not interact with TRPV1, suggesting that 2-AG is a true natural ligand for cannabinoid receptors (7). To examine cannabinoid receptor-dependent effects, therefore, we used 2-AG rather

than anandamide as an endocannabinoid. As shown in Fig. 2, PGN (10 μg/mL)-induced phosphorylation of NF-κB was significantly inhibited by 2-AG (1 μM).

2-AG inhibits PGN-induced phosphorylation of NF-κB via CB1 receptor

To test whether cannabinoid receptors are involved in the inhibitory effect of 2-AG, we first examined the expression of CB1 and CB2 mRNAs in U87 glioblastoma cells using reverse transcription-PCR. The CB1 receptor expression was detectable in these cells, but CB2 receptor expression was below the limit of detection (Fig. 3A). We then tested effects of the CB1 receptor antagonist AM281, and found that AM281 (1 μM) reversed the suppressing effect of 2-AG on PGN-induced NF-κB phosphorylation (Fig. 3B). These results indicate that CB1 receptors are involved in the effects of 2-AG on the NF-κB signaling pathway.

Cannabinoid agonist WIN55,212-2 affects the cell morphology via CB1 receptor

To further confirm the presence of functional CB1 receptors in U87 cells, we examined effect of cannabinoid receptor antagonists on cannabinoid-induced morphological changes. Cell morphology was assessed after 72 hr-treatment with drugs. Because of the long incubation time, we used the cannabinoid agonist WIN55,212-2 instead of 2-AG, which is easily degraded by endogenous enzymes such as monoacylglycerol lipase. Microscopic analysis showed that the treatment with WIN55,212-2 (1 μM) affected the cell morphology and increased the number of shrunk cells (Figure 4A). The CB1 antagonist AM281 (1 μM), but not the CB2 antagonist AM630 (1 μM), prevented the WIN55,212-2-induced morphological change (Fig. 4A, 4B). These results indicate that functional CB1 receptors are expressed in U87 cells, consistent with the above observation (Fig. 3).

WIN55,212-2 inhibits PGN-induced cell proliferation via CB1 receptor

Finally, we examined the ability of WIN55,212-2 to inhibit PGN-induced cell proliferation. When applied alone, WIN55,212-2 (0.01-1 μM) failed to significantly affect the cell proliferation (Fig. 5A). However, WIN55,212-2 (1 μM) significantly inhibited PGN-induced cell proliferation (Fig. 5B). This effect of WIN55,212-2 was reversed by AM281 (Fig. 5B). These results indicate that activation of CB1 receptors suppresses the PGN-induced cell proliferation in U87 human glioblastoma cells.

Discussion

Several lines of evidence show that cannabinoids exert a variety of physiological and pharmacological actions (7, 8, 12). Notably, cannabinoids inhibit tumor growth and have been proposed as potential anti-tumoral agents (8, 13, 14). However, the mechanisms by which cannabinoids prevent tumor progression are still not fully understood. Our data suggest the possibility that the anti-tumoral and protective action of cannabinoids against PGN-induced inflammatory assaults might be associated with their ability to suppress phosphorylation of NF-κB via CB1 receptor signaling.

PGN is a characteristic cell wall component of gram-positive bacteria that can stimulate inflammatory signaling in several types of cells. In the present study, we found that PGN potently induced NF-κB phosphorylation in human glioma cells (Fig. 1A) and enhanced cell growth (Fig. 1B). The NF-κB signal is involved in a variety of physiological and pathological events, including inflammation, immune responses, and apoptosis. Recently, it has become clear that NF-κB signal pathway also has a critical role in cancer development and progression (4, 5, 15, 16). Activation of NF-κB has been reported in a wide variety of tumor cells (16). Our study showed that 2-AG, one of the endocannabinoids, has an inhibitory effect on PGN-induced NF-κB phosphorylation (Fig. 2, 3B), meaning that cannabinoids are most likely to prevent tumor progression in inflammation. Indeed, the cannabinoid agonist WIN-55212-2 abolished PGN-induced cell proliferation (Fig. 5B). These results suggest the therapeutic potential of cannabinoids in inflammation-dependent tumor progression.

TLRs have been established to play an essential role in the activation of innate immunity by recognizing specific patterns of microbial components such as PGN, LPS, flagellin, CpG DNA and dsRNA derived from virus (1). LPS, an essential component of the cell wall of gram-negative bacteria, activates NF-κB signal pathway through TLR4. Effects of cannabinoids on LPS-induced NF-κB signal pathway have been investigated in several cell types. The prototypic cannabinoid Δ^9-tetrahydrocannabinol (THC) inhibits LPS-induced NF-κB activation in the macrophage cell line RAW 264.7 that expresses CB2 receptors (17), but does not in the microglial cell line BV-2 that also expresses CB2 (18). In hippocampal tissue including both neurons and glial cells, 2-AG inhibits LPS-induced phosphorylation of NF-κB via CB1 receptors (19). The present study demonstrated that cannabinoids also inhibit the NF-κB signal induced by the TLR2 agonist PGN

through a mechanism involving CB1 receptors (Fig. 2, 3). Because signaling pathways of TLR2 and TRL4 for activation of NF-κB are shared by other TLRs (1), it is likely that TLR-mediated NF-κB activation may be generally sensitive to cannabinoids. The mechanisms underlying suppression of NF-κB signaling by cannabinoids are not fully understood. A recent study demonstrated that the effect of 2-AG on LPS-induced NF-κB phosphorylation is mediated by peroxisome proliferator-activated receptor-γ in hippocampal neurons (20). Further studies are needed to determine the molecular mechanisms.

In conclusion, we showed that cannabinoids prevent PGN-induced pathological signal transduction and cell proliferation in U87-MG human malignant glioma cells. Our results provide a possibility of cannabinoids as a new therapeutic approach for inflammation-dependent tumor progression.

Acknowledgments

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References

1. Takeda K, Akira S: TLR signaling pathways. Semin Immunol 16: 3-9, 2004
2. Buchanan MM, Hutchinson M, Watkins LR, Yin H: Toll-like receptor 4 in CNS pathologies. J Neurochem 114: 13-27, 2010
3. Chaturvedi MM, Sung B, Yadav VR, Kannappan R, Aggarwal BB: NF-kappaB addiction and its role in cancer: 'one size does not fit all'. Oncogene 30: 1615-1630, 2011
4. Basseres DS, Baldwin AS: Nuclear factor-kappaB and inhibitor of kappaB kinase pathways in oncogenic initiation and progression. Oncogene 25: 6817-6830, 2006
5. Karin M: Nuclear factor-kappaB in cancer development and progression. Nature 441: 431-436, 2006
6. Nogueira L, Ruiz-Ontanon P, Vazquez-Barquero A, Moris F, Fernandez-Luna JL: The NFkappaB pathway: a therapeutic target in glioblastoma. Oncotarget 2: 646-653, 2011
7. Kano M, Ohno-Shosaku T, Hashimotodani Y, Uchigashima M, Watanabe M: Endocannabinoid-mediated control of synaptic transmission. Physiol Rev 89:

309-380, 2009
8. Guindon J, Hohmann AG: The endocannabinoid system and cancer: therapeutic implication. Br J Pharmacol 163: 1447-1463, 2011
9. Centonze D, Finazzi-Agro A, Bernardi G, Maccarrone M: The endocannabinoid system in targeting inflammatory neurodegenerative diseases. Trends Pharmacol Sci 28: 180-187, 2007
10. Cabral GA, Griffin-Thomas L: Cannabinoids as therapeutic agents for ablating neuroinflammatory disease. Endocr Metab Immune Disord Drug Targets 8: 159-172, 2008
11. Saito T, Sugimoto N, Ohta K, Shimizu T, Ohtani K, Nakayama Y, Nakamura T, Hitomi Y, Nakamura H, Koizumi S, Yachie A: Phosphodiesterase inhibitors suppress Lactobacillus casei cell wall-induced NF-κB and MAPK activations and cell proliferation through protein kinase A- or exchange protein activated by cAMP-dependent signal pathway. ScientificWorldJournal, in press, doi:10.1100/2012/748572.
12. Klein TW: Cannabinoid-based drugs as anti-inflammatory therapeutics. Nat Rev Immunol 5: 400-411, 2005
13. Guzman M: Cannabinoids: potential anticancer agents. Nat Rev Cancer 3: 745-755, 2003
14. Sarfaraz S, Adhami VM, Syed DN, Afaq F, Mukhtar H: Cannabinoids for cancer treatment: progress and promise. Cancer Res 68: 339-342, 2008
15. Aggarwal BB: Nuclear factor-kappaB: the enemy within. Cancer Cell 6: 203-208, 2004
16. Prasad S, Ravindran J, Aggarwal BB: NF-kappaB and cancer: how intimate is this relationship. Mol Cell Biochem 336: 25-37, 2010
17. Jeon YJ, Yang KH, Pulaski JT, Kaminski NE: Attenuation of inducible nitric oxide synthase gene expression by delta 9-tetrahydrocannabinol is mediated through the inhibition of nuclear factor- kappa B/Rel activation. Mol Pharmacol 50: 334-341, 1996
18. Kozela E, Pietr M, Juknat A, Rimmerman N, Levy R, Vogel Z: Cannabinoids Delta(9)-tetrahydrocannabinol and cannabidiol differentially inhibit the lipopolysaccharide-activated NF-kappaB and interferon-beta/STAT proinflammatory pathways in BV-2 microglial cells. J Biol Chem 285: 1616-1626, 2010
19. Zhang J, Chen C: Endocannabinoid 2-arachidonoylglycerol protects neurons by limiting COX-2 elevation. J Biol Chem 283: 22601-22611, 2008

20. Du H, Chen X, Zhang J, Chen C: Inhibition of COX-2 expression by endocannabinoid 2-arachidonoylglycerol is mediated via PPAR-gamma. Br J Pharmacol 163: 1533-1549, 2011

Figure Legends

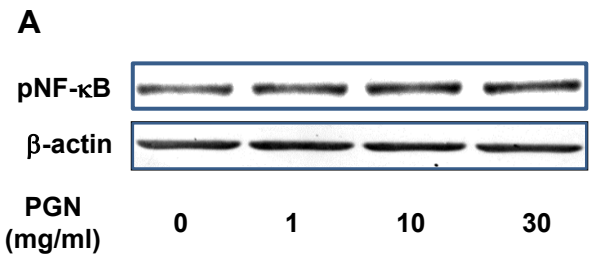
Fig. 1, Change in the level of phospho-NF-κB (A), and change in cell growth (B) in U87-MG human malignant glioma cells after treatment with PGN (1-30 μg/mL). Each column represents the mean ± SEM. *P < 0.05 vs untreated controls.

Fig. 2, Change in the level of phospho-NF-κB after treatment with PGN (10 μg/mL) or a combination of 2-AG (1 μM) in U87 cells. Each column represents the mean ± SEM. *P < 0.01 vs untreated controls. #P < 0.01 vs PGN treatment group.

Fig. 3, Expression of CB1 receptor (A), and change in the level of phospho-NF-κB after treatment with PGN (10 μg/mL), a combination of 2-AG (1 μM) and PGN (10 μg/mL), or a combination of 2-AG (1 μM), PGN (10 μg/mL), and AM281 (1 μM) (B) in U87 cells. Each column represents the mean ± SEM. *P < 0.05 vs untreated controls. #P < 0.05 vs PGN treatment group. ##P < 0.05 vs PGN and 2-AG treatment group.

Fig. 4, Change in the cell morphology (A) and % of shrunk cells 3 days after treatment with WIN55,212-2 (1 μM), a combination of WIN55,212-2 (1 μM) and AM281 (1 μM), or a combination of WIN55,212-2 (1 μM) and AM630 (1 μM) (B) in U87 cells. Each column represents the mean ± SEM. *P < 0.05 vs untreated controls. #P < 0.05 vs WIN55,212-2 treatment group.

Fig. 5, Change in cell growth after treatment with WIN55,212-2 (0-1.0 μM) (A), and change in cell growth after treatment with PGN (10 μg/mL), a combination of WIN55,212-2 (1 μM) and PGN (10 μg/mL), or a combination of WIN55,212-2 (1 μM), PGN (10 μg/mL) and AM281 (1 μM) (B) in U87 cells. Each column represents the mean ± SEM. *P < 0.05 vs untreated controls. #P < 0.05 vs PGN treatment group. ##P < 0.05 vs PGN and WIN55,212-2 treatment group.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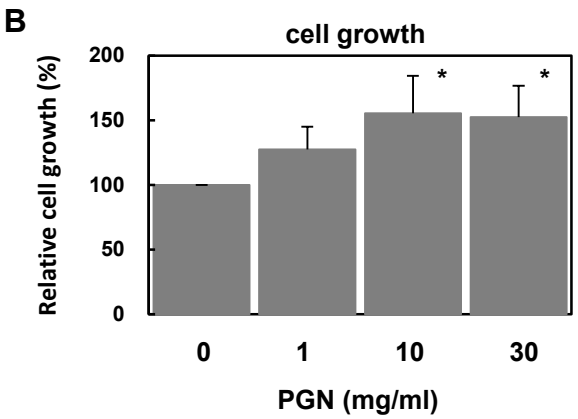
pNF-κB
β-actin
PGN
(mg/ml)
0
1
10
30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cell growth
Relative cell growth (%)
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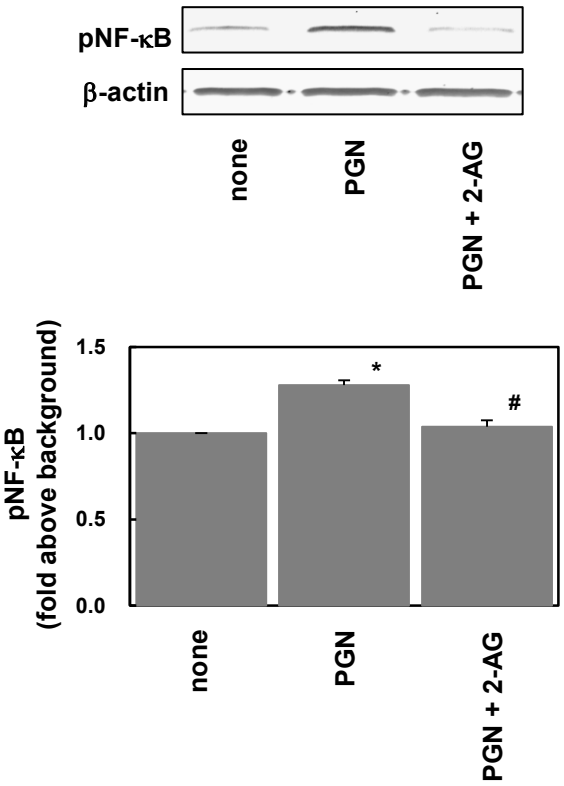
150
100
50
0
*
*
0
1
10
30
PGN (mg/ml)

Fig. 1


pNF-κB
β-actin
none
PGN
PGN + 2-AG
pNF-κB
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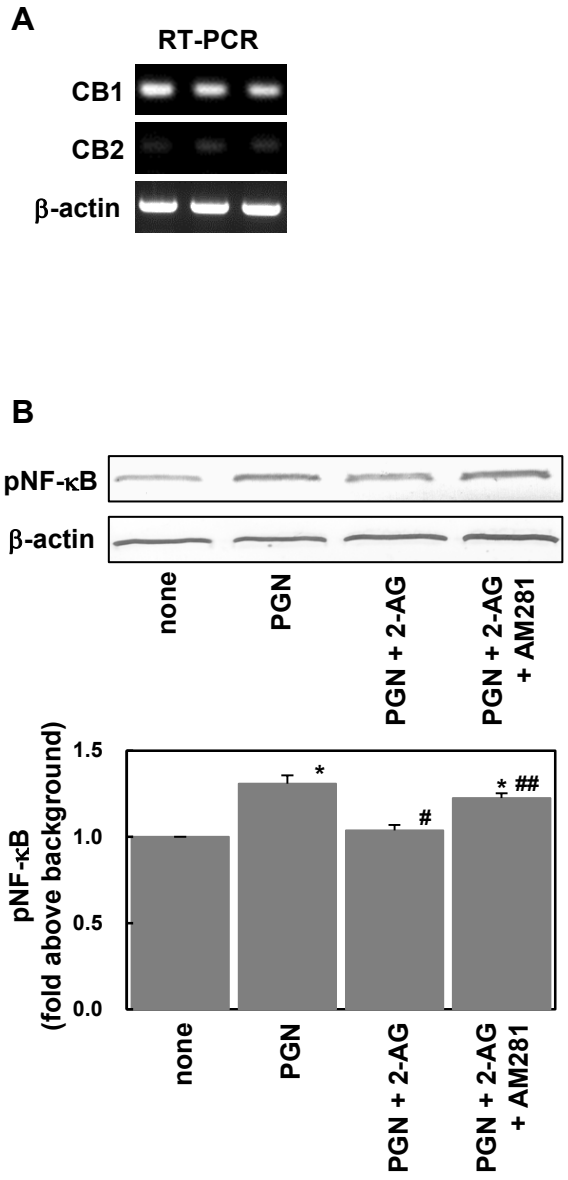
1.0
0.5
0.0
*
#
none
PGN
PGN + 2-AG

Fig. 2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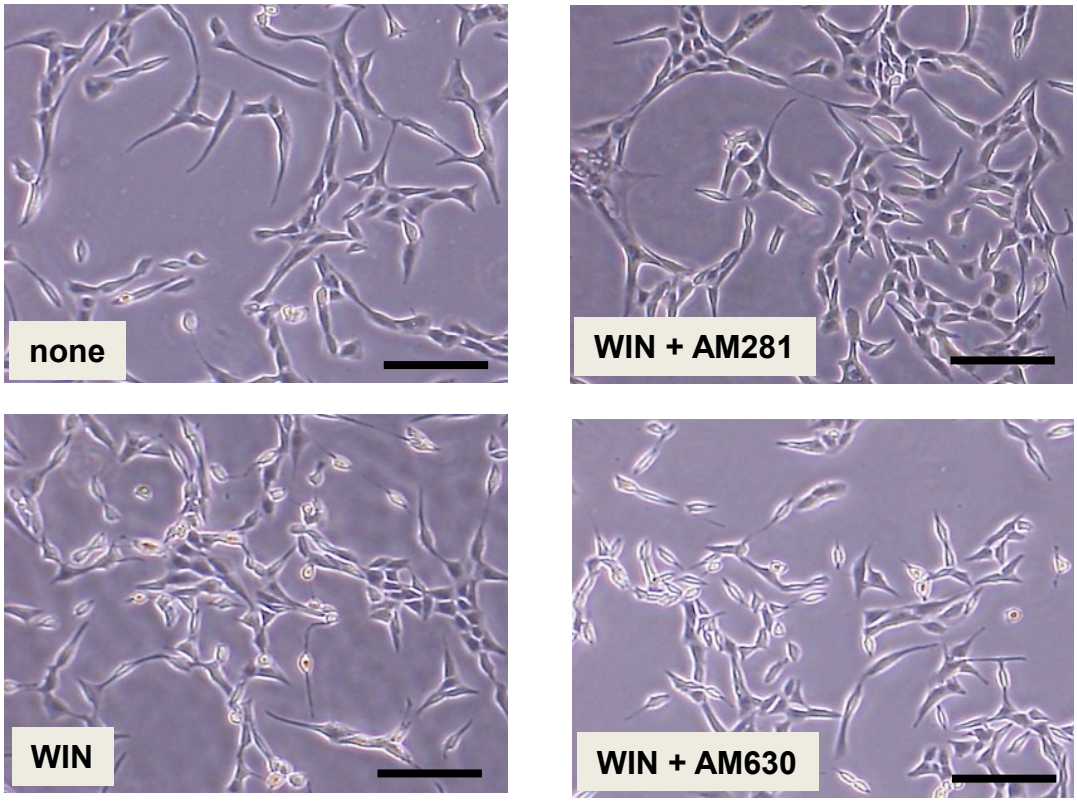
RT-PCR
CB1
CB2
β-actin
B
pNF-κB
β-actin
none
PGN
PGN + 2-AG
PGN + 2-AG + AM281
pNF-κB (fold above background)
1.5
1.0
0.5
0.0
*
#
* ##
none
PGN
PGN + 2-AG
PGN + 2-AG + AM281

Fig. 3

A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WIN + AM281
WIN
WIN + AM630

B

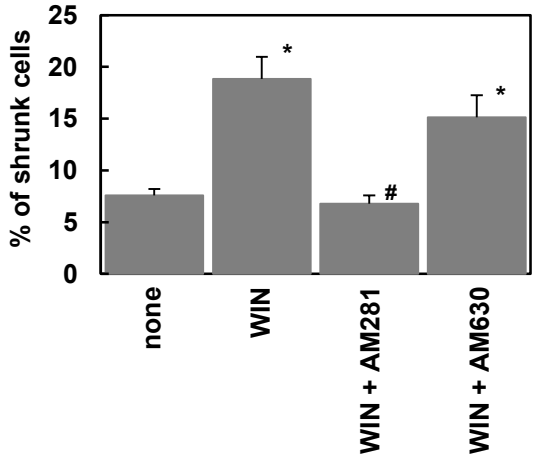
% of shrunk cells
25
20
15
10
5
0
*
*
#
none
WIN
WIN + AM281
WIN + AM630

Fig. 4

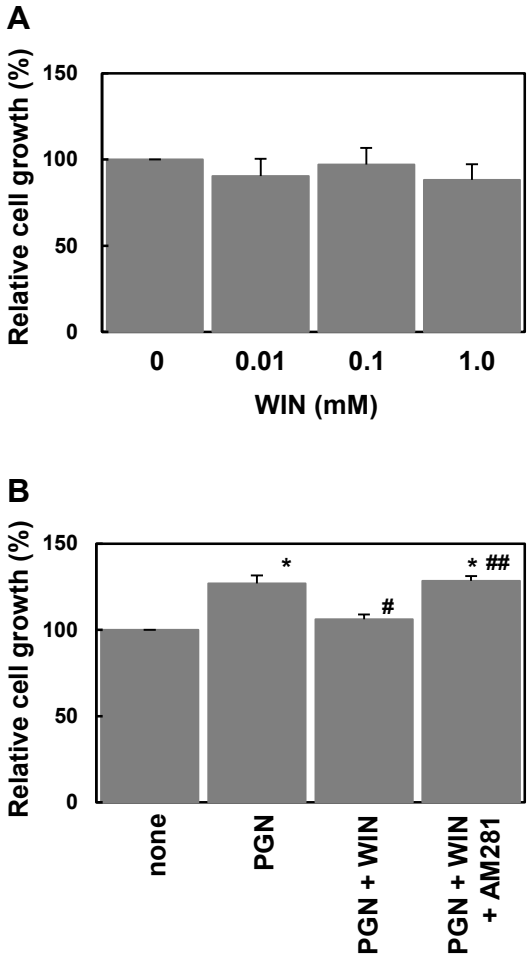
A
Relative cell growth (%)
150
100
50
0
0
0.01
0.1
1.0
WIN (mM)
B
Relative cell growth (%)
150
100
50
0
*
#
* ##
none
PGN
PGN + WIN
PGN + WIN + AM281

Fig. 5