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A novel tag-free probe for targeting molecules interacting with a flavonoid catabolite

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ABSTRACT

3,4-Dihydroxyphenylacetic acid (DOPAC) is one of the colonic microflora-produced catabolites of quercetin 4′-glucoside (Q4′G). Although the interaction of DOPAC with cellular proteins might be involved in its biological activity, the actual proteins have not yet been identified. In this study, we developed a novel tag-free DOPAC probe to label the targeted proteins by the copper(I)-catalyzed azide alkyne cycloaddition (CuAAC) and verified its efficacy. Various labeled proteins were detected by the DOPAC probe with the azide labeled biotin and a horseradish peroxidase (HRP)-streptavidin complex. Furthermore, a pulldown assay identified Keap1 and aryl hydrocarbon receptor (AhR) as the target proteins for the phase 2 enzyme up-regulation.

1. Introduction

Quercetin is one of the most widely distributed flavonoids in vegetables and fruits and has attracted much attention because of its beneficial biological effect, such as anti-oxidation [1,2] and anti-inflammation [3]. In general, quercetin exists in food stuffs as a glycoside form with a sugar moiety. Quercetin 4′-glucoside (Q4′G) is one of the major quercetin glycosides in onion and consumption of onions accounts for 29% of the total flavonol and flavone intake [4]. It is known that the fates of quercetin glycosides after intake are different depending on its sugar moiety. Mullen and his colleagues investigated the bioavailability of Q4′G in rats and proposed that Q4′G reaching the colon is subjected to hydrolysis by intestinal microbiota into 3,4-dihydroxyphenylacetic acid (DOPAC) [5]. DOPAC is subsequently converted into 3-hydroxyphenylacetic acid (OPAC) or 3,4-dihydroxybenzoic acid, which is also known as protocatechuic acid in the large intestine, and OPAC undergoes further conversion to hippuric acid [5]. Among the phenolic acid catabolites, DOPAC has been reported to have the significant ability to induce the phase 2 enzyme gene expression [6,7].

A previous study indicated that DOPAC is oxidized to form *o*-quinone, then covalently binds to sulfhydryls in GSH or protein due to its catechol structure [6]. Protein modification affects the protein function, consequently leading to effects on the cellular signaling pathway and gene expression. For example, covalent modification of Keap1 *via* cysteine residues subsequently causes activation of the Keap1/Nrf2/antioxidant response element (ARE) pathway [8]. The underlying mechanism in which DOPAC exerts an antioxidant effect *via* inducing the phase 2 enzyme is not fully understood. Therefore, developing a new probe of DOPAC provides an important clue to reveal the mechanism of its biological activity, including the phase 2 enzyme induction.

Click chemistry is a modular synthetic approach introduced by Sharpless and his co-workers in 2001 [9]. The reaction of this chemistry assembles molecules rapidly and efficiently under simple conditions and provides extremely high yields of the products, which can be easily isolated. The copper (I)-catalyzed alkyne azide 1,3-dipolar cycloaddition (CuAAC), which is known as the “cream of the crop” of click chemistry yields 1,4-disubstituted 1,2,3-triazols by conjugating the terminal alkyne and azide functional groups [10,11]. Over the last decade, it has been widely used as a major conjugating method in modern chemistry because of its high selectivity and tolerance to a wide range of reaction conditions.

In the present study, we designed a novel DOPAC probe that can be used for the CuAAC reaction. We introduced an alkyne moiety into DOPAC by esterification with 2-propyn-1-ol to afford the DOPAC propargyl ester (DPE, Fig. 1A). To confirm its efficacy as a protein thiol modifier, DPE was incubated with a model protein in the cell lysate, followed by CuAAC click reaction with an azide-

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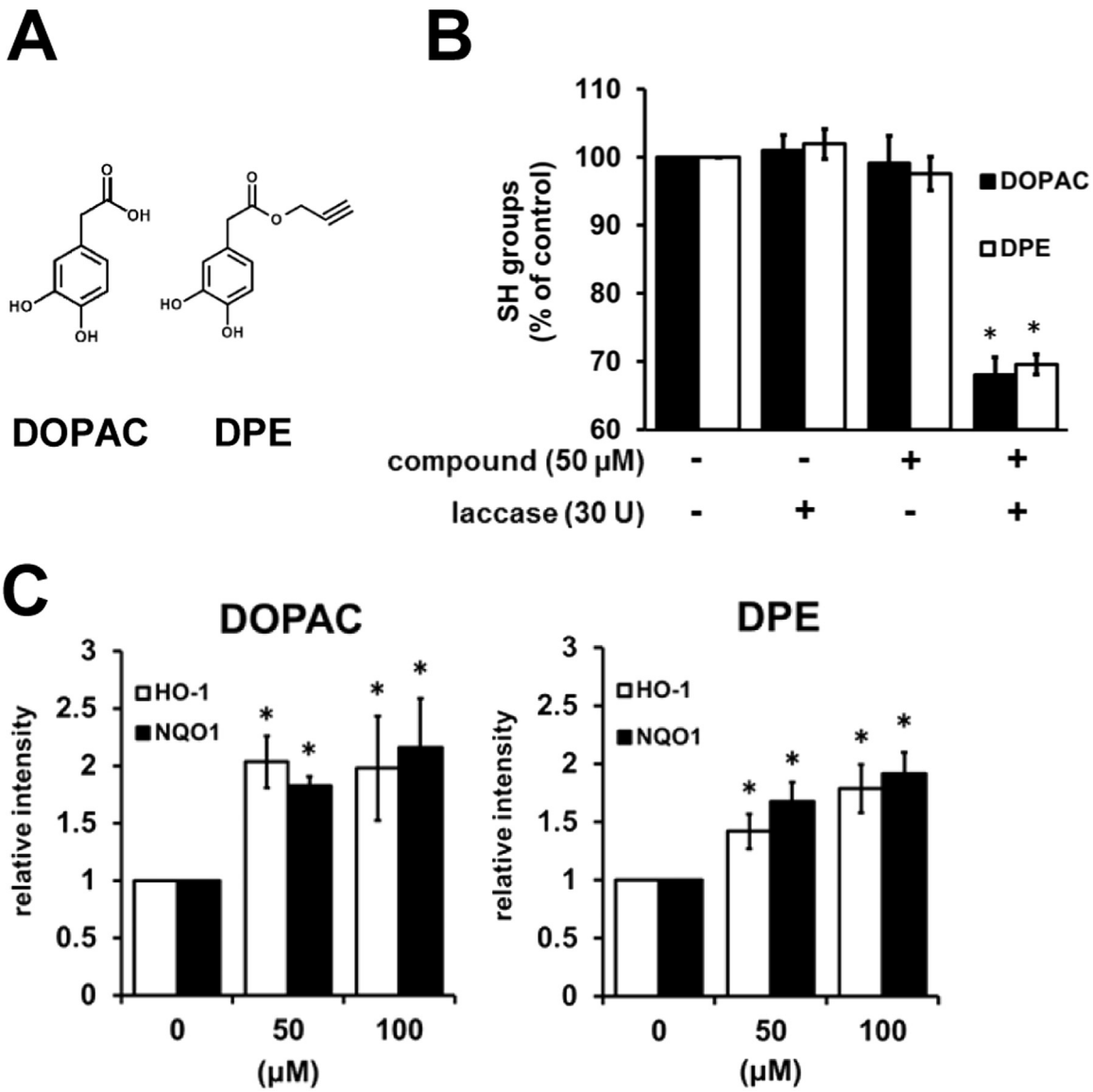

Fig. 1. DPE shows the similar chemical and biological properties to DOPAC. (A) Chemical Structures of DOPAC and DPE. (B) Modification of sulfhydryl groups in GAPDH by DOPAC and DPE. GAPDH (500 μg/ml) was incubated with DOPAC or DPE in 50 mM sodium phosphate buffer (pH 7.2) for 1 h at 37 °C in the presence or absence of laccase (30 units). The level of residual sulfhydryl groups in GAPDH was measured by the spectrophotometric method using DTNB. (C) Induction of the gene expression of HO-1 (black bars) and NQO1 (white bars) by DOPAC (left) or DPE (right). Hepa1c1c7 cells were treated with DOPAC or DPE for 24 h and total RNA was extracted. The values represent means ± S.D. of more than three separate experiments ($^*p < 0.05$ compared with control; Student's *t*-test.).

labeled biotin and finally detected using the horseradish peroxidase (HRP)-conjugated streptavidin. The use of DPE combined with the subsequent introduction with an azide-linked biotin by the CuAAC reaction is expected to enable the highly effective tagging of the DOPAC modified protein.

2. Materials and methods

2.1. Materials

DOPAC, laccase from *Rhus vernicifera*, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl]amine (TBTA), bis(4-nitrophenyl) phosphate (BNPP) and azide-PEG_3-biotin conjugate were obtained from Sigma Aldrich (St. Louis, USA). *p*-Toluensulfonic acid monohydrate (PTSA), *n*-butanol, toluene, copper (II) sulfate pentahydrate, protease inhibitor cocktail and Chemi-Lumi One Super were purchased from nacalai tasque (Kyoto, Japan). 2-Propyl-1-ol was obtained from Tokyo Chemical Industry (Tokyo, Japan). Streptavidin, HRP conjugate was purchased from Funakoshi (Tokyo, Japan). Anti-actin antibody, anti-aryl hydrocarbon receptor (AhR) antibody, anti-Keap1 antibody, horseradish peroxidase-linked anti-mouse IgG and horseradish peroxidase-linked anti-goat IgG were purchased from Santa Cruz Biotechnology (Santa Cruz, USA). Streptavidin Mag Sepharose was purchased from GE health care (Little Chalfont, UK). All other chemicals such as benzyl azide were purchased from Wako Pure Chemical Industries (Osaka, Japan).

2.2. General procedure

MS was recorded on ESI-mode by using Bruker MicrOTOF II and MicrOTOF Control 3.0. Data analysis was carried out using Data Analysis 4.0 SP2. ^{1}H NMR spectra were recorded on Varian Mercury 300. Chemical shift are described in parts per million (ppm) and coupling constants in Hz. Multiplicity and qualifier abbreviations are as follows: *s*=singlet, *d*=doublet, *t*=triplet, *quint*=quintet, *sext*=sextet, *m*=multiplet.

2.3. Cell culture

The mouse hepatoma cell line Hepa1c1c7, obtained from the American Type Culture Collection, was grown and maintained at 37 °C in α-minimum essential medium (MEM-α, Thermo SCIENTIFIC, Waltham, USA) containing 10% fetal bovine serum, 4 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. For experiments, cells were seeded in complete medium and treated with each reagent or DMSO vehicle.

2.4. Fisher esterification of DOPAC

DOPAC (30 mg, 0.18 mmol) and PTSA (6 mg, 0.03 mmol) were dissolved in the solution of dehydrated toluene (20 ml) and 2-propyl-1-ol (1 ml, 17 mmol), and stirred for 6 h at 40 °C, followed by cooling to room temperature. The reaction mixture was washed with 5% $NaHCO_3$ solution and water twice. The aqueous phase was extracted with 20 ml ethyl acetate and combined with the organic phase. The combined extract was dried over anhydrous magnesium sulfate, filtered, and evaporated to dryness. The product was purified by preparative thin-layer chromatography (TLC) ($CHCl_3$:MeOH=9:1) to afford 0.34 mg (0.07 mmol) of DOPAC propargyl ester (DPE). ^{1}H NMR (300 MHz, $CDCl_3$): 2.48 (1H, *t*, *J*=2.5 Hz, **H**≡C–), 3.56 (2H, *s*, –C**H**$_2$–Ar), 4.70 (2H, *d*, *J*=2.5 Hz, –O–C**H**$_2$-C≡), 6.67–6.78 (3H, *m*, **H**–Ar–); HR-ESI-MS (negative mode) *m*/*z* $[M–H]^-$ 205.0508 (205.0506, calcd for $C_{11}H_9O_4$).

DOPAC butanol ester (DBE) was synthesized in the same method as DPE, except that *n*-butanol 1.8 ml (20 mmol) was used instead of 2-propyl-1-ol. ^{1}H NMR (300 MHz, $CDCl_3$): 0.92 (3H, *t*, *J*=7.4 Hz, C**H**$_3$–CH_2–), 1.36 (2H, *sext*, *J*=7.4 Hz, CH_3–C**H**$_2$–CH_2–), 1.62 (2H, *quin*, *J*=3.5 Hz, –CH_2–C**H**$_2$–CH_2–), 3.50 (2H, *s*, –C**H**$_2$–Ar), 4.10 (2H, *t*, *J*=6.6 Hz, –CH_2–C**H**$_2$–O–), 6.63–6.72 (3H, *m*, **H**–Ar–).

2.5. CuAAC reaction with DPE and benzyl azide

DPE (23 mg, 112 μmol) and benzyl azide (14 μl, 112 μmol) were mixed in 2 ml of 50% *t*-butanol in water with copper (II) sulfate pentahydrate (0.28 mg, 1.12 μmol) and ascorbic acid sodium salt (2.22 mg, 11.2 μmol) at room temperature under the dark condition until the complete consumption of the reactants checked by TLC. The product was purified by preparative TLC (hexane:ethyl acetate=3:7), monitored by Dragendorff's reagent. Formation of 1,2,3-triazol was confirmed by NMR and mass spectrometry: ^{1}H NMR (300 MHz, $CDCl_3$): 3.46 (2H, *s*, Ar–C**H**$_2$–C(O)O–), 5.17 (2H, *s*, –O–C**H**$_2$–C_2N_3H–) 5.49 (2H, *s*, –C_2N_3H–C**H**$_2$–Bz), 6.55–6.78 (3H, *m*, **H**–Ar–), 7.24–7.38 (5H, *m*, **H**–Bz), 7.50 (1H, *s*, –C_2N_3**H**–), HR-ESI-MS (negative mode) *m*/*z* $[M–H]^-$ 338,1142 (338.1146, calcd for $C_{18}H_{16}N_3O_4$).

2.6. RNA extraction and RT-PCR

Confluent Hepa1c1c7 cells were treated with DOPAC or DPE at the indicated concentrations for 24 h. The total RNA was extracted from the cells with TRIzol reagent (Thermo Fisher Scientific, Waltham, MA, USA). The RNA concentration was determined by measuring the absorbance at 260 nm. cDNA was synthesized from total RNA (5 μg) and oligo (dT) primer using PrimeScript Reverse Transcriptase (Takara-Bio, Kusatsu, Japan) in accordance with manufacturer's instructions. The PCR reactions were performed using BIOTAQ DNA polymerase (BIOLINE, London, UK) and gene-specific primers: heme oxygenase-1 (HO-1), (F) 5′-ACATCGACAGCCCCACCAAGTTCAA-3′ and (R) 5′-CTGACGAAGTGACGCCATCTGTGAG-3′; NAD(P)H:quinone oxidoreductase 1 (NQO1), (F) 5′-TCGAAGAACTTTCAGTATCC-3′ and (R) 5′-TGAAGAGAGAGTACATGGAGCC-3′; β-actin, (F) 5′-GTCACCCACACTGTGCCCATCTA-3′ and (R) 5′-GCAATGCCAGGGTACATGGTGGT-3′. The PCR products were subjected to agarose gel electrophoresis (3%) stained with ethidium bromide and and imaged with an LAS3000 image analyzer (Fuji Film, Tokyo, Japan). Densitometric analysis of the bands was carried out using the Image J Software Program (National Institutes of Health, Bethesda, MD, USA).

2.7. DTNB assay

Loss of free sulfhydryls in GAPDH was measured as previously reported [6]. Briefly, GAPDH (500 μg/ml) and 50 μM DPE were dissolved in 70 mM sodium phosphate buffer (pH 7.2) and incubated for 1 h at 37 °C with or without 30 U laccase. An aliquot (0.1 ml) of the mixture was blended with an equal volume of 20% trichloroacetic acid (*w*/*v*) and centrifuged at 1500 rpm for 10 min at 4 °C. After the supernatant was removed, the pellet was washed with ethanol and ethyl acetate (1:1) twice and then centrifuged at 15,000 rpm for 10 min. The dried pellet was dissolved with 0.4 ml of 8 M guanidine hydrochloride and 13 mM EDTA in 133 mM Tris buffer (pH 7.6), followed by the incubation with 45 μl DTNB (10 mM) for 5 min. The absorbance was measured at 412 nm using a microplate reader (Bio-Rad Laboratories, Tokyo, Japan).

2.8. Detection of DPE-modified GAPDH and DPE-modified intracellular proteins

As for GAPDH experiment, GAPDH (1 mg/ml) and 50 μM DPE were dissolved in 500 μl of 70 mM sodium phosphate buffer (pH 7.2) and incubated for 1 h at 37 °C with or without 30 U laccase. As for the intracellular protein experiment, confluent Hepa1c1c7 cells were incubated with 100 μM BNPP or 0.1% DMSO in FBS-free MEM-α for 1 h, followed by the treatment with 100 μM DPE for 0, 1, 3, and 8 h. The treated cells were lysed with the lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10 mM NaF, 2 mM Na_3VO_4, 0.1% SDS, 1% Triton-X100, and protease inhibitor cocktail. The cell lysate was ultrafiltered with Vivacon® 500 (Sartorius, Gottingen, Germany) to remove the free-DPE. After protein quantification by a BCA assay (Thermo Fisher Scientific), the CuAAC reaction was performed as previously reported [12]. The GAPDH solution or cell lysate (100 μg proteins/50 μl) were incubated with 1 mM copper (II) sulfate pentahydrate, 1 mM sodium ascorbate, 0.1 mM TBTA and azide-PEG_3-biotin conjugate for 1 h at room temperature in the dark. After SDS-PAGE, the membrane was incubated with HRP-conjugated streptavidin for 1 h. Streptavidin-biotin complex was visualized by Chemi-Lumi One Super.

2.9. Competitive inhibition of DPE-modification in cells and in cell lysate

As for treatment of cells, confluent Hepa1c1c7 cells were pre-incubated with DOPAC or DBE for 30 min and incubated with 25 μM DPE for 3 h in serum-free MEM-α. As for treatment of cell lysate, cell lysate prepared from Hepa1c1c7 containing 100 μg proteins were co-incubated with 25 μM DPE and 0–200 μM DOPAC or DBE in the presence of 30 U laccase. In both experiments, CuAAC reaction, SDS-PAGE and western blot analysis were subsequently conducted as described before.

2.10. Pulldown assay and western blotting

Confluent Hepa1c1c7 cells were treated with 50 μM DPE or 0.1% DMSO in FBS-free MEM-α for 5 h. The treated cells were lysed, and the cell lysate containing 1000 μg of protein were subjected to ultrafiltration and the CuAAC reaction as mentioned above. The protein solution was incubated with 100 μM Streptavidin Mag Sepharose for 30 min at room temperature with constant shaking, washed and eluted according to the manufacture's

protocol. The eluted proteins were subjected to SDS-PAGE and transferred to membranes. After blocking, membrane was treated with anti-Keap1 antibody (1:200) or anti-AhR antibody (1:200), followed by incubation with appropriate secondary antibody and detection using Chemi-Lumi One Super.

2.11. Statisitics

All values were expressed as means ± SD. Statistical significance was assessed by Student's paired two-tailed *t*-test. A *p*-value of 0.05 was regarded to be statistically significant.

3. Results and discussion

Protein labeling using a probe with an alkyne handle and subsequent CuAAC click reaction were employed as an efficient method for molecular targeting. To introduce an alkyne functionality into DOPAC, we esterified DOPAC with 2-propyn-1-ol into DPE *via* the Fischer esterification method (Fig. 1A). This alkyne functionality could form a 1,2,3-triazol derivative with azide-functionalized tags such as biotin and fluorescent dyes, which allow detection in a later step. In order to examine whether the esterification of DOPAC influences the ability to bind to protein sulfhydryls, we compared the thiol modification ability of DPE with that of DOPAC. We used GAPDH as a model protein of the thiol modification, because GAPDH has four cysteine residues on each subunit and is vulnerable to a nucleophilic attack [13]. Laccase was used as a polyphenoloxidase to oxidize DOPAC and DPE into the *o*-quinone structures *via* a two-electron oxidation as previously reported [14]. As shown in Fig. 1B, the 1-h incubation of GAPDH with DOPAC or DPE in the presence of laccase resulted in reduction of the free sulfhydryl amount, whereas no change was observed under the condition without laccase. This result suggested that DPE had the same ability and phenol oxidase dependency to modify the protein sulfhydryls as DOPAC. Next, we examined the biological features of DPE such as the inducible expression of the phase 2 drug-metabolizing enzyme genes. As shown in Fig. 1C, DOPAC dose-dependently increased the gene expression of HO-1 and NQO1, which is consistent with a previous report [6]. DPE also enhanced their gene expression in a manner similar to DOPAC in the cultured cells. The significant biological activity of DPE comparable to the original DOPAC suggested that the esterification of DOPAC with 2-propyn-1-ol showed no effect on the modifying ability of the protein sulfhydryls.

We next confirmed the capability of DPE to undergo a CuAAC reaction with an azide-linked tag molecule. DPE and benzyl azide were incubated for 24 h at room temperature in the dark with copper (II) sulfate pentahydrate as the catalyst to afford the proposed product. The purification was done by preparative TLC according to the fluorescence excited at 254 nm. Also, the reaction of the isolated product with Dragendorff's reagent afforded an orange colored compound, suggesting that it contains nitrogen [15]. Based on the spectral data, the product was identified as a 1,2,3-triazol compound with the DOPAC ester and benzyl moiety (Fig. 2A).

To confirm the efficacy of DPE to tag the target protein, we tried to detect DPE-modified proteins using GAPDH as an *in vitro* model protein. DPE was incubated with GAPDH in the presence or absence of laccase, followed by the CuAAC reaction with the azide-labeled biotin and detection using the HRP-conjugated streptavidin. DPE-modified GAPDH was detected under the condition with laccase, affording strong evidence for effective tagging (Fig. 2B). The DPE-modified GAPDH was hardly observed in the absence of laccase, suggesting that DPE covalently binds to GAPDH through the oxidation-dependent electrophilic attachment to the sulfhydryl group.

Subsequently, we applied this probe to cultured cells to label the cellular proteins. DPE contains an ester bond, which would be hydrolyzed by carboxylesterase present in the cells. Therefore, we used BNPP as an esterase inhibitor to prevent cleavage of DPE in the cells [16]. After BNPP pretreatment, the cells were incubated with DPE for the indicated periods, then lysed to extract the cellular proteins. Free DPE in the cell lysate was removed by ultrafiltration to prevent additional binding during the CuAAC reaction. Then, CuAAC reaction was carried out to afford the DPE modified proteins having the biotin tags. After SDS-PAGE, the DPE-modified proteins were detected by the HRP-conjugated streptavidin. As shown in Fig. 3A, the DPE-modified proteins were observed in the groups with and without the BNPP treatment. These results

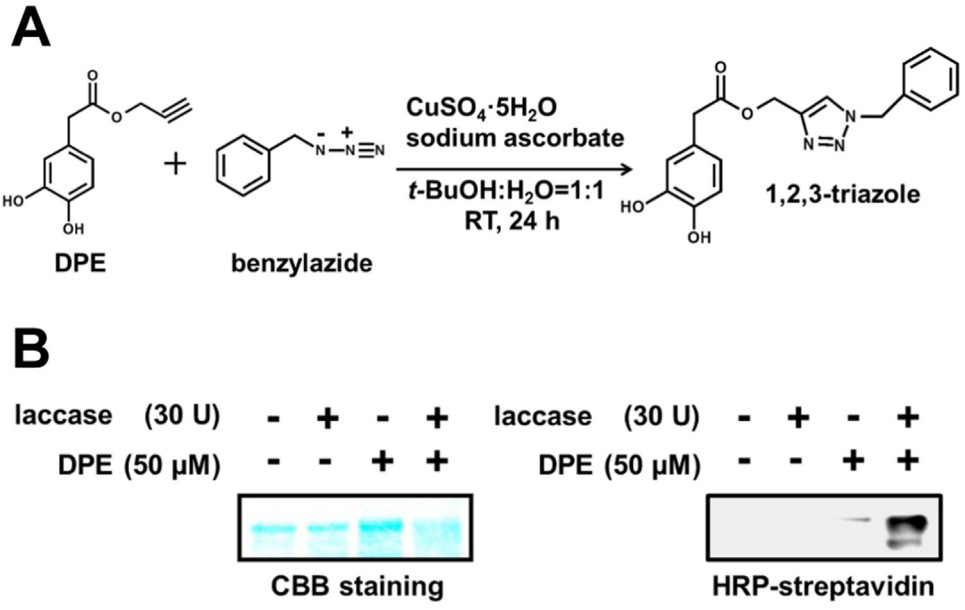

Fig. 2. CuAAC reaction of DPE with an azide-linked tag molecule. (A) Formation of 1,2,3-triazole by CuAAC reaction with DPE and benzyl azide. (B) Detection of GAPDH tagged by CuAAC reaction with DPE and the azide-labeled biotin. GAPDH (1 mg/ml) was incubated with or without 50 μM DPE in the presence or absence of 30 U laccase in 70 mM sodium phosphate buffer (pH 7.2) for 1 h at 37 °C. DPE-tagged GAPDH was detected by CBB staining (left) and HRP-streptavidin (right).

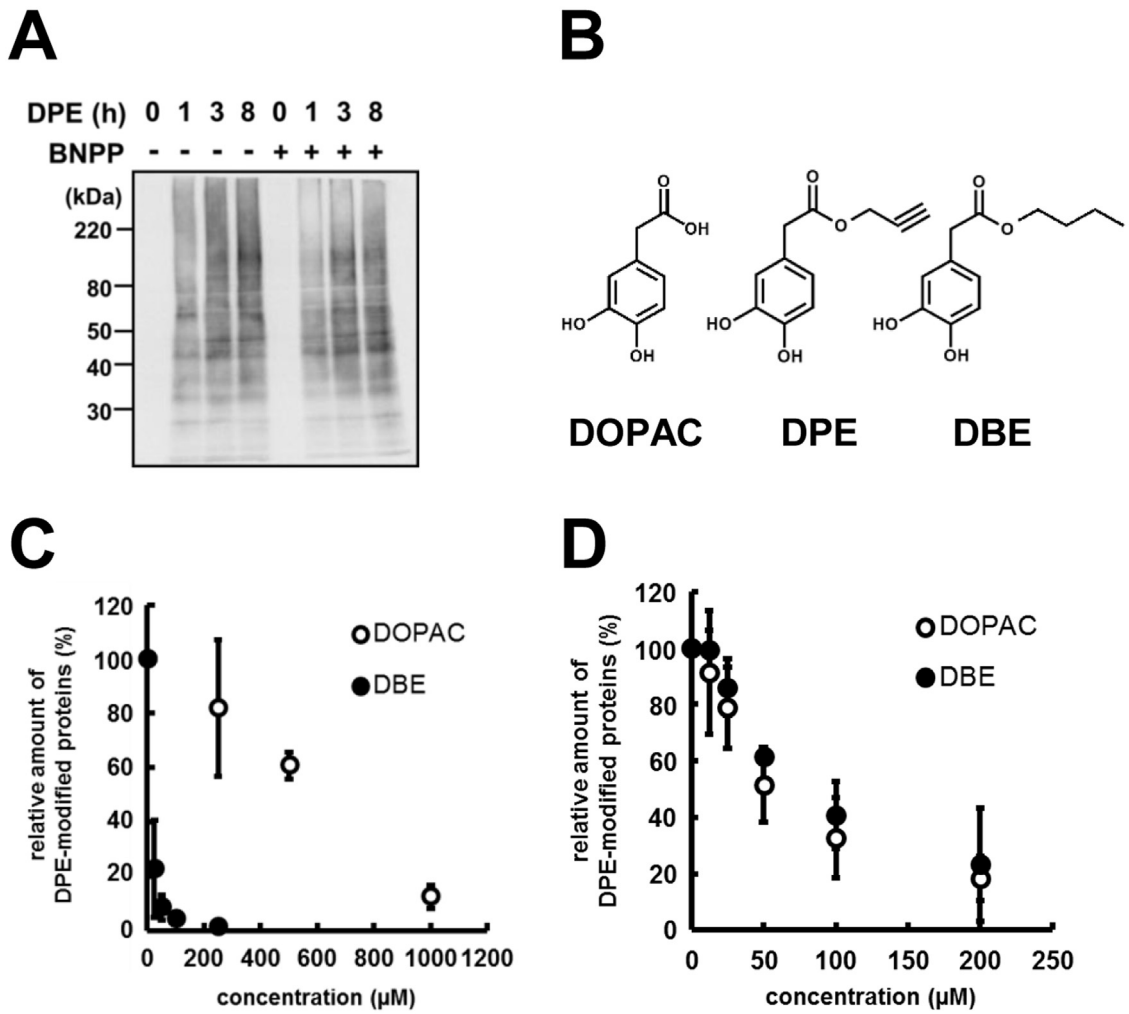

Fig. 3. Detection of the intracellular DPE-modified proteins. (A) Detection of the DPE-modified proteins in Hepa1c1c7. Confluent Hepa1c1c7 cells were pre-incubated with 0.1% DMSO or 100 μM BNPP and incubated with 100 μM DPE for indicated time periods in serum-free MEM-α. The DPE-tagged cellular proteins were detected by HRP-streptavidin. (B) Structural comparison of DBE with DOPAC and DPE. (C) Effect of the pre-treatment of DOPAC or DBE with DPE on the DPE-modified protein formation in Hepa1c1c7 cells. Hepa1c1c7 cells were pre-incubated with DOPAC (open circle) or DBE (closed circle) for 30 min and incubated with 25 μM DPE for 3 h in serum-free MEM-α. (D) Effect of the co-treatment of DOPAC or DBE with DPE on the DPE-modified protein formation in cell lysate. Cell lysate was incubated with 25 μM DPE in combination with DOPAC (open circle) or DBE (closed circle) in the presence of 30 U laccase for 1 h. The values represent means ± S.D. of three separate experiments.

suggested that the ester linkage in DPE is resistant to hydrolysis by carboxylesterase.

To verify whether the proteins modified by DPE correspond to the DOPAC targets, we examined the effect of the pre-incubation with DOPAC for 30 min on the incorporation of DPE into the cellular proteins. The pretreatment of DOPAC actually inhibited the formation of the DPE-modified proteins, though this was observed only at the higher concentration of DOPAC (Fig. 3C). To investigate the effect of the esterification, we next synthesized another DOPAC ester using *n*-butanol instead of 2-propyn-1-ol to afford DBE (Fig. 3B). DBE inhibited DPE-modified proteins even at a concentration lower than that of DOPAC (Fig. 3C). These data suggested that the esterification of DOPAC increases the frequency of competition with DPE possibly by the rise in the intracellular accumulation resulted from increased membrane permeability. To investigate this possibility, we compared the inhibitory ability of DOPAC and DPE using cell lysate. As shown in Fig. 3D, co-treatment of DOPAC with DPE in cell lysate exhibited almost the same inhibition against the formation of the DPE-modified protein as that of DBE. Taken together, these results suggest that DPE has higher ability to capture intracellular proteins, but targets the same proteins as DOPAC.

It should be noted that DPE with click chemistry is able to detect the targeted proteins having a wide range of molecular weights, which is consistent with the previous study using epigallocatechin-3-gallate with redox-cycle staining [17]. Nevertheless, another study demonstrated that the DOPAC probe directly tagged with biotin [6] modified a much lower amount of proteins than the tag-free DOPAC probe developed in the present study. This might be due to the difference in the steric hindrance; the biotin-tagged DOPAC probe might restrict its ability to access the protein cysteine residues. On the other hand, upon exposure of the cells to the biotin-tagged DOPAC probe in the presence of the catechol-type flavonoids, a significant decrease in the DOPAC-modified proteins occurred [6]. Furthermore, we observed that co-incubation of quercetin with DPE decreased amount of DPE-modified proteins compared to single incubation of DPE (Nakashima S and Nakamura Y, unpublished data), in agreement with the previous report [6]. Taken together, we concluded that the tag-free DOPAC with the compactness is a more useful probe for understanding its molecular targets of dietary polyphenols with catechol moiety.

We finally attempted to detect the modification of Keap1 and AhR in the cells exposed to DPE, because the expression of a phase

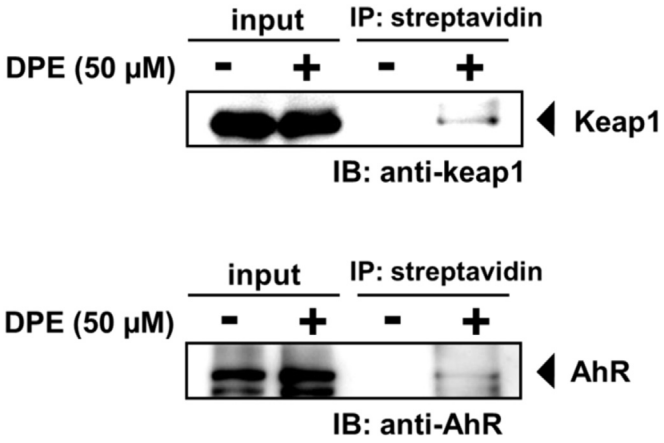

Fig. 4. Identification of the target proteins of DOPAC. Detection of the DPE-modified Keap1 and AhR. Confluent Hepa1c1c7 cells were incubated with 50 μM DPE for 5 h in serum-free MEM-α. The cell lysate was incubated with Streptavidin Mag Sepharose beads for 30 min. The DPE-modified Keap1 (left) or AhR (right) were detected by immunoblot analysis.

2 enzyme, NQO1, is regulated by the Keap1/Nrf2/ARE pathway and AhR/xenobiotic response element (XRE). Human Keap1 contains 27 cysteines, some of which are postulated to be the targets of electrophiles and oxidants that facilitate the derepression of Nrf2 and give rise to the phase 2 enzyme induction [18,19]. AhR is a transcription factor normally presents in the cytoplasm and upon ligand binding, it translocases into nucleus and bind to XRE and activates XRE-driven gene expression including some phase 2 enzyme like NQO1 [20]. Electrophilic quinones have reported to have capability to covalently bind to and activate AhR [21]. These led us to assume that direct modification of Keap1 and/or AhR of DOPAC may regulate the gene expression. To confirm this, Hepa1c1c7 cells were treated and Keap1 or AhR was detected by Western blot analysis. The pull-down assay successfully detected the modification of Keap1 and AhR by DPE (Fig. 4). These results indicate that Keap1 and AhR are potential targets of DOPAC for the up-regulation of phase 2 enzymes in Hepa1c1c7 cells.

In conclusion, this study provides an alternative approach to identify the molecular targets for not only a colonic microflora-produced catabolite of quercetin glycosides, DOPAC, but also the parent flavonoids. Click reactions afford the single reaction compound of interest quickly and irreversibly with high yield and specificity. The tag-free probe using click chemistry is extremely useful not only in the pulldown experiments using a biotin-tag and avidin-bound solid phase, but also histochemistry with the fluorescence tags to quantify or reveal the location. Since the catechol type polyphenols are potential modifiers of redox-dependent cellular events through sulfhydryl modification, the present results strongly encourage further investigation into chemical biology of polyphenols such as the molecular and biochemical mechanisms for biological activities.

Conflict of interest

The authors declare no competing financial interest.

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Transparency document. Supporting information

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