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Clarification of the Role of Quercetin Hydroxyl Groups in Superoxide Generation and Cell Apoptosis by Chemical Modification

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Accumulated data have suggested that the hydroxyl groups of flavonoids are important for their bioactive function. To directly demonstrate the role of hydroxyl groups, we synthesized a derivative of quercetin, 3,7,3′,4′-*O*-tetrabenzylquercetin (4Bn-Q) that substituted the hydroxyl groups of quercetin with benzyl groups, and then evaluated the ability to inhibit cell proliferation and cause apoptosis in human leukemia (HL-60) cells. The results reveal that quercetin, but not 4Bn-Q, inhibited cell proliferation and induced apoptosis as characterized by DNA fragmentation, activation of caspase-3, and PARP cleavage. Treatment with 4Bn-Q reduced the intracellular level of quercetin-induced superoxide, and the scavenger of superoxide, Mn (III) tetrakis (4-benzoic acid) porphyrin chloride (MnTBAP), reduced the superoxide level and apoptosis induced by quercetin. These findings directly demonstrate that the hydroxyl groups of quercetin contributed to the generation of intracellular superoxide, consequently inhibiting proliferation and inducing apoptosis in HL-60 cells.

Key words: quercetin; chemical modification; superoxide; apoptosis

Flavonoids are polyphenolic compounds that are widely distributed in many edible plants. Accumulated data have revealed flavonoids to have strong antioxidative effects[1] and a variety of bioactivities.[2–4] Furthermore, structure-activity relationships have been suggested by investigating the structural diversity based on the different aglycone, glycosidation and acylation patterns of flavonoids.[5–8] In particular, the hydroxyl groups of flavonoids have been suggested to play a critical role in their bioactivities. For example, the hydroxyl number of a flavonoid is important for its radioprotective effects[9] and caspase-3 activation.[5] Our previous reports have indicated that the bioactivities of flavonoids appeared to be associated with the structure of the B-ring.[10] However, most of this speculation was made by comparing the different number of hydroxyl groups among flavonoids. Little evidence was obtained from direct structural modification. In this present study, we chose quercetin as an experimental material because it is one of the best-studied flavonoids and can inhibit cell proliferation[11,12] and induce apoptosis in a variety of cancer cells.[13–17] To directly clarify the role of the hydroxyl groups of quercetin in its bioactive functions, we synthesized the quercetin derivative, 3,7,3′,4′-*O*-tetrabenzylquerctin (4Bn-Q) which substituted the hydroxyl groups of quercetin with benzyl groups at the 3,7,3′,4′ positions. The bioactive function of these two compounds was then evaluated for the ability to inhibit cell proliferation and induce apoptosis in human leukemia (HL-60) cells which provide a valid model for testing antileukemic or general antitumoral compounds. Our results show that quercetin significantly inhibited cell proliferation by caspase-mediated apoptosis. However, 4Bn-Q lost this function. Intracellular reactive oxygen species (ROS) data show that the hydroxyl groups of quercetin might have contributed to superoxide generation, consequently inhibiting proliferation and inducing apoptosis in HL-60 cells.

Materials and Methods

Synthesis of 4Bn-Q. 4Bn-Q was synthesized as previously described.[18] Briefly, a solution of quercetin (1.0 equiv.) in *N*,*N*-dimethylformamide (DMF), K_2CO_3 (3.5 equiv.) and benzyl bromide (3.5 equiv.) were mixed. After vigorously stirring at 0 °C for 2 h, the reaction mixture was warmed to room temperature over 2 h and stirred for another 12 h. The resulting mixture was diluted with water (400 ml), and then extracted with EtOAc. The organic layer was washed with water and dried over $MgSO_4$. The residue was purified by column chromatography, using dichloromethane as the eluent, to afford the tetrabenzylether. The chemical structure of benzylated quercetin was determined by NMR, which was recorded on a spectrometer (ECA-600, 600 MHz for ^1H , Jeol, Japan) using [D_6] $CDCl_3$ as the solvent, and tetramethylsilane (TMS) as the internal standard. Chemical shifts and *J* values are given in Hz.

Materials and cell culture. Quercetin, protein kinase K, RNase A and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were from Sigma. The antibodies against poly (ADP) ribose polymerase (PARP) and caspase-3 were from Cell Signaling Technology. Fetal bovine serum (FBS) was from BioWhittaker Co., and Mn(III)tetrakis(4-benzoic acid)porphyrin chloride (MnTBAP) was from Calbiochem. The human leukemia (HL-60) cell line was obtained from Cancer Cell Repository (Tohoku University, Japan) and was cultured at 37 °C with 5% CO_2 in an RPMI-1640 medium

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Abbreviations: 4Bn-Q, 3,7,3′,4′-*O*-tetrabenzylquercetin; DHE, dihydroethidium; HL-60, human leukemia cell; HPF, hydroxyphenyl fluorescein; MnTBAP, Mn (III) tetrakis (4-benzoic acid) porphyrin chloride; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; $O_2^{-\bullet}$, superoxide; OH, hydroxyl radical; ROS, reactive oxygen species

containing 10% FBS and 1% penicillin-streptomycin glutamine (PSG).

Cell viability assay. The cell survival rate was measured by an MTT assay.[19] HL-60 cells were suspended at a density of 1.5×10^4 cells/well in an RPMI-1640 medium containing 10% FBS and 1% PSG. After 24 h of preculture, the cells were treated with 25–100 μM of quercetin or 4Bn-Q for 48 h. An MTT solution (0.5 mg/ml) was then added to each well, and the cells incubated for another 4 h. The resulting MTT-formazan product was dissolved by the addition of 100 μl of 0.04 N HCl-2-propanol and determined by measuring the absorbance at 595 nm with a micro-plate reader. The cell viability is expressed as the optical density ratio of the treated product to the control.

DNA fragmentation assay. The DNA fragmentation assay was carried out as described.[10] HL-60 cells (1.0×10^6 cells/3 ml/dish) were treated with 25–50 μM of quercetin or 4Bn-Q for 8 h. The cells were harvested by centrifugation and washed in ice-cold PBS. The resulting pellets were resuspended in a lysis buffer (50 mM Tris–HCl at pH 8.0, 10 mM EDTA, and 0.5% SDS) plus 0.1 mg/ml of RNase A. After 30 min of incubation at 37 °C, proteinase K was added and the mixture was incubated for another 3 h. DNA was separated by 2% agarose gel electrophoresis and digitally imaged after staining with ethidium bromide.

Determination of apoptosis by flow cytometry. Apoptotic DNA was measured by flow cytometry as described previously.[20] Briefly, after being treated with each sample, HL-60 (2.0×10^6) cells were fixed overnight in 70% ethanol at −20 °C and then resuspended in PBS. The cells were stained in 1 ml of PBS containing 10 μg/ml of RNase for 30 min in room temperature, and then 25 μg/ml of propidium iodide was added and the mixture stained in the dark. Fluorescence emitted from the propidium-DNA complex was detected with a flow cytometer (CyFlow, Partec). Apoptotic nuclei were distinguished by their hypodiploid DNA content from the diploid DNA content of normal nuclei.

Western blot analysis. After being treated with each sample, HL-60 (2.0×10^6) cells were lysed with a modified RIPA buffer containing 10 mM Tris–HCl (pH 8.0), 150 mM NaCl, 1 mM EDTA, 0.1% Nonidet P-40, 1% deoxycholate, 50 mM sodium fluoride, 50 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, and a proteinase inhibitor cocktail (Nacalai Tesque, Kyoto, Japan). The lysate was sonicated three times for 10 s and then centrifuged at 14,000 g for 15 min at 4 °C. Equal amounts of the lysate protein were run on SDS–PAGEs and electrophoretically transferred to a PVDF membrane (Amersham Pharmacia Biotech). After blotting, the membrane was first blocked with a TBST buffer (500 mM NaCl, 20 mM Tris–HCl (pH 7.4) and 0.1% Tween 20) containing 5% non-fat dried milk, and then incubated overnight with specific antibodies at 4 °C and further incubated for 1 h with the HRP-conjugated secondary antibody. Bound antibodies were detected by using the ECL system, and the relative amounts of proteins associated with the specific antibody were quantified by using Lumi Vision Imager software (TAITEC).

Intracellular ROS assay. Intracellular superoxide was measured by using dihydroethidium (DHE) fluorescein (Molecular Probes). DHE has been reported to be oxidized specifically by superoxide anions[21] and is one of the most frequently used fluorescence probes for measuring intracellular superoxide. The intracellular hydroxyl radical (•OH) was measured by using hydroxyphenyl fluorescein (HPF; Molecular Probes). HPF is a fluorescence probe to selectively detect the hydroxyl radical and partial ONOO–, and is frequently used to detect the intracellular hydroxyl radical.[22] In brief, HL-60 cells (1.0×10^6) were treated with quercetin or 4Bn-Q for various times and then incubated with 20 μM DHE or 10 μM HPF for 30 min at 37 °C. The cells (1.0×10^6) were analyzed at FL1 (530 nm) for HPF and FL2 (585 nm) for DHE with a flow cytometer (CyFlow, Partec).

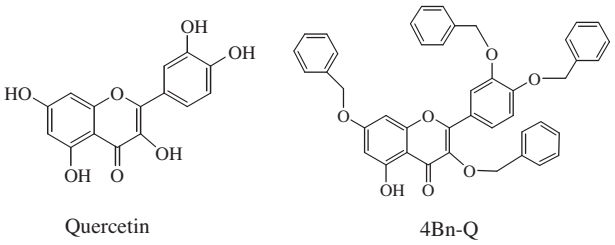

Fig. 1. Chemical Structures of Quercetin (left) and 3,7,3′,4′-*O*-Tetrabenzylquercetin (4Bn-Q) (right).

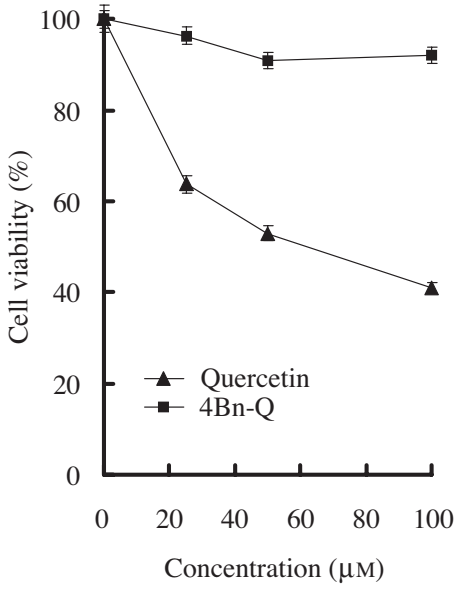

Fig. 2. Influence of Quercetin and 4Bn-Q on the Viability of HL-60 Cells.

HL-60 cells were placed in 96-well plates for 24 h, and then treated with or without different concentrations of each compound for another 48 h. The cell density was assessed colorimetrically after staining with MTT and is expressed as the optical density ratio of the treated to control cells at 595 nm. Cell survival is expressed as the optical density ratio of the treated to control cells. Data are presented as the mean ± SD of three independent experiments.

Results

Synthesis of quercetin derivative, 4Bn-Q

In order to replace the hydroxyl groups of quercetin, 3,7,3′,4′-*O*-tetrabenzylquercetin was synthesized and purified *via* column chromatography. The chemical structure of 4Bn-Q (Fig. 1) was determined by a ^{1}H-NMR analysis as follows: 4.98 (s, 2H, OCH_2Ph), 5.03 (s, 2H, OCH_2Ph), 5.11 (s, 2H, OCH_2Ph), 5.24 (s, 2H, OCH_2Ph), 6.42 (d, J = 1.8 Hz, 1H, aromatic H), 6.45 (d, J = 2.4 Hz, 1H, aromatic H), 6.94 (d, J = 9.0 Hz, 1H, aromatic H), 7.22–7.45 (m, 20H, aromatic H), 7.53 (dd, J = 9.0, 1.8 Hz, 1H, aromatic H), 7.70 (d, J = 2.4 Hz, 1H, aromatic H). Therefore, the position of the benzyl group was assigned to the 3,7,3′,4′-position of quercetin.

Effect of quercetin and 4Bn-Q on cell proliferation

To compare the effect of quercetin and 4Bn-Q on the proliferation of HL-60 cells, cells were treated with 25–100 μM of each compound. After a 48-h culture, the cell survival rate was investigated by an MTT assay. As

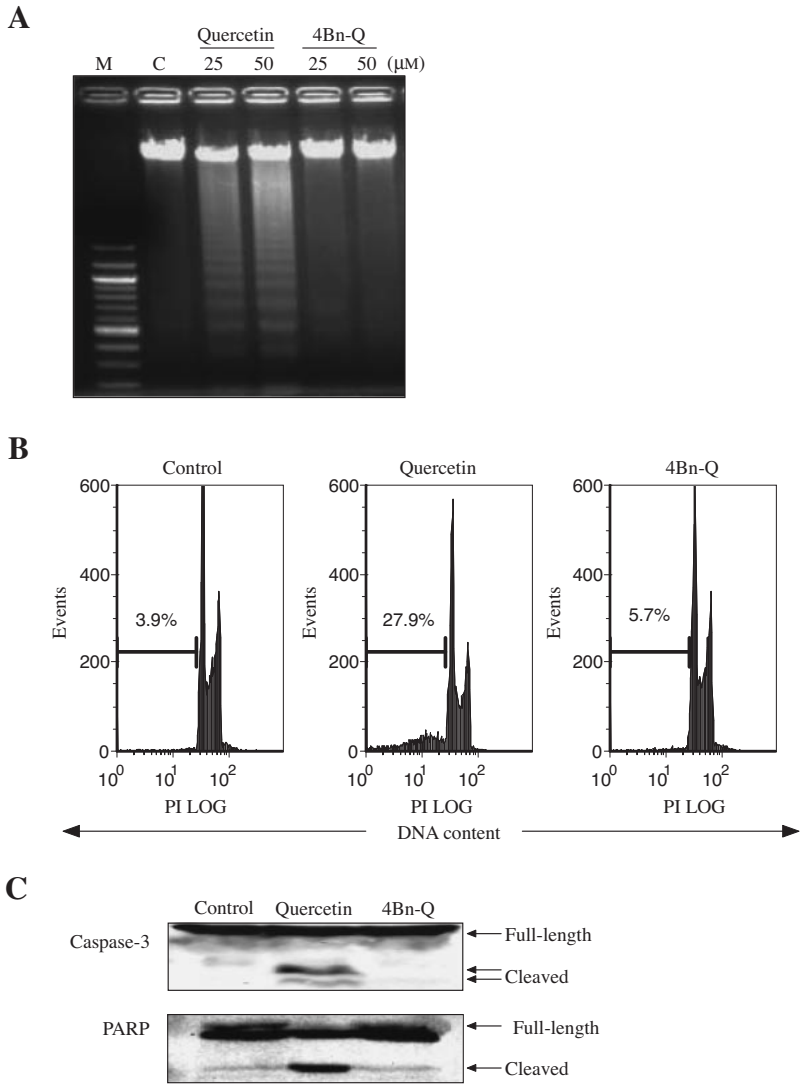

Fig. 3. Apoptosis Induction of HL-60 Cells by Quercetin and 4Bn-Q.

Cells were grown in the absence (control) or the presence of quercetin (25 μM) or 4Bn-Q (25 μM) for 8 h. A, DNA fragmentation. DNA was extracted and then separated by 2.0% agarose gel electrophoresis. The gel was visualized under ultraviolet light after staining with ethidium bromide (M, DNA marker; C, control). B, Quantification of apoptotic DNA. Harvested cells were fixed in 70% ethanol and stained with propidium iodide, before being subjected to a flow cytometric analysis. The percentages of cells with hypodiploid DNA content represent the fractions undergoing apoptotic DNA. C, caspase activation and PARP cleavage. Cellular lysate was applied to 15% or 10% of SDS–PAG caspases, and PARP were detected with the corresponding specific antibodies, being visualized by a chemiluminescence ECL kit.

shown in Fig. 2, quercetin inhibited cell proliferation in the concentration range of 25–100 μM with an IC_{50} (50% inhibitory concentration) value of 58 μM, while 4Bn-Q showed no inhibition under the same condition, losing the ability to inhibit cell proliferation.

Effect of quercetin and 4Bn-Q on cell apoptosis

To elucidate whether quercetin and 4Bn-Q could induce apoptosis in HL-60 cells, we characterized HL-60 cells by several approaches. DNA fragmentation was detected in the cells treated with 25–50 μM of quercetin, but not in the cells treated with 25–50 μM of 4Bn-Q (Fig. 3A). The percentage of cells with hypodiploid DNA was further quantified as 3.9% for the control, and 27.9% and 5.7% for the treatment by quercetin or 4Bn-Q (25 μM, 8 h), respectively (Fig. 3B). The significant difference in apoptotic DNA was observed between control and quercetin treatment, but not between control and 4Bn-Q treatment. Molecular data further revealed that the active form of caspase 3, an initiator caspase of apoptosis, and the cleavage of poly-(ADP ribose) polymerase (PARP), which responds to DNA strand breaks and is used as another hallmark of apoptosis, occured in the quercetin-treated cells (25 μM, 8 h), but not in the 4Bn-Q-treated cells (Fig. 3C). Therefore, quercetin might have suppressed cell proliferation by inducing apoptosis, and 4Bn-Q lost this ability.

Effect of quercetin and 4Bn-Q on the intracellular ROS level

Several lines of evidence have suggested that some flavonoids induce tumor cell apoptosis, in part, by enhancing the intracellular ROS level.[23,24] To know the mechanism underlying apoptosis induction by quercetin, we measured the intracellular levels of various ROS in quercetin- or 4Bn-Q-treated cells by using fluorescent probes. As shown in Fig. 4A, quercetin markedly increased the level of superoxide ($O_2^{-\bullet}$), while 4Bn-Q did not affect the level. EGCG was used as a negative control.[25] On the other hand, quercetin and 4Ben-Q did

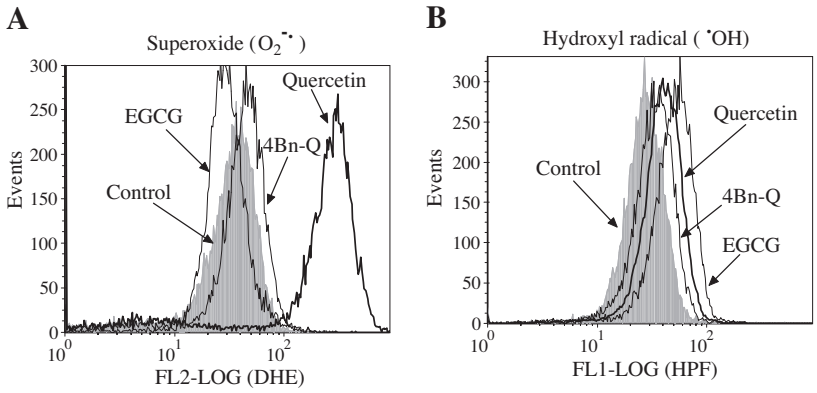

Fig. 4. Influence of Quercetin and 4Bn-Q on Intracellular ROS Levels.
HL-60 cells were grown in the absence (control) or presence of quercetin (25 μM) or 4Bn-Q (25 μM) for 30 min. The harvested cells were then respectively incubated with 20 μM of DHE or 10 μM of HPF for another 30 min and analyzed by flow cytometry as described in the Materials and Methods section.

not markedly affect the level of the hydroxyl radical ($^{\bullet}OH$) (Fig. 4B). As a positive control, EGCG markedly increased the intracellular level of $^{\bullet}OH$.[23] We also performed a time-course assay from 15 min to 120 min to measure the levels of these ROS, and obtained similar results to that with the treatment for 30 min (data not shown). These data suggest that the presence of hydroxyl groups might be essential for quercetin to generate superoxide, because 4Bn-Q, which substituted the hydroxyl groups of quercetin by benzalytion, lost this ability. It is also interesting that ROS generated by quercetin and EGCG were different, although both compounds could induce apoptosis in HL-60 cells, suggesting they may have a different mechanism for inducing apoptosis.

MnTBA, a scavenger of superoxide, reduces intracellular superoxide and cell apoptosis

To further demonstrate whether the increased superoxide level by quercetin was associated with cell proliferation inhibition and apoptosis induction, we used the scavenger of superoxide, MnTBAP, to scavenge quercetin-induced intracellular superoxide. The cells were treated with quercetin in the presence or absence of MnTBAP, and the intracellular superoxide level and cell apoptosis were then determined by flow cytometry. As shown in Fig. 5A, pretreatment with MnTBAP reduced the level of quercetin-induced superoxide, although the superoxide was not completely reduced to the control level. Moreover, the percentage of apoptotic DNA in the cells pretreated with MnTBAP pretreatment was 13.9%, lower than that in the cells treated by quercetin alone (21.5%) (Fig. 5B).

Discussion

Accumulated data have suggested that the hydroxyl groups of flavonoids play critical roles in their bioactive functions. To directly demonstrate the role of hydroxyl groups, we synthesized quercetin derivative 4Bn-Q, which substituted the hydroxyl groups with benzyl groups, and then evaluated its ability to suppress cell proliferation in HL-60 cells, this being a valid model for testing antileukemic or general antitumoral compounds and for clarifying the molecular mechanism. Substitution of the hydroxyl groups of quercetin has been done by acylation[26] and methylation.[27] It has been reported that the acylated derivative of quercetin was hydrolyzed in cultured cells,[28] and that the methylated-derivative of quercetin was easily metabolized by glycosylation.[29] To substitute the hydroxyl group of quercetin by a stable group, we synthesized a benzylated derivative of quercetin (4Q-B). Our results reveal that quercetin inhibited cell proliferation and induced apoptosis, but that 4Bn-Q had lost this ability. These data indicate that the bioactivity of quercetin appears to be associated with the presence of the hydroxyl groups. The question remains as to that how the hydroxyl groups of quercetin affect cell proliferation or apoptosis induction. It has been reported that flavonoids such as apigenin, quercetin, myricetin and kaempferol were able to induce apoptosis in HL-60 cells by elevating the intracellular ROS level, although it is unclear which ROS were essential for apoptosis induction by quercetin.[5] Superoxide and hydroxyl radicals are major intracellular ROS. To demonstrate whether these ROS would be involved in quercetin-induced apoptosis, we examined the intracellular levels of superoxide and hydroxyl radicals with specific fluorescent probes. The data revealed that treatment with quercetin increased the level of superoxide, and the 4Bn-Q had no such effect. On the other hand, there was no significant change in the levels of the hydroxyl radicals between the two compounds. Thus, the hydroxyl groups of quercetin might contribute to the generation of superoxide, rather than the hydroxyl radical.

To further clarify whether the increased superoxide level by quercetin was linked to cell proliferation inhibition and apoptosis induction, a scavenger of superoxide (MnTBAP) was used. The results revealed that MnTBAP reduced quercetin-induced superoxide, although the superoxide was not completely reduced to the control level. Moreover, the percentage of apoptotic DNA (13.9%) in the cells pretreated with MnTBAP was lower than that in the quercetin-treated cells (21.5%) (Fig. 5B). Our data demonstrate, at least in part, that superoxide contributed to quercetin-induced apoptosis.

Finally, the hydroxyl group at position 5 could not be benzylated in the present study. This may have been due

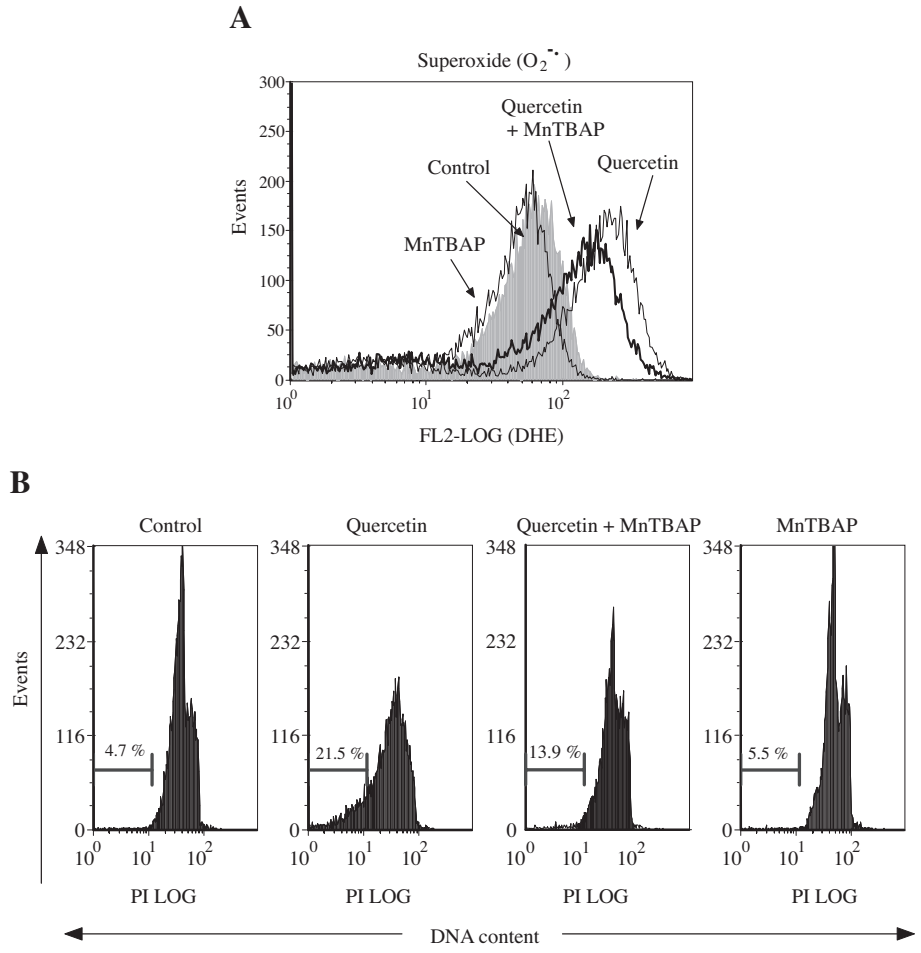

Fig. 5. Effect of MnTBAP on Quercetin-Induced Superoxide and Apoptosis.

A, Intracellular superoxide production in HL-60 cells. Cells were pretreated with MnTBAP (100 μM) for 1 h, and then treated with quercetin (25 μM) for another 30 min. The harvested cells were incubated with 20 μM of DHE for 30 min and analyzed by flow cytometry as described in the Materials and Methods section. B, Quantification of apoptotic DNA. HL-60 cells were pretreated with MnTBAP (100 μM) for 1 h and then treated with quercetin for 8 h. The harvested cells were fixed in 70% ethanol and stained with propidium iodide, before being subjected to flow cytometric analysis. The percentages of cells with hypodiploid DNA contents represent the fractions undergoing apoptotic DNA.

to its poor reactivity, because the hydrogen bond between the hydroxyl group at position 5 and the keto group at position 4 was quite stable under the present synthetic conditions. On the other hand, we also attempted to benzylate the hydroxyl groups at only positions 3′ and 4′ to clarify the role of the B-ring hydroxyl groups in intracellular ROS generation. However, the hydroxyl groups at positions 3 and 7 revealed reactivity as high as that at positions 3′ and 4′ under the present synthetic conditions. We therefore obtained a tetrabenzylquercetin (4Bn-Q) which is a benzylated derivative of quercetin at 3,7,3′,4′ positions. Based on our data and other reports, we can conclude that these hydroxyl groups, at least, contributed to generation of intracellular ROS. To clarify the role of the hydroxyl groups at individual positions, we intend to obtain the different derivatives of quercetin by modifying the synthetic conditions.

In summary, chemical modification of quercetin by substituting the hydroxyl groups with benzyl groups directly demonstrated that the hydroxyl groups of quercetin were linked to the generation of intracellular superoxide, cell proliferation inhibition and apoptosis induction. These findings will provide new insight into the cancer chemopreventive properties of quercetin.

Acknowledgments

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