Original Article

Suppressive effect of resveratrol, catechin and their conformationally constrained analogs on neutrophil extracellular trap formation by HL-60-derived neutrophils

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Neutrophil extracellular trap (NET) formation is a unique self-defense mechanism of neutrophils; however, it is also involved in many diseases, including atherosclerosis. Resveratrol and catechin are antioxidants with anti-atherosclerotic properties. Here, we examined the effects of resveratrol, catechin, and other related compounds on NET formation. HL-60-derived neutrophils were pretreated with resveratrol and other compounds before stimulation with phorbol-myristate acetate (PMA). DNA and myeloperoxidase released from neutrophils were determined. Resveratrol suppressed the DNA release from neutrophils in a dose-dependent manner. NET formation was enhanced by 1-palmitoyl-2-oxovaleroyl phosphatidylcholine (POVPC), a truncated form of oxidized phospholipid, and resveratrol suppressed NET formation induced by POVPC and PMA. Furthermore, we designed several analogs of resveratrol or catechin whose conformation was restricted by the inhibition of the free rotation of aromatic rings. The conformationally constrained analogs were more effective at inhibiting NET formation; however, their inhibitory function decreased when compound was a large, hydrophobic analog. The most potent compounds, planar catechin and resveratrol, suppressed myeloperoxidase release from activated neutrophils. In addition, these compounds suppressed DNA release from neutrophils stimulated with calcium ionophore. These results suggest that resveratrol, catechin and their analogs exert anti-NET effects, and that constraining the geometry of these compounds enhanced their inhibitory effects.

***Key Words*: neutrophil extracellular trap, resveratrol, catechin, planar analog, HL-60**

Neutrophil extracellular trap (NET) formation, in which neutrophils release decondensed DNA strings extracellularly, was first reported in 2004 as a novel self-defense function of neutrophils.(1) DNA strings in NETs are associated with several neutrophil-derived proteins such as neutrophil elastase (NE), myeloperoxidase (MPO), and histones.(2) NETs are an effective defense system since neutrophil-derived proteins are toxic to bacteria via their proteolytic activity and reactive oxygen species (ROS). NETs are found in different parts of the human body both at non-infectious sites as well as at sites of bacterial infection, suggesting their involvement in diseases by evoking the inflammatory responses.(3) Plasma concentrations of NET markers, including MPO-DNA complex, increases in patients with cardiovascular diseases, liver cancer, or acute thrombosis associated with the coronavirus disease 2019.(4–6)

We previously reported that NET formation by neutrophils following phorbol ester stimulation is enhanced in the presence of oxidized low-density lipoprotein (oxLDL) or oxidized phosphatidylcholine (oxPC).(7,8) OxLDL is a risk factor for cardiovascular disease that promotes foam cell formation in macrophages, which is a typical characteristic of atherosclerotic lesions. Various oxPC species are generated in oxLDL, and these oxidation products are potent stimulants for vascular cells to induce inflammatory responses.(9) However, recent studies on cardiovascular diseases have indicated endothelial erosion is another type of vascular damages that leads to thrombus formation, in which neutrophils may play active roles.(10) Endothelial erosion is characterized by low lipid accumulation and the presence of inflammatory proteins derived from neutrophils, such as MPO or NE. An increase in plasma MPO levels in patient circulation after acute myocardial infarction suggests the involvement of neutrophils in cardiovascular events.(11) Therefore, oxLDL promotes atherosclerosis via two mechanisms. First, oxLDL facilitates foam cell formation and lipid accumulation in lesions after recognition by scavenger receptors. Second, oxLDL acts on neutrophils to enhance NET formation, which promotes endothelial erosion and thrombosis.(12)

Resveratrol, a flavonoid found in red wine, exerts anti-atherosclerotic, antioxidant, and antiaging effects.(13) Some flavonoids, such as catechins, also perform similar functions.(14) We previously demonstrated that catechin analogs with planar conformations exhibited improved antioxidant properties.(15) As various flavonoids, such as resveratrol and catechins, inhibit NET formation,(16–18) we investigated whether planar analogs of flavonoids have any beneficial effects on neutrophils. In another study, we found that conjugated compounds of catechin and silibinin with planar structure had exhibited improved antioxidant function and cytoprotective effect on cells from amyloid-β-induced apoptosis.(19) In this study, we examined the effects of resveratrol, catechins, and their planar analogs on NET formation by HL-60-derived neutrophils.

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Materials and Methods

Materials. All-*trans* retinoic acid (AtRA), phorbol 12-myristate 13-acetate (PMA), resveratrol, and catechin were purchased from Fujifilm Wako Pure Chemical Co. (Osaka, Japan). Silibinin and poly-L-lysine solution (P4707) were purchased from Sigma-Aldrich (St. Louis, MO). Planar catechin (PCat) was prepared from (+)-catechin and acetone via the oxa-Pictet-Spengler reaction, and PCat phytyl (PCat-P) was prepared from (+)-catechin and teprenone according to the synthesis method of PCat.[15] Dimethyl resveratrol was synthesized from the starting materials 3,5-dimethoxybenzaldehyde and 4-methoxybenzyl chloride in five steps (see Supplemental Material 1*). All other planar analogs were synthesized as previously described.[15,19] Next, 1-palmitoyl-2-(5-oxovaleroyl)-phosphatidylcholine (POVPC) was purchased from Avanti Polar Lipids Inc. (Alabaster, AL). Micrococcal nuclease (MNase) was purchased from TAKARA Bio, Inc. (Shiga, Japan). SYTOX Green was purchased from Thermo Fisher Scientific (Waltham, MA). Anti-human MPO antibody was purchased from Dako (A0398; Carpinteria, CA). Horseradish peroxidase-conjugated anti-rabbit IgG was purchased from GE Healthcare (NA934; Buckinghamshire, UK).

Induction of NETs by HL-60 cells-derived neutrophils. HL-60 cells were treated with AtRA to induce their differentiation into neutrophil-like cells, as previously described.[7] Briefly, 2 μM AtRA were added to HL-60 cells (2.0×10^6 cells/dish) cultured in RPMI-1640 medium supplemented with 5% deactivated (for 30 min at 56°C) fetal bovine serum, 50 U/ml penicillin, and 50 μg/ml streptomycin in 10-cm dishes for four days. HL-60-derived neutrophil-like cells were collected and washed once with serum-free RPMI-1640 (phenol red free) medium, seeded in a 24-well poly-L-lysine-coated plate (5.0×10^5 cells/well), and cultured for 30 min. The cells were treated with aliquots of flavonoids or their planar analogs at 37°C under 5% CO_2 for 30 min, followed by treatment with 20 or 50 μM PMA at 37°C under 5% CO_2 for 2 h. In some experiments, 10 μM POVPC was added together with PMA, as previously described.[8]

Calcium ionophore-induced NET formation was carried out according to the method of Manda-Handzlik *et al.*[20] Briefly, HL-60 cells were cultured in RPMI containing 1.25% DMSO for five days to differentiate into neutrophils, then the neutrophils were seeded in 24-well poly-L-lysine-coated plate. The cells were treated with either resveratrol or PCat for 30 min followed by treatment with calcium-ionophore for 2 h.

Fluorometric quantitation of NET-DNA. NET-DNA was quantified using the SYTOX Green fluorescent dye to detect extracellular DNA, as described in our previous reports.[7,8] In the original procedure (referred to "method A"), the culture medium recovered at the end of cell culture was treated with 1 U/ml MNase to partially degrade the DNA and was centrifuged at 1,800 × *g* for 10 min. The supernatant was then transferred to a 96-well plate and mixed with 1 μM SYTOX Green.[7] In some experiments we used a modified procedure ("method B") in which the culture medium was treated with 1 U/ml MNase to degrade the DNA exposed extracellularly upon NET formation in the culture dishes. The culture medium from each dish was recovered and centrifuged at 1,800 × *g* for 10 min. After mixing the supernatant with 1 μM SYTOX Green, the fluorescence intensity was measured as previously described.[8] Fluorescence (ex: 485 nm, em: 525 nm) was measured using Varioskan Flash (Thermo Fisher Scientific).

Detection of MPO released upon NET formation. Western blotting for MPO was performed as previously described.[8] Briefly, the culture medium recovered from the stimulated neutrophils was separated on a 10% polyacrylamide gel and transferred to a polyvinylidene fluoride membrane. After blocking the membrane with 2% skim milk for 1 h, the membrane was first treated with the anti-MPO antibody (dilution 1:10,000), and then with the anti-rabbit IgG antibody (dilution 1:3,000). Bands were detected using the ECL Prime Western Blotting Detection Reagent (RPN2232; Cytiva, Waltham, MA) and ImageQuant LAS 500 (GE Healthcare). Intensity of each immunoreactive band was measured using the ImageJ software.

Data analysis. Data analysis was performed using the JMP Pro 16.0.0 software. Normal distribution of values was evaluated using the Shapiro–Wilk test, equal variance was confirmed using the O'Brien test, and statistical significance was calculated using analysis of variance. Tukey–Kramer test was used to examine the differences, whereas the Wilcoxon test was used for samples without normal distribution. Statistical significance was set at $p<0.05$.

Results

HL-60 (human promyelocytic leukemia) cells were differen-

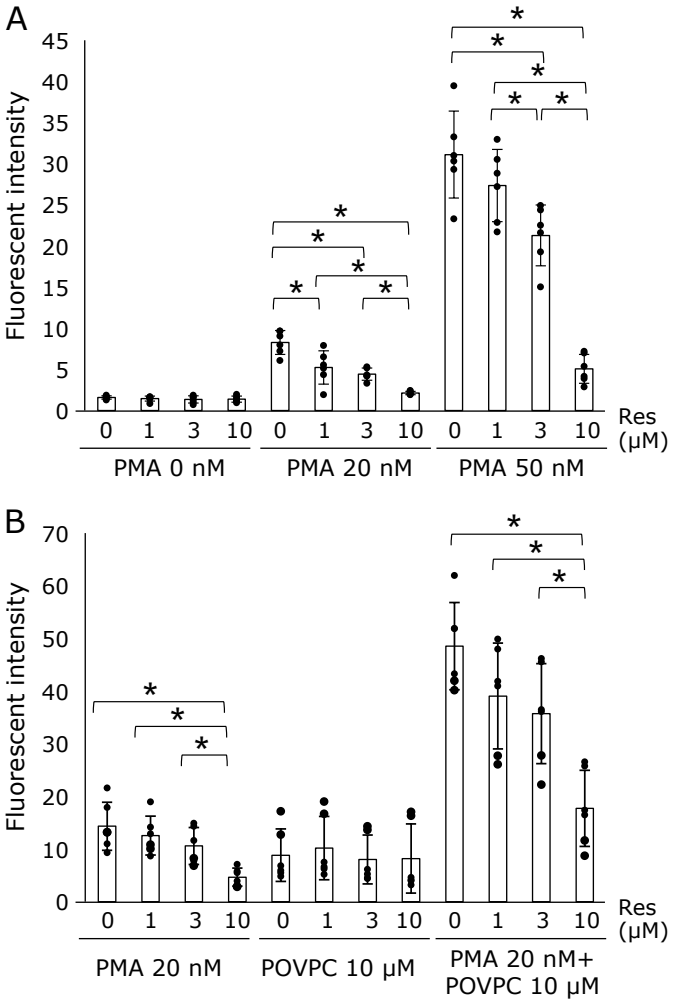

Fig. 1. Resveratrol suppresses phorbol myristate acetate (PMA)-induced neutrophil extracellular trap (NET) formation by HL-60 neutrophils. (A) To HL-60-derived neutrophils, 0–10 nM resveratrol was added 30 min before stimulation with 20 or 50 nM PMA. After the cells were incubated for 2 h, DNA released from the cells was fluorometrically measured with SYTOX Green using the "method A" as described in the Materials and Methods section. (B) HL-60-derived neutrophils were stimulated with 20 nM PMA, 10 nM 1-palmitoyl-2-(5-oxovaleroyl) phosphatidylcholine (POVPC), or both to induce NET formation. Resveratrol (0–10 nM) was added 30 min before stimulation, and DNA released from the cells after 2 h of incubation was fluorometrically measured using the "method A". $n = 6$, $^*p<0.05$.

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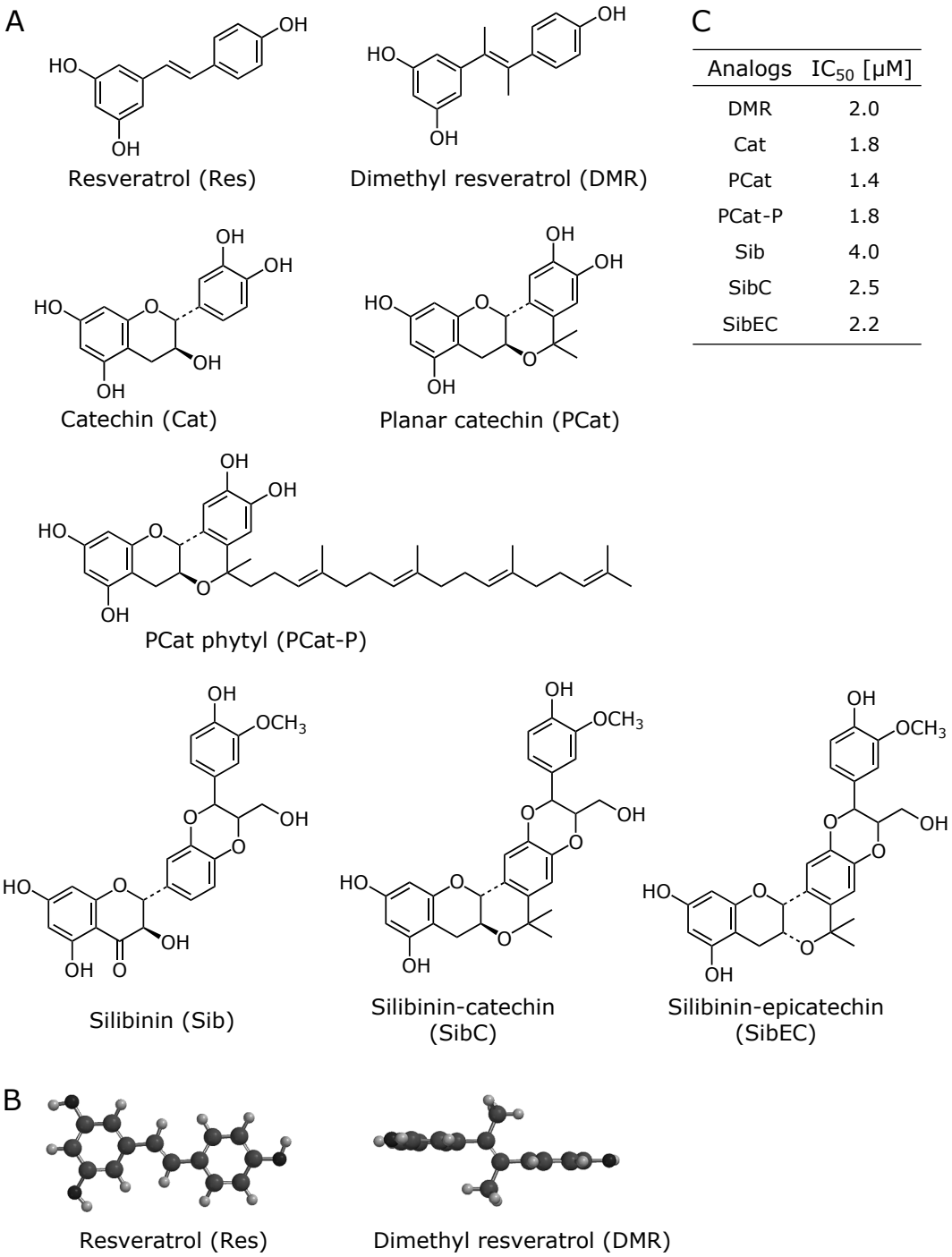

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Analogs	IC_{50} [μM]
DMR	2.0
Cat	1.8
PCat	1.4
PCat-P	1.8
Sib	4.0
SibC	2.5
SibEC	2.2

Fig. 2. Planar analogs of resveratrol and catechin effectively inhibit NET formation. (A) Structures of resveratrol, catechin, and their planar analogs examined in this study. (B) Optimized geometry of resveratrol and dimethyl resveratrol (DMR) from density functional theory (DFT) calculations at B3LYP/6-31G(d) level. (C) Inhibitory effects of these compounds on NET formation by HL-60-derived neutrophils were determined using the "methods B" described in the Materials and Methods section. Half-maximal inhibitory concentration (IC_{50}) values of these compounds for PMA-induced NET formation. $n = 3$.

tiated into neutrophils by treatment with all-*trans*-retinoic acid, then NET formation was induced by the addition of 20 or 50 nM of PMA. Addition of resveratrol 30 min before PMA stimulation suppressed NET formation when the concentration of resveratrol was higher than 3 μM (Fig. 1A).

Oxidized phospholipids stimulate inflammation in many cells. POVPC is a phospholipid produced during the oxidation of low-density lipoprotein.[9] Stimulation of HL-60-derived neutrophils with POVPC alone induced NET formation, and the same dose of POVPC enhanced PMA-induced NET formation (Fig. 1B). Resveratrol suppressed NET formation when the cells were stimulated with PMA, or PMA and POVPC, but it did not show a suppressive effect when cells were treated with POVPC alone.

Resveratrol is a polyphenol with two aromatic rings connected to a bridge containing a −C=C− double bond to form a planar structure with conjugated dienes. Here, we synthesized dimethyl

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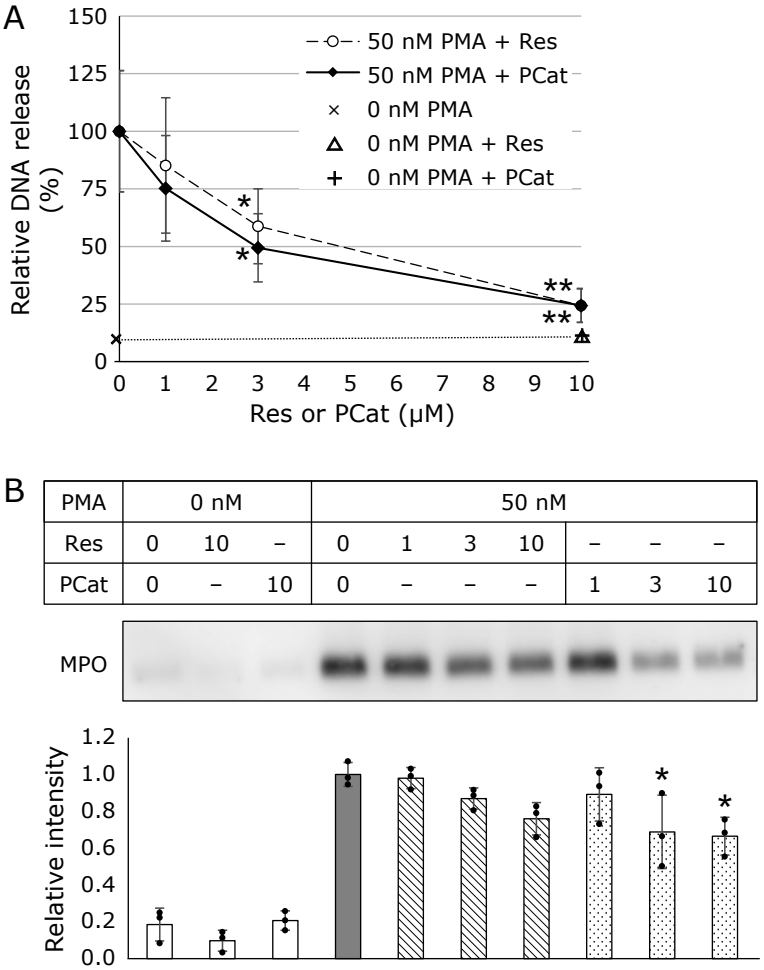

Fig. 3. Comparison of the effects of resveratrol and planar catechin (PCat) on PMA-induced NET formation. (A) HL-60-derived neutrophils were pretreated with either resveratrol or PCat for 30 min and stimulated with 50 nM PMA for 2 h. DNA released from the cells was fluorometrically measured with SYTOX Green using the "method A" described in the Materials and Methods section. (B) HL-60-derived neutrophils were treated under the same conditions as (A). MPO released in the cell culture supernatant was detected using Western blotting. Band intensity was determined using the ImageJ software. $n = 3$, *$p<0.05$ compared to without inhibitors, **$p<0.01$ compared to without inhibitors.

resveratrol (DMR) with a conformationally constrained structure by introducing a methyl group at the double bond (Fig. 2A and B, and Supplemental Material 1*). DMR showed an approximately 2-fold stronger suppressive effect on NET formation, with a half-maximal inhibitory concentration (IC_{50}) of 2.0 μM (Fig. 2C).

Catechin is a polyphenol with a flavonoid structure, that exerts anti-oxidant and anti-atherosclerotic effects. Catechins contain two aromatic ring moieties. We previously designed planar analogs of catechin, PCat, PCat-T and SibC by introducing an additional ring to prevent any free rotation between the two aromatic rings.[15,19] Catechin was found to be a potent inhibitor of NET formation (Fig. 2C). Among all analogs, IC_{50} value of PCat was lower than that of catechin. Silibinin is a polyphenol found in the seeds of *Silybum marianum* (also known as milk thistle or Mary thistle) that exerts antioxidant, anti-inflammatory, and anticancer effects. Inhibitory effect of silibinin on NET formation was weaker than that of catechin. However, sterically constrained SibC and SibEC improved the inhibitory activity of silibinin by decreasing its IC_{50} value. Notably, the anti-NET efficacies of SibC and SibEC were similar under these experimental conditions (Fig. 2C).

IC_{50} values of the analogs in Fig. 2C were calculated using method B, in which MNase was added after collecting the culture medium, whereas the effect of resveratrol was measured using method A. The suppressive effect of the strongest analog, PCat, was compared with that of resveratrol, side-by-side, under the same assay conditions. Both compounds suppressed DNA release from neutrophils in a dose-dependent manner, with PCat being slightly more effective than resveratrol (Fig. 3A). IC_{50} values of PCat and resveratrol were found to be 2.9 and 4.0 μM, respectively. Then, effects of these two compounds on MPO release from neutrophils was examined using Western blotting. Stimulation of neutrophils with 50 nM PMA increased the extracellular MPO release, whereas 10 μM PCat and resveratrol reduced MPO release by 40 and 30%, respectively (Fig. 3B).

Since many flavonoids including resveratrol are reported to inhibit protein kinase C (PKC), we examined the levels of activated PKC in the presence and absence of resveratrol and PCat. HL-60-derived neutrophils were stimulated with PMA with or without the compounds, the whole cell lysate was subjected to Western blotting against anti-PKC and anti-phospho-PKC antibodies (Supplemental Material 2*). Either resveratrol or PCat did not change the levels of phosphorylated form of PKC significantly under the current conditions.

Effects of resveratrol and PCat on NET formation were examined using another type of inducer of NETosis. HL-60 cells were differentiated into neutrophils by treatment with DMSO, then

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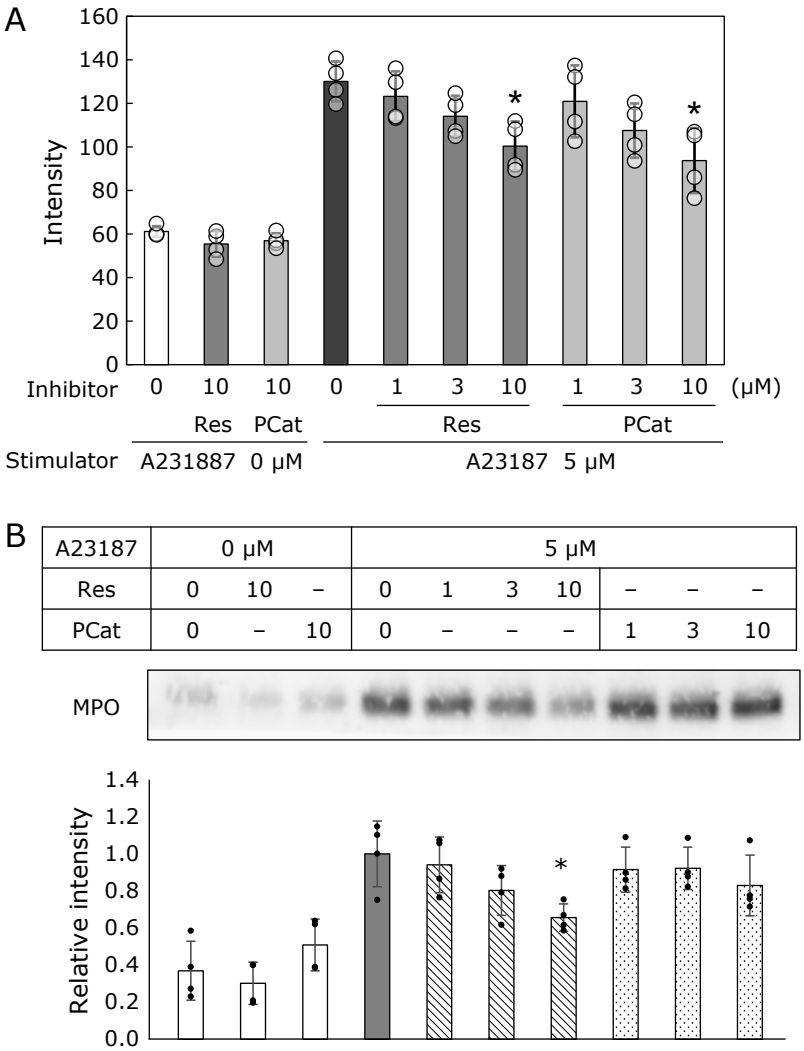

Fig. 4. Resveratrol and planar catechin (PCat) suppressed A23187-induced NET formation by HL-60 neutrophils. To HL-60-derived neutrophils differentiated with DMSO, either resveratrol of PCat (0–10 nM) was added 30 min before stimulation with 5 μM A23187. After the cells were incubated for 2 h, DNA released from the cells was fluorometrically measured with SYTOX Green using the "method A" as described in the Materials and Methods section. $n = 4$, $^*p<0.05$ compared to without inhibitors.

stimulated with a calcium ionophore, A23187. It was reported that HL-60-derived neutrophils respond to A23187 when differentiated with DMSO but not differentiated with AtRA.[8] Both resveratrol and PCat reduced DNA release from HL-60-derived neutrophils in a dose dependent manner (Fig. 4A). Resveratrol suppressed MPO release from A23187-stumilated neutrophils, although PCat was less effective in inhibiting MPO release than resveratrol (Fig. 4B).

Discussion

In this study, we found that resveratrol, and catechin suppressed NET formation by stimulated HL-60 neutrophils. In addition, their planar analogs exerted stronger suppressive effect on NET formation than the parent flavonoids.

Analogs tested in this study were designed to restrict the free rotation of aromatic rings by introducing additional rings or methyl groups. We previously reported that planar catechins exhibited improved ROS-scavenging property.[15] Interestingly, a highly planar analog, SibC, produced by introducing PCat structure in silibinin, inhibited the aggregation of amyloid-β and prevented the apoptosis of SH-SY5Y neuroblastoma cells damaged by amyloid-β.[19] The anti-apoptotic activity was not observed in a non-planar isomer, SibEC, although it showed even better antioxidative efficacy. As resveratrol and catechin inhibit NET formation, we speculated that their planar analogs may improve their ability to inhibit NET formation.

The two planar analogs, DMR and PCat, showed lower IC_{50} values for NET formation by HL-60-induced neutrophils than the original compounds. However, PCat-P did not enhance the inhibitory effect of catechin. PCat-P was modified by adding a long C-20 phytyl chain, suggesting that increase in the hydrophobicity and/or molecular size negatively affects NET inhibition. Moreover, we found that SibC, a unique planar analog inhibiting amyloid aggregation, inhibited NET formation more potently than silibinin but not as potently as catechin. In addition to the planar structure, its hydrophobicity and molecular size may affect inhibitory effect on NET formation. These findings suggest that planar conformation, hydrophobicity, and size of molecules influence the inhibition of NET formation.

PMA is a widely used neutrophil stimulator that activates protein kinase C (PKC), and its subsequent signaling cascades are diverse.[21] Polyphenol compounds are reported to inhibit PKC reactions,[22] thus these flavonoids may suppress NET

formation via PKC inhibition.[22–24] However, we could not detect alteration of phospho-PKC levels after resveratrol or PCat treatment under the current experimental conditions. In addition, resveratrol and PCat reduced DNA release from the neutrophils stimulated with A23187, although efficacy of inhibition by these compounds seems to be slightly milder than the cells stimulated with PMA. These results suggest that resveratrol and the catechin analog suppress NET formation at least in part through mechanisms other than PKC inhibition. In the literatures, Das *et al.*[23] reported that 100 μM resveratrol reduces the membrane translocation of PKCα by 80% in HEK293 cells. Nosál' *et al.*[24] reported that 10 μM resveratrol significantly reduced the membrane translocation of PKC in isolated neutrophils. On the other hand, even 20 μM of catechin resulted in only 20% inhibition of PKC activation in THP-1-derived neutrophils.[25] It is noted that even lower concentrations of flavonoids were examined in this study.

As intracellular ROS generation is an important step in NET induction by neutrophils,[3] these flavonoids and their analogs may inhibit NET formation by scavenging intracellular ROS. ROS generation was detected in PMA-stimulated HL-60-derived neutrophils by ESR using DMPO as a probe, however, 10 μM resveratrol did not reduce ROS production in PMA-stimulated neutrophils (Supplemental Material 3*). Previously, we reported the other planar analogues, SibC and SibE, had less radical scavenging potency against hydroxyl radical (•OH) than catechin.[19] We do not think ROS scavenging can explain anti-NET formation, although further examinations would be needed to confirm this possibility.

Another possibility to be considered is that resveratrol and planar catechin analogs may have some kind of stabilizing effect of cellular membranes. It was reported previously that resveratrol had a stabilizing effect on phospholipid lipid bilayer.[26] Addition of resveratrol to a solid membrane composed of phosphatidylcholine, sphingomyelin and cholesterol reduced membrane fluidity, while resveratrol increased the membrane fluidity of a soft membrane prepared by egg phosphatidylcholine. This biphasic effect of resveratrol on the membrane fluidity is just similar to the membrane stabilization by cholesterol.[27] Cholesterol is an amphipathic lipid containing rigid rings; thus it aligns together with phospholipids in membranes and interact with hydrophobic parts of phospholipids that leads to regulate molecular packing in the membranes. Resveratrol has hydroxyl groups on both aromatic rings, and the hydroxyl groups of resveratrol could make hydrogen bonds with multiple phospholipids in membranes. A study with molecular dynamics simulation showed resveratrol could either penetrate into the hydrophobic space in membranes to align with phospholipids or place at the interphase of hydrophobic layer and hydrophilic head groups depending on the membrane conditions.[28] Some larger catechin analogs with more hydroxyl groups, such as epigallocatechin gallate and theaflavin, were shown to interact with head group of phospholipids from the aqueous phase.[29] Planar structure and limited numbers of hydroxyl group may contribute to interaction with hydrophobic parts of membranes. In our experiments, we examined POVPC, a truncated product of oxPC with a strong cytotoxic effect due to membrane disruption, which was reported to have many biological effects on vascular cells.[9,30,31] Interestingly, POVPC greatly enhanced PMA-induced NET formation,[8] suggesting membrane conditions could influence NET formation. We showed in this study that resveratrol suppressed the increase in NET formation by addition of POVPC. If this is due to the membrane stabilizing effect of resveratrol, it may explain with the discrepancy between the anti-NET efficacy and ROS scavenging property of the compounds studied in this study.

In conclusion, our findings revealed the efficacy of the conformationally constrained analogs of resveratrol, and catechin, DMR and PCat, in inhibiting NET formation.

Author Contributions

Conceptualization, HI and KF; Data curation, HO, TO, YW, and TM; Synthesis of materials, MM and KF; Experiments, HO, WP, TO, and YW; Data analysis, HO, WP, and TO; Draft preparation, HO and HI; Review and editing, HO, TO, KF, and HI.

Conflict of Interest

No potential conflicts of interest were disclosed.

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